METHODS AND COMPOSITIONS FOR IMPROVED ENZYME ACTIVITY IN TRANSGENIC PLANTS

FIG. 1

Abstract: Compositions and methods for increasing enzyme activity across a broad physiological spectrum in plants, plant cells, tissues and seeds are provided. Compositions include plants or plant parts comprising two or more polynucleotides encoding polypeptides that are active across a broader physiological spectrum than when either polynucleotide is expressed alone. Vectors comprising these polynucleotide molecules as well as host cells comprising the vectors are further provided. Compositions also comprise transformed bacteria, plants, plant cells, tissues, and seeds. In addition, methods are provided for producing the plants, plant cells, tissues and seeds of the invention. Methods for increasing plant yield and methods for conferring resistance to an herbicide in a plant are further provided.
METHODS AND COMPOSITIONS FOR IMPROVED ENZYME ACTIVITY IN TRANSGENIC PLANTS

RELATED APPLICATIONS

Priority is claimed to U.S. Provisional Patent Application No. 60/891,977, filed February 28, 2007, which is incorporated by reference in its entirety herein.

FIELD OF THE INVENTION

The present invention relates generally to molecular plant biology.

BACKGROUND OF THE INVENTION

Diurnal and seasonal variations regulate plant growth. Many of the effects of seasonal variation on plant metabolism, such as the effects of temperature change, can be attributed to altered enzymatic rates. In the case of transgenic plants, the catalytic activity of an enzyme may be further influenced by the genetic source of the transgene rather than the plant in which it is expressed (Oliver et al. (1993) Mol. Gen. Genet. 239:425-434).

Current commercially available transgenic plants are made tolerant of herbicides by expression of a single enzyme with a narrow functional range of effective temperature optima. See e.g., Light et al. (1999) Weed Sci. 47:644-650; Light et al. (2001) Weed Sci. 49:543-548. These limitations on herbicide resistance are well documented and have been accounted for in the formulations and application procedures of many herbicides. For example, glyphosate tolerant plants harboring the EPSP synthase enzyme CP4 are not fully tolerant of glyphosate for the duration of the growing season. If the glyphosate is used at a wrong time, the plants suffer and yields drop.

Resistance phenotypes may also be subject to additional environmental variations and/or to differential regulation of a resistance gene within plant tissues, organs, cellular compartments etc. Accordingly, methods are needed in the art to improve transgenes such that the encoded enzymes are functional across a broader spectrum of environmental conditions (e.g., temperature, soil acidity, etc.) and/or physiological conditions (e.g., pH, concentration of an enzyme substrate or cofactor, etc.). To meet this need, the present invention provides methods of
expressing two or more enzymes that perform a same or similar function in a plant, wherein the two or more enzymes have difference kinetic parameters, to achieve optimal enzyme activity across a range of environmental and/or physiological conditions.

SUMMARY OF THE INVENTION

The present invention provides methods and compositions for improved enzyme activity in transgenic plants. The invention is useful as applied to plants such as agricultural plants, including both monocots and dicots.

In one aspect of the invention, transgenic plants are provided, wherein the plants have improved enzyme activity. For example, a transgenic plant may comprise at least (a) a first heterologous polynucleotide encoding a first polypeptide capable of conferring a trait of interest, and (b) a second heterologous polynucleotide encoding a second polypeptide capable of conferring said trait of interest, wherein said first and second polynucleotides are stably expressed in said plant, and wherein said plant shows said trait of interest over a broader range of a physiological or environmental condition as compared to a plant comprising either said first or second polynucleotide expressed alone and subject to said range of a physiological or environmental condition. As another example, a transgenic plant of the invention may comprise at least (a) a first heterologous polynucleotide encoding a first polypeptide capable of conferring herbicide resistance, and (b) a second heterologous polynucleotide encoding a second polypeptide capable of conferring said herbicide resistance, wherein said first and second polynucleotides are stably expressed in said plant, and wherein said plant shows said herbicide resistance over a broader range of a physiological or environmental condition as compared to a plant comprising either said first or second polynucleotide expressed alone and subject to said range of a physiological or environmental condition.

In another aspect of the invention, methods are provided for preparing transgenic plants with improved enzyme activity, which thereby confer a trait of interest to the plant. For example, such a method may comprise introducing into said plant at least (a) a first heterologous polynucleotide encoding a first polypeptide capable of conferring said trait of interest, and (b) a second heterologous polynucleotide encoding a second polypeptide capable of conferring said trait of interest, wherein said first and second polynucleotides are stably expressed in said plant, and wherein said plant shows said trait of interest over a broader spectrum of a physiological or
environmental condition as compared to a plant comprising either said first or second polynucleotide expressed alone and subject to said spectrum of a physiological or environmental condition. As another example, such a method may comprise (a) providing a transgenic plant comprising a first heterologous polynucleotide encoding a first polypeptide capable of conferring said trait of interest, and (b) introducing into said plant at least a second heterologous polynucleotide encoding a second polypeptide capable of conferring said trait of interest, wherein said first and second polynucleotides are stably expressed in said plant, and wherein said plant shows said trait of interest over a broader spectrum of a physiological or environmental condition as compared to a plant comprising either said first or second polynucleotide expressed alone and subject to said spectrum of a physiological or environmental condition.

For conferring herbicide resistance to a plant, a representative method of the invention comprises introducing into said plant at least (a) a first heterologous polynucleotide encoding a first polypeptide capable of conferring resistance to said herbicide, and (b) a second heterologous polynucleotide encoding a second polypeptide capable of conferring resistance to said herbicide, wherein said first and second polynucleotides are stably expressed in said plant, and whereby said plant is herbicide resistant over a broader spectrum of a physiological or environmental condition as compared to a plant comprising either said first or second polynucleotide expressed alone and subject to said range of a physiological or environmental condition. In an additional representative method for conferring herbicide resistance to a plant, the method may comprise (a) providing a transgenic plant comprising a first heterologous polynucleotide encoding a first polypeptide capable of conferring resistance to said herbicide, and (b) introducing into said plant at least a second heterologous polynucleotide encoding a second polypeptide capable of conferring resistance to said herbicide, wherein said first and second polynucleotides are stably expressed in said plant, and whereby said plant is herbicide resistant over a broader spectrum of a physiological condition as compared to a plant comprising either said first or second polynucleotide expressed alone and subject to said range of a physiological or environmental condition.

In another aspect of the invention, methods are provided for increasing plant vigor or yield by (a) providing a plant having improved enzymatic properties as described herein; and (b) treating the plant with an effective amount of said herbicide.

In a particular aspect of the invention, a plant having improved enzyme activity can
encode first and second polypeptides that each confer resistance to glyphosate. For example, such a plant can comprise first and second polypeptides that encode EPSP synthase enzymes, e.g., any one of SEQ ID NOs: 2, 4, 6, or 8, or any one of the amino acid sequences depicted in Figures 5A-5P.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is the activity of glyphosate resistant EPSP synthase enzymes derived from Brevundomonas vesicularis (GRG8, SEQ ID NO:2), Arthrobacter globifortnis (GRG23, SEQ ID NO:4), Enterobacteriaceae sp. (GRGl, SEQ ID NO:6) and Sulfolobus solfataricus (GRG20, SEQ ID NO:8) across a broad temperature spectrum. EPSP synthase enzyme activity at each temperature is plotted as a percentage of the maximal activity.

Figure 2 is a graph depicting the activity of glyphosate resistant EPSP synthase enzymes derived from Arthrobacter globifortnis (GRG23, SEQ ID NO:4) and Enterobacteriaceae sp. (GRGl, SEQ ID NO:6) as a function of the concentration of salt, expressed in mM.

Figure 3 shows a graph depicting the rate of product formation as a function of substrate concentration for a plant expressing equal amounts of one or both of two enzymes with complementary properties. The 'El' curve represents the rate of product formation from a plant expressing a said quantity of enzyme 1, where enzyme 1 has a Km for its substrate of 5 µM and a kcat of 25 sec⁻¹. The 'E2' curve represents the rate of product formation from a plant expressing a said quantity of E2, where E2 has a Km for its substrate of 30 µM and a kcat of 50 sec⁻¹. The 'El +E2' curve represents the rate of product formation from a plant expressing a said quantity of both E1 and E2.

Figure 4 is a graph depicting the rate of product formation as a function of inhibitor concentration for a plant expressing equal amounts of one or both of two enzymes with complementary properties. The 'El' curve represents the rate of product formation from a plant expressing a said quantity of enzyme 1, where E1 has a ki of 250 µM, a Km for its substrate of 10µM, and a kcat of 25 sec⁻¹. The 'E2' curve represents the rate of product formation from a plant expressing a said quantity of E2, where E2 has a ki of 50 µM, a Km for its substrate of 10 µM, and a kcat of 50 sec⁻¹. The 'El +E2' curve represents the rate of product formation from a plant expressing a said quantity of both E1 and E2 as a function of the concentration of inhibitor. The concentration of substrate is fixed (in this case, 20 µM).
Figures 5A-5P depict nucleotide and amino acid sequences of the grg8, grg23, grgl, and grg20 sequences described in Example 1.

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides methods of expressing two or more enzymes that perform a same or similar function in a plant, wherein the two or more enzymes have difference kinetic parameters, to achieve optimal enzyme activity across a range of environmental and/or physiological conditions. For example, an increase in activity of certain metabolic enzymes can result in increased or accelerated plant growth and development. Similarly, an increase in activity of an enzyme that confers resistance to herbicides, insects, or disease, enhances plant protective mechanisms to enable plant growth. Plants, plant tissues, and seeds prepared by the disclosed method are also provided.

1. COMPOSITIONS

Compositions of the invention include plants or plant parts comprising a first heterologous polynucleotide encoding a first polypeptide and a second heterologous polynucleotide encoding a second polypeptide that performs a same or similar enzymatic function as the first polypeptide, and wherein expression of the first and second polynucleotides increases enzyme activity of the polypeptides over a broader range of environmental and/or physiological conditions than expression of either polynucleotide alone.

Relevant environmental and/or physiological conditions include any conditions that may result in variable activity of an enzyme of interest in a plant. The descriptors environmental and physiological are not mutually exclusive as changes in the environment can also affect physiological conditions within a plant. In addition, enzymes in different plant organs (e.g., leaves, buds, stems, flowers, fruits, tubers, rhizomes), plant tissues (e.g., dermal, ground, or vascular tissues), and/or plant cellular compartments (e.g., cytoplasm, chloroplast, mitochondria) are subject to different biochemical environments that impact optimum enzyme activity. Representative conditions include temperature, pH, concentration of an enzyme substrate or cofactor, salt concentration, concentration of free radicals or free radical donors, and concentration of an enzyme inhibitor or catalyst. The range of conditions at which each
individual enzyme is active (i.e., a range of temperature or pH) can be exclusive or overlapping. For two or more enzymes which are active in an overlapping range of a environmental and/or physiological condition, the optimal enzyme activity (e.g., the percentage of maximum activity) is different among the individual enzymes such that the combined enzyme activity is increased over a broader range of conditions as compared to the enzyme activity of any one of the individual enzymes.

As used herein, an increase in enzyme activity includes any significant increase of activity or function of the polypeptide of interest, for example, an increase in the inhibition or stimulation of biological or chemical reactions within a cell or organism that can lead to enhanced or diminished metabolic activity, growth, or development. Assays to measure enzyme activity are well-known in the art. An increase in enzyme activity observed in the disclosed multiple-component expression system (i.e., plants expressing two or more polynucleotides encoding polypeptides with a same or similar function but different kinetic properties) can comprise a level of activity that is about 1% or more greater than a level of activity observed with any single component, for example, an increase of 5%, 10%, 15%, 20%, 25%, 30%, 40%, 50%, 80%, 100%, 120%, or greater. Alternatively, an increase in enzyme activity observed in the disclosed multiple-component expression system can comprise a level of activity that is about 2-fold or more greater than a level of activity observed with any single component, for example, about 5-fold or more, or about 10-fold or more, or about 25-fold or more, or about 50-fold or more, or about 100-fold or more, or about 200-fold or more, or about 500-fold or more, or about 1000-fold or more. Enzymes of a multiple component expression system may be referred to as first and second enzymes (or first, second, and third enzymes, etc.). The designations of one enzyme as a "first" enzyme and another enzyme as a "second" enzyme are merely arbitrary, the significance of the two enzymes being the differential enzymatic properties as described herein.

A broader range of environmental and/or physiological conditions, as used to describe conditions in which an enzyme of interest is active according to the disclosed methods, refers to any extension in the range of the condition, either above or below or both, within which an enzyme performs its biological function. For example, expression of two polynucleotides encoding a polypeptide of interest, wherein the first and second polypeptide have different temperature optima, resulting in an increase in enzyme activity of the polypeptide of interest.
over a temperature range that is at least about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65°C or more than when either of the polynucleotides is expressed alone. In non-limiting examples, each polypeptide employed in the methods of the invention can have enzyme activity with a temperature optimum within the temperature range of about 0°C to about 10°C, from about 10°C to about 20°C, from about 20°C to about 30°C, from about 30°C to about 40°C, from about 40°C to about 50°C, from about 50°C to about 60°C, or from about 60°C to about 70°C. Plants expressing the two or more polynucleotides encoding polypeptides with different temperature optima will have an elevated level of enzyme activity based upon the combined activities of the enzymes as the temperature changes throughout the day or season.

For example, a transgenic plant of the invention may comprise a plant expressing a first polypeptide having optimal enzyme activity within a temperature range of about 5°C to about 35°C, or a plant expressing a second polypeptide having optimal enzyme activity at a higher temperature as compared to the first polypeptide and within a temperature range of about 20°C to about 60°C. As another example, a transgenic plant of the invention may comprise a plant expressing a first polypeptide having optimal enzyme activity within a temperature range of about 10°C to about 30°C and a second polypeptide having optimal enzyme activity at a higher temperature as compared to the first polypeptide and within a temperature range of about 25°C to about 50°C. Representative plants having the above-noted features include a plant expressing a first polypeptide having an amino acid sequence of SEQ ID NO:2, and a second polypeptide having an amino acid of SEQ ID NO:4, 6, or 8, and a plant expressing a first polypeptide having an amino acid sequence of SEQ ID NO:4, and a second polypeptide having an amino acid of SEQ ID NO: 6 or 8. See Figure 1.

One non-limiting method of increasing the temperature spectrum within which an enzyme performs its biological function is to introduce into an organism one or more polynucleotides encoding an enzyme of interest derived from psychrophilic and/or thermophilic organisms. Psychrophilic organisms, such as Bacillus globisporus and Shewanella sp., are organisms that thrive at temperatures as low as or lower than 0°C. Thermophilic organisms, such as Bacillus cadiotenax, Thermus thermophilus, and Aquifex aeolicus, thrive at higher temperatures, typically above 120°C. Several metabolic enzymes have been isolated from such organisms (see e.g., U.S. Patent Nos. 6,727,084; 6,902,915; 6,921,641). U.S. Patent No. 6,385,546 further describes a method to increase the thermophilicity (e.g., the ability to function
at or above the optimal temperature for the native polypeptide) or psychrophilicity (e.g., the ability to function at or below the optimal temperature for the native polypeptide) of any enzyme by changing amino acid residues that affect the stability of the polypeptide without modifying or affecting the active or binding sites of the polypeptide. The present invention encompasses such modifications when intended to increase the desired activity of an enzyme by introducing into a plant at least two polynucleotides encoding at least two polypeptides, wherein expression of the at least two polynucleotides increases enzyme activity over a broader spectrum of temperatures than expression of either polynucleotide alone.

To address differential enzyme substrate concentrations within a plant, a two-component expression system according to the invention can comprise a first enzyme with a relatively low Km for substrate, and a relatively low catalytic rate constant ("turnover number"; Kcat), and a second enzyme having a relatively higher Km for substrate and a relatively higher catalytic rate constant as compared to the first enzyme. Optionally, the enzyme inhibition constant for a competitive inhibitor may also vary between the first and second enzymes. Plants that stably express polynucleotides encoding both enzymes will show higher levels of enzyme activity over a broader spectrum of substrate concentration than provided by either enzyme alone.

