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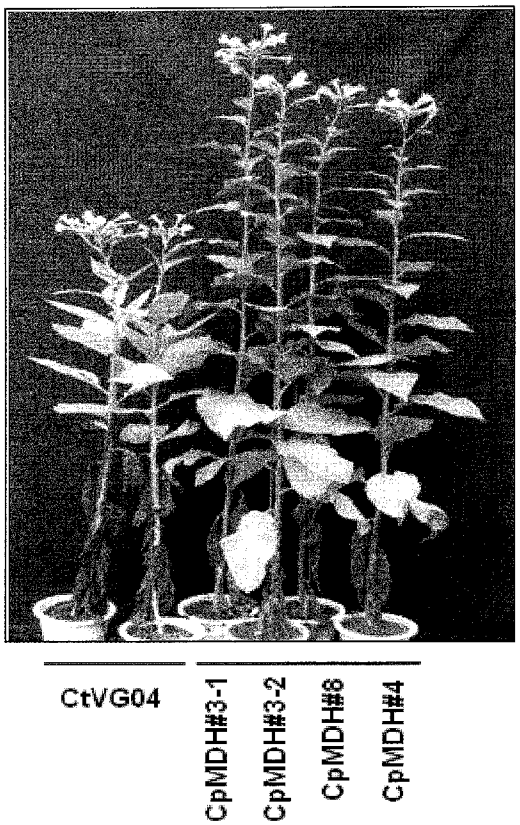

(57) Abstract: The present invention relates to a method for enhancement of photosynthesis and biomass of a plant by plastid transformation with MDH gene, more precisely a method for enhancement of photosynthesis and biomass of C3 plant by plastid transformation system with MDH gene. The MDH plastid transgenic plant prepared by the method of the present invention exhibits not only increased photosynthesis efficiency but also increased growth rate, leaf area, stem diameter and biomass of the plant, compared with the control plant. Therefore, the plastid transformed C3 plant prepared by C4 type gene introduction can be effectively used for enhancing photosynthesis and biomass of the plant.

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【DESCRIPTION】

【Invention Title】

THE METHOD FOR ENHANCEMENT OF PHOTOSYNTHESIS AND BIOMASS OF PLANT BY PLASTID TRANSFORMATION OF MALATE DEHYDROGENASE

【Technical Field】

The present invention relates to a method for enhancement of photosynthesis and biomass of plant by plastid transformation with *MDH* gene, more precisely a method for enhancement of photosynthesis and biomass of C3 plant by plastid transformation system with *MDH* gene.

【Background Art】

According to environmental disruption and population increase, it has been a very important issue to improve plant productivity. In parallel with the method for increasing the production of crops by the conventional breeding techniques, attempts to increase the production of crops and biomass using molecular breeding technique have been tried. Particularly, in the field of agriculture and forestry, there has been an attempt to introduce a recombinant nucleic acid molecule prepared by manipulation of a gene related with photosynthesis into a plant to express the molecule therein. This attempt can be made not

only in a specific kind of a plant but also in various kinds of plants.

Most of terrestrial higher plants including major agricultural crops such as rice, wheat, barley, soybean and potato assimilate CO_2 in the air through C3 photosynthesis pathway (Calvin cycle). Rubisco (ribulose 1,5-bisphosphate carboxylase/oxygenase), the first enzyme of CO_2 fixation, reacts not only with CO_2 but also with O_2 as well, leading to photorespiration consuming the assimilated carbon (Matsuoka *et al., Annu Rev Plant Physiol Plant Mol Biol* 52, 297-314, 2001). In the meantime, C4 plants such as corn, sorghum, and sugar cane have an additional C4 photosynthesis pathway increasing CO_2 concentration near Rubisco, which inhibits photorespiration, so that photosynthesis efficiency is much higher than in C3 plants (von Caemmerer and Furbank, *Photosynth Res* 77, 191-207, 2003; Sage, *New Phytologist* 161, 341-370, 2004).

Major enzymes involved in C4 photosynthesis pathway are PEP carboxylase, NADP-dependent malate dehydrogenase, NADP-dependent malic enzyme and pyruvate phosphate dikinase. In plants, NADP-dependent malate dehydrogenase (NADP-MDH; EC 1.1.1.82) is a very important enzyme for photosynthesis and exists only in chloroplasts (Sheen, *Auun Rev Plant Physiol Plant Mol Biol* 50, 187-217, 1999). NADP-dependent MDH converts oxaloacetate into malate in chloroplasts,

suggesting that it is involved in photosynthesis (carbon dioxide assimilation) along with NADP-dependent malic enzyme and phosphoenolpyuvate carboxykinase (PCK) and at the same time regulates the release of malate from chloroplasts to cytoplasm (von Caemmerer and Furbank, *Photosynth Res* 77, 191-207, 2003).

Recently, studies have been focused on enhancement of photosynthesis efficiency in C4 or C3 plants by manipulating genes of the major enzymes involved in C4 plant photosynthesis (Bailey *et al., J Exp Bot* 51 Spec No, 339-346, 2000; Matsuoka *et al., Annu Rev Plant Physiol Plant Mol Biol* 52, 297-314, 2001; Hausler *et al., J Exp Bot* 53, 591-607, 2002; Jeanneau *et al., J Exp Bot* 53, 1837-1845, 2002; Leegood, *J Exp Bot*, 53, 581-590, 2002; Rademacher *et al., Plant J* 32, 25-39, 2002; Miyao, *J Exp Bot* 54, 179-189, 2003; Chen *et al., Planta* 219, 440-449. 2004; Izui *et al., Annu Rev Plant Biol* 55, 69-84, 2004).

Plastid transformation has advantages over nucleus transformation, which is able to increase foreign gene expression dramatically and is a pro-environmental technique by which foreign gene does not exist in pollen, so that this plastid transformation has been a main subject in the field of plant biotechnology. Plastid can be divided into chloroplast involved in photosynthesis, amyloplast involved in amyloid storage, luekoplast not

containing pigments, and chromoplast involved in color of flowers and fruits. A plant cell has approximately 200 plastids, and a plastid has 100 genomes and accordingly has 10,000-50,000 copies of genes, while a plant nucleus has generally 1 - 2 copies of genomes. Thus, the copy number of genome in plastid and the copy number of genome in nucleus are independently maintained. Therefore, if a foreign gene is introduced into plastid as a form of episome, which means plastid is transformed with a foreign gene, a target protein will be mass-produced efficiently, which is incomparable with when nucleus is transformed.

Most reports concerning plastid transformation are made in tobacco and some succeeded the transformation in Arabidopsis thaliana, potato and tomato. However, the transformation efficiency is very low in those plants except tobacco. And even in the transformation of tobacco chloroplast, the promoter/terminator system used for a vector for transformation is originated from tobacco, so that the first homologous recombination induced during the generation of a transformant is followed by another recombination once again in those homologous sequences between terminator region and the tobacco chloroplast genome, resulting in a plant having an abnormal chloroplast genome (Staub JM, Maliga P, *Proc. Natl. Acad. Sci.* 91, 7468-7472, 1994 ;Svab Z, Maliga P, *Proc. Natl. Acad. Sci.*

90, 913-917, 1993). According to the conventional method using the typical vector for plastid transformation, the chances of the secondary recombination that might produce abnormal plastid transformant are approximately 50%. In these cases, successful introduction of a foreign gene and stable expression thereof cannot be guaranteed (Korean Patent Application No. 2006-12477).

The present inventors transformed C3 plant with *Corynebacterium glutamicum MDH* gene by using plastid transformation system. And the inventors completed this invention by confirming that photosynthesis and biomass of plant were enhanced by plastid transformation.

【Disclosure】

【Technical Problem】

It is an object of the present invention to provide a transgenic plant with enhanced photosynthesis and biomass, a method for preparing the transgenic plant and a method for enhancing photosynthesis and biomass.

【Technical Solution】

To achieve the above object, the present invention provides a transgenic plant generated by inserting MDH (malate dehydrogenase) gene into plastid genome of C3 plant.

The present invention also provides a method for preparing the transgenic plant with *MDH* gene in its plastid genome, which comprises the following steps: 1) constructing a vector for MDH plastid transformation by inserting *MDH* (malate dehydrogenase) gene sequence which is active in plastid into a vector for plastid transformation; 2) Preparing a transformant by introducing the vector for MDH plastid transformation into C3 plant or C3 plant culture cell; 3) culturing the transformant of step 2); and 4) re-differentiating the cultured transformant of step 3) by tissue culture.

The present invention further provides a method for enhancing photosynthesis and biomass, which includes the step of cultivating the transgenic plant in outdoors or greenhouse under high luminosity condition.

【Advantageous Effect】

The transgenic plant with enhanced photosynthesis or biomass generated by MDH plastid transformation of the present invention exhibits remarkably increased oxygen generation, CO_2 absorption, growth rate, leaf area, stem diameter and biomass, compared with control plants, so that it can be effectively used for enhancing photosynthesis and biomass of a plant by providing effective plastid transformed C3 plant.

【Description of Drawings】

The application of the preferred embodiments of the present invention is best understood with reference to the accompanying drawings, wherein:

Fig. 1 is a schematic diagram of a recombinant expression vector for plastid transformation containing rice *clp* promoter and *E. coli rrnB1/B2* terminator:

(a): *trnI-trnA* region of tobacco plastid genome where a foreign gene is inserted;

(b): CtVG04, the recombinant expression vector for plastid transformation using tobacco *rrn* promoter and *psbA* terminator;

(c): pTIA, the basic vector for the construction of the vector for plastid transformation containing tobacco *trnI-trnA*;

(d): pRclPADGHT, the recombinant vector containing rice *clp* promoter, *aadA* gene, *gfp* gene and *rrnB1/B2* terminator; and

(e): pRclPGAH, the recombinant vector in which MCS (multicloning site) is inserted in between rice *clp* promoter and *gfp* gene and *aadA* gene and *rrnB1/B2* terminator are linked stepwise.

