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Expressing the Saccharomyces cerevisiae SUC2 gene encoding invertase apoplastic localization in the transgenic potato (*Solanum tuberosum* L.) does not contribute to microtubers production of identical physiological age

Alexander N. Deryabin

Timiryazev Institute of Plant Physiology of the Russian Academy of Sciences, Moscow, Russian Federation

Authors' Contribution	**Deryabin, A. N** has conducted the research	
*Corresponding Author's Email Address	anderyabin@mail.ru	**Review Proccess: peer review**
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ABSTRACT

The purpose of the study was to evaluate the function of apoplastic invertase in the in vitro synchronisation of microtuber formation in potato (Solanum tuberosum L.) plants. A non-transgenic potato plants (hereinafter nonTPs) and a line of potato plants, which expressed the *SUC2* gene of *Saccharomyces cerevisiae* encoding invertase apoplastic localization under the control of the B33 promoter of the patatin gene (hereinafter TPs), was used to achieve this purpose. Cell divisions in the axillary meristems of single-node cuttings were synchronized by exposing to 7°C for 24 h in a MS medium (without agar). After the low-temperature, the synchronisation (LTS) of proportion of simultaneously dividing cells in meristems of nonTPs was four-fold greater than without chilling. The LTS did not change the number of dividing cells in the axillary meristems of TPs. The LTS contributed to an increase in the mass and size of microtubers in both potato lines. All microtubers of nonTPs were of physiological maturity. At the same time, microtubers of TPs were physiologically immature, with high glucose content. During the tuber formation, the activity of acidic invertases in microtubers of TPs was higher than that of nonTPs. Microtubers of TPs were larger than the nonTPs, more hydrated with low starch amount. It is possible that the glucose that builds up in the TPs' microtubers acts as a signal to start cell division. It reveals how sugars and apoplastic invertase function as regulators. The high activity of apoplastic invertase plays a negative role in the production of potato microtubers of identical physiological age.

Keywords: *Saccharomyces cerevisiae*, *Solanum tuberosum*, 2,3,5-triphenyltetrazolium chloride, low temperature, invertase, microtubers, synchronisation, sugars, transgenic plants, tuber formation.

INTRODUCTION: The potato (*Solanum tuberosum* L.) is the most important non-cereal food crop in the world. Potato as a vegetable is very important for its high-quality proteins, starch, minerals, trace elements, considerable amounts of essential vitamins, as well as very low-fat content). The sowing time and fertilizers play a vital role on potato yield and quantity (Malik *et al.*, 2018; Rehman *et al.*, 2020). Potato plants, unfortunately, are susceptible to infection by many viral pathogens, which the decreases the yield of tubers (Urooj *et al.*, 2016). Micropropagation of virus-free potato plants is the most effective method of biotechnology (Kane, 2018). Then, the plants obtained *in vitro* can be used to produce microtubers (MT). MT plays an important role in the technology of seed potato production, since it has great advantage in storage, transport, and mechanization due to their little size and reduced weight. The use of MT technology in seed tuber production, breeding programs, germplasm conservation, and research appear to have enormous potential (Sadawarti *et al.*, 2016). The development of methods for large-scale production of MT potatoes acutely raises the problem of their quality, and especially, the alignment of physiological age (Caldiz, 2009).

Tuberization (TB) is one of the means for plant vegetative propagation. TB in potatoes is a complicated developmental process, involving anatomical, biochemical, and hormonal changes at ontogeny stages (Aksenova *et al.*, 2014). During the stolon growth, cell divisions are confined mainly to subapical zone, perpendicular to the axis of the stolon growth accompanied by reorganization in the microtubular cytoskeleton (Aksenova *et al.*, 2014). As the elongation of the stolon-tip ceases, the direction of cell division changes from transverse to longitudinal one and causes swelling of the sub-apical region of the stolon (Kondhare *et al.*, 2020). Along with the tuber growth, there is observed active transport of assimilates from the source organs. The assimilates are further converted into the storage compounds, especially the starch and proteins.

