Charles University, Faculty of Science

Department of Experimental Plant Biology

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Mgr. Hana Ševčíková

Regulation of morphogenic processes in potato – the role of sugar metabolism

Regulace morfogenních procesů u brambor – role sacharidového metabolismu

Doctoral thesis

Supervisor: doc. RNDr. Helena Lipavská, Ph.D.

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Prohlašuji, že jsem předloženou disertační práci zpracovala samostatně a použité zdroje jsem řádně ocitovala. Tato práce ani její podstatná část nebyla předložena k získání jiného nebo stejného akademického titulu.

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Podpis

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Abstrakt (in Czech)

Tuberizace bramboru (*Solanum tuberosum*) je komplexní morfogenní proces, ovlivňovaný jak vnitřními faktory, tak podmínkami vnějšího prostředí. Vzhledem k tomu, že brambor je jednou z nejdůležitějších plodin na světě, pochopení regulace tohoto procesu získává význam nejen z teoretického, ale i z praktického hlediska. Tato práce se soustředí na roli sacharidů, které jsou nejen důležitým zdrojem energie a stavebním materiálem, ale zároveň slouží i jako signální molekuly regulující vývojové procesy včetně tuberizace. Hlavním používaným experimentálním materiálem byly rostliny bramboru odrůdy Lada (WT) a její spontánně tuberizující mutant (ST). Protože předchozí výsledky naznačovaly, že ST rostliny mají pozměněné rozdělování cukrů mezi jednotlivé části rostliny a také signifikantně vyšší celkový obsah sacharidů než WT (Fischer et al. 2008), cílem prvního předkládaného manuskriptu bylo detailně prozkoumat cukerný metabolismus ST rostlin rostoucích *in vitro* za mixotrofních (MT) a fotoautotrofních (PA) podmínek. Byly pozorovány změny v alokaci rozpustných sacharidů a v depozici škrobu, s převahou ukládání do bazálních částí stonku u ST rostlin. Přestože jsou gibereliny (GAs) považovány za jedny z hlavních inhibitorů tuberizace, ST rostliny měly hladiny GAs překvapivě vysoké. Studium exprese genů zodpovědných za iniciaci tvorby hlíz odhalilo zvýšené hladiny *StSP6A* (bramborový homolog *FT*) v listech ST rostlin. Druhý manuskript je srovnáním fyziologických reakcí čtyř rostlinných druhů (bramboru, tabáku, řepky olejky a jahodníku) kultivovaných *in vitro* za MT a PA podmínek. Studie odhalila signifikantní rozdíly v sacharidovém metabolismu studovaných rostlin mezi oběma typy kultivace a co je nejzajímavější, významně druhově specifickou reakci na MT kultivaci. Kompletně odlišné zacházení se zdroji, pravděpodobně odráží různé strategie, kterými se jednotlivé druhy vyrovnávají s nadbytkem asimilátů. Ve třetím manuskriptu jsme zkoumali ST kořenové kultury, které i po oddělení od zbytku rostliny stále vykazují podobné změny ve fenotypu a sacharidovém metabolismu jako kořeny celistvých rostlin. Hlavním cílem předkládané práce je přispět k porozumění role sacharidového metabolismu v síti regulačních vztahů řídících rostlinou morfogenezi.

Klíčová slova: brambor, fotoautotrofní kultivace, kultivace *in vitro*, metabolismus sacharidů, tuberizace

Abstract

Potato (*Solanum tuberosum*) tuberization is a complex, strictly regulated morphogenic process. Since potato is one of the most important crops in the world, understanding the regulation of this process is gaining in importance not only from a theoretical but also from a practical point of view. This work focuses on the role of sugars, which can serve not only as an energy and building material sources, but also as an important signal regulating many developmental processes including the tuberization. As a primary experimental material potato cv. Lada (WT) and its spontaneously tuberizing (ST) mutant was used. Since the previous results with ST plants suggested altered carbohydrate partitioning between plant organs and significant difference in total carbohydrate contents between ST plants and the WT (Fischer et al. 2008), in the first manuscript presented, the aim was to examine in detail the sugar metabolism of ST plants grown *in vitro* mixotrophically (MT) and photoautotrophically (PA). We observed changes in soluble carbohydrate allocation and starch deposition, favouring basal stem part in ST. Even thought, gibberellins (GAs) are considered to be the main tuberization inhibitors, ST potato plants had surprisingly high GAs levels. The determination of tuber-inducing marker gene expressions revealed increased levels of *StSP6A* (potato *FT* homologue) in ST leaves. The second manuscript presented is focusing on a comparison of physiological reactions of four plant species (potato, tobacco, rapeseed and strawberry) under MT and PA cultivation *in vitro*. We found significant differences in plant carbohydrate metabolism between MT and PA cultivation systems. Moreover, each plant species under study had its own specific reaction to MT cultivation, completely altering the way of using its resources, probably due to the different strategies each plant uses to deal with the high amount of assimilates. In the third manuscript presented we investigated ST potato root cultures, which eventhough separated from the rest of the plant body showed similar changes in the phenotype and sugar metabolism as the whole ST plant. The main aim of the thesis is to contribute to the understanding of the role of the carbohydrate metabolism in the network of regulatory relationships governing the plant morphogenic processes.

Key words: carbohydrate metabolism, cultivation *in vitro*, photoautotrophic cultivation, potato, tuberization

Abbreviations

CO	CONSTANCE
FT	FLOWERING LOCUS T
GA2ox	GA2-oxidase
GAs	gibberellins
GI	GIGANTEA
LD	long day
Lox	LIPOXIGENASE
MSP	manganese-stabilizing protein
MT	mixotrophic cultivation
PA	photoautotrophic cultivation
PHYB	PHYTOCHROME B
POTH1	POTATO HOMEOBOX 1
PS II	photosystem II
SD	short day
ST	spontaneously tuberizing potato mutant
StSP6A	SELF PRUNING 6A
SUT	sucrose transporter
WT	wild type

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1. Introduction

Potato *(Solanum tuberosum*) is one of the most important crops worldwide and to understand the regulation mechanisms responsible for potato tuber formation, a morphogenic process called tuberization, is important both from theoretical and practical point of view. Therefore, many researchers focus their studies on the mechanisms of potato tuber formation, from the initiation phase to the control of tuber development.

Plant growth and development is a continuous process going on throughout the lifetime of a plant, contrasting to that in animals where the development of all the major organs is defined at an early developmental stage. New organs formation and their morphogenesis, is intimately connected with cell division and there are numerous gene products that contribute to the plant development. The intrinsic logic of genetics has traditionally been interpreted as the one of the hierarchical command chains. While we were searching for the 'missing links' in such hierarchical chains, the results indicated that in fact the feedbacks and cross-talks are essential for controlling the delicate and precise processes such as new organs formation.

Morphogenesis is therefore regulated on many levels, starting with the external factors and environmental conditions having huge impact on this process. Photoperiod, light quality, temperature, and water availability are the most important. Plant development is also known to be affected by nutritional factors, such as carbon or nitrogen availability. For example, the leaf shape can be affected by different concentrations of sucrose (Le Hir et al. 2006), and the sexuality of flowers can be influenced by the nitrogen levels in the soil in some species (e.g. zucchini, Tak-Cheung and Stephenson 1993).

However, we cannot forget other important factor having impact on the developmental processes including tuberization which is the developmental state of the plant. Unless it reaches certain age or size the plant is not ready for tuber formation even in the perfect tuber-inducing conditions (Ewing and Struik 1992). The physiological state of the plant is another factor we cannot overlook, including the metabolic balance (e.g. the state of sugar metabolism) and certain phytohormone levels (for review see e.g. Hannapel et al. 2004, Rodríguez-Falcón et al. 2006, Sarkar 2008).

It is not uncommon that the developmental change taking place at certain part of the plant body (e.g. stolon in the case of tuberization) can be triggered by signal(s) coming from completely different and spatially distant plant organ (e.g. leaves). The spatial separation of these plant parts emphasizes the need for a long-distance signalling in the regulation of the morphogenetic processes.

It is clear now that, similarly to other morphogenic processes, there is more than one path controlling the tuber formation; particular pathways provide signals in response to various endogenous as well as exogenous factors and cooperatively govern the process (Fig. 1).

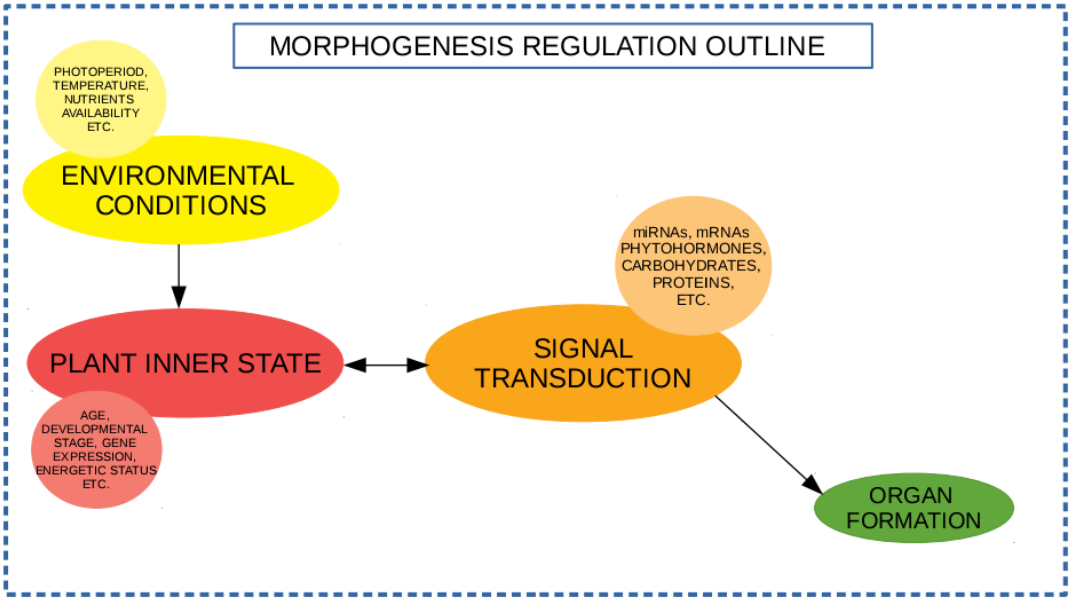

Fig.1: Schematic outline of plant morphogenesis regulation. Various environmental conditions are mirrored in the plant inner state which is interacting with the signal transduction elements and when all conditions are favourable, the organ formation can begin.

2. Current state of knowledge

2.1. Potato tuberization process

Potato tuberization is a complex developmental process consisting of a series of closely linked steps – stolon formation, initiation of tuberization, tuber formation itself, and storage compounds deposition. As the first tuberization event is considered the stolon formation which is, similarly to tuberization itself, regulated by photoperiod and phytohormones (Carrera et al. 1999, Koda and Kazawa 1983, Macháčková et al. 1998). The stolon formation occurs in both tuber-inducing and non-inducing conditions; however, the stolon growth extent has been negatively correlated with the strength of the tuber-inductive signal. Very strong induction results in a formation of sessile tubers with no prior stolon growth (Van Den Berg et al. 1995).

The tuber is usually formed at the stolon tip by its swelling. One of the early signs of tuber formation is significant increase of the cell division in the sub-apical stolon part accompanied by reorganization of the microtubular cytoskeleton (Sanz et al. 1996). The swelling occurs when the stolon ceases to elongate and the cells in the pith and cortex enlarge and divide transversely. Later, the cells in the perimedullary region enlarge and divide in random orientations to form a bulk of the tissue of the mature tuber (Xu et al. 1998a), the details can be seen in the Fig. 2. Along with the tuber growth, the active transport of the assimilates from the source organs occurs. Assimilates are further transformed to the storage compounds, especially the starch and storage proteins (e.g. patatine).

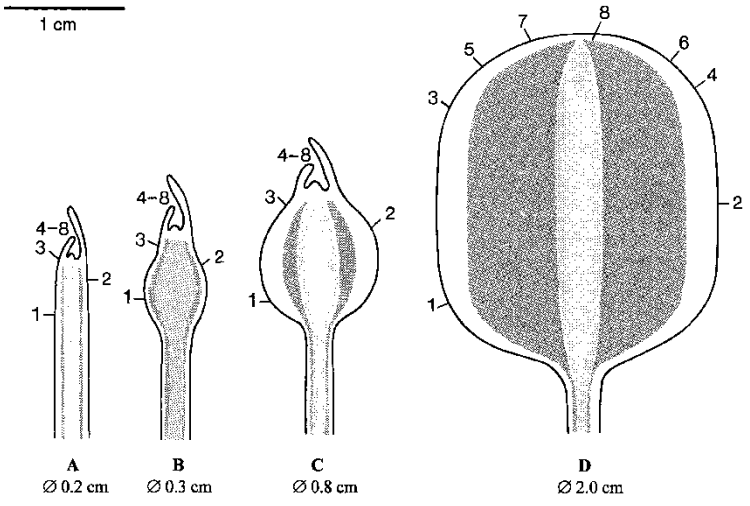

Fig. 2: Diagram of longitudinal sections through potato tubers, showing the morphology of the stolon and tuber and the thickening of the perimedullary zone (dark-shaded area). Positions of nodes are indicated schematically. The numbers indicate the nodes. Bar=1 cm. (A) 0.2 cm stolon, showing the continuous vascular bundle. (B) 0.3 cm tuber, showing the growth of pith (light-shaded area). (C) 0.8 cm tuber, showing the onset of growth of the perimedullary region. (D) 2.0 cm tuber, showing the thickening perimedullary region (figure from Xu et al. 1998).

2.2. External and internal factors regulating tuberization

As was mentioned earlier, the environmental conditions in which the plant is growing play an important part in affecting the morphogenic processes onset and consecution. Temperature and light conditions, especially photoperiod, belong among the most substantive environmental factors having impact on tuber formation (Jackson et al. 1996). The tuberization is induced by low temperatures and short-day photoperiod, although we can find both, potato plants strictly short-day-dependent (spp. andigena) and photoperiod neutral (modern commercially used potato cultivars) (for review see e.g. Jackson 1999, Sarkar 2010).

Recently, great progress has been made in finding the particular agents cooperatively governing the pathway associated with the photoperiod (Kloosterman et al. 2013, Navarro et al. 2011). However, domesticated potatoes are cultivated all around the world, at different latitudes in contrast to the original native potato and during the domestication process and further commercial cultivation the photoperiod dependence was weakened or completely eliminated by breeders over the years. So, the knowledge of the day-length control of the tuberization is mostly theoretical and less practical which puts in urgence the need to study other regulatory pathways, such as those that reflect the metabolic and energetic state of the plant.

Exposure to the external tuber-inducing conditions results in significant changes in the plant metabolism. Thus, the external conditions are partially mirrored in the internal factors regulating the tuber induction. The plants under tuber-inducing conditions show extensive metabolical and morphogenic changes of both the underground and above-ground organs. For example, plant transition to the induced state is characterized by increased photosynthetic activity and starch synthesis and also by increased export of sucrose from the leaves (Lorenzen and Ewing 1992). These metabolic changes must be accompanied by multiple changes in gene expression.

2.2.1. Gene expression

Although modulation of gene expression leading to the different morphogenetic processes is a typical consequence of the environmental signals perception and also reflects the plant inner state (e.g. developmental status), very few genes involved in the control of the induction of the tuberization have been identified and their possible functions have not been fully investigated yet. Most of the gene products and proteins known to have a role in the induction of tuberization are also having signal transduction function and their overview can be found in the chapter devoted to the signal transduction mechanisms (Chapter 2.3.3).

A bit more is known about the genes governing the process of the tuber development itself, not only the initiating steps. The greatest dynamics of gene expression, comparing to the developmental state where there tuberization is absent, was observed during the induction of the tuberization and during the first days of the tuber formation. It indicates some coordination of the process with the metabolism status (especially sugar metabolism) and redistribution of the assimilates at that time. No wonder, that the largest group of the genes expressed in this plant's development period are the genes that control carbohydrate metabolism (AGPase, sucrose synthase) and the biosynthesis of lipids (Bachem et al. 2000, Ronning et al. 2003).