Fig. 2 is a diagram showing the map of expression

vectors for MDH plastid transformation.

Fig. 3 is a diagram showing the results of PCR confirming the presence of MDH in the plastid transgenic plant.

Fig. 4 is a diagram showing the results of Southern blotting confirming that MDH exists in plastids of the T_0 and T_1 generation plastid transgenic plants in the form of homoplasmy.

Fig. 5 is a diagram showing the results of Northern blotting illustrating the expression levels of MDH in plastids of the T_0 and T_1 generation plastid transgenic plants.

Fig. 6 is a graph showing the enzyme activity of MDH in plastid of the T_1 generation plastid transgenic plant.

Fig. 7 is a graph showing the light-dependent CO_2 absorption rate in T_1 generation plastid transgenic plant.

Fig. 8 is a graph showing the Fv/Fm values obtained from the greenhouse cultivation according to the different light conditions (high luminosity, medium luminosity and low luminosity).

Fig. 9 is a diagram showing the phenotype of the period of floral axis development of the T_1 generation plastid transgenic plant cultivated in greenhouse.

Fig. 10 is a graph showing the growth rate of the T_1 generation plastid transgenic plant cultivated in

greenhouse from the cultivation to the period of floral axis development.

Fig. 11 is a graph showing the leaf area of the T_1 generation plastid transgenic plant cultivated in greenhouse, measured in the period of floral axis development.

Fig. 12 is a graph showing the stem diameter of the T_1 generation plastid transgenic plant cultivated in greenhouse, measured in the period of floral axis development.

Fig. 13 is a graph showing the live weight and dry weight of biomass (leaves and stems) of the T_1 generation plastid transgenic plant cultivated in greenhouse, measured in the period of floral axis development.

【Best Mode】

Hereinafter, the present invention is described in detail.

The present inventors constructed MDH expression vector by inserting *Corynebacterium glytamicum* originated *MDH* gene into the expression vector for plastid transformation (see Fig. 2). Then, seeds of wild type tobacco plant (*Nicotiana tabacum, Samsun*) were germinated. Leaves of the young plant were separated and explanted in MS medium for further use in plastid transformation. Re-

differentiation was repeated in selection medium to induce resistant young shoot to grow a transgenic plant. DNA was extracted from the cultivated plant, followed by PCR to confirm *MDH* gene introduction (see Fig. 3). Southern blotting was performed with the cultivated T_0 and T_1 generation transformants to investigate whether *MDH* gene was successfully delivered to the next generation as homoplasmy (see Fig. 4). Northern blotting was also performed with the cultivated T_0 and T_1 generation transformants to confirm the transcription of a target gene was normally induced in plastid genome of the T_0 and T_1 generation transformants (see Fig. 5). After confirming the successful transformation of a plant with *MDH* gene, MDH enzyme activity was measured. As a result, MDH enzyme activity in the MDH transgenic plant was significantly increased, compared with the control plant (see Fig. 6). CO_2 absorption by photosynthesis was also investigated in the transgenic plant. As a result, CO_2 absorption rate of the transgenic plant was approximately 1.4 fold increased, compared with the control plant (see Fig. 7). Chlorophyll fluorescence is generally released from photosystem 2 (PSⅡ). So, Fv/Fm (the ratio of fluorescence variation to maximum chlorophyll fluorescence) of the dark adapted leaf indicates the maximum value of quantum yield of photochemical reaction. The present inventors measured

photosystem II efficiency according to the increase of luminosity in plastid transgenic plants. As a result, Fv/Fm values of plants growing under medium luminosity and low luminosity, regardless of the plastid transgenic plants or the control plants, were all maintained as 0.8. However, when plants were cultivated under high luminosity, the plastid transgenic plant maintained Fv/Fm value as 0.8 but the control plant reduced Fv/Fm value to 0.42. From the results, it was confirmed that photoinhibition by photosystem 2 was not induced in the MDH transgenic plants even under high luminosity condition (see Fig. 8). The present inventors measured growth rate and biomass of the plastid transgenic plants. As a result, growth rate of the transgenic plants was 40 - 55% increased, compared with the control plants (see Figs. 9 and 10). In addition, the MDH transgenic plants were confirmed to have 86% increased leaf area, 35% increased stem diameter, 63% increased live weight and 14% increased dry weight (see Figs. 11 - 13).

As explained hereinbefore, the introduction of MDH into C3 plant resulted in the enhancement of photosynthesis and biomass of the plant.

The present invention provides a transgenic plant generated by inserting *MDH* gene into plastid genome of a target plant.

The *MDH* gene is preferably C4 plant type NADP-dependent MDH gene, but not always limited thereto. The *MDH* gene is preferably originated from eukaryotes, but not always limited thereto.

The *MDH* gene herein is preferably originated from one of *Corynebacterium glutamicum, Rhodococcus,* Oceanobacillus, Aspergillus, Methanothermus, Chaetomium, Methanopyrus, Bacillus, Methanocaldococcus, Magnaporthe, Phaeosphaeria, Gibberella, Desulfitobacterium, coccidioides, Thermus, Candidatus, Pyrococcus, Solibacter, Aurantimonas, Syntrophus, Enterococcus, Methanosphaera, Anopheles, Entamoeba, Yersinia, Mesorhizobium, Tribolium, Salmonella, Aurantimonas, Amycolatopsis, Escherichia coli, Apis, Burkhoderia, Bordetella, Pseudomonas and Aedes, and more preferably originated from *Corynebacterium glutamicum*, but not always limited thereto. And any *MDH* gene originated from other bacteria can be used as long as it has at least 30% homology with the amino acid sequence of *Corynebacterium glutamicum* originated *MDH*.

A target plant for the transformation is preferably C3 plant, which is selected from the group consisting of tobacco; cereals including rice; beans including mung bean, kidney bean and pea; starch-storing plants including potato, cassava and sweet potato; oil-storing plants including soybean, rape, sunflower and cotton plant; vegetables

including tomato, eggplant, carrot, hot pepper, Chinese cabbage, radish, water melon, cucumber, melon, crown daisy, spinach, cabbage and strawberry; garden plants including chrysanthemum, rose, carnation and petunia; and Arabidopsis, but not always limited thereto, and any C3 plant well-known to those in the art can be used.

The present invention also provides a method for preparing the transformant comprising the following steps: 1) constructing a vector for MDH plastid transformation by inserting *MDH* (malate dehydrogenase) gene sequence which is active in plastid into a vector for plastid transformation; 2) preparing a transformant by introducing the vector for MDH plastid transformation into C3 plant or C3 plant culture cell; 3) culturing the transformant of step 2); and 4) re-differentiating the cultured transformant of step 3) by tissue culture.

In this method, the *MDH* gene of step 1) is preferably originated from one of *Corynebacterium glutamicum, Rhodococcus,* Oceanobacillus, Aspergillus, Methanothermus, Chaetomium, Methanopyrus, Bacillus, Methanocaldococcus, Magnaporthe, Phaeosphaeria, Gibberella, Desulfitobacterium, coccidioides, Thermus, Candidatus, Pyrococcus, Solibacter, Aurantimonas, Syntrophus, Enterococcus, Methanosphaera, Anopheles, Entamoeba, Yersinia, Mesorhizobium, Tribolium,

Salmonella, Aurantimonas, Amycolatopsis, Escherichia coli, Apis, Burkhoderia, Bordetella, Pseudomonas and Aedes, and more preferably originated from *Corynebacterium glutamicum*, but not always limited thereto. And any *MDH* gene originated from other bacteria can be used as long as it has at least 30% homology with the amino acid sequence of *Corynebacterium glutamicum* originated *MDH*.

In this method, the expression vector for plastid transformation of step 1) is the recombinant expression vector containing the construct composed of a plant plastid originated promoter that has low homology with the polynucleotide of the genome of a target plant plastid, rbs sequence, and a terminator that has low homology with the polynucleotide of the genome of a target plant plastid in that order (see Fig. 2). The target plant is preferably C3 plant, which is selected from the group consisting of tobacco; cereals including rice; beans including mung bean, kidney bean and pea; starch-storing plants including potato, cassava and sweet potato; oil-storing plants including soybean, rape, sunflower and cotton plant; vegetables including tomato, eggplant, carrot, hot pepper, Chinese cabbage, radish, water melon, cucumber, melon, crown daisy, spinach, cabbage and strawberry; garden plants including chrysanthemum, rose, carnation and petunia; and Arabidopsis, but not always limited thereto, and any C3 plant well-known

to those in the art can be used.

The plastid originated promoter is preferably *clp* promoter originated from rice (*Oryza sativa*), but not always limited thereto. The terminator herein is preferably *rrnB1/B2* terminator originated from *Escherichia coli* pHCE19 vector, but not always limited thereto. The vector for plastid transformation herein additionally contains a selection gene which is preferably *aadA* or *gfp*, but not always limited thereto, and the vector is preferably the recombinant vector containing *trnI* gene sequence, *trnA* gene sequence, *aadA* gene sequence, *gfp* gene sequence and *rbs* sequence (see Fig. 1).

The present invention further provides a method for enhancing photosynthesis and biomass, which includes the step of cultivating the transgenic plant in outdoors or greenhouse under high luminosity condition.

Practical and presently preferred embodiments of the present invention are illustrative as shown in the following Examples.

