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IMPROVED PROCEDURES FOR TRANSFORMATION OF SUGAR BEET

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ABSTRACT

Sugar beet gene manipulation has the potential to improve crop productivity, alleviate biotic or abiotic stress and reduce the environmental impact of crop growth. We have considered the procedures for transformation which have been developed and optimized several aspects so as to achieve efficient protocols for the delivery of foreign genes, recovery of transformants and regulation of expression of the foreign genes in transgenic plants.

Sugar beet has been regenerated through organogenesis directly from explants or *via* an intervening callus phase. Somatic embryos were induced at various frequencies from seedlings of several genotypes on MS or PGo medium containing a range of concentrations of BAP (N-6-benzylaminopurine) or TDZ (thidiazuron). The embryos were multiplied during subcultures which involved mechanical wounding.

Several marker genes were tested for the detection of early and stable transformation events. It was found that the *E.coli gus*A gene can be expressed in sugar beet cells but the GUS assay is invasive. The red coloration which indicates that the maize *C1/Lc* genes have been expressed was never observed in sugar beet. A synthetic jellyfish green fluorescent protein gene (*sgfp-S65T*) was expressed at a high level in sugar beet and was used to non-invasively monitor gene expression *in situ*.

Factors affecting the efficiency of *Agrobacterium*-mediated transformation were investigated, using the *gus*A gene as a reporter. The best result was for sugar beet line SDM 10 (Lion Seeds, UK) precultured on BAP and TIBA (2,3,5-triiodobenzoic acid) supplemented medium, nearly 50% of the explants showed GUS activity with 1 to 60 blue spots per explant (size 4-16 mm^2) transformed with EHA101 (pBECKS400). The effects of plasmids containing part of the reverse transcriptase gene of a sugar beet *LINE* (non-LTR retrotransposon) and satellite DNA repeats of sugar beet or *B. procumbens* on transformation frequency and transgene expression in stable transformants were compared.

Transgenic sugar beet cells and plants have been obtained from cocultivated explants and callus and analyzed by a range of methods. We are using two strategies for optimizing the morphology and anatomy of sugar beet storage roots so as to achieve a high sucrose/high juice purity/low soil tare crop. The first strategy involves the manipulation of storage root cytokinin levels by deployment of the *A. tumefaciens ipt* gene and the second strategy involves direct manipulation of the *cdc* cascade of the storage roots.

RESUME - AMELIORATION DES TECHNIQUES DE TRANSFORMATION DE LA BETTERAVE SUCRIERE

La manipulation génétique de la betterave sucrière peut potentiellement améliorer sa productivité, sa réponse aux stress biotiques et abiotiques et réduire l'impact de l'environnement sur sa croissance. Les techniques de transformation de la betterave sucrière ont ainsi été mise au point. Nous avons considéré les différentes possibilités d'amélioration des protocoles de transfert de gènes étrangers, d'obtention de plantes transgéniques et de l'expression de ces gènes étrangers dans ces plantes.

La betterave sucrière peut être régénérée soit par organogenèse directe d'explants primaires soit par une phase cal. Les embryons somatiques ont été induits à des fréquences différentes à partir de plantules de plusieurs génotypes sur milieu MS ou PGo avec une gamme de BAP (6-6-benzylaminopurine) ou TDZ (thidiazuron). Les embryons ont été multipliés par des repiquages associant des blessures mécaniques.

Différents gènes marqueurs ont été comparés pour la détection d'événements de transformations précoces et stables. Il a été trouvé que le gène *gus*A d'*E.coli* peut s'exprimer dans les cellules de betteraves mais la coloration du test GUS est envahissante. La coloration rouge caractéristique de l'expression du gène *C1/Lc* du maïs n'a jamais été observée chez la betterave sucrière. Un gène synthétique (*sgfp-S65T*) de méduse conférant une fluorescence verte s'est exprimé à un niveau très élevé dans la betterave sucrière et, sa coloration n'étant pas envahissante, il a pu être utilisé pour contrôler l'expression génétique *in situ*.