Throughout the tuberization process an increased expression of two lipoxygenase (*Lox*) genes was found. One was expressed from the moment of induction of the tuber formation to the beginning of visible tuberization, the increased expression of the latter occurred during the growth and development of the tuber (Bachem et al. 1996). *Lox* genes are known to code the growth regulators (they are closely connected with jasmonic and tuberonic acid) and their expression varies according to the stage of the development of the plant (Siedow 1991). *Lox* genes expressions can be induced by exogenous phytohormones, e.g. by the addition of ABA or by plant injury (Geerts et al. 1994). Lipoxygenase genes, in particular *Lox1* class genes, have been studied deeper by Kolomiets et al. (2001). Using *in situ* hybridization, they found that *Lox1* mRNA is accumulated in apical and sub-apical parts of the newly emerging tubers with a specific occurrence in the vascular tissue, that is, in the areas with the highest cell division activity. The authors believe that Lox1 is involved in the regulation of the cell division and growth during the development of the tuber via its signalling pathway.

2.3. Tuberization signal transduction and execution

As was already said, nowadays we have quite a knowledge about the transmission of photoperiodic tuber inducing signal. It starts with the perception of the appropriate environmental stimuli in the leaves. The signal is further mediated by phytochrome and GAs. On long days (LDs), phytochrome B (PHYB) inhibits tuberization (Jackson et al. 1996). The fact that this effect is graft transmittable, has been interpreted as evidence that PHYB induces the formation of a mobile tuberization inhibitor (Jackson et al. 1998), which must be produced in the leaves in response to LD. Another proof of this notion is that phyB-antisense plants easily tuberize under LDs similarly to the GA-deficient mutant (Carrera et al. 2000). High GAs levels correlate with the inhibition of the tuberization, whereas low levels are associated with the tuber induction (Rodríguez-Falcón et al. 2006, Kloosterman et al. 2007), similar behaviour was observed with strigolactones (Roumeliotis et al. 2012). More on the role of the gibberellins in the regulation of tuberization is in the chapter 2.3.2.

Unfortunately, the modern potato cultivars used all around the world are mostly photoperiodic insensitive. They are still very sensitive to the GAs though, to their inner levels and also to the externally added ones (Tizio 1971). Therefore, we must turn our interest to other factors, that might play an important regulatory role in the initiation of the tuberization and the tuber development itself, for example plant energetic status and its possible interplay with GAs.

2.3.1. Plant energetic status

Similarly to other morphogenic processes, tuber formation is highly energy and carbon demanding, so it should not begin when the carbohydrate availability is poor. Therefore, we can assume that plant must carefully monitor its energetic status so that it can make the right decision whether to form the tubers or not. For this purpose, a highly sensitive set of sensors, strategically placed all over the plant body is needed, because detection of the overall carbohydrate balance is not a simple task. The next almost equally important step would be the integration of the signal from these sensors. Scientists work on the unveiling of the particular components of this signalling network for some time now and the knowledge in this field is getting bigger every day, but the definitive proofs are often still lacking. For example, there is no doubt that the

level of sucrose, as one of the crucial energy and carbon storing molecules, must be precisely monitored. But so far, no direct sensor of the sucrose level was found in plants. So, there is an idea that sucrose metabolites may play the role of signalling molecules. For example, signal can arise via sucrose cleavage products, glucose or UDP-glucose and fructose (Price et al. 2004, Christopher et al. 2018). Another sugar molecules known to have the signalling function are disaccharide trehalose, and its metabolic intermediate, trehalose-6-phosphate (Eastmond and Graham 2003, Paul 2008, Smeekens et al. 2010). It seems that the idea of some complex system monitoring the plant energetic status is gradually building up but which components of this signalling network are involved in the initiation of tuberization is still a question. And an answer to this question is of rising importance, since potato is grown all around the world and especially in the developing countries it is one of the main parts of the people's diet.

Scientists have been studying the sugars involvement in tuberization for a long time. As early as in the 1930s, there has been a hypothesis that the levels of the carbohydrates at the top of the stolon control the onset of tuberization. It is also well known that increasing concentrations of sucrose in *in vitro* media for plants increase tuberization. In much more recent decades, several experiments have been carried out to measure the current sucrose content of the stolons and other parts of the *in vitro* and *in vivo* cultivated plants but the results have been quite often contradictory. For example, Ross et al. (1994) observed increasing levels of sucrose in the stolon tips and developing tubers, while Vreugdenhil et al. (1998) found a decrease in the sucrose level prior to the visible swelling of the stolons. Later, Viola (2001) measured the concentration of sugars along the entire length of the stolon tip during the three stages of development of the stolon (non-swelling, small swelling and large swelling of the stolons). All three situations showed a similar pattern of sucrose concentration at the top of the stolon tip (more swelled stolon, more abundant sucrose) and its concentration gradually decreased along the stolon longitudinal axis. The apoplastic unloading of the phloem compounds (mainly sucrose) predominating during the stolon extension is replaced by symplastic unloading during the tuber induction, and this may be responsible for the up-regulation of several genes involved in the sucrose metabolism (Viola 2001). This phenomenon is coincident with a biochemical switch from an invertase-sucrolytic pathway to a sucrose synthase (SuSy)-sucrolytic pathway in the subapical region of the stolon tip. SuSy provides an energetically less costly

route because it conserves oxygen and allows a higher cellular energy state to be maintained (Bologa et al. 2003, Christopher et al. 2018).

Sugar metabolism enzymes, e.g. ADP-glucose pyrophosphorylase and invertase can also play an important role. Antisense expression of the former and the overexpression of the latter lead to increased tuber formation (Müller-Röber et al. 1992, Sonnewald et al. 1997). Hexokinases, on the other hand, probably do not play any important role in the tuberization regulation (Veramendi et al. 1999, 2002).

Currently, the role of sugars in the regulation of potato tuberization is relatively overlooked, except for the experiments with potato sucrose transporter StSUT4, which is expressed mainly in the sink potato organs and the inhibition of its expression leads to early tuberization and increased yield of tubers along with a higher efflux of the soluble sugars from the source leaves to the growing tuber. An interesting detail is that a signal generated by SUT4 is of a mobile nature (Chincinska et al. 2007). Although the SUT4 function in plants is still not fully understood, there are some indications that it may not be active predominantly as a sucrose transporter. The current model assumes the signalling role of SUT4, based on the idea that SUT4 could bind SUT1 (the major sucrose exporter in source tissues) to produce an inactive heterodimer, thereby regulating the assimilation flow into the sink tissues, consequently inhibiting the initiation of tubers (Chincinska et al. 2007).

Evidently there are still many unresolved issues considering the involvement of sugars in the regulation of tuberization. In the stage of tuber growth, it is of course the main role of the carbohydrates to serve as a source of carbon and energy for the further growth of the tuber, and in particular the synthesis of the storage substances that are stored in the tuber. However, the involvement of the carbohydrates in the initiation of the tuber formation is unclear, although a number of indirect evidence, such as those obtained in the study of induction of *in vitro* tuberization, shows the essential role of the carbohydrates at this stage of the process (Donnelly 2003).

2.3.2. Gibberellins

Phytohormones play a prominent role in the regulation of the morphological events of tuberization activated in the stolon apex. Gibberellins (GAs), cytokinins, auxin and jasmonate-like compounds have all been implicated to have a role in regulating the tuber development (for review see e.g. Hannapel et al. 2004; Rodríguez-Falcón et al. 2006; Sarkar 2008).

As the most important, and also the most studied phytohormones regulating tuberization, are considered gibberellins – a broad group of biologically active cyclic diterpenes and their derivates of natural or artificial origin. GAs synthesis takes place mostly in the actively growing plant organs, such as buds, young leaves, and developing fruits. The GAs levels are affected by both external and internal plant conditions – light, temperature, other phytohormones (e.g. auxins) and also GAs own feedback loop involving biosynthesis and degradation. Each plant species has its own unique reaction to GAs but in general they are considered to have a positive effect on the shoot growth (by stimulation of the longitudinal cell growth), and to play an important role in the juvenile to the generative phase transition (for review see e.g. Yamaguchi 2008). GAs are well known to have strong inhibitory effect on potato tuberization (Vreugdenhil and Sergeeva 1999) and their effect is usually connected with plant photoperiodic reactions. For example, strictly SD induced potato spp. andigena mutants with blocked GAs synthesis are tuberizing not only in the SD conditions but also in the non-inductive LD (van den Berg et al. 1995).

The connection between GAs and tuberization has been studied in the 1970s, e.g. by Kumar and Wareing (1974), who investigated the role of the various growth regulators on the tuberization of potato spp. andigena under induction and non-induction conditions. While growing in SD induction conditions, the studied plants had significantly lower GAs levels then the LD grown ones. Moreover, the plants growing for 3 weeks in SD conditions which were moved for another 2 weeks into the LD conditions had the same levels of GAs as the plants growing in SD for the whole 5 weeks. Authors concluded that once the tuberization is induced, it cannot be reversed by a simple change of conditions from inducing to non-inducing (Kumar and Wareing 1974). In another study Koda and Kazawa (1983) reported quite high GAs levels in the growing stolons followed by a sharp drop once they started to swell after tuberization induction. The connection between GAs and photoperiod in tuberization

control was studied e.g. by Macháčková et al. (1998). They used potato spp. andigena grown in various light conditions from continuous light to SD and also in SD with dark phase interrupted by a one hour of light in the middle. The SD grown plants showed 4-10x lower GAs levels in all plant organs than LD and SD with light break grown plants (Macháčková et al. 1998). With the first studies done with more precise methods capable to determine individual GAs (e.g. GC-MS), new questions emerged – especially whether all the GAs have the same function in tuberization regulation. Measuring the levels of GA_1, GA_{20}, GA_4 and GA_9 during various stages of potato tuberization, the levels of GA_4 a GA_9 remained more or less the same during the stolon and tuber development and the level of GA_{20} was under the detection limit to distinguish any significant changes. GA_1 on the other hand, showed significant differences during the stolon growth and tuber growth, stating GA_1 as the main bioactive gibberellin governing this process (Xu et al. 1998b). Second GA established as tuber induction relevant is GA_3 which level was also sharply dropping under induction conditions (Malkawi et al. 2007).

Inactivation of GAs usually goes through 2β-hydroxylation by GA2oxidase (GA2ox). The role of *StGA2ox1* gene in tuberization was studied by Kloosterman et al. (2007). *StGA2ox1* showed higher expression during the early stages of potato tuber development prior to plant visible swelling localized to the subapical stolon region and the tuber growth took place. Plants overexpressing *StGA2ox1* had a dwarf phenotype, reduced stolon growth and earlier tuberization. Transgenic plants with reduced expression levels of *StGA2ox1* showed normal plant growth, an altered stolon swelling phenotype and delayed tuberization. The authors thereby proposed a role for StGA2ox1 in early tuber initiation through modifying GAs levels in the sub-apical stolon region at the onset of tuberization (Kloosterman et al. 2007).

Detailed measurements of *S. tuberosum* spp. andigena chlorophyll, anthocyanins and GAs contents along with various physiological measurements led to the conclusion that there are in fact two independent pathways controlling the tuber induction, photoperiod-dependent and GAs-dependent. These pathways are subjected to a vast crosstalk and their interplay is needed for the tuberization to be induced (Martínez-García et al. 2002). Not only GAs synthesis and inactivation but also their transport across the plant to a particular organ might be significant factor, similarly to the other molecules functioning as the morphogenesis inner regulators. Unfortunately, we don't have a precise knowledge of the GAs distribution within the plant. In this

context it may be interesting that the SWEET facilitators, usually considered as sugars transporters, were proposed to be involved in GA transport as well (Kanno et al. 2016). So, there we have yet another connection between the plant energetic status and the phytohormone balance that needs to be considered when trying to uncover the pathways responsible for the tuberization regulation.

2.3.3. Mobile elements

The last but definitely not the least players involved in the potato tuberization, or any other morphogenic process in that matter, are the mobile elements. Molecules especially designed to transport information across the plant to an executing organ. The nature of these signals can be various – as mobile elements can serve proteins, mRNAs or microRNAs (miRNAs) and others.

Photoperiodic signal transduction during initiation of tuberization studied on potato spp. andigena can serve us as a good example of how these molecules function even though we already know that this pathway is strongly reduced in most commercially used potatoes. The photoperiodic signals are sensed by the phytochrome B receptor which further interacts with GA20-oxidase (the key regulatory enzyme of GA biosynthesis) and the homeodomain protein StBEL5. *StBEL5* overexpression promotes tuberization and its mRNA is graft transmissible (Chen et al. 2003, Banerjee et al. 2006) as well as mRNA of one of its binding partners, POTH1 (Potato Homeobox 1; Mahajan et al. 2012, Sharma et al. 2016). StBEL5 and POTH1 form a tandem dimer that binds to a specific TTGAC motif in the GA20-oxidase promoter and down-regulates its activity, thus allowing to induce tuberization (Chen et al. 2004, Rodriguez-Falcón et al. 2006, Hannapel 2013).

Since the photoperiod is not the only factor with the power to regulate tuberization other factors get to play their part here. One of them is the miR172 miRNA which is also mobile and can increase the levels of *StBEL5* mRNA in the leaves and stolons (Martin et al. 2009). Moreover, the overexpression of miR172 leads to suppression of its interaction partner RAP1, factor serving as StSP6A (SELF PRUNING 6A) inhibitor. StSP6A, transcription factor FT (FLOWERING LOCUS T) potato orthologue, is an example of the protein dependent positive signalling mechanisms controlling the induction of tuberization (Navarro et al., 2011) and target gene of transcription factors StBEL5 and POTH1 too. StSP6A and *StBEL5* both serve

as phloem mobile signals, although one of them, StSP6A, is exported from the leaves as a protein, while the other, *StBEL5*, moves as mRNA. It is worth mentioning that the target StSP6A stolon genes may also include *GA2-oxidase* (GAs inactivation enzyme) although it also probably further complicates the picture (Navarro et al., 2011). Simplified model of the described tuber induction pathways and possible sugars involvement is pictured in the Figure 3.

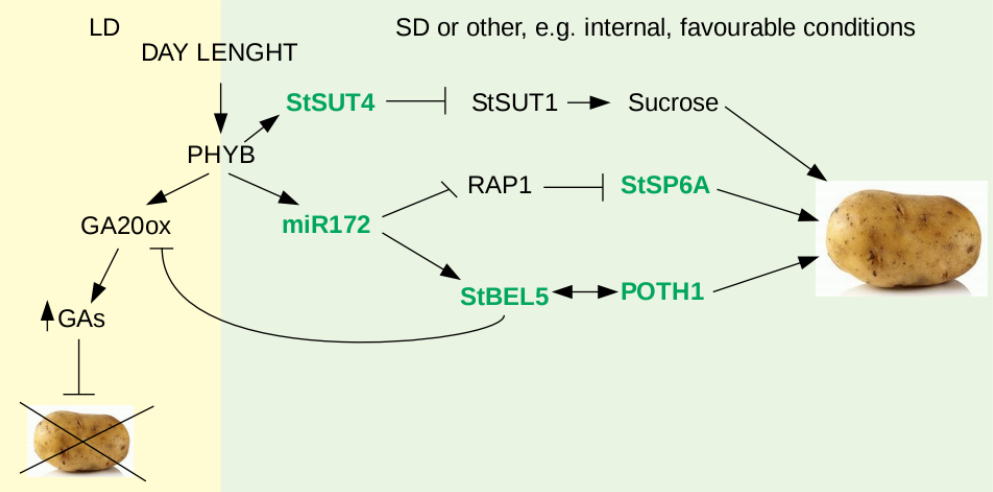

Fig. 3: Simplified model of the initiation of tuberization regulation. Day length is sensed by phytochrome B (PHYB). During long days (LD), PHYB activates GA-oxidase (GA20ox) and the gibberellins (GAs) synthesis is induced and tuberization repressed. During short days (SD) or other favourable conditions several pathways leading to tuberization induction can be activated. StSUT4 (sucrose transporter 4) might play a role as an inhibitor of StSUT1 (sucrose transporter 1), and increased sucrose efflux from leaves induces tuberization. PHYB activates miR172 that promotes tuberization likely through inhibition of the RAP1 repressor which releases the StSP6A (self-pruning 6A) repression. BEL5 is induced by miR172 and thought to promote tuber formation through changes in hormonal levels via interaction with POTH1 (potato homeobox 1). Green molecules are known to be mobile. Arrows and blunted lines indicate activation and repression, respectively.