For example, the second enzyme may have an affinity for substrate that is at least about 2-fold greater than that of the first enzyme, for example, at least about 5-fold greater, or at least about 10-fold greater, or at least about 20-fold greater, or at least about 50-fold greater, or at least about 100-fold greater, or at least about 200-fold greater, or at least about 500-fold greater, or at least about 1000-fold greater, or more. As additional examples, the first enzyme may have a Km for substrate of about 1-100 µM, and the second enzyme may have a higher Km for substrate of about 30-300 µM; or the first enzyme may have a Km for substrate of about 1-100 µM, and the second enzyme may have a higher Km for substrate of about 50-500 µM. As further examples, the first enzyme may have a Km for substrate of about 1-10 µM, and the second enzyme may have a higher Km for substrate of about 5-50 µM; or the first enzyme may have a Km for substrate of about 5-10 µM, and the second enzyme may have a higher Km for substrate of about 10-50 µM; or the first enzyme may have a Km for substrate of about 5-10 µM, and the second enzyme may have a higher Km for substrate of about 15-30 µM. Relevant Km values will depend on the particular enzyme and substrate of interest, and enzyme pairs having the differential Km values as described herein can be readily identified by one of skill in the art.
Similarly, the second enzyme may have a higher catalytic rate that is increased by at least about 2-fold greater than that of the first enzyme, for example, at least about 5-fold greater, or at least about 10-fold greater, or at least about 20-fold greater, or at least about 50-fold greater, or at least about 100-fold greater, or at least about 200-fold greater, or at least about 500-fold greater, or at least about 1000-fold greater, or more. Relevant \( K_{\text{cat}} \) values will depend on the particular enzyme and substrate of interest, and enzyme pairs having the differential \( K_{\text{cat}} \) values as described herein can be readily identified by one of skill in the art.

As a specific example, two or more polynucleotides encoding an EPSP synthase can be used to confer herbicide resistance across a broader range of substrate conditions than either EPSP alone. EPSP synthase is involved in the penultimate step in the shikimic acid pathway for the biosynthesis of aromatic amino acids and many secondary metabolites, including tetrahydrofolate, ubiquinone and vitamin K (Gruys et al. (1999) Inhibitors of Tryptophan, Phenyalanine, and Tyrosine Biosynthesis as Herbicides, Dekker, New York). EPSP synthase converts phosphoenolpyruvic acid (PEP) and 3-phosphoshikimic acid (S3P) to 5-enolpyruvyl-3-phosphoshikimic acid (Amrhein et al. (1980) Plant Physiol. 66:830-834). To achieve optimal EPSP activity, a two-component expression system according to the invention can comprise a first EPSP enzyme with a substantially low \( K_m \) for PEP (\textit{e.g.}, less than about 1 \( \mu \text{M} \)) and a high \( K_i \) for glyphosate (\textit{e.g.}, 200 \( \mu \text{M} \)), and a second EPSP enzyme with a more modest \( K_m \) for PEP (\textit{e.g.}, about 40 \( \mu \text{M} \)) and a higher \( K_i \) for glyphosate (\textit{e.g.}, greater than about 2 \( \text{mM} \)). Plants that stably express polynucleotides encoding both EPSP synthases will show high levels of EPSP synthase activity over a broader spectrum of substrate concentration than provided by either enzyme alone.

For example, expression of two polynucleotides encoding a polypeptide of interest, wherein the first and second polypeptide have different \( K_m \) and \( K_{\text{cat}} \), can result in an increase in enzyme activity of the polypeptide of interest over a range of substrate concentration that is at least about 2-fold greater than when either of the polynucleotides is expressed alone, for example at least about 5-fold greater, or at least 10-fold greater, or at least about 25-fold greater, or at least about 50-fold greater, or at least about 100-fold greater. Similarly, the multiple-component expression system of the invention similarly provides for broadening of an effective range of enzyme activity limited by concentration of a cofactor, concentration of free radicals or free radical donors, concentration of an enzyme inhibitor or catalyst, or maximal catalytic rate.
Enzyme activity can also be regulated by differential pH in plant parts, plant tissues, and plant cellular compartments. In particular H⁺/K⁺ and/or H⁺/Na⁺ pumps maintain a higher pH in the chloroplast stroma (approximately pH 8.0) when compared to the cytoplasm (approximately pH 7.0 - 7.5). Alkalization of the chloroplast stroma is light-induced and allows for efficient function of photosynthetic carbon reduction cycle enzymes. See Wu et al. (1992) Plant Physiol. 98:666-672. For expression of heterologous enzymes in chloroplast, enzymes having optimal activity at elevated pH will be more effective in conferring the trait of interest. Several polypeptides conferring herbicide resistance are encoded in the chloroplast (e.g., the protein conferring atrazine resistance), or are encoded in the nuclear genome but function within chloroplasts (e.g., enol-pyruvylshikimate-phosphate synthase, which confers resistance to glyphosate) or mitochondria (e.g., aryl acylamidase, which confers resistance to propanil). See Della-Cioppa et al. (1986) Proc. Natl. Acad. Sci. USA 83,6873-6877; Daniell et al. (1981) Weed Res. 21, 171-177; Gaynor et al. (1983) Plant Physiol. 72, 80-85. Furthermore, enzymes with varied pH optima are useful in different compartments and may confer superior properties to plants. For example, it is known in the art that the toxicity of glufosinate herbicides is due, at least in part, to inhibition of glutamine synthases (OS's) in both cytoplasm and chloroplasts. Thus, development of plants expressing multiple glufosinate resistant GS’s with differing pH optima (i.e., optimized for the pH environment of cytoplasm and alternatively chloroplast) can confer superior resistance to glufosinate by achieving maximal reaction rate in each cellular compartment. Other enzymes may also benefit from expression in multiple cellular compartments, and thus make use of the invention.

The multi-enzyme expression system of the present invention provides improved enzyme activity over a broader range of temperature as compared to the individual enzymes of the combined expression system. The pH optima of the two or more enzymes of a multi-enzyme expression system can vary as described herein above with respect to fold or percentage differences of an environmental and/or physiological condition. For example, a transgenic plant prepared as described herein may comprise a first polypeptide having optimal enzyme activity within a pH range of about pH 4.0 to about pH 6.5, and a second polypeptide having optimal enzyme activity at a higher pH as compared to the first polypeptide and within the range from about pH 6.0 to about pH 8.5. A representative plant having improved enzyme activity over a broader pH range may comprise a plant expressing a first polypeptide having an amino acid
sequence of SEQ ID NO:4, and a second polypeptide having an amino acid of SEQ ID NO: 6. See Table I.

Additional examples include a plant expressing a first polypeptide having optimal enzyme activity within a pH range of about pH 5.0 to about pH 5.5, and a second polypeptide having optimal enzyme activity at a higher pH as compared to the first polypeptide; a plant expressing a first polypeptide having optimal enzyme activity within a pH range of about pH 5.5 to about pH 6.0, and a second polypeptide having optimal enzyme activity at a higher pH as compared to the first polypeptide; a plant expressing a first polypeptide having optimal enzyme activity within a pH range of about pH 6.0 to about pH 6.5, and a second polypeptide having optimal enzyme activity at a higher pH as compared to the first polypeptide; a plant expressing a first polypeptide having optimal enzyme activity within a pH range of about pH 6.5 to about pH 7.0, and a second polypeptide having optimal enzyme activity at a higher pH as compared to the first polypeptide; a plant expressing a first polypeptide having optimal enzyme activity within a pH range of about pH 7.0 to about pH 7.5, and a second polypeptide having optimal enzyme activity at a higher pH as compared to the first polypeptide; a plant expressing a first polypeptide having optimal enzyme activity within a pH range of about pH 7.5 to about pH 8.0, and a second polypeptide having optimal enzyme activity at a higher pH as compared to the first polypeptide; and a plant expressing a first polypeptide having optimal enzyme activity within a pH range of about pH 8.0 to about pH 8.5, and a second polypeptide having optimal enzyme activity at a higher pH as compared to the first polypeptide.

Enzyme activity can also be strongly affected by salt concentration. It is known in the art that plant cells in different plant tissues are subject to large variations in salt concentration. These conditions can vary with environmental conditions. For example, stoma cells have widely varying intracellular salt levels depending on environmental conditions. Thus, the multi-enzyme expression methods of the invention are useful for generating plants with improved enzyme activity over a broader range of physiological salt concentrations than a single enzyme alone. For example, transgenic plants of the invention may comprise a first polypeptide having optimal enzyme activity within a salt concentration range of about 50 mM to 150 mM, and a said second polypeptide having optimal enzyme activity at a higher salt concentration as compared to the first polypeptide and within a range from about 100 mM to 200 mM. A representative plant having improved enzyme activity over a broader pH range may comprise a plant expressing a
first polypeptide having an amino acid sequence of SEQ ID NO:4, and a second polypeptide having an amino acid of SEQ ID NO: 6. See Table II and Figure 2. Additional examples include plants expressing a first polypeptide and a second polypeptide, wherein the second enzyme has optimal activity at a salt concentration that is at least about 2-fold greater than a salt concentration at which the first enzyme shows optimal activity, for example, at least about 5-fold greater, or at least about 10-fold greater, or at least about 20-fold greater, or at least about 50-fold greater, or at least about 100-fold greater, or at least about 200-fold greater, or at least about 500-fold greater, or at least about 1000-fold greater.

It may also be advantageous to express herbicide resistance genes in both cytoplasm and organelles. For example, rice plants have a robust capability for nitrogen utilization when glutamine synthetase (GS) is expressed in both cytoplasm and chloroplast. See Sun et al. (2005) J. Plant Physiol. Mol. Biol. 31(5): 492-498. GS is the target for the herbicide glufosinate, suggesting that mechanisms for glufosinate tolerance will be more effective if carried out in both subcellular compartments. The methods disclosed in the instant application can be used to optimally express a first and second polynucleotide encoding first and second polypeptides having optimal activities in the different pH and/or salt environments of the chloroplast and cytoplasm, respectively.

The expression of genes conferring traits of interest in chloroplasts, or in chloroplasts as well as cytoplasm, is also useful in other instances, including for example, to improve production of essential amino acids in grains (see e.g., U.S. Patent Nos. 7,026,527 and 7,071,383); to regulate photosynthetic pathways, to regulate synthesis of lipids and plant growth regulatory hormones; to enhance a plant's ability to respond to stress conditions such as ultraviolet AB radiation, extreme temperatures, infection and/or high doses of irradiation; (see e.g., U.S. Patent No. 6,781,034); to modulate carbon allocation and starch synthesis (see e.g., U.S. Patent No. 6,716,474). See also Mullet, J. E. (1988) Ann. Rev. Plant Physiol. Plant Mol Biol. 39:475-502.

The methods of the instant invention can further be combined with methods of targeting proteins to the desired subcellular locations, such as cytoplasm, chloroplasts, and mitochondria. Thus, a heterologous polypeptide, which is optimized as disclosed herein for elevated activity in the alkaline environment of the chloroplast stroma, can further comprise a chloroplast transit peptide, as is known in the art. See e.g., U.S. Patent No. 6,130,366 and disclosure entitled "Polynucleotide Constructs" herein below. Alternatively, the polynucleotides encoding
polypeptides of interest may be stably integrated into an organelle genome. For example, representative techniques for transforming chloroplasts are described in U.S. Patent No. 6,642,053. Polynucleotides expressed in plant organelles may also comprise promoters with specific and/or elevated expression in organelles, for example, as described in U.S. Patent Nos. 4,710,461 and 5,391,725. See also, disclosure entitled "Polynucleotide Constructs" herein below.

LA. TRAITS

Methods and compositions can be used to broaden the range of environmental and/or physiological conditions at which any enzyme of interest is active. Representative desired traits include improved crop yield; insect resistance; tolerance to broad-spectrum herbicides; resistance to diseases caused by viruses, bacteria, fungi, and worms; and enhancement of mechanisms for protection from environmental stresses such as heat, cold, drought, and high salt concentration. Additional desired traits include output traits that benefit consumers, for example, nutritionally enhanced foods that contain more starch or protein, more vitamins, more anti-oxidants, and/or fewer trans-fatty acids; foods with improved taste, increased shelf-life, and better ripening characteristics; trees that make it possible to produce paper with less environmental damage; nicotine-free tobacco; ornamental flowers with new colors, fragrances, and increased longevity; etc. Still further, desirable traits that may be used in accordance with the invention include gene products produced in plants as a means for manufacturing, for example, therapeutic proteins for disease treatment and vaccination; textile fibers; biodegradable plastics; oils for use in paints, detergents, and lubricants; etc. Enzyme activity relevant to any of the above-noted traits, or any other desirable plant trait, can be optimized by selection of multiple polynucleotides having different kinetic properties, as described herein.

In one aspect of the invention, the polynucleotide of interest encodes a polypeptide capable of conferring herbicide resistance, i.e., an ability to tolerate a higher concentration of an herbicide, or to tolerate a certain concentration of an herbicide for a longer period of time than plants that are not tolerant or resistant to the herbicide. Techniques for measuring herbicide resistance activity are well known in the art. See e.g., U.S. Patent Nos. 4,535,060 and 5,188,642, each of which is herein incorporated by reference in their entirety.

Herbicides for which several resistant or tolerant transgenes have been identified include,
but are not limited to, the following:

(a) An herbicide of interest includes one that inhibits the growing point or meristem, such as an imidazolinone or a sulfonylurea. Exemplary polynucleotides that impart herbicide resistance to this class of herbicides include mutant ALS and AHAS enzymes as described, for example, by Lee et al. (1988) EMBO J. 7:1241, and Miki et al. (1990) Theor. Appl. Genet. 80: 449, respectively. See also, U.S. Patent Nos. 5,198,599; 5,605,011; 5,013,659; 5,141,870; 5,767,361; 5,731,180; 5,304,732; 4,761,373; 5,331,107; 5,928,937; and 5,378,824; and PCT International Publication No. WO 96/33270, each of which is incorporated herein by reference.

(b) Additional herbicides of interest include glyphosate (resistance imparted by mutant 5-enolpyruvyl-3-phosphikimate synthase (EPSP synthase) and aroA genes). See e.g., U.S. Patent No. 4,940,835, which discloses the polynucleotide of a form of EPSP synthase which can confer glyphosate resistance. U.S. Patent No. 5,627,061 also describes genes encoding EPSP synthase enzymes. See also U.S. Patent Nos. 6,566,587; 6,338,961; 6,248,876 Bl; 6,040,497; 5,804,425; 5,633,435; 5,145,783; 4,971,908; 5,312,910; 5,188,642; 4,940,835; 5,866,775; 6,225,114 Bl; 6,130,366; 5,310,667; 4,535,060; 4,769,061; 5,633,448; 5,510,471; RE 36,449; RE 37,287 E; and 5,491,288; PCT International Publication No. WO 01/66704; European Patent Application Publication Nos. EP1 173581A and EP1 173580A; and European Patent No. EPI 173582, each of which is incorporated herein by reference. Additional representative EPSP synthase polynucleotides are set forth as SEQ ID NOs: 1, 3, 5, and 7, which encode the EPSP synthase polypeptides of SEQ ID NOs: 2, 4, 6, and 8, respectively, and are also shown in Figures 5A-5P.

Glyphosate resistance is also imparted to plants that express a gene encoding a glyphosate oxidoreductase enzyme as described more fully in U.S. Patent Nos. 5,776,760 and 5,463,175, which are incorporated herein by reference. In addition, glyphosate resistance can be imparted to plants by the over expression of polynucleotides encoding glyphosate N-acetyltransferase. See e.g., U.S. Patent Application Publication Nos. 20040082770 and PCT International Publication No. WO 01/46227. A DNA molecule encoding a mutant aroA gene, which also confers glyphosate resistance, can be obtained under American Type Culture Collection (ATCC) Accession No. 39256, and the polynucleotide of the mutant gene is disclosed in U.S. Patent No. 4,769,061.

EPSP synthases have been isolated from plants, bacteria and fungi, including E. coli (Duncan et al. (1984) FEBS Lett. 170:59-63), Staphylococcus aureus (Horsburgh et al. (1996)

EPSP synthase is the target of the herbicide glyphosate, i.e., any herbicidal form of N-phosphonomethylglycine (including any salt thereof) and active derivatives thereof that result in the production of the glyphosate anion in planta. Inhibition of EPSP synthase by glyphosate has been shown to proceed through the formation of an EPSP synthase-S3P-glyphosate ternary complex and the binding is ordered with glyphosate binding to the enzyme only after the formation of a binary EPSP synthase-S3P complex. Binding of glyphosate to EPSP synthase has been shown to be competitive with PEP and uncompetitive with respect to S3P (Kishore et al. (1988) Ann. Rev. Biochem. 57:627-663). By binding to EPSP synthase, glyphosate shuts down the shikimic acid pathway, thereby leading to a depletion of aromatic amino acid biosynthesis and death or severe growth reduction of the plant.

