Transgenic plants and fungi capable of metabolizing phosphite as a source of phosphorus

Inventors: Luis Rafael Herrera-Estrella, Guanajuato (MX); Damar Lizbeth Lopez-Arredondo, Guanajuato (MX); Alfredo Heriberto Herrera-Estrella, Guanajuato (MX)

Publication Classification

Abstract

System, including methods and compositions, for making and using transgenic plants and/or transgenic fungi that metabolize phosphite as a source of phosphorus for supporting growth.
Fig. 7

<table>
<thead>
<tr>
<th></th>
<th>WT</th>
<th>PTXD-3</th>
<th>PTXD-5</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 PI / 0 Phi</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>50 µM Phi</td>
<td></td>
<td></td>
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<tr>
<td>100 µM Phi</td>
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<td></td>
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<tr>
<td>1 mM Phi</td>
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<td></td>
<td></td>
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<tr>
<td>50 µM Pi</td>
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<td></td>
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</tbody>
</table>
Fig. 12

Fig. 13
TRANSGENIC PLANTS AND FUNGI CAPABLE OF METABOLIZING PHOSPHITE AS A SOURCE OF PHOSPHORUS

CROSS-REFERENCE TO PRIORITY APPLICATION

[0001] This application is based upon and claims the benefit under 35 U.S.C. §119(e), the Paris Convention priority right, and any and all other applicable law of U.S. Provisional Patent Application Ser. No. 61/199,784, filed Nov. 19, 2008, which is incorporated herein by reference in its entirety for all purposes.

INTRODUCTION

[0002] Phosphorus is an essential element for plant and fungal growth. This element, in oxidized form, is incorporated into many of the biomolecules in a plant or fungal cell, such as to provide genetic material, membranes, and molecular messengers, among others.

[0003] Inorganic phosphate (Pi) is the primary source of phosphorus for plants. Accordingly, phosphate-based fertilizers offer a cheap and widely used approach to enhancing plant growth. However, phosphate-based fertilizers come from a non-renewable resource that has been projected to be depleted in the next seventy to one hundred years, or sooner if the usage rate increases faster than expected.

[0004] The phosphate-based fertilizers common to modern agriculture generally cannot be used efficiently by cultivated plants, due to several important factors. First, phosphate is highly reactive and can form insoluble complexes with many soil components, which reduces the amount of available phosphorus. Second, soil microorganisms can rapidly convert phosphate into organic molecules that generally cannot be metabolized efficiently by plants, which reduces the amount of available phosphorus further. Third, growth of weeds can be stimulated by phosphate-based fertilizers, which not only reduces the amount of available phosphorus still further but which also can encourage the weeds to compete with the cultivated plants for space and other nutrients. Losses due to the conversion of phosphate into inorganic and organic forms that are not readily available for plant uptake and utilization, and competition from weeds, implies the use of excessive amounts of phosphate fertilizer, which not only increases production costs but also causes severe ecological problems. Therefore, there is an urgent need to reduce the amount of phosphate fertilizer used in agriculture.

[0005] A reduced form of phosphate, phosphite (Phi), is also used in cultivation of plants. It has been shown that treatment with phosphite can increase plant production (as measured by fruit size and biomass) in avocado and citrus fruits. Phosphite may be transported into plants using the same transport system as phosphate and may accumulate in plant tissues for extended periods of time. However, there apparently are no reports of any enzymes in plants that can metabolize phosphite into phosphate, the primary source of phosphorus in plants. Moreover, even during phosphate starvation, phosphite apparently cannot satisfy the phosphorus nutritional requirements of the plant. Accordingly, in spite of similarities to phosphate, phosphite is a form of phosphorus that generally cannot be metabolized directly by plants, and thus is not a plant nutrient. Nevertheless, phosphite “fertilizers” are sold commercially, even though there appears be no proof or even an indication in the scientific literature that plants can assimilate phosphite.

[0006] Phosphite can promote plant growth indirectly. For example, phosphite is used as an anti-fungal agent (a fungicide) on cultivated plants. Phosphite is thought to prevent diseases caused by oomycetes (water molds) on such diverse plants as potato, tobacco, avocado, and papaya, among others. Phosphite thus may promote plant growth, not directly as a plant nutrient, but by protecting plants from fungal pathogens that would otherwise affect plant growth. Nevertheless, phosphite-based fungicides are often labeled as fertilizers. This mislabeling may be encouraged by government regulations that make the approval process shorter and less complex if manufacturers characterize fungicides as fertilizers.

[0007] The proposed mechanisms for phosphate acting as a fungicide are manifold. For example, phosphite may act on fungi by inhibiting phosphorylation reactions through an increment in the accumulation of inorganic pyrophosphate (PPI), which in turn can interrupt phosphate pathways that are metabolically critical. Alternatively, or in addition, phosphite may induce a natural defense response in plants. In any event, the efficacy of phosphite as a fungicide may be influenced by several factors, including environment, type of pathogen, type of plant, and concentration.

[0008] The concentration of phosphite in contact with plants may be a critical factor for phosphite effectiveness because too much phosphite can be toxic to plants. In particular, phosphite may compete with phosphate for entry into plant cells, since phosphite may be transported into plants via the phosphate transport system. Phosphite toxicity may be due to (1) reduced assimilation of phosphate by plants, in combination with (2) an inability to use phosphite as a source of phosphorus by oxidation to phosphate, which causes phosphate accumulation in the plants. Also, phosphite may be sensed in plants as phosphate, which prevents the plants from inducing a phosphorus salvage pathway that promotes plant survival under conditions of low phosphate. Phosphite toxicity affects such diverse plants as Brassica napus, Allium cepa (onion), Zea mays L... (corn), and Arabidopsis thaliana. Accordingly, the exposure of plants to phosphite may need to be controlled very carefully. Therefore, a better system is needed for exploiting the benefits of phosphate to plants while reducing its drawbacks.

[0009] Generation of transgenic plants has been instrumental in creating improved agricultural systems. At least four selection systems have been established for identifying transgenic plants by selective growth. Each selection system is based on resistance to an antibiotic (kanamycin or hygromycin) or an herbicide (glyphosate or phosphinothricin). However, each selection system has disadvantages. For example, each selection system can have problems with toxicity. Also, selection with antibiotics may be inefficient since plants can have alternate resistance mechanisms. Furthermore, except for the selection system using phosphinothricin, none of the selection “systems” provides a “universal” selectable marker for most or all plants. Therefore, a new selectable marker is needed for use in generating transgenic plants.

SUMMARY

[0010] The present disclosure provides a system, including methods and compositions, for making and using transgenic
plants and/or transgenic fungi that metabolize phosphite as a source of phosphorus for supporting growth.

**BRIEF DESCRIPTION OF THE DRAWINGS**

[0011] FIG. 1 is a schematic flowchart of an exemplary method of (i) making a transgenic plant (or fungus) that is capable of metabolizing a reduced form of phosphorus, such as phosphite, as a source of phosphorus for supporting growth, and/or (ii) using, as a selectable marker, a nucleic acid that confers a capability to metabolize a reduced form of phosphorus, such as phosphite, as a source of phosphorus for supporting growth, in accordance with aspects of the present disclosure.

[0012] FIG. 2 is a schematic representation an exemplary nucleic acid for use in the method of FIG. 1, in accordance with aspects of the present disclosure.

[0013] FIG. 3 is a proposed mechanism for oxidation of hypophosphate to phosphate in bacteria using enzymes expressed from the ptxD and htxA genes of *Pseudomonas stutzeri*, in accordance with aspects of the present disclosure.

[0014] FIG. 4 is a schematic representation of an exemplary chimeric gene constructed for use in generating a transgenic plant that metabolizes phosphate to phosphite, in accordance with aspects of the present disclosure.

[0015] FIG. 5 is a schematic diagram of a portion of a strategy followed to create the chimeric gene of FIG. 4, in accordance with aspects of the present disclosure.

[0016] FIG. 6 is a pair of photographs showing exemplary data obtained with the chimeric gene of FIG. 4 used as a selectable marker by selection of transgenic plants for their ability to grow on a phosphate-containing medium in the absence of phosphate, in accordance with aspects of the present disclosure.

[0017] FIG. 7 is a series of photographs of data obtained from growth tests of control and transgenic (ptxD) *Arabidopsis* lines germinated and cultivated in a liquid growth medium, with or without phosphate (Ph) or phosphate (Pi) as the source of phosphorus, in accordance with aspects of the present disclosure.

[0018] FIG. 8 is a bar graph of data obtained from tests of the ability of the *Arabidopsis* lines of FIG. 7 to extract phosphorus from their growth media, with the plants cultivated for 45 days in growth media containing different concentrations of phosphate (Ph) as the source of phosphorus, in accordance with aspects of the present disclosure.

[0019] FIG. 9 is a schematic representation of the distribution of control (WT) and ptxD transgenic (PTXD) *Arabidopsis* plants across a growth substrate, as used for the experiments of FIGS. 10 and 11, in accordance with aspects of the present disclosure.

[0020] FIG. 10 is a photograph of parental and ptxD transgenic plants distributed according to FIG. 9 and tested for growth on a substrate containing phosphate (Pi) as the source of phosphorus, in accordance with aspects of the present disclosure.

[0021] FIG. 11 is a photograph of parental and ptxD transgenic plants distributed according to FIG. 9 and tested for growth on a substrate containing phosphate (Pi) as the source of phosphorus, in accordance with aspects of the present disclosure.

[0022] FIG. 12 is a bar graph of data obtained from tests of the ability of the *Arabidopsis* lines of FIG. 7 to increase in weight when cultivated in the absence or presence of phosphate and/or phosphite as the source of phosphorus, in accordance with aspects of the present disclosure.

[0023] FIG. 13 is a set of photographs of control (WT) and ptxD transgenic lines of *Nicotiana tabacum* (tobacco) 25 days after germination in the presence of phosphate or phosphite as the source of phosphorus, in accordance with aspects of the present disclosure.

[0024] FIG. 14 is a set of photographs of another growth experiment performed with the control and transgenic tobacco lines of FIG. 13, in accordance with aspects of the present disclosure.

**DETAILED DESCRIPTION**

[0025] The present disclosure provides a system, including methods and compositions, for making and using transgenic plants and/or transgenic fungi that metabolize phosphite as a source of phosphorus for supporting growth. The plants and/or fungi optionally may also metabolize hypophosphate as a source of phosphorus. The system disclosed herein may substantially change the way a more reduced form of phosphorus (relative to phosphate), such as phosphite, is utilized as a fertilizer and/or fungicide. The system also may provide a new selectable marker for use in generating transgenic plants and/or fungi. The system further may substantially change the way at least one reduced form of phosphorus is removed from waste water, such as industrial/municipal effluents.

[0026] A nucleic acid is provided. The nucleic acid may be used for generating a transgenic plant and/or fungus. The nucleic acid, which may be termed a construct, may comprise at least one chimeric gene that confers on a plant cell and/or fungal cell a capability to metabolize at least one reduced form of phosphorus to phosphate. In some embodiments, the nucleic acid may comprise a gene that expresses a phosphite dehydrogenase enzyme, a gene that expresses a hypophosphate dehydrogenase enzyme, or both.

[0027] The nucleic acid may comprise a chimeric gene including a coding region and a transcription promoter. The coding region may encode a phosphite dehydrogenase enzyme, such as PtxD from *Pseudomonas stutzeri*, a homolog of PtxD from the same or another bacterial species, or a derivative of either, among others. In some examples, the coding region may be at least 80%, 90%, or 95% (or completely) identical to the PtxD coding sequence of *Pseudomonas stutzeri*. The promoter may be functional in plants, fungi, or both and may be operatively linked to the coding region. The promoter may be heterologous with respect to the coding region. The chimeric gene may be capable of promoting sufficient expression of the enzyme, in a plant or fungal cell containing the nucleic acid, to confer an ability on the cell to metabolize phosphite (Ph) as a phosphorus source for supporting growth, thereby enabling growth of the cell without an external source of phosphate (Pi). The promoter may (or may not) be a plant promoter or a viral promoter of a plant virus and may be capable of promoting the sufficient expression of the enzyme in a plant cell. For example, the promoter, such as a promoter obtained from the PLDZ2 gene of *Arabidopsis thaliana*, may be inducible by low phosphate availability. Alternatively, or in addition, the promoter may be a root-specific promoter. In other cases, the promoter may be constitutive and may correspond to the 35S promoter of Cauliflower Mosaic Virus. In some embodiments, the nucleic acid may include a transcription terminator that is functional in the plant cell and/or fungal cell and that is operatively linked to the promoter and coding region. In some embodiments, the
promoter may be a fungal promoter capable of promoting the 
sufficient expression of the enzyme in a fungal cell.  

[0028] The nucleic acid may provide expression of one or 
more polypeptides that metabolize at least one reduced form 
of phosphorus to phosphate, to enable a transgenic plant (or 
fungus) to use a reduced form of phosphorus as a nutrient. The 
expression of the one or more polypeptides may be heritable. 
For example, the nucleic acid may be integrated into the 
genome of the plant (or fungus). Furthermore, the expression 
of at least one of the polypeptides may be under control of a 
constitutive promoter or an inducible promoter (e.g., inducible 
by low phosphate, such as by use of a promoter from a 
PLD2 gene of Arabidopsis or a plant AtPT1 gene for a high 
affinity phosphate transporter), under control of a tissue-spe 
cific promoter (e.g., leaf-specific or root-specific), or a 
combination thereof, among others. 

[0029] A plant cell or a fungal cell is provided that 
expresses a phosphite dehydrogenase enzyme from a chimeric 
gene. The cell may be isolated from other cells or may be 
associated with other cells in a multi-cellular structure 
(e.g., a plant or a mycelium). The cell may (or may not) also 
express a hypophosphite dehydrogenase enzyme from a chimeric 
gene. Accordingly, the cell may metabolize phosphite, 
hypophosphite, or both, as a phosphorus source for supporting 
growth. In some embodiments, the cell may be a plant cell 
and expression of the phosphite dehydrogenase enzyme, the 
hypophosphite dehydrogenase enzyme (if present), or both 
may be controlled by a root-specific promoter. The plant cell 
may be from any suitable species. For example, the plant cell 
may be a eukaryotic algal cell, such as a Chlamydomonas 
cell. In other cases, the plant cell may be from a species of 
vascular plant. In some embodiments, the cell may be a fungal cell 
that belongs to a species of Trichoderma or that belongs to a 
mycorrhizal species of fungus capable of forming a symbiotic 
relationship with a plant. 

[0030] A transgenic plant (or plant part) is provided that 
expresses a phosphite dehydrogenase enzyme, and, option 
ally, a hypophosphite dehydrogenase enzyme from one or 
more chimeric genes. The plant may, through expression of 
the enzyme(s), metabolize phosphite and/or hypophosphite 
as a source of phosphorus for supporting growth. The plant 
may be a vascular plant, such as a crop plant, for example, a 
species of crop plant selected from the group consisting of 
misit, soybean, rice, potatoes, tomatoes, sugarcane, and wheat. A seed that germinates to produce the transgenic plant 
also is provided. 

[0031] A method of reducing fungal infections in plants is 
provided. A plurality of fungal cells may be applied to a seed 
form of plants, the plants themselves, soil in which the plants 
are or will be disposed, or a combination thereof. The fungal 
cells may express a phosphite dehydrogenase enzyme from a 
chimeric gene and may belong to a species of Trichoderma. 

[0032] A plant associated with a plurality of fungal cells to 
form mycorrhizae is provided. The fungal cells may express 
a phosphite dehydrogenase enzyme from a chimeric gene. 
The fungal cells may render the plant capable of growth on 
phosphate (and/or hypophosphate) as a phosphorus source by 
oxidizing phosphate to phosphate. 

[0033] A method is provided of fertilizing a crop plant 
using hypophosphite and/or phosphate as a phosphorus 
source for supporting growth. The crop plant may express a 
phosphite dehydrogenase enzyme, a hypophosphite dehydro 
genase enzyme, or both. Alternatively, or in addition, the crop 
plant may form mycorrhizae with a plurality of fungal cells 
expressing a phosphite dehydrogenase enzyme, a hypophos 
phite dehydrogenase enzyme, or both. At least one reduced 
form of phosphorus, such as phosphate and/or hypophosphate, 
may be applied to the plant and/or to soil adjacent the plant, 
such that the reduced form is metabolized to phosphate by the 
plant and/or the mycorrhizae to support growth and produc 
tivity of the plant. 

[0034] A method is provided of treating liquid waste (e.g., 
an effluent) to lower its content of reduced phosphorus. Con 
tact is created between (i) water containing hypophosphate 
and/or phosphate as a contaminant and (ii) a plurality of plant 
cells and/or fungal cells expressing a phosphite dehydrogen 
ase enzyme, a hypophosphite dehydrogenase enzyme, or both, 
such that at least a portion of the hypophosphate and/or 
phosphate is oxidized to phosphate and/or phosphate. In some 
cases, the contact may be created between the water and a 
plurality of vascular plants expressing one or both of the 
enzymes. The method may provide a bioremediation system 
for rivers, reservoirs, soils, holding tanks, and the like that 
are contaminated due to industrial manufacturing. For example, 
phosphate is a common polluting agent in rivers and lakes near 
industrial sites, such as manufacturers of optical discs (e.g., 
DVDs and CDs) that use hypophosphate to reduce metal ions 
in chemical plating processes. Transgenic plants and/or fungi 
disclosed herein thus may help remove hypophosphate and/or 
phosphate from contaminated water by taking up and convert 
ing the hypophosphate and/or phosphate into phosphate. Use 
of plants and/or fungi may be more efficient than using a 
bacterial-based system. 