Utilisant le gène *gus*A comme gène rapporteur, nous avons exploré différents facteurs limitants la transformation génétique via *Agrobacterium*. Le meilleur résultat a été obtenu à partir de SDM 10 (Lion Seeds, UK) mis en préculture dans un milieu complété en BAP et en TIBA (2,3,5-triiodo benzic acid). Environ 50% des explants ont montré une activité GUS avec 1 à 60 tachetures par explant (4-16 mm^2) issu de transformation avec EHA101 (pBECKS400). Les effets de l'utilisation de plasmides, contenant une partie du gène de la transcryptase reverse issu de la betterave sucrière *LINE* (rétrotransposon non-LTR) et de l'ADN répété de séquences satellites issues de betteraves sucrières ou *B. procumbens*, ont été testés pour la fréquence des événements de transformation et le niveau d'expression des transgènes dans des plantes transgéniques stabilisées.

Des cellules et des plantes transgéniques de betteraves sucrières ont été obtenues après co-culture d'explants et de cals, pour être ensuite analysées par toute une gamme de technique. Afin d'améliorer la morphologie et l'anatomie des racines de betteraves sucrières stockées ainsi que d'obtenir une haute teneur en sucre avec une bonne pureté de jus et une faible tare terre, nous avons développé deux stratégies. La première stratégie consiste en la modification des niveaux de cytokinines endogènes par l'introduction du gène *ipt* d'*Agrobacterium tumefaciens*, et la seconde consiste en la modification de la cascade *cdc* dans les racines stockées.

ÜBERSICHT - VERBESSERTE VERFAHREN ZUR TRANSFORMATION VON ZUCKERRÜBE

Die Genmanipulation in der Zuckerrübe (*Beta vulgaris*) hat das Potential, die Ernteerträge zu verbessern, biotischen und abiotischen Streß zu mindern und die Höhe des Umwelteinflußes auf Wuchs und Ertrag zu verringern. Es sind verschiedene Verfahren für die Transformation von Zuckerrübe entwickelt worden. Wir haben verschiedene Aspekte, die Einfluß auf die Optimierung der Verfahren zur Transgenübertragung, der Rückgewinnung von Transformanten und der Expressionsregulierung der Fremdgene in den transformierten Pflanzen haben, betrachtet.

Zuckerrübe kann über die Organogenese direkt von Pflanzenteilen oder mit einer zwischenzeitlichen Kallusphase regeneriert werden. Somatische Embryonen werden mit unterschiedlicher Frequenz von Sämlingen verschiedener Genotypen auf MS oder PGo Medium, die verschiedene Konzentrationenan BAP (N-6-benzylaminopurine) oder TDZ (thidiazuron) enthalten, induziert. Die Embryonen werden durch das Anlegen von Subkulturen, das mit mechanischer Verletzung gekoppelt ist, vervielfältigt.

Für die Erkennung früher und stabiler Transformationsereignisse wurden verschiedene Markergene getestet. Das *gus*A Gen von *E. Coli* kann in Zuckerrübenzellen exprimiert werden, der GUS-Test ist jedoch invasiv und zerstört das Gewebe. Die Expression des *C1/Lc* Genes von Mais, die zu einer Rotfärbung führt, konnte in Zuckerrübe nicht beobachtet werden. Das synthetische Gen (*sgfp-S65T*) der Qualle für grüne Fluoreszenz wird in Zuckerrübe stark exprimiert und wurde für die *in situ* (nicht invasiv) Beobachtung der Genexpression genutzt.