However, it will be appreciated that those skilled in the art, on consideration of this disclosure, may make modifications and improvements within the spirit and scope of the present invention.

Example 1: Construction of basic vector for plastid transformation

The present inventors modified CtV2 vector for chloroplast transformation (Guda G, *et al.*, *Plant Cell Rep* 19:257-26, 2000) provided by professor Daniell's lab, USA, and then constructed the typical vector CtVG04 for chloroplast transformation containing GFP by the same manner as used for the construction of pT1G vector (Jeong SW, *et al.*, *Plant Cell Rep*, 22:747-751, 2004) (Fig. 1b). PCR amplification was performed using chromosome DNA of *Oryza sativa* as a template with rclpP5 primer (SEQ. ID. NO: 1) and rclpP3 primer (SEQ. ID. NO: 2), followed by cloning into pCR2.1-TOPO vector (Invitrogen, USA). Particularly, PCR was performed with exTaq enzyme (TaKaRa, Japan) by using 10 - 100 ng of genomic DNA of *Oryza sativa* as a template as follows; predenaturation at 94℃ for 5 minutes, denaturation at 94℃ for 1 minute, annealing at 55℃ for 1 minute, polymerization at 72℃ for 1 minute, 30 cycles from denaturation to polymerization, and final extension at 72℃ for 10 minutes. 800 bp *rrnB1/B2* terminator (SEQ. ID. NO: 3) was separated from the commercial *Escherichia coli* expression vector pHCE19 (TaKaRa, Japan) by using *Pst*I/*Hinc*II and used in this

invention. The nucleotide sequence of *Oryza sativa clp* promoter was confirmed by sequencing (SEQ. ID. NO: 4).

To construct pTIA vector, PCR was performed with 10 - 100 ng of tobacco genomic DNA using I-L1 primer (SEQ. ID. NO: 5) and I-R2 primer (SEQ. ID. NO: 6) to amplify 1.95 kb trnI-trnA boarder DNA fragment. PCR was performed with exTaq enzyme (TaKaRa, Japan) as follows; predenaturation at 94℃ for 5 minutes, denaturation at 94℃ for 1 minute, annealing at 55℃ for 1 minute, polymerization at 72℃ for 2 minutes, 30 cycles from denaturation to polymerization, and final extension at 72℃ for 10 minutes. PCR product was digested with *Xba*I/*Kpn*I and the ends were blunted by using Klenow enzyme, which was inserted into *Pvu*II site of pUC18 vector (Fermentase, USA), resulting in the basic vector pTIA (Fig. 5c).

To construct pRclPADGHT vector (Fig. 5d), aadA-GFP DNA fragment separated from pTIG vector was introduced into *Sal*I/*Pst*I site of pBluescript KSII vector (Stratagene, USA), which was then digested with *BamHI*/*Sma*I. The *rrnB1/B2* terminator DNA fragment was introduced into the end blunted by Klenow enzyme. Then, *rclp* promoter was inserted into *Xho*I/*Sal*I site to construct pRclPADGHT vector.

To construct pRclPGAH vector (Fig. 5e), PCR was performed using pRclPADGHT vector as a template with aadA-PstI 5' primer (SEQ. ID. NO: 7) and aadA-HindIII 3' primer

(SEQ. ID. NO: 8), followed by cloning into pCR2.1 TOPO TA vector (Invitrogen, USA) to construct pCR2.1 aadA(H/P) vector. PCR was performed with exTaq enzyme (TaKaRa, Japan) as follows; predenaturation at 94℃ for 5 minutes, denaturation at 94℃ for 30 seconds, annealing at 50℃ for 30 seconds, polymerization at 72℃ for 30 seconds, 27 cycles from denaturation to polymerization, and final extension at 72℃ for 5 minutes. DNA fragment obtained from pCR2.1 aadA(H/P) vector by digesting with *Pst*I/*Hind*III was introduced into pRclPADGHT predigested with *Pst*I/*Hind*III to construct double aadA vector. In the meantime, rclp promoter was amplified by PCR using pRclPADGHT vector as a template with rclp-XhoI primer (SEQ. ID. NO: 9) and rclp-MCS 3′ primer (SEQ. ID. NO: 10) containing *Sal*I, *Xba*I, *EcoR*V and *Sac*I sites in that order, followed by cloning into pCR2.1 TOPO TA vector (Invitrogen, USA) to construct pCR2.1 Rclp-MCS. PCR was performed by the same conditions as provided for the construction of pCR2.1 aadA(H/P). aadA vector was digested with *Xho*I/*Sac*I to cut off the original rclp promoter and *aadA* gene, and instead rclp-MSC DNA fragment obtained by digesting Rclp-MCS with XhoI/*Sac*I was inserted into the region, resulting in the construction of pRclPGAH.

Example 2: Construction of vector for plastid transformation containing *MDH*

The basic vector shown in Fig. 1 was used as a vector for plastid transformation containing *MDH*. First, PCR was performed using chromosome DNA of *Corynebacterium glutamicum* as a template with MDH 5′ primer (SEQ. ID. NO: 11) and MDH 3′ primer (SEQ. ID. NO: 12), followed by cloning into pGEM T-easy vector (Promega, USA). The constructed vector was named "TvecMDH". PCR was performed with exTaq enzyme (TaKaRa, Japan) as follows; predenaturation at 94℃ for 5 minutes, denaturation at 94℃ for 30 seconds, annealing at 55℃ for 30 seconds, polymerization at 72℃ for 1 minute, 27 cycles from denaturation to polymerization, and final extension at 72℃ for 5 minutes. TvecMDN vector was digested with *Sal*I/*Hind*Ⅲ to cut MDH DNA, which was inserted into *Sal*I/*Hind*III site of pRclPADGHT vector to construct RclpMDH vector. The RclpMDH vector was digested with *Xho*I/*Eco*RI and the ends were blunted by treating Klenow enzyme. The vector was then inserted into CtVG04 vector predigested with *Pst*I (Fig. 1b) to construct the vector for plastid transformation (Fig. 2A). The vector was designed to have *Corynebacterium glutamicum MDH* gene in front of selection markers if necessary (Fig. 2B). The vectors shown in Fig. 2A and Fig. 2B have monocistronic structure in which *MDH*

gene is transcribed by a single promoter. A vector designed to have polycistronic structure in which *Corynebacterium glutamicum MDH* gene is transcribed by *clp* promoter together with *aadA* and *gfp* selection genes was also constructed (Figs. 2C, D, E and F). P1 is the probe used for Southern blotting and P2 is the probe used for Northern blotting. To perform Southern blotting, chromosome was digested with *Bgl*Ⅱ와 *BamH*I and the sizes of bands presented by P1 probe were indicated in schematic diagram of each vector.

Example 3: Construction of plastid transformed tobacco plant by using the vector for plastid transformation containing *MDH*

Plastid transformation was performed by using the vector constructed in Example 2 and the vector CtVG04 constructed in Example 1 was used for the control group.

After germinating wild type tobacco (*Nicotiana tabacum, Samsun*) seeds in a chamber for 8 weeks, leaves were separated from the young plant and explanted in MS medium supplemented with 1 mg/ℓ BAP and 0.1 mg/ℓ NAA for further use in plastid transformation.

Gold particles (0.6 ㎛ in diameter) were coated with the vectors for plastid transformation constructed in Examples 1 and 2 by using $CaCl_2$ and spermidine, followed by

transformation using PDH-1000/He gene delivery system (BioRad, USA) under the conditions of 100 psi acceleration power, 9 cm target distance and 28 in/Hg vacuum.

The tobacco plant leaves treated above were cultured for 2 days at 25℃ under dark condition with 2,000 lux of light. The leaves were cut into 2 - 5 mm sections, which were cultured in MS medium supplemented with 1 mg/ℓ BAP, 0.1 mg/ℓ NAA and 500 mg/ℓ spectinomycin for 6 - 7 weeks to induce resistant young shoot. The young shoot induced in the spectinomycin resistant medium was cut into 3 mm × 3 mm pieces, followed by redifferentiation in the same selection medium to induce resistant young shoot again. The repeat of the redifferentiation in the selection medium resulted in the increase of homoplasmy of the introduced gene.

To confirm whether the redifferentiated plant (T_0) induced in the antibiotic containing medium was the transformant having the target gene or not, DNA was extracted from each induced plant, followed by PCR using vector specific *trnI* F3 primer (SEQ. ID. NO: 13) and *trnA* R1 primer (SEQ. ID. NO: 14). As a result, the target gene was successfully delivered into the transgenic plant. PCR was performed as follows; predenaturation at 95℃ for 5 minutes, denaturation at 95℃ for 45 seconds, annealing at 55℃ for 45 seconds, polymerization at 72℃ for 5 minutes, 30 cycles from denaturation to polymerization,

and final extension at 72℃ for 15 minutes. Approximately 800 bp sized band amplified from a part of *trnI* and *trnA* was detected in wild type, while both about 800 bp sized band amplified from a part of *trnI* and *trnA* and about 5.1 kb sized band resulted from the introduction of a foreign gene in between *trnI* and *trnA* were detected at the same time in MDH transgenic plant (Fig. 3).

Example 4: Southern blotting with plastid transformed tobacco plant

Southern blotting was performed using *trnA* gene as a marker to investigate homoplasmy of chloroplast in the T_0 and T_1 transformants obtained in Example 3. For the Southern blotting, total chromosome DNA was extracted by using a DNA extraction kit (DNeasy Plant Mini Kit, QIAGENE, Germany). 4 μg of the extracted DNA was digested with *BamH*I and *Bgl*II, which proceeded to electrophoresis on 1% agarose gel, followed by blotting on a blotting membrane (Zeta-Probe GT blotting membrane: Bio-Rad, USA). To prepare a probe, 0.6 kb *BamHI/Bgl*II DNA fragment containing *trnA* was labeled with [α-^{32}P]dCTP. Prehybridization and hybridization were performed using 0.25 M sodium phosphate buffer (pH 7.2) containing 7%(w/v) SDS at 65℃ for overnight. Upon completion of the reaction, the membrane was washed with 20 mM sodium phosphate buffer (pH 7.2)

containing 5%(w/v) SDS at 65℃ for 30 minutes, which was then exposed for 3 hours.