[0035] A method is provided of utilizing a coding sequence 
for a phosphite dehydrogenase as a selectable marker for 
production of a transgenic plant. The method may be used to 
obtain a plant transformed with a nucleic acid encoding a 
phosphite dehydrogenase enzyme that is expressible from 
the nucleic acid as a selectable marker. Plant cells and a com 
position including the nucleic acid may be contacted under 
conditions that promote introduction of the nucleic acid into 
the plant cells. The plant cells may be cultured in a medium 
containing phosphite as a primary or exclusive phosphorus 
source for growth of the plant cells. Selection may be per 
fomed of transformed plant cells produced by the steps of 
contacting and culturing and that express the phosphite dehy 
drogenase enzyme as evidenced by growth in the medium. At 
least a portion of the transformed plant cells may be regener 
ated into a transgenic plant. 

[0036] The transgenic plants disclosed herein may provide 
substantial benefits. For example, in some cases, the plants 
may metabolize phosphate using NAD+ as an electron accept 
or, to generate NADH and phosphate, which are both useful 
molecules for the plant. The transgenic plants also or alter 
atively may provide development of a new agricultural system 
based on phosphate. Phosphate may be less reactive in the soil 
than phosphate and therefore may create fewer insoluble 
compounds that the plant cannot utilize. Also, since most soil 
microorganisms are unable to metabolize phosphate, less of 
the phosphate (relative to phosphate) is converted into organic 
forms that plants cannot utilize. Furthermore, phosphate may 
have less impact on the bacterial ecosystem around the plants 
related to phosphate. Competition from weeds also may be 
reduced substantially since the weeds should not be able to 
utilize phosphate. The use of phosphate thus should decrease 
fertilizer costs and reduce the negative impact of fertilizer on 
the environment.
The transgenic plants disclosed herein also may offer increased effectiveness of phosphite as a fungicide, while acting as a fertilizer on the transgenic plants. When used as a fungicide on non-transgenic plants, phosphite generally needs to be used very carefully, to avoid plant toxicity. However, in the transgenic plants disclosed herein, phosphite may be metabolized by the plant to become non-toxic.

The system disclosed herein may provide substantial advantages for generating transgenic plants. A selectable marker of the system may function at least substantially universally in plants. Furthermore, the selective agent (e.g., hypophosphite or phosphite) may be nontoxic for transgenic plants, since the reaction products may be innocuous (e.g., NADH and phosphate), and also may be less expensive than in other selection schemes.

Further aspects of the present disclosure are provided in the following sections: (I) definitions, (II) generation of transgenic plants and fungi, (III) use of transgenic plants and fungi, and (IV) examples.

I. DEFINITIONS

The various terms used in the present disclosure generally each have a meaning recognized by those skilled in the art, consistent with the context in which each term is used. However, the following terms may have additional and/or alternative meanings, as described below.

Plant—a member of the Plantae kingdom of eukaryotic organisms, which may be described as a tree, bush, grass, shrub, herb, vine, fern, moss, a eukaryotic alga, or a combination thereof, among others. A plant typically possesses cellulose cell walls and is capable of carrying out photosynthesis. The plant may be a vascular plant. In some embodiments, the plant may be an annual or a perennial. The plant may be a flowering plant, such as a monocotyledon or a dicotyledon. In some embodiments, the plant may produce a grain, tuber, fruit, vegetable, nut, seed, fiber, or a combination thereof, among others. Furthermore, the plant may be a crop plant, which may be cultivated in a field. Exemplary crop plants that may be suitable for generation of transgenic plants according to the present disclosure include tobacco (e.g., N. tabacum), potato, maize, rice, wheat, alfalfa, soybean, tomato, sugarcane, and the like.

Plant part—any portion of a plant that is less than a whole plant and that includes at least one plant cell. A plant part thus may be a plant tissue, such as leaf tissue, root tissue, stem tissue, shoot tissue, callus tissue, flower tissue, or any combination thereof, among others. A plant part may be an isolated plant cell or a colony or set of plant cells. A plant cell may be a protoplast or may include a cell wall, among others.

Transgenic—comprising a nucleic acid construct. The construct may be integrated into an organism’s (and/or cell’s) genome (e.g., nuclear or plastid genome), in any subset or at least substantially all of the cells of the organism. For example, the construct may be present in a plant’s germine. Accordingly, the construct may be heritable, that is, inherited by at least one or more members, or at least substantially all members, of a succeeding generation of the organism, or in descendants of a cell. A plant or fungus (or plant or fungal part (e.g., a cell)) that is “transformed” with a construct has been modified to contain the construct in the current generation or in any preceding generation(s) of the plant or fungus (or plant or fungal part). A transgenic plant may be provided by a seed that germinates to form the transgenic plant. Also, a transgenic plant may produce one or more seeds that germinate to produce transgenic progeny plants.

Nucleic acid—a compound comprising a chain of nucleotides. The chain may be composed of any suitable number of nucleotides, such as at least about 10, 100, or 1000, among others. A nucleic acid may be termed a polynucleotide, and may, for example, be single-stranded, double-stranded, or a combination thereof.

Gene—a nucleic acid or segment thereof that provides an expressible unit for expression of a polypeptide and/or a functional RNA (e.g., a messenger RNA, an interfering RNA, or an enzymatic RNA, among others). A gene thus may include (a) a coding region (also termed a coding sequence, which may be continuous or interrupted (such as by one or more introns)) to define the sequence of the polypeptide and/or functional RNA, (b) at least one transcription promoter (also termed a promoter sequence) and, (c) optionally, at least one transcription terminator (also termed a termination sequence), with the transcription promoter and the transcription terminator operatively linked to the coding region. A gene optionally may include one or more other control regions and/or untranslated regions, such as at least one 5’ untranslated region, 3’ untranslated region, intron, or any combination thereof, among others.

Promoter—a nucleic acid region that controls (i.e., promotes, regulates, and/or drives) transcription of a gene to produce a primary transcript and/or a messenger RNA. A promoter may operate, for example, by determining, at least in part, the rate of transcriptional initiation of a gene by RNA polymerase. The promoter also or alternatively may determine the rate of transcriptional elongation after transcription is initiated. The promoter may be functional in plants and/or fungi and thus may be a plant promoter and/or a fungal promoter.

Chimeric gene—a gene with sequence elements, such as a transcription promoter and a coding region, that are heterologous with respect to one another. The term “heterologous” means that the sequence elements (e.g., the promoter and coding region) originate and/or are derived from respective different sources, such as different species of organisms. A chimeric gene also may comprise a transcription terminator, which may originate from a source distinct from the coding region, and from the same source as, or a source distinct from, the promoter. Exemplary terminators that may be used in the chimeric genes include the 35S terminator of Cauliflower Mosaic Virus, the nopaline synthase terminator of Agrobacterium tumefaciens, or the like.

Construct—a nucleic acid created, at least in part, using techniques of genetic engineering. A construct thus may be termed a nucleic acid construct.

Expression—a process by which a product, namely, an RNA and/or a polypeptide, is formed from information provided by a nucleic acid and/or gene, generally in the form of DNA. Accordingly, the nucleic acid/gene may be expressed to form an RNA and/or polypeptide, which means that the RNA and/or polypeptide is expressed from the nucleic acid/gene.

Reduced forms of phosphorus—any phosphorus-containing compounds and/or ions in which phosphorus has an oxidation state of less than +5, such as +3 or +1. Accordingly, reduced forms of phosphorus may, for example, include phosphite and hypophosphite, among others. A reduced form of phosphorus may be abbreviated “RP.”
[0051] Phosphate—phosphoric acid (H₃PO₄), its dibasic form (H₃PO₄⁻), its monobasic form (HPO₄²⁻), its triply ionized form (PO₄³⁻), or any combination thereof. Phosphate may be provided as any suitable phosphate compound or combination of phosphate compounds. Exemplary forms of phosphate include phosphate salts of sodium, potassium, lithium, rubidium, cesium, ammonium, calcium, or magnesium, or any combination thereof, among others. In phosphate, four oxygens are bonded directly to a phosphorus atom. Phosphate also or alternatively may be called “orthophosphate” and/or “inorganic phosphate” and may be abbreviated as “Pi.” Phosphate is distinct from “organophosphate,” which is an organic version of phosphate in which one or more of the phosphate oxygens are bonded to organic moieties, generally to form a phosphate ester.

[0052] Phosphorus—phosphoric acid (H₃PO₄), its conjugate base/singly ionized form (H₂PO₄⁻), or its doubly ionized form (HPO₄²⁻), or any combination thereof. In phosphorus, three oxygens and one hydrogen are bonded directly to a phosphorus atom. Phosphorus may be provided as any suitable phosphate compound or combination of phosphate compounds. Exemplary forms of phosphorus include phosphorus salts of sodium, potassium, lithium, rubidium, cesium, ammonium, calcium, or magnesium, or any combination thereof, among others. Phosphorus can be oxidized to phosphorus. Phosphorus also or alternatively may be called “inorganic phosphorus” and may be abbreviated as “Pi.” Phosphorus is distinct from “organophosphate,” which is an organic version of phosphorus in which one or more of the phosphorus oxygens are bonded to organic moieties, generally to form a phosphorus ester.

[0053] Hypophosphite—hypophosphorous acid (H₂PO₃) and/or its conjugate base (H₂PO₃⁻), which may be provided as any suitable hypophosphite compound or combination of hypophosphite compounds. In hypophosphite, two oxygens and two hydrogens are bonded directly to a phosphorus atom. Exemplary forms of hypophosphite include hypophosphite salts of sodium, potassium, lithium, rubidium, cesium, ammonium, or a combination thereof, among others. Hypophosphite can be oxidized to phosphorus and/or to phosphate. Hypophosphite also or alternatively may be called “inorganic hypophosphite” and may be abbreviated as “H₂Pi.”

[0054] Nutrient—any substance that is metabolized to promote growth and reproduction, and/or is required for survival.

[0055] Fertilizer—any composition that includes one or more nutrients for plants (and/or fungal associated with the plants).

[0056] External Source—a supply that is outside of a plant and accessible to the plant, generally by contact with the plant. Exemplary external sources that may be suitable for the transgenic plants described herein may include an external source of phosphorus, an external source of phosphate, or an external source of reduced phosphorus, among others.

[0057] Selectable Marker—a construct or segment thereof and/or a gene that confers a growth advantage on a plant or plant part (and/or a fungus and/or fungal cell) that contains the construct/gene, when growth of the plant or plant part (and/or fungus and/or fungal cell) is tested by contact with a suitable culture medium.

[0058] Effluent—water carrying and/or mixed with waste material. An effluent may or may not be flowing. An exemplary effluent may, for example, be industrial refuse and/or sewage, which may be combined with a larger body of water, such as a stream, river, pond, lake, swamp, wetland, or the like.

[0059] Remediation—any process that modifies water (e.g., waste water and/or an effluent) to a more desired composition, such as to make the water less toxic, more environmentally friendly, in better conformation with government standards, etc.

[0060] Enzyme that oxidizes a reduced form of phosphorus—an enzyme that catalyzes or promotes oxidation of a reduced form of phosphorus (e.g., with an oxidation state of +1 or +3) to a more oxidized state (e.g., +1 to +3, +1 to +5, and/or +3 to +5). For example, the enzyme may oxidize hypophosphite to phosphate, phosphate to phosphate, and/or hypophosphite to phosphate, among others. For convenience, the enzyme may be termed an “oxidase,” since it catalyzes/ promotes an oxidation reaction, or may be called a “phosphorus oxidoreductase” or “enzyme of reduced phosphorus metabolism,” and may be abbreviated, for convenience herein, as “RP-OxR.” Exemplary enzymes that oxidize a reduced form of phosphorus may include a phosphate dehydrogenase enzyme (which may, for example, be called NAD: phosphate oxidoreductase, phosphonate dehydrogenase, NAD-dependent phosphate dehydrogenase, or the like), a hypophosphite dehydrogenase (e.g., hypophosphite:2-oxoglutarate oxidoreductase), or the like. The enzyme may oxidize a reduced form of phosphorus using any suitable cofactor (s), coenzyme(s), and/or substrate(s) present in and/or near a cell. Furthermore, the enzyme may originate and/or be derived from bacteria, fungi, plants, or animals.

[0061] Phosphate dehydrogenase enzyme—an enzyme that catalyzes oxidation of phosphate to phosphate. The enzyme generally catalyzes the oxidation with sufficient efficiency to enable growth of a plant cell and/or fungal cell in the presence of phosphate as a phosphorus source to support growth. The enzyme may be of bacterial origin. The enzyme may be a PtxD polypeptide (i.e., PtxD) or PtxD-like, which is any polypeptide that is capable of catalyzing oxidation of phosphate to phosphate and that is (a) at least 90%, 95%, or completely identical to PtxD (SEQ ID NO:1; GenBank: AAC71709.1) of Pseudomonas stutzeri WM 88, (b) a derivative of PtxD of SEQ ID NO:1, (c) a homolog (i.e., a paralog or ortholog) of PtxD (SEQ ID NO:1) from the same or a different bacterial species, or (d) a derivative of (c). Homologs of PtxD (SEQ ID NO:1) have substantial similarity to PtxD of Pseudomonas stutzeri, which may, for example, be determined by the blastp algorithm (e.g., program BLASTP 2.2.18), as described in the following two references, which are incorporated herein by reference: Stephen F. Altshul et al. (1997), “Gapped BLAST and PSI-BLAST: a new generation of protein database search programs,” ConStructs Res. 25:3389-3402; and Stephen F. Altshul et al. (2005) “Protein database searches using compositionally adjusted substitution matrices,” FEBS J. 272:5101-5109. Examples of substantial similarity include at least 50%, 60%, 70%, 80% sequence identity, a similarity score of at least 200 or 250, and/or an E-Value of less than 1e-40, 1e-60, or 1e-80, among others, using the blastp algorithm, with optimal alignment and, if needed, introduction of gaps.

[0062] Exemplary homologs of PtxD of Pseudomonas stutzeri may be provided by Actinobacter radiouriensis SK82 (SEQ ID NO:2; GenBank: EET83888.1); Alcaligenes faecalis (SEQ ID NO:3; GenBank AA127791.1); Cyanobdaceae sp. CCY0110 (SEQ ID NO:4; GenBank: EAZ89932.1);
Gallionella ferruginea (SEQ ID NO:5; GenBank EES62080.1); Janthinobacterium sp. Marseille (SEQ ID NO:6; GenBank ABR91484.1); Klebsiella pneumoniae (SEQ ID NO:7; GenBank ABR80271.1); Marinobacter algicola (SEQ ID NO:8; GenBank EDM49754.1); Methylobacterium extorquens (SEQ ID NO:9; NCBIYIP_003606079.1); Nostoc sp. PCC 7120 (SEQ ID NO:10; GenBank BAB77417.1); Oxalobacter formigenes (SEQ ID NO:11; NCBI ZP_04579760.1); Streptomyces svices (SEQ ID NO:12; GenBank EDY59675.1); Thioalkalivibrio sp. HL-ElGR7 (SEQ ID NO:13; GenBank ACL72000.1); and Xanthobacter flavus (SEQ ID NO:14).

[0063] GenBank ABG73582.1, among others. Further aspects of PtxD homologs are described in U.S. Patent Application Publication No. 2004/0091985 (the ‘985 publication”) to Metcalf et al., which is incorporated herein by reference. The phosphate dehydrogenase may have an amino acid sequence with at least 50%, 60%, 80%, 90%, or 95% or 100% sequence identity to one or more of SEQ ID Nos: 1-14.

[0064] Exemplary derivatives of PtxD of Pseudomonas stutzeri that may be suitable are described in the ‘985 publication and in U.S. Pat. No. 7,402,419 to Zhao et al., which is incorporated herein by reference. The derivatives may provide, for example, altered cofactor affinity/specifcity and/or altered thermostability.

[0065] The phosphate dehydrogenase enzyme may contain a sequence region with sequence similarity or identity to any one or any combination of the following consensus motifs: a NAD-binding motif having a consensus sequence of VĢILG-MGAIQK (SEQ ID NO:15); a conserved signature sequence for the D-isomer specific 2-hydroxycacid family with a consensus sequence of XPAGALLVNP<CRGWSW (SEQ ID NO:16), where X is K or R, or a shorter consensus sequence within SEQ ID NO:16 of RGWSW (SEQ ID NO:17); and/or a motif that may enable hydrogenases to use phosphate as a substrate, with a general consensus of GWQPQ<SGYTCGL (SEQ ID NO:18), but that can be better defined as GWX, PX, X, YX, X<GR, GL (SEQ ID NO:19), where X is R, Q, T, or K; X2 is A, V, Q, R, K, H, or E; X3 is L or F; X4 is G, F, or S; and X5 is T, R, M, L, A, or S. Further aspects of consensus sequences found by comparison of PtxD and PtxD homologs are described in U.S. Patent Application Publication No. 2004/0091985 to Metcalf et al., which is incorporated herein by reference.

