(12) PATENT **(11)** Application No. **AU 200059458 B2**
(19) AUSTRALIAN PATENT OFFICE **(10)** Patent No. **737600**

(54) Title
Early-maturing sugarcane with high sugar content

(51)[7] International Patent Classification(s)
A01H 005/00 **C12N 015/52**

(21) Application No: **200059458** (22) Application Date: **2000.09.14**

(43) Publication Date : **2001.05.17**
(43) Publication Journal Date : **2001.05.17**
(44) Accepted Journal Date : **2001.08.23**

(62) Divisional of:
199953553

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Abstract

A transgenic sugarcane of a high sugar content and/or an early-maturing transgenic sugarcane and a method for producing such an transgenic sugarcane are provided. The sugar content of the transgenic sugarcane of the present invention increased by about 5 to about 15%. The sugarcane of the present invention grew in remarkably accelerated manner and reached in full growth in a period of as short as about 7 to 8 months. According to the present invention, the sugarcane capable of supplying more stably a large amount of sugar and a starting material for microorganism-culture liquid are obtained.

AUSTRALIA

Patents Act 1990

COMPLETE SPECIFICATION

STANDARD PATENT

Applicant(s):

AJINOMOTO CO., INC.

Invention Title:

EARLY-MATURING SUGARCANE WITH HIGH SUGAR CONTENT

The following statement is a full description of this invention, including the best method of performing it known to me/us:

Early-Maturing Sugarcane with High Sugar Content

Background of the Invention

The present invention relate to transgenic sugarcane of a high sugar content and/or early-maturing transgenic sugarcane, which contains a glycinebetaine-synthesizing enzyme genes genetically transferred thereinto to increase the sugar content and/or to increase the weight, length and diameter of the stems of the sugarcane in the harvest time that has a shorter period; and a method for obtaining a high sugar content and/or early maturing property of sugarcane.

Sugarcane is important as a main source of sugar and it is cultivated in various places in the world. Further, in some areas, sugarcane is the main agricultural product and the production thereof is economically very important. Although the cultivation environments in these countries are various, sugarcane having a high sugar content per plant is generally desired from the viewpoint of the reduction of the labor. The sugarcane of a high sugar content and/or early-maturing sugarcane is advantageous because of the efficient use of the land. Also from the viewpoint of the amount of fertilizers necessitated in the course of the cultivation or the effect on the environment including the discharge of the residue remaining after obtaining the sugar, sugarcanes having the above-described properties are desired. To obtain such early-maturing sugarcane plants of a high sugar content, the cross-fertilization of sugarcanes of different varieties, species or genera was conducted and, particularly, the resulting hybrids with sorghum is considered to be hopeful. For example, it was reported that a hybrid between a sugarcane strain (RKS96) and sweet sorghum (species: Collier) had a sugar content increased by 2.1 % about 8 months after the planting. The report says that the cross-fertilized product had "a remarkable early-maturing

property and high sugar content" [Akira Sugimoto *"Nettai Nogyo* (Tropical Agriculture)" Vol. 40, No. 4, 229-236). Although sugarcane strains which are hopeful to some extent could be thus obtained by the cross-fertilization between different varieties, species or genera, it is not yet satisfactory in the sugar content and early-maturing property.

In addition to the breeding by means of the cross-fertilization in the prior art, breeding by means of the genetic manipulation has also been developed. Methods for producing transgenic plants including the transformation techniques of plants and the regeneration system from callus into plants are now being established in various plant species after the investigation and development of the molecular biological techniques. It was reported that such techniques can be employed also for sugarcane. For example, the transformation system of sugarcane using Agrobacterium is reported in Center for Genetic Engineering and Biotechnology (CIGB) in Cuba. However, no gene suitable for use for producing the early maturing and high sugar content sugarcane has not yet be found, and the production of transgenic sugarcane plants having a high sugar content and/or early maturing property has not yet been reported.

