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Apoplastic expression and functional role of *Saccharomyces cerevisiae* yeast invertase in transgenic potato plants

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ABSTRACT

Apoplastic invertase is the key enzyme of carbohydrate metabolism in plants. The application of transgenic plants, which possess genes of heterologous organisms, expands understanding of the functional role of products of these genes. The purpose of this study was to characterise the invertase of S. cerevisiae from transgenic potato plants (Solanum tuberosum L., cv. Désirée), which expressed the suc2 gene of yeast under the control of the patatin class I B33 promoter. The suc2 gene presence in the plant genome and its expression were shown using PCR and RT-PCR. Yeast invertases were identified by MALDI-TOF MS analysis. Our results demonstrated the transit of the mature protein of the yeast invertase to the apoplast. A soluble form of the yeast invertase was present in the apoplast, and it was weakly adsorbed onto the cellular wall. Glucose and fructose levels in the apoplastic fluid of the transgenic plants increased by 40-55% as compared to those in the wild-type potato plants. Increased acid invertase activity in the transgenic plant leaves was observed as the result of the suc2 gene expression. Moreover, the increased total sugar contents in the transgenic plants led to some changes in their morphometric parameters (decreased offshoot length, less developed root system, and larger water content in tissues). The potato line with the integrated suc2 gene is a convenient tool for studying the role of the apoplastic invertase and the products of its activity in plant growth regulation and adaptation to unfavorable environment (in particular, to hypothermia).

Keywords: *Solanum tuberosum*, *B33* promoter, apoplast, invertase, *suc2* gene.

INTRODUCTION

The expression of heterologous genes in plants is an effective method to improve our understanding of plant resistance mechanisms [1]. Transformed plant lines were previously used to investigate the role of carbohydrate metabolism in the development of cold resistance in potato plants [2-6]. Of particular interest is a potato line whose carbohydrate metabolism is altered by the integration of the target *suc2* gene. This gene encodes the extracellular *Saccharomyces cerevisiae* yeast invertase (β-fructofuranosidase, E.C. 3.2.1.26) under the control of the patatin class I promoter (*B33* promoter). Since class I patatin is the main storage protein of potato tubers, the *B33* promoter mainly provides tuber-specific gene expression of the controlled *suc2* gene [7]. Gene expression regulation occurs chiefly at the transcriptional level where the regulatory region (promoter) plays a major role. Hence, we investigated the activity of the patatin *B33* promoter in potato plant roots, stems and leaves. The results confirmed the high tissue specificity of the *B33* promoter and revealed its limited activity in vegetative organs [2, 8].

In higher plants, an invertase has several isoforms, which differ in the subcellular localization and biochemical properties. Alkaline/neutral invertases are soluble proteins localized in the cytoplasm, mitochondria, and chloroplasts with optimum activity at pH 7.0-7.8 [9-11]. Acid invertases have an optimum activity at pH 4.5-5.0 and are localized as soluble proteins in the vacuole and apoplast [9, 12]. Importantly, invertases localized in the apoplast

are subdivided into soluble and insoluble; they are usually ionically bound to the cell wall [13]. Potato invertases localized in the apoplast are covalently bound to the cell wall [14]. Therefore, potato lines with the *suc2* gene expression offer unique opportunities for research since the integrated *suc2* gene encodes the yeast invertase with an *N*-end-connected potato proteinase II inhibitor signal peptide, which provides apoplastic localization of foreign invertase [15].

Apoplastic acid invertase is a key enzyme of carbohydrate metabolism involved in important physiological processes, including phloem unloading, cell differentiation control, sucrose level regulation in free cell spaces, and sucrose transport across the plasmalemma [12, 14]. Moreover, the potato plant apoplastic invertase coordinates donor-acceptor bonds between the main photosynthetic organs (leaves) and acceptor organs, i.e. tubers [16]. Thus, the potato line with the integrated *suc2* gene is a convenient tool to study the role of the apoplastic invertase and the products of its activity during tuber growth, development and formation.