As a result, 0.8 kb sized DNA band was detected in wild type, approximately 3.0 kb sized band was detected in the vector control group transformed with CtVG04 of Example 3, and 1.5 kb sized DNA band was detected in the MDH transformant. From the above results, it was confirmed that a target gene was introduced into the chloroplast genome of the redifferentiated transgenic plant and well-preserved during the transformation and this introduced foreign gene was delivered to the next generation as homoplasmy (Fig. 4).

Example 5: MDH expression in plastid transformed tobacco plant

Northern blotting was performed to investigate whether the *MDH* gene introduced into the T_0 and T_1 transformants obtained in Example 3 was successfully transcribed. Total RNA for the Northern blotting was extracted using TRizol reagent (Invitrogen, USA). 2 μg of the extracted RNA proceeded to electrophoresis on 1.2% agarose gel supplemented with 5.1%(v/v) formaldehyde, followed by blotting on a blotting membrane (Zeta-Probe GT blotting membrane: Bio-Rad, USA). 1.044 bp *MDH* was amplified by PCR and the amplified product was labeled with

[α-^{32}P]dCTP to prepare a probe. Prehybridization and hybridization were performed using 0.25 M sodium phosphate buffer (pH 7.2) containing 7%(w/v) SDS at 65℃ for overnight. Upon completion of the reaction, the membrane was washed with 20 mM sodium phosphate buffer (pH 7.2) containing 5%(w/v) SDS at 65℃ for 30 minutes, which was then exposed for 30 minutes.

As a result, no RNA bands were detected in wild type and the vector control plants, 1.8 kb sized RNA bands containing *MDH* and *rrnB1/B2* terminator were detected in the redifferentiated MDH transformed T_0 and T_1 plants. In addition, various sized mRNAs linked by operon were also detected. The foreign gene introduced into plastid genome of the redifferentiated T_0 and T_1 transformants were well-preserved during the transcription, suggesting that the inserted gene can be translated as a protein functioning as an enzyme (Fig. 5).

Example 6: MDH enzyme activity of plastid transformed tobacco plant

NADP-dependent MDH enzyme activity of the T_0 and T_1 transformants obtained in Example 3 was measured with a spectrophotometer (Shimadzu UV-VIS Spectorophotometer, Japan). 6 hours after the sun rise, the 4^{th} - 5^{th} fresh leaves from the top were taken and prepared in the size of

1.5 cm in diameter. Leaf tissues were separated using extraction buffer [100 mM HEPES-KOH (pH7.3), 10 mM $MgCl_2$, 2 mM K_2HPO_4, 1 mM EDTA, 10% glycerol, 10 mM β-mercaptoethanol, 10 mM NaF, 2 mM PMSF]. The leaves were homogenized fast by using a mortar at a low temperature, followed by centrifugation at 12000 rpm (4℃) for 15 minutes. The supernatant was transferred in a new tube. When there were floating substances, centrifugation was performed one more time. The leaf extract which was standing at 4℃ for at least 30 minutes proceeded to Sephadex G-25 (1*3 cm) column (GE Healthcare Bio-Science AB, Sweden) to eliminate salts in order to be used for enzyme reaction. Enzyme reaction solution [50 mM HEPES-KOH (pH7.3), 5 mM $MgCl_2$, 0.2 mM NADPH, 2 U MDH, 5 mM EDTA, 2 mM OAA, 0.2 mM NADPH] was prepared. 1 ㎖ of NADP-dependent MDH enzyme reaction solution containing a proper amount of the leaf extract was treated at 30℃ and NADPH absorption rate was measured at 340 nm. The amount of protein included in the leaf extract was quantified by using protein assay kit (BIoRad, USA). As a result, the NADP-dependent MDH enzyme activity of the MDH transgenic plant was remarkably increased, compared with that of the vector control plant (Fig. 6).

Example 7: Photosynthesis dependent CO_2 absorption in plastid transformed tobacco plant

The T_0 and T_1 transformants prepared in Example 3 were cultivated in greenhouse. CO_2 absorption rate of a leaf without damage was measured with Ll-6400 photosynthesis system (Li-Cor, Inc., Lincoln, NE, USA). Red and blue LED lights were used as a light source and 6400 CO_2 mixture was used as CO_2 herein. To measure the CO2 absorption rate according to luminosity, a normal healthy leaf was fixed in a chamber provided with 400 μmol photons CO_2 mol^{-1}air CO_2 at 25℃ and then CO_2 absorption rate was measured with changing light conditions (0, 100, 300, 500, 700, 1000 and 1500 μmol photons CO_2 $mol^{-1}s^{-1}$).

As a result, CO_2 absorption rate of the MDH transgenic plant was approximately 1.41 fold increased at 1500 μmol photons compared with the control plant (Fig. 7).

Example 8: Photosystem II efficiency according to the increase of luminosity in plastid transformed tobacco plant

Chlorophyll fluorescence is generally released from photosystem 2 (PSⅡ). So, Fv/Fm (the ratio of fluorescence variation to maximum chlorophyll fluorescence) of the dark adapted leaf indicates the maximum value of quantum yield of photochemical reaction. That is, the value means the maximum, potential value to carry out photosynthesis in a leaf, which can also be an index of measuring the damage of photosystem 2. Normal healthy leaf maintained the Fv/Fm

value as 0.8. The T_0 and T_1 transformants prepared in Example 3 were cultivated in greenhouse with different light conditions (high luminosity; 800 ~ 1600 μmol photons/m^2/sec, medium luminosity; 300 ~ 800 μmol photons/m^2/sec, low luminosity; 30 ~ 300 μmol photons/m^2/sec). The 5th leaf from the top of the plant was taken and clipped in a potable chlorophyll fluorescence meter (Hansatech, King's Lynn, UK). After the dark treatment for 30 minutes, Fv/Fm value was measured at saturated luminosity. As a result, Fv/Fm values of plants growing under medium luminosity and low luminosity, regardless of the MDH transgenic plant or the control plant, were all maintained as 0.8. However, under high luminosity, the MDH transgenic plant maintained Fv/Fm value as 0.8 but the vector control plant exhibited reduced Fv/Fm value to 0.42. From the results, it was confirmed that photoinhibition by photosystem 2 was not induced in the MDH transgenic plant even under high luminosity condition (Fig. 8).

Example 9: Growth rate and biomass of plastid transformed tobacco plant

The T_0 and T_1 transformants prepared in Example 3 were cultivated in greenhouse and biomass increase was observed every 2 - 3 days until floral axis was developed. To

evaluate biomass increase rate, growth rate, leaf area, stem diameter, live weight and dry weight of a plant were all considered. To measure the leaf area, the 13th leaf from the bottom of a plant was selected and the leaf area was measured by using a soft ware. Stem diameter was measured 3 cm up from the bottom of a plant using a ruler (Manostat, 15-100-500). Roots, stems and leaves were separated from a plant and live weight of each was measured by a balance. They were dried in an 80℃ oven for 6 days, and dry weight of each was measured. Growth rate of the MDH transgenic plant was rapidly increased from the 38th day after transplantation to the soil and 40 - 55% increased on around the 65th day when floral axis was developed, compared with the vector control plant (Figs. 9 and 10). Compared with the vector control plant, leaf area of the MDH transgenic plant was 88% increased, stem diameter was 35% increased, live weight was 63% increased and dry weight was 14% increased (Figs. 11 - 13).

Those skilled in the art will appreciate that the conceptions and specific embodiments disclosed in the foregoing description may be readily utilized as a basis for modifying or designing other embodiments for carrying out the same purposes of the present invention. Those

skilled in the art will also appreciate that such equivalent embodiments do not depart from the spirit and scope of the invention as set forth in the appended claims.

【CLAIMS】

【Claim 1】

A transgenic plant prepared by inserting *MDH* (malate dehydrogenase) gene into the plastid genome of a target plant.

【Claim 2】

The transgenic plant according to claim 1, wherein the *MDH* gene is originated from one of *Corynebacterium glutamicum, Rhodococcus,* Oceanobacillus, Aspergillus, Methanothermus, Chaetomium, Methanopyrus, Bacillus, Methanocaldococcus, Magnaporthe, Phaeosphaeria, Gibberella, Desulfitobacterium, coccidioides, Thermus, Candidatus, Pyrococcus, Solibacter, Aurantimonas, Syntrophus, Enterococcus, Methanosphaera, Anopheles, Entamoeba, Yersinia, Mesorhizobium, Tribolium, Salmonella, Aurantimonas, Amycolatopsis, Escherichia coli, Apis, Burkhoderia, Bordetella, Pseudomonas and Aedes.

【Claim 3】

The transgenic plant according to claim 1, wherein the *MDH* gene is originated from *Corynebacterium glutamicum.*

【Claim 4】

The transgenic plant according to claim 1, wherein the target plant is C3 plant.

【Claim 5】

The transgenic plant according to claim 4, wherein the C3 plant is selected from the group consisting of tobacco; cereals including rice; beans including mung bean, kidney bean and pea; starch-storing plants including potato, cassava and sweet potato; oil-storing plants including soybean, rape, sunflower and cotton plant; vegetables including tomato, eggplant, carrot, hot pepper, Chinese cabbage, radish, water melon, cucumber, melon, crown daisy, spinach, cabbage and strawberry; garden plants including chrysanthemum, rose, carnation and petunia; and Arabidopsis.