[0066] A phosphate dehydrogenase enzyme may (or may not) be a NAD-dependent enzyme with high specificity for phosphate as a substrate (e.g., Km ~50 μM) and/or with a molecular weight of about 36 kilodaltons. The dehydrogenase enzyme may, but is not required to, act as a homodimer, and/or have an optimum activity at 35°C and/or a pH of about 7.25-7.75.

[0067] Hypophosphite dehydrogenase—an enzyme that catalyzes oxidation of hypophosphite to phosphate. The enzyme may, for example, be a bacterial enzyme, such as HtXA from Pseudomonas stutzeri WM 88 (SEQ ID NO:20; GenBank AAC71711.1) or Alcaligenes faecalis (GenBank AAT17775.1).

[0068] An HtXA polypeptide may, but is not required to, be a Fe-dependent enzyme with high specificity for hypophosphite as a substrate (e.g., Km ~0.54-0.62 mM) and/or with a molecular weight of about 32 kilodaltons. The HtX polypeptide may, but is not required to, act as a homodimer, and/or to have an optimum activity at 27°C and/or a pH of about 7.0.

[0069] A polypeptide or HtX coding region—a sequence encoding a PtxD polypeptide (i.e., a phosphite dehydrogenase enzyme) or an HtX polypeptide (i.e., a hypophosphite dehydrogenase enzyme), respectively. An exemplary PtxD coding region is provided by ptxD of Pseudomonas stutzeri (SEQ ID NO:21; GenBank AF601070.1). In other examples, a ptxD-like coding region with at least 50%, 60%, 80%, 90%, 95% or complete identity to one or more of the polypeptides of SEQ ID NO:1-14 may be utilized.

II. GENERATION OF TRANSGENIC PLANTS

NAD FUND

[0070] The present disclosure provides methods of making transgenic plants and transgenic fungi that have a modified metabolism of phosphorus. The methods may be used to create, as a primary goal, transgenic plants and/or fungi (or at least one plant or fungal cell) carrying a nucleic acid construct encoding an enzyme of phosphorus oxidation, such as for better growth on a phosphate and/or hypophosphate fertilizer in agriculture. Alternatively, or in addition, the methods may be used to create, as a primary goal, transgenic plants and/or fungi carrying a construct including another gene of interest, with the construct also including a gene encoding an enzyme of phosphorus oxidation acting as a selectable marker to facilitate identification and/or isolation of the transgenic plants or fungi. The method steps disclosed in this section and elsewhere in the present disclosure may be performed in any suitable combination, in any suitable order, and repeated any suitable number of times.

[0071] FIG. 1 shows a schematic flowchart of an exemplary method 20 of (i) making a transgenic plant (and/or fungus) that metabolizes at least one reduced form of phosphorus (“RP”) to phosphate and/or (ii) using, as a selective marker, a nucleic acid that confers a capability to metabolize a reduced form of phosphorus to phosphate.

[0072] At least one construct (or nucleic acid) may be obtained, as indicated at 22. At the least one construct 23 may include at least one first gene 24, which may be at least one chimeric gene encoding at least one enzyme (“RP-OXRe”), such as a phosphite dehydrogenase, that catalyzes oxidation of a reduced form of phosphorus, such as oxidation of phosphate to phosphate. Construct 23 also may include at least one second gene 26 (“Gene2”), which also may (or may not) be a chimeric gene. In some embodiments, the at least one first gene may be a pair of genes encoding at least two distinct polypeptides that each catalyze oxidation of at least one reduced form of phosphorus. The at least two polypeptides may act to oxidize phosphorus substrates in series (e.g., catalyzing oxidation of hypophosphate to phosphate with a first polypeptide and then catalyzing oxidation of phosphate to phosphate with a second polypeptide). In some examples, the at least one second gene may include a selectable marker for use in plants and/or fungi and/or may include a gene(s) of primary interest, among others. First gene 24 and second gene 26 may be linked, such as being present in the same polynucleotide, or may be present on respective discrete polynucleotides. Each gene may be constructed, at least in part, outside of plants, such as in vitro and/or in a microorganism (e.g., bacteria, yeast, etc.). Furthermore, each gene may be capable of expression in plants, fungi, or both that contain the gene.
The at least one gene (24 and/or 26) may be introduced into at least one recipient plant 28 (or fungus), plant or fungal tissue, and/or plant or fungal cell, indicated at 30. The at least one plant, tissue, or cell, prior to introduction of the at least one gene, may at least substantially require phosphate as an external source of phosphorus for growth. In other words, the plant, tissue, or cell may be at least substantially unable to metabolize directly a reduced form of phosphorus (such as phosphate) as a nutrient. 

Introduction of the at least one gene may be performed by contacting (a) the at least one plant/fungus, tissue, and/or cell and (b) a composition (a modifying agent) that includes a nucleic acid comprising the at least one gene, under conditions that encourage introduction of the nucleic acid into the plant, tissue, and/or cell. The step of contacting may be performed by any mechanism that creates contact between the at least one plant/fungus, tissue, and/or cell and the composition. The composition may, for example, include one or more polynucleotides containing the at least one gene, with the polynucleotides in and/or on a carrier. Exemplary carriers that may be suitable include biological cells (e.g., bacterial cells), plant viruses, inert particles, lipids (in micelles and/or liposomes), and/or the like. Exemplary contact created with a composition including the gene may include contacting a plant, plant tissue, or plant cells with a bacterium (e.g., an Agrobacterium species, such as Agrobacterium tumefaciens or Agrobacterium rhizogenes) carrying the at least one gene, or with one or more projectiles carrying the at least one gene (e.g., particles coated with a polynucleotide including the at least one gene and fired at the plant, tissue, or cell from a gene gun). More generally, introducing the at least one gene may be performed on a plant/fungus, plant or fungal tissue, and/or plant or fungal cells by injection, injection, particle bombardment, electroporation, cell fusion, lipofection, calcium-phosphate mediated transfection, any combination thereof, or the like.

Transgenic candidates 34 (also termed transformation candidates) may be generated, indicated at 36, by and/or after creating contact between the plant, tissue, and/or cells and the composition. The transgenic candidates may be the plant/fungus, tissue, and/or cells used for contacting, or may be derived from any later generation (i.e., progeny or division products) of the plant/fungus, tissue, and/or cells. In any event, the transgenic candidates may be seeds, plants, tissues, explants, isolated cells, cell colonies, aggregates, and/or the like.

Selection for growth (i.e., a growth advantage) of transgenic candidates 34 in a selective medium 37 may be performed, indicated at 38. Candidates 34 that possess a growth advantage on the selective medium, such as transgenic plants 40, generally are substantially larger than the other candidates. In other examples, the selection may be performed with transformed plant (or fungal) cells and may include culturing the plant (or fungal) cells in a selective medium. In these cases, culturing the cells may permit selection and/or isolation of one or more colonies of cells formed by the step of culturing. The colonies may be expressing an enzyme, such as a phosphate dehydrogenase, that oxidizes a reduced form of phosphorus, as evidenced by formation of the colony in the medium.

Any suitable selective medium 37 may be utilized according to a selectable marker provided by the at least one first gene and/or a selectable marker (second) gene that was introduced. For example, the selective medium may include a reduced form of phosphorus, such as hypophosphite and/or phosphate. The reduced form of phosphorus may be a primary external source of phosphorus and/or may be at least substantially the only phosphorus present in the medium, which means that the medium is at least substantially without phosphate (i.e., a low phosphate or no phosphate medium). Alternatively, or in addition, the selective medium may include another selectable agent, such as hygromycin or phosphinothricin, if the selection for growth is based on second gene 26 (e.g., bar or hph). In some embodiments, the selection may be performed based on a marker gene (NCBI NM_123703.1; GeneID:834355) or the promoter of the MtPT1 gene or MtPT2 gene (GenBank: AF000354.1 and
AF000355.1) of *Medicago truncatula* (Xiao, et al, Plant Biology, 2006, 8:439-449, the disclosure of which is incorporated herein by reference).

Furthermore, first gene 24 may include transcribed but untranslated regions, such as a 5' leader sequence and/or 5' untranslated region 58, a 3' untranslated region 60, and/or one or more introns 62. First gene 24 may be provided by nucleic acid 23 that includes any other suitable sequences, such as at least one second gene 26, replication control sequences for replication in bacteria or another non-plant species, a selectable marker for another species (e.g., bacteria), or any combination thereof, among others. In some embodiments, nucleic acid 23 may be any combination of linear or circular (i.e., a closed loop), at least mostly double-stranded or at least mostly single-stranded, and DNA or RNA.

FIG. 3 shows a proposed mechanism 70 for oxidation of hypophosphate to phosphate in bacteria catalyzed by enzymes expressed from ptxD and htxA genes. The proposed mechanism presented here is for illustration purposes only, and is not intended to limit the definition of any of the components shown, such as ptxD or htxA genes or PtxD or HtxA polypeptides, or limit the scope of the invention.

Mechanism 70 shows a hypophosphate ion 72 may be oxidized to a phosphate ion 74 by the action of an HtxA polypeptide 76 (hypophosphate:2-oxoglutarate dioxygenase) encoded by an htxA gene. HtxA polypeptide 76 may use Fe^{2+} 78 as a cofactor and 2-oxoglutarate 80 as an electron donor. In addition, enzyme 76 may convert 2-oxoglutarate 80 to succinate 82, and molecular oxygen 84 to carbon dioxide 86.

Phosphate ion 74, in turn, may be oxidized to a phosphate ion 88 by the action of a PtxD polypeptide 90. Polypeptide 90 may use NAD^+ 92 as an electron acceptor that is reduced to NADH 94.

III. USE OF TRANSGENIC PLANTS AND FUNGI

The transgenic plants disclosed herein may be used for any suitable purpose. Exemplary purposes include production of a commercial product (e.g., food, wood, pharmaceuticals, dyes, oils, lubricants, inks, rubber, cotton, fibers, biofuels, etc.), and/or water remediation. Water remediation, as used herein, generally includes any removal of pollution or at least one contaminant from a body of water and/or soil that has contacted contaminated water.

A method of water remediation is provided. Any transgenic plant, fungi, or both disclosed herein may be used in the method. The method steps disclosed in the section and elsewhere in the present disclosure may be performed in any suitable order, in any suitable combination, with each step performed any suitable number of times.

One or more transgenic plants may be obtained. The transgenic plants may have been transformed, in the current generation or in any preceding generation, with a construct that confers a capability of oxidizing at least one reduced form of phosphorus.

Obtaining the one or more transgenic plants may include any suitable procedures. For example, the step of obtaining may include introducing into the current generation or, more typically, an earlier generation of the transgenic plants, one or more constructs encoding one or more polypeptides that oxidize a reduced form of phosphorus to phosphate.

The one or more transgenic plants may be contacted with water to be remediated. Contacting plants to water may include any combination of bringing the water to plants, bringing the plants to water, and germinating seeds for the plants in contact with the water. The water may be substantially stationary or may be flowing with respect to the plants. In some embodiments, the step of contacting may include contacting the plants with an industrial and/or municipal effluent.

IV. EXAMPLES

The following examples describe selected aspects and embodiments of the present disclosure, such as exemplary methods of making transgenic plants (including algae) and transgenic fungi that metabolize phosphate as a source of phosphorus, exemplary transgenic plants and transgenic fungi, and exemplary methods of using a gene encoding a phosphite dehydrogenase enzyme as a selectable marker for selection of transgenic plants and transgenic fungi. The examples are presented for illustration only and are not intended to define or limit the scope of the present disclosure.

Example 1

Exemplary Generation of Transgenic Plants Expressing a Bacterial Phosphate Dehydrogenase Enzyme

This example describes an exemplary method of generating transgenic plants with modified phosphorus metabolism; see FIGS. 4-6.

FIG. 4 shows an exemplary nucleic acid, a chimeric gene 100, constructed for use in generating a transgenic plant that metabolizes phosphite to phosphate, to permit growth on phosphate in the absence of phosphate. The gene was constructed using the Gateway® system (Gateway® Technology, 2003, Invitrogen) as described in the following paragraphs.

Gene 100 includes a 35S promoter sequence 102 from Cauliflower Mosaic Virus (CaMV) operatively linked to a coding sequence 104 (SEQ ID NO:21) from ptxD of *Pseudomonas stutzeri* WM88. Expression of gene 100, indicated at 106, to produce the PtxD polypeptide (a phosphite dehydrogenase enzyme) is thus controlled/driven by 35S promoter 102. Gene 100 optionally may include a terminator sequence 107, such as a 35S terminator from CaMV, disposed downstream of and operatively linked to the coding sequence (and promoter sequence). The gene further may include a 5' untranslated sequence disposed between the promoter sequence and the ptxD coding sequence, and/or a 3' untranslated sequence disposed between the ptxD coding sequence and the termination sequence. Furthermore, the gene may include an intron that is transcribed along with ptxD coding sequence and that is removed from the transcript by post-transcriptional splicing.

FIG. 5 shows a schematic diagram of a portion of a strategy used to create gene 100 of FIG. 4. A forward primer 108 (SEQ ID NO:22) and a reverse primer 110 (SEQ ID NO:23) were synthesized. Each primer has a hybridization region 112, 114 that hybridizes, indicated at 116, 118 in the lower part of the figure, in either a forward or reverse orientation to the ends of ptxD coding region 104. Each primer has an attB site 120, 122 (attB1 or attB2) positioned 5-prime to hybridization region 112 or 114. The primers were utilized to amplify coding sequence 104 from a plasmid (pWPM302) using the polymerase chain reaction, to create a ptxD amplified product. A construct of the expected size, about 1000 base pairs, was generated, as detected by gel electrophoresis and staining of the amplified product. The primers alterna-
tively may be designed to amplify additional untranslated sequences from upstream and/or downstream of the ptxD coding sequence.

The ptxD amplified product next was incorporated into a plasmid vector using site-specific recombination provided by the Gateway® system. The amplified product was recombined with plasmid pDONR221, via the attP1 and attP2 sites of pDONR 221 and the attB1 and attB2 sites of the amplified product, to create a ptxD-derived of pDONR221, “initial clone” pDONR221. The initial clone has the full-length ptxD coding sequence oppositely flanked by attL1 and attL2 sites.

The ptxD sequence of the initial clone then was moved into an acceptor vector by further site-specific recombination to produce an expression construct, pB7WG2D-pxtD. The acceptor vector was pB7WG2D.1, which includes, in order around the vector, (1) a 3SS promoter, (2) attR1 and attR2 sites disposed downstream of the 3SS promoter, (3) a 3SS terminator, (4) a “bar” gene (confers phosphonothiocin resistance) as a selectable marker in plants, (5) a gene, SmSp⁶, as a selectable marker in bacteria, particularly Agrobacterium (confers spectinomycin (Sp) and streptomycin (Sm) resistance), and (6) an EgfpER gene. Gateway®-system directed recombination formed expression clone (pB7WG2D-pxtD) including gene 100 (see FIG. 4), bar, SmSp⁶, and EgfpER.

The expression construct was used to transform electrocompliant Agrobacterium tumefaciens by electroporation. A transformed Agrobacterium clone carrying the expression construct was selected for subculture. The transformed Agrobacterium clone was used to transform Arabidopsis thaliana (ecotype Col-0) (generally described herein as “wild-type” (WT)) using a modified floral dip method. Transformed T0 progeny were selected using phosphonothiocin resistance. In particular, screening was performed with MS 0.1x media containing phosphonothiocin (20 mg/L). Twenty-eight resistant lines were identified through PCR amplification of the ptxD gene. Each resistant line was analyzed via T1 progeny using MS 0.1x media containing phosphonothiocin (20 mg/L) to look for 3:1 (resistant:sensitive) segregation of the T1 progeny, to identify plants that showed Mendelian transmission of the ptxD gene. Ten homozygous ptxD transgenic plants were established from T2 progeny of T1 progeny exhibiting 3:1 transmission.

The ptxD transgenic plants were tested for their ability to grow in media containing only phosphate (e.g., about 0.1 to 5 mM) as an external source of phosphorus. Control plants showed no substantial growth in this media (i.e., showed growth limited to the internal phosphorus reserves accumulated in the seed), whereas the transgenic plants grew efficiently, thereby demonstrating that the transgenic plants are able to metabolize a reduced form of phosphorus (phosphate) as a source of phosphorus.

The ptxD expression construct also was used to provide a selectable marker for selection of transgenic plants with modified phosphorus metabolism. Wild-type Col-0 plants were transformed using Agrobacterium containing the ptxD expression construct. T0 progeny (seeds) were plated on a medium with phosphate (5 mM) as the source of phosphorus. FIG. 6 shows exemplary data for growth of the TO progeny, relative to wild type plants, on the phosphate medium. Transgenic plants 130 (circled in the right panel) have a substantial growth advantage relative to wild type plants 132 (left panel) and relative to other T0 progeny 134 that apparently were not transformed with the expression construct and/or that did not efficiently express the PtxD polypeptide from the introduced construct.

