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Trehalose-6-phosphate synthase as an intrinsic selection marker for plant transformation

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Abstract

Insertion of foreign DNA into plant genomes occurs randomly and with low frequency. Hence, a selectable marker is generally required to identify transgenic plants. Until now, all selection systems have been based on the use of non-plant genes, derived from microorganisms and usually conferring antibiotic or herbicide resistance. The use of microorganism-derived genes however has raised biosafety concerns. We have developed a novel selection system based on enhancing the expression of a plant-intrinsic gene and the use of a harmless selection agent. Selection takes advantage of the reduced glucose sensitivity of seedlings with enhanced expression of *AtTPS1*, a gene encoding trehalose-6-P synthase. As a result, transformants can be identified as developing green seedlings amongst the background of small, pale non-transformed plantlets on high glucose medium. In addition, vegetative regeneration of tobacco leaf explants is very sensitive to high external glucose. Overexpression of *AtTPS1* in tobacco allows selecting glucose insensitive transgenic shoots.
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Keywords: Plant transformation; Intrinsic selection marker; *AtTPS1*; Trehalose-6-phosphate synthase; Glucose resistance; *Arabidopsis thaliana*; *Nicotiana tabacum*

1. Introduction

The selection systems for plant transformation that are currently most widespread make use of dominant marker genes conferring resistance against herbicides or antibiotics. Ecological concerns as well as worries over food safety have been raised with

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respect to the use of these resistance genes as selection markers in plant transformation (WHO, 1993). Consequently, alternative selection systems have recently been developed which no longer result in the presence of resistance genes in transgenic plants (for a review see Miki and McHugh, 2004). The first strategy has employed markers, which allow the selection of transgenic plants under innocuous conditions. However, all these marker genes have been derived from microorganisms. Introduction of such heterologous genes into foodstuffs has met with opposition from the general public due to ethical and/or biosafety concerns. The second strategy has been to remove the resistance markers from the transgenic plants after their use. Methods such as co-transformation and subsequent crossing out of the marker, use of a Cre/Lox system to excise the marker gene after transformation and a transposase driven removal of the marker gene all require genetic segregation. For various crops, it would require many years of breeding to find the proper genotype (Ebinuma et al., 1997; Corneille et al., 2001; Hare and Chua, 2002; Breitler et al., 2004; Erikson et al., 2004; Miki and McHugh, 2004). In addition, concern over the complete removal of the markers has hindered swift acceptance of these methods. Transformation without any selectable marker has been successfully applied in the case of potato plants (de Vetten et al., 2003). However, the selection procedure consists of many PCR reactions and is very laborious especially when it has to be applied to other crop plants where the transformation efficiency is generally lower than 1%.

In order to develop a purely plant based and environmentally friendly selection system, we have explored the usefulness of the trehalose-6-P synthase gene *AtTPS1*. Trehalose is a non-reducing disaccharide that is abundantly present as stress protectant in microorganisms and some desert plants such as *Selaginella lepidophilla*. In higher plants such as *Arabidopsis thaliana* however, trehalose is only detected in small quantities (Vogel et al., 2001). In the yeast *Saccharomyces cerevisiae*, trehalose is synthesized in two reactions from UDPglucose and glucose-6-phosphate by trehalose-6-phosphate (T6P) synthase (Tps1) and T6P phosphatase (Tps2). Deletion of *TPS1* eliminates growth on glucose because both Tps1 and T6P are indispensable for the regulation of glucose influx into glycolysis (Thevelein and Hohmann, 1995). The Arabidopsis genome encodes 11 *TPS1* homologues that fall into 2 subfamilies displaying most similarity either to yeast *TPS1* (Class I, *AtTPS1–4*) or *TPS2* (Class II, *AtTPS5–11*) (Leyman et al., 2001). *AtTPS1* complements the yeast *tps1*Δ strain for its growth defect on glucose, which suggests a possible regulatory role for *AtTPS1* in plant carbon metabolism (Blazquez et al., 1998; Van Dijck et al., 2002). Indeed, the *Attps1-1* knockout mutant is embryo lethal because *AtTPS1* appears to be essential in the regulation of sugar metabolism during embryo development (Eastmond et al., 2002). Sugar homeostasis is tightly regulated in plants. Plantlets germinated in the presence of high concentrations of glucose remain white and petite because the external sugar switches off the photosynthetic machinery. Previously, we showed that *AtTPS1* plays a regulatory role in this phenomenon, as ectopic expression of *AtTPS1* in Arabidopsis renders plants significantly less sensitive to glucose compared to wild type plants (Avonce et al., 2004). Based on this trait, we exploited *AtTPS1* as selection marker for plant transformation in combination with glucose as selection agent.