Glyphosate-resistant EPSP synthase polypeptides have been identified and used to increase glyphosate tolerance in plants. Glyphosate resistance polypeptides confer upon a cell an ability to tolerate a higher concentration of glyphosate than cells that do not express the polypeptide, or to tolerate a certain concentration of glyphosate for a longer time than cells that do not express the polypeptide. Tolerance refers to an ability to survive, or to carry out essential cellular functions such as protein synthesis and respiration in a manner that is not readily discernable from untreated cells. An example of a naturally-occurring glyphosate-resistant EPSP synthase includes the bacterial gene from Agrobacterium tumefaciens strain CP4 which has been used to confer herbicide resistance on plant cells following expression in plants. Mutated EPSP synthase polypeptides have been identified through random mutagenesis and selection for herbicide resistance, including a mutated EPSP synthase from Salmonella typhimurium strain CT7 that confers glyphosate resistance in bacterial cells, and confers glyphosate resistance on
plant cells (U.S. Patent Nos. 4,535,060; 4,769,061; and 5,094,945 and U.S. Appl.
Nos. 60/669,686 and 20040177399). These enzymes contain amino acid substitutions in their active
sites that prevent the binding of glyphosate without affecting binding by PEP or S3P. Mutations
that occur in the hinge region between the two globular domains of EPSP synthase have been
shown to alter the binding affinity of glyphosate but not PEP (He et al. (2003) Biosci.
Biotechnol. Biochem. 67(6):1405-1409). Therefore, such enzymes have high catalytic activity,
even in the presence of glyphosate.

In one aspect of the invention, the present invention provides transgenic plants having
two or more polypeptides conferring glyphosate resistance, for example, two or more glyphosate
resistant EPSP synthase polypeptides, wherein the plants have an increased resistance to
glyphosate and/or an increased yield over a broader range of environmental and/or physiological
conditions than when only one of the polypeptides is expressed in the plant. For example, plants
expressing two or more EPSP synthase polypeptides have a broader temperature spectrum of
resistance to glyphosate or increased yield of about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15,
20, or 25, 35, 45, 55, 65°C or greater than when either EPSP synthase polypeptides are expressed
alone. Also provided are plants expressing two or more EPSP synthase polypeptides, wherein at
least one of the two EPSP synthase polypeptides is active between about 0°C to about 10°C,
about 10°C to about 20°C, about 20°C to about 30°C, about 30°C to about 40°C, about 40°C to
about 50°C, about 50°C to about 60°C, or about 60°C to about 70°C.

A variety of techniques can be used to assay EPSP synthase activity. For example,
Lewendon et al. (1983) Biochem J. 213:187-91 describes two assays which couple the EPSP
synthase reaction with other enzymes which produced detectable products. In the forward
direction, EPSP synthase can be coupled with chorismate synthase, the enzyme in the shikimate
acid pathway which converts EPSP to chorismate; as EPSP synthase produces EPSP, chorismate
synthase can convert EPSP to chorismate which can be detected at 275 nm. Since EPSP
synthase can also proceed in the reverse direction, activity can also be assayed with coupling to
pyruvate kinase and lactate dehydrogenase which oxidize NADH in the breakdown of pyruvate,
allowing the detection of NADH loss at 340 nm which corresponds to pyruvate evolution by
EPSP synthase. EPSP synthase activity can also be assayed by measuring an increase in
resistance of a plant to glyphosate when glyphosate-resistant EPSP synthase is present, or by
measuring an increase in plant yield when glyphosate-sensitive and/or -tolerant EPSP synthase is
expressed.

An increase in activity of glyphosate-sensitive or glyphosate-tolerant EPSP synthase across a broader range of temperatures (such as those that span night and day in many climates, and those that extend beyond typical growing seasons in some climates) will result in increased production of metabolic components necessary for growth and development and, hence, improve plant yield. EPSP synthase sequences derived from plants (both monocots and dicots) and other microorganisms (such as bacteria, fungi or yeast) are also included in this invention. For example, glyphosate-tolerant EPSP synthase enzymes derived from *Brevundomonas vesicularis*, *Arthrobacter globiformis*, *Enterobacteriaceae sp.*, and *Sulfolobus solfataricus* are enzymatically active at varying temperatures. Representative glyphosate resistant plants of the invention include plants expressing glyphosate-tolerant EPSP synthase polypeptides derived from at least *Brevundomonas vesicularis* and *Arthrobacter globiformis; Brevundomonas vesicularis* and *Enterobacteriaceae sp.; Brevundomonas vesicularis* and *Sulfolobus solfataricus; Arthrobacter globiformis* and *Enterobacteriaceae sp.; Arthrobacter globiformis* and *Sulfolobus solfataricus;* or *Enterobacteriaceae sp.* and *Sulfolobus solfataricus*. Additional plants of the invention express glyphosate-tolerant EPSP synthase polypeptides derived from at least *Brevundomonas vesicularis, Arthrobacter globiformis, and Enterobacteriaceae sp.; Brevundomonas vesicularis, Enterobacteriaceae sp.* and *Sulfolobus solfataricus; Arthrobacter globiformis, Enterobacteriaceae sp., and Sulfolobus solfataricus;* or *Brevundomonas vesicularis, Arthrobacter globiformis, and Sulfolobus solfataricus*. Still additional plants of the invention express glyphosate tolerant EPSP synthase polypeptides derived from at least *Brevundomonas vesicularis, Arthrobacter globiformis, Enterobacteriaceae sp., and Sulfolobus solfataricus*. It is contemplated that other glyphosate-tolerant EPSP synthase polypeptides can be used in the present invention in addition to those described above, or in any combination with any number of the polypeptides described above such that EPSP synthase is active across a broader range of environmental and/or physiological conditions than expression of either EPSP synthase polypeptide alone.

As discussed in further detail herein below, variants of any known EPSP synthase enzymes or those disclosed herein can be employed in the methods and compositions of the invention. Functional variants of EPSP synthase which are tolerant to glyphosate are known. See e.g., Kishore and Shah (1988) *Ann. Rev. Biochem.* 57:627-63; Wang et al. (2003) *J. Plant*
An herbicide that inhibits photosynthesis, such as a triazine (psbA and gs+genes) and a benzonitrile (nitrilase gene) is also of interest. Przibilla et al. (1991) *Plant Cell* 3:169 describes the transformation of Chlamydomonas with plasmids encoding mutant psbA genes. Polynucleotides for nitrilase genes are disclosed in U.S. Patent No. 4,810,648 and DNA molecules containing these genes are available under ATCC Accession Nos. 53435, 67441, and 67442. Cloning and expression of DNA coding for a glutathione S-transferase is described by Hayes et al. (1992) *Biochem. J.* 285:173.

(d) Acetohydroxy acid synthase, which has been found to make plants that express this enzyme resistant to multiple types of herbicides, has been introduced into a variety of plants (see e.g., Hattori et al. (1995) *Mol. Gen. Genet.* 246:419). Other genes that confer resistance to herbicides include: a gene encoding a chimeric polypeptide of rat cytochrome P4507A1 and yeast NADPH-cytochrome P450 oxidoreductase (Shiota et al. (1994) *Plant Physiol.* 106:17), genes for glutathione reductase and superoxide dismutase (Aono et al. (1995) *Plant Cell Physiol.* 36:1687), and genes for various phosphotransferases (Datta et al. (1992) *Plant Mol. Biol.* 20:619).

(e) Protoporphyrinogen oxidase (protox) is necessary for the production of chlorophyll, which is necessary for all plant survival. The protox enzyme serves as the target for a variety of herbicidal compounds. These herbicides also inhibit growth of all the different species of plants present, causing their total destruction. The development of plants containing altered protox activity which are resistant to these herbicides are described in U.S. Patent Nos. 6,084,155; 6,288,306; 6,282,837; and 5,767,373; and PCT International Publication No. WO 01/12825.

(f) Additional herbicides of interest include phosphono compounds such as glufosinate (resistance provided by phosphinothricin acetyl transferase (PAT) and *Streptomyces hygroscopicus* phosphinothricin acetyl transferase (bar) genes). European Patent Application Publication No. 0333033A and U.S. Patent No. 4,975,374 disclose polynucleotides of glutamine synthetase genes which confer resistance to herbicides such as L-phosphinothricin. The polynucleotide of a phosphinothricin acetyl transferase gene is provided in European Patent Nos. 0242246 and 0242236. De Greef et al. (1989) *BioTechnology* 7: 61 describe the production of transgenic plants that express chimeric bar genes coding for phosphinothricin acetyl transferase activity. See also U.S. Patent Nos. 5,969,213; 5,489,520; 5,550,318; 5,874,265; 5,919,675;
5,561,236; 5,648,477; 5,646,024; 6,177,616; and 5,879,903, which are incorporated herein by reference.

(e) Additional herbicides are pyridinoxy or phenoxy propionic acids and cyclohexones (resistance conferred by ACCase inhibitor-encoding genes). Exemplary polynucleotides conferring resistance to phenoxy propionic acids and cyclohexones, such as sethoxydim and haloxyfop, are the AceI-S1, Accl-S2 and AceI-S3 genes described by Marshall et al. (1992) *Theor. Appl. Genet.* 83: 435.

L.B. Polynucleotide and Polypeptide Variants

The present invention further contemplates variants and fragments of the herbicide resistance polynucleotides and polypeptides described herein. Various methods can be employed to modify the various polypeptides which confer resistance to an herbicide such that the new polypeptide will have the desired activity at a different temperature optimum. For example, gene shuffling or sexual PCR procedures (for example, Smith (1994) *Nature* 370:324-25; U.S. Patent Nos. 5,837,458; 5,830,721; 5,811,238; and 5,733,731, each of which is herein incorporated by reference) can be used to identify additional polynucleotides that encode polypeptides that perform similar functions as those described herein (for example, polypeptides that confer herbicide resistance at varying temperature optima). Gene shuffling involves random fragmentation of several mutant DNAs followed by their reassembly by PCR into full length molecules. Examples of various gene shuffling procedures include, but are not limited to, assembly following DNase treatment, the staggered extension process (STEP), and random priming *in vitro* recombination. In the DNase mediated method, DNA segments isolated from a pool of positive mutants are cleaved into random fragments with DNaseI and subjected to multiple rounds of PCR with no added primer. The lengths of random fragments approach that of the uncleaved segment as the PCR cycles proceed, resulting in mutations in different clones becoming mixed and accumulating in some of the resulting sequences. Multiple cycles of selection and shuffling have led to the functional enhancement of several enzymes (Stemmer (1994) *Nature* 370:398-91; Stemmer (1994) *Proc. Natl Acad. Sci. USA* 91:10747-51; Crameri et al. (1996) *Nat. Biotechnol.* 14:315-19; Zhang et al. (1997) *Proc. Natl. Acad. Sci. USA* 94:4504-09; and Crameri et al. (1997) *Nat. Biotechnol.* 15:436-38). Such procedures could be performed, for example, on polynucleotides derived from cryophilic and/or thermophilic
organisms to generate polypeptides that are active at lower and higher temperature ranges, as well as those that confer a desired enzyme activity (i.e., herbicide resistance).

Fragments or biologically active portions include polypeptide fragments comprising a portion of an amino acid sequence encoding a polypeptide and that retains biological activity (i.e., herbicide resistance or EPSP synthase activity such as increased yield and/or resistance to glyphosate). A fragment of a polynucleotide may encode a biologically active portion of a polypeptide, or it may be a fragment that can be used as a hybridization probe or PCR primer using methods disclosed elsewhere herein. Polynucleotides that are fragments of a polynucleotide comprise at least about 15, 20, 50, 75, 100, 200, 300, 350, 400, 450, 500, 550, 600, 650, 700, 750, 800, 850, 900, 950, 1000, 1050, 1100, 1150, 1200, 1250, 1300, 1350, 1400, 1450, 1500, 1550, 1600, 1650, 1700, 1750, 1800, 1850, 1900, 1950 contiguous nucleotides, or up to the number of nucleotides present in a full-length polynucleotide disclosed herein depending upon the intended use. Contiguous nucleotides are immediately adjacent to one another.

Fragments of a polynucleotide can encode polypeptide fragments that retain the biological activity of the full-length polypeptide (e.g., herbicide resistance or EPSP synthase activity such as increased yield and/or resistance to glyphosate). For example, a fragment retains a biological activity of the full-length polypeptide if it has at least about 30%, at least about 50%, at least about 70%, or at least about 80% of the activity of the full-length polypeptide.

A fragment of a polynucleotide that encodes a biologically active portion of a polypeptide of the invention can encode at least about 15, 25, 30, 50, 75, 100, 125, 150, 175, 200, 250, 300, 350, 400 contiguous amino acids, or up to the total number of amino acids present in a full-length polypeptide of the invention.

The invention also encompasses variant polynucleotides, including naturally occurring variants as well as recombinantly produced variants. For example, variants of the EPSP synthase polypeptides disclosed herein include polypeptides that differ conservatively because of the degeneracy of the genetic code, as well as those that are sufficiently identical. Sufficiently identical polypeptides refer to polypeptides having an amino acid sequence that has at least about 60% or 65% sequence identity, about 70% or 75% sequence identity, about 80% or 85% sequence identity, about 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% sequence identity compared to a reference sequence using one of the alignment programs using standard
parameters. One of skill in the art recognizes that these values can be appropriately adjusted to
determine corresponding identity of polypeptides encoded by two polynucleotides by taking into
account codon degeneracy, amino acid similarity, reading frame positioning, and the like. Amino acid substitutions that are made to increase the thermophilicity, psychrophilicity, or thermostability of an enzyme are also encompassed by the present invention.

To determine the percent identity of two amino acid sequences or of two polynucleotides, the sequences are aligned for optimal comparison purposes. The percent identity between the two sequences is a function of the number of identical positions shared by the sequences (i.e., percent identity = number of identical positions/total number of positions (e.g., overlapping positions) x 100). The two sequences may be the same length. The percent identity between two sequences can be determined using techniques similar to those described below, with or without allowing gaps. In calculating percent identity, typically exact matches are counted.

The determination of percent identity between two sequences can be accomplished using a mathematical algorithm. A nonlimiting example of a mathematical algorithm utilized for the comparison of two sequences is the algorithm of Karlin and Altschul (1990) Proc. Natl. Acad. ScL USA 87:2264, modified as in Karlin and Altschul (1993) Proc. Natl. Acad. ScL USA 90:5873-5877. Such an algorithm is incorporated into the BLASTN and BLASTX programs of Altschul et al. (1990) J. Mol. Biol. 215:403. BLAST nucleotide searches can be performed with the BLASTN program, score = 100, wordlength = 12, to obtain polynucleotides homologous to herbicide resistance-encoding polynucleotides used in methods of the invention. BLAST polypeptide searches can be performed with the BLASTX program, score = 50, wordlength = 3, to obtain amino acid sequences homologous to polypeptide molecules expressed using the methods of the invention. To obtain gapped alignments for comparison purposes, Gapped BLAST can be utilized as described in Altschul et al. (1997) Nucleic Acids Res. 25:3389. Alternatively, PSI-Blast can be used to perform an iterated search that detects distant relationships between molecules. See Altschul et al. (1997) supra. When utilizing BLAST, Gapped BLAST, and PSI-Blast programs, the default parameters of the respective programs (e.g., BLASTX and BLASTN) can be used. See www.ncbi.nlm.nih.gov. Another non-limiting example of a mathematical algorithm utilized for the comparison of sequences is the ClustalW algorithm (Higgins et al. (1994). Nucleic Acids Res. 22:4673-4680). ClustalW compares sequences and aligns the entirety of the amino acid or DNA sequence, and thus can provide data
about the sequence conservation of the entire amino acid sequence. The ClustalW algorithm is
used in several commercially available DNA/amino acid analysis software packages, such as the
ALIGNX module of the vector NTi Program Suite (Informax, Inc). After alignment of amino
acid sequences with ClustalW, the percent amino acid identity can be assessed. A non-limiting
example of a software program useful for analysis of ClustalW alignments is GENEDOC™.
GENEDOC™ (Karl Nicholas) allows assessment of amino acid (or DNA) similarity and identity
between multiple polypeptides. Another non-limiting example of a mathematical algorithm
utilized for the comparison of sequences is the algorithm of Myers and Miller (1988) CABIOS
4:1 1-17. Such an algorithm is incorporated into the ALIGN program (version 2.0), which is part
of the GCG sequence alignment software package. When utilizing the ALIGN program for
comparing amino acid sequences, a PAM120 weight residue table, a gap length penalty of 12,
and a gap penalty of 4 can be used.