On the other hand, glycinebetaine (hereinafter referred to as "betaine") is known to be a low-molecular weight compound which is related to the resistance to the environmental stress in plants. Genes relating to betaine synthesis were isolated from some biological species. These genes will be collectively called "glycinebetaine-synthesizing enzyme genes" herein. Examples of them include choline dehydrogenase genes (bet A) and betaine aldehyde dehydrogenase genes (bet B) from Escherichia coli (Lamark et al., Mol. Microbiol. 5, 1049-1064, 1991). It is known that in Escherichia coli, betaine is synthesized by converting choline into betainealdehyde by choline dehydrogenase (CDH) encoded by bet A and further converting it into betaine

by betainealdehyde dehydrogenase (BADH) encoded by betB. It is also known that betaine is biosynthesized in the similar way also in higher plants.

It was reported that some transgenic plants containing such betaine-synthesizing enzyme genes introduced thereinto were produced and that they actually had a resistance to stress (G. Lilius, N. Holmberg and L. Bulow, Bio/Technolog., 14, 177-180, 1996). In particular, it was reported that when betA from Escherichia coli was introduced into a rice plant, the rice plant accumulated betaine (5 μmol/gFW) and exhitibed a salt resistance (Hayashi et al., "The 14th Biotechnology Symposium Proceedings, 14, 263-268, 1996).

Summary of the Invention

The object of the present invention is to provide a transgenic sugarcane of a high sugar content and/or an early-maturing transgenic sugarcane, which has an increased sugar content and/or which is has an accelerated growth rate.

Another object of the present invention is to provide a method for obtaining a sugarcane having such a high sugar content and/or imparting the early-maturing property to the sugarcane.

The inventors have unexpectedly found that betaine-synthesizing genes can be used for the purpose of the present invention. The inventors have succeeded in the production of a sugarcane of the present invention by introducing betaine-synthesizing genes into the sugarcane. Namely, the objects of the present invention can be attained by introducing betaine-synthesizing enzyme genes into the sugarcane to express the genes in the plant cells thereby to accumulate a large amount of betaine in the plant cells. The betaine-synthesizing enzyme genes are introduced into sugarcane plant cells together with elements necessitated for the expression, and thereby expressed. The transgenic sugarcane plants of the present invention thus

obtained may mature in a short period and/or the maximum sugar content of this sugarcane is higher than that of a corresponding non-transgenic sugarcane, because a large amount of the betaine is accumulated in the cells thereof,.

The term "early-maturing" herein means that the sugar content or brix of the transgenic sugarcane plants reaches the maximum earlier than a corresponding non-transgenic sugarcane plants by about 4 to 6 months, particularly about 5 to 6 months. Since the growing period of sugarcanes is usually 13 months, the period is thus shortened by about 31 to 46 %, particularly by about 38 to 46 %. The term "high sugar content" means that the maximum sugar content is higher than that of corresponding non-transgenic sugarcane plants by about 3 to 15 % or more, preferably by 5 to 15 % or more, and particularly by about 10 to 15 %.

Preferred Embodiments of the Present Invention

As described above, the transgenic sugarcane of the present invention contains a large amount of glycinebetaine accumulated in the cells thereof due to the glycinebetaine-synthesizing enzyme gene introduced into them and expressed. In this connection, one or more glycinebetaine-synthesizing enzymes can be introduced into them. In short, the sugarcane of the present invention is produced in the following steps:

a) a step of cloning the betaine-synthesizing enzyme genes;

b) optionally, a step of (re)cloning the obtained betaine-synthesizing enzyme genes into a suitable binary vector (shuttle vector);

c) a step of introducing the vector into the sugarcane cells to obtain the transformed cells; and

d) a step of regenerating the sugarcane plants from the obtained transformed cells and cultivating the plants.

The term "betaine-synthesizing enzyme genes" means a group of genes concerning the betaine synthesis. Any of those capable of increasing the total biosynthesis amount of betaine in the host can be used for the production of the transgenic sugarcane plants of the present invention. Among them, choline dehydrogenase(betA), betainealdehyde dehydrogenase, choline oxidase(codA), etc. are preferable. Choline dehydrogenase is particularly preferred.

Although the source of the betaine-synthesizing enzyme genes is not limited to a particular biological species, preferably, these genes are from a organisms essentially having a high resistance to stress because betaine is known to concern the stress resistance. The illustrative examples of betaine-synthesizing enzymes suitable for the present invention are betA from E.coli, from Rhizobium meliloti and from Sinorhizobium meliloti(Osteras M, et al., Proc Natl Acad Sci USA(1998) 95(19), 11394-11399) and codA from Arthrobacter tumefaciens (Patcharaporn D, et al., Plant Molecular Biology (1995), 29, 897-907). .In one embodiment of the present invention, betA gene from E.coli is used.