Further aspects of generating transgenic plants with modified phosphorus metabolism are described in U.S. Provisional Patent Application Ser. No. 61/199,784, filed Nov. 19, 2008, which is incorporated herein by reference.

Example 2

Characterization of Arabidopsis Plants Expressing PtxD

This example presents an investigation of the growth characteristics of the parental (“wild-type” (WT) or control) Arabidopsis line, Col-0, and two of the transgenic Arabidopsis lines described in Example 1 and comprising the ptxD expression construct of Example 1; see FIGS. 7-12.

Two transgenic Arabidopsis lines, dubbed PTXD-3 and PTXD-5, were prepared and isolated as described in Example 1. Each line is homozygous for the ptxD expression construct of Example 1.

The parental line and the PTXD-3 and PTXD-5 transgenic lines were tested for the ability to grow on a liquid medium, with or without inorganic phosphate (Pi) as the source of phosphorus. Seeds from the parental and transgenic lines were germinated in liquid media and tested for growth. In the absence of phosphate (and phosphate), neither the parental line nor the transgenic lines showed significant growth beyond germination. (Each line exhibited paltry growth for a short time, which apparently was permitted by phosphate stores in the seeds, which are quickly depleted from the seeds.) In contrast, both the parental (WT) line and the transgenic lines grew efficiently in the presence of 50, 100, and 1000 µM phosphate. FIG. 7 shows photographs of data obtained from tests of the growth of the parental (WT) line and the transgenic PTXD-3 and PTXD-5 lines on a liquid growth medium, with or without phosphate (Pi) or phosphate (Pi) as the source of phosphorus. In FIG. 7, the absence or presence of sustained plant growth (beyond the germination stage) is identified with a minus (−) or a plus (+) symbol, respectively. Both the parental line and the transgenic lines grew efficiently in the presence of 50 µM inorganic phosphate (bottom row). Also, neither the parental line nor the transgenic lines showed detectable growth in the absence of both phosphate and phosphate. However, both transgenic lines, but not the parental line, grew efficiently in the presence of 50, 100, and 1000 µM inorganic phosphate as phosphorus source. Therefore, the transgenic lines acquired the ability to metabolize phosphate as a phosphorus source to support plant growth.

FIG. 8 shows a bar graph of data obtained from tests of the ability of the wild-type and transgenic Arabidopsis lines of FIG. 7 to reduce the amount of total phosphorus in a growth medium containing different concentrations of phosphate (50, 100, and 1000 µM) as the source of phosphorus.

Wild type and the two transgenic Arabidopsis lines, PTXD-3 and PTXD-5, were germinated and cultivated in one-liter plastic containers with 0.1x Murashige and Skoog liquid medium lacking phosphate and supplemented with either 50, 100 or 1000 micromolar phosphorus acid (H₂PO₄). One hundred plants per plastic container were allowed to grow for 45 days in the plastic container in a growth chamber with a 16:8 light:dark cycle for each 24-hour period. The plants were covered to avoid moisture loss. A
double layer of plastic mesh was placed where seeds were sown to germinate on top of liquid media in each plastic container.

[0108] After 45 days of growth the total phosphorus content was determined in the liquid media, after removing the plants, using a vanadium-molybdate method. Briefly, 5 mL of liquid media from each sample was digested with nitric acid-perchloric acid (HNO₃-HClO₄; 5:1). Then, the phosphorus content was determined with a colorimetric method based on the addition of a solution of ammonium molybdate (20 mM) and ammonium metavanadate (10 mM) in 70% perchloric acid. After a 20-minute incubation at room temperature, the absorbance at 400 nm was measured with a spectrophotometer.

[0109] In FIG. 8, the first three bars labeled as “initial” represent the initial concentration of total phosphorus in the media without plants. The sets of bars labeled as WT (Col-0), PmTXD-3, and PmTXD-5 represent the total phosphorus content in the media (initially 50 μM, 100 μM, or 1000 μM, respectively) after 45 days of incubation in the presence of the corresponding Arabidopsis lines. The transgenic plants (PmTXD-3 and PmTXD-5), but not the wild-type plants, diminished the phosphorus content in the media by more than 50%. The decrease in phosphorus content, which in this case represents a removal of phosphate from the media, is due to the uptake of phosphate by the plants. The transgenic lines have a high capacity to remove phosphate from the media because they are able to convert it into phosphate, which sustains plant growth. This ability to remove phosphate from an aqueous medium may be exploited to remove phosphate from waste water, such as effluents produced by CD/DVD factories.

[0110] FIG. 9 shows a schematic representation of the distribution of parental (WT) and ptxD transgenic (PTXD) Arabidopsis plants used for the experiments of FIGS. 10 and 11.

[0111] FIGS. 10 and 11 show photographs of parental and ptxD transgenic plants distributed according to FIG. 9 and tested for growth on a substrate containing added phosphate (P)(FIG. 10) or phosphate (Pi)(FIG. 11) as the source of phosphorus. The presence or absence of sustained growth (beyond the germination stage) is indicated by a plus (+) or a minus (−) symbol, respectively. FIG. 10 shows similar growth of wild-type and transgenic plants on phosphate. In contrast, FIG. 11 shows that only the transgenic plants were capable of sustained growth on phosphate. The plants here and in FIG. 11 were grown in a sand:vermiculite mixture (1:1) and received water and nutrient solutions (lacking any other phosphorus source except as previously indicated) periodically.

[0112] FIG. 12 shows a bar graph of data obtained from tests of the ability of the Arabidopsis plant lines of FIG. 7 to increase in weight when cultivated in the presence of various sources of phosphorus. The dry weight of three plants cultured in sand:vermiculite (1:1) as substrate is plotted in the figure with respect to each plant line and source(s) of phosphorus. Wild type plants did not grow substantially with phosphate as the source of phosphorus, while the transgenic lines grew similarly or better on phosphate (Pi) relative to phosphate (P).

Example 3
Transgenic Tobacco Plants Expressing PtxD

[0113] This example describes the creation and characterization of transgenic Nicotiana tabacum (tobacco) comprising the ptxD expression construct of Example 1; see FIG. 13.

[0114] Nicotiana tabacum was transformed with the expression construct described in Example 1. In particular, tobacco leaf explants were co-cultivated with an Agrobacterium strain harboring a 35S::PtxD construct (Example 1) within its T-DNA. Leaf discs were allowed to regenerate in MS media containing 1 mM phosphate as the only phosphorus source. Plants regenerated from these leaf discs on phosphate-containing media were transferred to soil and allowed to set seed under greenhouse conditions.

[0115] FIG. 13 shows photographs of T2 transgenic tobacco seeds, homozygous for the 35S::PtxD gene, and control tobacco seedlings taken 25 days after germination in MS media containing either phosphate (1 mM P) or phosphate (1 mM Pi) as the only phosphorus source. The presence or absence of growth (after depletion of seed-furnished phosphorus) is indicated by a plus (+) or a minus (−) symbol, respectively. It can be seen that the control seedlings germinated but were unable to sustain normal growth in phosphate-containing media, compared to when phosphate is supplied as a phosphorus source. In contrast, tobacco plants from each transgenic line showed sustained growth in the presence of phosphate or phosphate as the source of phosphorus. These experiments demonstrate the ability to modify phosphorus metabolism in tobacco.

[0116] FIG. 14 shows photographs of additional growth experiments performed with the control and transgenic tobacco lines of FIG. 13. Seedlings were germinated and maintained in MS media supplemented with 1 mM phosphate as the only phosphorus source for 25 days. The seedlings then were transferred to culture flasks containing MS with 1 mM phosphate as the only phosphorus source and were allowed to grow for 25 additional days in a plant growth chamber at 23°C, with a photoperiod of 18 h light, followed by 6 h darkness for each 24-hour period. It can be observed that the PTXD transgenic plants are able to sustain rapid growth in media containing phosphate as the only phosphorus source, whereas the control plant is unable to use phosphate for its growth and development.

Example 4
Transgenic Algae with Modified Phosphorus Metabolism

[0117] This example describes a method of creating a transgenic line of algae expressing a phosphate dehydrogenase enzyme that enables growth of the algae on phosphate as a source of phosphorus.

[0118] Photosynthetic algae have been adapted genetically for many applications, such as production of biofuels, pharmaceuticals, and antigens, and the like. The algae can be cultured in large fermentation tanks that incorporate a light system to support photosynthesis and promote growth. Generally, the fermentation tanks must be protected from contamination with undesirable algae (or other organisms). Toward this end, the algae are grown under artificial light rather than sunlight, to reduce the risk of contamination. Accordingly, growth of the algae with exposure to sunlight in open tanks or fields (e.g., in ponds), which would be much cheaper, is not feasible currently because of the high risk of contamination.

[0119] The present disclosure enables the use of sunlight and open fields for growth of target algae by modifying the target algae for growth on phosphate as a source of phosphorus. The modified target algae would be capable of thriving in
a medium containing phosphate and lacking phosphate, which would not support growth of unwanted (contaminant) algae because they would require phosphate. Accordingly, contamination by the unwanted algae would be reduced or eliminated, permitting the target algae to be cultured at a lower cost in an open tank or field with photosynthesis driven by sunlight.

[0120] An expression construct for transformation of an algae species, such as *Chlamydomonas reinhardtii*, is generated. The construct can express any suitable phosphate dehydrogenase (and, optionally, a hypophosphate dehydrogenase, too). In the present illustration, the construct expresses PtxD from the ptxD coding sequence. The construct utilizes a hybrid promoter sequence to drive expression while avoiding gene silencing: the HIS70A promoter is fused upstream of the RB62S promoter (each promoter is provided by *C. reinhardtii*; Schrodal et al, 2000, Plant J. 21: 121-131). The hybrid promoter sequence drives expression of the first intron of RB62S of *C. reinhardtii*, which is fused to the coding sequence of the ptxD gene (*Pseudomonas stutzeri*), which, in turn, is fused to the transcription termination sequence of the RB62S gene. To enhance expression of PtxD polypeptide from the construct, the ptxD coding sequence may be modified to have a G or C in the third position of codons that permit this change (via degeneracy of the genetic code), to optimize codon usage in *C. reinhardtii*.

[0121] The ptxD expression construct may be provided as a plasmid containing an origin of replication functional in *E. coli*; a selectable marker for *E. coli* (e.g., an ampicillin-resistance gene), and a selectable marker functional in *C. reinhardtii*, among others. An exemplary selectable marker for *C. reinhardtii* encodes a zeomycin binding protein that confers resistance to zeomycin and phleomycin (Lumbrales et al., 1998, Plant J. 14: 441-447).

[0122] The ptxD expression construct is introduced into *C. reinhardtii* by any suitable mechanism, such as particle bombardment (Debchuy et al., 1989, EMBO J. 8: 2803-2809) or with the aid of glass beads (Kindle et al., 1991, PNAS 88: 1721-1725), among others.

[0123] Transformation of *C. reinhardtii* with glass beads can be carried out as described by Kindle (1990, PNAS 87: 1228-1232). Cell walls are removed from *C. reinhardtii* cells by incubating them in undiluted autolysin for 30-60 min at room temperature. The effectiveness of treatment is monitored by sensitivity to 0.004% Nonidet P-40 detergent (Sigma). Cells are harvested by centrifugation, resuspended in liquid medium, and transformed immediately to avoid cell-wall regeneration. Glass beads (0.45-0.52 mm) are washed with concentrated sulfuric acid, then rinsed thoroughly with distilled water, dried, and sterilized by baking at 250°C for 2-3 h. Glass beads (300 mg) are added to 0.4 mL of cells, 2 micrograms of plasmid DNA is added, and cells agitated at top speed on a Fisher Vortex Genie II mixer in 15-mL conical disposable polycarbonate centrifuge tubes. The beads are allowed to settle, and cells are spread on selective agar plates with a glass spreader. For direct selection of zeomycin-resistant transformants, cells are agitated with glass beads and DNA, diluted in 20 mL TAP liquid medium and left to express the *b* gene by incubating at 25°C in the light (80 μE m⁻² s⁻¹) for 15-18 h with gentle shaking. Cells are then pelleted by centrifugation, resuspended in 5 mL of TAP containing 0.5% molten agar, and poured onto the surface of a TAP/2% agar plate containing zeomycin at 20 mg/mL.

[0124] Zeomycin-resistant colonies are then spread in TAP media lacking any source of phosphate, but supplemented with 1 mM phosphate as a phosphorus source. Plates are incubated for 18 to 24 h at 25°C. In light and colonies that grow are able to use phosphate as a phosphorus source.

Example 5

Transgenic Trichoderma Expressing a Phosphate Dehydrogenase

[0125] This example describes a method of creating a fungus of the genus *Trichoderma* modified to express a phosphate dehydrogenase enzyme, to render the fungus capable of growing on phosphite as a source of phosphorus.

[0126] A. Introduction

[0127] *Trichoderma* species are free-living fungi that are common in soil and root ecosystems. Recent discoveries show that they behave as avirulent plant symbionts, as well as being parasites of phytopathogenic fungi. Some strains establish robust and long-lasting colonization of root surfaces and penetrate into the epidermis. As ubiquitous soil inhabitants and rhizosphere-compotent fungi, *Trichoderma* species have been used successfully as biological control agents for the management of plant pathogens. Several mechanisms of bio-control have been proposed for *Trichoderma*, including competition, mycoparasitism, and the induction of plant defense responses due to colonization of plant root intercellular spaces (Howell, 2003; Yedidia et al., 1999). Root colonization by *Trichoderma* species also frequently enhances root growth and development, crop productivity, resistance to abiotic stresses, and the uptake and use of nutrients.

[0128] *Trichoderma* species may be modified to express PtxD or an ortholog or derivative thereof, to render the *Trichoderma* capable of growth on phosphite. Optionally, the *Trichoderma* also may be modified to express a hypophosphate dehydrogenase (e.g., Hxa). In any event, these transgenic *Trichoderma* may be put to various uses. For example, they may be used for bioremediation purposes, such as to eliminate phosphate (and/or hypophosphate) from waste water discharge of the CD and DVD industry. The transgenic *Trichoderma* can be utilized for bioremediation alone, or in combination with a transgenic plant (e.g., Example 1). Use of a combined transgenic plant/fungal system for removal of phosphate (and/or hypophosphate) may be more efficient than the use of either alone. Alternatively, the transgenic *Trichoderma* can be associated with plants to protect them from pathogen fungi. In this case, the plants may be non-transgenic such that they require phosphate as a source of phosphorus, or may be transgenic plants that can grow on phosphite as a source of phosphorus (e.g., Example 1). In any event, the transgenic *Trichoderma* may function as a powerful fungicide, since both the *Trichoderma* itself, and its utilization of phosphite may protect the plants.

[0129] B. Protocol

[0130] Transformation of *Trichoderma atroviride* (IMI 206040) protoplasts is carried out using methods known to the art, such as the PEG-CaCl₂ method (Herrera-Estrella et al., 1990; Back & Kenerley, 1998), biolistics (Corito et al., 1993), or electroporation (Goldman et al., 1990), among others. The transforming DNA is a plasmid or a PCR product carrying a gene encoding a phosphate dehydrogenase enzyme (e.g., PtxD) under control of the *Trichoderma reesei* pki promoter or the *Aspergillus nidulans* trpC promoter, and the *T. atroviride* blu17 or the *A. nidulans* trpC terminator. Plasmids
are purified using the Qiagen Plasmid Midi Kit or cesium chloride gradients. For selection, 100, 200, and 500 μL aliquots are plated using an agar overlay containing 1.2 M sorbitol and 200 mM H₃PO₄ as sole phosphorus source, immediately after treatment or after a 2-4 hour incubation period in 1.2 M sorbitol. After three to four days of incubation at 28°C, colonies capable of growth on phosphate as the phosphorus source should appear on the plates. Transformants should appear only when transformed with constructs carrying the ptxD coding sequences. Transformants are subjected to three rounds of monosporic culture to obtain homokaryons. Alternatively, *Trichoderma* transformants may be obtained by co-transformation using an antibiotic resistance marker for selection (such as hph, which confers hygromycin resistance), in combination with a construct carrying the ptxD gene. Under the latter strategy, hygromycin-resistant transformants carrying the ptxD gene are first selected, and strains capable of using phosphite as a phosphorus source can be selected at a later stage as mentioned above, or are identified in a screen by testing expression of the ptxD gene.

[0131] Conidia of transformants carrying a phosphite-utilization cassette are produced by solid or submerged fermentation processes known in the art (Cavalcanete et al., 2008). The conidia may be applied to plants, seeds thereof, or to soil, among others. For example, the conidia may be applied to seeds (e.g., with a latex sticker, such as Rhoplex B-151), directly to plant roots as a spore suspension (e.g., with a sticker), or to soil in water as a spore suspension or in a wheat bran/peat preparation mixture (0.5%, w/w), among others.

