Recent Patents on Plant Transgenic Technology

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Abstract: Plant transformation technology plays an ever-increasing role in improving crop plant traits and addressing research questions. In recent years, a number of important patents on plant transgenic technologies have been published. These patents represent significant new progresses, many representing novel approaches in the field of plant transgenic technology. This review highlights selected as well as the most significant advancements made mostly within the past 2 years in plant transgenic research. These advancements include the improvements of plant transformation systems, development of new plant selectable markers, engineering for plant stress tolerance, production of vaccines in transgenic plants, etc. Of these, the engineering for plant stress tolerance accounts for a high percentage of the transgenic field of inventions. Some results are drastic and unexpected, having a great potential in their applications towards a wider range of plant species. This review also tries to avoid terms and languages pertaining to the patent and legal documents to ease readings.

Keywords: Agrobacterium, T-DNA, transformation, selectable marker, stress tolerance, transgenic plant, vaccine.

INTRODUCTION

Plant transformation technology plays a unique role in improving crop plant traits as well as addressing research questions which otherwise would be very difficult to achieve by conventional approach. From the 1980’s to the 1990’s, world-wide efforts in plant transformation focused on establishment of regeneration and transformation protocols. Afterwards, major efforts in plant transformation has been towards developing high-through-put regeneration and transformation processes for major crop species, as well as establishing reproducible regeneration and transformation systems in highly recalcitrant plant species. In recent years, transformation technology has been advancing so rapidly and is now widely applied to benefiting society that numerous patents have been filed and published. To date there have been around 1,889 patents in the field of plant transgenic technology filed or published either in the U.S.A., Europe, or worldwide. Most patents on plant transgenenic technology describe the improvements of Agrobacteriummediated transformation methods and it applications. These include, but are not limited to, transformation methods and efficiency, selectable markers and selective agents, engineering of plants for stress tolerance, and vaccine production, etc. Of these patents, development of new or improved selectable marker genes and selective agents, as well as engineering of plants for stress tolerance, account for a major category of patent inventions. Of these, there are a higher number of patent applications for plant transformation technology addressing crop production constraints such as herbicide resistance, abiotic and biotic tolerance, and enhancing yield, etc. This review will highlight selected and the most significant advancement in plant transgenic technology with particular focus on the patents filed or published over the past two years. Some inventions will still be presented to reflect new approaches, even though these patents do not provide phenotypic results.

IMPROVING PLANT TRANSFORMATION METHODS AND EFFICIENCY

Post-infection desiccation enhances plant transformation (Patent EP1137790B1, Monsanto Technology LLC): Agrobacterium infection, including both inoculation and cocultivation, of explants is a critical stage in determining transformation efficiency of a target plant species. It is this stage that major cell or tissue damages take place, due to the Agrobacterium infection or tissue maceration. Cheng and Fry (2006) discovered that post-infection (i.e., after Agrobacterium infection) desiccation of target explants promotes transformation frequencies substantially [1]. The key to this invention is the use of Whatman filter paper to generate a relatively dry culture environment for the explants during cocultivation after explant inoculation. Such post-infection desiccation treatment can reduce from about 20% to 35% of the weight of the plant cell or tissue prior to coculture. Therefore, the mechanism of such desiccation could be similar to the osmotic treatment medium used in biolistic transformation, though the exact mechanism of osmotic treatment is still unknown, which presumably reduces cell turgidity, minimizing the damages caused by infection. Data from Cheng and Fry’s stable transformation experiments (in ten replicates), showed that the transformation efficiency in wheat ranged from 3.6% to 37.7%, with an average of 10%. This is by far the highest transformation frequency in wheat reported to date using Agrobacterium-tumefaciens. The transformation vectors used in these experiments were standard binary vectors in which Figwort Mosaic Virus [2] or enhanced CaMV35S drives npt II as a selectable marker or GUS reporter gene. Such desiccation-enhanced transformation has been reproducible in different plant species such as maize (Zea mays), wheat (Triticum aestivum), and
soybean (Glycine max) [1] as well as rice (Oryza sativa) [3] and Brachypodium [4].

Vectors carrying repABC origin for improved plant transformation (Patent WO2007030432 A3, Monsanto Technology LLC): The Agrobacterium-mediated T-DNA integration into the plant genome generally starts at the right border (RB) and ends at left border (LB), respectively [5]. In some cases, however, the endonucleases do not nick equally at both RB and LB. In this case, the integrated T-DNA often contains partial or entire plasmid vector DNA. This problem is named "border read-through" and the non-T-DNA region is referred to as the vector backbone. Apparently, integration of the vector backbone into the plant genome is undesirable for transgenic crop plants because backbone DNA contains undesirable plasmid elements. Thus, it would be highly desirable to generate transgenic plants that are free from vector backbone DNA with one or two copies of the transgenes.

The replication origin for maintaining a low copy number of a DNA construct in a plant-cell-transforming bacterial cell is comprised of one or more copies of repA, repB, and repC [6, 7]. Ye et al. (2007) tackled the backbone integration problem by employing repABC element such as oriRi from Agrobacterium rhizogenes and repABC origin from plasmid p42b of Rhizobium etli [8]. These origins of the repABC element have been shown not only to reduce the frequency of backbone integration but also to increase the frequency of one- or two-copy T-DNA transformation events. In addition, combined use of both origins enabled to generate transformation events having lower frequencies of backbone DNA and higher frequencies of one- or two-copy T-DNA integration.

The patent illustrated the reduced backbone integration and low transgene copy numbers in soybean transformation experiments. Soybean meristem axis excised from mature tissues were transformed with a control DNA plasmid carrying oriV (from vector pCGN1589 with DraI digestion) and a plasmid containing oriRi (this invention), then regenerated into whole plants. Out of 39 plants tested, only 3 contained vector backbone sequences, a frequency of 7.7%. In comparison, the control plasmid (an oriV plasmid) exhibited a vector backbone frequency of 21% to 25%. The experiments also showed that this invention (oriRi-containing plasmid) resulted in 56.4% one-copy events and 33.3% two-copy events, or a total of nearly 90% of one- and two-copy events combined.

Use of oriRi also improved 2T-DNA (two separate T-DNA regions in the same vector) transformation. When the oriRi-containing plasmid carrying 2 separate T-DNA regions (oriRi 2T) was compared side-by-side with the oriV 2T vector control, the result demonstrated that one and/or two copy transformants were increased from 35% in the control oriV vector, to more than 40% in oriRi vectors. The frequency of marker-free transgenic plants in T0 plants (marker free events/total T0 plants) was increased from 4.76% in the oriV control vector to 16.4% and 18.4%, respectively, in the two oriRi vectors. Overall, the marker-free transformation frequency (marker free events/initial explant number) more than doubled in the two oriRi 2T vectors compared to the oriV 2T control vector.

The beneficial impact of the oriRi replication origin on corn transformation was also investigated using Agrobacterium containing the oriRi and the oriV vectors in parallel. Although the overall transformation efficiency with oriRi and oriV vectors was the same, about 95% of the transgenic plants derived from oriRi vector were backbone-free, which is significantly better than oriV vector control showing about 78% backbone free frequency. Results also revealed that most vector backbones transferred contained entire vector backbone sequence. Importantly, the oriRi vector significantly increased single copy plant frequency, compared to the oriV vector control, while the frequency of 2 or more copy events in oriRi vector was decreased.

Use of multiple enhancer sequences to enhance Agrobacterium-mediated plant transformation (Patent WO2008011465A3, Monsanto Technology LLC): These enhancer sequences include “overdrive” [9] or “T-DNA transfer stimulator sequence” (TSS) [10]. Both overdrive and TSS are cis-acting sequences next to the right border (RB, a 24-bp imperfect repeat) and are present in many Agrobacterium strains including A. tumefaciens (termed overdrive) and A. rhizogenes (termed TSS). The overdrive and TSS are binding sites for VirC1 protein. Such binding is thought to improve VirD2 nicking [11-13]. These two proteins are essential for the T-strand production during the early events of the T-DNA transfer [5]. Either the overdrive or TSS contains an 8-bp core sequence, which is similar but not identical to each other. This 8-bp core sequence repeats different times in different Agrobacterium strains with shorter repeats being likely insufficient for T-DNA transfer.