Unless otherwise stated, GAP Version 10, which uses the algorithm of Needleman and
Wunsch (1970) supra, will be used to determine sequence identity or similarity using the
following parameters: % identity and % similarity for a nucleotide sequence using GAP Weight
of 50 and Length Weight of 3, and the nwsgapdna.cmp scoring matrix; % identity or % similarity
for an amino acid sequence using GAP weight of 8 and length weight of 2, and the BLOSUM62
scoring program. Equivalent programs may also be used, including any sequence comparison
program that, for any two sequences in question, generates an alignment having identical
nucleotide residue matches and an identical percent sequence identity when compared to the
corresponding alignment generated by GAP Version 10.

Naturally occurring polynucleotide variants can be identified using well-known
molecular biology techniques, such as polymerase chain reaction (PCR) and hybridization
techniques as outlined below. Variant polynucleotides also include synthetically derived
diversity polynucleotides that have been generated, for example, by using site-directed mutagenesis but
which still encode the polypeptide having the desired biological activity. Variant polypeptides
encompassed by the present invention are biologically active, that is they retain the desired
biological activity of the native polypeptide, i.e., herbicide resistance activity or EPSP synthase
activity such as increased yield and/or resistance glyphosate. Biologically active variants have at
least about 30% of the activity of the native polypeptide, for example, at least about 50%, or at
least about 70%, or at least about 80%, or at least about 90%, or at least about 95%. Methods for
measuring enzyme activity are specific to individual enzymes of interest.

The skilled artisan further appreciates that changes can be introduced by mutation into the polynucleotides of the invention thereby leading to changes in the amino acid sequence of the encoded polypeptides, without altering the biological activity of the polypeptides. Thus, variant isolated polynucleotides can be created by introducing one or more nucleotide substitutions, additions, or deletions into the corresponding polynucleotide disclosed herein, such that one or more amino acid substitutions, additions or deletions are introduced into the encoded polypeptide. Mutations can be introduced by standard techniques, such as site-directed mutagenesis and PCR-mediated mutagenesis, or shuffling techniques, which are described in further detail elsewhere herein. Such variant polynucleotides are also encompassed by the present invention.

For example, conservative amino acid substitutions may be made at one or more nonessential amino acid residues, i.e., a residue that can be altered from the native sequence of a polypeptide without altering the biological activity. In contrast, an essential amino acid residue is required for biological activity. A conservative amino acid substitution is one in which the amino acid residue is replaced with an amino acid residue having a similar side chain. Families of amino acid residues having similar side chains have been defined in the art. These families include amino acids with basic side chains (e.g., lysine, arginine, histidine), acidic side chains (e.g., aspartic acid, glutamic acid), uncharged polar side chains (e.g., glycine, asparagine, glutamine, serine, threonine, tyrosine, cysteine), nonpolar side chains (e.g., alanine, valine, leucine, isoleucine, proline, phenylalanine, methionine, tryptophan), beta-branched side chains (e.g., threonine, valine, isoleucine) and aromatic side chains (e.g., tyrosine, phenylalanine, tryptophan, histidine). Amino acid substitutions may be made in nonconserved regions that retain function. In general, such substitutions would not be made for conserved amino acid residues, or for amino acid residues residing within a conserved motif, where such residues are essential for polypeptide activity. However, one of skill in the art would understand that functional variants may have minor conserved or nonconserved alterations in the conserved residues.

Alternatively, variant polynucleotides can be made by introducing mutations randomly along all or part of the coding sequence, such as by saturation mutagenesis, and the resultant mutants can be screened for the ability to confer herbicide resistance activity to identify mutants
that retain activity. Following mutagenesis, the encoded polypeptide can be expressed recombinantly, and the activity of the polypeptide can be determined using standard assay techniques.

LC. Expression Constructs

The polynucleotides employed in the methods and compositions of the invention may be modified to obtain or enhance expression in plant cells. The polynucleotides of the invention may be provided in expression cassettes for expression in the plant of interest. Plant expression cassettes include a DNA construct that is capable of resulting in the expression of a polynucleotide in a plant cell. The cassette can include in the 5'-3' direction of transcription, a transcriptional initiation region (i.e., promoter) operably linked to one or more polynucleotides of interest, and a translation and transcriptional termination region (i.e., termination region) functional in plants. The cassette may additionally contain at least one additional polynucleotide to be introduced into the organism, such as a selectable marker gene or the second polynucleotide of interest with different temperature optima for activity. Alternatively, the additional polynucleotide(s) can be provided on multiple expression cassettes. Such an expression cassette is provided with a plurality of restriction sites for insertion of the polynucleotide(s) to be under the transcriptional regulation of the regulatory regions.

A heterologous polynucleotide or polypeptide is one that is not endogenous to the cell or is not endogenous to the location in the native genome in which it is present, and has been added to the cell by infection, transfection, microinjection, electroporation, microprojection, or the like. For expression of a heterologous polynucleotide, such polynucleotide is operably linked to a promoter sequence that initiates and mediates its transcription. It is recognized that operably linked polynucleotides may or may not be contiguous. Where used to reference the joining of two polypeptide coding regions, operably linked polypeptides are expressed in the same reading frame.

The promoter may be any polynucleotide sequence which shows transcriptional activity in the chosen plant cells, plant parts, or plants. The promoter may be native or analogous, or foreign or heterologous, to the plant host and/or to the DNA sequence of the invention. Where the promoter is native or endogenous to the plant host, it is intended that the promoter is found in the native plant into which the promoter is introduced. Where the promoter is foreign or
heterologous to the DNA sequence of the invention, the promoter is not the native or naturally occurring promoter for the operably linked DNA sequence of the invention. The promoter may be inducible or constitutive. It may be naturally-occurring, may be composed of portions of various naturally-occurring promoters, or may be partially or totally synthetic. Guidance for the design of promoters is provided by studies of promoter structure, such as that of Harley and Reynolds (1987) *Nucleic Acids Res.* 15:2343-61. Also, the location of the promoter relative to the transcription start may be optimized. See e.g., Roberts, et al. (1979) *Proc. Natl. Acad. Sci. USA*, 76:760-4. Many suitable promoters for use in plants are well known in the art.


The promoter may include, or be modified to include, one or more enhancer elements. Promoters containing enhancer elements provide for higher levels of transcription as compared to promoters that do not include them. Suitable enhancer elements for use in plants include the PCISV enhancer element (U.S. Patent No. 5,850,019), the CaMV 35S enhancer element (U.S. Patent Nos. 5,106,739 and 5,164,316) and the FMV enhancer element (Maiti et al. (1997) *Transgenic Res.* 6:143-156). *See also* PCT International Publication No. WO 96/23898.

Often, such constructs can contain 5' and 3' untranslated regions. Such constructs may contain a 'signal sequence' or 'leader sequence' to facilitate co-translational or post-translational transport of the peptide of interest to certain intracellular structures such as the chloroplast (or other plastid), endoplasmic reticulum, or Golgi apparatus, or to be secreted. For example, the construct can be engineered to contain a signal peptide to facilitate transfer of the peptide to the endoplasmic reticulum. A signal sequence is known or suspected to result in cotranslational or post-translational peptide transport across the cell membrane. In eukaryotes, this typically involves secretion into the Golgi apparatus, with some resulting glycosylation. A leader sequence refers to any sequence that, when translated, results in an amino acid sequence sufficient to trigger co-translational transport of the peptide chain to a sub-cellular organelle. Thus, this includes leader sequences targeting transport and/or glycosylation by passage into the endoplasmic reticulum, passage to vacuoles, plastids including chloroplasts, mitochondria, and the like. Plant expression cassettes may also contain an intron, such that mRNA processing of the intron is required for expression.

A 3' untranslated region is a polynucleotide located downstream of a coding sequence. Polyadenylation signal sequences and other sequences encoding regulatory signals capable of affecting the addition of polyadenylic acid tracts to the 3' end of the mRNA precursor are 3' untranslated regions. A 5' untranslated region is a polynucleotide located upstream of a coding sequence.

Other upstream or downstream untranslated elements include enhancers. Enhancers are
polynucleotides that act to increase the expression of a promoter region. Enhancers are well known in the art and include, but are not limited to, the SV40 enhancer region and the 35S enhancer element.

The termination region may be native with the transcriptional initiation region, may be native with the sequence of the present invention, or may be derived from another source. Convenient termination regions are available from the Ti-plasmid of A. tumefaciens, such as the octopine synthase and nopaline synthase termination regions. See also Guerineau et al. (1991) Mol. Gen. Genet. 262:141-144; Proudfoot (1991) Cell 64:671-674; Sanfacon et al. (1991) Genes Dev. 5:141-149; Mogen et al. (1990) Plant Cell 2:1261-1272; Munroe et al. (1990) Gene 91:151-158; Ballas et al. (1989) Nucleic Acids Res. 17:7891-7903; and Joshi et al. (1987) Nucleic Acid Res. 15:9627-9639.

Where appropriate, the polynucleotide(s) may be optimized for increased expression in the transformed host cell. That is, the sequences can be synthesized using host cell-preferred codons for improved expression, or may be synthesized using codons at a host-preferred codon usage frequency. Generally, the GC content of the polynucleotide will be increased. See e.g., Campbell and Gowri (1990) Plant Physiol. 92:1-11 for a discussion of host-preferred codon usage. Methods are known in the art for synthesizing host-preferred polynucleotides. See e.g., U.S. Patent Nos. 6,320,100; 6,075,185; 5,380,831; and 5,436,391, U.S. Published Application Nos. 20040005600 and 20010003849, and Murray et al. (1989) Nucleic Acids Res. 17:477-498, herein incorporated by reference.

In one aspect of the invention, polynucleotides of interest are targeted to the chloroplast for expression. In this manner, where the polynucleotide of interest is not directly inserted into the chloroplast, the expression cassette may additionally contain a polynucleotide encoding a transit peptide to direct the nucleotide of interest to the chloroplasts. Such transit peptides are known in the art. See e.g., Von Heijne et al. (1991) Plant Mol. Biol. Rep. 9:104-126; Clark et al. (1989) J. Biol. Chem. 264:17544-17550; Della-Cioppa et al. (1987) Plant Physiol. 84:965-968; Romer et al. (1993) Biochem. Biophys. Res. Commun. 196:1414-1421; and Shah et al. (1986) Science 233:478-481.

The polynucleotides of interest to be targeted to the chloroplast may be optimized for expression in the chloroplast to account for differences in codon usage between the plant nucleus and this organelle. In this manner, the polynucleotides of interest may be synthesized using
chloroplast-preferred codons. See e.g., U.S. Patent No. 5,380,831, herein incorporated by reference.

This plant expression cassette can be inserted into a plant transformation vector, which allows for the transformation of DNA into a cell. Such a molecule may consist of one or more expression cassettes, and may be organized into more than one vector DNA molecule. For example, binary vectors are plant transformation vectors that utilize two non-contiguous DNA vectors to encode all requisite cis- and trans-acting functions for transformation of plant cells (Hellens and Mullineaux (2000) Trends in Plant Science 5:446-451). Vectors are polynucleotide constructs designed for transfer between different host cells. Expression vectors are a type of vector having an ability to incorporate, integrate and express heterologous DNA sequences or fragments in a foreign cell.

The plant transformation vector comprises one or more DNA vectors for achieving plant transformation. For example, it is a common practice in the art to utilize plant transformation vectors that comprise more than one contiguous DNA segment. These vectors are often referred to in the art as binary vectors. Binary vectors as well as vectors with helper plasmids are most often used for Agrobacterium-mQdiatQd transformation, where the size and complexity of DNA segments needed to achieve efficient transformation is quite large, and it is advantageous to separate functions onto separate DNA molecules. Binary vectors typically contain a plasmid vector that contains the cis-acting sequences required for T-DNA transfer (such as left border and right border), a selectable marker that is engineered to be capable of expression in a plant cell, and a polynucleotide of interest (i.e., a polynucleotide engineered to be capable of expression in a plant cell for which generation of transgenic plants is desired). Also present on this plasmid vector are sequences required for bacterial replication. The cis-acting sequences are arranged in a fashion to allow efficient transfer into plant cells and expression therein. For example, the selectable marker sequence and the sequence of interest are located between the left and right borders. Often a second plasmid vector contains the trans-acting factors that mediate T-DNA transfer from Agrobacterium to plant cells. This plasmid often contains the virulence functions (Vir genes) that allow infection of plant cells by Agrobacterium, and transfer of DNA by cleavage at border sequences and vir-mediated DNA transfer, as in understood in the art (Hellens and Mullineaux (2000) Trends in Plant Science, 5:446-451). Several types of Agrobacterium strains (e.g., LBA4404, GV3101, EHA101, EHA105, etc.) can be used for plant
transformation. The second plasmid vector is not necessary for introduction of polynucleotides into plants by other methods such as microprojection, microinjection, electroporation, polyethylene glycol, etc.

The present invention further provides transgenic plants comprising more than one of the polynucleotides of the invention to elevate the activity of the desired enzyme over a broad temperature spectrum. One of skill in the art recognizes that several different strategies can be utilized to generate such a plant or plant part including, but not limited to the following:

Fused enzyme. In this aspect of the invention, the first polynucleotide is positioned in the plant vector directly downstream of at least the second polynucleotide under the direction of a single promoter. Alternatively, the first and at least the second polynucleotides can be separated and put under the direction of different promoters within a single construct.

Two or more polynucleotides, one construct. In this expression vector, both the first and at least the second polynucleotides are placed under the control of separate promoters, in a single plasmid construct. This enables the expression of each polynucleotide as a separate entity; however, the tandem would behave in the plant progeny as a single locus.

Two or more polynucleotides, one promoter. The maize streak virus promoter is a bi-functional promoter able to express genes in two directions. Using this promoter, transcription can be initiated on opposite strands in the vector and in opposite directions. Therefore, each polynucleotide can be expressed from a single promoter.

Two or more polynucleotides, two or more constructs. In another approach, two or more separate vector constructs are made, each containing one of the polynucleotides under the direction of different promoters. This approach requires that the plant be doubly transformed.

Cells modified according to the present invention are contemplated at each stage of the invention. This invention further contemplates the introduction of at least one polynucleotide whose enzyme activity is optimal within a temperature range that extends beyond that of the native enzyme and thereby broadens the temperature range in which the enzyme is active in the cell.

Host cells are useful for making, storing, reproducing or manipulating polynucleotide constructs of the invention. Contemplated host cells are eukaryotic cells, such as yeast or plant cells. Prokaryotic host cells containing constructs and/or vectors according to the invention are also contemplated (i.e., E. coli).
LD. Plants and Plant Parts

As used herein, a plant refers to a whole plant, a plant organ (e.g., leaves, stems, roots, etc.), a seed, a plant cell, a propagule, an embryo, and progeny of the same. Plant cells can be differentiated or undifferentiated (e.g., callus, suspension culture cells, protoplasts, leaf cells, root cells, phloem cells, pollen). The present invention may be used for introduction of polynucleotides into any plant species, including, but not limited to, monocots and dicots. Examples of plants of interest include, but are not limited to, corn (maize), sorghum, wheat, sunflower, tomato, crucifers, peppers, potato, cotton, rice, soybean, sugarbeet, sugarcane, tobacco, barley, and oilseed rape, Brassica sp., alfalfa, rye, millet, safflower, peanuts, sweet potato, cassava, coffee, coconut, pineapple, citrus trees, cocoa, tea, banana, avocado, fig, guava, mango, olive, papaya, cashew, macadamia, almond, oats, vegetables, ornamentals, and conifers.

Vegetables include, but are not limited to, tomatoes, lettuce, green beans, lima beans, peas, and members of the genus Curcumis such as cucumber, cantaloupe, and musk melon. Ornamentals include, but are not limited to, azalea, hydrangea, hibiscus, roses, tulips, daffodils, petunias, carnation, poinsettia, and chrysanthemum. Crop plants are also of interest, including, for example, maize, sorghum, wheat, sunflower, tomato, crucifers, peppers, potato, cotton, rice, soybean, sugarbeet, sugarcane, tobacco, barley, oilseed rape, etc.

This invention is suitable for any member of the monocot plant family including, but not limited to, maize, rice, barley, oats, wheat, sorghum, rye, sugarcane, pineapple, yams, onion, banana, coconut, and dates.