2. Materials and methods

2.1. Constructs

The pBIN*AtTPS1* construct is a pBIN19 derivative and was prepared as described in Avonce et al., (2004). The pTPSM construct is created by inserting the *AtTPS1* ORF in a pBIN19 derivative without the *NPTII* gene. The pBIN-35S-nos construct was digested with the restriction enzymes *Nhe*I and *Apa*I, sticky ends were made blunt using the T4 DNA polymerase and the vector recircularized with T4 DNA ligase resulting in p35S-nos. *AtTPS1* was ligated in this empty vector between the *Kpn*I–*Xba*I endonuclease sites. This resulted in the pTPSM construct, free of any classical marker. The vector pTPSMGW is a pTPSM derivative. pTPSM was linearized with the *Nhe*I restriction site and the ends were made blunt with the T4 DNA polymerase. The gateway cassette (Invitrogen) was ligated into the pTPSM vector to create the pTPSMGW vector. The vectors were transformed via electroporation into *Agrobacterium tumefaciens* strain C58C1 for Arabidopsis transformation or LBA4404 for Nicotiana transformation.

2.2. Plant transformation and growth conditions

Arabidopsis *AtTPS1* overexpressing lines were obtained using a *35S*::*AtTPS1* cassette that was inserted into the Arabidopsis genome via *Agrobacterium* transformation using pBIN*AtTPS1* (Avonce et al., 2004). *Arabidopsis thaliana* ecotype Columbia and Landsberg plants were incubated in water at 4 °C for 3 days under constant light for stratification. Plants were germinated and grown on moisturized soil (Osmocote, Asef) at 22 °C/day and 18 °C/night with a photoperiod of 12 h/12 h. After 4 weeks, inflorescences were clipped and 4 days later plants were transformed by the "flower dip" method with some modifications (Clough and Bent, 1998). *Nicotiana tabacum* overexpressing *AtTPS1* lines were obtained from O. Goddijn (Leiden, The Netherlands). Seeds of *N. tabacum* SR1 were surface sterilized by vapor and germinated on Murashige and Skoog (MS) medium supplemented with vitamins and 1% sucrose and solidified with 0.8% phytagar (Murashige and Skoog, 1962). For transformation, leaf disks of 1 cm^2 were co-cultivated with *Agrobacterium* strains and regenerated (see below).

2.3. Glucose sensitivity and selection of transformants

To test Arabidopsis and Nicotiana for glucose sensitivity, seeds were surface sterilized and after stratification, germinated on MS medium as above, supplemented by either 1% sucrose (control conditions) or 6% glucose. Glucose sensitive phenotype was noted 6–10 days after imbibition.

In a similar way Arabidopsis transgenic lines were selected after flower dip transformation. Seeds were collected and dried, and germinated on 6% glucose containing MS medium. Six days later the seedlings were scored for insensitivity to glucose. To test gene expression during time course experiments, sterilized seeds were grown in liquid culture (0.5× MS, 0.25% glucose) for 10 days. A 2% glucose or 2% sorbitol were added for 1, 3 and 6 h. Regeneration of *N. tabacum* leaf cells occurred on two subsequent MS based media. The first regeneration MS medium contains 2% sucrose, 0.2 mg/l NAA and 2 mg/l BAP and 100 mg/l augmentin (control) or 5% glucose (or 7% glucose with 0.05 mg/l IAA as indicated in the text) 2 mg/l BAP and 100 mg/l augmentin. Calli and shoots were grown in 12 h/12 h dark/light period at 22/18 °C, the calli were transferred to the second root inducing MS medium containing 1% sucrose without addition of hormones. When roots became visible, the explants were moved to soil.