Es wurden Faktoren, die die Effizienz der *Agrobakterium*-vermittelten Transformation beeinflußen, untersucht, wobei das *gus*A Gen als Reportergen genutzt wurde. Die besten Ergebnisse konnten mit der Linie SDM 10 (Lion Seeds, UK), vorkultiviert in mit BAP und TIBA (2,3,5-triiodo benzic acid) angereichertem Medium, erzielt. Etwa 50% des Materials, das mit dem *Agrobakterium*-Stamm EHA101 (pBECKS400) transformiert wurde, zeigte GUS-Aktivität mit 1 bis 60 blauen Punkten pro Gewebeteil (4-6 mm^2). Verschiedene Plasmide, die repetitive DNA-Elemente enthalten, wurden konstruiert. So wurden ein Teil des Reverse Transkriptase-Genes eines Zuckerrüben-*LINEs* (non-LTR-Retrotransposon) und Satelliten-DNA-Repeats von Zuckerrübe bzw. *B. procumbens* eingeführt und deren Effekt auf die Transformationsfrequenz und Transgenexpression in stabilen Transformanten untersucht.

Transgene Zuckerrübenzellkulturen und Pflanzen konnten von verschieden Ausgangsmaterialien regeneriert werden. Die transgenen Pflanzen wurden mit verschieden Methoden untersucht. Momentan führen wir Promotoren von Genen des Zellzyklus ein, um die Zellteilungskompetenz und Zellteilungsaktivität zu testen. Weiterhinwerden Gene der Cytokininsynthese auf ihr Potential, die Morphologie und Anatomie der Zuckerrübenwurzel zu optimieren, getestet.

INTRODUCTION

Sugar beet production is greatly affected by agricultural policies worldwide. In order to increase its competitiveness, it is necessary to achieve further improvements of crop productivity with reduced inputs and a lower environmental impact of crop growth. The sugar yield of current varieties developed by conventional breeding appears to have reached a plateau. Traditional breeding also confronts problems dealing with pest and disease resistance and stress tolerance. Transgenic technology offers the prospect of solving these problems by introducing genes from a wide range of organisms.

Sugar beet is recalcitrant to *in vitro* culture and genetic transformation (TETU *et al*, 1987; D'HALLUIN *et al*, 1992). Transgenic sugar beets were obtained by *Agrobacterium*-mediated transformation (D'HALLUIN *et al*, 1992; ELLIOTT *et al* 1992; KRENS *et al*, 1996) and PEG-mediated guard cell protoplast transformation (HALL *et al*, 1996). Such experiments are sometimes difficult to repeat and the techniques are difficult to transfer to other laboratories. There are problems related to transgene position effects, multiple integration and frequent gene silencing in transgenic plants. We have considered several aspects of the procedures for transformation so as to achieve efficient protocols for the delivery of foreign genes, recovery of transformants and regulation of expression of the foreign genes in transgenic plants.

MATERIALS AND METHODS

PLANT MATERIALS: Sugar beet breeding lines SDM 1-11 (supplied by Lion seeds, UK), HB 526 (from Hilleshog AB, Sweden) and CMS 22003 were used in this investigation. *In vitro* cultures were established by inoculating seeds on MS or PGo medium containing BAP, thidiazuron, TIBA and NAA alone or in combination (ELLIOTT *et al*, 1992). Somatic embryogenic, organogenic as well as non-morphogenic calli were induced from seedlings, cotyledons, hypocotyls and leaves by culturing them on medium supplemented with various combinations of phytohormones. The frequencies of shoot formation from explants or calli were calculated as the number of responsive explants or calli during the 6 weeks of culture divided by the total number used.

AGROBACTERIUM STRAINS AND PLASMIDS: *Agrobacterium* strains EHA101 and LBA4404 which carry the binary vectors pBECKS400 (McCORMAC *et al*, 1997) or pTOK233 (HIEI *et al*, 1994) were used. These vectors contain the *gus*A gene, *npt*II and *bar* or *hyg*ʳ genes. Additional plasmids were constructed by replacing the *gus*A gene with either m*gfp*, s*gfp*S65T (CHIU *et al*, 1996) or *C1/Lc* genes. Other plasmids have also been made by inserting a sugar beet genomic DNA fragment encoding either part of reverse transcriptase gene of a *LINE* retrotransposon (SCHMIDT *et al*, 1995) or the repetitive DNA from sugar beet and *B. procumbens* (SCHMIDT *et al* 1991, 1996).