It is known that the cell cycle in plants consists of four stages (G1, S, G2, and M) with two major check points, namely, the G1 check point and G2 check point, that ensure normal cell division (Qi and Zhang, 2020). The cell cycle progression is retarded or arrested at different stages and depends on external factors. For example, high or low temperatures trigger G2 arrest in maize and Arabidopsis (Shimotohno *et al.*, 2021). The arrested cells can continue proliferation if the optimal temperature is restored. The resistance of plant cells to low temperatures (LT) is reported to depend on the stage of the cell mitosis cycle: the lowest resistance was observed at stages G2 and the highest was at G1 one. We have assumed that it is possible to achieve simultaneous initiation of the TB individual stages by synchronizing cell cycles at definite key stages of potato stolons. Our study showed that exposing the single-node cuttings to LT synchronizes the cell division in axillary meristems and contribute to the formation of MT of identical physiological age (Deryabin and Yur'eva, 2008). In addition, similar exposure of the single-node cuttings to LT made it possible to prolong dormancy of the produced MT. In nature, plants are an autotrophic culture, while *in vitro* culture is predominantly a heterotrophic one. Therefore, for growing plants *in vitro* sucrose (2-3%) is used as the source of carbohydrates. It is noteworthy to say that sucrose can't be utilized in the cells. It

must be split into hexoses (fructose+glucose) by invertase (D-fructofuranoside fructohydrolase, EC 3.2.1.26) before being used. In addition, biochemical changes in the potato cells with developed MT are concerned with the synthesis of starch and depend on the invertase activity (Hajirezaei *et al.*, 2000). Invertases differ in the biochemical properties and localization in the cell. Alkaline invertases with an optimum activity at pH 7.0-7.8 are localized mainly in the cytoplasm but are also found in chloroplasts and mitochondria (Roitsch and González, 2004). Acid invertases show an optimum activity at pH 4.5-5.0 and are localized in the vacuoles and extracellular space (apoplast) of cells. Invertases change the sugar composition in the compartments and the expression of the genes sensitive to the changes in the hexose/sucrose ratio (Halford and Paul, 2003). The way, the potato plants transport and store the food reserves is crucial to improving crop productivity. It is noteworthy that apoplastic invertase is involved in unloading the phloem and coordinating the source-sink relationship between potato leaves and tubers (Roitsch and González, 2004). Transgenic potato plants with the integrated target *SUC2* gene encoding apoplastic invertase in the yeast, under the control of the promoter of a class I patatin gene (B33 promoter) is of a fundamental interest. Patatin, a ~40 kDa glycoprotein, encoded by a multigene family refers to the most abundant tuber protein (Aminedi and Das, 2014). Since the 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 (Kolachevskaya *et al.*, 2015). In these plants, the *SUC2* gene expression affects the carbohydrate metabolism, TB and resistance to LT was studied. It was shown that, the results of the constitutive expression of the *SUC2* gene are a higher activity of acid invertase and glucose and fructose contents in the apoplast of transgenic potato plants compared to the non-transformed plants (Deryabin and Trunova, 2014). In comparison with the non-transformed potato plants, transgenic plants formed a minimal number of tubers per plant, but tubers had a higher mass. Transgenic potato plants were characterized by reduced sensitivity to low temperature stress (Deryabin *et al.*, 2005) and reduced the threshold concentration of sucrose required for TB. We assume that the transgenic potato plants are a convenient tool to studying a functional role of the apoplastic invertase in the production of MT of identical physiological age due to simultaneous initiation of both stages of tuberization.

OBJECTIVES: The objective of this study were as follows: to study the effect of synchronisation of cellular division in axillary meristems of single-node cuttings by LT on MT formation using potato plants, which express the *SUC2* gene of yeast encoding acidic apoplastic invertase. We highlights: i) the importance of LT to achieve synchronisation in tuberization of potato tubers; ii) the activity of apoplastic invertase plays a negative role in the production of potato MT of identical physiological age. This work offers understanding of mechanisms that regulate TB and its synchronisation in potatoes.