2.4. DNA analysis and quantitative PCR

DNA was prepared from leaf discs of Arabidopsis and tobacco plants and PCR primers to test the presence of the *35S-AtTPS1-nos* cassette in the genome were the following: *35S* forward primer: aagaagacgttccaaccacg; *AtTPS1* reverse primer: cgctcagaacaactatggtt. Seeds were germinated in liquid MS medium (1/2 strength) supplemented with 0.25% sucrose at 24 h light. After 9 days, seedlings were put in the dark for 8 h. On day 10, in 1 h light, 2% glucose (final concentration) was added. Seedlings were harvested at 1, 3 and 6 h after glucose induction. Total RNA was extracted from Arabidopsis seedlings and after DNAse treatment (Gibco BRL), cDNA was synthesized using M-MLV reverse transcriptase (Promega). QPCR was carried out using an ABI-prism 7700 Sequence Detection System (SDS, Applied Biosystems) as prescribed by the manufacturer. UBQ4 was used as housekeeping reference, WT before addition of glucose was used as calibrator. The following primers and probes were used. *UBQ4*: forward primer: gcttctgagcttttgtgatgtgat; reverse primer: gaaaccaaaccaggtgaagatctc; probe: FAM-tgttcgagtctcatgcac-TAMRA. *AtCAB1*: forward primer: gccaaagggcccatcag-3′; reverse primer: tcggtgattcgccagagaa; probe: FAM-tgaccgtgtcaagtact-TAMRA.

3. Results and discussion

We constructed a new transformation vector containing the *AtTPS1* gene as a selection marker. For this purpose, we first prepared an empty vector by deleting the *NPTII* gene out of a pBIN19 derivative that contains the CaMV 35S promoter and the nos terminator resulting in the p35S-nos construct (Fig. 1). In order to increase the expression level of the *AtTPS1* gene in Arabidopsis, the ORF was inserted in a pBIN19 vector (pBINAtTPS1) (Avonce et al., 2004) and into the empty p35S-nos vector between the 35S promoter and the nos terminator (pTPSM, Tre-6-P Synthase as Marker) (Fig. 1). In this pTPSM construct the *AtTPS1* ORF is

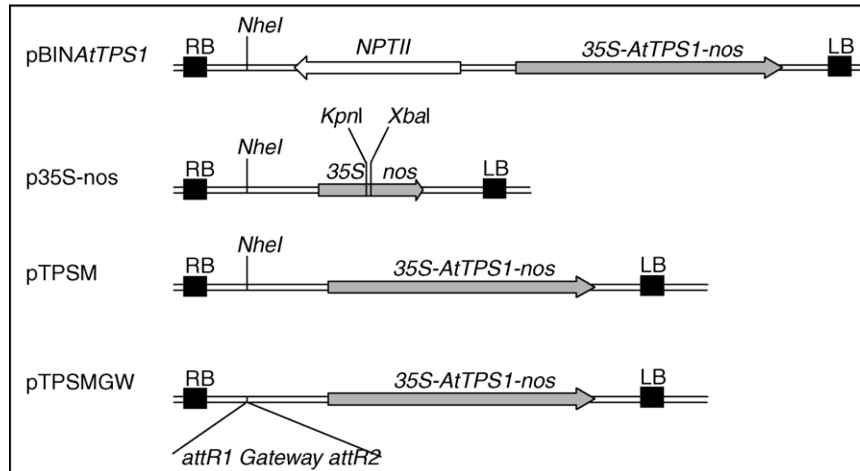

Fig. 1. Map of the T-DNA regions of different pBIN1 9 derived constructs.

the only coding sequence in the T-DNA region. This construct can be used to ligate a second gene of interest into the unique *Nhe*I restriction site. To facilitate gene incorporation and for high throughput purposes, we inserted a gateway cassette into this *Nhe*I site, to obtain the pTPSMGW construct (Fig. 1).

Stable *35S*::*AtTPS1* transgenic Arabidopsis plants were generated using the *pBINAtTPS1* construct. Those plants were tested for their glucose sensitivity. Increased levels of sugar in the growth medium causes stunted growth of roots and hypocotyl and inhibition of photosynthesis and greening combined with accumulation of anthocyanin in cotyledons of wild type seedlings (Sheen et al., 1999; Rolland et al., 2001, 2002). At a molecular level, expression of the CAB genes, which encode the chlorophyll a/b binding proteins and which are indispensable for the operation of the photosynthesis apparatus, is reduced in response to glucose. A transgenic Arabidopsis line strongly overexpressing *AtTPS1* germinated on high external glucose and displayed green, normal sized plantlets similar to the ones germinated in the absence of glucose (Fig. 2a). Glucose insensitivity by overexpression of *AtTPS1* was confirmed in different transgenic lines (Avonce et al., 2004). We underscored this phenotype at the molecular level by measuring the transcription