PREPARATION OF *AGROBACTERIUM* CULTURES, INOCULATION AND COCULTIVATION WITH PLANT MATERIALS AND SELECTION OF TRANSFORMANTS: *Agrobacterium tumefaciens* was cultured on solid LB medium containing 100-200 mg/L spectinomycin or 50 mg/L hygromycin. *Agrobacterium* suspension cultures were established by inoculating the bacteria cells into liquid LB or AB into medium containing the appropriate antibiotics. Sugar beet cells or tissues were inoculated with *Agrobacterium* and cocultivated for 2-3 days. The cocultivated

materials were transferred to medium supplemented with geneticin, kanamycin or PPT for selection of stable transformants and cefotaxime to prevent growth of bacteria.

ANALYSIS OF TRANSIENT AND STABLE TRANSGENE EXPRESSION:

GUS assay: After 10 days the cocultivated materials and putative transformants were sampled for histochemical GUS assay. The assays were performed according to a modified protocol of Jefferson (1987) by addition of 20% (v/v) methanol. Materials were submerged in GUS solution containing X-Gluc and incubated overnight at 37°C. Leaf segments were washed twice in 99% ethanol for 2 h, then examined for staining. The transformation frequencies based on transient *gus*A gene expression were calculated as the number of explants showing colour, divided by the total number of explants analyzed.

GFP observation: The expression of GFP was observed after cocultivation and during selection. GFP activity was visualized using a Nikon epi-fluorescence microscope with filter 3-3A, providing excitation wave lengths 420-490nm; photographs were taken with Kodak Elite 400ASA film using exposure times of 5-20 seconds.

PCR and Southern blotting: PCR analysis and Southern blot hybridization using *npt*II, *gus*A and *bar* coding sequences as primers and probes were done on the primary transformants to confirm integration of the genes. The transformation frequencies were based on the total number of transgenic shoots or plants dividing by the total number of explants or callus tissues cocultivated with *Agrobacterium*.

Herbicide resistance tests: Transgenic plants containing the *bar* gene were identified by the glufosinate paint assay. A filter paper containing 1mg/ml of glufosinate solution was attached to a leaf with a clip. Ten days after the application, the filter paper was removed and the leaf area under the filter paper was observed. Leaves of plants without the *bar* gene showed chlorosis or necrosis under the filter paper. Transgenic plants were resistant or showed a mild toxic reaction.

RESULTS AND DISCUSSION

REGENERATION OF SUGAR BEET PLANTS THROUGH *IN VITRO* CULTURE

Sugar beet has been regenerated through organogenesis directly from explants or *via* an intervening callus phase. Direct shoot formation from leaf, petiole and shoot base segments was obtained on BAP or TDZ contained media. This procedure was less genotype dependent and the frequencies varied from 50-80%. Regeneration from calli induced on cotyledon, hypocotyl and shoot base segments was dependent on genotype and combinations of phytohormones. SDM 10 and 3 showed better responses on medium containing BAP and NAA or TIBA than SDM 11 and 9.

Somatic embryos were induced from seedlings of several genotypes on MS or PGo medium containing a range of concentrations of BAP (N-6-benzylaminopurine) or TDZ (thidiazuron). There were significant differences between genotypes. HB 526 and SDM 3 performed better than SDM 9 and 10. The embryos were multiplied during subcultures which involved mechanical wounding. The multiplied embryos were used in transformation experiments.

COMPARISON OF REPORTER GENES IN SUGAR BEET

Several marker genes were tested for the detection of early and stable transformation events. The *E.coli gus*A gene can be expressed in sugar beet cells but the GUS assay is invasive. The red coloration which indicates that the maize *C1/Lc* genes have been expressed and that the relevant biosynthetic pathway is functional, was never observed in sugar beet. A synthetic jellyfish green fluorescent protein gene (*sgfp-S65T*) was expressed at a high level in sugar beet and was used to non-invasively monitor gene expression *in situ*.

Calli of SDM 3 and 10 were cocultivated with EHA101 (pBECKS. s*gfp*). Stable transformed calli which showed green fluorescence at a range of densities were obtained at frequencies of 5.3% and 11.1% respectively on selection medium. Shoots which expressed the *sgfp* gene were also obtained from cocultivated shoot explants and derived calli of 22003 and SDM 3 at frequencies of 3.1% and 3.9% respectively.