MATERIALS AND METHODS: Plant growth conditions: The research material was non-transgenic potato (*Solanum tuberosum* L., cv. Désirée) plants (hereinafter referred to as nonTPs) and the potato line transformed with a vector containing the *SUC2* gene of *Saccharomyces cerevisiae* (hereinafter referred to as TPs). In vitro potato plantlets were grown at 22-24°C with dinnual 16-h light (illuminating intensity of 100 µmol photons/ (m^2 s) on Murashige and Skoog medium, containing 2% sucrose, 0.7% agar, 0.5 mg/L HCl-thiamine, 0.5 mg/L pyrydoxine, 60 mg/L *myo*-inositol, pH 5.8 (MS medium) (figure 1).

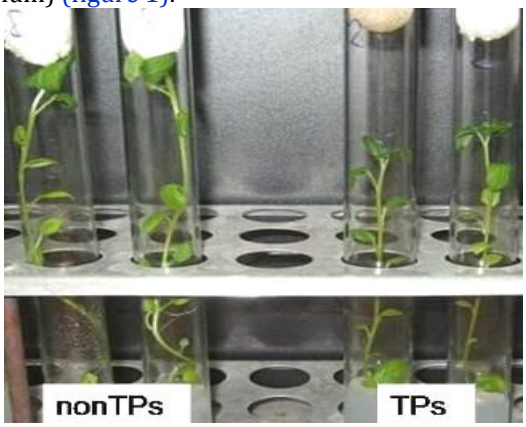

Figure 1: Potato (*S. tuberosum* L., cv. Désirée) plants (age 5 week) on standard MS medium with 2% sucrose without growth regulators (22-24°C, 16 h light, illuminating intensity of 100 µmol photons·(m^2·s)$^{-1}$): nonTRs non-transgenic potato plants; TPs–a potato line with altered carbohydrate metabolism caused by the integration of the target *SUC2* gene, encoding the invertase of *S. cerevisiae*, under the control of the *B33* promoter of the patatin gene, class I.

Before, we showed that the TPs had some decreased growth parameters (fresh biomass of leaves, shoot length, the number of internodes) compared to those of the nonTPs (Deryabin and Trunova, 2014). Plants were propagated by the cuttings method using single-node cuttings with one axillary bud.

Construction of the expression vector and plant transformation: The TPs were obtained from the Max-Planck Institute of Molecular Plant Physiology (Golm, Germany). The TPs were obtained using an Agrobacterium-mediated transformation. The invertase construct was prepared using an *Asp718/Sal*I fragment prepared from the PI-3-INV plasmid. Fragment containing the sequence of the *SUC2* gene fused to the signal sequence of proteinase inhibitor II of potato, provides apoplastic localization of protein in potato plants. Fragment was cloned between the B33 promoter and octopine synthase terminator in binary vector derivatives of pBin19. The TPs were selected on MS medium with kanamycin.

Complementary DNA synthesis and reverse transcriptase-polymerase chain reaction (RT-PCR) analysis: Total RNA was extracted from leaves of potato plants using the Plant Total RNA Kit Spectrum (Sigma, USA). Complementary DNA synthesis was performed with the Agilent Low RNA Input Fluorescent Linear Amplification Kit (Agilent, USA). Primers were designed using the Vector NTI program based on the *S. cerevisiae SUC2* gene sequence, presented in the NCBI database: 5'-TCC AAG ACA AAG ATG CGT TGC G-3' (F) and 3'-TGA AGG AAC CGC CAG CAG GT-5' (R). RT-PCR was performed in a Mastercycler gradient (Eppendorf, Germany). The amplified DNA fragments were separated using 1% (w/v) 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 GelPilot DNA Molecular Weight Marker (Giagen, USA) was used as a molecular-weight size marker.

Low-temperature synchronisation (LTS) of cell divisions in axillary meristems of single-node cuttings: The experiments were conducted with single-node cuttings excised from the middle part of potato plants. Cell divisions in the axillary meristems of cuttings were synchronized by exposing to 7°C for 24 h in a MS medium (without agar). This regime was selected in the preliminary experiments (Yurieva and Deryabin, 2008). The process of cell divisions in the axillary meristems was analyzed one day after LT-treatment. Control cuttings were kept on a MS medium at 22-24°C.