Several researchers who worked with this potato line focused mainly on the following: i) functional activity of the invertase protein encoded by the *suc2* gene under heterologous expression, and ii) the ability of the protein to cause physiological and biochemical changes in the potato plant metabolism. It was found that potato plants expressing the yeast invertase gene had an increased activity of acid invertase. These plants also featured higher sugar content in tubers (specifically, higher glucose and fructose content) [17] and leaves (specifically, higher glucose and sucrose content) [2, 18] as compared to the control plants. The transformants were characterized by reduced sensitivity to hypothermia [2, 3] and oxidative stress induced by paraquat [19], and they reduced the threshold concentration of sucrose required for tuber initiation *in vitro* [20]. Moreover, unlike the control plants, the transformed plants formed a minimal number of tubers per one plant, but the tubers were heavier [15].

Our choice of the *suc2* gene from *S. cerevisiae* as a target gene was determined by two reasons. Firstly, the yeast invertase is foreign to the potato plants, and hence its activity is not inhibited by common plant inhibitors. Secondly, the yeast invertase has a wider pH range as compared with the plant invertase [21]. In order to prevent possible transgene expression termination after some period of time, the presence and expression of the target gene must be periodically monitored. For this purpose, we conducted appropriate long-term molecular-biochemical studies to identify and characterize some properties and functional features of the yeast invertase encoded by the *suc2* gene under heterologous expression in potato plants.

MATERIALS AND METHODS

Plant materials and growth conditions

The materials used in this study were potato plants (*Solanum tuberosum* L., cv. Désirée) (hereinafter referred to as wild-type plants) and the potato line transformed with a vector containing the *suc2* gene, under the control of the tuber-specific patatin *B33* promoter of class I (hereinafter referred to as transformed plants).

Potato plants were grown *in vitro* at 22 ^{o}C under a 16-h light/8-h dark regime and 100 µmol m^{-2} s^{-1} light intensity for 5 weeks on a Murashige-Skoog medium [22] containing 0.7% agar and 2% sucrose, pH 5.8. Leaves taken from the middle of the plants were used as the research materials.

Construction of the expression vector and plant transformation

The apoplastic invertase construct was prepared using an *Asp718/Sal*I fragment (containing the sequence of the *suc2* gene encoding the mature invertase protein fused to the signal sequence of proteinase inhibitor II) prepared from the PI-3-INV^{5} plasmid [15]. Transformed plants were obtained via *A. tumefaciens*-mediated transformation and, selected *in vitro* on kanamycin containing medium. They were tested for transgene expression using the Northern-blot hybridization [15, 23].

Isolation of plant DNA and RNA, and cDNA Synthesis

Genomic DNA was isolated by using cetyl trimethyl ammonium bromide [24]. Total RNA was extracted using the Plant Total RNA Kit Spectrum (Sigma, USA) and cDNA synthesis was performed with the Agilent Low RNA Input Fluorescent Linear Amplification Kit (Agilent, USA) following the manufacturer's recommendations.

PCR analysis

The presence of the invertase-encoding *S. cerevisiae suc2* gene in the transgenic plant genome was confirmed by polymerase chain reaction (PCR). Primers were designed using the Vector NTI program based on the *S. cerevisiae suc2* gene sequence, presented in the NCBI database (www.ncbi.nlm.nih.gov): 5'-TCCAAGACAAAGATGCGTTGCG-3' (forward primer (F)) and 3'-TGAAGGAACCGCCAGCAGGT-5' (reverse primer (R)). A 20-µl PCR reaction volume was prepared using 150 ng plant genomic DNA, 0.2 mM dNTP, 0.02 nM