The betaine-synthesizing enzyme genes used in the present invention are preferably obtained from a microorganism, such as Escherichia coli, Rhizobium meliloti and Arthrobacter,especially Rhizobium meliloti ATCC9930 and Arthrobacter tumefaciens ATCC8010, which is easily handled in the experimental operations, particularly Escherichia coli which has already been molecular-biologically well analyzed.

The betaine-synthesizing enzyme genes are cloned into a suitable vector by a standard method known in the art. For ordinary techniques, refer to, for example, Sambrooks et al., Molecular cloning – Laboratory manual, the second edition (Cold Spring Harbor Laboratory Press) 1989. The cloned DNA fragments may be, if necessary, re-cloned in a more suitable

gene-transferring vector. Further, nucleic acid fragments capable of hybridizing with the originally cloned nucleic acid fragment under stringent conditions are also suitable for the purpose of the present invention. These DNA fragments include nucleic acid fragments capable of encoding the glycine-betaine sysnthesizing enzyme proteins having one or more amino acids deletion, one or more amino acids addition or one or more amino acids substitution. The term "stringent conditions" means standard conditions such as described in the above-mentioned book by Sambrook et al. (1989) and well-known to those skilled in the art. The nucleic acid sequence which can hybridize with the clone under the stringent conditions will have usually at least 60 %, preferably at least 80 %, and particularly preferably at least 90 % homology with the originally cloned betaine-synthesizing enzyme genes. Further, the betaine-synthesizing enzyme used in the present invention may be expressed as fusion genes with a suitable transit peptide depending on host cells used. As the transit peptides, those from various living organisms are usable in the present invention. The non-restrictive examples of the transit peptides suitable for the present inventino are mitochondria transit peptides (MAtthes P. Purnell, et al., Gene (1997) 186, 249-254 (tomato)) and choroplast transit peptides (Sugita M., et al., Mol. Gen. Genet. (1987) 209(2), 247-256 (tomato), Xie Y, et al., Nucleic Acids Res. (1988) 16(15), 7749).

Among them, mitochondria transit peptides are preferred, and those for tomatoes are particularly preferred. The sequence of the betaine-synthesizing enzyme genes in the present invention may be altered so as to have an optimum codon depending on the codon usage of the hosts used.

For the production of the transgenic sugarcane plants of the present invention, a gene transfer method usually employed for the transformation of plants can be employed. The gene transfer methods include, for example, agrobacterium method, electroporation method and methods using a particle

gun. For the purpose of the present invention, the electroporation method or Agrobacterium method is preferred. Since the protoplasts may not be easily handled, the Agrobacterium method wherein the protoplasts are not used is usually particularly preferred. In Examples described hereinafter, the Agrobacterium method is employed. Agrobacterium microorganisms usable herein include Agrobacterium tumefaciens and Agrobacterium rhizogenes. Agrobacterium tumefaciens is preferred.

The genes can be transferred into the whole plants, leaves, stems, calli, protoplasts, etc. For the purpose of the present invention, callus is preferably used because they can be kept as plant cell cultures and the regeneration from them into the plant has been already established. Although the species of sugarcane usable in the present invention is not limited, it is preferred that the induction and culture of the calli and the regeneration thereof are relatively easy. Thus, sugarcane strain Nco310 is particularly preferred. Although the culture period of the calli used is not particularly limited so far as the regeneration potency is kept, calli growing in logarithmic phase are preferred. The calli of day 4 or 5 after the passage are particularly preferably used. The calli used for the transformation are cut into small pieces of, preferably less than 5 mm, particularly about 1 mm, in order to increase the efficiency of infection of Agrobacterium and the efficiency of selection of the transformed cells.