【Claim 6】

A method for preparing a transgenic plant with the insertion of *MDH* gene in its plastid genome, comprising the following steps:

1) constructing a vector for MDH plastid transformation by inserting *MDH* (malate dehydrogenase) gene sequence which is active in plastid into a vector for plastid transformation;

2) preparing a transformant by introducing the vector for MDH plastid transformation into C3 plant or C3 plant culture cell;

3) culturing the transformant of step 2); and

4) re-differentiating the cultured transformant of step 3) by tissue culture.

【Claim 7】

The method for preparing a transgenic plant according to claim 6, wherein the *MDH* gene of step 1) is originated from one of *Corynebacterium glutamicum, Rhodococcus,* Oceanobacillus, Aspergillus, Methanothermus, Chaetomium, Methanopyrus, Bacillus, Methanocaldococcus, Magnaporthe, Phaeosphaeria, Gibberella, Desulfitobacterium, coccidioides, Thermus, Candidatus, Pyrococcus, Solibacter, Aurantimonas, Syntrophus, Enterococcus, Methanosphaera, Anopheles, Entamoeba, Yersinia, Mesorhizobium, Tribolium, Salmonella, Aurantimonas, Amycolatopsis, Escherichia coli, Apis, Burkhoderia, Bordetella, Pseudomonas and Aedes.

【Claim 8】

The method for preparing a transgenic plant according to claim 6, wherein the *MDH* gene of step 1) is originated from *Corynebacterium glutamicum*.

【Claim 9】

The method for preparing a transgenic plant according to claim 8, wherein the *MDH* gene is represented by SEQ. ID. NO: 16.

【Claim 10】

The method for preparing a transgenic plant according to claim 6, wherein the expression vector for plastid transformation of step 1) contains the gene construct composed of a plant plastid originated promoter that has low homology with the polynucleotide of the genome of a target plant plastid, rbs sequence, and a terminator that has low homology with the polynucleotide of the genome of a target plant plastid in that order.

【Claim 11】

The method for preparing a transgenic plant according to claim 10, wherein the target plant is C3 plant.

【Claim 12】

The method for preparing a transgenic plant according to claim 11, wherein the C3 plant is selected from the group consisting of tobacco; cereals including rice; beans including mung bean, kidney bean and pea; starch-storing plants including potato, cassava and sweet potato; oil-

storing plants including soybean, rape, sunflower and cotton plant; vegetables including tomato, eggplant, carrot, hot pepper, Chinese cabbage, radish, water melon, cucumber, melon, crown daisy, spinach, cabbage and strawberry; garden plants including chrysanthemum, rose, carnation and petunia; and Arabidopsis.

【Claim 13】

The method for preparing a transgenic plant according to claim 10, wherein the plastid originated promoter is *clp* promoter originated from rice (*Oryza sativa*).

【Claim 14】

The method for preparing a transgenic plant according to claim 10, wherein the terminator is *rrnB1/B2* terminator originated from *Escherichia coli* pHCE19 vector.

【Claim 15】

The method for preparing a transgenic plant according to claim 10, wherein the vector for plastid transformation additionally includes a selection gene.

【Claim 16】

The method for preparing a transgenic plant according to claim 15, wherein the selection gene is *aadA* or *gfp*.

【Claim 17】

A method for enhancing photosynthesis and biomass of a plant, which includes the step of cultivating the transgenic plant of claim 1 in outdoors or greenhouse under the high luminosity condition.

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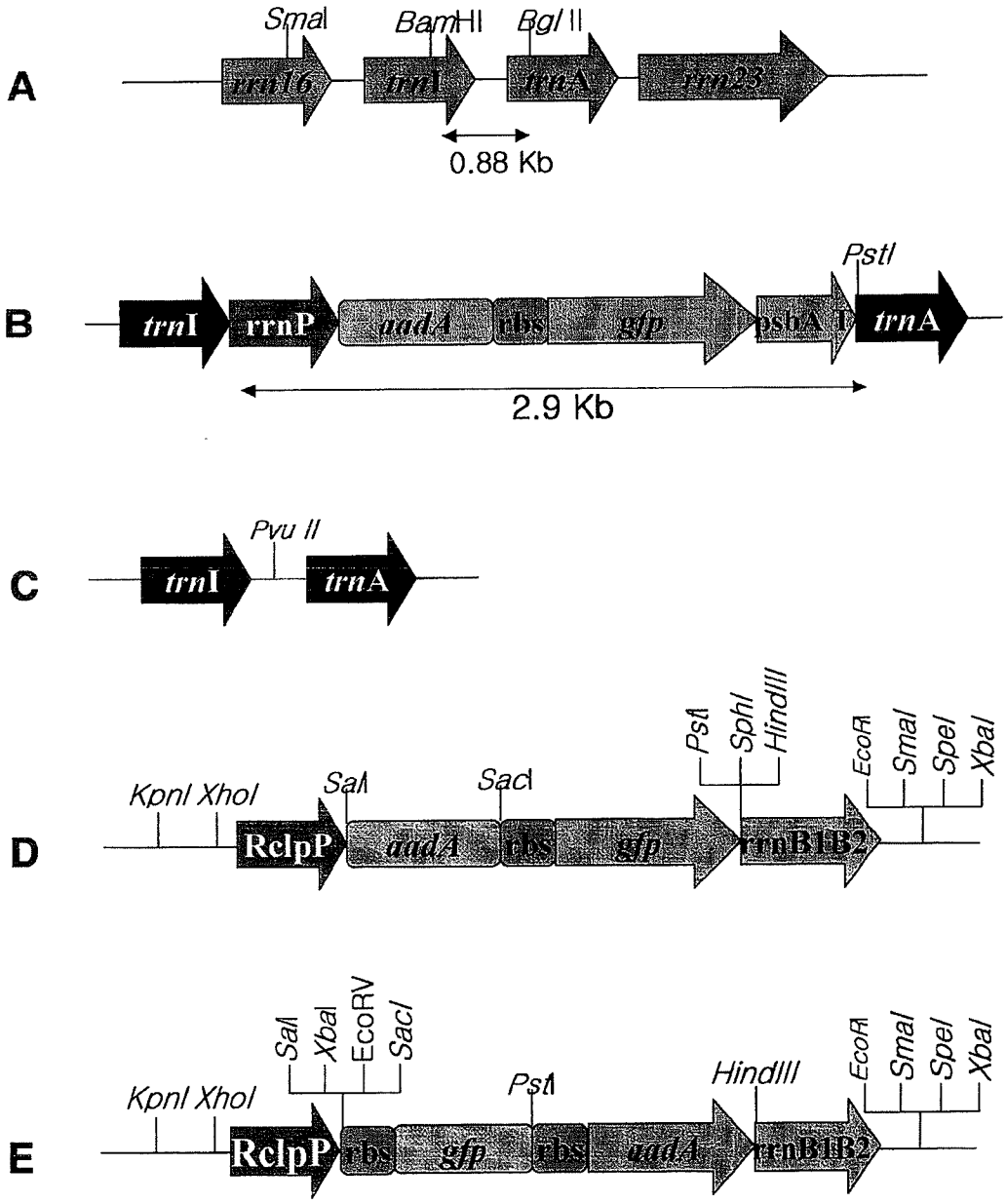
Figures

Fig. 1



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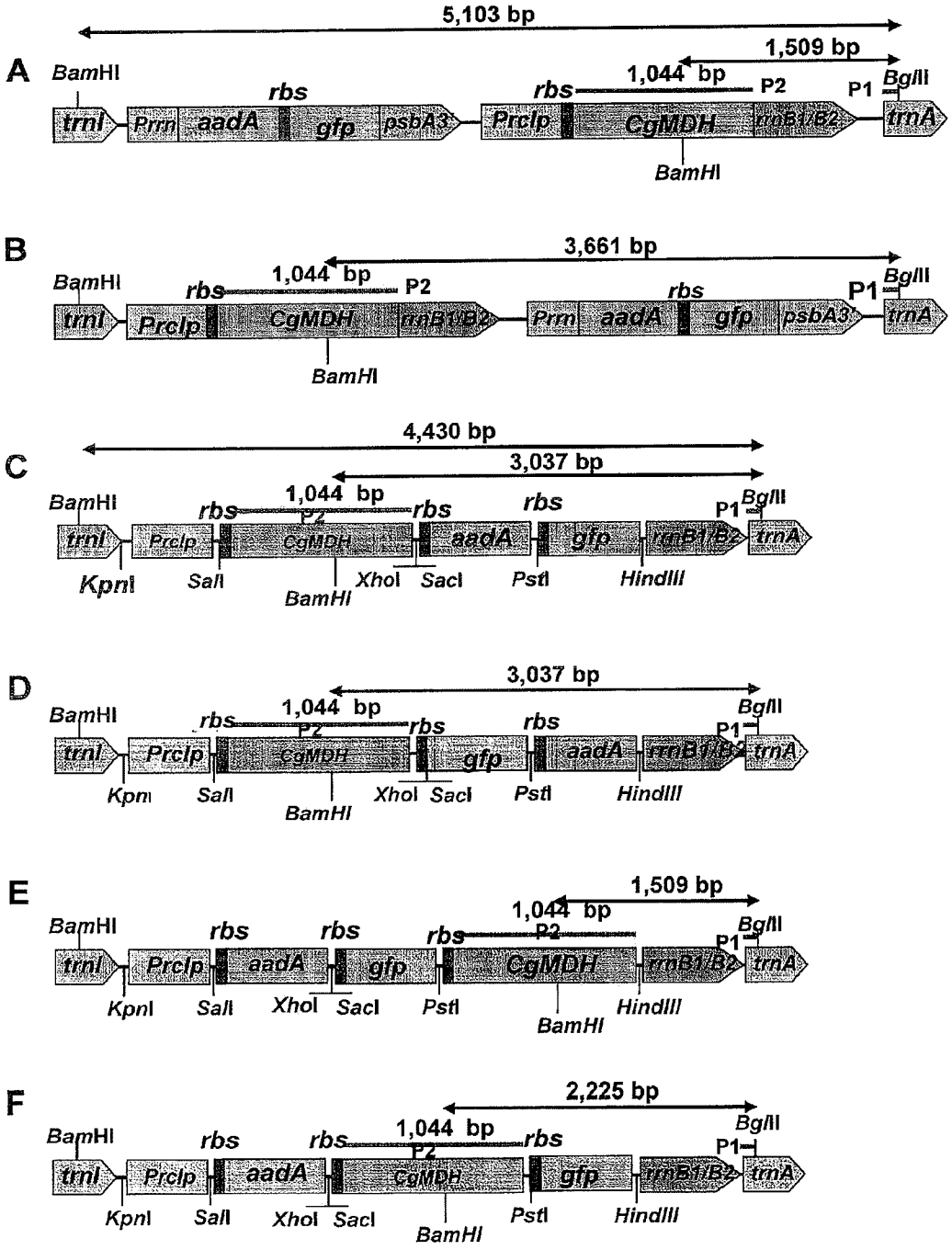
Figures