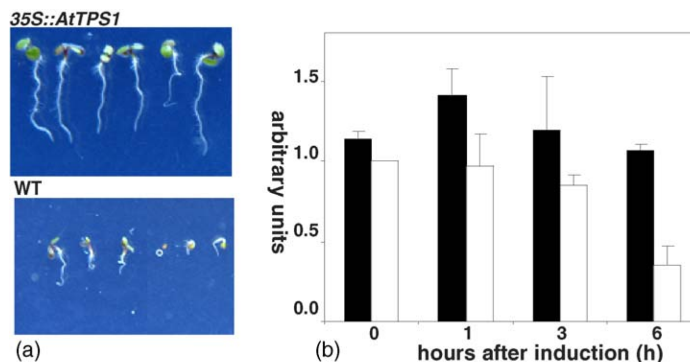

Fig. 2. Arabidopsis overexpressing plants *35S*::*AtTPS1* are less sensitive to glucose than wild type: (a) 5-day old Arabidopsis wild type (WT) and *35S*::*AtTPS1* transgenic seedlings grown on 6% glucose; (b) normalized QPCR expression analysis of *35S*::*AtTPS1* (black bars) and WT (white bars) plantlets grown in liquid medium. *CAB1* expression was measured 1, 3 and 6 h after the addition of 2% glucose and normalized to expression after addition of 2% sorbitol for the same time period.

level of the *CAB1* gene using QPCR. Wild type and *35S*::*AtTPS1* seedlings were grown for 10 days in liquid MS medium in constant light. A 2% glucose was added and seedlings were collected at various time points after glucose induction. In wild type plants, *CAB1* expression remained the same 1 and 3 h after glucose addition, but it was severely reduced after 6 h. In *35S*::*AtTPS1* transgenic seedlings, *CAB1* transcript levels were already higher in the control condition before adding glucose. Moreover, no glucose-induced reduction was observed in *CAB1* expression after 6 h (Fig. 2b). These results confirm the regulatory connection between trehalose metabolism and glucose regulated gene expression.

We used this characteristic of *AtTPS1* to develop a plant derived selection marker, which we first tested in Arabidopsis transformation. Plants were transformed using the flower dip method with the *A. tumefaciens* strain C58C1(pBIN*AtTPS1*). Seeds were collected and dried, and transgenic lines were selected on medium containing 6% glucose. To further enhance the difference in appearance between wild type and *35S*::*AtTPS1* transgenic seedlings, the plants were germinated in 24 h light. Six days after imbibition, the few green, normal sized plantlets were selected among the large majority of small, colourless plants. PCR analysis of the genomic DNA of the selected seedlings confirmed the presence of *35S*::*AtTPS1* using specific *35S* forward and *AtTPS1* reverse primers. PCR results from different experiments showed that in average 80% of the selected seedlings contained the *35S*::*AtTPS1* construct in their genome (Fig. 3a). Sequence analysis of the purified PCR bands confirmed the correct amplification of the *35S*::*AtTPS1* fragment (data not shown). This strong enrichment for transgenic plants is sufficient to easily isolate a transgenic line. Integration of the T-DNA into the Arabidopsis genome using the flower dip method occurred with a frequency of approximately 1%. For four independent lines the *35S*::*AtTPS1* construct was stably expressed over three generations. We compared the general appearance of the *35S*::*AtTPS1* transgenic Arabidopsis plants with wild type plants. Thorough visual analysis revealed no morphological differences during vegetative growth under normal conditions (Fig. 3b). Detailed analysis of the growth rate also revealed no difference between wild type Arabidopsis and several independent *35S*::*AtTPS1* lines. During the vegetative stage, leave numbers and sizes were measured. The transition to generative stage was evaluated by the time of flowering and the number and composition of the flowers. For all these morphological traits transgenic plants and wild type scored identical (Fig. 3c). To assess yield performance, we analysed the weight of the seeds produced by individually grown transgenic or wild type plants ($n \geq 10$). In addition, the average mass of 1000 seeds was determined. Seed numbers as well as seed weight were of comparable levels between transgenic plants and wild type plants (data not shown). The only detectable physiological phenotype caused by *35S*::*AtTSP1* apart from glucose insensitivity is the ability to endure environmentally imposed drought conditions better than wild type plants (Avonce et al., 2004). This trait is generally beneficial and would therefore not impair the use of *AtTPS1* as a marker gene for plant transformation. These results demonstrate that the use of the *AtTPS1* gene as a marker will not have any negative effect on the growth and yield of economically important crop plants, a crucial aspect for agronomic performance.