OPTIMIZATION OF TRANSFORMATION PROCEDURES

Using the *gus*A gene as a marker, factors affecting the efficiency of *Agrobacterium*-mediated transformation were investigated. Among the genotypes tested SDM 10 and SDM 3 gave the highest transformation frequencies. Leaf blade tissue was more readily transformed than petiole tissue. Highest transformation frequencies were obtained when explants were derived from shoots cultured on cytokinin (BAP) and antiauxin (TIBA)-containing medium compared to those from medium containing cytokinin alone or hormone-free medium. The *Agrobacterium* strain and concentration also affected transformation frequency. On the basis of *gus*A expression in the cocultivated explants, EHA101 (pBECKS400) showed the highest transformation frequency followed by LBA4404 (pTOK233), then LBA4404 (pBECKS400). Nearly 50% of explants of SDM 10 showed GUS activity with 1 to 60 blue spots per explant (size 4-16 mm^2) transformed with EHA101 (pBECKS400).

EFFECTS OF DNA SEGMENTS OF *BETA* ON TRANSFORMATION AND TRANSGENE EXPRESSION

Part of the reverse transcriptase gene of a sugar beet *LINE* (non-LTR retrotransposon) sequence and satellite DNA repeats of sugar beet or *B. procumbens* were constructed into the plant expression vector pBECKS400. The effects of these plasmids on transformation frequency and transgene expression in stable transformants were compared. Preliminary results showed no significant difference in transformation efficiency. Cell lines transformed with the partial *LINE* sequence all showed more GUS activity than the corresponding controls.

ANALYSIS OF TRANSGENIC SUGAR BEET

Putatively transformed shoots have been obtained on selection medium containing kanamycin, geneticin or PPT singly or in combinations, from cocultivated shoot segments of SDM 3 and SDM 10 and embryogenic callus of SDM 2, 3 and CMS 22003. Some of the shoots have already developed to whole plants and have been established in soil in a growth room. The presence and expression of the transgenes in

transformed calli and transgenic plants was confirmed using a histochemical GUS assay, observation of GFP activity, the polymerase chain reaction assay and Southern blot hybridization. Transformation frequency averaged 3% but varied from 0 to 9% in a range of experiments.

Two SDM 2 and one SDM 3 transgenic *bar* plants were subjected to a herbicide resistance test. The transgenic plants were normal or showed mild toxicity response while plants without the *bar* gene showed chlorosis or necrosis after application of glufosinate.

CONCLUSIONS AND FUTURE WORK

Sugar beet plants have been regenerated *in vitro* through a range of pathways. These techniques have enabled us to obtain transgenic sugar beets *via Agrobacterium*-mediated genetic transformation. The effect of DNA segments of *Beta* on transgene integration and gene expression needs to be further investigated with attempts to reduce position effects and gene silencing. The procedure developed here will facilitate development of high yield/low environmental impact/stress tolerant/pest and disease resistant sugar beet.

Soil tare is a great burden to sugar beet growers and processors. The manipulation of sugar beet storage organ development will lead to reduce soil tare and increase productivity (ELLIOTT *et al*, 1993; 1996). We are using two strategies for optimizing the morphology and anatomy of sugar beet storage roots so as to achieve a high sucrose/high juice purity/low soil tare crop. The first strategy involves the manipulation of storage root cytokinin levels by deployment of the *A. tumefaciens ipt* gene so as to enhance the cytokinin level in the outer zone of the storage root where inactive cambia are localised. The hormone profile will then favour division of the cells and production of new zones of storage tissue (ELLIOTT *et al*, 1996). The alternative strategy exploits recent advances in understanding of the regulatory cascade of cell division cycle (*cdc*) control genes. Several key genes have been isolated from beet tissue (FOWLER *et al*, in press) and approaches which will accelerate steps which are currently rate-limiting for division are being developed.

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