Cytological studies: Axillary buds were fixed in a mixture of 96% ethanol and glacial acetic acid (3:1). The cells of axillary meristems were macerated in acetic acid at heating. Squashed preparations were produced and stained with of acetocarmine. No less than 350–400 cells were examined through a BIOLAM light microscope (LOMO, Russia). Total number of the cells was counted as well as the number of the cells in metaphase, anaphase, and telophase. From 10 to 12 meristems were examined for each experiment variant.

Induction of tuberization: The tuberization was induced on the 7th day of culturing cuttings by replacing the liquid MS medium with identical MS medium with 8% sucrose, in the dark at 22°C.

Determination of the activity of acid invertases in MT: The activity of acid invertases in MT was estimated at 3, 6 and 10 weeks of TB by the amount of glucose formed by hydrolysis of sucrose in the incubation MS 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 (Deryabin *et al.*, 2005). Since there is a weak adsorption of yeast invertase to the cell wall of the TPs (Deryabin and Trunova, 2014), the total values of data for the activity of acid invertases are shown in the figure. It was established that potato invertases localized in the apoplast are bound covalently to the cell wall.

Determination of sugars and starch: The fructose and sucrose content in MT was determined by the reaction of ketoses with resorcinol (Deryabin *et al.*, 2005). Starch content in MT was carried out by a method based on hydrolytic cleavage of starch to glucose. The glucose content was determined by the glucose oxidase method using the Olvex diagnosticum Kit (Vital Diagnostics, Russia).

Determination of the physiological maturity of MT: The physiological maturity of MT was determined by the tetrazolium test in 70 days after the induction of TB. Method based on the ability of 2,3,5-triphenyltetrazolium chloride (TTC) to be reduced in the tissues of the MT (Sacher and Iritani, 1982). Authors of test found that in young potato tubers the rate of TTC reduction in the apical zone was greater than in the basal zone. As the tubers became older, reduction of TTC became more pronounced in the basal part and weakened in the apical zone. We cut the MT strictly along the central axis and placed the halves in 0.5% solution of TTC (Sigma-Aldrich Chem. Corp., USA). After 2h, the degree of physiological maturity of MT was determined. If TTC reduction was more pronounced in the basal part than in the apical part, the MT was considered physiologically mature. For each variant, at least 30-35 MT were examined.

Determination of dry matter content in MT and their morphometric analysis: The diameter (mm) of MT, fresh weight (mg) and dry matter content (%) determined in 70 days after the induction of TB.

Statistical analysis: Experiments was replicated at least three times. The t-tests software (ISI, USA) was used for the analysis of the data. The table and figures shows the mean values of the typical experiment ± SE. Bars having the same letter are not significantly different at the 0.05 level. We discuss only the differences significant with a 95% significance level.

RESULTS AND DISCUSSION: RT-PCR analyses of expression of *SUC2* targeted gene in TPs: The activity of a yeast invertase is not known to be inhibited by plant inhibitors, and it is of a wider pH than plants invertase (Von Schaewen *et al.*, 1990). It is the main reasons why the yeast *SUC2* gene was used. To detect the *SUC2* gene expression, we used RT-PCR (figure 2).

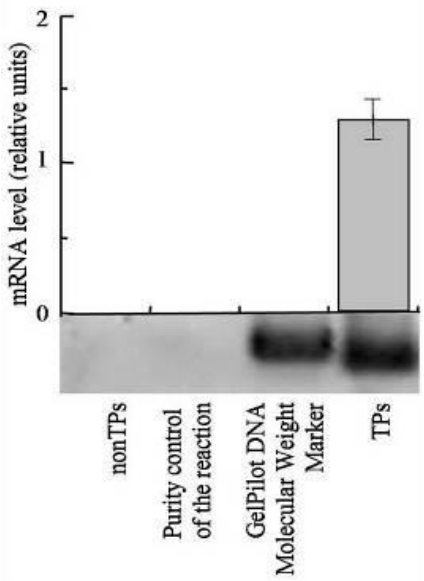

Figure 2: Results of RT-PCR analyse of expression of *SUC2* gene in potato line transformed with a vector containing the *SUC2* gene of *S. cerevisiae* encoding of invertase apoplastic localization (TPs). Quantified results of RT-PCR are presented by bars.