The flower dip transformation procedure results in transgenic seeds and glucose sensitivity of seed germination in general is well established. Despite the simplicity of the flower dip transformation procedure, it has never been successfully used for other plants so far. The transformation procedures used for other plants result in vegetative transgenic shoots of which the sensitivity to glucose is not clear. Hence, we tested our *AtTPS1* selection system also in *N. tabacum*, a model plant for vegetative transformation. Initially, plants overexpressing *AtTPS1* were scored for glucose sensitivity during germination and during shoot regeneration from a leaf cell. Germinated Nicotiana seedlings are not as sensitive to external sugar as Arabidopsis seedlings. They are also smaller in the presence of glucose but the leaves remain green. Overexpression of *AtTPS1* clearly reduced the sensitivity to glucose, as the seedlings remained larger than wild type seedlings when germinated on 6% glucose (Fig. 4a–d). This is not due to osmotic resistance as seedlings of both *35S*::*AtTPS1* and wild type lines grew at the same rate on 6% mannitol (data not shown).

We observed that regeneration of a new shoot from a leaf cell is very sensitive to external glucose. Adding 7% glucose to the medium kills wild type leaf discs in suboptimal auxin concentrations. However, when leaf discs of the *N. tabacum (35S*::*AtTPS1*) transgenic

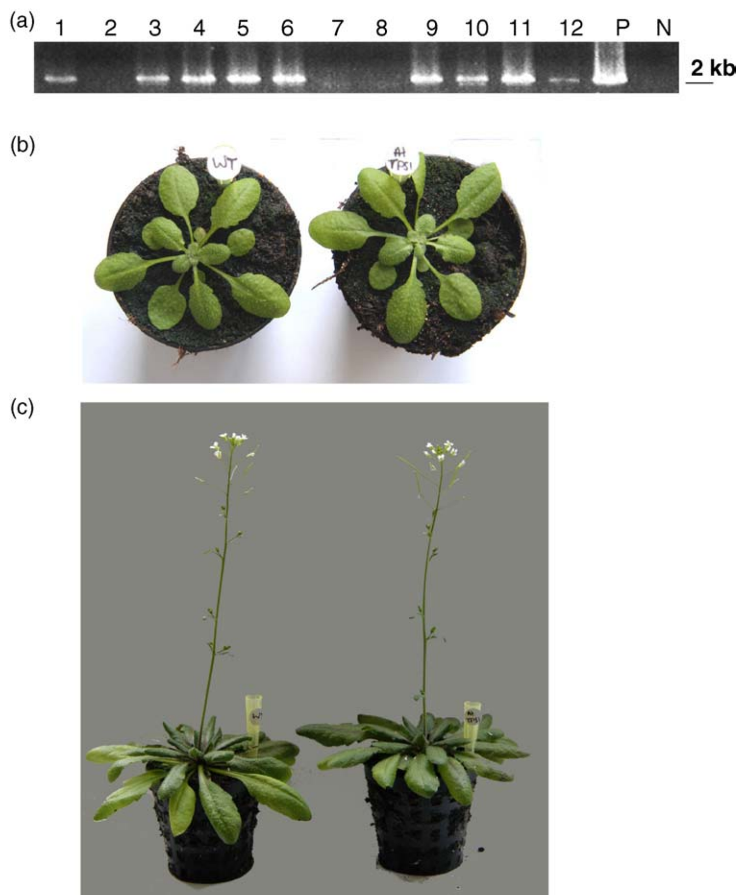

Fig. 3. Plant transformation with *35S*::*AtTPS1*: selection of transformants and morphological comparison of transgenic and wild type plants: (a) PCR analysis of *35S*::*AtTPS1*; genomic DNA was obtained of 12 selected seedlings (1–12) after flower dip transformation of Arabidopsis with *Agrobacterium* (pTPSM), in comparison with a stable *35S*::*AtTPS1* transgenic line as a positive control (P) and wild type plant as a negative control (N); ((b) and (c)) plant morphology during the vegetative (b) and generative (c) stage of wild type (left) and *35S*::*AtTPS1* transgenic (right) plants.