[0132] The following references are incorporated herein by reference:


**Example 6**

**Mycorrhizae Formed with a Fungus Expressing a Bacterial Phosphite Dehydrogenase Enzyme**

**[0140]** This example describes a method of creating a mycorrhizal-type fungus modified transgenically to express a phosphite dehydrogenase enzyme (and/or a bacterial hypophosphite dehydrogenase enzyme), which renders the transgenic fungus capable of growing on phosphate (and/or hypophosphite) as the source of phosphorus. A method is also disclosed of forming mycorrhizae by associating the transgenic fungus with a plant. Mycorrhizae formed with these transgenic fungi and the plant can supply the plant with phosphate for growth. Accordingly, the plant itself would not need to be transgenic, since the mycorrhizae would do all the work of converting phosphate (and/or hypophosphite) into phosphate.

**[0141] A. Introduction**

**[0142]** Phosphorus (P) is an essential nutrient that can limit plant productivity in natural and agricultural ecosystems. A plant can form a natural symbiotic relationship with a mycorrhizal fungus, which acts as an extension of the plant’s root system to provide the plant with mineral nutrients, particularly phosphate, in exchange for carbon-containing molecules derived from the plant’s photosynthetic activity (Smith and Read, 1997). Mycorrhizal fungi penetrate root cells of the plant, with the plasma membranes of the fungi and plant establishing a close association to form so-called arbuscular structures. Mineral nutrients, particularly phosphate, can be transferred from fungal cells to plant cells in the arbuscular structures. In addition to mineral nutrients, mycorrhizae can also improve the ability of the plant to uptake water and can protect it from heavy metals (Khan, A.G., 2006; Forbes et al., 1998).

**[0143]** Mycorrhizae have to compete with other microorganisms for phosphate availability. Therefore, transgenic mycorrhizal strains that express a gene encoding a phosphate dehydrogenase enzyme capable of converting phosphate into phosphate can be used to supply plants with phosphate. In this case, the mycorrhizal fungus will convert phosphate into phosphate, which then may be transferred to the roots of non-transgenic plants unable to metabolize phosphate. Alternatively, to make the system more efficient, an association of transgenic mycorrhizal fungi and transgenic plants both expressing a gene encoding phosphate dehydrogenase can be used. The association of transgenic mycorrhizal fungi with non-transgenic or transgenic plants can be used to enhance plant productivity using fertilizers in which phosphate has been replaced by phosphite, or to bioconcentrate emissions from CD or DVD producing factories or soils in which phosphate has been used as a fungicide (Ohtake, H., 1995).

**[0144] B. Protocol**

**[0145] This example utilizes the ptxD coding sequence from *Pseudomonas stutzeri*. However, any suitable coding sequence for a phosphate dehydrogenase may be exploited.**

**[0146] A gene construct is created by placing the ptxD coding sequence under control of the *Aspergillus nidulans* glyceraldehyde-3-phosphate dehydrogenase (gpd) promoter and the transcription terminator region of the *A. nidulans* tryptophan synthetase (trpC) gene. A selectable marker such as the aph gene from *E. coli*, which confers resistance to hygromycin, or the ble gene, which confers resistance to phleomycin, is also included in the transforming molecule (Barrett et al., 1990).**

**[0147] For transformation, protoplasts of a mycorrhizal fungus (e.g., Laccaria bicolor, Conococcum geophilum, Hebeloma cylindrosporum, Paxillus involutus, Gigaspora rosea, Glomus mosseae, Glomus aggregatum, Glomus intraradices, Pisolithus tinctorius, etc.) are obtained according to the protocol of Barrett et al. (1990). To isolate proto-
plasts, mycelia are collected and washed several times with sterile water and then treated with hydrolytic enzymes (a mixture of cellulase, chitinase, and proteases, with 5 to 10 mg/mL of each enzyme) in an osmotic solution (PDCl; potato-dextrose-broth with 0.8 M mannitol or 0.6 M sucrose) to degrade the cell walls. The mycelia are incubated with the enzymes for 1 to 3 hours at 32°C, with constant agitation (100 rpm). The protoplast suspension is filtered and washed with the osmotic solution. Protoplasts are recovered by centrifugation for 10 min at 800 rpm and the protoplast pellet resuspended in PDCl buffer and the number of protoplasts determined by counting under a microscope.

[0148] Protoplasts (1×10^6 in 250 μL) are mixed with 5 to 20 micrograms of the gene construct and incubated in PEG transformation solution (25-60% polyethylene glycol 4000, 10-25 mM CaCl2, 10 mM Tris-HCl, pH 7.5) for 45 minutes at 4°C. One mL of additional PEG transformation solution is added and incubation is continued at room temperature for 20 minutes. Protoplasts are allowed to regenerate cell walls in liquid media and transformants are selected in solid media. The solid media (Potato dextrose agar) contains 100 μg/mL hygromycin or 100 μg/mL of phleomycin, depending on the selectable marker used for the transformation. Growing colonies are transferred to solid media three times to isolate stably transformed mycelia. The presence of the selectable marker as well as the ptuD gene is confirmed by PCR. Once stable transformants are isolated, a 2 mm portion of mycelium is transferred to PDA media lacking phosphate and supplemented with 1 mM phosphate to identify colonies that express the ptuD gene construct. Southern blot analysis is used to confirm the presence of the corresponding genes.

[0149] To confirm that the transgenic fungus can provide phosphate to plants, soil is inoculated with mycelia of the ptuD-transformed fungus, and tobacco seed is germinated in the soil. The soil is fertilized with a normal concentration of nitrogen and potassium, with phosphate as the phosphorous source. Growth of tobacco plants from the seed in soil inoculated with the ptuD transgenic fungus is compared to growth in control soil that has not been inoculated.

[0150] The following references are incorporated herein by reference:


B. Identification, Cloning, and Characterization of RP-Oxidoreductases


C. Transformation of Plants


Example 8

Selected Embodiments I

[0190] This example describes selected embodiments of the invention, presented as a series of indexed paragraphs.

[0191] A transgenic plant capable of utilizing at least one reduced form of phosphorus as a phosphorus fertilizer. The transgenic plant of this paragraph may be further characterized as follows: (A1) wherein the plant expresses a bacterial coding sequence encoding an enzyme capable of oxidizing phosphate to phosphate, thereby permitting use of phosphite as a phosphorus fertilizer (and a source of phosphorus); (A2) wherein the bacterial coding sequence of A1 is ptxD from Pseudomonas stutzeri, Alcaligenes faecalis, or Xanthobacter flavus; (A3) wherein the transgenic plant of A1 or A2 expresses htxA and ptxD coding sequences, thereby permitting use of hypophosphate and/or phosphate as a phosphorus fertilizer; (A4) wherein each or both of the bacterial coding sequences of A3 is from Pseudomonas stutzeri, Alcaligenes faecalis, or Xanthobacter flavus; (A5) wherein at least one of the bacterial coding sequence(s) of any of A1 through A4 is under control of a constitutive promoter, a leaf-specific promoter, a tissue-specific promoter, a root-specific promoter, a promoter inducible by low phosphate, or the 3SS promoter from the Cauliflower Mosaic Virus; (A6) any combination of A1 through A5.

[0192] B. The use of a transgenic plant capable of oxidizing hypophosphate to phosphate, and/or phosphate to phosphate, to eliminating hypophosphate and/or phosphate from an industrial or municipal effluent.

[0193] C. The use of one or more bacterial coding sequences that oxidize hypophosphate to phosphate, and/or phosphate to phosphate, as a selectable marker for the production of transgenic plants.

[0194] D. The use of recombinant DNA molecules composed of one or more bacterial coding sequences encoding enzymes that oxidize hypophosphate to phosphate, and/or phosphate to phosphate, and a promoter sequence functional in plants as a selectable marker for the production of transgenic plants.

[0195] E. A chimeric gene functional in a plant cell, which chimeric gene comprises: (1) a plant-expressible promoter sequence; (2) a terminator signal sequence; and (3) a coding region of a bacterial gene that oxidizes phosphite into phosphate, which coding region: encodes a functional NAD:phosphate oxidoreductase enzyme, and is positioned between such plant-expressible promoter sequence and such terminator signal sequence so as to be expressible, wherein expression of such coding region in a plant cell confers the capacity of using phosphite as a phosphorus source on such plant cell and wherein such capacity to use phosphite as a phosphorus source is capable of providing a basis for selection of such plant cell. The chimeric gene of this paragraph may be further characterized as follows: (E1) wherein the coding region is from the ptxD gene from Pseudomonas stutzeri, Alcaligenes
faecalis, or Xanthobacter flavus; (E2) wherein the promoter sequence is a constitutive promoter; (E3) wherein the promoter sequence is the 35S promoter from Cauliflower Mosaic Virus; (E4) wherein the terminator signal sequence is a nopine synthetase terminator signal sequence; (E5) wherein the terminator signal sequence is a Cauliflower Mosaic Virus terminator signal sequence; or (E6) any combination of E1 through E5.

0196 F. A transgenic plant that expresses at least one foreign enzyme at a level enabling the plant to metabolize a reduced form of phosphorus as a phosphorus fertilizer.

Example 9
Selected Embodiments II

0197 A. A transgenic plant comprising a construct that confers (1) a growth advantage on the plant for growth using a reduced form of phosphorus as a nutrient and/or (2) a capability to metabolize at least one reduced form of phosphorus. The transgenic plant of this paragraph may be further described as follows: (A1) wherein the construct confers a growth advantage on the plant if phosphate is an at least substantially exclusive external source of phosphorus for the plant; (A2) wherein the construct confers a growth advantage on the plant if hypophosphate is an at least substantially exclusive external source of phosphorus for the plant; (A3) wherein the construct confers a growth advantage on the plant if hypophosphate is an at least substantially exclusive external source of phosphorus for the plant and if hypophosphate is an at least substantially exclusive external source of phosphorus for the plant; (A4) wherein the transgenic plant is capable of growth without phosphate as an external source of phosphorus, and wherein a non-transgenic variety of the transgenic plant lacking the construct is at least substantially unable to grow without phosphate as an external source of phosphorus; (A5) wherein the transgenic plant was transformed initially with the construct in a progenitor of the transgenic plant; (A6) wherein the construct encodes expression of one or more polypeptides that confer on the plant a capability to metabolize at least one reduced form of phosphorus to phosphate, and, optionally, wherein at least one of the polypeptides oxidizes phosphate to phosphate and, optionally, wherein at least one of the polypeptides is capable of using nicotinamide adenine dinucleotide (NAD+) and/or nicotinamide adenine dinucleotide phosphate (NADP+) as an electron acceptor, and, optionally, wherein the one or more polypeptides include a PtxD polypeptide, which, optionally, is encoded by a coding region originating at least substantially from Pseudomonas stutzeri, Alcaligenes faecalis, or Xanthobacter flavus; (A7) wherein the one or more polypeptides of A6 include an HtxA polypeptide; (A8) wherein expression of at least one of the one or more polypeptides of A6 or A7 is inducible; (A9) wherein expression of at least one of the polypeptides of any of A6 through A8 is inducible by low phosphate; (A10) wherein expression of at least one of the polypeptides of any of A6 through A9 is under control of a constitutive promoter; (A11) wherein expression of at least one of the polypeptides of any of A6 through A10 is under control of a leaf-specific promoter; (A12) wherein expression of at least one of the polypeptides of any of A6 through A11 is under control of a root-specific promoter; (A13) wherein expression of at least one of the polypeptides of any of A6 through A12 is under control of a promoter that is not tissue specific; or (A14) any combination of A1 through A13.

0199 B. A transgenic plant comprising a construct encoding a bacterial polypeptide that confers on the plant a capability to metabolize phosphite to phosphate.

0200 C. A seed that germinates to produce, or any plant part used to produce or vegetatively reproduce, the transgenic plant of paragraph A or B.

0201 D. A nucleic acid for generating a transgenic plant, comprising: a chimeric gene capable of conferring on a plant (1) a growth advantage for growth using a reduced form of phosphorus as a nutrient and/or (2) a capability to metabolize at least one reduced form of phosphorus. The nucleic acid of this paragraph may be further described as follows: (D1) wherein the chimeric gene includes a promoter operatively linked to a coding region, and wherein the promoter is capable of controlling expression of the coding region in a plant, and, optionally, wherein the coding region encodes one or more polypeptides that oxidize phosphite to phosphate and, optionally, wherein the coding region is provided at least substantially by a ptxD gene; (D2) wherein the chimeric gene includes a promoter operatively linked to a coding region, and wherein the promoter originated at least substantially in a plant and/or a plant virus, and, optionally, wherein the promoter includes a 35S promoter from Cauliflower Mosaic Virus; (D3) further comprising a transcripational terminator that is functional in a plant and operatively linked to the promoter and the coding region of D1 or D2; (D4) wherein the coding region of any of D1 through D3 encodes a polypeptide that oxidizes phosphite to phosphate; (D5) wherein the nucleic acid is disposed in a microorganism; (D6) wherein the nucleic acid is isolated from cells; (D7) wherein the nucleic acid is disposed in a transgenic plant; or (D8) any combination of D1 through D7.

0202 E. A method of generating a transgenic plant, comprising: selecting for transformation of a plant or plant part using, as a selectable marker, a nucleic acid that confers a capability to metabolize a reduced form of phosphorus. The method of this paragraph may be further described as follows: (E1) wherein the step of selecting for transformation includes a step of selecting for a growth advantage of the plant or plant part, relative to other plants or plant parts, with one or more reduced forms of phosphorus as an external source of phosphorus for the plants or plant parts; (E2) wherein the step of selecting for a growth advantage in E1 is performed in the plants or plant parts in contact with a medium containing phosphite, hypophosphate, or both; (E3) wherein the step of selecting for a growth advantage of E1 or E2 is performed in the medium containing at least substantially no phosphite; (E4), further comprising a step of contacting the plant or plant part, a progenitor thereof, or both, with a modifying agent including a construct that provides the selectable marker; (E5) wherein the modifying agent of E4 includes Agrobacterium cells containing the construct; (E6) wherein the construct of E4 or E5 encodes a polypeptide that oxidizes phosphite to phosphate; (E7) wherein the construct of any of E4 through E6 encodes a polypeptide that oxidizes hypophosphite to phosphate; (E8) wherein the step of contacting of any of E4 through E7 includes a step of firing projectiles at the plant or plant part, a progenitor thereof, or both; (E9) wherein the step of selecting for transformation is performed with a plant part, and wherein the plant part is a tissue explant or an isolated plant cell; or (E10) any combination of E1 through E9.
[0203] F. A method of fertilizing the transgenic plant of paragraph A or B, wherein a reduced form of phosphorus is used as foliar fertilizer or added to amend soil composition to provide a source of phosphate to sustain plant growth and reproduction.

[0204] G. A method of water remediation, comprising: contacting (i) an effluent including phosphate and (ii) a transgenic plant comprising a construct that confers a capability to metabolize phosphate to phosphate, thereby reducing a level of phosphate in the effluent.

Example 10
Selected Embodiments III

[0205] This example describes selected embodiments of the invention, presented as a series of indexed paragraphs.

[0206] A. A nucleic acid, comprising: a chimeric gene including (a) a coding region that encodes a phosphate dehydrogenase enzyme and (b) a transcription promoter operatively linked to the coding region, wherein the promoter is heterologous with respect to the coding region and is functional in plants, fungi, or both, and wherein the chimeric gene provides sufficient expression of the enzyme, in a plant or fungal cell containing the chimeric gene, to confer an ability on the cell to metabolize phosphate (PPi) as a phosphorous source for supporting growth, thereby enabling growth of the cell without an external source of phosphate (Pi). The nucleic acid of this paragraph may be described further as follows: (A1) wherein the phosphate dehydrogenase enzyme is of bacterial origin; (A2) wherein the phosphate dehydrogenase enzyme is PtxD of Pseudomonas stutzeri (SEQ ID NO:1), an analog or derivative of PtxD (SEQ ID NO:1), or a PtxD-like homolog from another bacterial species; (A3) wherein the bacterial phosphate dehydrogenase enzyme has an amino acid sequence with at least 50%, 60%, 80%, 90%, or 95% sequence identity to at least one of SEQ ID NO:1-14; (A4) wherein the phosphate dehydrogenase enzyme has an amino acid sequence including a first sequence region having an NAD-binding motif with sequence similarity or identity to VGILGMGAIG (SEQ ID NO:15), a second sequence region having sequence similarity or identity to XPGALLVNPRCG-SWD (SEQ ID NO:16), where X is K or R, a third sequence region having sequence similarity or identity to GWX, PX2YX2X2X2X2X2 (SEQ ID NO:19), where X1 is R, Q, T, or K, X2 is A, V, Q, R, K, I, L, or E, X3 is L or F, X4 is F, G, F, or S, and X5 is R, M, I, A, or S, or includes any combination of the second, third, and second sequence regions; (A5) wherein the phosphate dehydrogenase enzyme has an amino acid sequence that is at least 90% identical to PtxD from Pseudomonas stutzeri (SEQ ID NO:1); (A6) wherein the chimeric gene further includes a transcription terminator that is operatively linked to the coding region and heterologous with respect to the coding region; (A7) wherein the promoter is a plant promoter or a viral promoter of a plant virus and is capable of promoting the sufficient expression of the enzyme in a plant cell; (A8) wherein the promoter of A7 corresponds to the 35S promoter of Cauliflower Mosaic Virus; (A9) wherein the promoter of A7 is inducible by low phosphate availability; (A10) wherein the promoter of A9 corresponds to a promoter of the PLDZZ gene of Arabidopsis thaliana; (A11) wherein the chimeric gene is capable of promoting the sufficient expression of the enzyme both in a plant cell and in a fungal cell each containing the chimeric gene; (A12) wherein the promoter is a fungal promoter capable of promoting the sufficient expression of the enzyme in a fungal cell; (A13) wherein one or more codons of the coding region have been changed in vitro to improve translational efficiency in plants and/or fungi; (A14) further comprising an intron connected to the coding region and configured to be transcribed with the coding region and removed by splicing after transcription, wherein the intron is optionally disposed within the coding region; (A15) wherein the coding region has at least 90% sequence identity with SEQ ID NO:21; or (A16) any combination of A1 through A15.