primers and 1 U Taq DNA polymerase (Celanese, Russia), and the buffer was prepared using 50 mM KCl, 20 mM Tris-HCl (pH 9), 0.1% Triton X-100 and 2.5 mM $MgCl_2$. PCR was performed in a My Cycler TM Thermal Cycler (Bio-Rad, USA) under the following conditions: predenaturation at 94 ^oC for 4 min followed by 30 cycles consisting of denaturation at 94 ^oC for 45 s, annealing at 61 ^oC for 1 min, and synthesis at 72 ^oC for 1 min; then there was a final elongation at 72 ^oC for 5 min. The amplified DNA fragments were separated using 1.5% agarose gel electrophoresis in a Tris-acetate buffer, identified by staining with ethidium bromide and visualized under ultraviolet light, using the Gel Doc XR System (Bio-Rad, USA). The GeneRuler TM Express DNA Ladder Set (Fermentas, Lithuania) was used as a molecular-weight size marker.

Detection of invertase activity

Soluble invertase fractions and fractions associated with the cell wall were isolated according to Krishnan [25]. These fractions were used for electrophoretic separation of proteins in polyacrylamide gel (PAGE) without Na-dodecyl sulfate (Ds-Na) (Native electrophoresis) according to Davis [26].

Acid invertase activity in the gel was visualized according to the method of [27], based on hydrolysis of sucrose by the enzyme and subsequent oxidation of the formed glucose during the reduction of 2,3,5-triphenyltetrazolium chloride (TTC) in an alkaline medium into a red colored compound, formazan. The gel obtained from Native-PAGE was washed with a 0.1 M phosphate-citrate buffer (pH 5.5) and left in the dark for 1 hour at 37 ^oC in the same buffer, containing 0.1 M sucrose. Following a brief wash in distilled water, gels were developed by incubation in a boiling solution of 4% NaOH containing 0.2% (w/v) TTC. Red band coloration in the gel appeared at the positions of invertase activity within 1 minute.

The activity of different forms of invertase was estimated by the amount of glucose formed by hydrolysis of sucrose in the incubation medium containing 0.2 ml of the enzyme fraction and 0.3 ml buffer with sucrose (the final sucrose concentration was 150 mM). Invertase fractions were prepared as previously described [3]. To determine the acidic (vacuolar or apoplast) invertase activity, we used the incubation medium which contained a 1 M acetate buffer (pH 4.7), whereas for the determination of the alkaline/neutral (cytoplasmic) invertase activity, we used the incubation medium which contained a phosphate-citrate buffer (pH 7.5). The enzyme activity was expressed as μmol of glucose formed by hydrolysis of sucrose in the incubation medium per volume of sample taken for analysis.

Apoplastic fluid was prepared according to Hone [28]. Proteins of apoplastic fluid were precipitated with acetone and separated by denaturing electrophoresis in 12.5% Ds-Na-PAGE. After electrophoresis, the gel was stained with Coomassie R-250 to visualize the proteins. The Precision Plus Protein TM Standards Set (Bio-Rad, USA) was used as molecular-weight size marker.

MALDI-TOF

Yeast invertases were identified by means of Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) analysis. The mass spectra were measured with a Bruker Ultraflex MALDI-TOF mass spectrometer (Germany). Protein identification was accomplished using the Mascot software (www.matrixscience.com). The search was performed using the NCBI database.

Determination of carbohydrates

The content of fructose in the apoplastic fluid was determined according to Roe by the reaction of ketoses with resorcinol and subsequent recalculation of the sucrose content [29]. The glucose content was determined by the glucose oxidase method using the Agat-glucose Kit (Russia).

Statistical analysis

Statistical processing of data was performed by the T-tests program (ISI, USA) and visualized using the graphical mathematical package Microcal Origin (Microcal Software Inc., USA). The figures show the mean values of the typical experiment and their standard errors. Subject to the method used, the experiments were repeated three to seven times to obtain similar reproducible results.
$P < 0.05$ was considered to be statistically significant.