plants were cultivated with the same level of glucose, calli were formed and later shoots appeared that grew into plants upon shifting them to root inducing medium and eventually soil (Fig. 4e and f). We exploited this phenotype to generate transgenic shoots after vegetative transformation. Leaf discs of wild type plants were co-cultivated with the *Agrobacterium* strain LBA4404 containing the pTPSM or the pTPSMGW vector. Typically leaf discs were incubated on a MS medium containing 5% glucose without auxin. After 3 weeks, the emerging shoots were transferred to root inducing medium and plantlets were finally grown into soil (Fig. 5a and b). In contrast, transformation of tobacco explants with *Agrobacterium* containing the empty vector p35S-nos also generated calli, but no shoots (Fig. 5c). Genomic DNA isolated from the freshly generated plants after pTPSM transformation was checked by PCR for the presence of the *35S*::*AtTPS1* cassette. All 16 shoots except one contained the *35S*::*AtTPS1* insertion in the genome (Fig. 5d). Similar results with near to 100% selection efficiency were obtained using pTPSMGW as transformation vector (data not shown).

In conclusion, we have developed a novel intrinsic marker gene for plant transformation based on the observation that overexpression of the Arabidopsis *AtTPS1* gene strongly reduces the glucose sensitivity

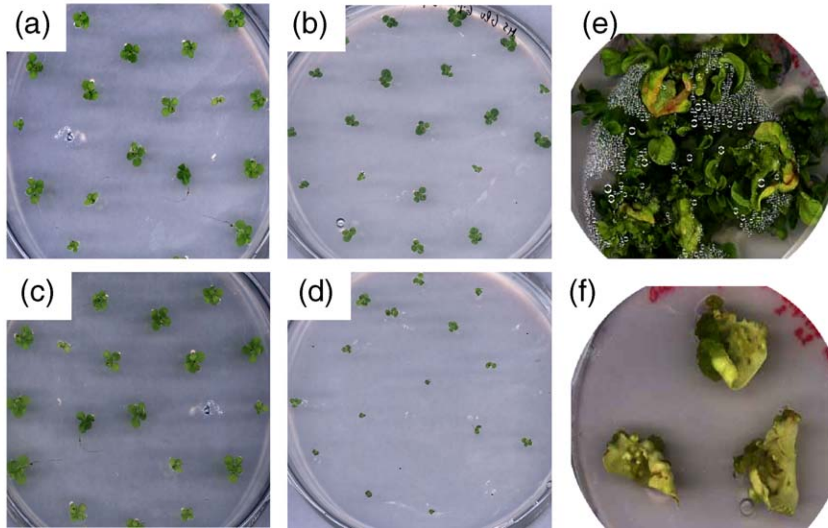

Fig. 4. *Nicotiana tabacum 35S*::*AtTPS1* is less sensitive to glucose containing medium than wild type plants. A 20-day old Nicotiana seedlings were grown on 1% sucrose ((a) and (c)) or 6% glucose ((b) and (d)). *35S*::*AtTPS1* transgenic lines ((a) and (b)) were compared with control plantlets ((c) and (d)). Explants of *35S*::*AtTPS1* transgenic lines (e) and wild type (f) were cultivated on 7% glucose with 0.05 mg/l IAA for 5 weeks at 24 h light.

of transgenic plants without causing any detectable negative side-effects. Increasing the transcript levels of *AtTPS1* makes the plant less sensitive to glucose at germination (Arabidopsis) or upon shoot regeneration from explants (Tobacco). This trait enabled us to distinguish plants that carry a transgene from non-transformed plants. We have shown that the selection protocol works very efficiently for the two most