3. Material and methods

3.1. Plant material

As primary experimental material served potato plants (*Solanum tuberosum* L.) cv. Lada and spontaneously tuberizing (ST) potato mutant lacking one isoform of manganese-stabilizing protein I (MSP) of photosystem II (PSII). The ST plants were originally transformed via. *Agrobacterium* with a gene-trap construct for random gene activation. Proteomic analyses revealed that the lack of one of MSP isoform in the mutant plants is the only known explanation of its complex phenotypic changes, like delayed senescence, increased carbohydrate content, basal branching, reduced rooting, and enhanced tuberization (Fischer et al. 2008). Brief information about the MSP is in the next chapter.

Tobacco (*Nicotiana tabacum* L.) cv. Samsun, rapeseed (*Brassica napus* L.) cv. Asgard, and strawberry (*Fragaria vesca* L.) plants were used along with potato in the second manuscript presented. And the root cultures derived from ST potato and Lada potato were used in the third manuscript.

3.1.1. Manganese-stabilizing protein

Manganese-stabilizing protein (MSP, also denoted as PsbO) is an essential extrinsic subunit of photosystem II (PS II) localised on the luminal side of the thylakoid membrane. Its function is to stabilize the water-splitting Mn_4CaO_5 cluster on oxygen-evolving complex catalysing the oxidation of water to molecular oxygen (Gururani et al. 2015). MSP was found in all photosynthetic organism so far and there are proofs that it is crucial for PS II proper function. For example, Arabidopsis with silenced expression of both MSP paralogs is neither able to assemble PS II nor growth photoautotrophically (Yi et al. 2005).

MSP protein is highly conserved among higher plants (Thornton et al. 2005). Arabidopsis contains two genes for the MSP as do other plants including potato (Murakami et al. 2002). The two MSPs do not have the same function in plant, as far as our knowledge can reach MSP-1 is responsible for stabilizing of the Mn_4CaO_5 cluster and the facilitation of the water oxidation reaction, and MSP-2 regulates the turnover of D1 subunit (one of the PS II subunits; Lundin et al. 2008). Interestingly, the loss-of-function MSP mutants can still grow photoautotrophically if there is still

one isoform present. Arabidopsis mutants lacking one of the two MSP isoforms showed just slightly retarded growth and reduced PSII activity (Lundin et al. 2007) but as was already mentioned cannot grow autotrophically when both MSP isoforms are silenced (Yi et al. 2007).

The ST plants lack one of three isoforms of MSP-1 (Fischer 2005, doctoral thesis) However, what can possibly be the role of potato MSP-1 in the regulation of tuberization is still beyond our understanding. Gururani et al. (2012) worked with potato plants with altered MSP expression (both enhanced and reduced), although they did not report which isoform they used. Plants with reduced MSP expression were photosynthetically more active and also showed enhanced tuberization and increased carbohydrate content, similarly to the previous work of our team published in Fischer at al. 2008 and publication presented in this thesis (Ševčíková et al. 2017). Even though we are still lacking some basic information, their results strongly suggest that altered photosynthetic machinery can be possibly connected with early tuberization (Gururani et al. 2012).

3.2. Cultivation methods

The plants under study were cultivated mostly *in vitro* (with few exceptions of pot cultivation mentioned in the text) using two types of cultivation arrangements:

1. Mixotrophic *in vitro* cultivation

This very widespread method has its advantages, such as strictly controlled environment inside the cultivation vessel, relatively low demand for cultivation chamber technologies, and there is a large range of standardized cultivation protocols for a huge variety of plants. The methods features (high sugar concentration in the cultivation medium, high relative humidity in a closed system with minimal gas exchange and usually also reduced photosynthetic photon flux), however, are also a source of many disadvantages. Firstly, for all plants it is highly artificial to uptake high quantity of sucrose through the roots. This can cause an osmotic stress (Dubuc and Desjardins 2007) or sucrose and starch accumulation in leaves which can lead to chlorophyll synthesis reduction (Kirdmanee et al. 1992). Secondly, the high relative humidity in the cultivation vessels can cause hyperhydricity of cultivated plants, leading to non-functional stomata (Chakrabarty et al. 2006). These conditions together are causing that plants under this type of cultivation are forced to be heterotrophic or

at least mixotrophic, which is obviously no good start for any physiological research to conduct on them.

2. Photoautotrophic *in vitro* cultivation

By the alteration of the culture environment we can induce a photoautotrophic (PA) metabolism even in *in vitro* cultures. Literature is talking mostly about enhancing of the illumination, enabling more gas exchange and removing of sugars from the cultivation media. We chose the last two approaches and combined them. Since the low offer of suitable solutions for PA *in vitro* cultivation on the recent market, we used the proven method (used e.g. by Haisel et al. 2002) to which the material is no longer available and improved it by using common laboratory consumables. The resulting improved sun caps are very easy to be made, cheap, durable and most importantly functioning alternative to commercially sold laboratory material. In fact, we let this method to be officially certified by Czech ministry of agriculture. Full text of this method is in the appendix number 2 to this thesis (in Czech).

The above-mentioned methods of cultivation were used in the published paper and one of the manuscripts presented below. The third manuscript deals with the root organ-cultures and the methodology is described in it.

4. Aims of the thesis

The main aim of the thesis was to contribute to the understanding of the role of carbohydrate metabolism in the network of regulatory relationships governing plant morphogenic processes. To narrow this question dawn, I postulated specific aims:

1) to examine in detail the sugar metabolism of ST plants grown both mixotrophically and photoautotrophically and compare it to WT plants under the same conditions and to check possible influence of metabolic changes on gibberellin levels and levels of key tuberization regulators (paper 1).

2) to characterize selected physiological reactions of four plant species widely used to basic plant physiology research, potato (*Solanum tuberosum* 'Lada'), tobacco (*Nicotiana tabacum* 'Samsun'), rapeseed (*Brassica napus* 'Asgard') and strawberry *(Fragaria vesca*), to photoautotrophic cultivation *in vitro* in comparison with standard mixotrophic cultivation *in vitro* (paper 2) with special interest in:

a) the differences in carbohydrate content and distribution,

b) the growth changes related to specific cultivation conditions,

c) the shifts in selected gas-exchange parameters.

3) to study selected phenotypical and metabolic characteristics of ST plants root cultures (paper 3).

5. Summary of published and unpublished results

5.1. Publication 1 – Ševčíková et al. (2017), published

Original research article, Journal of Plant Physiology 214, 53-63 (IF_{2016}=3.121)

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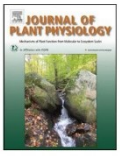

Original article

Carbohydrates and gibberellins relationship in potato tuberization

Hana Ševčíková[a,*], Petra Mašková[a], Danuše Tarkowská[b], Tomáš Mašek[c], Helena Lipavská[a]

[a] Department of Experimental Plant Biology, Faculty of Science, Charles University, Viničná 5, CZ-12844 Prague, Czech Republic
[b] Laboratory of Growth Regulators, Centre of the Region Hand for Biotechnological and Agricultural Research, Institute of Experimental Botany Academy of Sciences of the Czech Republic and Palacký University, Šlechtitelů 27, CZ-78371 Olomouc, Czech Republic
[c] Department of Genetics and Microbiology, Faculty of Science, Charles University, Viničná 5, CZ-12844 Prague, Czech Republic

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ABSTRACT

Potato represents the third most important crop worldwide and therefore to understand regulations of tuber onset is crucial from both theoretical and practical points of view. Photosynthesis and related carbohydrate status along with phytohormone balance belong to the essential factors in regulation of plant development including storage organ formation. In our work we used potato (*Solanum tuberosum*) cv. Lada and its spontaneously tuberizing mutant (ST plants) grown *in vitro* under low carbohydrate availability (non-inductive conditions). Small plant phenotype and readiness to tuberization of ST plants was, however, not accompanied by lower gibberellins levels, as determined by UHPLC–MS/MS. Therefore, we focused on the other inducing factor, carbohydrate status. Using HPLC, we followed changes in carbohydrate distribution under mixotrophic (2.5% sucrose in medium) and photoautotrophic conditions (no sucrose addition and higher gas and light availability) and observed changes in soluble carbohydrate allocation and starch deposition, favouring basal stem part in mutants. In addition, the determination of tuber-inducing marker gene expressions revealed increased levels of *StSP6A* in ST leaves. Collectively these data point towards the possibility of two parallel cross-talking pathways (carbohydrate − and gibberellin- dependent ones) with the power of both to outcompete the other one when its signal is for some reason extraordinary strong.

Introduction

Potato (*Solanum tuberosum*) tuberization is a complex process governed by a tight regulatory network of environmental as well as internal factors. Understanding the mechanisms of tuber induction and tuber formation is of great significance since potato is the third most important food crop in the world and its production is still rising mainly in developing countries.

Temperature and light conditions, especially photoperiod, belong among the most substantive environmental factors having impact on tuber formation (Jackson et al., 1996). Tuberization is induced by low temperatures and short-day photoperiod, although we can find both, potato plants strictly short-day-dependent (spp. andigena) and photoperiod neutral (modern commercially used potato cultivars) (Jackson, 1999). Tuber development on the stolon tip requires a precise synchronization of the biochemical and morphological processes that are strongly affected by differential gene expression (Bachem et al., 2000; Hannapel, 1991a, 1991b). Developmental stage of the plant is also an important factor we cannot ignore. Plants have to reach a certain size or age to be able to respond to environmental factors (Ewing and Struik, 1992). Once the plant is capable of responding to the external and internal conditions, an integration and translation of the stimuli come into play. In the plant language it means production of signaling molecules responsible for tuber onset induction. These signals can be of various nature such as recently identified signaling proteins, mRNAs and miRNAs (e.g. Hannapel, 2013) or long-time known phytohormones and sugars (Sarkar, 2008).

Protein-dependent positive signaling mechanisms involve mainly a potato *FT* (*FLOWERING LOCUS T*) orthologue *StSP6A (SELF PRUNING 6A)* (Navarro et al., 2011), and *StBEL5* and *POTH1* (*Potato Homeobox 1*) transcription factors and a lot of their target genes (e.g. *POTM1*, *FPF*, *CDC2*). Both, *StSP6A* and *StBEL5* serve as phloem mobile signals, though one of them, *StSP6A*, is exported from leaves as a protein, while the other, *StBEL 5*, moves as mRNA to the stolon where its action is conditioned by formation of a heterodimer with *POTH1*, other supposedly phloem mobile signal (Mahajan et al., 2012; Sharma et al., 2016). It is worth mentioning that *StSP6A* stolon target genes include also *GA2-oxidase* (*GA2ox*) and also sucrose transporter *SUT1*

Abbreviations: GA, gibberellin; GA20ox, GA20-oxidase; GA2ox, GA2-oxidase; MSP, manganese-stabilizing protein; PSII, photosystem II; ST, spontaneously tuberizing potato mutant; PHYB, phytochrome B; *StSP6A*, potato *FT* (*FLOWERING LOCUS T*) ortholog

* Corresponding author.
E-mail address: hana.sevcikova@natur.cuni.cz (H. Ševčíková).

(Navarro et al., 2011).

The role of individual phytohormones in the regulation of tuberization is not yet fully proved. Over the years, the role of abscisic acid, cytokinins and auxins was considered to be the most significant, in the positive tuberization control, usually in synergy with an appropriate photoperiod (Machackova et al., 1998). Also tuberonic acid, a jasmonic acid derivative, has long been regarded as the significant tuber-inducing agent (Yoshihara et al., 1996). On the contrary, there is the widely accepted idea of gibberellins being the main negative tuberization regulators of phytohormonal nature. The main bioactive gibberellins (GAs), acting in delaying the tuberization onset, are GA_1 and GA_3, because their levels are high during stolon growth and get rapidly down before the tuber formation starts (Malkawi et al., 2007; Xu et al., 1998). Another evidence of GAs role in tuberization is that adding inhibitors of GAs biosynthesis (such as chlorocholine chloride, paclobutrazol, or ancymidol) enhances the tuber formation (Abdala et al., 1995; Vreugdenhil et al., 1994). Not only GAs synthesis/inactivation but also their transport within a plant or a particular organ might be significant factor. However, no clear picture of the mechanisms responsible for GA distribution is available. Quite recently, the SWEET facilitators, preferentially responsible for sugars transport, were proposed to be involved in GA transport (Kanno et al., 2016), which further complicates as yet unclear picture. GAs regulation of plant growth and development also interacts closely with other external or internal factors, *e.g.* temperature or photoperiod. For example the strictly photoperiod-dependent andigena potato plants with blocked GAs biosynthesis tuberize not only under short day photoperiod but also under non-inductive long days (Vandenberg et al., 1995). Moreover, the synthesis of GAs itself is photoperiodicaly regulated. GA20-oxidase (GA20ox) controls the crucial step in the bioactive GAs synthesis (Hedden and Thomas, 2012) and *StGA20ox1* gene expression is probably under control of phytochrome B (PHYB) since the level of *StGA20ox1* transcript was raised in the transgenic potato plants that have reduced the level of PHYB (Jackson et al., 2000). Inactivation of GAs usually goes through 2β-hydroxylation by GA2oxs. The role of *StGA2ox1* gene in tuberization was studied by Kloosterman et al. (2007). *StGA2ox1* showed higher expression during the early stages of potato tuber development prior visible swelling localized to the sub-apical stolon region and tuber growth took place. Plants overexpressing *StGA2ox1* had a dwarf phenotype, reduced stolon growth and earlier tuberization. Transgenic plants with reduced expression levels of *StGA2ox1* showed normal plant growth, an altered stolon swelling phenotype and delayed tuberization. The authors thereby proposed a role for *StGA2ox1* in early tuber initiation through modifying GAs levels in the sub-apical stolon region at the onset of tuberization (Kloosterman et al., 2007). In 2002, Martinez-Garcia et al. hypothesized that potato plants have two main tuber formation controlling pathways. The first one is GAs dependent and the second one photoperiod dependent (Martinez-Garcia et al., 2002). The above mentioned facts show that these two pathways are subjects of a vast crosstalk.

When we think about regulation of tuberization in its complexity we cannot neglect the plant energetic status since tuberization itself is energetically extremely demanding process. At this point photoassimilates come into play. Since the 1930s, there is a hypothesis that carbohydrate levels in the stolon tip control the onset of tuberization. Further, it becomes well known that increasing concentration of sucrose in the medium of *in vitro* cultivated plants securely enhances tuberization and sugars serve not only as the important source of energy but also as regulating agents (Claassens and Vreugdenhil, 2000). In the last decades, several experiments measuring actual sucrose content in stolons and in other parts of both *in vitro* and *in vivo* cultivated plants were performed with contradicting results. Ross et al. (1994) observed an increasing level of sucrose in stolon tips and developing tubers, whereas Vreugdenhil et al., (1998) found a decrease of sucrose level before visible stolon swelling. Later, Viola et al. (2001) measured sugar concentration along the whole length of the stolon tip during three developmental stages of the stolon (non-swelling, small swelling and large swelling stolons). All three situations showed similar pattern of sucrose concentration being highest at the stolon tip (more swelled stolon, more abundant sucrose) and its concentration gradually declining along the stolon longitudinal axis (Viola et al., 2001). Nowadays, the sugar role in potato tuberization regulation is relatively overlooked, with the exception of experiments with potato sucrose transporter StSUT4 which is expressed mainly in sink organs of potato and inhibition of its expression leads to early tuberization and increased tuber yield accompanied with higher efflux of soluble sugars from the source leaves to the growing tuber (Chincinska et al., 2008). Other recent studies touching the question of sugar involvement in tuber induction are mainly focused on the effect of phytohormone levels and their interactions (Aksenova et al., 2000; Kolachevskaya et al., 2017).

All the known facts taken together, there is an urging question remaining, concerning the two signals we are focusing at. Is gibberellin in charge or are sugars the main regulators? Xu et al. (1998) presumed that these two agents are controlling each other but their precise relationship remains unclear. After series of *in vitro* experiments comparing potato plants growing on various sucrose concentrations and measuring their GAs levels authors presented a hypothesis that it is in fact sucrose regulating internal GAs levels during the onset of tuberization (Xu et al., 1998).