RESULTS AND DISCUSSION

The presence of target gene in the transformed potato plant genome was shown using PCR with gene-specific primers and subsequent product separation was done using agarose gel electrophoresis. A 636 bp band was amplified from DNA isolated from all Km-resistant potato plants (Figure 1a). The above band was not amplified from DNA of the wild-type plants. Consequently, the target heterologous *suc2* gene was localized in the transformant genome. Since the presence of *suc2* gene in the genome of the transgenic plants might not signify its

expression, the next task was to identify the *suc2* gene expression in the transformed potato plant genome. For this purpose, we used three independent methods: 1) reverse transcription followed by PCR (RT-PCR); 2) qualitative reaction in the gel (based on the invertase, a protein product of the *suc2* gene) and 3) quantitative analysis of the invertase enzymatic activity based on the determination of the amount of glucose resulted from the sucrose decomposition in the incubation medium.

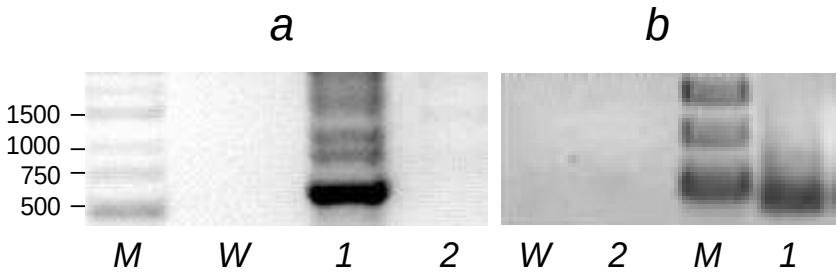


Figure 1. Results of (*a*) PCR and (*b*) RT-PCR analyses of the wild-type potato plants and plants transformed with the vector containing the *suc2* gene of the yeast *S. cerevisiae* with gene specific primers. *M* - GeneRuler TM Express DNA Ladder, *W* - purity control of the reaction, *1* and *2* - transformed and wild-type plants, respectively.

RT-PCR is the most sensitive method used to detect low-abundance mRNA and obtain evidence of a particular gene expression. All PCR-positive transformants showed the *suc2* gene expression as evidenced by RT-PCR (Figure 1b).

Native protein electrophoresis followed by the reaction product (acid invertase) visualization is one of the most reliable methods for identifying protein products formed as a result of the target gene operation [17]. It is known that TTC can be reduced in an alkaline medium by both dehydrogenases and via a nonenzymatic pathway [30] by sugars (glucose, fructose, etc.). We therefore performed protein electrophoresis without Ds-Na and determined the acid yeast invertase localization in order to confirm the *suc2* gene expression. Invertase activity gels showed that there was considerable invertase activity in the soluble fraction but no activity in the cell wall fraction (Figure 2, lane 3 and 4). No invertase activity was detected in the soluble or cell wall fractions in the wild-type plants (Figure 2, lane 1 and 2). Yeast invertase activity in the leaf homogenates of the transformants showed active expression of the *suc2* gene and poor adsorption of the heterologous protein in the cell walls of potato plants.

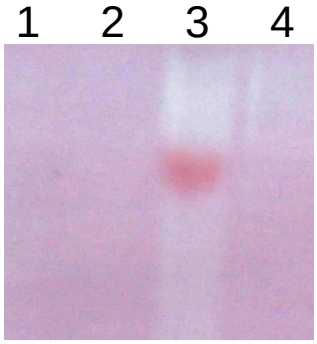


Figure 2. Visualization of the invertase protein activity in the gel under non-denaturing conditions (the results of a native PAGE). *1* and *2* - wild-type plants (supernatant and cell wall fractions, respectively), *3* and *4* - transformed plants (supernatant and cell wall fractions, respectively).

Subsequent analysis of the different forms of the invertase activity in the potato plant leaves showed the increased activity of apoplastic and vacuolar invertase forms in the transformed plants by 30% and 40%, respectively (Figure 3). A slight increase in the cytoplasmic invertase activity in the transformed plants was probably due to the residual yeast invertase activity in the growth medium with pH 7.5. The observed increase in the vacuolar invertase activity in the transformed plants confirmed our assumption on the partial adsorption of yeast invertase in the cell wall, as well as evidenced the presence of invertase in the tissues of the transformed plants in soluble form (Figure 2). Thus, obtained data indicated that a higher constitutive activity of the acid invertase in the transformed plants compared to that in the wild-type plants was a result of the *suc2* gene expression.