As it can be seen in the picture the PCR-positive TPs showed the *SUC2* target gene expression. These data confirm the presence and expression of the inserted *SUC2* gene in the potato plants.

Intensity of cell divisions in axillary meristems of the single-node cuttings after LTS: A widespread interest in cell synchronisation is maintained by the studies of control mechanisms involved in cell cycle regulation. During the passage of the mitosis phases, the cells of axillary meristems react to LT differently: the prolongation of mitosis and the entire cycle changes. Therefore, distribution of the cells on mitosis phases changes, too. A day after 24-hour-exposure of single-node cuttings of nonTPs to LT, the frequency of the cells in the metaphase was 24%, whereas this parameter without LTS was 6% (figure 3). Therefore, the share of synchronously dividing cells of axillary meristems of single-node cuttings nonTPs four-fold increased. The TPs showed no difference in the number of dividing cells in meristems of single-node cuttings between the pretreated LT and control variants. According to our study, on the 7th day of culturing in the dark in the MS medium single-node cuttings produced etiolated shoots with rudimentary leaves, which corresponded morphologically to stolons.

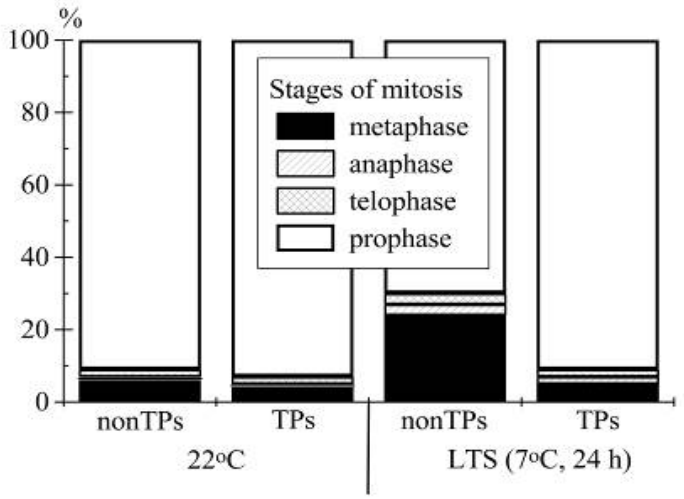

Figure 3: The intensity of cell divisions in axillary meristems of one-bud cuttings of non-transgenic potato plants (nonTPs) and line transformed with a vector containing the *SUC2* gene of *S. cerevisiae* encoding apoplastic invertase (TPs) one day after low-temperature synchronisation (LTS) of cell divisions in axillary meristems of one-bud cuttings with exposing to 7°C for 24 h in MS medium (without agar).

Effect of LTS on the activity of acid invertases in MT: Before, we showed that a yeast invertase synthesized by the *SUC2* gene is slightly adsorbed on the cell-wall of TPs (Deryabin and Trunova, 2014). Besides, the analysis of invertase activity showed the increased activity of apoplastic invertase in TPs by 30%. According to the received data, during the TB the potato plant differed in the activity of acidic invertases in MT (figure 4a). At 3-week TB stage, the activity of acid invertases in MT of TPs was 1.5 times higher than that in nonTPs. At the 6-week TB stage in both plant lines, the enzyme activity was minor, and at the 10-week one, it was not detected. It is important to note that higher activity of the acidic invertases in the TPs compared to that in the nonTPs resulted from the *SUC2* gene expression. There was no influence of cell divisions LTS in axillary meristems of single-node cuttings on acidic invertase activity in MT.

Effect of LTS on biological and chemical parameters of MT: The changes in the TPs metabolism caused by the expression of the *SUC2* gene influenced the biological and chemical parameters of MT. The analysis of the sugars amount 10 weeks after induction of TB showed an 8-fold increase in glucose content in the MT of TPs, compared to that in nonTPs (figure 4b). No influence of LTS on content sugars was shown in MT of nonTPs. At the same time, in both potato lines no sucrose and fructose amount differed. The results of our study on the study of acidic invertase activity and sugar concentration in the MT provide some additional information on the apoplastic localization of yeast invertase in TPs.