Manganese-stabilizing protein (MSP) of photosystem II (PSII), also called PsbO, was found in two isoforms in potato (MSP-1 and MSP-2). MSP is crucial for PSII integrity as its function is to stabilize the Mn_4CaO_5 cluster and it is also needed for efficient water splitting (Yi et al., 2005). Interestingly, the loss-of-function *MSP* mutants can still grow photoautotrophically if there is still one isoform present. Arabidopsis mutants lacking one of the two *MSP* isoforms showed just slightly retarded growth and reduced PSII activity (Lundin et al., 2007) but cannot grow autotrophically when both *MSP* isoforms are silenced (Yi et al., 2007). In the previous study we characterized in detail a phenotypical features of spontaneously tuberizing potato mutant (ST) that was transformed with a gene-trap construct for random gene activation. Proteomic analyses revealed that the lack of one of *MSP* isoform in the mutant plants is the only known explanation of such complex phenotypic changes, like delayed senescence, increased carbohydrate content, basal branching, reduced rooting, and enhanced tuberization (Fischer et al., 2008). This phenomenon was further investigated by Gururani et al. (2012). They worked with potato plants with altered *MSP* expression (both enhanced and reduced). Plants with reduced *MSP* expression were photosynthetically more active and also showed enhanced tuberization and increased carbohydrate content. The results strongly suggest that altered photosynthetic machinery is possibly connected with early tuberization (Gururani et al., 2012).

Since the previous results with ST plants suggested altered carbohydrate partitioning between plant organs and also significant difference in total carbohydrate contents between ST plants and the control (Fischer et al., 2008), in the present work, we aimed to examine the sugar metabolism of ST plants grown mixotrophically, along with autotrophically cultivated *in vitro* plants. Parallel analysis of gibberellin content and crucial tuber-inducing marker gene expressions was performed that allow us to propose well-founded hypothesis of regulatory importance of these two key factors involved in the tuberization regulatory pathway/s.

1. Materials and methods

1.1. Plant material and growth conditions

Potato plants (*Solanum tuberosum* L.) cv. Lada and spontaneously tuberizing (ST) potato mutant (Fischer et al., 2008) lacking one isoform of manganese-stabilizing protein (MSP) of photosystem II (PSII) were used. The plants were cultivated *in vitro* from single node cuttings taken from four-week-old plants and *in vivo* from sprouting tubers. Hetero-

trophic plants were cultivated in 100 ml Erlenmeyer flasks covered with aluminium foil on solid LS medium (Linsmaier and Skoog, 1965) containing 2.5% sucrose. Photoautotrophic plants were cultivated in 250 ml Erlenmeyer flasks covered with PP autoclavable transparent foil bearing 2–3 PP filter disc (diameter 8 mm, porosity 0.04 μm) (Sigma-Aldrich, St. Louis, USA) enabling better gas exchange and light transmissibility for photosynthesis of the cultivated plants. The photoautotrophic plants were grown on solid LS medium with 0% sucrose. All plants were cultivated under a 16 h photoperiod with PPFD approximately 400–500 μmol m^{-2} s^{-1} (daylight fluorescent tubes; Osram, Wintherthur, Switzerland) in temperature range from 18 to 21 °C.

1.2. Chlorophyll a fluorescence

The chlorophyll a fluorescence was measured on mature leaves of 6–8 weeks old greenhouse cultivated plants grown from tubers. The measuring proceeded after 20 min dark adaptation, when leaves were covered with aluminium foil, with the portable fluorometer FluorPen FP100max (Photon System Instruments, Brno, Czech Republic). Chlorophyll a fluorescence efficiency of Photosystem II was quantified as F_m-F_0/F_m.

1.3. Photosynthetic pigments content

The 5–6 weeks old photoautotrophically cultivated plants were used. Cutouts from leaf blades 5 mm in diameter were plunged in dimethylformamide to extract the photosynthetic pigments (chlorophyll a and b and total carotenoids). During the extraction procedure the samples were stored in dark and cold (4 °C) environment. The concentration of pigments was determined using spectrophotometer Helios λ (Unicam, Cambridge, UK), at wavelengths 480, 647, 664, and 710 and calculated with the equations reported by Wellburn (1994).

1.4. Gibberellin content and distribution

The gibberellin contents were determined in basal and apical halves of the plants (ST and WT) grown *in vitro* on standard LS medium (2.5% sucrose and 0% sucrose) for five weeks. Samples of the weight 0.2–1 g were frozen in liquid nitrogen and transported to analysis on dry ice. The samples were analyzed for GAs contents using the modified method described in Urbanova et al. (2013). Briefly, fresh potato tissue samples of 15 mg were ground to a fine consistency using 3-mm zirconium oxide beads (Next Advance Inc., Averill Park, NY, USA) and a MM 301 vibration mill at the frequency of 30 Hz for 3 min (Retsch GmbH & Co. KG, Haan, Germany) with 1 ml of ice-cold 80% acetonitrile containing 5% formic acid as extraction solution. The samples were then extracted overnight at 4 °C using a benchtop laboratory rotator Stuart SB3 (Bibby Scientific Ltd., Staffordshire, UK) after adding 17 internal gibberellins standards ($[^2H_2]GA_1$, $[^2H_2]GA_3$, $[^2H_2]GA_4$, $[^2H_2]GA_5$, $[^2H_2]GA_6$, $[^2H_2]GA_7$, $[^2H_2]GA_8$, $[^2H_2]GA_9$, $[^2H_2]GA_{15}$, $[^2H_2]GA_{19}$, $[^2H_2]GA_{20}$, $[^2H_2]GA_{24}$, $[^2H_2]GA_{29}$, $[^2H_2]GA_{34}$, $[^2H_2]GA_{44}$, $[^2H_2]GA_{51}$ and $[^2H_2]GA_{53}$; purchased from professor Lewis Mander, Australia). The homogenates were then centrifuged at 36 670g and 4 °C for 10 min, corresponding supernatants further purified using ion exchange SPE cartridges (Waters, Milford, MA, USA) and analyzed by ultra-high performance chromatography-tandem mass spectrometry (UHPLC–MS/MS; Micromass, Manchester, U.K). GAs were detected using multiple-reaction monitoring mode of the transition of the ion $[M-H]^-$ to the appropriate product ion. Masslynx 4.1 software (Waters, Milford, MA, USA) was used to analyze the data and the standard isotope dilution method (Rittenberg and Foster, 1940) was used to quantify the GAs levels.

1.5. Carbohydrate content determination

The 5–6 weeks old plants were cut to individual parts – apex (apical part of shoot with 2–3 not fully developed leaves), developed leaves, medial part of the stem, basal part of the stem and roots. Samples (50–100 mg fresh weight) were taken from each part of the plants (3–5 plants per sample). The samples were immediately frozen in liquid nitrogen than freeze-dried, boiled in 80% methanol at 75 °C for 15 min, the solvent was then vacuum-evaporated and the residue was resuspended in ultrapure water. Then, the sonicated samples were purified by centrifugation and filtration. The content of non-structural soluble carbohydrates was determined using high-performance liquid chromatography (HPLC, flow rate 0.5 ml min^{-1}, column temperature 80 °C) with refractometric detection (refractive index range 1–1.75; refractometer Shodex RI-71; Spectra Physics – Newport Corporation, Irvine, USA), columns assembly: either Hema-Bio 1000 Q + SB pre-column and IEX Ca2+ column (Watrex, Prague, Czech Republic) or Shodex Sugar Column SC-LG 6 × 50 mm pre-column and Shodex sugar SC1011 column (Shodex, Tokio, Japan) according to Vitova et al. (2002). The starch in pellets remaining after the extraction of soluble carbohydrates was hydrolyzed by α-amylase (Fluka Sigma-Aldrich, St. Louis, USA, 30U) and amyloglucosidase (Fluka Sigma-Aldrich, St. Louis, USA, 60U) in Na-acetate buffer (pH 4.5), and the glucose content was measured by the HPLC (for details see Steinbachova-Vojtiskova et al., 2006).

1.6. Gene expression analysis

Total RNA was isolated from leaves of photoautotrophically cultivated plants (in three biological replicates) using the TRI reagent isolation protocol (Sigma Aldrich). RNA integrity was checked by RNA denaturing agarose gel electrophoresis (Masek et al., 2005). Eight micrograms of total RNA and a mixture of oligodT (0.5 μg)/random hexamers (0.1 μg) primers were employed for cDNA synthesis using 1 μl of RevertAid Reverse Transcriptase (Thermo Scientific) in 20 μl reactions.

StBEL5, *POTM1* (*AGL8*) and *StSP6A* relative mRNA levels were determined by qPCR in LightCycler 480 (Roche) using LightCycler 480 SYBR Green I Master (Roche) in 10 μl reactions; each sample in 3 technical replicates. We utilized *polyubiquitine* and *EF1alpha-like* mRNAs as reference transcripts for data normalization. To avoid possible genomic DNA amplification, we designed primers spanning over exon junctions whenever possible or annealing to distantly positioned exons. Moreover, we supplied control reactions without reverse transcriptase in each experiment in order to check no genomic DNA amplification took place. We used the following primer sets: *StBEL5*, Acc. No.: AF406697, forward primer: GCT AAC AAG GAG CCA GGT GT, reverse primer: ATG TTT CTC TTC ATT TGG AGC ACT; *POTM1*, Acc. No.: NM_001288213, forward primer: CAG CTT TCC AAG AAG GTG AAG, reverse primer: CCA AGG TGA GGA GAG TCC AAT; *StSP6A*, Acc. No.: DQ111027, forward primer: CAA CTT TTA CAC TCT GAT TAT GGT G, reverse primer: GCT TGT ATT TGT AGT TGC TGG GA; *EF1alpha-like*, Acc. No.: NM_001288491, forward primer: GAT TTG CTG CTG TAA CAA GAT GG, reverse primer: GGA GAT GGG GAC GAA TGG G; *polyubiquitine*, Acc. No.: XM_006360024, forward primer: CTT CAA ATT TCT CTT TCA AGA TGC AG, reverse primer: AGC CTT TGC TGA TCC GGG G. The following amplification programme was applied: initial template denaturation, 95 °C 5 min; then 40 cycles of denaturation, 95 °C 10 s, primer annealing, 58 °C 20 s, and extension, 72 °C 15 s, followed by melting curve analysis, 97 °C 10 s, 65 °C 1 min, then data acquisition during slow warming to 97 °C (ramp rate: 0.11 °C/s, 5 signal reads per °C). The LightCycler 480 sofware (version 1.5) was applied to perform relative quantification analysis with the following settings: second derivative max quantification algorithm, mean Cps used, all-to-mean pairing mode, and standard curves in-run. The specificity of primer pairs and expected lengths of amplicons were verified by both melting curve analyses and agarose electrophoresis.

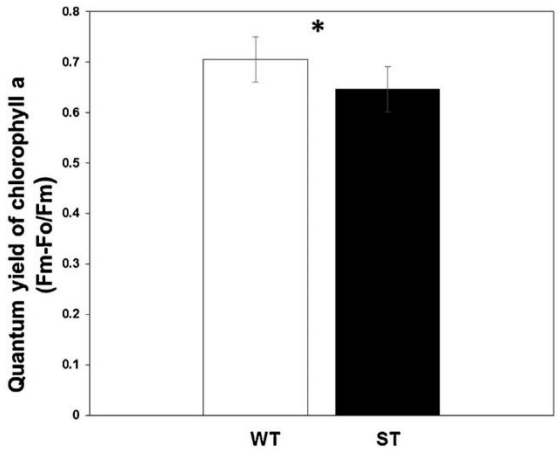

Fig. 1. Chlorophyll a fluorescence of *in vivo* cultivated WT and ST plants, bars refer to standard errors, * = significant difference at $\alpha = 0.05$, n = 8.

1.7. Statistical analysis

All data were analyzed with NCSS 9 statistical software (NCSS, LLC. Kaysville, Utah, USA). Analysis of variance (ANOVA) and Tukey-Kramer Test (for normally distributed data) or Kruskal-Wallis Multiple-Comparison Z-Value Test (for data not normally distributed) were used. The differences were examined at $P \leq 0.05$ and $P \leq 0.01$ levels.

2. Results

2.1. Chlorophyll a fluorescence under in vivo conditions

The spontaneously tuberizing (ST) potato plants (Fischer et al., 2008) used in this study lack the mangan stabilizing protein (MSP) of photosystem II (PS II). Therefore, we started our analyses with the measurement of chlorophyll a fluorescence of greenhouse cultivated WT and ST plants and found that ST plants had significantly lower quantum yield of chlorophyll a fluorescence (Fig. 1). That indicated that primary photosynthetic reactions are affected and ST plants have lower numbers of open reaction centers.

2.2. Growth parameters of in vitro cultivated plants

Previously conducted experiments (Fischer et al., 2008) showed the effect of various sucrose concentrations on ST tuberization potential. There was a dramatic gap between 3% sucrose and 2.5% sucrose content in the cultivation media where on the former majority of plants formed tubers or basal tuber like swellings (Fig. 2) and on the latter only 12 percent of plants formed tuber. We used plants growing under *in vitro* conditions with sugar supply (2.5% sucrose) to reduce the stress related changes in photosynthesis and still we found big differences in the phenotypes of WT and ST plants. ST plants had very short shoots (Fig. 2A) of significantly lower fresh weight than WT (Fig. 2B). Their stems and leaves were dark green to purple whereas those of WT plants were light green. Big difference was also found in the root system size and morphology (Fig. 2A). WT plants formed more branched adventive roots than ST and their whole root system was well-developed while ST plants barely formed any roots (Fig. 2A).

2.3. Gibberellins content of in vitro cultivated plants

Short plant phenotype and frequent tuber forming could indicate lower gibberellins (GAs) content. We measured GAs contents in the basal and apical parts of WT and ST plants cultivated under the standard *in vitro* conditions. Surprisingly, the difference in the average total GAs content we found was orderly higher in both basal and apical parts of ST plants than in WT plants (the detected GAs were following: GA_1, GA_3, GA_4, GA_5, GA_7, GA_8, GA_9, GA_{15}, GA_{19}, GA_{20} and GA_{29}) (Fig. 3A). We divided detected GAs into three groups – precursors of bioactive GAs ($GA_{9,\ 19}$ and $_{20}$), bioactive GAs ($GA_{1,\ 3,\ 4,\ 5}$ and $_7$) and inactivated GAs (GA_8 and $_{29}$). Most abundant group comprised inactivated GAs followed by precursors of active GAs. When we examined bioactive GAs contents only, we found the ST plants to contain more GA_1, GA_3, GA_4, GA_5 and GA_7 than those of WT plants (Fig. 3B). In conclusion, we found substantially higher GAs contents in spontaneously tuberizing potato in all categories, most importantly also in the group of bioactive compounds. Further, we tested the response of ST plants to exogenous application of GA_3. The experiment was performed under moderate sucrose supply (3%), leading to no tuber formation in WT. Under these conditions approx. 75% of ST plants formed tubers. Importantly, ST tuberization was completely inhibited by application of GA_3 in all concentrations tested (0.1, 0.05 and 0.01 M).

2.4. Growth parameters of photoautotrophically cultivated plants under in vitro conditions

In the next experiments, we attempted to exclude the effects of external energy and carbon supply. We used our innovated system of air permeable vessel closure for Erlenmeyer flasks which enabled autotrophic *in vitro* cultivation of plants and could provide useful information on experimental material grown photoautotrophically with still making use of the benefits of *in vitro* cultivation. Although the two genotypes resembled each other more, there were still differences in plant phenotypes: ST plants had shorter shoots with deeper color (Fig. 2C). The ST plant root system was better developed than under mixotrophic conditions but still with significantly less and shorter adventitious roots (Fig. 2C).