Fig. 2



Figures

Fig. 3

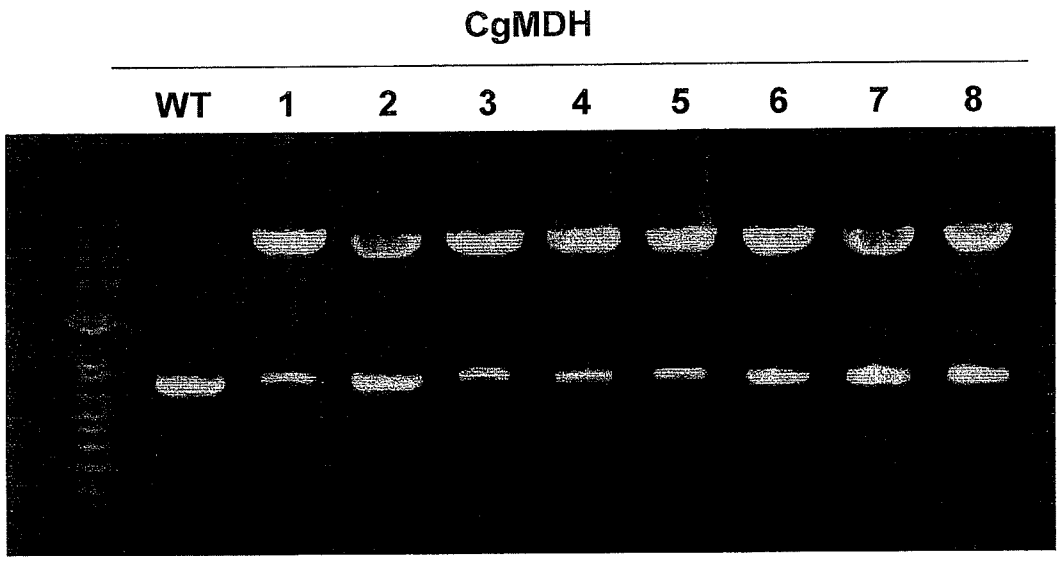
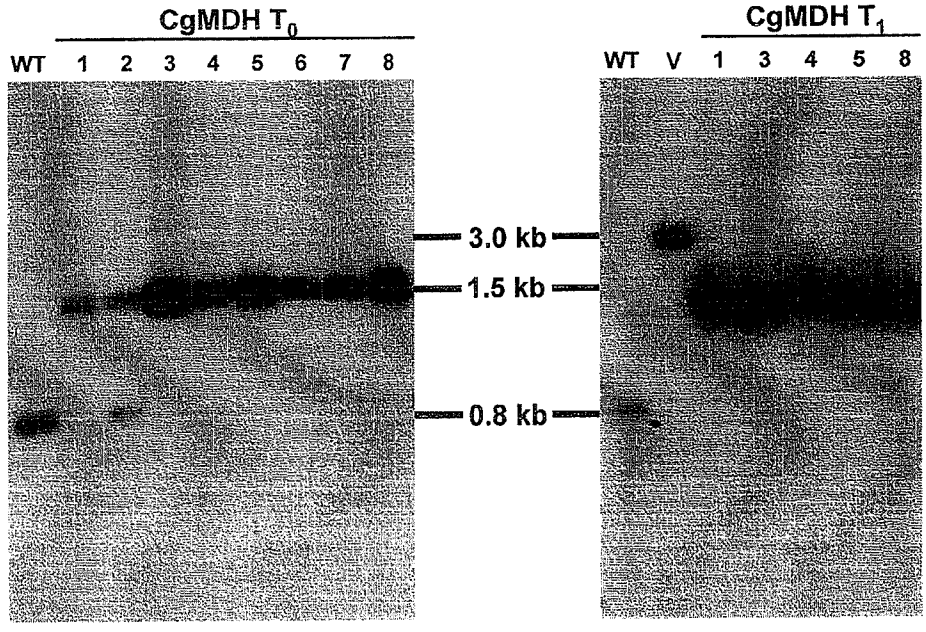


Fig. 4



Figures

Fig. 5

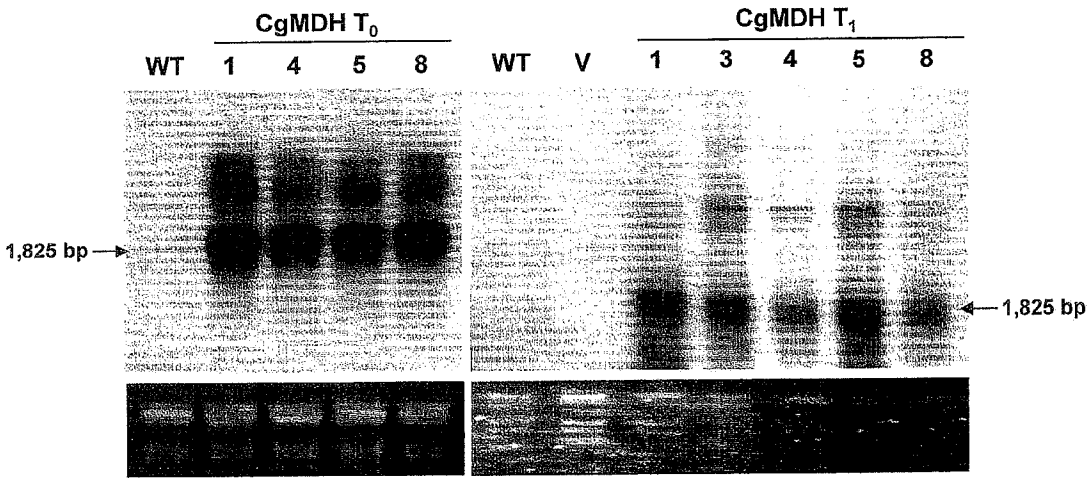
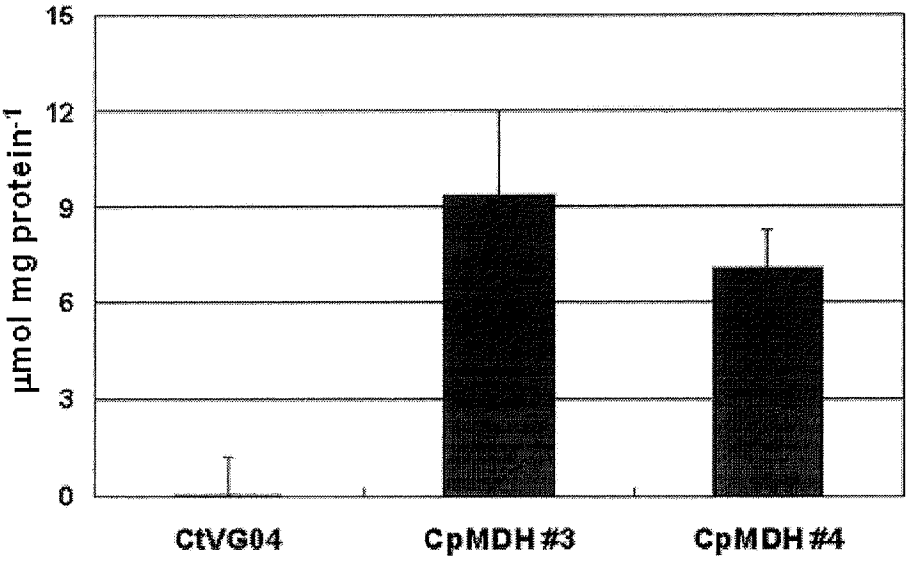