[0208] B. A plant cell comprising a nucleic acid that expresses a phosphate dehydrogenase enzyme in the plant cell and capable of metabolizing phosphate as a source of phosphorus for supporting growth. Optionally, the nucleic acid is according to paragraph A. The plant cell of this paragraph may be described further as follows: (B1) further comprising an other nucleic acid that expresses a hypophosphite dehydrogenase enzyme, optionally of bacterial origin, in the plant cell; (B2) the plant cell of B1 wherein the nucleic acids collectively confer an ability on the cell to metabolize hypophosphite (Hphi) as a phosphorus source for supporting growth; (B3) wherein the other nucleic acid of B1 or B2 encodes a polypeptide with at least 95% sequence identity to HtxA of Pseudomonas stutzeri (SEQ ID NO:20); (B4) the plant cell of any of B1 through B3, wherein the nucleic acids are integrated adjacent one another in the genome of the plant cell; (B5) wherein expression of the phosphate dehydrogenase enzyme, the hypophosphite dehydrogenase enzyme, or both are controlled by a root-specific promoter; (B6) wherein the plant cell is homoygous for the nucleic acid; (B7) wherein the plant cell is a eukaryotic algal cell; (B8) wherein the algal cell of B7 is a Chlamydomonas cell; (B9) wherein the plant cell is from a species of vascular plant; or (B10) any combination of B1 through B9.

[0209] C. A plant composed of a plurality of plant cells according to paragraph B. The plant of this paragraph may be described further as follows: (C1) wherein the plant is a vascular plant, such as a species of crop plant, (C2) wherein the species of crop plant of C1 is selected from the group consisting of maize, soybean, rice, potatoes, tomatoes, sugarcane, and wheat.

[0210] D. A fungal cell comprising a nucleic acid that expresses a phosphate dehydrogenase enzyme in the fungal cell and capable of metabolizing phosphate as a source of phosphorus for supporting growth. Optionally, the nucleic acid is according to paragraph A. The fungal cell of this paragraph may be described further as follows: (D1) further comprising a nucleic acid that expresses a bacterial hypophosphite dehydrogenase enzyme in the fungal cell; (D2) the fungal cell of D1, wherein the nucleic acids collectively confer an ability on the cell to metabolize hypophosphite (Hphi) as a phosphorus source for supporting growth of the fungal cell; (D3) wherein the fungal cell is from a species of Trichoderma; (D4) wherein the fungal cell is a member of a species of mycorrhizal fungus capable of forming a symbiotic relationship with a plant; or (D5) any combination of D1 through D4.

[0211] E. A method of reducing fungal infections in plants, comprising: applying a plurality of the fungal cells of paragraph D to a seed form of plants, the plants themselves, soil in which the plants are disposed, or a combination thereof. In some cases, the fungal cells may be spores.

[0212] F. A plant associated with a plurality of fungal cells according to paragraph D to form mycorrhizae. Optionally,
the fungal cells render the plant capable of growing on a medium containing phosphite (Phi), hypophosphite (Hphi), or both, as a phosphorus source for supporting growth.

0213] G. A method of fertilizing a crop plant using hypophosphate and/or phosphite as a phosphorus source for supporting growth, the crop plant (a) including a plurality of cells comprising the nucleic acid of paragraph A, (b) forming mycorrhizal with a mycorrhizal fungus comprising the nucleic acid of paragraph A, and/or (c) being associated with a Trichoderma fungus comprising the nucleic of claim 1, the method comprising: applying at least one reduced form of phosphorus to the plant and/or to soil adjacent the plant, such that the reduced form is metabolized to phosphate by the plant and/or the fungus to support growth and productivity of the plant.

0214] H. A method of fertilizing the plant of paragraph C, the method comprising: applying at least one reduced form of phosphorus to the plant and/or to soil adjacent the plant, such that the reduced form is metabolized to phosphate by the plant to support growth and productivity of the plant. Optionally, the reduced form may be applied as foliar fertilizer or added to amend soil to provide a source of phosphate to sustain plant growth and reproduction of the plant.

0215] I. A method of treating water to lower its content of reduced phosphorus, the method comprising: contacting water containing hypophosphate and/or phosphite with a plurality of the plant cells and/or fungal cells comprising the nucleic acid of paragraph A, such that at least a portion of the hypophosphate and/or phosphite is oxidized to phosphate and/or phosphate. Optionally, the step of contacting includes a step of contacting the water with a plurality of vascular plants composed of plant cells comprising the nucleic acid of paragraph A.

0216] J. A method of treating liquid waste to lower its content of reduced phosphorus, the method comprising: contacting (i) water containing hypophosphate and/or phosphite as a contaminant and (ii) a plurality of the plant cells and/or fungal cells comprising the nucleic of paragraph A, such that at least a portion of the hypophosphate and/or phosphite is oxidized to phosphate and/or phosphate.

0217] K. A method of utilizing the nucleic acid of paragraph A for production of a transgenic plant, comprising: selecting for growth of plant cells comprising the nucleic acid of paragraph A as a selectable marker during production of a transgenic plant.

0218] L. A method of obtaining a plant transformed with a nucleic acid encoding a phosphite dehydrogenase enzyme that is expressible from the nucleic acid as a selectable marker, comprising: contacting plant cells and a composition including the nucleic acid under conditions that promote introduction of the nucleic acid into at least a subset of the plant cells; culturing the plant cells in a medium containing phosphite as a primary or exclusive phosphorus source for growth; selecting transformed plant cells produced by the steps of contacting and culturing, and expressing the phosphite dehydrogenase enzyme as evidenced by growth in the medium; and regenerating at least a portion of the transformed plant cells into a transgenic plant. The method of this paragraph may be described further as follows: (1.1) wherein the composition includes Agrobacterium cells that supply the nucleic acid during the step of contacting; or (1.2) wherein the composition includes projectiles that are fired at the plant cells in the step of contacting.

0219] M. A plant, comprising: a nucleic acid including a chimeric gene expressing a phosphite dehydrogenase enzyme such that the plant is capable of metabolizing phosphite (Phi) as a phosphorus source for supporting growth, thereby enabling growth of the plant without an external source of phosphate (Pi). The plant of this paragraph may be described further as follows: (M1) wherein the nucleic acid is stably integrated into the genome of the plant; (M2) wherein the plant is a vascular plant; (M3) wherein the plant is a species of algae; (M4) wherein the phosphite dehydrogenase enzyme has any of the features of paragraph A; or (M5) any combination of M1 through M4.

0220] N. A fungus, comprising: a nucleic acid including a chimeric gene expressing a phosphite dehydrogenase enzyme such that the fungus is capable of metabolizing phosphite (Phi) as a phosphorus source for supporting growth, thereby enabling growth of the fungus without an external source of phosphate (Pi). The fungus of this paragraph may be described further as follows: (N1) wherein the nucleic acid is stably integrated into the genome of the fungus; (N2) wherein the fungus is a species of Trichoderma; (N3) wherein the fungus is a mycorrhizal species capable of forming a symbiotic relationship with a plant; (N4) further comprising a plant associated with the fungus to form mycorrhizae; (N5) wherein the phosphite dehydrogenase enzyme has any of the features of paragraph A; or (N6) any combination of N1 through N5.

0221] The disclosure set forth above may encompass multiple distinct inventions with independent utility. Although each of these inventions has been disclosed in its preferred form(s), the specific embodiments thereof as disclosed and illustrated herein are not to be considered in a limiting sense, because numerous variations are possible. The subject matter of the inventions includes all novel and nonobvious combinations and subcombinations of the various elements, features, functions, and/or properties disclosed herein. The following claims particularly point out certain combinations and subcombinations regarded as novel and nonobvious. Inventions embodied in other combinations and subcombinations of features, functions, elements, and/or properties may be claimed in applications claiming priority from this or a related application. Such claims, whether directed to a different invention or to the same invention, and whether broader, narrower, equal, or different in scope to the original claims, also are regarded as included within the subject matter of the inventions of the present disclosure.

SEQUENCE LISTING

<160> NUMBER OF SEQ ID NO'S: 23

<210> SEQ ID NO 1

<211> LENGTH: 336
<212> TYPE: PRT
<213> ORGANISM: Pseudomonas stutzeri

<400> SEQUENCE: 1

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1  5  10  15

Gln Leu Leu Ala Pro His Cys Glu Leu Met Thr Asn Glu Thr Asp Ser

20  25  30

Thr Leu Thr Arg Glu Glu Ile Leu Arg Arg Cys Arg Asp Ala Gln Ala

35  40  45

Met Met Ala Phe Met Pro Asp Arg Val Asp Ala Asp Phe Leu Gln Ala

50  55  60

Cys Pro Glu Leu Arg Val Val Gly Cys Ala Leu Lys Gly Phe Asp Asn

65  70  75  80

Phe Asp Val Asp Ala Cys Thr Ala Arg Gly Val Trp Leu Thr Phe Val

95  100  105  110

Pro Asp Leu Leu Thr Val Pro Thr Ala Glu Leu Ala Ile Gly Leu Ala

115  120  125

Val Gly Leu Gly Arg His Leu Arg Ala Ala Asp Ala Phe Val Arg Ser

130  135

Gly Phe Glu Gln Gly Trp Glu Pro Glu Phe Tyr Gly Thr Gly Leu Asp

140

Asn Ala Thr Val Gly Ile Leu Gly Met Gly Ala Ile Gly Leu Ala Met

145  150  155  160

Ala Asp Arg Leu Gln Gly Trp Gly Ala Thr Leu Gln Tyr His Glu Ala

165  170  175

Lys Ala Leu Asp Thr Glu Thr Glu Gln Arg Leu Arg Gly Leu Arg Val

180  185  190

Ala Cys Ser Glu Leu Phe Ala Ser Ser Asp Phe Ile Leu Leu Ala Leu

195  200  205

Pro Leu Asn Ala Asp Thr Glu His Leu Val Asn Ala Glu Leu Leu Ala

210  215  220

Leu Val Arg Pro Gly Ala Leu Leu Val Asn Pro Cys Arg Gly Ser Val

225  230  235  240

Val Asp Glu Ala Ala Val Leu Ala Ala Leu Glu Arg Gly Glu Leu Gly

245  250  255

Gly Tyr Ala Ala Asp Val Phe Glu Met Glu Asp Trp Ala Arg Ala Asp

260  265  270

Arg Pro Arg Leu Ile Asp Pro Ala Leu Ala His Pro Asn Thr Leu

275  280  285

Phe Thr Pro His Ile Gly Ser Ala Val Arg Ala Val Arg Leu Glu Ile

290  295  300

Glu Arg Cys Ala Ala Asn Ile Ile Glu Val Leu Ala Gly Ala Arg

305  310  315  320

Pro Ile Asn Ala Ala Asp Arg Leu Pro Lys Ala Glu Pro Ala Ala Cys

325  330  335

<210> SEQ ID NO 2
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<212> TYPE: PRT
<213> ORGANISM: Acinetobacter radioresistens

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<210> SEQ ID NO 3
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<212> TYPE: PRT
<213> ORGANISM: Alcaligenes faecalis
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| Ala Leu Leu Glu Thr Ala Ala Glu Val Ile Ser Asn Gin Ser Asp Ser | 20 25 30 |
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Thr Met Ser Arg Glu Glu Val Leu Arg Thr Asn Asp Ala Asp Gly 35 40 45
Met Met Val Phe Met Pro Asp Ser Ile Asp Ala Phe Leu Ser Ala 50 55 60
Cys Pro Asn Leu Lys Val Ile Gly Ala Ala Leu Lys Gly Tyr Asp Asn 65 70 75 80
Phe Asp Val Glu Ala Cys Thr Arg His Gly Ile Thr Phe Thr Ile Val 85 90 95
Pro Asp Leu Leu Thr Ser Pro Thr Ala Glu Leu Thr Ile Gly Leu Leu 100 105 110
Leu Ser Ile Thr Arg Asn Met Leu Gln Gly Asp Asn Tyr Ile Arg Ser 115 120 125
Arg Gln Phe Asn Gly Thr Trp Thr Pro Arg Phe Tyr Gly Thr Gly Leu Thr 130 135 140
Gly Lys Thr Ala Gly Ile Ile Gly Thr Gly Ala Val Gly Arg Ala Val 145 150 155 160
Ala Lys Arg Leu Ala Ala Phe Asp Met Gln Ile Gln Tyr Thr Asp Pro 165 170 175
Gln Pro Leu Pro Gln Glu Ser Glu Arg Ala Trp Asn Ala Ser Arg Thr 180 185 190
Ser Leu Asp Gln Leu Leu Ala Thr Ser Asp Phe Ile Ile Pro Met Leu 195 200 205
Pro Met Ser Ser Asp Thr His His Thr Ile Asn Ala Arg Ala Leu Asp 210 215 220
Arg Met Lys Pro Gly Ala Tyr Leu Val Asn Ala Cys Arg Gly Ser Ile 225 230 235 240
Val Asp Glu Arg Ala Val Val Ala Leu Arg Thr Gly His Leu Gly 245 250 255
Gly Tyr Ala Ala Asp Val Phe Glu Met Glu Glu Trp Ala Arg Pro Asp 260 265 270
Arg Pro His Ser Ile Pro Asp Glu Leu Leu Asp Pro Ala Leu Pro Thr 275 280 285
Phe Phe Thr Pro His Leu Gly Ser Ala Val Lys Ser Val Arg Met Glu 290 295 300
Ile Glu Arg Glu Ala Ala Leu Ser Ile Leu Glu Ala Leu Gln Gly Arg 305 310 315 320
Ile Pro Arg Gly Ala Val Asn His Val Gly Ala Gly Arg 325 330

<210> SEQ ID NO: 4
<211> LENGTH: 332
<212> TYPE: PRT
<213> ORGANISM: Cyanothece sp. CCYO110

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Lys Glu Thr Leu Ser Arg Glu Glu Val Ile Asn Arg Ser Arg Asn Ala 35 40 45
Gln Gly Leu Met Val Phe Met Pro Asp His Ile Asp Val Lys Phe Leu
Glu Ala Cys Pro Asn Leu Lys Val Ile Ser Gly Ala Leu Arg Gly Tyr
65  70  75  80
Asp Asn Phe Asp Val Glu Ala Cys Thr Lys His Asn Ile Trp Phe Thr
95  95
Ile Val Pro Asp Leu Leu Ala Ala Pro Thr Ala Glu Leu Thr Ile Gly
100  105  110
Leu Leu Ile Ile Leu Ala Arg Arg Met Leu Glu Gly Asp Arg Leu Ile
115  120  125
Arg Ser Asp Asn Phe Glu Gly Trp Lys Pro Gin Leu Tyr Gly Thr Gly
130  135  140
Leu Leu Asn Lys Ser Leu Gly Ile Ile Gly Met Gly Lys Leu Lys Lys
145  150  155  160
Ala Leu Ala Gly Arg Val Leu Gly Phe Asp Met Asn Leu Leu Tyr Thr
165  170  175
Asp Pro Ile Ser Leu Thr Asn Gin Gin Glu Lys Asp Trp Lys Ile Ser
180  185  190
Lys Thr Ser Leu Glu Glu Leu Ser Lys Ser Asp Tyr Val Val Leu
195  200  205
Met Val Ser Leu Val Pro Asp Thr Tyr His Leu Ile Asn Glu Asn Thr
210  215  220
Leu Lys Leu Met Lys Pro Lys Ser Phe Leu Ile Asn Pro Cys Arg Gly
225  230  235  240
Ser Val Val Asp Glu Asn Ala Ala Asp Ala Ile Lys Ser Gly His
245  250  255
Leu Ala Gly Tyr Ala Ala Asp Phe Glu Met Glu Asp Trp Ala Ile
260  265  270
Ala Asn Arg Pro Lys Ser Ile Asn Gin Thr Leu Thr Thr Asp Ile Lys
275  280  295
His Thr Phe Phe Thr Pro His Leu Gly Ser Ala Ile Asn Asp Val Arg
290  295  300
Arg Glu Ile Ala Ile Glu Ala Ala Lys Asn Ile Ile Glu Val Phe Ser
305  310  315  320
Asp Asp Arg Pro Lys Ser Ala Asn Asn Ile Ile
325  330