The *S. cerevisiae* invertase activity varies depending on the substrate concentration. The maximal activity was previously observed at 62 mM sucrose in the cultivation medium, pH 4 [31], which correlated with the sucrose concentration in the plant growth medium used in our studies. However, there was a need for some specific

experiments to confirm the presence of *S. cerevisiae* invertase protein in the extracellular space (apoplast) of the transformed plants. This need was determined by the following: a) the increased yeast invertase activity in the supernatant fraction in the gel (Figure 2) and b) the increased activity of apoplastic and vacuolar invertase forms (Figure 3). The *suc2* gene sequence encoding the mature invertase protein was fused with the signal sequence of potato proteinase inhibitor II in order to provide the apoplast localization of foreign invertase. Then, in order to confirm extracellular localization of yeast invertase, the apoplastic fluid from the wild-type and transformed potato plant leaves was obtained, and one-dimensional denaturing electrophoresis of soluble apoplastic proteins was carried out. The SDS-PAGE electrophoresis of apoplastic proteins showed the presence of a protein band with a molecular weight of about 60 kDa in the transformed plants, but not in the wild-type plants (data not shown). The *S. cerevisiae* yeast invertase in the transformed potato plants was identified using MALDI-TOF mass spectrometry (Figure 4). The extracellular *S. cerevisiae* invertase is mainly a homodimer with a molecular weight of 270 kDa [32]. Polymannan accounted for approximately 50% of the enzyme carbohydrate, while glucosamine accounted for just 3%. The removal of the enzyme carbohydrate revealed that the yeast invertase consisted of two identical subunits with a molecular weight of 60 kDa [33]. Thus, the results of one-dimensional denaturing electrophoresis and MALDI-TOF MS analysis are consistent with the published data and indicate the presence of the yeast invertase in the apoplastic space of the transformed potato plants.

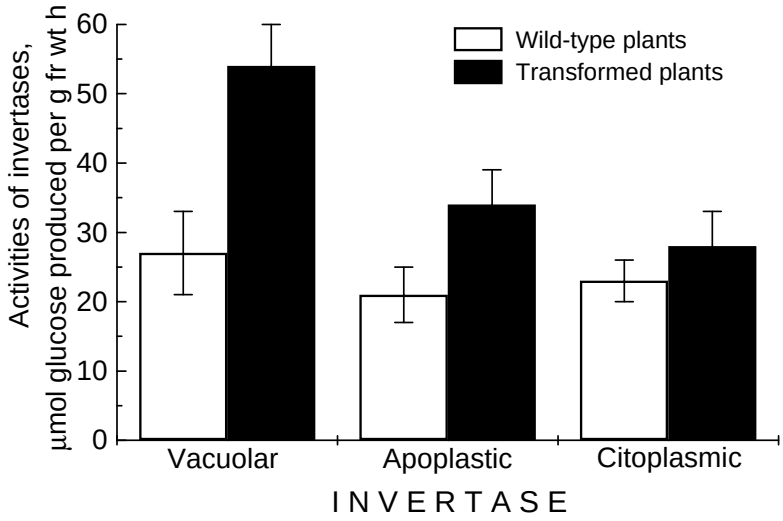


Figure 3. Activity of different invertase forms in the leaves of the wild-type and transformed plants.