Fig. 6



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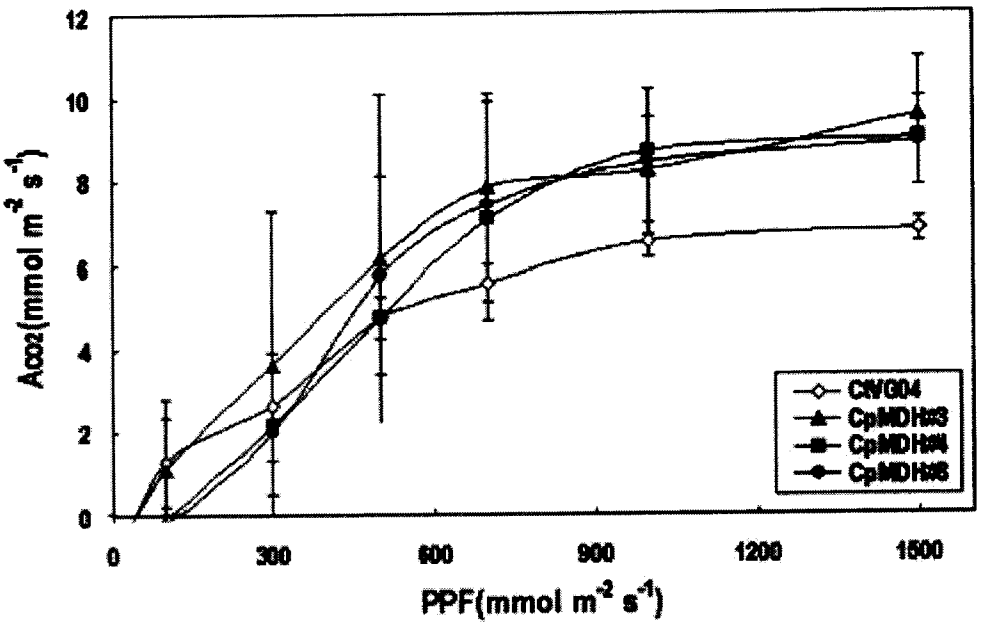
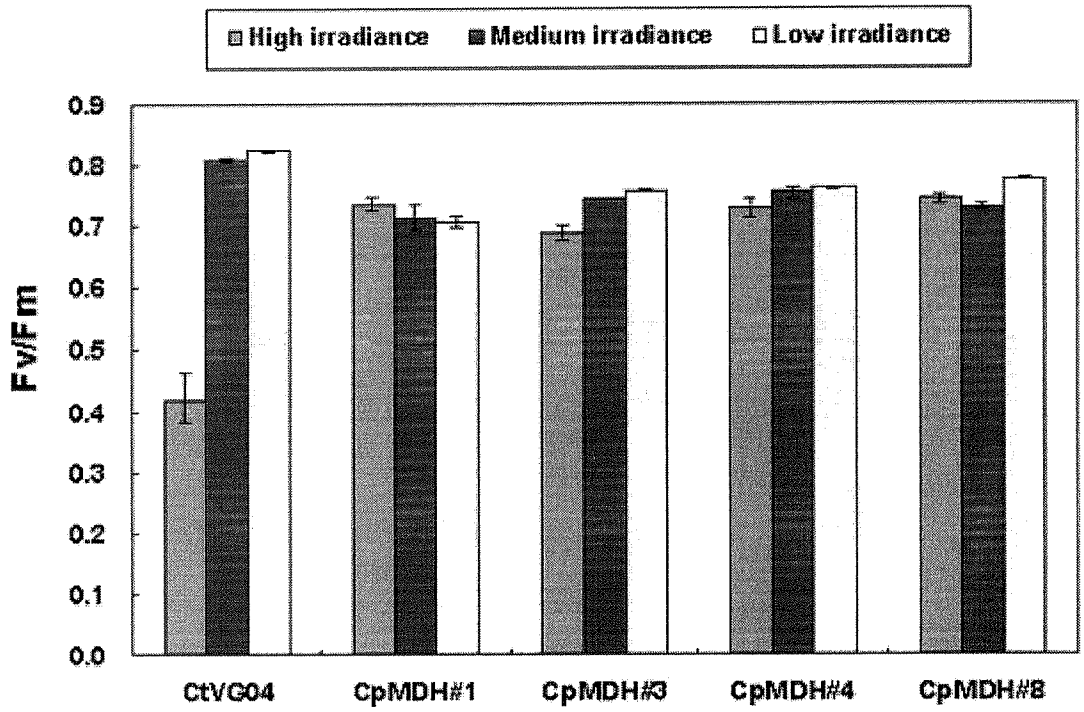
Figures

Fig. 7



Figures

Fig. 8



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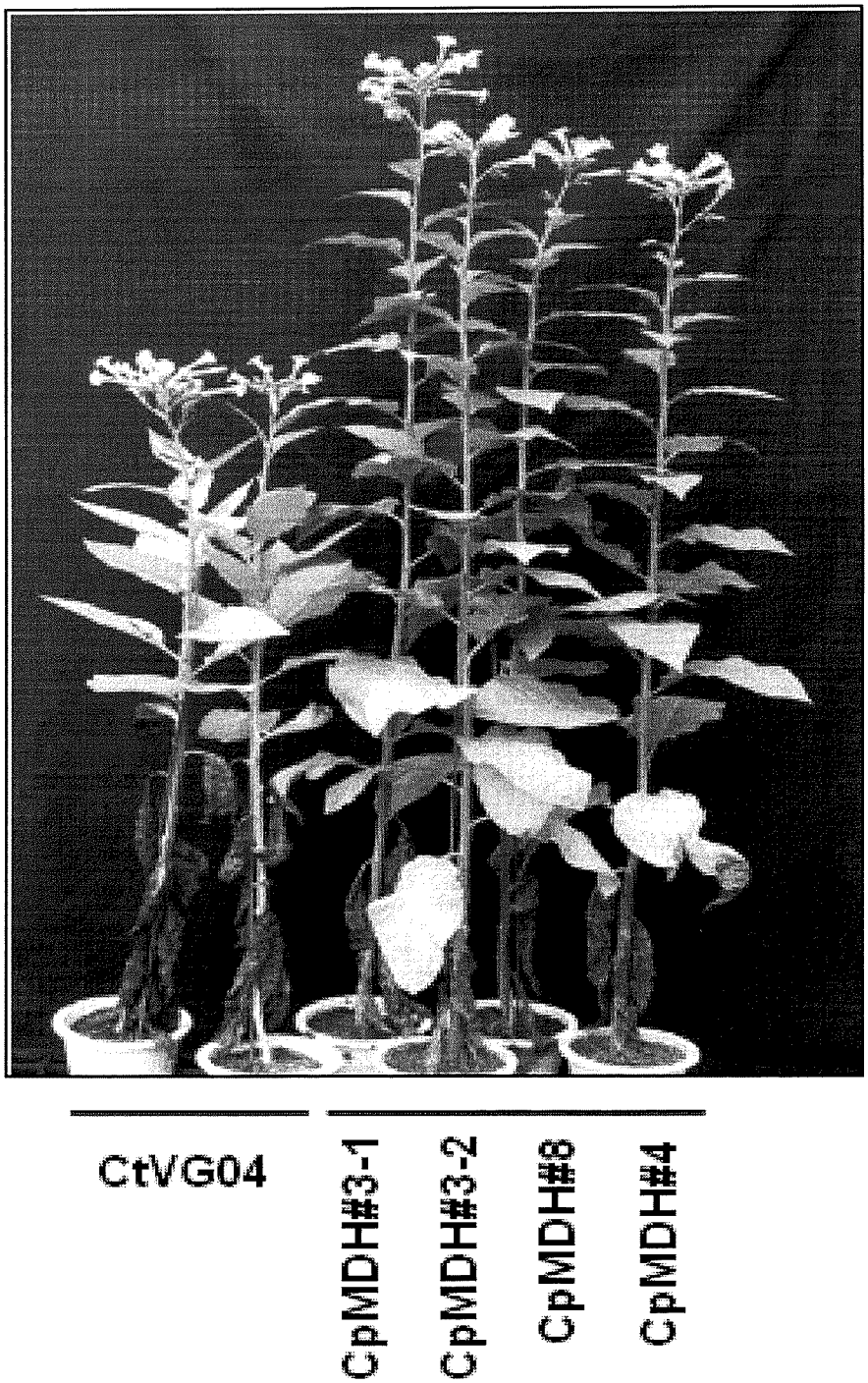
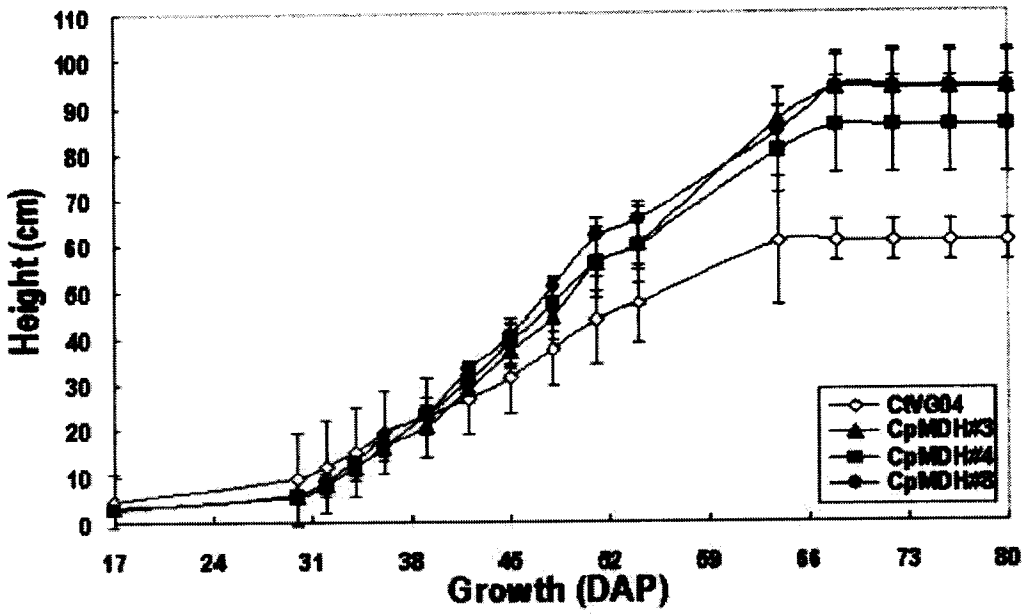
Figures