II. METHODS

The present invention provides methods of conferring a trait of interest to a plant by introducing into a plant at least (a) a first heterologous polynucleotide encoding a first polypeptide capable of conferring said trait of interest, and (b) a second heterologous polynucleotide encoding a second polypeptide capable of conferring said trait of interest, wherein said first and second polynucleotides are stably expressed in said plant, and wherein said plant shows said trait of interest over a broader spectrum of a physiological or environmental condition as compared to a plant comprising either said first or second polynucleotide expressed alone and subject to said spectrum of a physiological or environmental condition. In other
aspects of the invention, a second heterologous polynucleotide is introduced into a plant that already harbors a first heterologous polynucleotide. Accordingly, a method of conferring a trait of interest can also comprise (a) providing a transgenic plant comprising a first heterologous polynucleotide encoding a first polypeptide capable of conferring said trait of interest, and (b) introducing into said plant at least a second heterologous polynucleotide encoding a second polypeptide capable of conferring said trait of interest, wherein said first and second polynucleotides are stably expressed in said plant, and wherein said plant shows said trait of interest over a broader spectrum of a physiological or environmental condition as compared to a plant comprising either said first or second polynucleotide expressed alone and subject to said spectrum of a physiological or environmental condition. Relevant traits of interest are described herein above, including for example, herbicide resistance and plant yield.

In one such method, at least two polynucleotides encoding polypeptides that confer resistance to an herbicide of interest are introduced into a plant, wherein expression of the at least two polynucleotides increases enzyme activity over a broader spectrum of temperatures than expression of either polynucleotide alone. As discussed elsewhere herein, various polynucleotides can be employed in this method including, but not limited to, EPSP synthase sequences that can confer resistance to glyphosate.

The disclosed methods can also be used to improve plant yield, i.e., the quality and/or quantity of biomass produced by the plant. Biomass is a measurable amount or weight of a plant product. An increase in biomass production is any improvement in the yield of the measured plant product. Increasing plant yield has several commercial applications. For example, increasing plant leaf biomass may increase the yield of leafy vegetables for human or animal consumption. Additionally, increasing leaf biomass can be used to increase production of plant-derived pharmaceutical or industrial products. An increase in yield can comprise any statistically significant increase including, but not limited to, at least a 1% increase, at least a 3% increase, at least a 5% increase, at least a 10% increase, at least a 20% increase, at least a 30%, at least a 50%, at least a 70%, at least a 100% or a greater increase.

Plants produced using the disclosed methods can be treated with an effective concentration of an herbicide, where the herbicide application results in enhanced plant yield. An effective concentration is a concentration which allows the increased yield in the plant. Such effective concentrations for herbicides of interest are generally known in the art. The herbicide
may be applied either pre- or post emergence in accordance with usual techniques for herbicide application to fields comprising crops which have been rendered resistant to the herbicide.

ILA. Plant Transformation

Methods of the invention involve introducing one or more polynucleotides into a plant by presenting to the plant the polynucleotide in such a manner that the polynucleotide gains access to the interior of a cell of the plant. The methods of the invention do not require that a particular method for introducing a polynucleotide into a plant is used, only that the polynucleotide gains access to the interior of at least one cell of the plant.

Introduction of a polynucleotide into plant cells is accomplished by one of several techniques known in the art, including but not limited to electroporation or chemical transformation (See e.g., Ausubel, ed. (1994) Current Protocols in Molecular Biology, John Wiley and Sons, Inc., Indianapolis, Indiana. Markers conferring resistance to toxic substances are useful in identifying transformed cells (having taken up and expressed the test polynucleotide sequence) from non-transformed cells (those not containing or not expressing the test polynucleotide sequence). In one aspect of the invention, genes are useful as a marker to assess introduction of DNA into plant cells. Transgenic plants, transformed plants, or stably transformed plants, or cells, tissues or seed of any of the foregoing, refer to plants that have incorporated or integrated exogenous polynucleotides into the plant cell. Stable transformation refers to introduction of a polynucleotide construct a plant such that it integrates into the genome of the plant and is capable of being inherited by progeny thereof.

In general, plant transformation methods involve transferring heterologous DNA into target plant cells (e.g., immature or mature embryos, suspension cultures, undifferentiated callus, protoplasts, etc.), followed by applying a maximum threshold level of appropriate selection (depending on the selectable marker gene) to recover the transformed plant cells from a group of untransformed cell mass. Explants are typically transferred to a fresh supply of the same medium and cultured routinely. Subsequently, the transformed cells are differentiated into shoots after placing on regeneration medium supplemented with a maximum threshold level of selecting agent (i.e., temperature and/or herbicide). The shoots are then transferred to a selective rooting medium for recovering rooted shoot or plantlet. The transgenic plantlet then grow into mature plant and produce fertile seeds (e.g., Hiei et al. (1994) Plant J. 6:271-282; Ishida et al.
A general description of the techniques and methods for generating transgenic plants are found in Ayres and Park (1994) *CRC Crit. Rev. Plant Sci.* 13:219-239, and Bommineni and Jauhar (1997) *Maydica* 42:107-120. Since the transformed material contains many cells, both transformed and non-transformed cells are present in any piece of subjected target callus or tissue or group of cells. The ability to kill non-transformed cells and allow transformed cells to proliferate results in transformed plant cultures. Often, the ability to remove non-transformed cells is a limitation to rapid recovery of transformed plant cells and successful generation of transgenic plants. Then molecular and biochemical methods can be used for confirming the presence of the integrated nucleotide(s) of interest in the genome of transgenic plant.


There are three common methods to transform plant cells with *Agrobacterium*. The first method is co-cultivation of *Agrobacterium* with cultured isolated protoplasts. This method requires an established culture system that allows culturing protoplasts and plant regeneration from cultured protoplasts. The second method is transformation of cells or tissues with *Agrobacterium*. This method requires (a) that the plant cells or tissues can be transformed by *Agrobacterium* and (b) that the transformed cells or tissues can be induced to regenerate into whole plants. The third method is transformation of seeds, apices or meristems with *Agrobacterium*. This method requires micropropagation.

The efficiency of transformation by *Agrobacterium* may be enhanced by using a number of methods known in the art. For example, the inclusion of a natural wound response molecule such as acetylsyringone (AS) to the *Agrobacterium* culture has been shown to enhance transformation efficiency with *Agrobacterium tumefaciens* (Shahla et al. (1987) *Plant Molec. Biol.* 8:291-298). Alternatively, transformation efficiency may be enhanced by wounding the target tissue to be transformed. Wounding of plant tissue may be achieved, for example, by
punching, maceration, bombardment with microprojectiles, etc. See e.g., Bidney et al. (1992) *Plant Molec. Biol.* 18:301-313.

In another aspect of the invention, the plant cells are transfected with vectors via particle bombardment (i.e., with a gene gun). Particle mediated gene transfer methods are known in the art, are commercially available, and include, but are not limited to, the gas driven gene delivery instrument described in McCabe, U.S. Patent No. 5,584,807, the entire contents of which are herein incorporated by reference. This method involves coating the polynucleotide sequence of interest onto heavy metal particles, and accelerating the coated particles under the pressure of compressed gas for delivery to the target tissue.

Other particle bombardment methods are also available for the introduction of heterologous polynucleotide sequences into plant cells. Generally, these methods involve depositing the polynucleotide sequence of interest upon the surface of small, dense particles of a material such as gold, platinum, or tungsten. The coated particles are themselves then coated onto either a rigid surface, such as a metal plate, or onto a carrier sheet made of a fragile material such as mylar. The coated sheet is then accelerated toward the target biological tissue. The use of the flat sheet generates a uniform spread of accelerated particles that maximizes the number of cells receiving particles under uniform conditions, resulting in the introduction of the polynucleotide sample into the target tissue.

Specific initiation signals may also be used to achieve more efficient translation of sequences encoding the polypeptide of interest. Such signals include the ATG initiation codon and adjacent sequences. In cases where sequences encoding the polypeptide of interest, its initiation codon, and upstream sequences are inserted into the appropriate expression vector, no additional transcriptional or translational control signals may be needed. However, in cases where only coding sequence, or a portion thereof, is inserted, exogenous translational control signals including the ATG initiation codon should be provided. Furthermore, the initiation codon should be in the correct reading frame to ensure translation of the entire insert. Exogenous translational elements and initiation codons may be of various origins, both natural and synthetic. The efficiency of expression may be enhanced by the inclusion of enhancers that are appropriate for the particular cell system that is used, such as those described in the literature (Scharf et al. (1994) *Results Probl. Cell Differ.* 20:125).

The cells that have been transformed may be grown into plants in accordance with
conventional ways. See e.g., McCormick et al. (1986) *Plant Cell Rep.* 5:81-84. These plants
may then be grown, and either pollinated with the same transformed strain or different strains,
and the resulting hybrid having constitutive expression of the desired phenotypic characteristic
identified. Two or more generations may be grown to ensure that expression of the desired
phenotypic characteristic is stably maintained and inherited and then seeds harvested to ensure
expression of the desired phenotypic characteristic has been achieved. In this manner, the
present invention provides transformed seed (also referred to as transgenic seed) having a
polynucleotide of the invention, for example, an expression cassette of the invention, stably
incorporated into their genome.

Transgenic plants of the invention can be homozygous for the added polynucleotides; *i.e.*, a
transgenic plant that contains two added sequences, one sequence at the same locus on each
chromosome of a chromosome pair. A homozygous transgenic plant can be obtained by sexually
mating (selfing) an independent segregant transgenic plant that contains the added sequences
according to the invention, germinating some of the seed produced and analyzing the resulting
plants produced for enhanced enzyme activity (*i.e.*, herbicide resistance) and/or increased plant
yield relative to a control (native, non-transgenic) or an independent segregant transgenic plant.

It is to be understood that two different transgenic plants can also be mated to produce
offspring that contain two independently segregating added, exogenous polynucleotides. Selfing
of appropriate progeny can produce plants that are homozygous for all added, exogenous
polynucleotides that encode a polypeptide of the present invention. Back-crossing to a parental
plant and outcrossing with a non-transgenic plant are also contemplated.

**ILB. Evaluation Transformed Plants**

Following introduction of DNA into plant cells, the transformation or integration of the
polynucleotide into the plant genome is confirmed by various methods such as analysis of
polynucleotides, polypeptides and metabolites associated with the integrated sequence.

PCR analysis is a rapid method to screen cells, tissue or shoots for the presence of
incorporated gene at the earlier stage before transplanting into the soil (Sambrook and Russell
Spring Harbor, New York. PCR is carried out using oligonucleotide primers specific to the
nucleotide of interest or *Agrobacterium* vector background, *etc.*
Introduction of DNA may be confirmed by Southern blot analysis of genomic DNA (Sambrook and Russell (2001) supra). In general, total DNA is extracted from the cell or organism, digested with appropriate restriction enzymes, fractionated in an agarose gel and transferred to a nitrocellulose or nylon membrane. The membrane or blot then is probed with, for example, radiolabeled $^{32}$P-labeled target DNA fragment to confirm the integration of introduced DNA into the plant genome according to standard techniques (Sambrook and Russell (2001) supra).

In Northern analysis, RNA is isolated from specific tissues of the cell or organism, fractionated in a formaldehyde agarose gel, and blotted onto a nylon filter according to standard procedures that are routinely used in the art (Sambrook and Russell (2001) supra). Expression of RNA encoded by the polynucleotide of the present invention is then tested by hybridizing the filter to a radioactive probe derived from the sequence of interest, by methods known in the art (Sambrook and Russell (2001) supra).

Western blot and biochemical assays and the like may be carried out on the transgenic plants to determine the presence of polypeptide encoded by the nucleotide(s) of interest by standard procedures (Sambrook and Russell, 2001,) using antibodies that bind to one or more epitopes present on the herbicide resistance polypeptide.

EXAMPLES

Example 1

EPSP Synthases for Glyphosate Resistance

The DNA coding sequence and the amino acid sequence of the grg8 open reading frame are provided in U.S. Provisional Patent Application No. 60/640,195, filed December 29, 2004, and set forth as SEQ ID NO:1 and SEQ ID NO:2 of this application, respectively.

The DNA coding sequence and the amino acid sequence of the grg23 open reading frame are provided in U.S. Provisional Patent Application No. 60/741,166, filed December 1, 2005, and set forth as SEQ ID NO:3 and SEQ ID NO:4 of this application, respectively.

The DNA coding sequence and amino acid sequence of the grgl open reading frame are provided in U.S. Patent Application No. 10/739,610, filed December 18, 2003, and set forth as SEQ ID NO:5 and SEQ ID NO:6 of this application, respectively.

The DNA coding sequence and amino acid sequence of the grg20 open reading frame are
set forth as SEQ ID NO:7 and SEQ ID NO:8 of this application, respectively. This EPSPS is described in the art as from Sulfolobus solfataricus (American Type Culture Collection Accession No. 35092D). U.S. Provisional Patent Application 60/658,320, filed January 12, 2006, describes the discovery of its use as conferring herbicide resistance and teaches domains predictive of such resistance.

Each of the patent applications identified in this example, and in particular the above-noted named sequences, are expressly incorporated by reference into the instant application.

Example 2

EPSP Synthase Activity for Temperature Optima Determination

Individual glyphosate-resistant EPSP synthase enzymes were overexpressed in E. coli and purified to homogeneity by standard methods. To measure enzyme activity, each enzyme was diluted to an appropriate assay concentration in buffer containing HEPES (50 mM, pH 7) and 50 mM KCl, and then incubated for 15 minutes at 10, 20, 30, 40, 50 or 60°C. Following incubation, each enzyme was heated to 90°C for 1 minute to denature the enzyme, and then cooled to 4°C. The phosphate generated by each reaction was then added to a second assay containing inosine, purine nucleoside phosphorylase, xanthine oxidase, horseradish peroxidase, and the fluorescent substrate Amplex Red (see U.S. Provisional Patent Application No. 60/741,166, filed December 1, 2005). Following incubation for 15 minutes at room temperature, fluorescent product was quantified using a Gemini XPS spectrofluorometer (Molecular Devices Corporation of Sunnyvale, California). EPSP synthase product formation was plotted as a percentage of the temperature which yielded maximal activity, as shown in Figure 1.

Example 3

EPSP Synthase Activity for pH Optimum Determination

Individual glyphosate-resistant EPSP synthase enzymes were overexpressed in E. coli and purified to homogeneity by standard methods. To measure enzyme activity, each enzyme was diluted to an appropriate assay concentration in buffer containing HEPES (50 mM, pH 7) and 50 mM KCl, and then incubated for 15 minutes with buffers calibrated to pHs ranging from pH 6.0 to pH 8.0. Following incubation, each enzyme was heated to 90°C for 1 minute to denature the enzyme, and then cooled to 4°C. The phosphate generated by each reaction was then added to a second assay containing inosine, purine nucleoside phosphorylase, xanthine
oxidase, horseradish peroxidase, and the fluorescent substrate Amplex Red (see U.S. Provisional Patent Application No. 60/741,166, filed December 1, 2005). Following incubation for 15 minutes at room temperature, fluorescent product was quantified using a Gemini XPS spectrofluorometer (Molecular Devices Corporation of Sunnyvale, California). EPSP synthase product formation was plotted as a percentage of the temperature which yielded maximal activity, as shown in Figure 2 and in Table 1 below. GRG23 has a pH optimum at or below pH 6, while GRG1 attains maximum activity at pH 7.0. At pH 7.5, GRG1 has a higher percentage activity then GRG23. Thus, a plant cell expressing both GRG1 and GRG23 will have improved activity at a pH range between pH 6.0 and pH 7.5 as compared to a plant cell expressing GRG1 or GRG2 alone.

Table 1

<table>
<thead>
<tr>
<th>EPSPS</th>
<th>pH</th>
<th>Percent Maximum Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>GRG1</td>
<td>6.5</td>
<td>96.41</td>
</tr>
<tr>
<td></td>
<td>7.0</td>
<td>100.00</td>
</tr>
<tr>
<td></td>
<td>7.5</td>
<td>79.11</td>
</tr>
<tr>
<td></td>
<td>8.0</td>
<td>59.41</td>
</tr>
<tr>
<td>GRG23</td>
<td>6.5</td>
<td>100.00</td>
</tr>
<tr>
<td></td>
<td>7.0</td>
<td>92.45</td>
</tr>
<tr>
<td></td>
<td>7.5</td>
<td>71.61</td>
</tr>
<tr>
<td></td>
<td>8.0</td>
<td>43.81</td>
</tr>
</tbody>
</table>

Example 4

EPSP Synthase Activity for Determination of Optimum Salt Concentration

Individual glyphosate-resistant EPSP synthase enzymes were overexpressed in E. coli and purified to homogeneity by standard methods. To measure enzyme activity, each enzyme was diluted to an appropriate assay concentration in buffer containing HEPES (50 mM, pH 7) and 50 mM KCl, and then incubated for 15 minutes with buffers supplemented with various amounts of KCl ranging from 50 mM to 200 mM. Following incubation, each enzyme was heated to 90°C for 1 minute to denature the enzyme, and then cooled to 4°C. The phosphate generated by each reaction was then added to a second assay containing inosine, purine nucleoside phosphorylase, xanthine oxidase, horseradish peroxidase, and the fluorescent substrate Amplex Red (see U.S. Provisional Patent Application No. 60/741,166, filed December
Following incubation for 15 minutes at room temperature, fluorescent product was quantified using a Gemini XPS spectrofluorometer (Molecular Devices Corporation of Sunnyvale, California). EPSP synthase product formation was plotted as a percentage of the KCl concentration which yielded maximal activity, as shown in Table 2 below. GRG23 has maximal activity at a lower KCl concentration than GRGl. Therefore, a plant expressing both GRGl and GRG2 will have a higher enzyme activity over a broader range of salt concentrations as compared to a plant expressing GRGl or GRG2 alone.