By the same analogy, repeated use of the overdrive or TSS as a transformation enhancer would improve T-DNA transfer in plants and prevent the transfer of vector backbone sequence caused by insufficient nicking by VirD2 at RB region. This invention (Ye and Gilbertson, 2008) provides a new strategy of enhancing Agrobacterium-mediated plant transformation through introducing multiple overdrive or TSS into a plant transformation vector at the T-DNA border regions [14]. Such a technique may be extended to bacterially-mediated transformation including not only Agrobacterium- or Rhizobium-mediated transformation, but also Sinorhizobium-, Mesorhizobium- or Bradyrhizobium-mediated transformation [15], with as many as 18 copies of enhancer sequence repeats linked to T-DNA borders (either LB or RB) from various types of T plasmids. The improved transformation efficiencies by this technique were achieved in both maize and soybean. In maize, transformation frequencies (TFs) were from 22.8-25.3% with more than 10% quality events and over 40% one or two copies events, as compared to 6.4% quality events and 39% one or two copies events in control. In soybean, TFs were more than 4% with 12-34% single-copy events, as compared with 2.8% TF with only 4% single-copy events in control.

PLANT SELECTABLE MARKER GENES AND SELECTIVE AGENTS

Selectable marker genes play an essential role in discriminating very few transformed cells from most non-transformed cells in order to obtain transgenic plants and cut off the workload in tissue culture work. Therefore, except for few cases where no selection is required, most of trans-
formation systems need efficient selection process. It is important that the expression cassette for selectable marker gene is placed adjacent to the T-DNA left border. This strategy for cassette arrangement is to ensure the presence of intact gene of interest GOI cassette once marker gene is confirmed to be present, since the marker gene and GOI are co-integrated into the host plant genome. The most widely used selectable marker genes are herbicide resistance (bar, PAT, CP4 EPSPS) and antibiotic resistance (npt II, hpt) genes. However, new selectable marker genes have been recently deployed for various transformation purposes.

**Spectinomycin as a selective agent for plant transformation** (Patent WO2008112645, Monsanto Technology LLC): Martinell *et al.* (2008) described the use of selectable marker, *aadA* for plant transformation [16]. This gene encodes a aminoglycoside-3'-adenyltransferase (*aadA*). The *aadA* is for conferring resistance to spectinomycin. The *aadA* was driven by CaMV35S promoter or other promoters and can be fused to a chloroplast transit peptide to target this gene product to this organelle. Transformation was through *Agrobacterium tumefaciens* strain C58 and targeted explants derived from soybean (*Glycine max*) and cotton (*Gossypium spp*). Seeds for selection and shoot induction, the explants were subcultured to the same liquid medium but with different levels of spectinomycin (25-250 mg/L and up to 1000mg/L). The average transformation frequency was 18.6%, ranging from 12.6-26.1%, a significant increase from the approximate 2% transformation frequency obtained in comparable experiments utilizing glyphosate or dicamba as a selective agent. This increase is equivalent to 2-10 or 5-10 fold. However, the authors did not define the frequencies of transformation derived from the suspension culture, when it is used. Such a culture system could over-estimate the transformation frequency because of the possible breakup of the cell clusters.

The spectinomycin-selection-based protocols also demonstrated a shorter time frame needed to complete each protocol (inoculation to next generation seed), as compared with the glyphosate selective protocol. For example, the time from inoculation to the time for development of transformed rooted shoots was about 8 weeks, and subsequent T1 seed harvest was typically <6 months. Additional advantages of using this selection system are simple, time-saving, and streamlining in plant handling, leading to lower costs. The system enables stacking traits by transformation into existing transgenic germplasm through alternative selection system.

The spectinomycin selection system has been utilized to transform dry-excised soybean explants, of which split and viable meristem explants were recovered. Using these dry-excised explants, the transformation frequency was about 20-25% with over 10% routinely. The same selection system was also used in cotton and corn transformation employing either one or 2 T-DNA binary vector. Finally, we want to point out that the spectinomycin selection has been used extensively for plastid transformation via biolistics (not in this invention).

**AmGSTFI for multiple herbicide resistance** (Patent WO2009034395A1, University of Durham, United Kingdom): Crop plants can be engineered to display multiple herbicide resistance (MHR) (Edwards and Cummins, 2008) [17]. Such an approach has taken advantages of the mechanisms exiting in certain plant species such as black-grass (*Alopecurus myosuroides*), wild oat (*Avena fatua*), and annual rye-grass (*Lolium rigidum*) that are capable of acquiring multiple herbicide resistance. In these plants, the activities of glutathione transferases (GSTs) detoxify herbicides through responses to oxidative stress. The gene, *AmGSTFI*, encoding this enzyme has been cloned from black-grass, placed under the control of CaMV35S or inducible promoter, and subcloned into plant transformation vector pCAMBIA3300. Transgenic *Arabidopsis* plants expressing the *AmGSTFI* display MHR, i.e., three distinct classes of herbicide, atrazine, alachlor, chlortoluron. In these plants, overexpression of *AmGSTFI* was also found to up-regulate other enzymes of antioxidant metabolism.

**DSM-2 as a novel selectable marker** (Patent WO2008070845A3, Dow AgroSciences LLC): DSM-2, a novel selectable marker gene, was created which confers plant resistance to both glufosinate and 2,4-dichlorophenoxyacetic acid (2,4-D) (Lira *et al.*, 2008) [18]. Interestingly, the DSM-2 was identified from the NCBI database (see the ncbi.nlm.nih.gov website) as a homologue with only 30% amino acid identity to PAT (phosphinothrin acetotransferase) [19] and 28% to BAR (bialaphos resistance) [20], respectively. Both PAT and BAR encoded by pat and bar detoxify phosphinothrin (PPT) or bialaphos which is a competitive inhibitor of glutamine synthetase (GS). Structurally, PPT lacks two alanine amino acids, unlike bialaphos. GS in many plants is an essential enzyme for the development and life of plant cells. GS converts glutamate into glutamine and is also involved in ammonia assimilation and nitrogen metabolism. GS is involved in a pathway in most plants for the detoxification of ammonia released by nitrate reduction. Therefore, potent inhibitors of GS are very toxic to plant cells. Breakdown or modification of the herbicide inside the plant could lead to resistance.

Percent identity of DSM-2 as compared with PAT or BAR was determined by first translating the nucleotide sequences. The DSM-2 is then codon-optimized, namely DSM-2 (v2) (codon-optimized gene coding sequence) and was fused with ubiquitin promoter which is flanked upstream by Rb7 MAR. The codon-optimization is to ensure the high enzymatic activity of the DSM-2 (v2) in different plant species and use of Rb7 MAR (Matrix Attachment Region) [21] to ensure high and consistent transgene expression independent of chromosomal location. The resulting binary transformation vector is referred to as pDAB3778. The control binary transformation vector is pDAB3779 in which PAT instead of DSM-2 (v2) gene is used as selectable marker. Additional binary vectors were further built by using different promoters. These promoters include *Arabidopsis* Cassava Vein Mosaic Virus (CvMV) promoter, *Ze a mays* Ubiquitin 1 (ZmUbi1) promoter, and *Arabidopsis thaliana* Ubiquitin 10 (AtUbi10) promoter. These vectors were carried by *Agrobacterium tumefaciens* strains ZIOs, EHA101, and LBA4404 and *Arabidopsis* was transformed using the floral dip method. Transformants (T1) were selected using 2,4-D post-emergence spray seven days after planting (DAP) and again 11 DAP with a 0.016% solution of 2,4-D herbicide. Over 100,000 T1 seed were screened and 260 transgenic plants resistant to 2,4-dichlorophenoxyacetic...
acid (2,4-D) were identified, leading to a transformation frequency of 0.26%. Many DSM-2 (v2) transgenic individuals survived 1,120 g/ha glufosinate (DL-Phosphinothricin) with little or no injury. The potential of DSM-2 (v2) as a plant selectable marker using glufosinate as the selection agent was evaluated with Arabidopsis transformed as described above. DSM-2 (v2) transgenic plants received the twice applications of 280 g/ha glufosinate at 7 DAP and 11 DAP, respectively. Plants were identified as Resistant or Sensitive 17 DAP. The inheritability of DSM-2 (v2) was assayed by following the segregation of T1 events through spraying glufosinate (200g/ha) to T2 siblings. The segregation data show that DSM-2 (v2) is stably integrated and inherited in a Mendelian fashion to at least three generations. These results indicate DSM-2 (v2) can effectively be used as an alternative selectable marker.