The vectors used for the production of the transgenic sugarcane of the present invention can be selected depending on the gene transfer method. For example, small vectors for Escherichia coli such as pBR322 and pUC series plasmids are suitable in the electroporation method and particle gun method. On the other hand, relatively small vectors containing at least right boundary region of T-DNA are used, and vectors containing both right and left boundary regions are particularly preferably used in the Agrobacterium

method. Further, infectious gemini virus vectors are also suitable in any gene transfer method. These vectors are well known in the art, and various vectors are commercially available. The vectors usually contain a promoter which can function in the plant cells such as 35S promoter of cauliflower mosaic virus (CaMV), a suitable terminator such as a nopaline synthase enzyme gene terminator, and elements useful for controlling the expression.

The promoter may be either an inducible promoter or constitutive promoter. Among them, a strong constitutive promoter is preferred, and 35S promoter of CaMV is particularly preferred. These vectors typically contain marker genes for screening the transformants, such as genes resistant to herbicides or genes resistant to antibiotics, e. g. glyphosate resustabt genes, methotrexate resistant genes, G418 resistant genes, hygromycin resistant genes and kanamycin resistant genes, and they may contain other markers suitable for confirmation of transformed cells such as GUS (β-glucuronidase) genes. Among the genes suitable for screening the transgenic sugarcane cells and/ or plants of the present invention, the genes resistant to antibiotics are preferred, and hygromycin resistant genes are particularly preferred. In the Agrobacterium method, either binary vector (shuttle vector) or intermediate vector may be used. In view of the easiness of the operation, the binary vector is preferred, and that having a relatively small size is particularly preferred. The introduced genes are not necessarily integrated into the genome of the plant cells, and they may be present as autonomous molecules in the cell as well. However, it is preferred that they are finally integrated into the genome and thus stably maintained in the chromosome of the cells. Binary vector pE2113 used in Examples is particularly suitable for such a purpose. Anyway, the introduced genes must be sufficiently expressed in the sugarcane plants or sugarcane cells to achieve the purpose of the present invention.

The obtained transformed cells may be regenerated into the plants by any suitable method known in the art, and they can be cultured by the standard methods known in the art. Although the transformed cells may be kept as the cultured plant cells, they must be kept under such conditions that the regeneration potency is maintained, for the purpose of the present invention. The properties of the plants thus obtained are examined. Since the vegetative propagation of sugarcane is generally possible, the sugarcane having desired characters can be propagated in a short time. In the actual mass production, such a method is employed. Further, the descendants of the transgenic sugarcane plants may be cultivated for two or three generations for evaluating the stability of the properties in some cases. If necessary, the mating of the transformants, interspecies cross with non-transformants, or genus cross may be repeated until desired properties are obtained. The sugarcane plant produced by such a method may be either homozygous or heterozygous for the introduced betaine-synthesizing genes.

From such a sugarcane, the sugar having the same quality as that of the corresponding non-transgenic sugarcane can be obtained by essentially the same treatment. Further, the residue remaining after the extraction of the sugar can be treated in the same manner as that of the corresponding non-transgenic sugarcane to prepare pulps or the like.

Examples

1. Cloning of betaine-synthesizing enzyme genes:

1-1. Isolation of chromosome DNA:

E. coli was cultured in an LB medium containing the ingredients given below at 37°C for 12 hours. The cells were collected with a centrifugal separator. From the cells, a chromosomal DNA solution was prepared with a chromosomal DNA isolation kit (a product of Biotechnology Co.). The final

concentration of the solution was 0.1 μg/ml. The chromosomal DNA was confirmed by 0.8 % agarose gel electrophoresis. A Tris-acetate buffer was used as the electrophoresis buffer. DNA was stained with ethidium bromide (0.5 μg/ml), and visualized with long wavelength ultraviolet light.

LB medium:

Bactotrypton	1 g/l
Yeast extract	0.5 g/l
NaCl	1 g/l.

pH: adjusted to 7.2.

1-2. Isolation of E. coli betA:

To isolate betA genes from E. coli by PCR method, two primers, i.e. primer 1 (SEQ ID NO:1: 5' ggc taa att cca gtc cat att ct 3') and primer 2 (SEQ ID NO:2: 5' ctc aat ctg atc ggt tcc tgc gt 3') were synthesized. With these two primers, bet A genes from E. coli were amplified using chromosomal DNA obtained as described above as the template using DNA Amplifier (Takara Co.). The PCR reaction conditions used were as described below. For the gene amplification, the reaction was conducted in 100 μl with Pyrobest polymerase (a product of Takara Co.).