Analysis of sugar concentration in the leaf apoplast provides additional information on the apoplastic localization of yeast invertase in the transformed plant tissues. It is known that the *Solanum* genus comprises a group of plants which use the apoplast as intermediate storage of assimilates (especially sugars) [19]. Our results demonstrated that glucose and fructose concentrations in the apoplast of the transformed plants, as compared to those in the wild-type plants, were 55% and 40% higher, respectively (Figure 5a). The total content of sucrose, glucose, and fructose in the apoplast was also 40% higher in the transformants. In our experiments we used potato plants with apoplastic localization of yeast invertase. It is known that environmental conditions in the apoplast (pH 4.5-4.7) comply with the conditions of maximum activity of extracellular *S. cerevisiae* invertase (pH optimum 3.5-5) [17, 34].

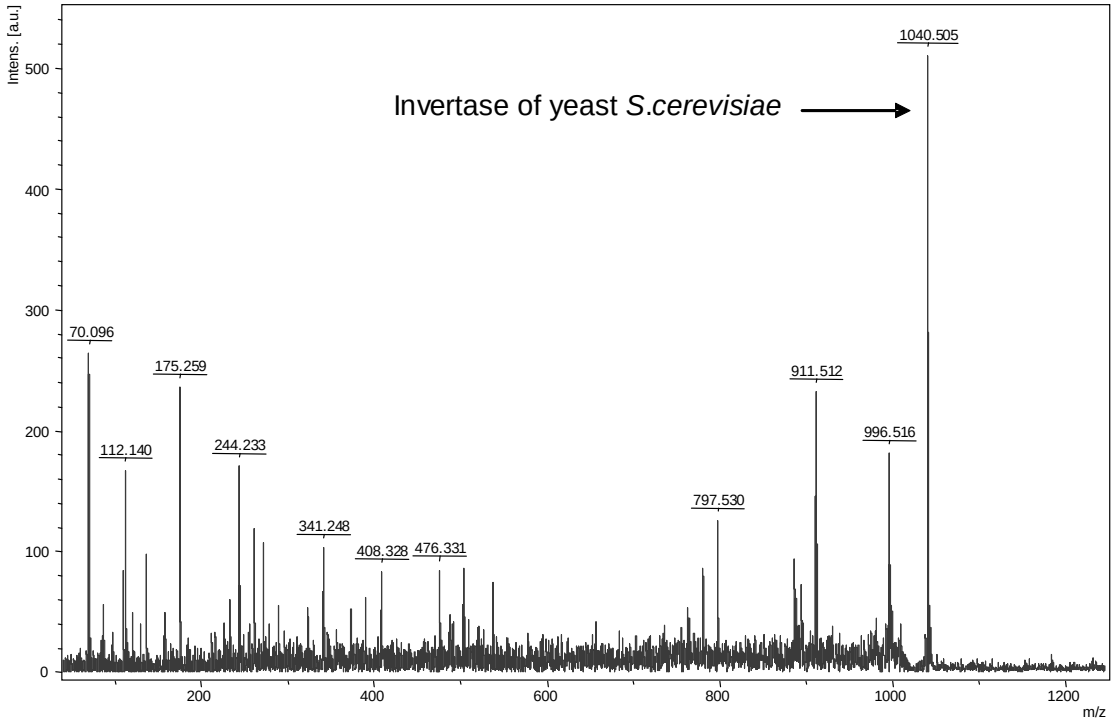
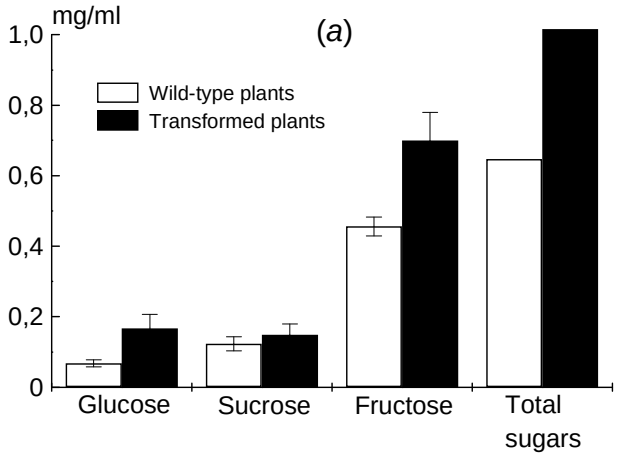


Fig. 4. MALDI-TOF mass spectra of proteins isolated from the apoplastic fluid of the transformed plants. The arrow points to the invertase of the yeast *S. cerevisiae*.