**Use of R-gene as plant selectable marker** (Patent WO2008091154A1, Cooperatie Avebe UA, Netherlands): One of the interesting inventions is the use of R-gene as plant selectable marker by De Vetten et al. (2008) [22]. R-genes can be isolated from Solanum species (potato) or other plants. Currently 4 R-genes for late blight resistance have been cloned and all belong to the NB-LRR class of plant R-genes; Rl and R3a from S. demissum [23, 24] and Rpi-blbl and Rpi-blb2 from S. bulbocastanum [25]. Examples of these R-genes include; potato R3a and Rpi-blb1, soybean Rps1b, Arabidopsis Rpp1, tomato Cj2, Cj4, and Cj9, Pto. Agrobacterium tumefaciens was employed to carry the binary vector pHINmf::R3 containing an expression cassette encompassing the coding sequence (CDS) of gene R3a. The cassette is regulated by the original promoter and terminator of R3a. The genomic fragment containing the R-gene cassette is isolated from the diploid potato clone SH83-92-488 [24]. Transgenic potato plants were developed with the above binary vector by Agrobacterium tumefaciens. There was no selection employed during the entire culture stages. Instead, recovered plants were challenged with pathogens by pipetting 10 μl droplets of a zoospore suspension of 2.5 x 10⁴ spores/ml on the adaxial side of fully-expanded leaves and transgenic plants displayed resistance response (no symptom or localized HR-like necrosis or trailing HR necrosis) while non-transgenic plants showed susceptible reaction (spreading lesion with massive sporulation or with no or little sporulation). These results suggested that the R-gene can be used as plant selectable marker. However, a possible limitation of this invention is that a compatible interaction between pathogens and host plants (to be transformed) will be prerequisite for this technology to be applicable.

**Soybean selection system based on AEC-resistance** (Patent US20080235824A1): Gabriel and Reddy (2008) discovered that fatty acid ester detergents, including Tween-20 and Span-20, strongly inhibit plant regeneration including shoot or root formation in both dicot and monocot, and can be used as a selective agent [33]. In tobacco and tomato, for instance, the inhibitory concentration is 1% Tween-20 or 1% Span-20 where in geranium and rice, the inhibitory concentration is 0.5% Span-20. Therefore, genes encoding enzymes that can detoxify these ester detergents can be used as plant selectable markers. An esterase clone (see below) is fused within a plant gene expression cassette and driven by CaMV35S promoter. The cassette is carried within the T-DNA of binary vector pCAMBIA (Cambia, Canberra, AU). The cloned esterase include bovine pregastric esterase (PGE), nematode triglyceride lipase-cholesterol esterase, bacterial tributyrin esterase, amoeba acyloxyacyl hydrolase gene, and/or plant triglyceride lipase-cholesterol esterase include bovine pregastric esterase (PGE), nematode triglyceride lipase-cholesterol esterase, bacterial tributyrin esterase, amoeba acyloxyacyl hydrolase gene, and/or plant triglyceride lipase-cholesterol esterase. The role of AEC as a selective agent was also evaluated in other plant species in this invention. Experiments using 7 to 10 day old seedlings showed that 20 mM AEC is lethal to pigweed (Amaranthus spp.), foxtail (Setaria spp.) and tobacco (Nicotiana spp.). In spite of these results, it is difficult to compare this new selection system with competing ones because there was lack of side-by-side comparisons and no data on transformation frequency was available in the patent description.

**Use of esterase genes as selectable markers for transforming plant cells** (Patent WO2008091154A1): Gabriel and Reddy (2008) discovered that fatty acid ester detergents, including Tween-20 and Span-20, strongly inhibit plant regeneration including shoot or root formation in both dicot and monocot, and can be used as a selective agent [33]. In tobacco and tomato, for instance, the inhibitory concentration is 1% Tween-20 or 1% Span-20 where in geranium and rice, the inhibitory concentration is 0.5% Span-20. Therefore, genes encoding enzymes that can detoxify these ester detergents can be used as plant selectable markers. An esterase clone (see below) is fused within a plant gene expression cassette and driven by CaMV35S promoter. The cassette is carried within the T-DNA of binary vector pCAMBIA (Cambia, Canberra, AU). The cloned esterase include bovine pregastric esterase (PGE), nematode triglyceride lipase-cholesterol esterase, bacterial tributyrin esterase, amoeba acyloxyacyl hydrolase gene, and/or plant triglyceride lipase-cholesterol esterase. The role of AEC as a selective agent was also evaluated in other plant species in this invention. Experiments using 7 to 10 day old seedlings showed that 20 mM AEC is lethal to pigweed (Amaranthus spp.), foxtail (Setaria spp.) and tobacco (Nicotiana spp.). In spite of these results, it is difficult to compare this new selection system with competing ones because there was lack of side-by-side comparisons and no data on transformation frequency was available in the patent description.

This selection system developed by Hildebrand and Rao (2009) [32] is mainly used in soybean somatic embryo-genesis. The E. coli DHPS gene was cloned and inserted into the binary vector p1201 with the pea RBCs transit peptide under the control of CaMV 35S promoter. For the selection of transgenic soybean somatic embryos, AEC concentrations of 1.5 to 2.5 mM are effective for selection in presence of 0.075% MSO (methylated seed oil) as surfactant. Soybean somatic embryos and tobacco transformed with E. coli DHPS in the above construct were resistant to normally lethal concentrations (1.5 to 2.5 mM) of AEC. Green proliferating transgenic somatic embryos survived 2.5 mM AEC. A lower concentration of AEC (1.5 mM) was used to select for transgenic tobacco shoots. These transgenic soybean somatic embryos and tobacco shoots were GUS positive. These results indicated that DHPS coupled with AEC can be used as a selection system for soybean and tobacco. The role of AEC as a selective agent was also evaluated in other plant species in this invention. Experiments using 7 to 10 day old seedlings showed that 20 mM AEC is lethal to pigweed (Amaranthus spp.), foxtail (Setaria spp.) and tobacco (Nicotiana spp.). In spite of these results, it is difficult to compare this new selection system with competing ones because there was lack of side-by-side comparisons and no data on transformation frequency was available in the patent description.
assays and then in stable transformation process to validate the esterase genes as selection system. Table 1 lists stable transformation results from optimal concentrations of detergents in different plant species.

Apparently, different sources of esterase conferred different transformation frequencies under the same selection and culture conditions. Vice versa, Tween-20 and Span-20 offers different selection efficiencies.

All transgenic events of the above species passed the transgenes to their progeny plants and displayed classical 3:1 segregation ratio, as indicated by PCR and the phenol red lipase/esterase tests [35]. Overall, the use of esterase genes as selectable markers does provide an alternative selection system, but doesn’t seem to be as effective as conventional selectable marker such as npt II and pat in some plant species.

### ENGINEERED RESISTANCE TO ENVIRONMENTAL STRESSES

**Transgenic plants resistant to biotic and abiotic stresses by overexpression of protochlorophyllide oxidoreductase C and its isoforms** (Patent WO2008020454A1, Jawaharlal Nehru University, India.): Plants are vulnerable to biotic and abiotic stresses but at the same time have a potential to resist these stresses. Oxidative stress is the ultimate cause of almost all kinds of biotic and abiotic stresses that affect crop plants. Singlet oxygen is one of the reactive oxygen species and is produced in pigment bed of the photosynthetic apparatus through type II photosensitization reactions of tetrapyrroles. Singlet oxygen causes oxidative cellular damage and plant death. Unlike detoxification of superoxide, by superoxide dismutase, there is no enzymatic detoxification mechanism available to destroy singlet oxygen produced from chlorophyll biosynthetic intermediates in light stress. To overcome this limitation, Tripathy (2008) [36] discovered that in Arabidopsis thaliana the overexpression of porC gene that encodes for the light-inducible isoform of protochlorophyllide oxidoreductase C, resulted in reduced accumulation of the photosensitizer protochlorophyllide and minimal production of singlet oxygen in response to light stress and 5-aminolevulinic acid (ALA) treatment; it was also demonstrated to possibly control plant death induced by oxidative stress through regulating the steady state accumulation of chlorophyll biosynthetic 5 intermediates and generation of singlet oxygen.