<table>
<thead>
<tr>
<th>EPSPs</th>
<th>[KCl]</th>
<th>Percent Maximum Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>GRG1</td>
<td>50</td>
<td>94.69</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>200</td>
<td>81.15</td>
</tr>
<tr>
<td>GRG23</td>
<td>50</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>95.05</td>
</tr>
<tr>
<td></td>
<td>200</td>
<td>78.18</td>
</tr>
</tbody>
</table>

Example 5

Plant Transformation by Particle Bombardment

Maize ears are best collected 8-12 days after pollination. Embryos are isolated from the ears, and those embryos 0.8-1.5 mm in size are used for transformation. Embryos are plated scutellum side-up on a suitable incubation media. DN62A5S media is one such media and is prepared as follows: combine 3.98 g/L N6 salts; 1 mL/L (of 1000x stock) N6 vitamins; 800 mg/L L-asparagine; 100 mg/L myo-inositol; 1.4 g/L L-proline; 100 mg/L casamino acids; 50 g/L sucrose; 1 mL/L (of 1 mg/mL stock) 2,4-D); adjust to pH 5.8 with 1N KOH/1N KCl; add gelrite (Sigma) at a concentration of 3g/L; after autoclaving and cooling to 50°C, 2 ml/L of a 5 mg/ml stock solution of Silver Nitrate (Phytotechnology Labs) is added and media is poured into plates. Media and salts other than DN62A5S are also suitable and are known in the art. Embryos are incubated overnight at 25°C in the dark. Embryos may also be incubated for variable times as sufficient to achieve plant transformation.

The resulting explants are transferred to mesh squares (30-40 per plate), transferred onto osmotic media for about 30-45 minutes, then transferred to a beaming plate (see e.g., PCT
DNA constructs designed to express a first and second EPSP synthase with varying kinetic optima in plant cells are accelerated into plant tissue using an aerosol beam accelerator, using conditions essentially as described in PCT International Publication No. WO 01/38514. After beaming, embryos are incubated for about 30 minutes on osmotic media, and placed onto incubation media overnight at 25°C in the dark. Care is taken to avoid unduly damaging beamed explants, for example, by incubating embryos for at least 24 hours prior to transfer to recovery media. Embryos are then spread onto recovery period media, for about 5 days, 25°C in the dark, and then transferred to a selection media. Explants are incubated in selection media for up to eight weeks, depending on the nature and characteristics of the particular selection utilized. After the selection period, the resulting callus is transferred to embryo maturation media, until the formation of mature somatic embryos is observed. The resulting mature somatic embryos are then placed under low light, and the process of regeneration is initiated by methods known in the art. The resulting shoots are allowed to root on rooting media, and the resulting plants are transferred to nursery pots and propagated as transgenic plants. The plants are assayed for improved resistance to glyphosate over a broader range of a physiological or environmental condition (e.g., temperature, pH, concentration of a substrate for said first and second polypeptide, concentration of a cofactor for said first and second polypeptide, concentration of free radicals or free radical donors, concentration of an inhibitor of said first and second polypeptide, or concentration of a catalyst of said first and second polypeptide) than when only a single ESP sequence is expressed. Alternatively, the plants can be assayed for increase yield when compared to expression of only one of the EPSP synthase sequences.

Example 6

Transformation of Plant Cells by Agrobacterium-MQdiatQd Transformation

Ears are collected 8-12 days after pollination. Embryos are isolated from the ears, and those embryos about 0.8-1.5 mm in size are used for transformation. Embryos are plated scutellum side-up on a suitable incubation media, and incubated overnight at 25°C in the dark. Embryos may also be incubated for variable times as sufficient to achieve plant transformation. Embryos are contacted with an Agrobacterium strain containing the appropriate vectors having two EPSP synthase sequences that are capable of conferring resistance to glyphosate and having
varying kinetic optima for Ti plasmid mediated transfer for about 5-10 minutes, and then plated onto co-cultivation media for about 3 days (25°C in the dark). After co-cultivation, explants are transferred to recovery period media for about five days (at 25°C in the dark). Explants are incubated in selection media for up to eight weeks, depending on the nature and characteristics of the particular selection utilized. After the selection period, the resulting callus is transferred to embryo maturation media, until the formation of mature somatic embryos is observed. The resulting mature somatic embryos are then placed under low light, and the process of regeneration is initiated as known in the art. The resulting shoots are allowed to root on rooting media, and the resulting plants are transferred to nursery pots and propagated as transgenic plants. The plants are assayed for improved resistance to glyphosate over a broader range of a physiological or environmental condition (e.g., temperature, pH, concentration of a substrate for said first and second polypeptide, concentration of a cofactor for said first and second polypeptide, concentration of free radicals or free radical donors, concentration of an inhibitor of said first and second polypeptide, or concentration of a catalyst of said first and second polypeptide) than when only a single ESP sequence is expressed. Alternatively, the plants can be assayed for increase yield when compared to expression of only one of the EPSP synthase sequences.

All publications and patent applications mentioned in the specification are indicative of the level of skill of those skilled in the art to which this invention pertains. All publications and patent applications are herein incorporated by reference to the same extent as if each individual publication or patent application was specifically and individually indicated to be incorporated by reference.

Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, certain changes and modifications may be practiced within the scope of the appended claims. Many modifications of the inventions set forth herein will come to mind to one skilled in the art to which these inventions pertain having the benefit of the teachings presented in the foregoing descriptions and the associated drawings. Therefore, it is to be understood that the inventions are not to be limited to the specific examples disclosed and that modifications and other examples are intended to be included within the scope of the appended claims. Although specific terms are employed herein, they are used in a generic and descriptive sense only and not for purposes of limitation.
What is claimed:

1. A plant comprising at least (a) a first heterologous polynucleotide encoding a first polypeptide capable of conferring a trait of interest, and (b) a second heterologous polynucleotide encoding a second polypeptide capable of conferring said trait of interest, wherein said first and second polynucleotides are stably expressed in said plant, and wherein said plant shows said trait of interest over a broader range of a physiological or environmental condition as compared to a plant comprising either said first or second polynucleotide expressed alone and subject to said range of a physiological or environmental condition.

2. The plant of claim 1, wherein said trait of interest is resistance to an herbicide.

3. The plant of claim 1, wherein said trait of interest is yield.

4. The plant of claim 2, wherein said first and said second polynucleotide confer resistance to glyphosate.

5. The plant of claim 4, wherein said first and said second polynucleotide encode a first and a second EPSP synthase polypeptide.

6. The plant of claim 5, wherein said first or said second EPSP synthase is from a plant.

7. The plant of claim 5, wherein said first or said second EPSP synthase is from a bacterium.

8. The plant of claim 5, wherein said first or said second EPSP synthase polypeptide comprises an amino acid sequence of SEQ ID NO: 2, 4, 6, or 8.

9. The plant of claim 5, wherein said first and said second EPSP synthase polypeptide each comprise an amino acid sequence of SEQ ID NO: 2, 4, 6, or 8.
10. The plant of claim 9, wherein said first EPSP synthase polypeptide comprises an amino acid sequence of SEQ ID NO:2 or 4 and the second EPSP synthase polypeptide comprises an amino acid sequence of SEQ ID NO:6 or 8.

11. The plant of claim 1, wherein said physiological or environmental condition is temperature.

12. The plant of claim 11, wherein said first polypeptide has optimal enzyme activity within a temperature range of about 5°C to about 40°C.

13. The plant of claim 11, wherein said second polypeptide has optimal enzyme activity at a higher temperature as compared to said first polypeptide and within a temperature range of about 20°C to about 60°C.

14. The plant of claim 13, wherein said first polypeptide has optimal enzyme activity within a temperature range of about 10°C to about 30°C and said second polypeptide has optimal enzyme activity at a higher temperature as compared to said first polypeptide and within a temperature range of about 25°C to about 50°C.

15. The plant of claim 14, wherein said first polypeptide comprises an amino acid sequence of SEQ ID NO:2, and said second polypeptide comprises an amino acid of SEQ ID NO:4, 6, or 8.

16. The plant of claim 14, wherein said first polypeptide comprises an amino acid sequence of SEQ ID NO:4, and said second polypeptide comprises an amino acid of SEQ ID NO:6 or 8.

17. The plant of claim 11, wherein expression of said first and said second polypeptides confers said trait of interest from about 5°C to about 60°C.
18. The plant of claim 1, wherein said physiological or environmental condition is pH.

19. The plant of claim 18, wherein said first polypeptide has optimal enzyme activity within a pH range of about pH 4.0 to about pH 6.5, and wherein said second polypeptide has optimal enzyme activity at a higher pH as compared to said first polypeptide and within the range from about pH 6.0 to about pH 8.5.

20. The plant of claim 19, wherein said first polypeptide comprises an amino acid sequence of SEQ ID NO:4, and said second polypeptide comprises an amino acid of SEQ ID NO: 6.

21. The plant of claim 1, wherein said physiological or environmental condition is salt concentration.

22. The plant of claim 21, wherein said first polypeptide has optimal enzyme activity within a salt concentration range of about 50 mM to 150 mM, and wherein said second polypeptide has optimal enzyme activity at a higher salt concentration as compared to said first polypeptide and within a range from about 100 mM to 200 mM.

23. The plant of claim 21, wherein said first polypeptide comprises an amino acid sequence of SEQ ID NO:4, and said second polypeptide comprises an amino acid of SEQ ID NO: 6.

24. The plant of claim 1, wherein said physiological or environmental condition is concentration of a substrate or concentration of a cofactor for said first and second polypeptide.

25. The plant of claim 24, wherein said first polypeptide has (a) an affinity for substrate or cofactor that is at least about 2-fold greater than an affinity for said substrate or cofactor by said second polypeptide, and (b) a catalytic activity that is at least about 2-fold greater than a catalytic activity of said second polypeptide.
26. The plant of claim 25, wherein said first polypeptide has an affinity for substrate or cofactor that is at least about 5-fold greater than an affinity for said substrate or cofactor by said second polypeptide.

27. The plant of claim 25, wherein said first polypeptide has an affinity for substrate or cofactor that is at least about 10-fold greater than an affinity for said substrate or cofactor by said second polypeptide.

28. The plant of claim 25, wherein said first polypeptide has a catalytic activity that is at least about 5-fold greater than a catalytic activity of said second polypeptide.

29. The plant of claim 25, wherein said first polypeptide has a catalytic activity that is at least about 10-fold greater than a catalytic activity of said second polypeptide.

30. The plant of claim 25, wherein said first polypeptide has (a) an affinity for substrate or cofactor that is at least about 10-fold greater than an affinity for said substrate or cofactor by said second polypeptide, and (b) a catalytic activity that is at least about 10-fold greater than a catalytic activity of said second polypeptide.

31. The plant of claim 1, wherein said first polypeptide has optimal enzyme activity in cytoplasm, and said second polypeptide has optimal enzyme activity in chloroplasts.

32. The plant of claim 1, wherein said plant is a monocot.

33. The plant of claim 1, wherein said plant is a dicot.

34. A transformed seed of the plant of claim 1.

35. A method of conferring a trait of interest to a plant, said method comprising introducing into said plant at least (a) a first heterologous polynucleotide encoding a first
polypeptide capable of conferring said trait of interest, and (b) a second heterologous polynucleotide encoding a second polypeptide capable of conferring said trait of interest, wherein said first and second polynucleotides are stably expressed in said plant, and wherein said plant shows said trait of interest over a broader spectrum of a physiological or environmental condition as compared to a plant comprising either said first or second polynucleotide expressed alone and subject to said spectrum of a physiological or environmental condition.

36. A method of conferring a trait of interest to a plant, said method comprising (a) providing a transgenic plant comprising a first heterologous polynucleotide encoding a first polypeptide capable of conferring said trait of interest, and (b) introducing into said plant at least a second heterologous polynucleotide encoding a second polypeptide capable of conferring said trait of interest, wherein said first and second polynucleotides are stably expressed in said plant, and wherein said plant shows said trait of interest over a broader spectrum of a physiological or environmental condition as compared to a plant comprising either said first or second polynucleotide expressed alone and subject to said spectrum of a physiological or environmental condition.

37. A method of increasing plant vigor or yield comprising:
   (a) providing a plant according to claim 2; and,
   (b) treating the plant with an effective amount of said herbicide to thereby increase plant vigor or yield.
**FIG. 5A**