During the TB fructose and glucose are phosphorylated by fructokinases (EC 2.7.1.4) and hexokinases (EC 2.7.1.1) and involved in the cell metabolism (including synthesis of sucrose and cell-wall polymers) (Büssis *et al.*, 1997). Meristems cells prefer to use monosaccharides (glucose, fructose), while the differentiated ones prefer sucrose. Sucrose at a concentration of 6–8% is optimal for the TB because it is the source of energy for plant growth and MT.

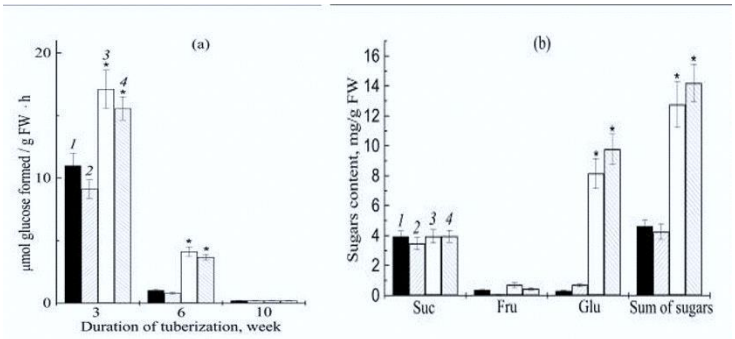

Figure 4: **:** Influence of low-temperature synchronisation (LTS, 7°C for 24 h) of cell divisions in axillary meristems of one-bud cuttings on (a) the activity of acid invertases and (b) content of sugars (Suc, sucrose; Fru, fructose; Glu, glucose) in microtubers of non-transgenic potato plants (nonTPs) and line transformed with the vector containing the *SUC2* gene of *S. cerevisiae* encoding apoplastic invertase (TPs).

The concentration of sucrose less than 4% and more than 8% in the medium slows down TB and/or MT forms less, and their number and size are smaller (Islam *et al.*, 2017). The participation of sugars in the regulation of the cell cycle is due to intracellular metabolic reactions, including those involving hexokinases (as a glucose sensor) that respond to the endogenous monosaccharides concentration (Wingler, 2018). Sugars are known to be an essential ingredient for cell division; as it affects cell division during the stolon swelling (Aksenova *et al.*, 2014). Monosaccharides not only provide primary substrates for the generation of starch and cellulose as biosynthetic processes, but also act as signaling molecules and control the stress situations (Ruan *et al.*, 2010). Besides, sugars play a protective role against LT, for example, as osmoprotectants and donors of carbon skeletons (Sami *et al.*, 2016). Potato plants differed in starch amount: the MT of TPs contained 50% less starch compared to that in nonTPs (table 1). According to the received data, the dry matter content of MT of TPs was 20% less than that in nonTPs (table 1).

Parameter	nonTPs		TPs	
	without LTS	LTS (7°C, 24 h)	without LTS	LTS (7°C, 24 h)
Starch content, %	15.3[a]	15.5[a]	9.9[b]	10.1[b]
Dry matter content, %	17.5[a]	17.8[a]	14.8[b]	14.6[b]
Microtubers mass, mg	46.4 ± 0.3[a]	58.5 ± 0.4[b]	52.2 ± 0.4[b]	67.4 ± 0.5[c]

Table 1: The effect of low-temperature synchronisation (LTS) of cell divisions in axillary meristems of single-node cuttings of potato on microtubers mass and biochemical parameters (10 weeks after tuberization induction).
In each row the values, which significantly differ at $p < 0.05$ are denoted by different letters.

The LTS of cell divisions in the axillary meristems of single-node cuttings contributed to an increase in the mass of MT in both potato lines. It is known that TB is associated with induction of meristems cell division in radial direction of the stolon subapical zone on the background of unchanged division in longitudinal direction (Aksenova *et al.*, 2014) therefore, simultaneousness of changing in the cell divisions direction, i.e., TB seems to result from initially synchronized stolons formation and growth. Indeed, the results of our experiments

demonstrated an four-fold increase in the share of synchronically dividing cells in meristems tissues of nonTPs exposed at 7°C for 24 h (figure 3). However, the TPs formed larger MT than that in the nonTPs, with a lower dry matter amount. The LTS of cell divisions in the axillary meristems of nonTPs cuttings contributed to an increase in the large MT share (with a diameter of ≥ 5 mm) (figure 5).