To achieve this, the porC cDNA was driven by CaMV35S promoter having omega (Ω) enhancer within the binary vector pCAMBIA1304. The Agrobacterium tumefaciens carrying this transformation vector was used to transform A. thaliana (ecotype CoI.). Southern blot confirmed the integration of single copy transgenes and Northern and Western blots revealed desirable levels of transgene expression (4-5 fold higher than WT controls) in T1 generation derived from independent T0 events. The transgenic plants were greener than control plants and two events have enhanced ChI contents (22% more in T12 & 27% more in T13), suggesting that porC is a limiting enzyme in ChI biosynthesis pathway. Increased accumulation of porC transcripts and PORC protein in PORC-plants resulted in efficient photoconversion of Pchlide to Chlβ and reduced accumulation of photosensitizer Pchlide under steady state illumination. Because of higher metabolic flux, the concentrations of other ChI biosynthetic intermediates, i.e., protoporphyrin IX (Proto IX), Mg-protoporphyrin plus its monoester (MPE) were also reduced in PORC-plants. The following assays were also performed to determine enhanced resistance to photo damage: Protochlorophyllide Oxidoreductase Activity, Anthocyanin Measurement, Estimation of lipid peroxide, Electrolyte Leakage, Reactive Oxygen Species (ROS) Staining, Singlet Oxygen Measurement, Fluorescence Spectra of Chloroplasts, Shibata Shift, and Pulse Amplitude Modulation Measurement (PAM).

**Transgenic disease resistant banana** (Patent US7534930, The State of Israel - Ministry of Agriculture & Rural Development, Israel.): It is estimated that over 80 million tons of bananas are produced worldwide each year, and serves as an essential food supply to around 400 million people in tropical and subtropical developing countries. In addition, banana production exceeds $3 billion US dollars annually, providing necessary income of foreign currency to

### Table 1. Use of Esterase Genes as Selectable Marker for Plant Transformation

<table>
<thead>
<tr>
<th>Plant Species</th>
<th>Sources of Esterase</th>
<th>Optimal Concentrations (%)</th>
<th>Transformation Frequency (%)</th>
<th>Antibiotic Coupled with</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Tween-20</td>
<td>Span-20</td>
<td></td>
</tr>
<tr>
<td>Tomato</td>
<td>Bovine PGE</td>
<td>1</td>
<td>-</td>
<td>7</td>
</tr>
<tr>
<td>Tobacco</td>
<td>Bovine PGE</td>
<td>0.5</td>
<td>-</td>
<td>6</td>
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<tr>
<td></td>
<td></td>
<td>-</td>
<td>0.5</td>
<td>12</td>
</tr>
<tr>
<td>Nematode lipase</td>
<td>Bovine PGE</td>
<td>-</td>
<td>1</td>
<td>39</td>
</tr>
<tr>
<td>geranium</td>
<td>Bovine PGE</td>
<td>-</td>
<td>0.5</td>
<td>4</td>
</tr>
<tr>
<td>rice</td>
<td>Bovine PGE</td>
<td>-</td>
<td>0.5</td>
<td>3</td>
</tr>
<tr>
<td>sugarcane</td>
<td>Bovine PGE</td>
<td>-</td>
<td>0.5</td>
<td>-</td>
</tr>
</tbody>
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*": Data are not available.

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many developing countries. However, banana production is currently threatened by two devastating diseases: the black leaf streak disease (BLSD) incited by *Mycosphaerella fijiensis* Morelet and the *Sigatoka disease* (SD) caused by *M. musculus* Leach ex Mulder. These pathogens attack all types of bananas and are widespread in most banana producing regions, leading to yield losses up to 30-50%. The invention is to improve bananas genetically to overcome the limitations of other field managements including fungicide sprays, etc (Vishnevetsky et al., 2009) [37]. The disease resistance was achieved through over-expression of a gene encoding endochitinase, stilbene synthase, or superoxide dismutase in banana. These genes were under the control of constitutive, inducible, or tissue-specific promoters. The constitutive promoters include CaMV35S [38], maize Ubi 1[39], rice actin [40], pEMU [41], and Synthetic Super MAS [42]. The inducible promoters can be pathogen-inducible such as the alfalfa PR10 promoter [43] and the promoters described before [44-48]. Suitable tissue-specific promoters include, but are not limited to, leaf-specific promoters such as described by Yamamoto et al. [49], Kwon et al. [50], Yamamoto et al. [51], Gotor et al. [52], and Orozco et al. [53], Matsuoka et al. [54]. Transgenic banana plants expressing the above gene were challenged with *Botrytis cinerea* and subsequently exhibited significantly reduced disease severity. The percent of spores that germinated on transgenic and control plants were less than 30% and over 50%, respectively while most of transgenic plants displayed disease severity of less than index 3 as compared with index 5 in control.

**Transgenic tomato plants resistant to yellow leaf curl virus** (Patent US7531716, University of Florida Research Foundation, Inc., USA): Tomato yellow leaf curl virus (TYLCV) has been devastating to crop production and causes increases in production costs due to increases in pesticide use. This invention described genetically-engineered resistance to tomato yellow leaf curl geminivirus, such as TYLCV-Israel (TYLCV-Is) (Polston and Hiebert, 2009) [55]. Transgenic tomato and tobacco plants display TYLCV-Is resistance. Such resistance is obtained by transforming a plant with a truncated version of the replication associated protein (Rep) gene of TYLCV, for instance, a truncated Rep gene from TYLCV-Is (Florida isolate). The full length Rep gene of TYLCV-Is encodes a virus replication related protein of approximately 357 amino acids; the Rep gene used in this invention was truncated at the 3’ end, leaving 508 nucleotides (nt) at the 5’ terminus which comprised of an 82-nt intergenic sequence and a 426-nt sequence that encodes a Rep protein fragment (N-terminus) of 142 amino acids. The resistance assay results showed that at harvest (12 weeks after inoculation), more than 41% of the TYLCV-Is tomato plants in eight out of 30 T1 lines remained free of virus symptoms, while 100% of the non-transformed control tomato plants developed severe symptoms; in tobacco, seven TYLCV-Is lines showed immunity to TYLCV in tobacco.

**Modification of plant disease resistance** (Patent WO2009015079A1, Samuel Roberts Noble Foundation, Inc., USA): Plant sterols, also referred to as phytosterols, are structural components of plant cell membranes. SQS encodes squalene synthase, an enzyme that catalyzes the first committed step of the sterol biosynthetic pathway. Sterol biosynthesis is suppressed in response to pathogen or elicitor challenge in various plant species [56-59]. The invention employed genes encoding SQS or sterol methyl transferase (SMT) which confer increased resistance to *P. syringae pv. glycinea* and *P. syringae pv. tomato* specifically (Wang et al., 2009) [60]. Promoters used to drive transgene SQS or SMT in this invention may be inducible, organelle-specific, tissue-specific, cell-specific, developmentally-specific, pest and/or pathogen-inducible or constitutive. Transgenic *Arabidopsis* plants overexpressing the SQS or SMT gene were produced and, when challenged with a pathogen, *P. syringae pv. tabaci*, were tolerant to these pathogens.

**Transgenic Bt-plants resistant to insects** (Patent US20090098099A1, Monsanto Technology LLC): Baum et al. (2009) [61] published the patent on using *Bacillus thuringiensis* strains 20 comprising novel crystal proteins which exhibit insecticidal activity against 20 lepidopteran insects with as high as 50% of larva displaying stunted growth (in cryET52-transgenics). Also described are novel *B. thuringiensis* genes and 20 their encoded crystal proteins, as well as methods of making and using 20 transgenic cells comprising the novel nucleic acid sequences of the invention. The promoters used to drive the transgene expression include constitutive CaMV35S, plant gene derived, or crystal protein gene derived promoters.