2.5. Photosynthetic pigments content

ST and WT plants showed phenotypic differences not only in their habitus but also in coloration, both under mixotrophic and photoautotrophic conditions. ST plants were dark green or sometimes even purple in contrast to light green WT plants indicating changes in pigment content and proportion. Photoautotrophically cultivated plants were expected to resemble the *in vivo* cultivated ones more, and therefore photosynthetic pigments contents were quantified per leaf area unit. We found significantly higher chlorophyll a, carotenoid and xanthophyll contents in ST plants. Although there was similar trend in chlorophyll b, the difference was not significant. The total chlorophylls content naturally proved to be significantly higher at ST plants (Fig. 4).

2.6. Gibberellins contents of photoautotrophically cultivated plants in vitro

As WT and ST plants cultivated autotrophically have more similar phenotypes than those cultivated mixotrophically we were interested whether there were any differences between ST and WT in GAs contents. Photoautotrophic ST plants had approximately the same average GAs contents as WT (detected GAs were following: GA_1, GA_3, GA_4, GA_5, GA_6, GA_7, GA_8, GA_9, GA_{13}, GA_{15}, GA_{19}, GA_{20}, GA_{24}, GA_{29}, GA_{34}, GA_{44}, GA_{51} and GA_{53}) (Fig. 5A). Unlike mixotrophically cultivated plants, both WT and ST cultivated photoautotrophically had GAs precursors as the most abundant group (Fig. 5B). There were large differences in the proportion of individual GAs belonging to particular GA groups under photo- and mixotrophic conditions (see supplementary Table 1). Importantly, no decrease in GA contents was detected in ST plants under photoautotrophic conditions compared to WT.

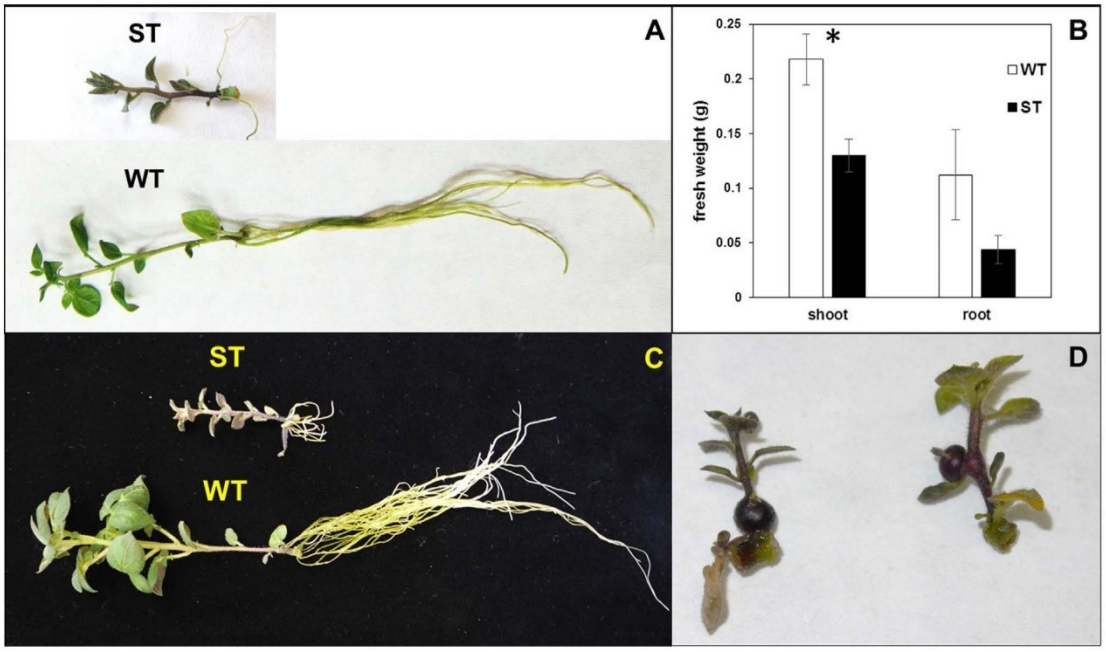

Fig. 2. WT and ST plants cultivated under various conditions *in vitro*. A) 5 weeks old mixotrophic plants, B) fresh weight of shoot and roots of mixotrophic plants, bars refer to standard errors, * = significant difference at α = 0.05, n = 5 (5 weeks old plants), C) 6 weeks old photoautotrophically cultivated plants), D) stem tubers and sessile tubers on 6 weeks old mixotrophic ST plants.

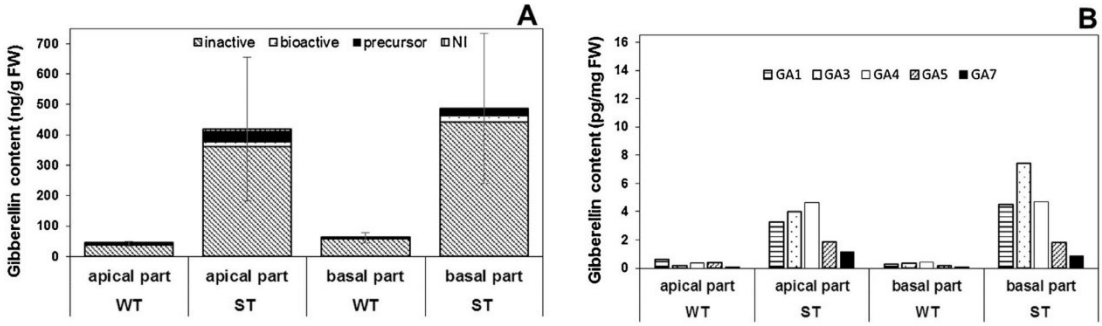

Fig. 3. Gibberellins contents in apical and basal parts of WT and ST plants cultivated *in vitro*. A) Contents of total GAs divided into functional groups, bars refer to standard deviations of total average GAs contents, NI = GAs without identified function, B) contents of bioactive gibberellins (GA_1, GA_3, GA_4, GA_5 and GA_7), n = 3-5.

2.7. Carbohydrate content and distribution

High C and energy source availability is regarded as an important condition for tuberization induction, which has been under *in vitro* provided by high sugar content in growth medium. The observed ability of ST to tuberize under low sugar supply led us to analyze carbohydrate balance and distribution in the material under study. In order to find out in detail how ST plants manage their carbohydrate metabolism, we determined sugar contents in individual parts of WT and ST plants grown mixotrophically *in vitro* on tuberization non-inducing medium. We did not find any significant differences in the total amounts of sugars in the individual above-ground parts (Fig. 6A). However, we observed trends showing that ST plants have higher total sugar content in apices and leaves. Medial and basal parts of the stem have the total sugar levels more or less similar in WT and ST. We found some differences in the relative proportions of individual sugars in particular organs (Fig. 6B) – substantial difference was found in basal parts of the shoots, where the ST plants accumulated significantly higher amount of sucrose than glucose and fructose. In leaves, ST plants had significantly more fructose than WT plants. Large differences were found in roots. It seems that ST plants preferably accumulated sucrose in the roots whereas WT plants preferred hexoses such as glucose and fructose.

We also searched for any deviation in starch contents and we found significant difference in starch deposition between individual parts of the ST and WT plants. Most distinctive difference was uncovered for leaves and apical parts of the plants (Fig. 7), where WT contained significantly more starch than ST plants. In contrast, most of ST plants deposited twice as much starch in the basal part of the shoot than in any other organ. In general view, we could see an interesting difference between ST and WT plants starch distribution – WT plants had more or less the same starch levels within the whole plant body whereas ST plants deposited starch unevenly, preferentially into basal part of the stem.

Sugar supply from the medium substantially influences carbohydrate distribution within a plant. In photoautotrophically cultivated cultures with no sugar added to the medium, plants contained

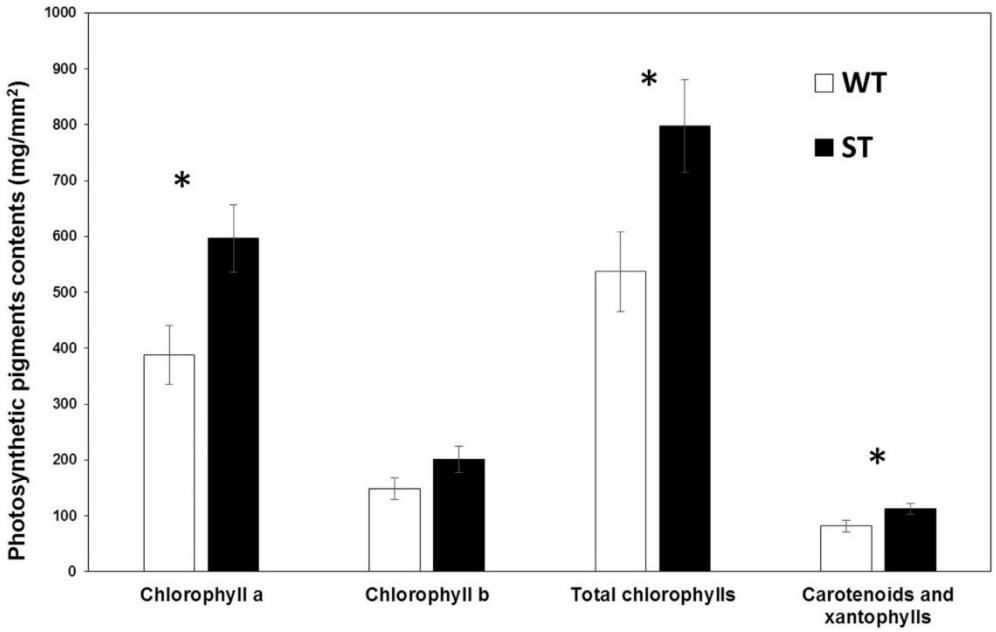

Fig. 4. Photosynthetic pigments contents in leaves of photoatotrophically cultivated WT and ST plants, bars refer to standard errors, * significant difference at $\alpha = 0.05$, n = 12.

significantly less sugars than their mixotrophically cultivated counterparts. Notable difference between ST and WT plants was found in the distribution to basal parts of the shoot and roots where we found significantly higher levels of sugars in ST plants (Fig. 8A). Significant difference was also observed in the proportions of carbohydrates in the spectra (Fig. 8B), ST plants contained more sucrose in the basal parts of the shoots while WT accumulated evenly sucrose and fructose. The opposite situation was in the leaves where we found a predominance of sucrose in WT plants.

In contrast to soluble carbohydrate levels differing substantially between mixo- and autotrophic cultures, the starch amounts and the pattern of starch deposition were very similar under both conditions (Fig. 7 and 9). In WT, the starch levels were comparable within plant body, while in ST the starch was distributed variably with the highest content in roots and basal parts of shoots and the lowest in leaves and apices.

2.8. Expression of key marker genes for tuber induction

As ST plants exhibit higher tuberization potential together with changed distribution of saccharides favouring starch accumulation in basal part of the plant we wondered whether there is also any change in expression of genes connected to tuber induction. For analysis, leaves of photoautotrophically cultivated plants were chosen as we assumed no/minimum deviations from natural conditions in phloem transport direction and intensity under these conditions. We determined two genes (*StSP6A* and *StBEL5*) producing mobile tuber inducing signals whose mobility is tightly bound to saccharide flow by phloem and one common downstream target gene (*POTM1*). *StSP6A* transcript level in ST plant leaves is significantly increased, while *StBEL5* transcript is decreased in ST plants (Fig. 10). The level of *POTM1* target transcript does not differ between WT and ST.

3. Discussion

The potato tuberization is characterized by significant morphological changes accompanied by biochemical switches on the whole plant level. The developing tubers become the largest plant sinks and therefore influence many of physiological processes of the plant (Oparka, 1985). The tuberization itself comprises complicated series of events which can be regulated independently but are also subjects of a vast crosstalk. Tuberization enhances CO_2 assimilation in the leaves

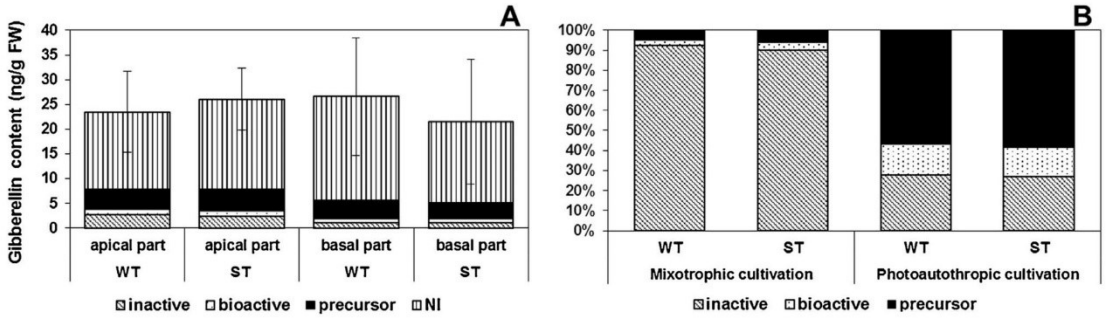

Fig. 5. A) Contents of GAs divided into functional groups, bars refer to standard deviations of total GAs contents, n = 3-5, B) relative proportions of gibberellins belonging to different functional groups.

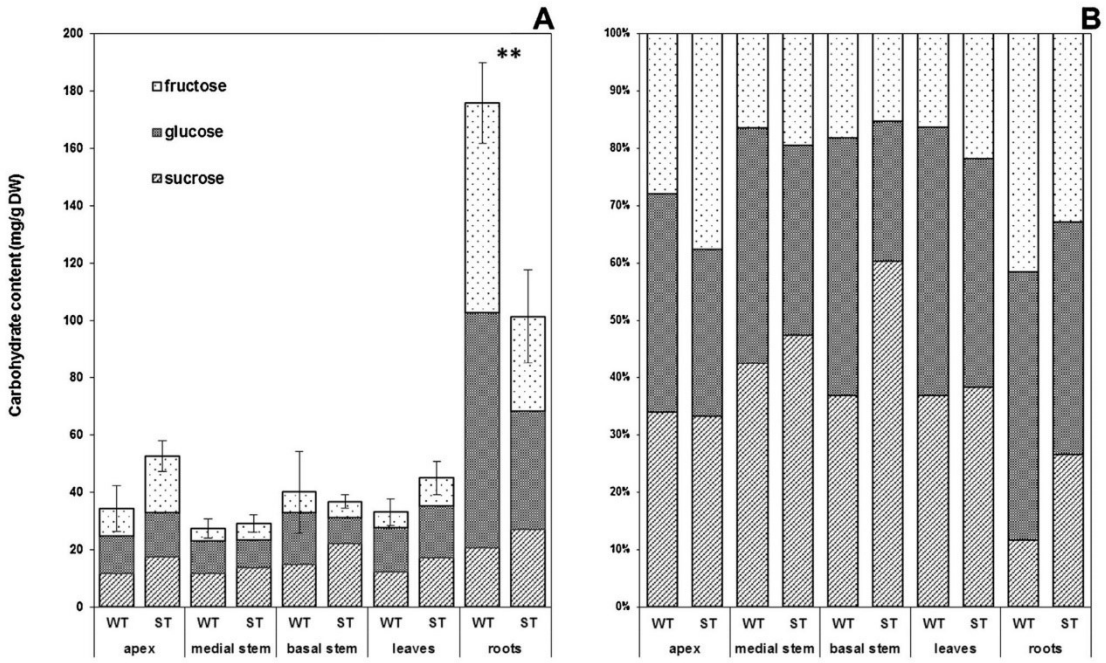

Fig. 6. A) Sugar content and B) relative sugar propotions in individual parts of WT and ST plants cultivated mixotrophically *in vitro*, bars refer to standard errors of the total carbohydrate content, ** significant difference at $\alpha = 0.01$, n = 5-9.

that enables increased transport of carbon to the developing tubers and subsequent starch deposition (Lorenzen and Ewing, 1992; Vanes and Hartmans, 1987), connected with an explicit switch from an invertase-dominated sucrolytic system to that dominated by sucrose synthase (Ross et al., 1994).

Our model – ST (spontaneously tuberizing) potato lacks one of the genes coding for mangan stabilizing proteins (MSP) (Fischer et al., 2008) and exhibit significant changes in chlorophyll a fluorescence (Fig. 1) pointing to disturbed early stages of photosynthesis light reactions. Similar results obtained Gururani et al. (2012) with potato plants with altered MSP expression. The ST plants cultivated under *in vitro* mixotrophic conditions showed no dramatic differences from WT in the contents of carbohydrates (Fig. 6A), nevertheless, there were striking differences in carbohydrate distribution. The ST plants were also markedly smaller with shorter internodia (Fig. 2A and C). These facts led us to a question whether other important regulators of tuberization cannot be involved – gibberellins.