**Nucleotide sequence of grg8 from Brevundomonas vesicularis (coding sequence nucleotides 296-1555):**

```
agt atg ggt aga gcc aaa ctc acg att atc c cg gc g gc aag cct tgg
Met Met Gly Arg Ala Lys Leu Thr Ile Ile Pro Pro Gly Lys Pro Leu
  5
  10
  15

acc gga cgc atg c cg gc g ga tcg aag tcg atc acc aac gc g ca
Thr Gly Arg Ala Met Pro Pro Gly Ser Lys Ser Ile Thr Asn Arg Ala
  20
  25
  30

ttg ctc ctc gcc ggc atc gcc gc c a g g c gc ac g gc gc gc
Leu Leu Leu Ala Gly Leu Ala Lys Gly Thr Ser Arg Leu Thr Gly Ala
  35
  40
  45

tgg aag acg gac gtt acc gc g c c g c t g c t g cg a g
Leu Lys Ser Asp Thr Arg Tyr Met Ala Glu Ala Leu Arg Ala Met
  50
  55
  60
  65

ggt gta acg atc gac gag ccc gac gac acc acg ttc atc gtc aa g gc
Gly Val Thr Ile Asp Glu Pro Asp Thr Thr Phe Ile Val Lys Gly
  70
  75
  80

agc gc g g a c gt c ag c cc gc c c g c g cc c tt cc tc g gc a a t gc
Ser Gly Lys Leu Leu Gln Pro Pro Ala Ala Pro Leu Phe Gly Asn Ala
  85
  90
  95

FIG. 5B

agc ggc gtc gat gac ggc ggc ctc agc cag tat gtc tgc 826
Ser Arg Val Gln Ile Asp Gly Gly Leu Ser Ser Gln Tyr Val Ser Ala
165 170

cgc gtc atg atg gcc ggc ggc ggc gat cgc gct gtc gat gtc gag ctt 874
Leu Leu Met Met Ala Gly Gly Asp Arg Val Asp Val Glu Leu
180 185 190

cgc gcc gaa cat atm ggc gct ctc ggc tat atc gac ctc gct gct gcc 922
Leu Gly Glu His Ile Gly Ala Leu Gly Tyr Ile Asp Leu Thr Val Ala
195 200 205

gcc gtc cgc gct ttc ggc gcc aag gtt gag cgt gtc gac cgc gcc gtc gcc 970
Ala Met Arg Ala Phe Gly Gly Ala Lys Val Arg Val Ser Pro Val Ala
210 215 220 225

tgg gtc gag ccc acc ggc tat cat gcc gcc gac ttc gtc gac gtt gcc 1018
Trp Arg Val Glu Pro Thr Gly Tyr His Ala Ala Asp Phe Val Ile Glu
230 235 240

cgc gtc gct gcc acc ttc gct ggc gcc gaa gtt ctc gtc gac ggc 1066
Pro Asp Ala Ser Ala Ala Thr Tyr Leu Trp Ala Ala Glu Val Leu Ser
245 250 255

gcc ggc aag atm gat ctc ggc acg ccc gcc gaa cag ttc tgc cca ccc 1114
Gly Gly Lys Ile Asp Leu Gly Thr Pro Ala Glu Gln Phe Ser Gln Pro
260 265 270

gat ggc aaa gcc tat gat ctc atg aat cca atg atg gtc ctc gcc 1162
Asp Ala Lys Ala Tyr Asp Leu Ile Ser Lys Phe Pro His Leu Pro Ala
275 280 285

gtc atc gag ggc tct gac atg cag gac gcc atc ccc gcc gtc gct gct 1210
Val Ile Asp Gly Ser Gln Met Glu Asp Ala Ile Pro Thr Val Ala Val
290 295 300 305

cgc gtc gct ttt gcc aat gtt gtc gct ggc gcc gtt ggt gtc gtc gtt 1258
Leu Ala Ala Phe Asn Glu Met Pro Val Arg Phe Val Gly Ile Glu Asn
310 315 320

cgc gtc aag gaa tgc gat ctc gtc ggc gcc gta cag ggc gct gtc 1306
Leu Arg Val Lys Glu Cys Asp Arg Ile Arg Ala Leu Ser Ser Gly Leu
325 330 335

tgc gtc gct gtt gtc gcc gcc ggc aag gaa gag gcc gac gtc gct gtc 1354
Ser Arg Ile Val Pro Asn Leu Gly Thr Glu Gly Asp Leu Ile
340 345 350

cgc gtc gct gtt gtc gcc ggc gcc gtc gac gtc gct gtc gct gtc 1402
Ile Ala Ser Asp Pro Ser Leu Ala Gly Lys Ile Leu Thr Ala Glu Ile
355 360 365
FIG. 5C

gat agc ttt gcc gat cac cgc atc gcc atg agc ttt gcg ctg gcc gcc 1450
Asp Ser Phe Ala Asp His Arg Ile Ala Met Ser Phe Ala Leu Ala Gly
370 375 380 385

c tg aag atc gcc ggc att acc att ctc gac ccc gac tgc gtc gcc aag 1498
Leu Lys Ile Gly Ile Gly Ile Thr Ile Leu Asp Pro Asp Cys Val Ala Lys
390 395 400

aca ttc ccc tcc tac tgg aat gtg ctc tac gtg ggg gtc gcc tcc 1546
Thr Phe Pro Ser Tyr Trp Asn Val Leu Ser Ser Leu Gly Val Ala Tyr
405 410 415

gaa gag tga cgctctgctc ctatagaggc ctcaggcggg atttatcttt 1595
Glu Asp *

Amino acid sequence of grg8 from Brevundomonas vesicularis:

Met Met Met Gly Arg Ala Lys Leu Thr Ile Ile Pro Pro Gly Lys Pro 1 5 10 15
Leu Thr Gly Arg Ala Met Pro Pro Gly Ser Lys Ser Ile Thr Asn Arg 20 25 30
Ala Leu Leu Ala Gly Leu Ala Gly Thr Ser Arg Leu Thr Gly 35 40 45
Ala Leu Lys Ser Asp Asp Thr Arg Tyr Met Ala Glu Ala Leu Arg Ala 50 55 60
Met Gly Val Thr Ile Asp Glu Pro Asp Thr Thr Phe Ile Val Lys 65 70 75 80
Gly Ser Gly Lys Leu Gln Pro Pro Ala Ala Leu Phe Leu Gly Asn 85 90 95
Ala Gly Thr Ala Thr Arg Phe Leu Thr Ala Ala Ala Leu Val Asp 100 105 110
Gly Lys Val Ile Val Asp Gly Asp Ala His Met Arg Lys Arg Pro Ile 115 120 125
Gly Pro Leu Val Asp Ala Leu Arg Ser Leu Gly Ile Asp Ala Ser Ala 130 135 140
Glu Thr Gly Cys Pro Pro Val Thr Ile Asn Gly Thr Gly Arg Phe Glu 145 150 155 160
Ala Ser Arg Val Gln Ile Asp Gly Gly Leu Ser Ser Gln Val Ser 165 170 175
Ala Leu Leu Met Met Ala Ala Gly Asp Arg Ala Val Asp Val Glu 180 185 190

2000
FIG. 5D

Leu Leu Gly Glu His Ile Gly Ala Leu Gly Tyr Ile Asp Leu Thr Val
195 200 205
 Ala Ala Met Arg Ala Phe Gly Ala Lys Val Glu Arg Val Ser Pro Val
210 215 220
 Ala Trp Arg Val Glu Pro Thr Gly Tyr His Ala Ala Asp Phe Val Ile
225 230 235 240
 Glu Pro Asp Ala Ser Ala Ala Thr Tyr Leu Trp Ala Ala Glu Val Leu
245 250 255
 Ser Gly Gly Lys Ile Asp Leu Gly Thr Pro Ala Glu Gln Phe Ser Gln
260 265 270
 Pro Asp Ala Lys Ala Tyr Asp Leu Ile Ser Lys Phe Pro His Leu Pro
275 280 285
 Ala Val Ile Asp Gly Ser Gln Met Gln Asp Ala Ile Pro Thr Leu Ala
290 295 300
 Val Leu Ala Ala Phe Asn Glu Met Pro Val Arg Phe Val Gly Ile Glu
305 310 315 320
 Asn Leu Arg Val Lys Glu Cys Asp Arg Ile Arg Ala Leu Ser Ser Gly
325 330 335
 Leu Ser Arg Ile Val Pro Asn Leu Gly Thr Glu Gly Asp Asp Leu
340 345 350
 Ile Ile Ala Ser Asp Pro Ser Leu Ala Gly Ile Leu Thr Ala Glu
355 360 365
 Ile Asp Ser Phe Ala Asp His Arg Ile Ala Met Ser Phe Ala Leu Ala
370 375 380
 Gly Leu Lys Ile Gly Gly Ile Thr Ile Leu Asp Pro Asp Cys Val Ala
385 390 395 400
 Lys Thr Phe Pro Ser Tyr Trp Asn Val Leu Ser Ser Leu Gly Val Ala
405 410 415
Tyr Glu Asp

Nucleotide sequence of grg23 from Arthrobacter globiformis, Strain ATX21308 (coding sequence nucleotides 109-1416; n = a, c, g, or t):

ggaccacat gctgtctctg atttcagggc tgctgccggt atgaccaggg gttttagagag 60
 ggacggcagc catcggggcc cttatcgagc caacgcacac agcggctcg gtc gcc tgt 117
 Val Ala Leu 1

165

gag cgg ggc cac gac ggc cga tca cgt aga ctc ttt gga gct tcg ctc
 Glu Arg Gly Gln His Gly Arg Ser Arg Arg Leu Phe Gly Ala Ser Leu
5 10 15

213

gaa agg atc acc atg gaa act gat cga cta gtt atc cca gga tcg aaa
 Glu Arg Ile Thr Met Glu Thr Asp Arg Leu Val Ile Pro Gly Ser Lys
20 25 30 35

261

agc atc acc aac cgg gct ttg ctt ttg gct gcc gca ggc aag ggc acg
 Ser Ile Thr Asn Arg Ala Leu Leu Leu Ala Ala Ala Lys Gly Thr
40 45 50
FIG. 5E

tcg gtc ctg aga cca ttg gtc agc gcc gat acc tca gca ttc aaa 309
Ser Val Leu Val Arg Pro Leu Val Ser Ala Asp Thr Ser Ala Phe Lys
  55   60   65
act gca att cag gcc ctc ggt gcc aac gtc tca gcc gac ggt gac aat 357
Thr Ala Ile Gln Ala Leu Gly Ala Asn Val Ser Ala Asp Gly Asp Asn
  70    75   80
tgg gtc gtt gaa ggc ctg gtt cag gca ccc cac ctc gac gcc gac ttc 405
Trp Val Val Glu Gly Leu Gly Gln Ala Pro His Leu Asp Ala Asp Ile
  85    90   95
tgg tgc gag gat gca ggt acc gtg gcc cgg ttc ctc cct cca ttc gtc 453
Trp Cys Glu Asp Ala Gly Thr Val Ala Arg Phe Leu Pro Pro Phe Val
 100  105  110  115
gcc gca gga cag ggg aag ttc acc gtc gag gga agc gag cag ctg cgg 501
Ala Ala Gly Glu Gln Tyr Val Asp Glu Gly Asp Glu Gln Leu Arg
  120   125  130
cgg cgc ccc ctt cgg ccc ctg gtc gac ggc atc cgc cac ctc ggc ggc 549
Arg Arg Pro Leu Arg Pro Leu Val Asp Gly Ile Arg His Leu Gly Ala
  135   140  145
cgc gtc tcc tcc gag cag ctg ccc cta cca att gaa ggc agc ggc ctg 597
Arg Val Ser Ser Glu Gln Leu Pro Leu Thr Ile Glu Ala Ser Gly Leu
  150   155  160
gca ggc ggg gag tac gaa att gaa gcc cat cag agc agc cag ttc ggc 645
Ala Gly Gly Glu Tyr Glu Ile Glu Ala His Gln Ser Ser Gln Phe Ala
  165   170    175
tcc ggc ctg atc atg gcc ccc cgg tac gcg cga cca ggc ctg cgt gtc 693
Ser Gly Leu Ile Met Ala Ala Asp Tyr Ala Arg Gln Gly Leu Arg Val
  180    185   190   195
cgg ata cca aat ccc gtg agc cag ccc tac ctc acg atg aca ctg cgg 741
Arg Ile Pro Asn Pro Val Ser Glu Pro Tyr Leu Thr Met Thr Leu Arg
  200  205  210
atg atg agg gac tcc ggc ctt gag acc agc acc gac gga gcc acc gtc 789
Met Met Arg Asp Phe Gly Leu Glu Thr Ser Thr Asp Gly Ala Thr Val
  215    220   225
agc gtc cct ccc ggg cgc tac aca gcc cgg cgg tat gaa att gaa cgg 837
Ser Val Pro Pro Gly Arg Tyr Thr Ala Arg Arg Tyr Glu Ile Glu Pro
  230  235  240
gac ggc tca act gcg tcc tac ttc gcc gcc gct tcc gcc gtc tct ggc 885
Asp Ala Ser Thr Ala Ser Tyr Phe Ala Ala Ala Ser Ala Val Ser Gly
  245   250   255
FIG. 5F

cga agc ttc gaa ttc cag gcc ctt ggc aca gac atc caa ggc gac 933
Arg Ser Phe Glu Phe Gln Gly Leu Gly Thr Asp Ser Ile Gln Gly Asp
260 265 270 275

ttc gca ttc ttc aat gta ctt ggg cgg ctc ggt gca gag gtc cac tgg 981
Thr Ser Phe Phe Asn Val Leu Gly Arg Leu Gly Ala Glu Val His Trp
280 285 290

gca ccc aac tcg gtc acc ata tcc gga ccc gaa agg ctg aac ggc gac 1029
Ala Pro Asn Ser Val Thr Ile Ser Gly Pro Glu Arg Leu Asn Gly Asp
295 300 305

tag gaa gtg gat atg ggc gag ata tcg gac acc ttc atg aca ctc gcg 1077
Ile Glu Val Asp Met Gly Glu Ile Ser Asp Thr Phe Met Thr Leu Ala
310 315 320

ggc att gcc cct cta gcc gat gga ccc atc acg ata acc aac att ggc 1125
Ala Ile Ala Pro Leu Ala Asp Gly Pro Ile Thr Ile Thr Asn Ile Gly
325 330 335

cat gca cgg ttg aag gaa tcc gac cgc atc tcg ggc atg gaa acc aac 1173
His Ala Arg Leu Lys Glu Ser Asp Arg Ile Ser Ala Met Glu Thr Asn
340 345 350 355

tgc gca acg ctc ggt gta cca acc gac gtc gga cac gac tgg atg cga 1221
Leu Arg Thr Leu Gly Val Gln Thr Asp Val Gly His Asp Trp Met Arg
360 365 370

tcc tac ccc tct acc ccc cac ggc gcc aga gtc aat tgc cac cgg gac 1269
Ile Tyr Pro Ser Thr Pro His Gly Gly Arg Leu Asn Cys His Arg Asp
375 380 385

ttt cac atg gcc aag cgc atg gtt tca tcc gga cgg ctc gca gtc gac 1317
His Arg Ile Ala Met Ala Phe Ser Ile Leu Gly Leu Arg Val Asp Gly
390 395 400

ttc cac tac ctt gga cgc ctt tcc cag aag cgg ctc tcc ctc ctc ccc 1365
Phe Asp Tyr Leu Gly Arg Leu Phe Pro Glu Lys Ala Ala Thr Leu Pro
405 410 415

ggc t atgtaccc ttcggcggcag acgctaggca tcggaacgc acatctgaca 1467
Gly
tgacccagt ccctcgctca cggcgtgtct gcgcgttacc aagcattctg ccctagggcc 1527
ttccggggcc ccttatgctt tcgggtgtgc cagatttcca tcgggtattg tcggctgacca 1587
tgacccggcg aatcaagtgtc tcagcactgt caaatggttg gcgccttgag gcgcgttctga 1647
tggctgcac gtcggcggcct ctcatcgtg tcaagcagcag cagattcggct tcacgggac 1707
FIG. 5G

gttcaggatc cgccctcgct gcgtgatctt gagcaaggc aatagttaga ttgacctcg 1767
ttgccggcca gacgcgaagc aataaggagt tttngcaggc cacccagatt cccgggttg 1827
aagccgatat gggctccatg ctaactattc gggctccgt ggaagtact 1887
tttcaacctt gcccc

Amino acid sequence of grg23 from Arthrobacter globiformis:

Met Ala Leu Glu Arg Gly Gln His Gly Arg Ser Arg Arg Leu Phe Gly 1 5 10 15
Ala Ser Leu Glu Arg Ile Thr Met Glu Thr Asp Arg Leu Val Ile Pro 20 25 30
Gly Ser Lys Ser Ile Thr Asn Arg Ala Leu Leu Ala Ala Ala Ala 35 40 45
Lys Gly Thr Ser Val Leu Val Arg Pro Leu Val Ser Ala Asp Thr Ser 50 55 60
Ala Phe Lys Thr Ala Ile Gln Ala Leu Gly Ala Asn Val Ser Ala Asp 65 70 75 80
Gly Asp Asn Trp Val Val Glu Gly Leu Gly Gln Ala Pro His Leu Asp 85 90 95
Ala Asp Ile Trp Cys Glu Asp Ala Gly Thr Val Ala Arg Phe Leu Pro 100 105 110
Pro Phe Val Ala Ala Gly Gln Gly Lys Phe Thr Val Asp Gly Ser Glu 115 120 125
Gln Leu Arg Arg Arg Pro Leu Arg Pro Leu Val Asp Gly Ile Arg His 130 135 140
Leu Gly Ala Arg Val Ser Ser Glu Gln Leu Pro Leu Thr Ile Glu Ala 145 150 155 160
Ser Gly Leu Ala Gly Gly Glu Tyr Glu Ile Glu Ala His Gln Ser Ser 165 170 175
Gln Phe Ala Ser Gly Leu Ile Met Ala Ala Pro Tyr Ala Arg Gln Gly 180 185 190
Leu Arg Val Arg Ile Pro Asn Pro Val Ser Gln Pro Tyr Leu Thr Met 195 200 205
Thr Leu Arg Met Met Arg Asp Phe Gly Leu Glu Thr Ser Thr Asp Gly 210 215 220
Ala Thr Val Ser Val Pro Pro Gly Arg Tyr Thr Ala Arg Arg Tyr Glu 225 230 235 240
Ile Glu Pro Asp Ala Ser Thr Ala Ser Tyr Phe Ala Ala Ala Ser Ala 245 250 255
Val Ser Gly Arg Ser Phe Glu Phe Gln Gly Leu Gly Thr Asp Ser Ile 260 265 270
Gln Gly Asp Thr Ser Phe Phe Asn Val Leu Gly Arg Leu Gly Ala Glu 275 280 285
Val His Trp Ala Pro Asn Ser Val Thr Ile Ser Gly Pro Glu Arg Leu 290 295 300
Asn Gly Asp Ile Glu Val Asp Met Gly Glu Ile Ser Asp Thr Phe Met 305 310 315 320
Thr Leu Ala Ala Ile Ala Pro Leu Ala Asp Gly Pro Ile Thr Ile Thr 325 330 335
Asn Ile Gly His Ala Arg Leu Lys Glu Ser Asp Arg Ile Ser Ala Met 340 345 350
**FIG. 5H**

Nucleotide sequence of *grg1* from *Enterobacteriaceae sp.* (coding sequence nucleotides 103-1398):