PCR reaction conditions:

94℃ for one minute;

and then 25 cycles of the reaction were conducted under the following conditions:

94℃ for 30 seconds;

55℃ for 30 seconds; and

72℃ for 2 minutes.

1-3. Cloning into a vector:

Vector plasmid pHSG399 (Takara Co.) for E. coli was used for cloning the obtained 1750 bp DNA fragments. 0.1 μg of pHSG399 was digested with a restriction enzyme SmaI (Takara Co.). The restriction enzyme reaction was conducted at 30°C for one hour. The completion of the reaction was confirmed by the 0.8 % agarose gel electrophoresis. The electrophoresis buffer used was Tris-acetate buffer. After the completion of the electrophoresis, the gel was stained with ethidium bromide (0.5 μg/ml), and visualized with long wavelenght ultraviolet light to confirm the completion of the digestion.

1-4. Transformation of E. coli:

The PCR reaction product obtained in step 1-2 was mixed with the SmaI digested vector plasmid pHSG399, and the mixture was ligated. The ligation reaction was conducted with a ligase solution (Takara Co.) in 50 μl at 16°C for one hour. The reaction liquid was mixed with competent cells of E. coli strain JM 109 (Takara Co.) on ice, and then they were left to stand for 30 minutes. After heating the mixture at 42°C for one minute, 800 μl of the above-described LB culture medium was added thereto, and the culture was incubated at 37°C for one hour. Then the mixture containing the bacterial cells are plated on the LB agar medium plates coated with 80 μl of 30 mg/ml X-gal solution and IPTG solution and containing 30 μg/ml of chloramphenicol. After 12 hours incubation at 37°C, white colonies were selected and the plasmids were extracted from the bacterial cells. The plasmid extraction was essentially conducted by alkali SDS method. In particular, the plasmid extraction was conducted with a DNA extraction kit Miniprep (Promega Co.). The obtained plasmids were finally suspended in 50 μl of sterilized water. The plasmid DNA was confirmed by determining the restricted incision site and the size by the electrophoresis according to the above-described method. Then the sequence of bet A genes in E. coli (SEQ ID NO:3) was confirmed by

sequencing the SmaI insert in the obtained plasmid DNA. The plasmid DNA was prepared in large scale from the clone thus determined, and used for further experiments described below.

2. Preparation of shuttle vector:

2-1. Isolation of tomato transit peptide gene:

DNA fragment (SEQ ID NO:6) of 69 bp in length which encodes a transit peptide for tomato mitochondria was isolated. Chromosomal DNA of tomato was obtained from tomato leaves. The chromosomal DNA was extracted with a DNA extraction kit for plants (QIAGEN Co.). Finally, 100 μl of 0.1 μg/μl DNA solution was obtained. The chromosomal DNA was confirmed by the electrophoresis method such as described in this pecification for the chromosomal DNA of E. coli. Primers were designed on the basis of a published sequence by using thus obtained chromosomal DNA as the template. From them, the DNA fragments which encode the desired transit peptide were amplified by PCR reaction. The sequences of the primers used for PCR are given below. PCR reaction was carried out under the conditions shown in Example 1. The PCR reaction product thus obtained was ligated into the SmaI restriction site of vector plasmid pHSG399 to transform E. coli JM109. The conditions of the preparation of vector plasmid pHSG399, ligation of the plasmid and PCR segment and transformation of E. coli. were substantially the same as those in Example 1.

Clones having the desired DNA fragments were selected from the transformants grown on the LB agar medium containing 30 μ g/ml of chloramphenicol. The clones were selected in the same manner as that in Example 1 wherein the cloning of E. coli betA genes is described. The obtained nucleotide sequence encoding transit peptide for tomato mitochondria is shown as SEQ ID NO: 6.