<210> SEQ ID NO 5
<211> LENGTH: 335
<212> TYPE: PRT
<213> ORGANISM: Gallionella ferruginea
<400> SEQUENCE: 5
Met Lys Pro Lys Ile Val Ile Thr Ser Trp Val Val His Pro Gin Thr Leu
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Asp Met Leu Arg Pro His Cys Asp Val Val Ala Asn Glu Thr Arg Glu
20  25  30
Arg Leu Ser Arg Glu Ile Lys Arg Cys Ser Asp Ala Val Ala
35  40  45
Val Met Thr Phe Met Pro Asp Ser Ile Asp Asp Ala Phe Leu Ala Glu
50  55  60
Cys Pro Glu Leu Arg Leu Val Ala Cys Ala Leu Lys Gly Tyr Asp Asn
65  70  75  80
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Tyr Asp Val Ala Ala Cys Thr Arg Arg Gly Val Arg Ile Thr Asn Val 85 90 95
Pro Asp Leu Leu Thr Ile Pro Thr Ala Glu Leu Thr Val Gly Leu Leu 100 105 110
Ile Gly Leu Thr Arg Lys Val Leu Gln Gly Asp Arg Phe Val Arg Ser 115 120 125
Gly Gln Phe Thr Gly Trp Arg Pro Met Leu Tyr Gly Ala Gly Leu Thr 130 135 140
Gly Arg Thr Leu Gly Ile Ile Gly Met Gly Ala Val Gly Arg Ala Ile 145 150 155 160
Ala Ala Arg Leu Gln Gly Tyr Met Glu Leu Tyr Thr Asp Pro 165 170 175
Gln Pro Leu Pro Pro Glu Leu Glu Ala Arg Leu Gly Leu Arg Lys Val 180 185 190
Gly Leu Val Gln Leu Leu Ala Glu Ser Asp Tyr Val Val Pro Met Val 195 200 205
Pro Tyr Thr Gln Asp Thr Leu His Met Ile Asn Ala Ala Ser Leu Ser 210 215 220
Ile Met Lys Pro Gly Ala Tyr Leu Val Asn Thr Cys Arg Gly Ser Val 225 230 235 240
Val Asp Glu Lys Ala Val Ala Asp Ala Leu Asp Ser Gly Lys Leu Ala 245 250 255
Gly Tyr Ala Ala Asp Ala Phe Glu Leu Glu Glu Trp Met Arg Pro Asp 260 265 270
Arg Pro Glu Ser Ile Ser Glu Arg Leu Ser Asp Thr Asp Leu Thr 275 280 285
Leu Phe Thr Pro His Ile Gly Ser Ala Val Asp Thr Val Arg Leu Ala 290 295 300
Ile Glu Met Glu Ala Ala Thr Asn Ile Leu Gin Val Val Leu Lys Gly Gin 305 310 315 320
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<210> SEQ ID NO 6
<211> LENGTH: 336
<212> TYPE: PRT
<213> ORGANISM: Janthinobacterium sp. Marseille

<400> SEQUENCE: 6

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

<210> SEQ ID NO 7
<211> LENGTH: 336
<212> TYPE: PRT
<213> ORGANISM: Klebsiella pneumoniae

<400> SEQUENCE: 7
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Gln Leu Leu Ala Pro His Cys Glu Leu Val Thr Asn Gln Thr Asp Ser
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Thr Leu Thr Arg Glu Ile Leu Arg Arg Cys Arg Asp Ala Gln Ala
30 35 40 45
Met Met Ala Phe Met Pro Asp Arg Val Asp Ala Asp Phe Leu Gln Ala
50 55 60
Cys Pro Glu Leu Arg Val Val Gly Cys Ala Leu Lys Gly Phe Asp Asn
65 70 75 80
Phe Asp Val Asp Ala Cys Thr Ala Arg Gly Val Trp Leu Thr Phe Val
85 90 95
Pro Asp Leu Leu Thr Val Pro Thr Ala Glu Leu Ala Ile Gly Leu Ala
100 105 110
Val Gly Leu Gly Arg His Leu Arg Ala Ala Asp Ala Phe Val Arg Ser
115 120 125
Gly Glu Phe Glu Gly Gly Trp Gln Pro Gln Phe Tyr Gly Thr Gly Leu Asp
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\[\text{Ala Cys Ser Glu Leu Phe Ala Ser Ser Asp Phe Ile Leu Leu Ala Leu} 195\]
\[\text{Pro Leu Asn Ala Asp Thr Gln His Leu Val Asn Ala Glu Leu Leu Ala} 210\]
\[\text{Leu Val Arg Pro Gly Ala Leu Leu Val Asn Pro Cys Arg Gly Ser Val} 225\]
\[\text{Val Asp Glu Ala Val Leu Ala Ala Leu Glu Arg Gly Gln Leu Gly} 245\]
\[\text{Gly Tyr Ala Ala Asp Val Phe Glu Met Glu Asp Trp Ala Arg Ala Asp} 260\]
\[\text{Arg Pro Arg Leu Ile Asp Pro Ala Leu Leu Ala His Pro Asn Thr Leu} 275\]
\[\text{Phe Thr Pro His Ile Gly Ser Ala Val Arg Val Leu Glu Ile} 290\]
\[\text{Glu Arg Cys Ala Ala Gln Asn Ile Ile Gln Val Leu Ala Gly Ala Arg} 305\]
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<210> SEQ ID NO: 8
<211> LENGTH: 336
<212> TYPE: PRT
<213> ORGANISM: Marinobacter algicola

<400> SEQUENCE: 8
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\[\text{Thr Leu Pro Pro Asp Ser Val Arg Ala Arg Ala Thr Ala Asp Ala} 35\]
\[\text{Met Met Ala Phe Met Pro Asp Arg Val Ser Glu Glu Phe Leu Val Ala} 50\]
\[\text{Cys Pro Asp Leu Lys Val Ile Gly Ala Ala Leu Lys Gly Phe Asp Asn} 65\]
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Ala Thr Arg Leu Gln Gly Trp Gly Ala Arg Val Leu Tyr Ser Gln Pro 165 170 175
Glu Ser Leu Pro Ala Ala Glu Gly Ala Leu Gly Leu Ser Arg Ser 180 185 190
Glu Leu Asp Asp Leu Leu Ala Glu Ser Asp Ile Val Ile Leu Ala Leu 195 200 205
Ala Leu Asn Glu His Thr Leu His Thr Leu Asn Ala Asp Arg Leu Arg 210 215 220
Gln Met Lys Arg Gly Ser Phe Leu Ile Asn Pro Cys Arg Gly Ser Val 225 230 235 240
Val Asp Glu Ala Ala Val Leu Gin Ser Leu Thr Tyr Gly His Leu Ser 245 250 255 260
Gly Tyr Ala Ala Asp Val Phe Glu Met Glu Asp Thr Ala Arg Pro Asp 265 270
Arg Pro Gin Arg Ile Asp Pro Ala Leu Leu Ala His Pro Asn Thr Leu 275 280 285 290
Phe Thr Ala His Thr Gly Ser Ala Val Arg Asp Val Arg Phe Ala Ile 295 300
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<210> SEQ ID NO 9
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<212> TYPE: PRT
<213> ORGANISM: Methylobacterium extorquens

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Leu Leu Ala Phe Met Thr Asp Cys Val Asp Ala Gly Phe Leu Glu Ala 50 55 60
Cys Pro Arg Leu Lys Val Val Ala Cys Ala Leu Lys Gly Trp Asp Asn 65 70 75 80
Phe Asp Val Glu Ala Cys Thr Arg Ser Gly Ile Trp Leu Thr Ala Val 85 90 95
Pro Asp Leu Leu Thr Glu Pro Thr Ala Glu Leu Ala Val Gly Leu Ala 100 105 110
Ile Gly Leu Cys Arg Asn Val Val Ala Gly Asp Arg Ala Val Arg Ala 115 120 125
Gly Phe Asp Gly Trp Arg Pro Arg Leu Tyr Gly Ser Gly Leu Tyr Gly 130 135 140
Ser Val Val Gly Val Ala Gly Met Gly Lys Val Gly Arg Ala Ile Thr 145 150 155 160
Arg Arg Leu Lys Gly Phe Gly Ala Arg Glu Leu Tyr Phe Asp Glu 165 170 175
Gln Ala Leu Pro Ala Ser Ala Glu Ala Glu Gly Ala Cys Arg Val 180 185 190
Ser Trp Asp Thr Leu Val Gly Arg Ser Asp Val Leu Ile Leu Ala Leu
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225 230 235 240
Val Asp Glu Ala Ala Val Ala Glu Ala Leu Ala Gly Arg Leu Gly
245 250 255
Gly Tyr Ala Ala Asp Val Phe Glu Met Glu Asp Thr Ala Leu Asp Asp
260 265 270
Arg Pro Arg Arg Ile Ala Pro Gly Leu Thr Val Glu Asp Arg Thr
275 280 285
Leu Phe Thr Pro His Leu Gly Ser Val Val Asp Thr Arg Arg Arg
290 295 300
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Val Pro Ala Asp Ser Ile Asn His Pro Glu Ser Leu Arg Gly Phe Asp
325 330 335
Gly Ala Asn

<210> SEQ ID NO 10
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<212> TYPE: PRT
<213> ORGANISM: Nostoc sp. PCC 7120
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Glu Leu Leu Lys Pro Ser Cys Glu Val Ile Ala Asn Pro Ser Lys Glu
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 Ala Leu Ser Arg Glu Ile Leu Gln Arg Ala Lys Asp Ala Glu Ala
35 40 45
Leu Met Val Phe Met Pro Asp Thr Ile Asp Glu Ala Phe Leu Arg Glu
50 55 60
Cys Pro Lys Leu Lys Ile Ile Ala Ala Ala Leu Lys Gly Tyr Asp Asn
65 70 75 80
Phe Asp Val Ala Ala Cys Thr His Arg Gly Ile Thr Phe Thr Ile Val
85 90 95
Pro Ser Leu Leu Ser Ala Pro Thr Ala Glu Ile Thr Ile Gly Leu Leu
100 105 110
Ile Gly Leu Gly Arg Gln Met Leu Glu Gly Asp Arg Phe Ile Arg Thr
115 120 125
Gly Lys Phe Thr Gly Trp Arg Pro Gln Phe Tyr Ser Leu Gly Leu Ala
130 135 140
Asn Arg Thr Leu Gly Ile Val Gly Met Gly Ala Leu Gly Lys Ala Ala
145 150 155 160
 Ala Gly Arg Leu Ala Gly Phe Glu Met Gin Leu Leu Tyr Ser Asp Pro
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(SEQ ID NO 11)

LENGTH: 331

ORGANISM: Oxalobacter formigenes

SEQUENCE: 11

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Lys Thr Phe Thr Arg Asp Glu Leu Glu Arg Asp Ala Ala Ala Asp
Ala Leu Met Ala Phe Met Pro Asp CyC Ile Asp Glu Asp Phe Leu Lys
Ala CyC Pro Lys Leu Lys Val Val Ala Ala Glu Lys Ala Lys Gly Tyr Asp
Asn Phe Asp Val Lys Ala CyC Thr Glu Arg Gly Val Trp Leu Thr Ile
Ala Pro Asp Leu Leu Thr Ile Pro Thr Ala Glu Leu Thr Val Gly Leu
Val Leu Ala Ile Thr Arg Asn Met Leu Glu Gly Asp Arg His Ile Arg
Ser Gly Glu Phe Asn Gly Trp Arg Pro Glu Leu Tyr Gly Leu Gly Leu
His Lys Arg Thr Ala Gly Ile Gly Met Gly Phe Val Gly Lys Ala
Val Ala Glu Arg Leu Lys Gly Phe Gly Met Asp Ile Leu Tyr Ala Asp
Gln Ser Pro Leu Ser Gln Glu Glu Glu Arg Glu Leu Gly Leu Thr Arg
Thr Gly Leu Pro Glu Leu Met His Ser Ser Asp Val Val Ile Pro Leu
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Gly Glu Met Lys Glu Gly Ser Tyr Leu Val Asn Ala Cys Arg Gly Ser
```
Val Val Asp Glu Lys Ala Val Val His Ser Leu Lys Thr Gly Gin Leu
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Ala Gly Tyr Ala Ala Asp Val Phe Glu Met Glu Asp Trp Ile Arg Ser
260 265 270
Asp Arg Pro Arg Glu Ile Pro Gin Glu Leu Leu Asp Arg Thr Ala Gin
275 280 285
Thr Phe Phe Thr Pro His Leu Gly Ser Ala Val Asp Glu Ile Arg Ile
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325 330

<210> SEQ ID NO 12
<211> LENGTH: 328
<212> TYPE: PRT
<213> ORGANISM: Streptomyces sviceus
<400> SEQUENCE: 12
Met Val Thr His Trp Ile His Pro Glu Val Val Asp Tyr Leu Arg Arg
1 5 10 15
Phe Cys Asp Pro Val Val Pro Val Glu Thr Glu Val Leu Gly Arg Arg
20 25 30
Gln Cys Leu Glu Leu Ala Ala Asp Asp Ala Leu Ile Met Cys Met
35 40 45
Ala Asp Arg Val Asp Asp Phe Leu Ala Glu Cys Pro Arg Leu Arg
50 55 60
Val Ile Ser Thr Val Val Lys Gly Tyr Asn Phe Asp Ala Glu Ala
65 70 75 80
Cys Ala Arg Arg Gly Val Trp Leu Thr Val Leu Pro Asp Leu Leu Thr
85 90 95
Ala Pro Thr Ala Glu Leu Ala Val Thr Leu Ala Val Ala Leu Gly Arg
100 105 110
Arg Ile Arg Glu Gly Asp Ala Leu Met Arg Ser Gly Arg Tyr Asp Gly
115 120 125
Trp Arg Pro Val Leu Tyr Gly Thr Leu Tyr Arg Ser Arg Ser Arg Val Gly
130 135 140
Val Val Gly Met Gly Leu Gly Arg Ala Val Ala Arg Leu Ser
145 150 155 160
Gly Phe Glu Pro Ser Glu Val Tyr Tyr Asp Lys Gin Pro Leu Gly
165 170 175
Ala Ser Glu Glu Arg Arg Gly Val Gly Ala Ala Gly Leu Glu Glu
180 185 190
Leu Met Gly Arg Cys Gin Val Val Leu Ser Leu Leu Pro Leu Ala Met
195 200 205
Asp Thr Arg His Leu Ile Gly Ser Asp Ala Ile Ala Ala Ala Arg Pro
210 215 220
Gly Gin Leu Leu Val Asn Val Gly Arg Gly Ser Val Val Asp Glu Asp
225 230 235 240
Ala Val Ala Ala Ala Leu Asp Cys Gly Pro Leu Gly Gly Tyr Ala Ala
245 250 255
Asp Val Phe Gly Cys Glu Asp Leu Thr Ala Pro Gly His Leu Arg Glu

Val Pro Arg Arg Leu Leu Thr His Pro Arg Thr Leu Thr Pro His

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Val Thr Ala Gly Leu Leu Arg Glu

<210> SEQ ID NO 13
<211> LENGTH: 336
<212> TYPE: PRT
<213> ORGANISM: Thioalkalivibrio sp. HL-EBGR7

<400> SEQUENCE: 13
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20 25 30
Thr Leu Pro Arg Glu Val Ile Leu Arg Arg Asp Ala Gln Ala

35 40 45
Met Met Ala Phe Met Pro Asp Arg Val Asp Ala Asp Phe Leu Gln Ala

50 55 60
Cys Pro Glu Leu Arg Val Val Gly Cys Ala Leu Lys Gly Phe Asp Asn

65 70 75 80
Phe Asp Val Asp Ala Cys Thr Ala Arg Gly Val Thr Phe Val

85 90 95
Pro Asp Leu Leu Thr Val Pro Thr Ala Glu Leu Ala Ile Gly Leu Ala

100 105 110
Val Gly Leu Gly Arg His Leu Arg Ala Ala Asp Ala Phe Val Arg Ser

115 120 125
Gly Glu Phe Gln Gly Trp Gln Pro Gln Phe Tyr Gly Thr Gly Leu Asp

130 135 140
Asn Ala Thr Val Gly Ile Leu Gly Met Gly Ala Ile Gly Leu Ala Met

145 150 155 160
Ala Asp Arg Leu Gln Gly Trp Ala Thr Leu Gln Thr His Glu Ala

165 170 175
Lys Ala Leu Asp Thr Gln Thr Glu Gln Arg Leu Gly Leu Arg Arg Val

180 185 190
Ala Cys Ser Glu Leu Phe Ala Sar Ser Asp Phe Ile Leu Leu Ala Leu

195 200 205
Pro Leu Asn Ala Asp Thr Gln His Leu Val Asn Ala Glu Leu Leu Ala

210 215 220
Leu Val Arg Pro Gly Ala Leu Val Asn Pro Cys Arg Gly Ser Val

225 230 235 240
Val Asp Glu Ala Ala Val Leu Ala Ala Leu Glu Arg Gly Gln Leu Gly

245 250 255
Gly Tyr Ala Ala Asp Val Phe Glu Met Glu Asp Trp Ala Arg Ala Asp

260 265 270
Arg Pro Arg Leu Ile Asp Pro Ala Leu Leu Thr His Pro Asn Thr Leu

275 280 285
-continued

Phe Thr Pro His Ile Gly Ser Ala Val Arg Ala Val Arg Leu Glu Ile  
  290 295 300

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Pro Ile Asn Ala Ala Asn Arg Leu Pro Lys Ala Glu Pro Ala Ala Cys  
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<210> SEQ ID NO 14
<211> LENGTH: 331
<212> TYPE: PRT
<213> ORGANISM: Xanthobacter flavus

<400> SEQUENCE: 14

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Pro Trp Pro Arg Asp Glu Ile Leu Arg Ala His Gly Ala Asp Ala  
35 40 45

Met Leu Ala Phe Met Thr Asp His Val Asp Ala Ala Phe Leu Asp Ala  
50 55 60

Cys Pro Glu Leu Lys Ile Val Ala Cys Ala Leu Lys Gly Ala Asp Asn  
65 70 75 80

Phe Asp Met Glu Ala Cys Arg Ala Arg Lys Val Ala Val Thr Ile Val  
85 90 95

Pro Asp Leu Leu Thr Ala Pro Thr Ala Glu Leu Ala Val Gly Leu Met  
100 105 110

Ile Thr Leu Gly Arg Asn Leu Leu Ala Gly Asp Arg Leu Ile Arg Glu  
115 120 125

Arg Pro Phe Ala Gly Trp Arg Pro Val Leu Tyr Gly Thr Gly Leu Asp  
130 135 140

Gly Ala Glu Val Gly Ile Val Gly Met Gly Ala Val Gly Gln Ala Ile  
145 150 155 160

Ala His Arg Leu Arg Pro Phe Arg Cys Arg Leu Ser Tyr Cys Asp Ala  
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Arg Pro Leu Ser Pro Ala Glu Asp Ala Gln Gly Leu Leu Arg Arg  
180 185 190

Asp Leu Ala Asp Leu Val Ala Arg Ser Asp Tyr Leu Val Leu Ala Leu  
195 200 205

Pro Leu Thr Pro Ala Ser Arg His Leu Ile Asp Ala Ala Ala Leu Ala  
210 215 220

Gly Met Lys Pro Gly Ala Leu Leu Ile Asn Pro Ala Arg Gly Ser Leu  
225 230 235 240

Val Asp Glu Ala Val Ala Asp Ala Leu Glu Ala Gly His Leu Gly  
245 250 255

Gly Tyr Ala Ala Asp Val Phe Glu Thr Glu Asp Trp Ala Arg Pro Asp  
260 265 270

Arg Pro Ala Ala Ile Glu Ala Arg Leu Leu Ala His Pro Arg Thr Val  
275 280 285

Leu Thr Pro His Ile Gly Ser Ala Val Asp Ser Val Arg Arg Asp Ile  
290 295 300

Ala Leu Ala Ala Arg Asp Ile Leu Arg His Leu Asp Gly Leu Gln
Gln Asp Pro Pro Ser Arg Asp Arg Ser Ala Ala 325 330

<210> SEQ ID NO 15
<211> LENGTH: 10
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Consensus sequence for NAD-binding motif

<400> SEQUENCE: 15
Val Gly Ile Leu Gly Met Gly Ala Ile Gly
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<210> SEQ ID NO 16
<211> LENGTH: 16
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Consensus signature sequence for the D-isomer specific 2-hydroxyacid family
<220> FEATURE:
<221> NAME/KEY: MISC_FEATURE
<222> LOCATION: (1)...(1)
<223> OTHER INFORMATION: Xaa presents Arg or Lys

<400> SEQUENCE: 16
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<210> SEQ ID NO 17
<211> LENGTH: 6
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Conserved signature sequence for the D-isomer specific 2-hydroxyacid family

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<210> SEQ ID NO 18
<211> LENGTH: 11
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Consensus motif that may enable hydrogenases to use phosphite as substrate

<400> SEQUENCE: 18
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<210> SEQ ID NO 19
<211> LENGTH: 11
<212> TYPE: PRT
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- **NAME/KEY:** MISC_FEATURE
- **LOCATION:** (3) .. (3)
- **OTHER INFORMATION:** Xaa represents X, which is Arg, Gln, Lys, or Thr

- **NAME/KEY:** MISC_FEATURE
- **LOCATION:** (5) .. (5)
- **OTHER INFORMATION:** Xaa represents X2, which is Ala, Val, Gln, Arg, Lys, His, or Glu

- **NAME/KEY:** MISC_FEATURE
- **LOCATION:** (6) .. (6)
- **OTHER INFORMATION:** Xaa represents X3, which is Leu or Phe

- **NAME/KEY:** MISC_FEATURE
- **LOCATION:** (8) .. (8)
- **OTHER INFORMATION:** Xaa represents X4, which is Gly, Phe, or Ser

- **NAME/KEY:** MISC_FEATURE
- **LOCATION:** (9) .. (9)
- **OTHER INFORMATION:** Xaa represents X5, which is Thr, Arg, Met, Leu, Ala, or Ser

SEQUENCE:

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```
Metr Phe Ala Glu Gin Gln Gin Arg Glu Tyr Leu Asp Lys Gly Tyr Thr Lys
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20     25
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35     40
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50     55
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65     70
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Arg Ser Glu Lys Ile Val Asp Leu Arg His Phe Leu Gly Glu Asn
85     90
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100    105
```

```
Pro Val Gln Trp His Gln Asp Thr Ala Phe Tyr Pro His Thr Asn Asp
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```