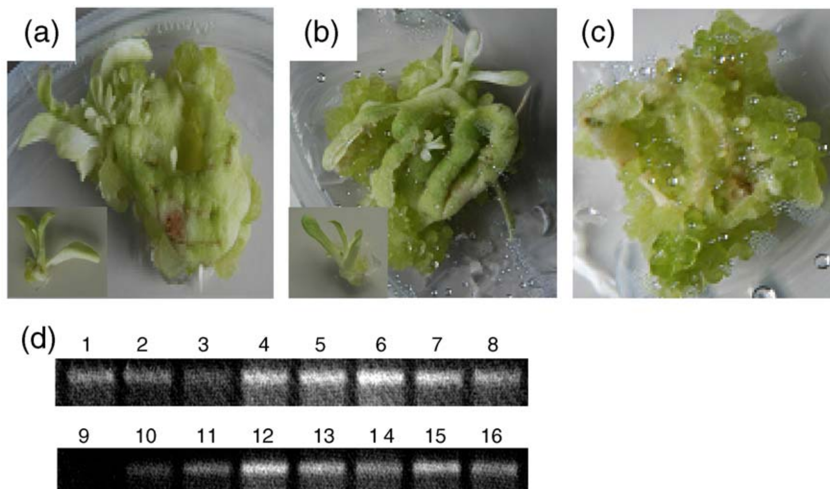

Fig. 5. Selection on glucose containing medium of tobacco shoots transformed with *35S*::*AtTPS1*: ((a)–(c)) callus and shoot generation after explant transformation of tobacco on 5% glucose without auxin, with pTPSM (a), pTPSMGW (b), and p35S-nos (c). Insets of G and H represent detached shoots. (d) PCR analysis of *35S*::*AtTPS1* in genomic DNA of 16 independent regenerated tobacco plants after transformation with pTPSM (positive and negative control as in Fig. 3a are not shown).

routinely used plant transformation procedures: the flower dip method in the model plant Arabidopsis and the vegetative transformation, which is used in tobacco. Many other crop plant transformation systems are based on Agrobacterium T-DNA transfer and experimental transformation protocols pursue regeneration of explants in a comparable method as tobacco. Hence, this selection system has a broad potential application in many crop plants. The *AtTPS1* selection method has major advantages in comparison to existing methods. Our method maintains the simplicity of a positive marker. One can screen for transgenic plants in the T1 generation. The method does not require complex procedures unlike marker-free transformation methods. And, no foreign gene needs to be inserted into the plant genome. Moreover, glucose is used as selection agent, which is non-toxic in the environment or to human beings. Hence, the *AtTPS1* transformation procedure can facilitate acceptance of genetically modified organisms by meeting the requirements of the WHO: "European legislation requires the phasing out of GM crops containing selectable markers conferring resistance to clinically used antibiotics by 2004" (WHO, 1993). On top of that, orthologues of the *AtTPS1* gene have been found in all completely sequenced plant genomes and in DNA from many other plants (Table 1). Hence, in all likelihood all plants contain an *AtTPS1* homologue. Combined with the general nature of glucose sensitivity of germinating and regenerating plants, this suggests that a species-specific *TPS1* plant transformation procedure could be applied universally.

Table 1
TPS1 orthologues in plants

Plant	Common name	EST/(patent no.)
A. thaliana	Tailcress	AC007260
Beta vulgaris	Sugar beet	AW777173
Saccharum sp.	Sugar cane	BU925699
Phaseolus coccineus	Scarlet runner bean	CA896670
Avicennia marina	Grey mangrove	CD777236
N. tabacum	Tobacco	(US20040093641)
Solanum tuberosum	Potato	BE340187
Lotus japonicus	Lotus	AV418750
Lycopersicon esculentum	Tomato	AI489004
Lycopersicon pennellii	Wild tomato	AW618571
Lycopersicon hirsutum	Wild tomato	AW616759
Malus x domestica	Apple	CO066804
Glycine max	Soybean	AI900187
Gossypium	Cotton	AF056946
Helianthus annuus	Sunflower	(US20040093641)
Zea Mays	Maize	AI855055
Oryza sativa	Rice	AU065233
Triticum aestivum	Wheat	BE402382
Hordeum vulgare	Barley	BE412694
Sorghum bicolor	Sorghum	AW286805
Medicago truncatula	Barrel medic	BE317084
Mesembryabthemum crystallinum	Common iceplant	BE033837
Thellungiella halophila	Salt cress	BQ060382
Dunaliella salina	Green algue	AAU00988
Prunus dulcis	Almond	BQ641089
S. lepidophilla	Rose of jericho	U96736

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