Primers used:

5' atg aat gct tta gca gca act aat aga aat 3' (sequence No. 4)

5' ctt tga gtc taa acc aag aag cct agc tgc 3' (sequence No. 5)

2-2. Fusion of transit peptide gene to betaine-synthesizing enzyme gene:

PCR was used to attach the nucleotide sequence encoding betaine-synthesizing enzyme obtained in Example 1 to the DNA fragment encoding the transit peptide obtained in Example 2-1. In this process, a primer (hereinafter referred to as "chimera primer") was prepared which has the sequence complementary to the 3'-terminal region of the nucleotide sequence encoding the transit peptide at its 3'-terminal half and complementary to the 5'-region of the betaine-synthesizing enzyme gene at its 5'-terminal half. Then the coding sequence of the transit peptide was amplified using the primer corresponding to 5'-terminal region of the transit peptide and the chimera primer. Separately, betaine-synthesizing enzyme gene was amplified using the chimera primer and the primer for 3'-terminal region of the betaine-synthesizing enzyme gene. The two amplified DNA segments were mixed, and the mixture was subjected to PCR reaction using 5'-primer for the transit peptide coding sequence and 3'-primer for the betaine-synthesizing enzyme gene to amplify the DNA segment in which the transit peptide code sequence is fused to the 5'-end of the betaine-synthesizing enzyme gene.

The obtained DNA segment which encodes the transit peptide-betaine synthesizing enzyme fusion protein was cloned into the SmaI site of vector plasmid pHSG399. The methods of the preparation of the vector plasmid and transformation into E coli. were the same as those described in Examples 1-3 and 1-4, respectively. The clone containing the desired DNA fragment was selected from the transformants. In particular, the clone was selected by

isolating plasmid DNA from the transformamts and confirming the inserted DNA by standard methods such as PCR amplification or restriction enzyme digestion. Finally, the nucleotide sequence of the obtained fragment was confirmed by sequencing it.

2-3. Construction of the shuttle vector plasmid pTmiEbetA:

XbaI polylinker (a product of Takara Co.) was linked at 5'-end and 3'-end of the DNA fragment endocing the transit peptide-betaine-synthesizing enzyme fusion protein obtained in above-described step 2-2. It was then digested with restriction enzyme XbaI. After agarose gel electrophoresis, the intended DNA fragment encoding the transit peptide-betaine-synthesizing enzyme fusion protein was isolated from the gel. The DNA fragment was ligated into Xbal site of Agrobacterium vector plasmid pE2113 [Yuko Ohashi et al., Plant Cell Physiol. 37(1): 45-59 (1996)] to obtain plasmid pTMiEbetA. This plasmid contains the DNA segment which encodes the fused protein, i.e. betaine-synthesizing enzyme (Bet A) protein from E. coli fused to the transit peptide for tomato mitochondria at the 3'-end of the transit peptide, in X baI site of pE2113. E.coli JM109 was transformed with the plasmid. The transformation method was the same as that of Example 1-4. However, LB agar medium plates containing kanamycin (25 μg/ml) were used for the selection. The clone containing the desired DNA segment was confirmed by extracting the plasmid, amplifying the DNA by PCR and then examining the size and restriction site thereof by agarose electrophoresis and restriction enzyme digestion.

2.4 Isolation and cloning of Rhizobium meliloti betA gene

To isolate betA genes from E.coli by PCR method, tow primers, i.e. primer 3(5' gca gcc gga tga ggg gca tgc cg 3', SEQ ID:7) and primer 4 (5' cgc tgg

atc ggt ttc ctc tcg ga 3', SEQ ID:8) were synthesized. These primer were designed based on previously described DNA sequence from Sinorhizobium meliloti (Osteras M, et al., Proc Natl AcadSci USA (1998) 95 (19), 11394-11399).

Choromosomal DNA from R. meliloti was isolated using the similar method described for isloation of E.coli choromosome DNA as described in Example 1-1.

With primers 4 and 5, betA gene from R. meliloti were amplified using chormosomal DNA from R.meliloti as the template. The condition used for PCR was the same as the condition used for amplifying E.coli betA gene which was described in Example 1-2.

The amplified DNA fragment was cloned into the SmaI site of plasmid pHSG399. then cloned into the XbaI-BamHI site of the vector for Abrobacterium containing 35S-promoter, according to the similar procedures described above for cloning of E.coli betA gene(Example 1-3 to 2-3).

The sequence of betA gene from R. meliloti was desribed in sequence listing as SEQ ID:9.