The role of gibberellins (GAs) as tuberization inhibitors was well established during the 1980s. The work of Koda and Okazawa (1983) showed that GAs endogenous levels were quite high at the stolon tips of potato plants (cv. Irish Cobbler) and were dropping drastically in the moment when the stolon tip started to swell and transform into a tuber and henceforth GAs levels remained low during tuber formation (Koda and Okazawa, 1983). These findings were further supported in the next decades due to fast development of quantitative and qualitative analytical methods (Malkawi et al., 2007; Xu et al., 1998) and GAs

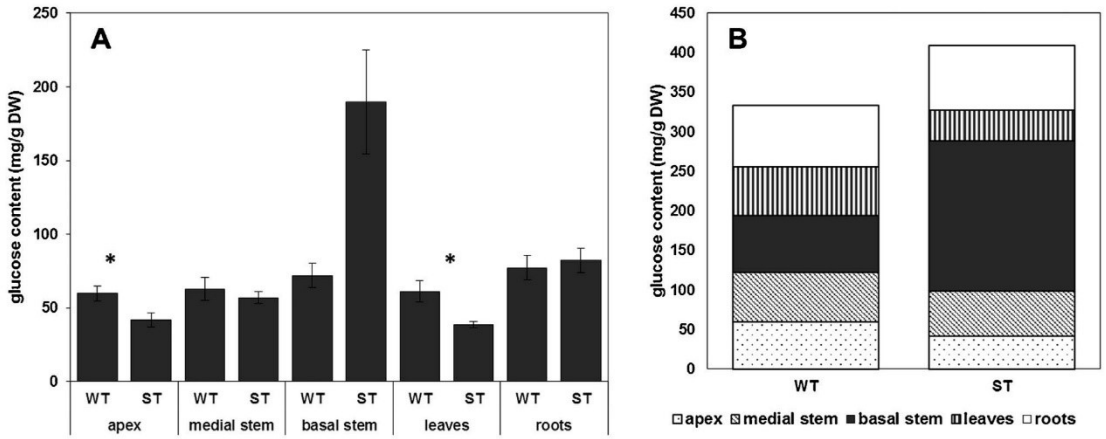

Fig. 7. A)Starch contents (given as glucose amount after enzymatic digestion) in individual parts and B) proportional view of starch content in individual parts of mixotrophically cultivated WT and ST plants, bars refer to standard errors, * significant difference at $\alpha = 0.05$, n = 5-9.

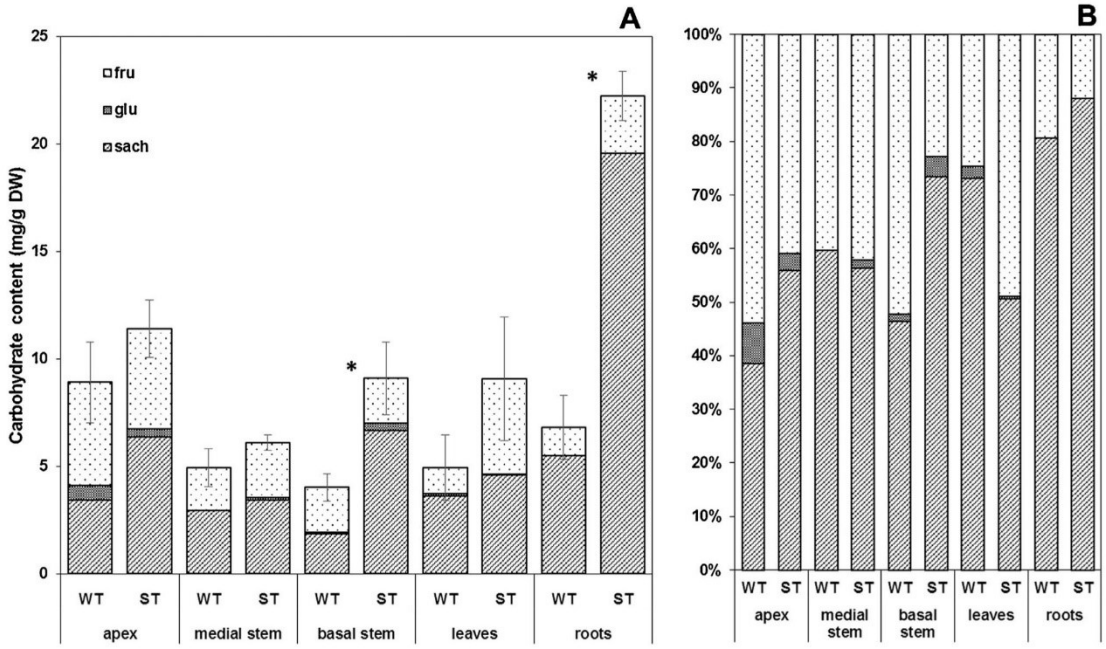

Fig. 8. A) Sugar content and B) relative sugar propotions in individual parts of photoatotrophically cultivated WT and ST plants, bars refer to standard errors of the total carbohydrate content, * significant difference at $\alpha = 0.05$, n = 3–14.

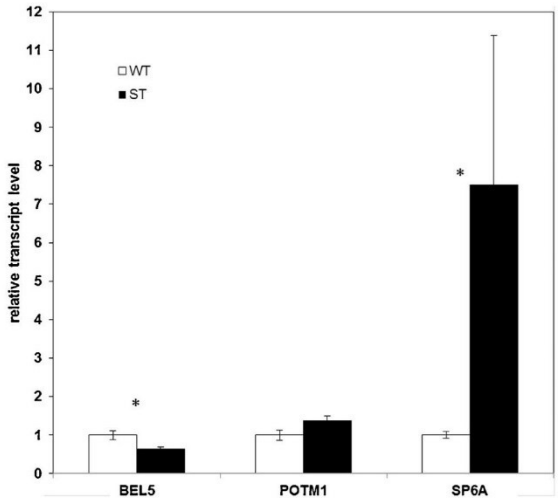

Fig. 10. Relative StBEL5, POTM1 and StSP6A transcript levels in ST to WT leaves of photoautotrophically cultivated plants. Each analyzed transcript was normalized against two reference mRNAs encoding for polyubiquitine and EF1alpha-like protein. Relative mean expression levels were normalized to 1 in WT plants for each target transcript. Bars refer to standard errors, * significant difference at $\alpha = 0.05$, n = 3 (biological replicates).

are now widely accepted as the main tuberization inhibitors. Therefore, the lower levels of gibberellins might be supposed as the most straightforward explanation of sugar-supply independent high potential of tuber formation in ST. Further, as showed by Kloosterman et al. (2007) potato plant readiness to form tubers can be associated with lower GAs due to higher *StGA2ox1* expression. Our measurements, however, clearly proved that ST plants have not lower GAs content than WT (Figs. 3 and 5). In the case of mixotrophically cultivated plants we found even four times higher GAs contents in both apical and basal parts of ST plants compared to those of WT plants (Fig. 3). However, the most distinct amount in ST plants (90% of all GAs) comprised inactivated ones, suggesting higher GAs turnover. Importantly, Xu et al. (1998) stated that in potato low GAs levels are tied with high sucrose levels and *vice versa*. Nevertheless, we found that ST plants contain not only high levels of GAs but also elevated levels of sucrose, at least in roots and basal parts of the stems. It looks like whatever connection between GAs, sucrose and tuberization is, it is somehow disturbed in ST plants, which makes them an interesting biological model. Functional connection of light, sugar and gibberellin networks in developmental processes regulation was recently proposed by Rabot et al. (2014). Our results on sucrose along with GAs levels thus endorse the role of sugars, which might beat GAs and start the tuberization process even though massive amounts of GAs are present. However, for technical reasons, the distribution of GAs was not followed as thoroughly as for sugars and thus the important question remains without clear and unambiguous answer.

The finding that in our model plants sugars might be of greater importance than GAs led us to decision to take a closer look on sugar distribution within the plant body. Precise knowledge of sugar action during the whole process of tuber onset and formation is important to have but not easy to obtain since sugars can play multiple roles in this process that is very difficult to distinguish one from the other. Sucrose represents the main driving force regulating the direction and intensity of phloem flow from source to sink (Carpaneto et al., 2005). On the other hand, sucrose itself can also generate signal (Smeekens et al., 2010) governing various developmental processes including tuberization and generally all carbohydrates can affect the transcription of carbohydrate metabolizing enzymes genes (Krapp et al., 1993; Rook et al., 2006).

Under *in vitro* conditions, the induction of tuberization can be achieved in most potato genotypes, including our WT plant, by increasing the availability of sucrose from the medium under short day photoperiod or darkness. ST potato mutant is, however, able to form tubers growing on much lower sucrose concentrations under LD

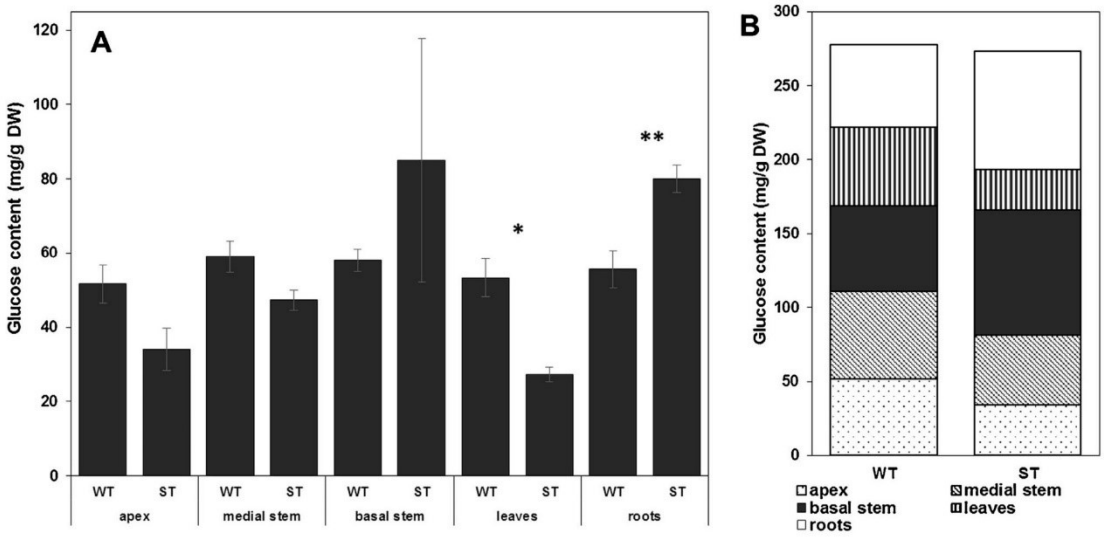

Fig. 9. A) Starch contents in individual parts and B) proportional view of starch content in idividual parts of photoautotrophically cultivated WT and ST plants, bars refer to standard errors, * and ** significant difference at $\alpha = 0.05$ and 0.01 respectively, n = 5-13.

photoperiod. As both, relatively photoperiod insensitive, ST line and SD-dependent cv. Taedong valley potatoes (Gururani et al., 2012) exhibit similar response to MSP absence, we propose that other part of tuberization control network, not photoperiod regulation, is affected. It has been shown that ST plants contain higher levels of both soluble sugars and starch than WT when cultivated mixotrophically on sugar enriched medium with 3% sucrose (Fischer et al., 2008). Under our slightly modified cultivation conditions, we observed similar trend, but the differences were not significant (Fig. 6A). The results on WT are consistent with our knowledge of how plants cultivated *in vitro* in general deal with the sucrose supply from the cultivation medium. Sucrose, similar to other nutrients, is usually added into the cultivation medium in excess to ensure the plants have sufficient carbon source for healthy growth. ST plants also have substantial amount of soluble sugars present in the roots although they translocate more of sucrose to the shoot, especially to the basal part where we found significantly higher sucrose content in ST plants compared to WT and where ST plants form stem tubers with high frequency (Fig. 2D). In consideration of the previously mentioned facts, the higher amounts of starch deposited in basal parts of ST plants (Fig. 7) is no surprise as Ross et al. (1994) suggested that the onset of starch synthesis in not yet tuberizing stolons correlated with sucrose import rather than with stored hexoses pool (Ross et al., 1994).

Our results combined with the fact that ST plants have impaired early steps of photosynthesis led us to creation of cultivation system that allowed to examine plants growing photoautotrophically under *in vitro* conditions where we expected deepening of the difference observed in both plant genotypes under mixotrophic conditions. Our improved cultivation system uses handmade gas and light permeable covers for Erlenmeyer flasks enabling us to study plants, including their carbohydrate status, in less artificial conditions than provide the standard *in vitro* cultivation systems. The plants cultivated under photoautotrophic conditions more resemble *in vivo* cultivated plants (Fig. 2C) and they can be used for a wide spectrum of analyses all year around, cultivated under the same other conditions as mixotrophic plants. Except for the roots, the pattern of distribution of soluble sugars was similar under both cultivation conditions (Fig. 8A). Especially interesting was sugar content in basal part of the stem where the differences were more pronounced – ST plants contained significantly higher amounts of glucose and especially sucrose than WT plants. ST plants were not only showing spontaneous tuberization but in addition they formed sessile tubers. Quite often stem basal parts of ST also exhibited tuber-like swellings. One can speculate that since sucrose itself can generate a signal regulating diverse morphogenetic processes like flowering and tuberization (Rolland et al., 2006; Smeekens et al., 2010) and sucrose signaling can be also involved in regulation of cell division leading to stolon swelling (Roitsch, 1999) there might be a possibility that we are observing sucrose driven tuber formation within ST plants. Knowing the regulatory mechanism behind increased sucrose effect is another burning question. It is possible that mobile signaling molecules are involved here, for example StSP6A – FT (FLOWERING LOCUS T) orthologue and *StBEL5,* which can alter gene expression at stolons, for example enhancing SUT1 expression, and enhancing the strength of stolon sink (Sharma et al., 2016). The swollen basal parts of stems can influence the whole plant physiology, in our case by limitation of sugar transport to apical parts of the plants, similarly to the previously observed phenomena of tubers in different stages of development on one and the same plant influencing the development of each other (Struik et al., 1991). Sucrose itself can act as signaling molecule, but the whole nature of sucrose signaling system remains unclear. Recently, sucrose synthase has been suggested as important component of sucrose signalisation. Nguyen et al. (2016), proposed the existence of a novel sucrose signaling pathway based on an experiment where they overexpressed sucrose synthase in tobacco and put their material through the conditions simulating a transition from heterotrophic to autotrophic growth. However, most points of this sucrose signaling pathway are still waiting for clarification. Sucrose transporters are another possible molecules used in sucrose signaling, for example SUT1 (Kuhn and Grof, 2010). What is also worth mentioning is the changed pattern of starch deposition within ST plants body (Fig. 9). Whereas WT plants store starch evenly across the plant body, ST plants have significantly lower starch contents in leaves and apical parts of the stem but most interestingly almost 3-fold higher starch amounts in basal parts of the stem compared to WT. This is consistent with the notion that the swellings at basal parts of ST stems, occurring frequently on mixotrophic and sometimes also on phototrophic plants, are of tuber nature. What makes ST plants quite different either from WT or usually observed plant behavior is the way they deal with carbohydrate stockpiles. Whereas plants usually choose to store either starch or soluble sugars, in some parts of photoautotrophic ST plants we

found high concentrations of both starch and soluble sugars simultaneously.

Shift in saccharide distribution along plant body may induce changes in transport or production of phloem-mobile signals and/or modification of mobile signal(s) production in leaves may strengthen saccharide allocation to stem base. Higher *StSP6A* transcript levels and slightly lower *StBEL5* transcript levels found in ST plants (Fig. 10) led us to following interpretation: Intervention in the primary photosynthetic phase in ST plants is inducing positive signalization towards *StSP6A* expression and negative signalization towards *StBEL5* expression in leaves. Interestingly, although promoter region of *BEL5* gene possesses numerous light-responsive cis-acting elements, *BEL5* transcript levels are influenced neither by photoperiod nor by irradiance level but by light quality (Chatterjee et al., 2007). Thus, to interpret the connection of *StBEL5* to primary photosynthesis in our system remains questionable.