```
aaaaaaggaatgtaacttgattgtgctggaa aaaaagttagggaagggagggta tgaagagtat
135
	cactggtt caatttagaa aaatcat tccagacc tca aggtacaca aa gtg aaa gta aca
Val Lys Val Thr
1

tta cag ccc gga gat ctg act gga att atc cag tca ccc gct tca aaa
162
Ile Gln Pro Gly Asp Leu Thr Gly Ile Ile Gln Ser Pro Ala Ser Lys
5 10 15 20

tag tcg atc cga gct tgt gct gca tcg gta gca aag gag atg
210
Ser Ser Met Gln Arg Ala Cys Ala Ala Ala Leu Val Ala Lys Gly Ile
25 30 35

tag gag atc att aat ccc ggt cat agc aat gat gat aag gct gcc agg
258
Ser Glu Ile Ile Asp Pro Gly His Ser Asn Asp Asp Lys Ala Ala Arg
40 45 50

tag att gta aag cgg ctt ggt gcc agg ctt gaa gat cag cct gat ggt
306
Asp Ile Val Ser Arg Leu Gly Ala Arg Leu Gly Asp Gln Pro Asp Gly
55 60 65

ttt ttc gat ata aca aat gaa ggc gta aag cct gtc gct cct ttt att
354
Ser Leu Gln Ile Thr Ser Gly Val Lys Pro Val Ala Pro Phe Ile
70 75 80

gag tgc ggt gaa ttt gtt cag cgg atg att ctc gtt cag ctt gtc
402
Asp Cys Gly Glu Ser Gly Leu Ser Ile Arg Met Phe Thr Pro Ile Val
85 90 95 100

tgg ttc agt aag gaa gag gtc acg atc aag gta tct gga agc ctt gtt
450
Ala Leu Ser Lys Glu Glu Val Thr Ile Lys Gly Ser Gly Ser Leu Val
105 110 115

cag cga cca atg gat ttc ttt gat gaa att ctt ccc gat cat ctc gtt gta
498
Thr Arg Pro Met Asp Phe Asp Glu Ile Leu Pro His Leu Gly Val
120 125 130
```
FIG. 5I

```
...aaa gtt aaa tct aac cag ggt aaa ttg cct ctc gtt ata cag ggg cca
Lys Val Lys Ser Asn Gln Gly Lys Leu Pro Leu Val Ile Gln Gly Pro
135
140
145

...ttg aaa cca gca gac gtt acg gtt gat ggg tcc tta agc tct cag ttc
Leu Lys Pro Ala Asp Val Thr Val Asp Gly Ser Leu Ser Ser Gln Phe
150
155
160

...ctt aca ggt ttg ttg ctt gca tat ggc gca gat gca agc gat gtt
Leu Thr Gly Leu Leu Ala Tyr Ala Ala Ala Asp Ala Ser Asp Val
165
170
175
180

...gcg ata aaa gta acg aat ctc aaa agc cgt ccg tat atc gat ctt aca
Ala Ile Lys Val Thr Asn Leu Lys Ser Arg Pro Tyr Ile Asp Leu Thr
185
190
195

...ctg gat gtt atg aag ccg ttt ggt ttg aag act ccc gag aat cga aac
Leu Asp Val Met Lys Arg Phe Gly Leu Lys Thr Pro Glu Asn Arg Asn
200
205
210

...tat gaa gag ttt tat ttc aaa gcc ggg aat gta tat gat gaa acg aab
Tyr Glu Glu Phe Tyr Phe Lys Ala Gly Asn Val Tyr Asp Glu Thr Lys
215
220
225

...atg cca gag tac acc gta gaa ggc gac tgg agc ggt gct ttt tta
Met Gln Arg Tyr Thr Val Glu Gly Asp Trp Ser Gly Gly Ala Phe Leu
230
235
240

...ctg gta ggc ggg gct att gcc ggg ccg atc acg gta aga ggt tgg gat
Leu Val Ala Gly Ala Ile Ala Gly Pro Ile Thr Val Arg Gly Leu Asp
245
250
255
260

...ata gct tcg acg cag gct gat aaa ggc ctc gtt cag gct tgg gat
Ile Ala Ser Thr Glu Ala Asp Lys Ala Ile Val Glu Ala Leu Met Ser
265
270
275

...gcc aac gca ggt att gcc att gat gca aaa gag atc aaa ctt cat cct
Ala Asn Ala Gly Ile Ala Ile Asp Ala Lys Glu Ile Lys Leu His Pro
280
285
290

...gct gat ctc aat gca ttt gaa ttt gat gct act gat tgc ccg gat ctt
Ala Asp Leu Asn Ala Phe Glu Phe Asp Ala Thr Asp Cys Pro Asp Leu
295
300
305

...ttt ccg cca ttc gtt gct tcc ttt ggc tct tat tgc aaa gga gaa gca aac
Phe Pro Pro Leu Val Ala Leu Ala Ser Tyr Cys Lys Gly Glu Thr Lys
310
315
320

...atc aaa ggc gta acg aag gct ggc cat aaa gaa aat gat gac aag gga tgg
Ile Lys Gly Val Ser Arg Leu Ala His Lys Glu Ser Asp Arg Gly Leu
325
330
335
340
```
FIG. 5J

acg ctg cag gac gag ttc ggg aaa atg ggt gtt gaa atc cac ctt gag
Thr Leu Gln Asp Glu Phe Gly Lys Met Gly Val Glu Ile His Leu Glu
345 350 355

gga gat ctg atg cgc gtg atc gga ggg aaa ggc gta aaa gga gct gaa
Gly Asp Leu Met Arg Val Ile Gly Gly Lys Gly Val Lys Gly Ala Glu
360 365 370

gtt agt tca agg cac gat cat cgc att gcg atg gct tgt gcg gtc gct
Val Ser Ser Arg His Asp His Arg Ile Ala Met Ala Cys Ala Val Ala
375 380 385

gct tta aaa gct gtg gtt gaa aca acc atc gaa cat gca gaa ggc gtt
Ala Leu Lys Ala Val Gly Thr Thr Ile Glu His Ala Glu Ala Val
390 395 400

aat aaa tcc tac cgc gat ttt tac agc gat ctt aac caa ctt ggc ggt
Asn Lys Ser Tyr Pro Asp Phe Tyr Ser Asp Leu Lys Gln Leu Gly Gly
405 410 415 420

gtt gta tct tta aac cat caa ttt aat ttc tca tga
Val Val Ser Leu Asn His Gln Phe Asn Phe Ser *
425 430 1389

Amino acid sequence of grgl from Enterobacteriaceae sp.: 

Met Lys Val Thr Ile Gln Pro Gly Asp Leu Thr Gly Ile Leu Gln Ser
1 5 10 15
Pro Ala Ser Lys Ser Ser Met Gln Arg Ala Cys Ala Ala Ala Leu Val
20 25 30
Ala Lys Gly Ile Ser Glu Ile Ile Asn Pro Gly His Ser Asn Asp Asp
35 40 45
Lys Ala Ala Arg Asp Ile Val Ser Arg Leu Gly Ala Arg Leu Asp
50 55 60
Gln Pro Asp Gly Ser Leu Gln Ile Thr Ser Glu Gly Val Lys Pro Val
65 70 75 80
Ala Pro Phe Ile Asp Cys Gly Glu Ser Gly Leu Ser Ile Arg Met Phe
85 90 95
Thr Pro Ile Val Ala Leu Ser Lys Glu Glu Val Thr Ile Lys Gly Ser
100 105 110
Gly Ser Leu Val Thr Arg Pro Met Asp Phe Phe Asp Glu Ile Leu Pro
115 120 125
His Leu Gly Val Lys Val Lys Ser Asn Gln Gly Lys Leu Pro Leu Val
130 135 140
Ile Gln Gly Pro Leu Lys Pro Ala Asp Val Thr Val Asp Gly Ser Leu
145 150 155 160
Ser Ser Gln Phe Leu Thr Gly Leu Leu Ala Tyr Ala Ala Ala Asp
165 170 175
Ala Ser Asp Val Ala Ile Lys Val Thr Asn Leu Lys Ser Arg Pro Tyr
180 185 190
Ile Asp Leu Thr Leu Asp Val Met Lys Arg Phe Gly Leu Lys Thr Pro
195 200 205
FIG. 5K

Glu Asn Arg Asn Tyr Glu Glu Phe Tyr Phe Lys Ala Gly Asn Val Tyr
210 215 220
Asp Glu Thr Lys Met Gln Arg Tyr Thr Val Glu Gly Asp Trp Ser Gly
225 230 235 240
Gly Ala Phe Leu Leu Val Ala Gly Ala Ile Ala Gly Pro Ile Thr Val
245 250 255 260
Arg Gly Leu Asp Ile Ala Ser Thr Gln Ala Asp Lys Ala Ile Val Gln
265 270
Ala Leu Met Ser Ala Asn Ala Gly Ile Ala Ile Asp Ala Lys Glu Ile
275 280 285
Lys Leu His Pro Ala Asp Leu Asn Ala Phe Glu Phe Asp Ala Thr Asp
290 295 300
Cys Pro Asp Leu Phe Pro Pro Leu Val Ala Ala Ser Tyr Cys Lys
305 310 315 320
Gly Glu Thr Lys Ile Lys Gly Val Ser Arg Leu Ala His Gly Ser
325 330 335
Asp Arg Gly Leu Thr Leu Gln Asp Glu Phe Gly Lys Met Gly Val Glu
340 345 350
Ile His Leu Glu Gly Asp Leu Met Arg Val Ile Gly Gly Lys Gly Val
355 360 365
Lys Gly Ala Glu Val Ser Ser Arg His Asp His Arg Ile Ala Met Ala
370 375 380
Cys Ala Val Ala Ala Leu Lys Ala Val Gly Glu Thr Thr Ile Glu His
385 390 395 400
Ala Glu Ala Val Asn Lys Ser Tyr Pro Asp Phe Tyr Ser Asp Leu Lys
405 410 415
Gln Leu Gly Gly Val Val Ser Leu Asn His Gln Phe Asn Phe Ser
420 425 430

Nucleotide sequence of _grg20_ from _Sulfolobus solfataricus_ (coding sequence nucleotides 10,578-11,822):

tgcagttt tctcactgtc atataaatct tgggttttgg aacctctcatt ttttattaca 60
tctcaacctc atctcctcttt acctgatgtt cctcttttcg gtattaaatc 120
cataaacatat ataaacactt atatctgtct atatatccat gcctttataac 180
actctcttctc tattctcttt tttttttcag gcagggtagt tagatgtcctaa 240
gtgaagtcct ctttttcttc atcctttcct tttttttttt tagattacat ccaaaac 300
ttcagattt cttttttatt gaagaggtt tggagaccat tggctttataac 420
ctctcattc atgctctctg atataaggag gagagacaa gatttgcattt ttcctaaata 500
tactcattat atattcctt ccatcatttc atatcattgcc gtcattttttt aatat 600
gttagaatttc cttctttcttc taatgttatt agctgtgcctt cattatcattttt 660
gattcttcat tgcgttcttct cttgctttttc aatattttttc tagcagtgtttag 720
atgcagattt tataagtgaattg tagagccccaa atatacgata aatccctgag ttaagttttta 820
tagcagctt tttcttttgatt cattgattaa ttttttctttttt ttccttttattttcctctcctc 920
ttgctatatc acctctattc atatcaatc atatcattttc cttctttttttt catttatttt 1020
FIG. 5L

ttagaataca cactcctaac actctcctttt attttcctgt attttgcatct tccttaaagaat 1200
actctccttta cactctctttttt aagatctcctt ttgacctattt ttaagcctatt ccaaagctcca 1260
tatcttcctttaa gaaattactat ccagacaggct ttgataataat atatgcataat atttgactca agttgacaa 1320
agactgtaagc cagctcattaa cagccatattt ccgctcattg gcctgctcgct gcctgctcgct 1380
gtagtttagtt cattatatgaac acatctgt gtaaatcttt ttccttcctgtgat 1440
atcgtgctatc tacatatctt ctaacccactt ataacggcata aacatattcata ctatcctgaata 1500
tataattaccta aaaaactctttt gttggtcccttt gctaaagttc cttattataaactacataa tccaataataa 1560
tcgttagcttattt acctgctatttt ttaagacttaa ctgctcatg 1620
atccagttgta caagccatttt ccagcttactt ccacatcctttt gtaaaacttcagtc 1680
cgacggattc aatagcgcct acctaagattg cccgctgcttctgt ccctgcttgcttctgct 1740
cccaactcatcg ttgaggtcctct ttggtccctc ttcgtaccata ccttggttattttt tcaatattaccatc 1800
atatctctctat ccttccttattttt tttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttt
FIG. 50

taagaatggga gaagaaggttc cgatatacga aagtcttaag aaatttggag atgttattat 10560
agttaggccct gtaagcttcttg atgtgaaaga tttatccatc aaagattagt ggaataaaat 10620
aagctctcca taacaaaaggt ctagctatta gtttaatttt tctttacactc ttcactag 10680
tatatctca taatgtggat ctttagtata agttatagaa gcgtcataaa ccagctt 10740
caatttggat aaagttaaa aacaattcttg aatattatcc tccagagaa tactgaagat 10800
aggagagtt ctataaatgtt ctagtttcatc tagaagatctt attctacttt 10860
tagccgcatc aagctgaggaaggagagaaagttgcaagta gatggttttttatttt 10920
taacagaaactg tctacataac gccgactcatc tatctttcaat ttcgctagaa 10980
ctttagctt aggttgagaa aaggtttaatgttcttggttctttccagcttctttccagtt 11040
gtcaatatat tctgctgtc ggaatttc aactggttc atctacatttttactgttcag 11100
attgctggc ctctgctactt ctttctatctt aataagttttt ggtttagcctt 11160
ttggttctgt gttgaatttt tattttttttt attttttttt 11220
aatttcttggg gagatgctttt gtttaggtttt gtttagttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttt
FIG. 5P

Thr Arg Val Tyr Leu His Asn Leu Val Leu Ser Glu Asp Val Ile Asp 35 40 45
Ala Ile Lys Ser Val Arg Ala Leu Gly Val Lys Val Lys Asn Asn Ser 50 55 60
Glu Phe Ile Pro Pro Glu Lys Leu Glu Ile Lys Glu Arg Phe Ile Lys 65 70 75 80
Leu Lys Gly Ser Ala Thr Thr Leu Arg Met Leu Ile Pro Ile Leu Ala 85 90 95
Ala Ile Gly Gly Glu Val Thr Ile Asp Ala Asp Glu Ser Leu Arg Arg 100 105 110
Arg Pro Leu Asn Arg Ile Val Glu Ala Leu Ser Asn Tyr Gly Ile Ser 115 120 125
Phe Ser Ser Tyr Ser Leu Pro Leu Thr Ile Thr Gly Lys Leu Ser Ser 130 135 140
Asn Glu Ile Lys Ile Ser Gly Asp Glu Ser Ser Gln Tyr Ile Ser Gly 145 150 155 160
Leu Ile Tyr Ala Leu His Ile Leu Asn Gly Gly Ser Ile Glu Ile Leu 165 170 175
Pro Pro Ile Ser Ser Lys Ser Tyr Ile Leu Leu Thr Ile Asp Leu Phe 180 185 190
Lys Arg Phe Gly Ser Asp Val Lys Phe Tyr Gly Ser Lys Ile His Val 195 200 205
Asn Pro Asn Asn Leu Val Glu Phe Gln Gly Val Ala Gly Asp Tyr 210 215 220
Gly Leu Ala Ser Phe Tyr Ala Leu Ser Ala Leu Val Ser Gly Gly Gly 225 230 235 240
Ile Thr Ile Thr Asn Leu Trp Glu Pro Lys Glu Tyr Phe Gly Asp His 245 250 255
Ser Ile Val Lys Ile Phe Ser Glu Met Gly Ala Ser Ser Glu Tyr Lys 260 265 270
Asp Gly Arg Trp Phe Val Lys Ala Lys Asp Lys Tyr Ser Pro Ile Lys 275 280 285
Ile Asp Ile Asp Ala Pro Asp Leu Ala Met Thr Ile Ala Gly Leu 290 295 300
Ser Ala Ile Ala Glu Gly Thr Ser Glu Ile Ile Gly Ile Gly Arg Leu 305 310 315 320
Arg Ile Lys Glu Ser Asp Arg Ile Glu Ser Ile Arg Lys Ile Leu Gly 325 330 335
Leu Tyr Gly Val Gly Ser Glu Val Lys Tyr Asn Ser Ile Leu Ile Phe 340 345 350
Gly Ile Asn Lys Gly Met Leu Asn Ser Pro Val Thr Asp Cys Leu Asn 355 360 365
Asp His Arg Val Ala Met Ser Ser Ala Leu Ala Leu Val Asn Gly 370 375 380
Gly Val Ile Thr Ser Ala Glu Cys Val Gly Lys Ser Asn Pro Asn Tyr 385 390 395 400
Trp Glu Asp Leu Leu Ser Leu Asn Ala Lys Ile Ser Ile Glu 405 410