**Transgenic plants with increased stress tolerance and yield** (Patent WO2009010460, BASF Plant Science, Germany): Shirley et al. (2009) have discovered that engineering of a plant with certain genes enhanced plant’s growth and environmental stress tolerance, thus increasing yield [62]. The genes capable of mediating such enhancements have been isolated from *Arabidopsis thaliana*, *Capsicum annuum*, *Escherichia coli*, *Physcomitrella patens*, *Saccharomyces cerevisiae*, *Triticum aestivum*, *Zea mays*, *Glycine max*, *Linum usitatissimum*, *Triticum aestivum*, *Oryza sativa*, *Helianthus annuus*, and *Brassica napus*. The gene encodes a prenyl-dependent CAAX protease, SAR8.2 protein precursor, a protein phosphatase 2C protein, a protein kinase, an ornithine decarboxylase, a glutathione reductase, a protein phosphatase 2A protein, or a MEK1 protein kinase. The promoter driving the expression of the above genes may be constitutive, inducible, developmental stage-preferred, cell type-preferred, tissue-preferred, or organ-preferred. Of these promoters, stress-inducible promoter is preferred for stress tolerance because its expression imposes a minimal impact on plant normal growth. Examples of promoters include CaMV 19S and 35S promoters, Sep1 promoter, rice actin promoter, *Arabidopsis* actin promoter, ubiquitin promoter, pEmu, and super promoter. Different binary constructs expressing the above proteins were introduced into different crop species including canola, soybean, rice, maize, and wheat. Expression of the proteins in the above plants resulted in increased tolerance to an environmental stress, and/or growth, and/or yield under normal and/or stress conditions as compared to wild type plants.

**GTP binding stress-related proteins confer plants stress tolerance** (Patent US20090031451A1, BASF Plant Science, Germany): This invention identified new, unique GTP-binding proteins capable of conferring stress tolerance to
plants through over-expression (da Costa e Silva et al., 2009) [63]. The invention provides transgenic plants expressing a GTP binding stress-related protein (GBSRP), displaying tolerance to environmental stress as compared to a wild type control plants. The described G-proteins include: (1) CTP binding protein-1 (CBP-1); (2) CTP binding protein-2 (CBP-2); (3) CTP binding protein-3 (GBP-3); (4) CTP binding protein-4 (GBP-4); and (5) CTP binding protein-5 (GBP-5), all from Physcomitrella patens. Background: Osmotic stress in plants is sensed by a two-component system comprised of a sensing protein and an effecting protein [64, 65]. Mitogen-activated protein kinase-dependent signal transduction pathways are tightly involved in these processes. Another major component of these signal transduction pathways is the GTP-binding protein (G-protein). Generally, there are at least three classes of G-proteins: (1) heterotrimeric (alpha, beta and gamma subunits), (2) monomeric (small) proteins, and (3) Dynains. GTP-binding proteins are named as such because each must bind GTP in order to be active. The functions of GTP-binding proteins are varied as they range from directly transmitting an external signal (by being associated with a membrane-bound receptor), to participating in vesicle traffic, to importing proteins into sub-cellular compartments.

Transgenic plants expressing these genes were from maize, wheat, rye, oat, triticale, rice, barley, soybean, peanut, cotton, rapeseed, canola, manihot, pepper, sunflower, tage
tes, solanaceous plants, potato, tobacco, eggplant, tomato, Vicia species, pea, alfalfa, coffee, cacao, tea, Salix species, oil palm, coconut, and perennial grass. Promoters driving the transgene expression are constitutive, tissue-specific or inducible. Examples of these promoters include Arabidopsis actin and RD29A. Transgenic Arabidopsis showed 44%-84% survival rate as compared with 28% of control plants under the same drought conditions; 60%-87% survival rate as compared with 2% survival rate of control plants under freezing conditions. These transgenic plants were also tolerant to salt conditions (data not available). However, the patent does not provide examples of stress tolerance in other plant species expressing the GTP-binding protein genes.

Phosphatase stress-related proteins (Patent US20080263725A1, BASF Plant Science, USA): Experimental evidence has shown that protein phosphatases are involved in the plant stress signaling cascade, and more particularly, in stress perception and signal transduction linked to physiological mechanism of adaptation in plants. For example, protein phosphatase 2C (PP2C) has been shown to be involved in stress responses in plants [66]. It has also been shown that, in yeast, the PPP2B phosphatase calcineurin (CaN) is a key component of a Ca++-dependent signal transduction pathway that mediates Na+, Li+, and Mn++ tolerance of Saccharomyces cerevisiae [67]. CaN functions to limit intracellular Na+ accumulation by regulating processes that restrict influx and enhance efflux of the cation across the plasma membrane. CaN also participates in cytosolic Ca++ homeostasis through the positive regulation of Golgi apparatus and vacuolar membrane-localized P-type ion

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a phosphatase substantially enhances plant stress tolerance [68].

Silva OC et al. (2008) identified new, unique phosphatases capable of conferring stress tolerance to plants upon over-expression [69]. The invention showed that transgenic plants overexpressing a gene coding for a phosphatase stress-related protein (PHSRP) results in increased tolerance to environmental stress. The described protein phosphatases include Protein Phosphatase 2A-2 (PP2A-2), Protein Phosphatase 2A-3 (PP2A-3), Protein Phosphatase 2A-4 (PP2A-4), Protein Phosphatase 2C-1 (PP2C-1), and Protein Phosphatase 2C-2 (PP2C-2), all from Physcomitrella patens. The full-length cDNA corresponding to the open-reading frames encoding the above proteins were isolated from Physcomitrella patens. These full-length cDNAs were then fused with Arabidopsis Actin2 promoter. Agrobacterium tumefaciens carrying binary vectors containing the expression cassettes of the above cDNAs were used to transform Arabidopsis thaliana ecotype C24 by flower dipping. T1 plants were confirmed and used for drought, freezing, and salt tolerance tests. For drought tolerance tests, T1 seedlings were desiccated under the in vitro dry conditions. The drought test results showed from 50 to 100% survival of stressed transgenic plants as compared with only 28% survival rate of non-transgenic controls. For freezing tolerance tests, seedlings were incubated under the in vitro cold (from 4°C through -5°C to 5°C) conditions. The results from freezing tolerance tests showed the 57-100% survival rate of transgenic plants as compared with 2% survival rate of non-transgenic controls. For the salt tolerance screening, seedlings were soaked in varying concentrations of NaCl from 50 mM through 200 mM to 600 mM. The transgenic plants are then screened for their improved salt tolerance demonstrating that transgene expression confers salt tolerance (data not available). The above tolerance tests were also conducted on transgenic soybean using similar stress tests.

Protein kinase stress-related proteins for stress tolerance (Patent US7442853 and US7521598, BASF Plant Science, Germany): Allen et al. (2008) [70] and da Costa e Silva et al. (2009) [71] reported that transgenic plants transformed with a gene encoding a protein kinase stress-related protein (PKSRP) results in increased tolerance to environmental stress. They also described agricultural products, including seeds, produced by the transgenic plants.

The discovered protein kinases included: (1) Ser/Thr Kinase and other type of kinases (PK-6, PK-7, PK-8 and PK-9), (2) Calcium dependent protein kinases (CDPK-1 and CDPK-2), (3) Casein Kinase homologs (CK-1, CK-2 and CK-3), and (4) MAP-Kinases (MPK-2, MPK-3, MPK-4 and MPK-5), all from Physcomitrella patens. The full-length cDNAs corresponding to PK-6, PK-7, PK-8, PK-9, CK-1, CK-2, CK-3, MPK-2, MPK-3, MPK-4, MPK-5, CPK-1 and CPK were isolated from Physcomitrella patens. Arabidopsis Actin2 promoter was used to drive each of the above genes. These expression cassettes were then subcloned into binary vector pGMSG and resultant vectors were used to transform Arabidopsis thaliana ecotype C24 following flower-dipping method [72, 73]. T1 seedlings were used to screen stress tolerance. The Materials and Methods for stress tests as
described in the previous session were used for stress tolerance tests. The results from drought stress screening showed that from 40 (vector PpPK-8) to 100% (vectors PpMPK-2 and PpPK-5) transgenic plants survived drought conditions. Freezing tolerance test results showed that 64 (vector PpCPK-2) to 100% (vectors PpPK-9 and PpPK-1) transgenic plants versus only 2% wild type plants survived freezing conditions. The transgenic plants were screened for their improved salt tolerance demonstrating that transgene expression confers salt tolerance (data not available). Transgenic soybean, rapeseed/canola, corn, and wheat were also developed for expressing the above genes for stress tolerance (data not shown in the patent description).