Further, *StSP6A*, a member of *FLOWERING LOCUS T* (FT) gene family, is known to be subjected to similar upstream regulatory pathways, so knowledge regarding *FT* gene expression regulation could be relevant. King et al. (2008) showed that in Arabidopsis, irradiance level as well as assimilation rate influence *FT* expression independently of phytochrome signalization, which demonstrates that there are two at least partly independent light-response mechanisms regulating flowering via *FT*. Consistently with this, very recently Feng et al. (2016) published a study documenting a retrograde signalization from chloroplast to nucleus leading to flower induction through repression of *FLC* floral integrator. This repressor regulates in Arabidopsis, besides others, also the *FT* expression. The published retrograde signalization is driven by the size of reduced plastochinone pool, directly reflecting photosystem II efficiency. When regarding analogous upstream regulatory pathways controlling potato *SP3D* (*FT* orthologue) and *StSP6A* induction, one can speculate also about such a possible chloroplast signalization towards *StSP6A* induction. Nevertheless, further detail studies are needed to verify such an attractive premise.

4. Conclusion

Our results, showing spontaneously tuberizing (ST) potato plant, cultivated both mixotrophically and photoautotrophically, with higher or similar GAs content comparing to WT and disrupted carbohydrates distribution do not support formerly proposed hypothesis, that GAs are inhibiting tuberization downstream of the inducing effect of sucrose (Fischer et al., 2008). Our recent findings, however, are rather pointing out to two parallel pathways by which GAs and sucrose regulate tuber onset. That way, once the plants are cultivated on high GAs concentration, the tuberization can be prevented completely. On the other hand, the addition of high amounts of sucrose to the media induces opposite reaction – massive tuber formation even in presence of high endogenous GA contents. Once GAs and sucrose are at physiological levels, very fragile balance arise. ST plants with strongly negatively affected early stages of photosynthesis may be trying to maintain this balance by investing most of newly synthesized sugars into GAs synthesis to promote growth with the aim to form new photosynthetically active leaves or simply outgrow the bad conditions. Once this strategy is not being successful, the plants resort to the only solution potato plants have, to survive bad conditions – forming tubers and therefore secure new plant generation.

Last two decades have brought many interesting findings and increased our knowledge about mechanisms regulating tuberization (*e.g.* potato FT orthologue involved in controlling tuberization and connection between StSP6A regulation and BEL5 family of transcription factors). However, interaction between carbohydrate balance, surely an important player in tuberization induction and other signaling pathways has not been thoroughly studied. Based on the achieved results, including those of elevated *StSP6A* in ST plants, it is tempting to speculate that the crosstalk between saccharide- and gibberellin-dependent pathways is mediated at least partly by *StSP6A*. This hypothesis, however, needs experimental validation.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jplph.2017.04.003.

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5.1.1. Statement of contribution

HL, HS and PM conceived and designed the experimental design. HS conducted the majority of the experiments and sample and data analyses. DT measured the GAs content. PM and TM performed the gene expression analysis. HS summarized the results and wrote the manuscript. HL and PM edited the manuscript and put valuable insights into it.

5.2. Publication 2 – Ševčíková et al., submitted

Original research article, submitted to Physiologia Plantarum (IF_{2017}=3.33)

The method of PA *in vitro* cultivation was officially confirmed as a certified methodology by Czech Ministry of Agriculture (Appendix 1).

Mixotrophic *in vitro* cultivations: the way to go astray in plant physiology

Hana Ševčíková*, Zuzana Lhotáková, Jaromír Hamet, Helena Lipavská

Department of Experimental Plant Biology, Faculty of Science, Charles University, Viničná 5, 12843, Prague, Czech Republic

** For correspondence. E-mail:* hana.sevcikova@natur.cuni.cz

Abstract:

Rate of photosynthesis and related plant carbohydrate status are crucial factors affecting plant vigour. It is a well-known fact, nowadays, that sugars not only provide the energy source and building blocks of the plant body but also serve as important signalling molecules governing plant growth and development (together with other signals) through a complex regulatory network. These facts are often neglected when mixotrophic cultivation of plants *in vitro* is used. Despite the undoubted advantages the technique has, there is one major disadvantage – artificial supply of sugar from cultivation media hinders studies of metabolism as well as sugar driven developmental processes. We compared the growth, selected gas-exchange parameters and sugar metabolism characteristics in four model plants, potato (*Solanum tuberosum* 'Lada'), tobacco (*Nicotiana tabacum* 'Samsun'), rapeseed (*Brassica napus* 'Asgard') and strawberry *(Fragaria vesca*), under both mixotrophic (MT) and photoautotrophic (PA) conditions. To ensure PA conditions we used our improved sun caps that serve as gas and light permeable covers for cultivation vessels. Using thorough comparison of numerous plant growth and carbohydrate metabolism characteristics (e.g. carbohydrate status, biomass allocation and photosynthesis activity of *in vitro* grown leaves) of plants cultivated under MT and PA conditions we found strongly species-

dependent reactions to exogenous sugar supply not allowing to create any general view on the overall impact of mixotrophic nutrition under *in vitro* conditions.

Abbreviations:

gs, stomatal conductance; MT, mixotrophic cultivation; PA, photoautotrophic cultivation; Pn, photosynthetic rate; PPFD, photosynthetic photon flux density; SnRK1, SNF1-related kinase 1

Introduction:

Plant mixotrophic (MT) *in vitro* cultivation is a widely used standard technique that is very important for commercial plantlet propagation of many plant species as well as a frequently used tool in the plant physiology research. The plants cultivated in this system have heterotrophic or mixotrophic metabolism mostly due to the high sugar concentration in the culture medium, the high relative humidity in tightly closed vessels without gas exchange and are often cultivated under low irradiance in cultivation chambers (Kozai and Kubota 2001, Kozai 2010). Carbohydrates supply metabolic energy and carbon skeletons for the biosynthesis of the organic compounds necessary for cell growth and act as an osmotic component in the culture medium. However, high sucrose amounts added to the culture medium can cause starch and sucrose accumulation in the leaves, which can inhibit the activity of the Rubisco enzyme (Hdider and Desjardins 1994) or reduce chlorophyll synthesis (Kirdmanee et al. 1992). Mixotrophically cultivated plants often tend to hyperhydricity due to the high relative humidity in tightly closed vessels, which is also closely connected with non-functional stomata (Chakrabarty et al. 2006). Importantly, these characteristics cause problems during transfer of plants to *ex vitro* conditions (Xiao et al. 2011). On the other hand, MT cultivation is considered to be economically efficient and has relatively low demand for cultivation chamber technologies, and there is a large range of standard cultivation protocols for a huge variety of plants.

Photoautotrophic (PA) cultivation is regarded as a more complicated arrangement of *in vitro* plant growing that requires ensuring a number of difficult conditions, such as proper light and gas exchange (Xiao et al. 2011). Nonetheless, it

provides several advantages over MT cultivation. When cultivating plants photoautotrophically, one can achieve enhanced growth of the plantlets, lower contamination levels (Zobayed et al. 2000), and reduced dependence on exogenous growth regulators (Khan et al. 2002). An improved plant photosynthetic capacity can also lead to optimization of the growth of the plant material propagated *in vitro* during acclimatization to *ex vitro* conditions (Xiao et al. 2011). A photoautotrophic metabolism can be induced in *in vitro* propagated plants by the alteration of the culture environment, for example by modification of the quantity and quality of the light that reaches the plantlets and by modifying the gas exchange (Aragon et al. 2010). That can be achieved by changing the type of closure on the culture flask. Under hermetic conditions and in smaller-sized flasks, ethylene accumulates, having mostly negative impacts on *in vitro* plant growth (Zobayed et al. 2001). Carbon dioxide (CO_2) concentration, on the other hand, fluctuates during the day cycle (Hoang et al. 2017). The PA cultivation vessel can be enriched with the carbon dioxide using membranes positioned in the flask lid (in a culture chamber with a high CO_2 concentration) or by direct CO_2 injection into the culture flask (Kitaya et al. 1995, Saldanha et al. 2012). Elevated CO_2 concentration can improve *in vitro* plant performance in ventilated systems (Cha-um et al. 2011).Another approach of altering the *in vitro* environment to improve photosynthetic competence is to reduce or exclude sucrose from the culture medium (Khan et al. 2002, Mosaleeyanon et al. 2004). Often a combination of the above mentioned is used, for example non-hermetical cultivation flask closure and reduced content of sucrose.

Mixotrophically cultivated plants usually face various environmental challenges during their transition to *ex vitro* growth. Van Huylenbroeck et al. (1998) distinguished two different behaviours of *in vitro* fully developed leaves according to their photosynthetic competence during the adaptation to autotrophic functioning: 1) the leaves function as storage organs with accumulated reserves, which are consumed during the first days *ex vitro*. These leaves never become completely photoautotrophic and only the newly developed leaves meet the plant assimilate demand *ex vitro*; 2) the leaves are fully photosynthetically competent and since very early after the transplantation they satisfy the sink demands of the plant.

Summary of published and unbublished results

The aim of this study was to characterize the selected physiological reactions of four plant species widely used to basic plant physiology research, potato (*Solanum tuberosum* 'Lada'), tobacco (*Nicotiana tabacum* 'Samsun'), rapeseed (*Brassica napus* 'Asgard') and strawberry *(Fragaria vesca*), to photoautotrophic cultivation in comparison with standard mixotrophic cultivation. The results we obtained clearly indicate huge differences in plant carbohydrate metabolism between MT a PA cultivation systems and moreover each plant species under study had its own specific reaction to mixotrophic cultivation. Therefore, there is no simple way to determine any specific plant metabolism alteration enabling to withstand the highly artificial MT conditions and its reaction to *ex vitro* transfer.

Materials and methods:

Plant material and growth conditions

Nicotiana tabacum 'Samsun' and *Brassica napus* 'Asgard' were cultivated from the seeds sterilized 10 min in 30% solution of hypochlorite. *Fragaria vesca* was cultivated from the shoot cuttings taken from at least six weeks old plants. *Solanum tuberosum* 'Lada' was cultivated from single node cuttings taken from four weeks old plants. All plants were cultivated *in vitro*. Mixotrophic (MT) plants were grown in 250 ml Erlenmeyer flasks covered with aluminum or polypropylene (PP) foil on semisolid MS medium (Murashige and Skoog Basal Salt Mixture, plant cell culture tested, Sigma-Aldrich, St. Louis, USA) containing 2.5% sucrose (potatoes) or 3% sucrose (tobacco, rapeseed and strawberry). Photoautotrophic (PA) plants were grown in 250 ml Erlenmeyer flasks covered with PP autoclavable transparent foil bearing two polypropylene filter discs (8 mm in diameter with porosity 0.04 μm, Sigma-Aldrich, St. Louis, USA) enabling sufficient gas exchange and light transmissibility for photosynthesis of the cultivated plants. The photoautotrophic plants were grown on semisolid MS medium with 0% sucrose. All plants were cultivated under a 16 h photoperiod with PPFD (photosynthetic photon flux density) approximately 100 μmol m^{-2} s^{-1} (daylight fluorescent tubes; Osram, Wintherthur, Switzerland). Tobacco, potato, strawberry and rapeseed were cultivated under given MT and PA conditions for 4, 6, 4 and 3 weeks, respectively.

Carbohydrate content determination

Summary of published and unbublished results

Samples (50-100 mg fresh weight) of the leaves or roots were collected (3-7 plants per each species per treatment). The samples were immediately frozen in liquid nitrogen than freeze-dried, boiled in 80% methanol at 75°C for 15 min, the solvent was then vacuum-evaporated and the residue was resuspended in Milli-Q ultrapure water (Millipore, Bedford, USA). Then, the samples were purified by centrifugation and filtration. The content of non-structural soluble carbohydrates was determined using high-performance liquid chromatography (flow rate 0.5 ml min^{-1}, temperature 80°C; sugar standards from Sigma-Aldrich) with refractometric detection (refractive index range 1–1.75; refractometer Shodex RI-71; Spectra Physics – Newport Corporation, Irvine, USA), pre-column: Shodex Sugar Column SC-LG 6x50mm; column: Shodex sugar column SC1011 8x300mm (Shodex, Tokio, Japan). The starch in pellets remaining after the extraction of soluble carbohydrates was hydrolyzed by α-amylase and amyloglucosidase, and the glucose content was measured by the HPLC as in Steinbachová-Vojtíšková et al. (2006).

Growth analysis

After the cultivation period mentioned above, PA and MT grown plants were used. All leaves were cut from the plants and scanned. Subsequently, the total leaf area was measured by ImageJ software (www.imagej.nih.gov). Parallel set of plants was used for biomass determination. The plants were cut into root and shoot parts which were placed onto aluminium foil dish and dried in an oven 130°C/18 hours and then weighted.

Photosynthetic pigments content

Leaves of PA and MT cultivated plants of appropriate age were used. Leaf discs of 5 mm diameter were plunged in dimethylformamide to extract the photosynthetic pigments (chlorophyll a and b and total carotenoids). The concentration of pigments was determined using spectrophotometer and calculated according to the equations reported by Wellburn 1994.

Photosynthesis rate, stomatal conductance and transpiration determination

The net photosynthetic rate (P_n), stomatal conductance (g_s) and transpiration rate were determined using single leaves still attached to the plant. For these

determinations, each leaf was placed in the measuring chamber of a LI-6400 portable gas exchange meter (LI-COR, Lincoln, NE, USA) with changing light intensity from 0 to 800 μmol m^{-2} s^{-1}. Before placing the leaf in the chamber, the culture medium was quickly removed from the plantlet base and the plantlets were put into a petri dish with water (preventing dehydration). Gas exchange was measured under laboratory temperature 25 °C and 600ppm of CO_2. The relative air humidity in leaf chamber was adjusted to at least 60% during the measurements. The measurement protocol consisted of 5 min initial measurement period (light intensity 100 μmol m^{-2} s^{-1}) followed by 10 min dark phase and 5 min of each light intensity in the following order: 100, 300, 400, 500, 700, 800, 100 μmol m^{-2} s^{-1}. The measured data were collected at 1 min intervals. Instantaneous water use efficiency (WUE) was calculated as a ration of P_n and transpiration rate under particular irradiance.

Statistical analysis

Analysis of variance (ANOVA) and Tukey-Kramer Test (for normally distributed data) or Kruskal-Wallis Multiple-Comparison Z-Value Test (for data not normally distributed) were used. Differences were examined at $P \leq 0.05$ and $P \leq 0.01$ levels. All data were analyzed with NCSS 9 statistical software (Hintze, J. (2013) NCSS 9. NCSS, LLC. Kaysville, Utah, USA).

Results:

Photoautotrophic plants were bigger and more developed

The most common way of plant cultivation *in vitro* uses agar-solidified media containing all essential nutrients including an easily accessible C and energy source, usually in the form of sucrose (considered as mixotrophic cultivation, MT). In this study, we cultivated plants, in addition to using this standard method, also under photoautotrophic (PA) conditions and compared the selected morphological and biochemical characteristics of the plants subjected to both types of cultivation (MT and PA). The comparison revealed dramatic differences in the plant phenotypes induced by the different way of nutrition. All studied PA plants showed similar

features in their phenotypes such as higher size and more developed shoot, particularly leaves compared to MT plants (Fig. 1).

Shoot biomass was significantly increased under PA cultivation

Photoautotrophic plants showed faster growth and had more developed leaves than MT ones. Among other analyses, we quantified the growth differences by measuring the dry weight of shoots and roots in PA and MT plants of the same age (Fig. 2A-D). The shoot biomass significantly increased under PA cultivation in all four species under study, the increase was more than 2 fold for all the plants but strawberry. The root biomass also increased significantly in tobacco and potato. The most dramatic dry weight increase under photoautotrophic condition was observed in tobacco. Considering all species studied, the PA cultivation induced a consistent stimulation of accumulation of photosynthesizing shoot biomass in comparison to an ambiguous effect on root biomass.