3. Production of transgenic sugarcane, and characteristics of obtained transgenic sugarcane:

3-1. Transformation of sugarcane:

A sugarcane calli (Saccharum officinarum NCo310) of day 4-5 after the passage were passed through a screen having a pore diameter of about 1 mm to reduce the diameter of the callus to about 1 mm. About 0.3 ml (PVC: packed cell volume) of the calli thus treated was suspended in10 ml of liquid MS medium [Murashige and Skoog medium, Murashige, T., and Skoog, F.: Physiol. Plant., 15,473 (1962)] containing 1 mg/ml of 2,4-D.

Agrobacterium tumefaciens LBA4404 containing plasmid pTMiEbetA

introduced thereinto by Tri-parental mating method was cultured by the shaking culture in 10 ml of YEP medium having a composition shown below at 28℃ until OD_{260} reached about 0.6, and the bacteria were harvested by centrifugation. Agrobacterium cells thus harvested were resuspended in 1 ml of MS medium. The resulting suspension was added to the callus suspension prepared as described above, and they were stirred together. The Agrobacterium/calli suspension was left to stand at 28℃ for 10 minutes. The calli were recovered on a sterilized filter paper to remove water as far as possible. Then the calli and the filter paper were placed on an MS agar plate containing 1 mg/l of 2,4-D, 500 mg/l of Cefotaxime and 80 mg/l of hygromycin, and cultured in a dark place at 28℃. The culture medium was exchanged every seven days.

YEP medium:

Bactopetone	10 g/l
Bactoyeast extract	10 g/l
NaCl	5 g/l
1 M $MgCl_2$	2.0 ml

pH: adjusted at 7.2 with NaOH.

3-2. Characteristics of transgenic sugarcane:

The infected calli were cultured for 4-5 weeks and the calli having resistance to antibiotics were moved into the agar MS medium containing 500 mg/l of Cefotaxime and 80 mg/l of hygromycin but free of 2,4-D, and cultured at 28℃ under conditions of (200 lux light for 16 hours) / (dark for 8 hours). The transgenic sprouts were obtained after culturing for about 4 weeks. After the micropropagation on MS agar plate containing 1.3 mg/l of IAA (indole acetic acid), 0.7 mg/l of kinetin and 0.2 mg/l of BAP (benzylaminopurine), the regenerated transgenic plants were cultivated and

examined in a closed system greenhouse.

Quaternary ammonium compounds in the transgenic sugarcane grown to a period of 5-7 leaves were analyzed by ^{1}H-NMR spectral method. A glycinebetaine peak which is not observed in non-transgenic sugarcane was observed in the transgenic sugarcane plants. The efficiency of appearance of the calli resistant to antibiotics and the efficiency of regeneration are shown in Table 1. The properties of the transgenic sugarcane obtained after the culture for a predetermined period are summarized in Table 2.

Table 1

Transformation efficiency of sugarcane callus,

and regenerationefficiency

Exp.	Amt. of co-cultured callus(ml)	Hygro-mycin-resistant callus	Efficiency of resist-ant callus	Hygro-mycin-resistant sugarcane	regeneration efficiency	Efficiency of hygromycin -resistant sugarcane
1	2.8	22	0.9×10^{-2}	19	0.86	0.8×10^{-2}
2	2.5	26	1.3×10^{-2}	24	0.92	1.2×10^{-2}
3	3.1	28	1.1×10^{-2}	25	0.899	1.0×10^{-2}

* 830 callus/ml PCV

** calculated as (hygromycin-resistant sugarcane plant) / (total number of calli used).

Table 2

Acceleration of growth of transgenic sugarcane and improvement in sugar accumulation

	Stem length (cm)	Stem diameter (mm)	Stem weight (g)	Brix degree	Sugar content
Nco310 (control)	215	19	501	14.5	12.4
Transgenic sugarcane	227	20	636	16.1	14.1

* All the data were obtained on day 245 after the planting.

** The data are the average of those of 20 samples.

The transgenic sugarcane plants grew sufficiently for harvesting in a period of as short as about 7 to 8 months, though the period of idividual plants varied. Brix degree and sugar content of the transgenic sugarcane plants were increased by about 5 % to about 15 % (maximum). As shown in Table 2, the acceleration of the growth of the plants was also remarkable and, in particular, the stem weight was increased by about 10 to 15 %.