The sucrose and glucose levels in the transgenic plant leaves exceed those in the control plants by 20% and 11%, respectively (Figure 5b). The fructose content in both lines was low. The low fructose content resulted from the high activity of fructokinase (E.C. 2.7.1.4.) that provided maximal use of free fructose in the glycolytic pathway.

The morphometric parameters of the transformed and control plants under *in vitro* conditions were different (Table 1 and Figure 6). The transformed plants exhibited decreased offshoot length, less developed root system, smaller number of internodes, and larger water content in tissues. These differences were caused by the introduction of the *suc2* gene and its expression in the transformed plants.



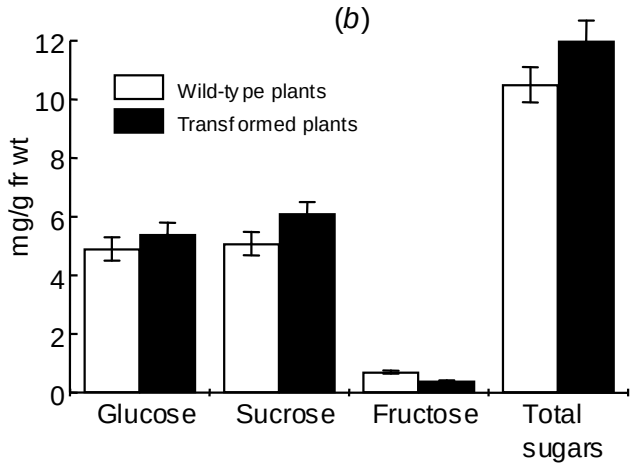


Figure 5. Sugar (glucose, fructose and sucrose) content in (*a*) apoplastic fluid and (*b*) leaves of the wild-type and transformed plants.

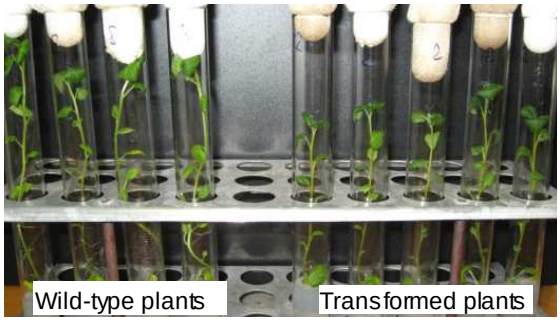


Figure 6. Wild-type potato plants and transformed potato plants (5 weeks old). Potato plants were grown *in vitro* on a Murashige-Skoog medium containing 2% sucrose.

Table 1. Morphometric parameters of the potato plants (5 weeks old)

Parameter	Wild-type plants	Transformed plants
Shoot length, cm	13.8 ± 0.3	10.6 ± 0.3
The number of internodes, *n*	13.0 ± 0.2	11.8 ± 0.3
Fresh roots, mg/plant	26.2 ± 5.2	10.6 ± 2.1
Fresh leaves, mg/plant	80.2 ± 6.4	59.1 ± 8.4
Contents of dried mass, % of the fresh plants	9.61	8.98

Data are mean values ±SE, n=30.

CONCLUSION

Our results show that the *suc2* gene encoding the extracellular invertase of the yeast *S. cerevisiae* is actively expressed in the genome of the transformed potato plants. The invertase protein is transported into the extracellular space of the transformed plants due to the presence of a signal peptide of the potato proteinase inhibitor II. The soluble form of the protein occurs in the apoplast and is weakly adsorbed onto the cellular wall. The constitutive *suc2* gene expression resulted in higher acid invertase activity and increased sugar contents in the apoplast and leaves of the transformed potato plants as compared to the wild-type plants.

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