```
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130    135
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```
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145    150
```

```
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165    170
```

```
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180    185
```

```
Pro Val Gly Thr Val Thr Leu His Val Arg Thr Leu His Gly Ser
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```
-continued

Gly  Pro  Asn  His  Ser  Thr  Ile  Arg  Arg  Arg  Phe  Leu  Leu  Ile  Gly  Tyr
   210  215
  220
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Tyr  Glu  Ser  Leu  Met  Val  Ser  Gly  Arg  Ser  Thr  Val  Phe  Pro  Arg  Met
   245  250  255
Val  Glu  Leu  Pro  Leu  Thr  Val  Pro  Tyr  Pro  Leu  Ser  Met  Tyr  Gly  Asp
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<210> SEQ ID NO 21
<211> LENGTH: 1011
<212> TYPE: DNA
<213> ORGANISM: Pseudomonas stutzeri

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  360
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  420
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gacacaccc agaaccagct cgcgtgcggc gaggtggtgtgc gcacactttt cttgcctgtc
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gacacaccc agaaccagct cgcgtgcggc gaggtggtgtgc gcacactttt cttgcctgtc
  780
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gacacaccc agaaccagct cgcgtgcggc gaggtggtgtgc gcacactttt cttgcctgtc
  840
tgggccacca cttgctgtgg ctgcctttgg tctggtctttt gacgcaacact ctggtcttttc
gacacaccc agaaccagct cgcgtgcggc gaggtggtgtgc gcacactttt cttgcctgtc
  900
tgggccacca cttgctgtgg ctgcctttgg tctggtctttt gacgcaacact ctggtcttttc
gacacaccc agaaccagct cgcgtgcggc gaggtggtgtgc gcacactttt cttgcctgtc
  960
cacactaacc cttgccctct ggccagcatc ggctcagagc ctcctgtgaa ctgcctctgc
 1011

<210> SEQ ID NO 22
<211> LENGTH: 56
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence

<400> SEQUENCE: 22
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  56
1. A nucleic acid, comprising:
a chimeric gene including (a) a coding region that encodes
a phosphite dehydrogenase enzyme and (b) a transcription
promoter operatively linked to the coding region,
wherein the promoter is heterologous with respect to the
coding region and is functional in plants, fungi, or both,
and
wherein the chimeric gene provides sufficient expression
of the enzyme, in a plant or fungal cell containing the
chimeric gene, to confer an ability on the cell to metabo-
lize phosphite (P) as a phosphorus source for supporting
growth, thereby enabling growth of the cell without
an external source of phosphate (P).

2. The nucleic acid of claim 1, wherein the phosphite
dehydrogenase enzyme is of bacterial origin.

3. The nucleic acid of claim 2, wherein the phosphite
dehydrogenase enzyme is PtxD of Pseudomonas stutzeri
(SEQ ID NO:1), an analog or derivative of the PtxD of SEQ
ID NO:1, or a PtxD-like homolog from another bacterial
species.

4. The nucleic acid of claim 2, wherein the bacterial phosphite
dehydrogenase enzyme has an amino acid sequence with
at least 80% sequence identity to at least one of SEQ ID
NOS:1-14.

5. The nucleic acid of claim 1, wherein the phosphite
dehydrogenase enzyme has an amino acid sequence includ-
ing a first sequence region having an NAD-binding motif with
sequence similarity or identity to VGIILGMAIG (SEQ ID
NO:15), a second sequence region having sequence similarity
or identity to XPGLNVPCRGSSVVD (SEQ ID NO:16),
where X is K or R, and a third sequence region having
sequence similarity or identity to GXW1IPX2X3YX4X5GGL
(SEQ ID NO:19), where X1 is R, Q, T, or K, X2 is A, V, Q, R,
K, H, or F, X3 is L, or F, X4 is G, F, or S, and X5 is T, R, M,
L, or A.

6. The nucleic acid of claim 1, wherein the phosphite
dehydrogenase enzyme has an amino acid sequence that is
at least 90% identical to PtxD from Pseudomonas stutzeri (SEQ
ID NO:1).

7. The nucleic acid of claim 1, wherein the chimeric gene
further includes a transcription terminator that is operatively
linked to the coding region and heterologous with respect to
the coding region.

8. The nucleic acid of claim 1, wherein the promoter is a
plant promoter or a viral promoter of a plant virus and is
capable of promoting the sufficient expression of the enzyme
in a plant cell.

9. The nucleic acid of claim 8, wherein the promoter corre-
sponds to the 35S promoter of Cauliflower Mosaic Virus.

10. The nucleic acid of claim 8, wherein the promoter is
inducible by low phosphate availability.

11. The nucleic acid of claim 8, wherein the promoter
corresponds to a promoter of the PLDZ2 gene of Arabidopsis
thaliana.

12. The nucleic acid of claim 1, wherein the chimeric gene
is capable of promoting the sufficient expression of the
enzyme both in a plant cell and in a fungal cell containing
the chimeric gene.

13. The nucleic acid of claim 1, wherein the promoter is a
fungal promoter capable of promoting the sufficient expres-
sion of the enzyme in a fungal cell.

14. A plant cell comprising the nucleic acid of any one of
claims 1-12 and capable of metabolizing phosphite as a
source of phosphorus for supporting growth.

15. The plant cell of claim 14, further comprising a nucleic
acid that expresses a bacterial hypophosphite dehydrogenase
enzyme in the plant cell.

16. The plant cell of claim 15, wherein the nucleic acids
collectively confer an ability on the cell to metabolize hypophos-
ite (Hphi) as a phosphorus source for supporting growth.

17. The plant cell of claim 16, wherein expression of the
phosphite dehydrogenase enzyme, the bacterial hypophos-
ite dehydrogenase enzyme, or both are controlled by a
root-specific promoter.

18. The plant cell of claim 14, wherein the plant cell is a
eukaryotic algal cell.

19. The plant cell of claim 18, wherein the algal cell is a
Chlamydomonas cell.

20. The plant cell of claim 14, wherein the plant cell is from
a species of vascular plant.

21. A plant composed of a plurality of plant cells according
to claim 14.

22. A plant composed of a plurality of plant cells according
to claim 20.

23. The plant of claim 22, wherein the plant is a species of
crop plant.

24. The plant of claim 23, wherein the species of crop plant is
selected from the group consisting of maize, soybean, rice,
potatoes, tomatoes, sugar cane, and wheat.

25. A fungal cell comprising the nucleic acid of any one of
claims 1-7, 12, and 13 and capable of metabolizing phosphite
as a source of phosphorus for supporting growth.

26. The fungal cell of claim 25, further comprising a
nucleic acid that expresses a bacterial hypophosphite dehy-
drogenase enzyme in the fungal cell.

27. The fungal cell of claim 26, wherein the nucleic acids
collectively confer an ability on the cell to metabolize hypophos-
ite (Hphi) as a phosphorus source for supporting growth.

28. The fungal cell of claim 25, wherein the fungal cell is
from a species of Trichoderma.

29. A method of reducing fungal infections in plants, the
method comprising:
applying a plurality of the fungal cells of claim 25 to a seed form of plants, the plants themselves, soil in which the plants are or will be disposed, or a combination thereof.

30. The fungal cell of claim 25, wherein the fungal cell is a member of a species of mycorrhizal fungus capable of forming a symbiotic relationship with a plant.

31. A plant associated with a plurality of fungal cells according to claim 30 to form mycorrhizae.

32. The plant of claim 31, wherein the fungal cells render the plant capable of growing on a medium containing phosphite (Phi) as a phosphorus source for supporting growth.

33. A method of fertilizing a crop plant using hypophosphate and/or phosphite as a phosphorus source for supporting growth, the crop plant (a) including a plurality of cells comprising the nucleic acid of claim 1, (b) forming mycorrhizae with a mycorrhizal fungus comprising the nucleic acid of claim 1, and/or (c) being associated with a Trichoderma fungus comprising the nucleic acid of claim 1, the method comprising:

- applying at least one reduced form of phosphorus to the plant and/or to soil adjacent to the plant, such that the reduced form is metabolized to phosphate by the plant and/or the fungus to support growth and productivity of the plant.

34. A method of fertilizing the plant of claim 22, the method comprising:

- applying at least one reduced form of phosphorus to the plant and/or to soil adjacent to the plant, such that the reduced form is metabolized to phosphate by the plant to support growth and productivity of the plant.

35. A method of treating liquid waste to lower its content of reduced phosphorus, the method comprising:

- contacting (i) water containing hypophosphate and/or phosphite as a contaminant and (ii) a plurality of the plant cells and/or fungal cells comprising the nucleic acid of claim 1, such that at least a portion of the hypophosphate and/or phosphite is oxidized to phosphate and/or phosphate.

36. The method of claim 35, wherein the step of contacting includes a step of contacting the water and a plurality of vascular plants composed of plant cells comprising the nucleic acid of claim 1.

37. A method of isolating transformed cells containing the nucleic acid of claim 1, the method comprising:

- contacting cells selected from plant cells and fungal cells with a composition comprising the nucleic acid of claim 1; and

- selectively proliferating one or more of the cells that have been transformed by the nucleic acid by culturing the cells in a medium containing phosphate and lacking sufficient phosphate to support growth.

38. A method of utilizing the nucleic acid of claim 1 for production of a transgenic plant, the method comprising:

- selecting for growth of plant cells comprising the nucleic acid of claim 1 as a selectable marker during production of a transgenic plant.

39. A method of obtaining a plant transformed with a nucleic acid encoding a phosphite dehydrogenase enzyme that is expressible from the nucleic acid as a selectable marker, comprising:

- contacting plant cells with a composition including the nucleic acid under conditions that promote introduction of the nucleic acid into at least a subset of the plant cells;

culturing the plant cells in a medium containing phosphite as a primary or exclusive phosphorus source for growth;

- selecting transformed plant cells produced by the steps of contacting and culturing, and expressing the phosphite dehydrogenase enzyme as evidenced by growth in the medium; and

- regenerating at least a portion of the transformed plant cells into a transgenic plant.

40. The method of claim 39, wherein the composition includes Agrobacterium cells that supply the nucleic acid during the step of contacting.

41. The method of claim 39, wherein the composition includes projectiles that are fired at the plant cells in the step of contacting.

42. A plant, comprising:

- a nucleic acid including a chimeric gene expressing a phosphite dehydrogenase enzyme such that the plant is capable of metabolizing phosphite (Phi) as a phosphorus source for supporting growth, thereby enabling growth of the plant without an external source of phosphate (P).

43. The plant of claim 42, wherein the nucleic acid is stably integrated into the genome of the plant.

44. The plant of claim 42, wherein the plant is a vascular plant.

45. The plant of claim 42, wherein the plant is a species of algae.

46. A fungus, comprising:

- a nucleic acid including a chimeric gene expressing a phosphite dehydrogenase enzyme such that the fungus is capable of metabolizing phosphite (Phi) as a phosphorus source for supporting growth, thereby enabling growth of the fungus without an external source of phosphate (P).

47. The fungus of claim 46, wherein the nucleic acid is stably integrated into the genome of the fungus.

48. The fungus of claim 46, wherein the fungus is a species of Trichoderma.

49. The fungus of claim 46, wherein the fungus is a mycorrhizal species capable of forming a symbiotic relationship with a plant.

50. The fungus of claim 46, further comprising a plant associated with the fungus to form mycorrhizae.

51. A nucleic acid, comprising:

- a chimeric gene including (a) a coding region that encodes an enzyme that catalyzes oxidation of phosphite to phosphate and (b) a transcription promoter operatively linked to the coding region, wherein the promoter is heterologous with respect to the coding region and is functional in a plant cell, and wherein the chimeric gene provides sufficient expression of the enzyme, in a plant cell containing the chimeric gene, to confer an ability on the plant cell to metabolize phosphite (Phi) as a phosphorus source for supporting growth.

* * * * *