**Transcription factors stress-related proteins** (Patent US20080178355A1, BASF Plant Science, Germany): Chen et al. (2008) [74] in this invention identified new and unique transcription factors that confer stress tolerance to plants through over-expression. The transgenic plants produced through this invention express a transcription factor stress-related protein (TFSRP), displaying increased tolerance to environmental stress as compared to a wild type plants. The invention described transcription factors including: leucine zipper-2 (LZ-2), leucine zipper-3 (LZ-3), DNA-binding factor-2 (DBF-2), and DNA-binding factor-3 (DBF-3) from *Physcomitrella patens*, *Brassica napus* DNA binding factor-1 (BnDBF-1) from *Brassica napus*, *Oryza sativa* DNA binding factor-1 (OsDBF-1), and *Oryza sativa* DNA binding factor-2 (OsDBF-2) from *Oryza sativa*.

In *Arabidopsis*, there are at least four different signal-transduction pathways leading to stress tolerance. These pathways are under the control of distinct transcription factors [75]. These transcription factors (TFs) involved in tolerance pathways are particularly suitable for engineering tolerance into plants, because a single TF gene can activate a whole cascade of genes leading to the tolerant phenotype.

*Arabidopsis*, soybean, and rapeseed/canola were engineered for over-expressing the LZ-2, LZ-3, DBF-2 or DBF-3 Gene. The integration and expression of these genes were detected using PCR and RT-PCR, respectively. Screening for drought, freezing, and salt tolerance was done using the same approaches as described in the previous sessions. Results showed that these TFs conferred various transgenic plants resistance to drought, cold and salt stresses (data not available in patent description).

**OTHER TRANSGENIC APPLICATIONS FOR NON-HUMAN/ANIMAL TARGETS**

**Phytoremediation** (Patent WO2009023347A2, University of Massachusetts, USA): Parkash and Dixit (2009) [76] discovered that over-expression of the gene encoding arsenite-inducible RNA-associated protein confers plant resistance to a metal, metal ion, metalloid, or metalloid ion.

Metal and metalloid pollutants such as arsenic (As) can adversely affect the health of millions of people worldwide because As is a toxic and carcinogenic. The metal and metalloid contaminated soil, sediment, and water supplies are major sources of contamination in the food chain. Plants grown in the contaminated soil can accumulate high levels of metal and metalloid pollutants in roots, shoots, and grain. The metal and metalloid pollutants can be also then accumulated exponentially in animals who consume them, causing increased metal and metalloid exposure in humans via a plant-animal-human pathway. In addition, metal and metalloid pollutants are phytotoxic and cause significant loss in crop yields. Arsenate is a phosphate analog and competes with phosphate for uptake in plants causing the inhibition of phosphate and other nutrients [76]. Therefore, arsenic pollution is an agricultural concern. Therefore, plants that are resistant to metal and metalloid pollutants and can accumulate a large biomass in the presence of metal and metalloid pollutants will be advantageous as biofuel plants. Such a plant could be grown on contaminated land to remove metal and metalloid pollutants.

Metals and metalloids are often present in the environment in different ionic forms. For instance, arsenic oxyanions, HAsO₄ and H₂AsO₄, are the most prevalent forms of arsenic in surface soil, water, and within cells, and these oxyanions contain arsenic in the pentavalent state (As(V)). Arsenate, which at neutral pH contains arsenic in the trivalent oxidation state (As(III)) and likely as the acid HAsO₃, is highly reactive and readily forms As (III)-thiol complexes. Plants utilize arsenic reductase to detoxify arsenic by reducing As (V) to As (III), which is subsequently detoxified via forming complexes with thiol-reactive peptides such as γ-glutamylcysteine (γ-EC), glutathione (GSH) and phytochelatins (PCs). These As (III)-thiol complexes are then sequestered into vacuoles by glutathione-conjugating pumps. It is also believed that plants trap arsenite in below ground tissues to prevent access to above ground reproductive tissues preventing possible mutagenic consequences. Because the binding of As (III) by the thiol-reactive peptides is stoichiometric, As(III) in excess of the binding capacity of the thiol-reactive peptides may not effectively be detoxified [76]. Therefore, it is desirable to identify novel As (III) binding protein. Similarly, it is desirable to identify metal and metalloid binding protein to develop engineered crops with resistant to these pollutants.

Arsenite-inducible RNA-associated protein (AIRAP) genes were first isolated from *Arabidopsis* and two putative AIRAPs were also identified from rice. The *Arabidopsis* AtAIRAP1, AtAIRAP2, AtAIRAP3, AtAIRAP4, and AtAIRAP5 genes are amplified and cloned into binary plant transformation vectors and transgenic *Arabidopsis* plants were produced with the above constructs via flower dipping method. These transgenes are driven by *Arabidopsis* actin ACT2 promoter. Transgenic rice plants were developed using binary vector pCAMBIA1300 harboring the AtAIRAP2 gene under rice ACT1 promoter. The expressions of AtAIRAP genes were confirmed by semi-quantitative RT-PCR analysis of *Arabidopsis* AtAIRAP1, AtAIRAP2, AtAIRAP4, and AtAIRAP5 mRNA from root tissue and rice OsAIRAP1 mRNA from root and shoot tissues. The transgenic plants are highly resistant to metal concentrations that inhibit growth in wild type control *Arabidopsis*. For metal resistance assays, transgenic plant lines were grown on 1/2x MS media supplemented with different metals. Constitutive expression of an arsenite-inducible putative zinc-finger protein (AtAIRAP2) from a promoter expression cassette, ACT2pt, confers strong resistance to toxic metals (500 micromolar Zn, 25 micromolar arsenite (As (III)), and...
75 micromolar Cd) in Arabidopsis. The T<sub>2</sub> homozygous transgenic seeds were grown on 1/2x MS media supplemented with metal concentrations as indicated and plants were allowed to grow for 3 weeks. The transgenic plants have a fresh or wet weight that is several fold greater than the wild type plants and had well-developed, longer roots. The overexpression of the AIRAP genes in plants also increased zinc accumulation which is highly desirable for crop improvement. However, whether these transgenic plants displayed any penalty in growth is not discussed in the patent description.

**Genes enhancing crop yield** (Patent WO2009054735A2, Vialactia Biosciences Limited, New Zealand; Puthigae et al. (2009) [77] discovered that overexpression of glutamate decarboxylase (GAD) can increase seed yield. They first identified an open-reading frame (ORF56) from a ryegrass (Lolium perenne) genomic library. The ORF56 encodes GAD which catalyses the reaction of glutamate into carbon dioxide and gamma-aminobutyrate (GABA). GAD is essential for regulating cytosolic pH as well as controlling basic morphology. The GABA biosynthesis is highly regulated under normal growth conditions and in response to stresses such as cold, heat, water or mechanical [78-81]. GABA stimulates ethylene production and may play a role in signaling [82]. High GABA impairs pollen tube growth, and consequently seed set, whereas lower levels are stimulatory [83]. The ORF56-encoded glutamate decarboxylase contains two major domains: the GAD catalytic domain and a Calmodulin binding domain (CaM). CaM stimulates GAD activity by binding to a C-terminal extension. Truncation of this domain results in shorter, more branched plants with delayed greening and lacking pollen [84, 85].

The above gene is driven by either constitutive or tissue-specific promoters, for instance, CaMV35S or Ubi promoters. Transgenic ORF56-plants were developed and were normal in all aspects assessed (data not available in patent description) except for seed yield, which was as high as 3.55 times the seed yield of the wild type control plants. Evidently, phenotypic analysis revealed that the increase in seed yield is the result of an increase in seed number rather than an increase in individual seed weight. Nonetheless, the invention does not discuss what promoter was used to drive the transgene expression and if increased seed yields are the results of more inflorescences or more flowers in particular.