According to the present invention, a sugarcane with an increased sugar content and/or an early maturing sugarcane is provided. The growth of this sugarcane is accelerated and the weight of the plant is increased in a short period. According to the present invention, the sugarcane capable of supplying a large amount of sugar or a starting material for microorganism-culture liquid can be obtained Therefore, sugarcane extracts containing sugar and molasses, foods made of purified products thereof, and microbiological products obtained from culture liquids containing them can be supplied in a large amount at low costs.

Those skilled in the art will readily recognize that various changes and

modifications may be made without departing from the spirit and scope of the present invention. The specific embodiments described herein are provided by way of example, and the specific embodiments and examples should not be considered as limiting the present invention.

SEQUENCE LISTING

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<120> An early maturating sugarcane with high sugar content

<130> OP99065-1

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<151> 1999-10-08

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<170> PatentIn Ver. 2.1

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<213> Artificial Sequence

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<223> Description of Artificial Sequence:PCR primer for tomat transit peptide

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<210> 5

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<212> DNA

<213> Artificial Sequence

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<223> Description of Artificial Sequence:PCR primer for tomato transit peptide

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<211> 69

<212> DNA

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gactcaaag 69

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<213> Artificial Sequence

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<223> Description of Artificial Sequence:PCR primer for
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<223> Description of Artificial Sequence:PCR primer for
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tattccgaga ggaaaccgat ccagcg 1886

We claim:

1. A transgenic sugarcane which contains a glycinebetaine-synthesizing enzyme gene genetically introduced thereinto to accumulate glycinebetaine in the cells and thereby to have a maximum sugar content higher than that of the non-transgenic sugarcane of the same variety in the harvest time.

2. The transgenic sugarcane of claim 1, which has a maximum stem weight higher than that of the non-transgenic sugarcane of the same variety in the harvest time.

3. The transgenic sugarcane of claim 1, which has shorter growing period until the harvest than that of the non-transgenic sugarcane of the same variety in the harvest time.

4. The transgenic sugarcane of claim 1, wherein the glycinebetaine-synthesizing enzyme gene is a choline dehydrogenase gene.

5. The transgenic sugarcane of claim 1, wherein the glycinebetaine-synthesizing enzyme gene is derived from Escherichia coli.

6. The transgenic sugarcane of claim 1, wherein the glycinebetaine-synthesizing enzyme gene is fused on 3'-end of mitochondria transit peptide gene.

7. A method of increasing the maximum sugar content of sugarcane in harvest time, which comprises the step of transferring a glycinebetaine-synthesizing enzyme gene into the sugarcane to express the genes and thereby to accumulate glycinebetaine in the cells.

8. A method of increasing the maximum stem weight of sugarcane in harvest time, which comprises the step of transferring a glycinebetaine-synthesizing enzyme gene into the sugarcane to express the genes and thereby to accumulate glycinebetaine in the cells.

9. A method of shortening the growing period of sugarcane, which comprises the step of transferring a glycinebetaine-synthesizing enzyme gene into the

sugarcane to express the genes and thereby to accumulate glycinebetaine in the cells.

10. The method of claim 7, wherein the glycinebetaine-synthesizing enzyme gene is a choline dehydrogenase gene.

11. The method of claim 7, wherein the glycinebetaine-synthesizing enzyme gene is derived from Escherichia coli.

12. The method of claim 7, wherein the glycinebetaine-synthesizing enzyme gene is fused to the 3'-end of mitochondria transit peptide gene.

13. A method of producing sugar, which comprises the step of cultivating a transgenic sugarcane which contains a glycinebetaine-synthesizing enzyme gene genetically introduced thereinto to accumulate glycinebetaine into the cells and thereby to have a maximum sugar content higher than that of the non-transgenic sugarcane of the same variety in the harvest time or which is obtained by transferring a glycinebetaine-synthesizing enzyme gene into sugarcane to express the genes and thereby to accumulate glycinebetaine in the cells, and extracting the sugar from the sugarcane.

14. A transgenic sugarcane substantially as herein described.

15. A method of producing sugar substantially as herein described.

Dated this 14th day of September 2000
AJINOMOTO CO., INC.
By their Patent Attorneys
GRIFFITH HACK