Fig. 9



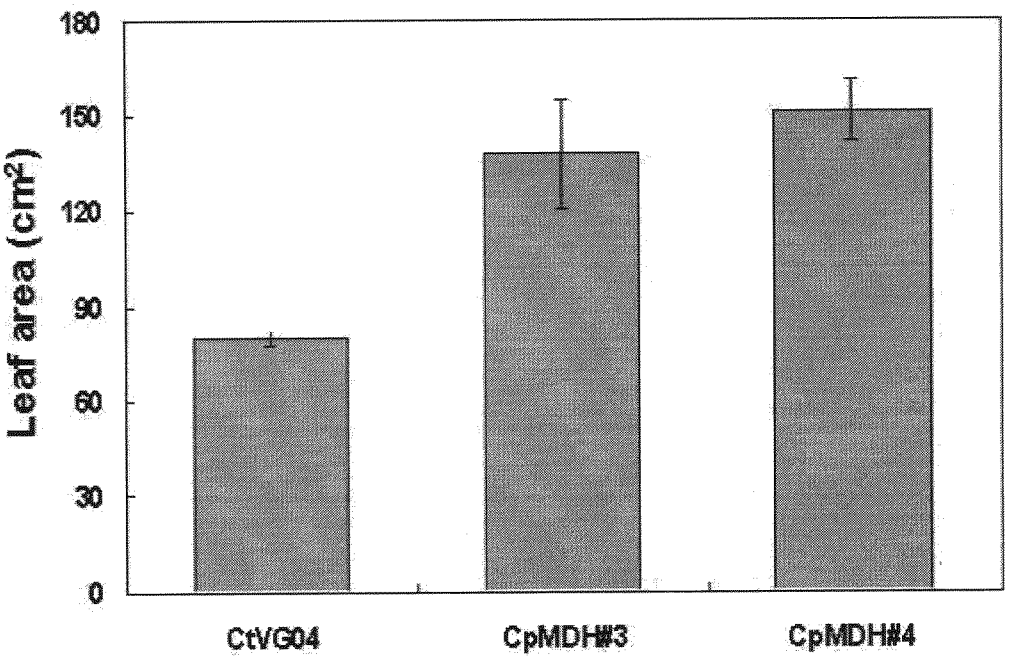
Figures

Fig. 10



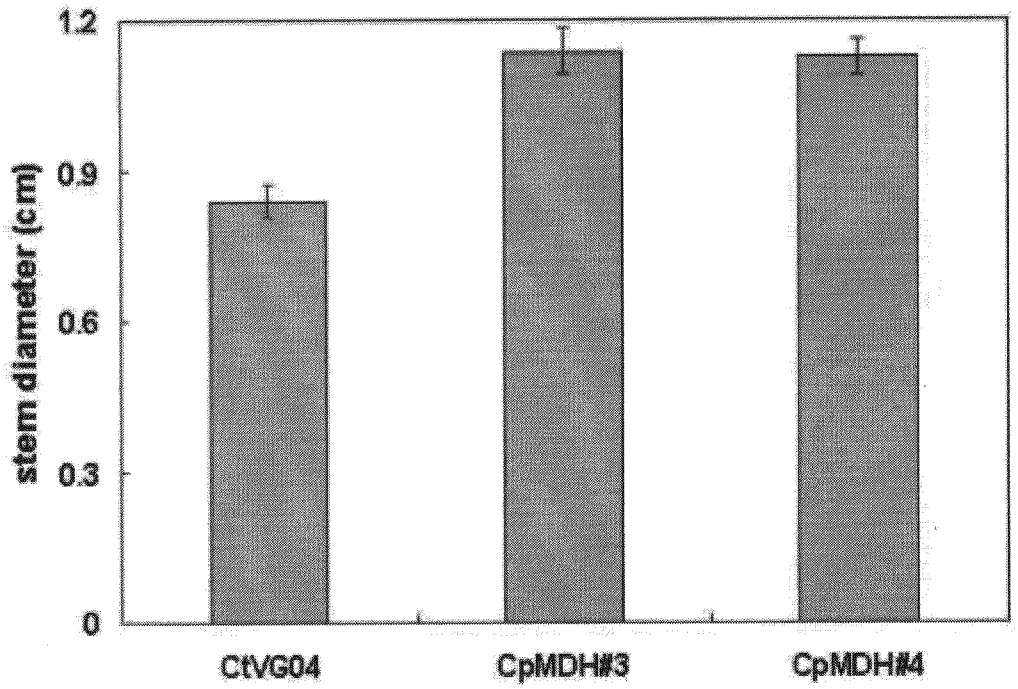
Figures

Fig. 11



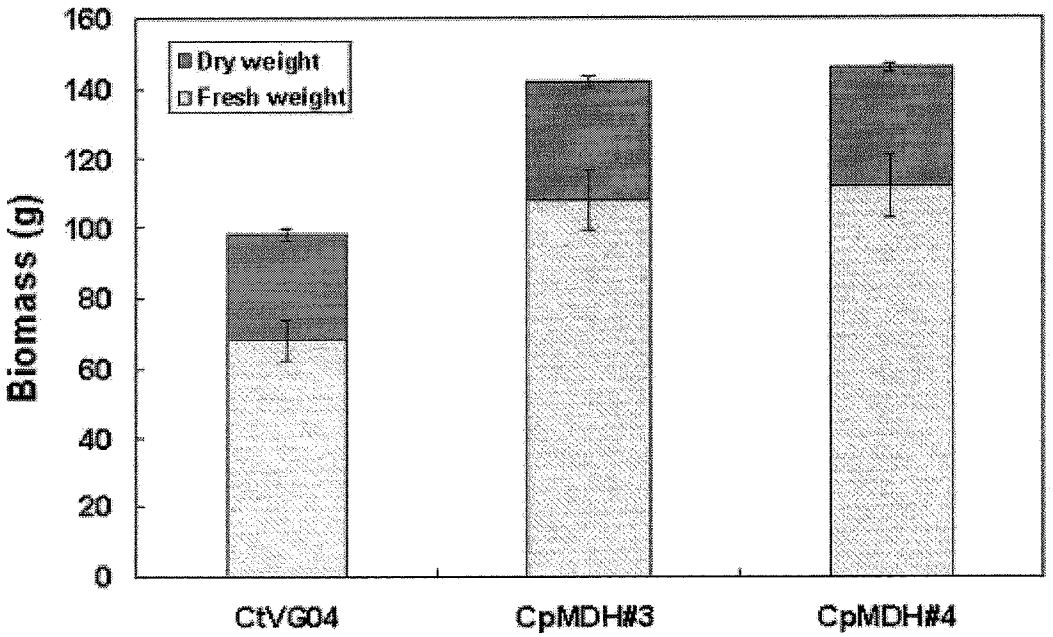
Figures

Fig. 12



Figures

Fig. 13



INTERNATIONAL SEARCH REPORT

International application No.
PCT/KR2007/005529

A. CLASSIFICATION OF SUBJECT MATTER

A01H 1/00(2006.01)i

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 8 A01H 1/00, A01H 5/00, C07H 21/00, C12N 15/09, C12N 15/11

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Korean Utility models and applications for Utility models since 1975

Japanese Utility models and applications for Utility models since 1975

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

eKIPASS, Delphion, NCBI, BLAST, PAJ, USPTO 'C3 plant' , 'C4 plant', 'MDH gene'

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	KR1020060023809 A(SEOUL NATIONAL UNIVERSITY INDUSTRY FOUNDATION) 15 Mar. 2006 See the abstract; Claims 1-11; Figures 1a-9	1-3, 17
Y	KR100275200 B1(DIRECTOR GENERAL OF NATIONAL INSTITUTE OF AGROBIOLOGICAL RESOURCES, MINISTRY OF AGRICULTURE, FORESTRY AND FISHERIES) 15 Dec 2000 See the abstract; Page4; Claims 1-54; Figures 1-7	1-3, 17
A	US5451513 A(Pal Maliga et al.) 19 Sep. 1995 See the abstract; Claims 1-13	1-17
A	KR100694389 B1(CJ CORP.) 12 Mar. 2006 See the abstract; Claims 1-6; Figures 1-7	1-17

☐ Further documents are listed in the continuation of Box C.

☒ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

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"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents,such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
12 FEBRUARY 2008 (12.02.2008)	**12 FEBRUARY 2008 (12.02.2008)**
Name and mailing address of the ISA/KR Korean Intellectual Property Office Government Complex-Daejeon, 139 Seonsa-ro, Seo-gu, Daejeon 302-701, Republic of Korea Facsimile No. 82-42-472-7140	Authorized officer CHO, Hyun Kyung Telephone No. 82-42-481-5629

Form PCT/ISA/210 (second sheet) (April 2007)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/KR2007/005529

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item1.b of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of :

   a. type of material
   - [x] a sequence listing
   - [ ] table(s) related to the sequence listing

   b. format of material
   - [ ] on paper
   - [x] in electronic form

   c. time of filing/furnishing
   - [x] contained in the international application as filed
   - [ ] filed together with the international application in electronic form
   - [ ] furnished subsequently to this Authority for the purposes of search

2. [ ] In addition, in the case that more than one version or copy of a sequence listing and/or table relating thereto has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that in the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments:

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.

PCT/KR2007/005529

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
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