**Compositions and methods for enhanced cellulosic ethanol production** (Patent US2009044292A1, Penn State Research Foundation, USA): Production of renewable energy such as ethanol from plant lignocellulosic materials represent next generation of energy sources. Plant lignocellulosic material is one of the most predominant natural compounds in the biosphere. It is also a common source of feed ingredient for livestock as well as pulp and paper in the wood industry. Plant lignocellulosic material includes cellulose, non-cellulosic polysaccharides and lignin. Lignin is an insoluble polymer which constitutes between 20-30% of the dry weight of wood. In addition to proving mechanical support for plant, lignin plays an important role in transporting water, minerals and defending plant from pathogen attacks. However, lignocellulosic material has been largely underutilized due to the difficulty of degrading lignin. Overcoming the lignin barrier is a limiting factor in accessibility of cellulosic substrates, such as cellulose and hemi-cellulose, to hydrolytic enzymes in biomass conversion. Pretreatment of lignocellulosic materials through physical, chemical, and biological processes have been used to break the lignin network before the next stage, i.e., hydrolytic enzymatic digestion and fermentation, can take place to convert sugars into ethanol. However, such pretreatment is a very expensive process in the lignocellulosic biomass conversion. Genetic engineering efforts to lower plant lignin content rarely succeed because reduced lignin content has pleiotropic, adverse effects on plant development and the ability to resist environmental stresses [86, 87]. Therefore, it is highly desirable to produce plants with improved processability of plant lignocellulosic materials but without undesirable side effects on plant development and tolerance to environmental stresses.

In this invention, Tien et al. (2009) [88] took a unique approach by using signal peptide which targets proteins to the cell wall of transgenic plants. The transgenes are controlled by either constitutive or tissue specific promoters, for example, CaMV35S or vascular- or xylem-specific promoters. Of total amino acids in these proteins, at least 5% are tyrosine, lysine, serine, threonine or cysteine. In some proteins, these amino acids account for 6-100%, or even 10-100%. In other proteins, the highest amino acid residues of tyrosine account for 5-100%. These modifications made a transgenic lignocellulosic plant increase release of fermentable sugars upon treatment with a cellulolytic agent compared to a wild-type plant while still maintaining substantially similar total lignin content compared to a wild-type plant of the same species. The fitness of transgenic plants was also evaluated. No significant morphological or growth differences were noted. Results showed that transgenic plants have the same fitness as wild-type plants.

Using above strategy, various plant species were engineered. They include: pines [89, 90]; several spruce species, including Picea glauca, P. mariana and P. abies [91]; alfalfa [92]; maize [93].

**Transgenic plants overexpressing a plant vacuolar H<sup>+</sup>-ATPase** (Patent US7534933, University of Connecticut, Whitehead Institute for Biomedical Research, and Beth Israel Deaconess Medical Center, Inc., USA): Gaxiola et al. (2009) [94] invented a novel approach of generating transgenic plants to overexpress vacuolar H<sup>+</sup>-PPase. Transgenic tobacco and petunia plants, overexpressing Arabidopsis AVP-1 are shown to have increased meristematic activity, resulting in larger leaves, stem, flower, fruit, root structures, increased salt tolerance, enhanced drought and freeze tolerance. The overexpression of transgene was achieved by using constitutive, inducible, or tissue-specific promoters. However, no specific example of promoters used was given.

**Transgenic plant capable of detecting environmental burden chemicals** (Patent EP1857550A4, Suntory LTD, Japan): To date, as industrialization is accelerating, various chemical pollutants were released to the environment. Many of these pollutants have accumulated in the environment and threaten the health of biological organisms including the human being. Particularly, dioxins/dioxin-like compounds and polycyclic aromatic hydrocarbons have extremely
adverse effects on the environment at a very low concentration. Among the environmental chemicals, dioxins/dioxin-like compounds and polycyclic aromatic hydrocarbons (including benzpyrene, methylcholanthrene, etc.) exhibit various types of in vivo toxicity such as immunotoxicity, teratogenicity, carcinogenicity and the like on mammals, and are considered to have adverse impact on the ecosystem as well as the human health [95]. Conventionally, dioxins/dioxin-like compounds and polycyclic aromatic hydrocarbons are monitored by collecting samples from many locations in question, transferring the samples to the experimental facilities, and then performing detection and quantification operations by analysis using instruments. Such analysis using instruments, regardless of high insensitivity and precision, requiring analytic equipment and skill, is time-consuming and costly due to the instruments and reagents. For these reasons, it is desirable to develop a simple, quick, highly-sensitive, and low-cost method.

Togami et al. (2009) [95] developed a strategy of using transgenic plants to monitor dioxins/dioxin-like compounds and polycyclic aromatic hydrocarbons. An aryl hydrocarbon receptor (AhR) gene was used in a plant transformation vector. The structure of the AhR gene is divided into three domains of (1) DNA-binding domain, (2) AhR ligand-binding domain, and (3) transcriptional activation domain. The AhR gene may be a recombinant AhR gene coding for an AhR fused protein which includes (a) an appropriate AhR ligand-binding domain derived from any of various species including human, mouse, rat, guinea pig and the like which can be used as a basic structural element, and (b) homologous or heterologous functional region fused thereto, such as an appropriate DNA-binding domain and an appropriate transcriptional activation domain. Clearly, the recombinant AhR gene does not need to encode a fused protein, and the above-mentioned three domains may be homologous. This AhR gene is then driven by AhR-ligand stimulus-inducible promoter. This promoter can be transcriptionally activated by the AhR-ligand which reacts with representative environmental chemicals in soil, including dioxins/dioxin-like compounds and/or polycyclic aromatic hydrocarbons. The promoter then drives dsRNA expression cassette to silence a flower pigmentation (anthocyanin biosynthesis) pathway, so that flower color will be changed to indicate the presence of the above pollutants.

Transgenic petunia plants were developed with the above vector and transgenic plants were grown in the soil containing dioxin or a dioxin-like compound. The result showed that the flower color of transgenic plants did change as a result of the above compounds, indicating the effectiveness of the transgenic plant as an indicator.

**VACCINE PRODUCTION IN TRANSGENIC PLANTS**

Conventional production of vaccine in animal cells can be costly and is difficult to scale up in a large quantity. Such production also suffers from various additional problems. By contrast, vaccine production in plants could be more cost-effective and easy to scale up. Below are three most recent inventions as examples on vaccine production in transgenic plants.

**Production of oral anti-viral vaccines in transgenic plants:** Background: In any vaccine production it is very important to develop the capacity of growing the disease-causing agent in large quantities. Presently, vaccines are normally produced from killed or live-attenuated pathogens. When the pathogen is a virus, large amounts of the virus must be grown in a living animal or cultured animal cells. It is critical to prove to lack virulence but retain the ability to establish infection and induce cell immunity if a live-attenuated virus is utilized. If a killed virus is utilized, the vaccine must have the capacity of surviving antigens to induce immunization. Additionally, surface antigens as the major viral particles which induce immunity may be isolated and administrated to offer immunity in order to utilize live-attenuated or killed viruses.

Vaccine production is costly because of the complex technology deployed for both the development and production of the vaccine. Vaccine needs to be concentrated and purified, whether it is made from the bacteria, virus, or other pathogenic organism. It costs even more for oral vaccine production due to the high cost of purifying a vaccine to comply with Food and Drug Administration (FDA) regulations since they require ten to fifty times more than the regular quantity of vaccine per dose, than a vaccine which is parenterally administered. As a result, of all the viral vaccines being produced today only a few are being produced as oral vaccines.