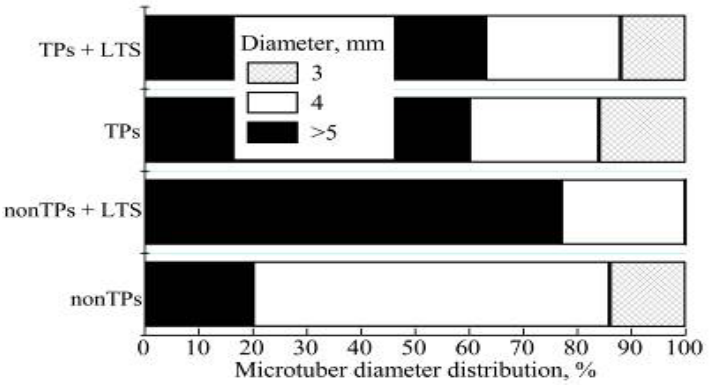

Figure 5: Influence of low-temperature synchronisation (LTS, 7°C for 24 h) of cell divisions in axillary meristems of one-bud cuttings in the distribution MT by diameter (10 weeks after the tuberization induction) of non-transgenic potato plants (nonTPs) and line transformed with a vector containing the *SUC2* gene of *S. cerevisiae* encoding apoplastic.

The influence of LTS on MT diameter distribution to TPs was not shown.

Determining the physiological maturity of the MT: In 70 days after the TB induction, we analyzed physiological maturity of MT. The results of the tetrazolium test showed that all the MT fractions for nonTPs produced from the single-node cuttings exposed to LT turned out physiologically mature (table 2, figure 6).

Plants	Microtubers diameter, mm	
	≤ 4	≥ 5
	22°C	
nonTPs	20	80
TPs	0	0
	LTS (7°C, 24 h)	
nonTPs	100	100
TPs	0	0

Table 2: The effect of low-temperature synchronisation (LTS) of cell divisions in the axillary meristems of single-node cuttings of potato on subsequent frequency of occurrence of microtubers at the stage of physiological maturity (according to tetrazolium test).

In addition, 80% of MT of nonTPs (without LTS) with a diameter of 5 mm and 20% of those with a diameter of 3–4 mm were at the stage of physiological maturity. However, all MT of TPs regardless of the size and presence of LTS showed a physiologically immature response.

CONCLUSION: In this study, the effect of synchronisation of cellular division in axillary meristems of single-node cuttings by LT on MT formation using potato plants, which express the *SUC2* gene of yeast encoding acidic apoplastic invertase, was explored. The results showed that the LT-treatment of single-node cuttings of nonTPs had a positive effect on synchronisation of TB and formation of MT identical physiological age. LTS not only promoted an increase in the frequency of occurrence of MT at the stage of physiological maturity, but also did an increase in the size and the average weight of MT in nonTPs.

Figure 6: Determination of the physiological maturity of microtubers by using a tetrazolium test. Method based on the ability of TTC to be reduced in the tissues of the tuber and change the color from light yellow to red. This process depends on the formation of insoluble, red-colored formazan, a product of TTC reduction by dehydrogenases. As the tubers became older, reduction of TTC became more pronounced in the basal part and weakened in the apical zone.

It was determined that large MT are more viable, while small MT are more vulnerable to damage during the storage. The TPs are not capable of LTS, but they form larger MT with lower dry matter content. The MT of TPs is physiologically immature enriched in glucose and low starch content (because of the higher activity of the acidic invertase during the TB). Perhaps, glucose accumulated in the MT of the TPs may act as well as the signaling molecule to promote cell division. It indicates the regulatory function of apoplastic invertase and sugars. It was concluded that the activity of apoplastic invertase plays a negative role in the production of potato MT of identical physiological age.

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