Problems can be encountered during the vaccine development and production, regardless of extreme precautions taken. For example, there is always a chance for one of the live viruses has survived from killed virus vaccines and vaccination may lead to isolated cases of the disease. Moreover, virus vaccines, either from the killed or live-attenuated type, are made from viruses grown in animal host cells, and therefore can be contaminated with cellular material from the animal host which can cause adverse, occasionally fatal, reactions in the vaccine recipients. The extra cost of vaccine will ultimately pass onto the consumers when the legal liability requires the vaccine manufacturer to take care, by insurance, of those who are harmed by a rare adverse reaction to a new or improved vaccine. Additional disadvantage of some vaccines, because of being prepared from whole killed virus, generally is the stimulation of the development of circulating antibodies (IgM, IgG), conferring such a limited degree of immunity that usually requires a boosting; the administration of additional doses of vaccine at specific time intervals. Live attenuated viral vaccines, though much more effective, have limited shelf-life and thus require maintaining vaccines though refrigeration during delivery to the site of application [96]. To date, vaccine production and administration are being made to be more cost-effective. In particular, engineered or mutants can be produced instead of live-virus vaccines. Specific deletion mutants can be developed which alter but do not inactive the virus so that vaccines can still replicate but cannot revert to virulence. For example, the gene coding for the immunizing protein of one virus can be introduced into the genome of a second, avirulent virus type, which can be administered as the vaccine. Such recombinant vaccines can be prepared in a vector virus, such as vaccinia virus or by other methods of gene splicing. Vectors may include not only avirulent viruses.
but also bacteria. Different avirulent viruses are being used as vectors and viral genes coding for a specific surface antigen to produce immunity in human or animal host can be cloned into plasmids. The cloned genes can then be expressed in prokaryotic or eukaryotic hosts if constructs are properly engineered and used.

**Vaccines expressed in plants** (US7504560 and US6034298, Prodigene, Inc., USA): Arntzen and Lam (2000, 2009) [97, 98], for the first time, invented the anti-viral vaccine that is produced in transgenic plants and then administered through either standard vaccine introduction method or through the consumption of the edible portion of those plants. A gene encoding a surface antigen of a viral pathogen is isolated and ligated to a promoter, which can regulate the production of the surface antigen in a transgenic plant. Example of the promoter used in this invention includes CaMV35S promoter for high levels of expression. The transgene cassette is then transferred to plant cells. The foreign gene is preferably expressed in a portion of the plant that is edible by humans or animals. These edible parts may include fruits, vegetable juice, or tubers which can be taken orally.

An example of this invention is the construction of expression vector pHVA-1 for Hepatitis B surface antigen. This is a binary plant transformation vector derived from pBI121 by subcloning the Hepatitis B surface antigen expression cassette into this vector. The cassette is driven by CaMV35S promoter. This vector was then mobilized into Agrobacterium tumefaciens which was used to transform tomato, tobacco and potato.

Transgene expression and vaccine production in transgenic plants were analyzed using Northern and immunoprecipitation blots, respectively. HBsAg vaccines were then purified through immunoaffinity from transformed plants. Leaf extracts from tomato or tobacco, or tuber extracts from potato transgenic plants were tested for the presence of material which reacts specifically with monoclonal antibody to serum-derived HBsAg. Further tests were conducted to determine if the recombinant HBsAg material in the transformed plant leaves was present as particles and the size determine if the recombinant HBsAg material in the transformed plant leaves was present as particles and the size range of the particles. Results showed that transgene mRNA and proteins were detected at various expression levels with anticipated molecular weight sizes.

**Oral anti-protozoiasis vaccines based on transgenic plants** (Patent US20080003243A1, Kitasato Institute, Japan): Chicken leucocytozoonosis is a disease infected by *Leucocytozoon caulleryi* that belongs to the suborder Haemosporina. *L. caulleryi* was carried in the blood of chicken as round gametocytes, and was named as a protozoan that belongs to the genus *Leucocytozoon*. This protozoan is biologically transmitted by a blood-sucking insect, *Culicoides arakawa* that belongs to the order Diptera and family Ceratopogonidae. Conventional vaccines against chicken leucocytozoonosis include live vaccines that use sporozoites, inactivated vaccines in which the antigen is a parasite-derived substance, and inactivated vaccines produced out of organ emulsions of protozoan-infected chicken. For the production of live vaccines, chickens are administered by injection with a small number of sporozoites separated from the salivary glands of *C. arakawa*. However, such live vaccine production is limited due to cost and inconvenience.

To solve the above-mentioned issues, Ito *et al.* (2008) [99] developed oral anti-protozoiasis vaccines produced in transformed plants. Specifically, the R7 immunogenic protein gene derived from second-generation schizonts of *Leucocytozoon caulleryi* was selected as the antigen gene, and a vector expressing this gene (driven by CaMV35S promoter) was introduced into potato tubers via *Agrobacterium*. Then, the leaves of the transformants were dried and mixed with formula feed for poultry, which was orally administered to chickens that had been vaccinated beforehand with a R7 immunogenic protein derived from second-generation schizonts of *Leucocytozoon caulleryi* obtained by expression in *E. coli*. As a result, the oral administration successfully increased antibody titer in chickens. This increase in antibody titer was particularly significant in chickens having a high antibody titer level at the start of the experiment. Similar techniques can be applied to other protective antigens against protozoans.

**Influenza immunogen and vaccine** (Patent US7361352, Acambis, Inc., USA and Vlaams Interuniversitair Instituut voor Biotechnologie, Belgium): Birkett and Fiers (2008) [100] published their invention on production of immunogen and vaccine against Hepatitis B virus (HBV). HBV belongs to a member of the family hepadnaviridae (DNA-containing animal viruses) that infects mammals. Hepatitis B core-protein monomers of the several hepadnaviridae self-assemble in infected cells into stable aggregates, so-called hepatitis B core protein particles (HBc particles). The hepatitis B viral core protein (HBc) functions as an immunogenic carrier moiety that stimulates the T cell response of an immunized animal. An important application of this carrier is its capability to present foreign or heterologous B cell epitopes at the site of the immunodominant loop. This loop is located at about residue positions 70-90 from the amino-terminus (N-terminus) of the protein [101]. HBc is highly immunogenic and specific, being suitable as a vaccine carrier. These are critical for influenza vaccine development, because they minimize genetic restriction and increase antibody titer.

This invention developed a hepadna virus-based immunogen for inducing antibodies to the extracellular domain of the influenza A M2 protein, and an inculum and a vaccine comprise of the immunogen dispersed in a diluent. The invented immunogen is a self-assembled particle comprised of recombinant HBc chimer protein molecules. Each of those molecules contains about 150 to about 375 amino acid residues, includes a sequence of 6 to about 24 residues, and carries four peptide-linked amino acid residue sequence domains from the N-terminus that are denominated Domains I, II, III and IV. The overexpression of the transgene coding for the above recombinant immunogen is under the control of bacterial lac promoter which is inducible for a high level of expression. The invention has several benefits and advantages: (1) use of the recombinant immunogen as a vaccine provides very high antibody titers against influenza A; (2) such high antibody titers have been produced with the aid of an adjuvant approved for use in humans; (3) the recombinant immunogen can be prepared easily and in large
quantities using well-established cell culture techniques to grow transformed host animal cells; (4) the immunogen is easily prepared using well-established recombinant techniques; (5) the selected immunogen exhibits greater stability at elevated temperatures than other HBc chimeras; (6) the invented immunogen is free of nucleic acid contaminants; (7) the resultant vaccine is easily administered, requiring minimal skills of operators.

CONCLUDING REMARKS AND FUTURE PERSPECTIVES

Recent advances in plant transgenic technology have been witnessing the dilemma of these technologies: On one hand, these patents were filed to protect new technologies; on the other hand, publishing patents will reveal the technology with certain critical and specific details. Meanwhile, because patents are usually material-, designed-, or procedure-based, it is often difficult, in reality, to protect the patents from being infringed unless a monitoring system is in place for monitoring patent violations. More in general, the issue on plant transgenic technology is complicated. New patents will certainly protect technology for commercial domains from being practiced but at the same time will restrict technology development and wide-spread uses which otherwise would benefit the entire society. Therefore, more freedom-to-operate rights should be granted to the non-profit organizations for them to use the plant transgenic technology as long as their uses of patented technologies are not for commercialization purposes. In any case, there is no doubt that new inventions aiming at improving and developing novel plant transformation systems will come to life as well as more extensive applications of transformation to solving various practical problems will be published in the years to come. As new transgenic technologies are being added to the tool box, the number of new inventions could be expected to increase at an accelerated speed, especially if the budgetary constraints for filing patents from public institutions could be lifted.

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CONFLICT OF INTEREST

The authors have no conflict of interest to declare.

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