Root/shoot ratio varies among studied plants

Root/shoot ratio, calculated from the plant dry weight, can give us valuable information about the plant resources allocation strategy under the given cultivation conditions. Potato, strawberry and rapeseed plants tend to invest more of their resources into shoots during PA cultivation, though the differences were not significant. The only significant change in biomass allocation was found in tobacco which, on the contrary, invested more resources into its root growth in PA plants than in MT ones (Fig. 2E-H).

Photoautotrophic plants had bigger whole leaf area

To further quantify the effect of PA cultivation on leaf size, we measured the whole leaf areas of plants. All photoautotrophically cultivated plants had significantly larger total leaf area than their MT counterparts. We observed an especially striking difference in the tobacco plants where the PA total leaf area was 6.5 fold larger than MT one (Fig. 3A-D). We conclude that PA cultivation unambiguously positively affected the leaf area expansion.

Summary of published and unbublished results

Photosynthetic pigments content was species specific

Leaf photosynthetic pigments content can serve as an indirect marker of the plant photosynthetic capacity, or photosynthetic readiness. Comparison of the photosynthetic pigments contents in PA and MT plants revealed marked species-dependent reactions (Fig. 3E-H). As expected, potato and strawberry showed a significant increase in all measured photosynthetic pigments contents (chlorophyll a, chlorophyll b and total carotenoids) under photoautotrophic conditions. In contrast, tobacco plants had almost equal photosynthetic pigments contents under both cultivation conditions. Surprisingly, rapeseed had significantly higher content of all photosynthetic pigments while growing on a media with sugar source (MT cultivation).

Sugar content differ among the plants under study

Using HPLC, we measured soluble sugar contents in leaves and roots of PA and MT plants (Fig. 4). The four studied plants showed different patterns of response of both sugar contents and spectra. In tobacco and potato leaves, the content of total sugars (sucrose, glucose and fructose) was significantly higher under MT conditions compared to PA ones, though in potato, all sugars were responsible for this increase, while in tobacco it was preferentially caused by sucrose. In strawberry and rapeseed, the opposite tendency was observed. Strawberry leaves contained significantly more total sugars under PA, which was mainly due to increased sucrose content. In rapeseed, we found a similar tendency in total sugars, caused by the increase of all three sugars determined; the increase, however, was not significant for any of them. As regards the root sugar contents, the species under study exhibited higher contents in MT plants with sucrose mainly responsible for the increase in tobacco, glucose and fructose in potato, and all three sugars in strawberry and rapeseed.

The significantly higher starch contents in MT plants were found only for leaves of tobacco and rapeseed and tobacco roots. All other comparisons of starch contents revealed no significant difference between PA and MT plants (Fig. 5).

All PA grown plants had higher photosynthetic activity

Summary of published and unbublished results

The gas-exchange measurements showed significantly higher rates of net photosynthesis (P_n) in all four PA plants compared to the MT ones. After 5 minutes of initial measurement in leaf chamber under the low irradiance close to the cultivation conditions (100 µmol photons m^{-2} s^{-1}) tobacco, strawberry and rapeseed did not show any difference in photosynthetic rates between PA and MT cultivated plants. On the contrary, potato plants exhibited a unique response: PA plants photosynthesized very actively while in MT plants respiration prevailed over photosynthesis (Fig. 6). The enhancement of assimilation rates in PA plants was present in all the species studied in a wide range of PAR (100-800 µmol photons m^{-2} s^{-1}) even under the higher irradiance than the cultivation conditions.

Stomatal conductance and transpiration rates were species-specific

After initial acclimation phase in leaf chamber, the light was switched off and a significant decrease in both, stomatal conductance (g_s) and transpiration rate was detected in all MT plants, which did not fully regenerate during next 30 minutes of measurement conditions. On the contrary, in PA plants the decrease of stomatal conductance between the initial measurement and the dark phase of light curve protocol was not so pronounced. After the dark phase, the majority of the plants of both PA and MT treatment kept the stomatal conductance almost constant even under the increasing irradiance. Note that the initial situation of the significantly higher g_s (except potato) in MT plants in comparison to PA ones did not endure across the following light curve protocol. In tobacco and strawberry, PA plants exhibited higher g_s under 300 photons m^{-2} s^{-1} onwards. On the other hand, PA grown potato and rapeseed tended to show lower g_s than their MT counterparts. The difference between PA and MT plants was significant in lower irradiances in potato (0-400 photons m^{-2} s^{-1}) and in rapeseed the difference between PA and MT was significant under 800 photons m^{-2} s^{-1} (Fig. 7).

The difference in transpiration rates between MT and PA plants was species-specific. In potato and rapeseed the transpiration rates were higher in MT plants in comparison with their PA counterparts. In potato, the difference was significant across the wide range of irradiance (100-700 photons m^{-2} s^{-1}), in rapeseed the difference was significant only under lower irradiance (100-400 photons m^{-2} s^{-1}). With the increasing irradiance, PA potato and rapeseed maintained the transpiration rates almost constant.

On the contrary, MT plants gradually decreased transpiration rates with increasing irradiance. These results suggest that even mixotrophically grown potato and rapeseed have functional stomata reacting to the changes in the leaf environment. On the other hand, strawberry showed higher transpiration rates in PA plants across the whole range of irradiance tested (100-800 photons m^{-2} s^{-1}). In tobacco the transpiration rates did not differ between PA and MT plants although there was a similar trend visible (Fig. 8).

Discussion:

The results of this study clearly show that creation of photoautotrophic (PA) well growing cultures *in vitro* is easier than has been assumed (Xiao et al. 2011). Our improved method consists of the ventilation system allowing higher CO_2 availability inside the vessel and it is neither technically nor economically difficult to use it within standard cultivation chambers (no changes in irradiance or light quality, no CO_2 level enhancement in growing chamber are necessary). The general opinion that higher PAR in the cultivation chamber is crucial for successful PA cultivation is not supported by our results. The growth rates of PA plants are higher compared to mixotrophic (MP) plants and all our studied PA plants are fully photosynthetically active.

The main sugar used in cultivation media *in vitro* is sucrose and its high availability via plant roots can cause numerous changes in plant metabolism and osmotic ratios. Sucrose serves as the main assimilate and the most transported sugar for most plants, therefore its levels are always carefully controlled. The current sucrose levels serve as a signal about plant well-being and are closely mirrored in the plant metabolism. When there is an imbalance caused by the externally added sucrose, plants can react unpredictably as they might interpret the conditions as either luxury C status or a stress load. Various types of stresses, however, influence the carbohydrate status in different ways; leading either to higher sucrose levels sensed e.g. by trehalose-6-phosphate signalling pathway (e.g. Figueroa and Lunn 2016) or to lower levels of sucrose which trigger SnRK1 (SNF1-related kinase 1) signalling pathway via glucose-6P shortage (e.g. Hulsmans et al. 2016). Sucrose can, in fact, influence plants more directly also, for example by changing the osmotic balance. There are even references about sucrose toxicity in high dosages to certain plants (Schmildt et al. 2015). Thus the question arises, why not just remove or reduce the sucrose supply during *in vitro* cultivation? Xiao (2011) stated that simply removing sugar from the culture medium

without increasing PPF (photosynthetic photon flux) and CO_2 concentration inside vessels would not promote the growth of plant culture. On the contrary, in our system, without sugar added and under standard PPF (100 μmol photons m^{-2} s^{-1}), the simple act of enabling higher gas exchange between the vessels and their surroundings led to similar phenotypical changes towards improved growth parameters in all four plants species under study. PA plants had more developed leaves and darker colouration. Measuring plant dry weight revealed a significant increase in the biomass of shoots in all studied PA plants. MT plants were remarkably smaller, which is consistent with a hypothesis that exogenous sugar reduces plant growth and photosynthesis (Hdider and Desjardins 1994). Higher growth rates of the PA potato plants, due to the increased ventilation, were observed also by other authors (Kubota and Kozai 1992, Badr et al. 2011) who obtained similar results with potatoes – the PA plants were taller and had more leaves with a higher surface area. Another study on various potato cultivars, however, showed plants with higher shoot height and fresh mass under MT conditions (Chanemougasoundharam et al. 2004). This may seem to be contradictory, but considering the presence of various morphological abnormalities in the hermetically closed MT cultivated plants, such as hooked stem apices, callusing in shoot bases and higher senescence index, the authors conclude that primarily non-hermetic flask closure leads to better potato plantlet quality.

Notably, the reaction of the roots to PA conditions differs among the plants followed in this study. Tobacco and potato, belonging to the same family (*Solanaceae*) had the same reaction – significantly more root biomass under PA conditions, which is in agreement with the findings on various plant species, e.g. *Billbergia zebrina* (Martins et al. 2015), *Wasabia japonica* (Hoang et al. 2017) and *Macadamia tetraphylla* (Cha-um et al. 2011). Strawberry and rapeseed on the other hand, showed no root response to PA cultivation. It has to be mentioned, that for many species propagated *in vitro* at least some degree of vessel ventilation is essential for root formation (e.g. *Macadamia tetraphylla*), if they are derived from the nodal explants (Cha-um et al. 2011). Some studies attribute the negative effects on root formation and growth in sealed systems to the accumulation of ethylene (Zobayed et al. 2001). Inhibition of the root growth in MT cultivated plants is often ascribed to sucrose in the media (Cha-um et al. 2011, Martins et al. 2015, Hoang et al. 2017). Even in the non-hermetically closed systems, increasing the content of sucrose in cultivation media led

to linear root dry mass reduction (Cha-um et al. 2011, Martins et al. 2015). When the growth parameters are shown as root/shoot ratio, only tobacco distinguished itself from the other studied plants by allocating significantly more resources to the roots under PA conditions. This shift in R/S ratio is in agreement with the study on *Wasabia japonica* (Hoang et al. 2017), where the PA cultivated plants developed more robust root system with larger xylem vessels. We also observed higher variability in R/S ratio in MT cultivated plants in all species, which we explain by the resource constraints during the root growth within the media, in comparison with PA plants that can effectively regulate the allocation of the assimilates to the belowground parts. However, R/S ratio reflects the source–sink interactions that modulate carbon assimilation, translocation, partitioning, and storage throughout the plant and have a pivotal role in specific plant strategy (Yu et al. 2016, Chang and Zhu 2017). Therefore, it can be expected that the remarkably different responses in C allocation resulting from the exchange of MT for PA nutrition will be observed in various plant species.

Concerning soluble sugars levels, we observed the same reaction to PA conditions in roots of all the plants under study. As expected, there were significantly lower levels of all the three major sugars (sucrose, glucose, and fructose) in roots of all studied PA plants. However, shoot sugar contents differed between tobacco and potato on one side and strawberry and rapeseed on the other. In the nightshades there were significantly more sugars at the MT plants (dominant sugar was sucrose in tobacco and glucose in potato), whereas strawberry and rapeseed showed the opposite reaction with higher sugar content in the PA ones. The potato plant results are consistent with the study performed by Badr et al. (2015), where principal component analysis and hierarchical cluster analysis of 108 metabolites identified by GC–MS revealed higher sugar content in MT plants with sucrose, fructose and glucose representing more than 97 % of the identified sugars in plantlets regardless of the culture conditions. In citrus plantlets, sucrose in the cultivation medium linearly increases the level of reducing sugars, starch, and total chlorophyll (Hazarika et al. 2000). In MT plants, we determined higher starch content in tobacco, potato and rapeseed (both in roots and shoots) and lower in strawberry shoots. Similarly, we observed higher contents of chlorophyll in MT cultivated rapeseed plants. Potato and strawberry on the other hand had significantly more photosynthetic pigments while

growing at PA conditions and there was no difference between PA and MT tobacco plants.

All the studied plants grown under PA conditions showed significantly higher net photosynthesis rate in comparison to the MT ones. It was shown in several studies that high sugar concentration in the culture medium caused feedback inhibition of photosynthesis by reducing the quantity and activation of Rubisco, e.g. in cultured strawberry, potato (Hdider and Desjardins 1994) and tobacco (Roh and Choi 2004). Kozai and Kubota (2001) attribute the low or negative net photosynthetic rates of plants *in vitro* to the low CO_2 concentration in the air-tight culture vessel during the photoperiod, not to a poor photosynthetic capacity of *in-vitro* developed leaves. Our PA cultivation system had increased air exchange and the rate of net photosynthesis was higher in all PA plants, especially potato, even during the initial low PAR (100 µmol photons m^{-2} s^{-1}), which is very similar to that in the cultivation chamber. MT potato plants even have no photosynthesis measurable similarly as in other studies on potato (Kubota and Kozai 1992, Badr et al. 2011). Badr et al. (2011) showed that lower photosynthetic rate of MT plants was caused by reduced stomatal conductance (g_s). However, in our case, the reduction of CO_2 assimilation in MT potato cannot be stomatally limited; MT-grown potato g_s was even significantly higher at 100, 300 and 500 µmol photons m^{-2} s^{-1} irradiance, in higher PAR values there was no difference in stomatal conductance between MT and PA. Regarding high contents of sugars in MT potato leaves, we suggest that P_n could be downregulated by hexokinase signalling. There is evidence that lasting higher sugar content in tissues suppress photosynthetic capacity through activation of a pathway involving hexokinase signalling leading to lower expressions of photosynthetic genes (e.g. Granot et al. 2014, Aguilera-Alvarado and Sanchez-Nieto 2017). We can also reject the stomatal limitation of photosynthesis in rapeseed, where g_s did not differ between MT and PA plants. On the contrary, the stomatal limitation could be considered in strawberry and tobacco MT plants where g_s under PAR 300 µmol photons m^{-2} s^{-1} and higher was reduced in comparison to PA. In addition to already mentioned potential Pn limiting factors in MT plants, light capture and efficiency of photochemical processes can play a role (Ruban 2015). Although we did not examine the primary photosynthesis directly, we can at least use chlorophyll contents as a proxy for primary photosynthetic processes. Our results on Pn, g_s, leaf

sugar and chlorophyll contents allow us to propose hypotheses about the limitation of photosynthesis under *in vitro* MT conditions in the four studied species (Tab. 1).

High relative humidity in closed cultivation systems influences the plant stomatal development and their ability to control water balance. Many authors demonstrated that one of the main constraints for MT cultivated plants during *ex vitro* transition is caused by ineffective or dysfunctional stomata (e.g. Pospisilova et al. 1987, Deccetti et al. 2008, Hoang et al. 2017) and weakly developed cuticle (e.g. Zobayed et al. 2000). Ventilated photoautotrophic cultivation can in many cases improve plant´s water control (Zobayed et al. 2000, Deccetti et al. 2008, Kozai 2010). The transpiration rates of all our plants under irradiance close to that used during cultivation were consistent with other studies, which showed significantly higher transpiration in MT plants in comparison to PA ones e.g. in *Ipomoea batatas*, tobacco and *Castanea sativa* (Zobayed et al. 2000, Haisel et al. 1999, Saez et al. 2015, respectively). However, we reject the idea that the stomata of our MT plants were completely dysfunctional. After initial measurement phase in leaf chamber (100 µmol photons m^{-2} s^{-1}), the light was switched off and a significant decrease in both, stomatal conductance and transpiration rate was detected in all MT plants, which did not fully regenerate during next 30 minutes of measurement conditions. The very high complexity of stomatal closure regulation (Daloso et al. 2016, Merilo et al. 2017) prevents us to clearly explain the mechanism of this stomatal reaction. However, we suppose that each plant species may emphasize a different part of this complex regulation network according to its evolutionary adaptations. During the gas exchange measurement, our MT plants had to integrate various external signals such as considerable decrease of RH in comparison to the growth conditions in the sealed vessel, faster airflow and thus a decrease of the boundary layer and a changing light conditions. In addition, the stomatal reaction further depends on the plant internal carbohydrate status. High sucrose levels were reported to be connected also with the hexokinase-dependent stomatal closure (e.g. Daloso et al. 2016) coordinating sugar availability to the rate of water loss through transpiration. Moreover, an overall distribution of water (and distribution of other compounds influenced by the water movement) is affected by changes in aquaporin based hydraulic conductance linked to the tissue sugar levels (once more also via hexokinase signalling) and coordinates the water distribution within the plant body (Kelly et al. 2017). Taking into account that

all the studied PA plants had higher instantaneous water use efficiency across the whole interval of 100-800 µmol photons m^{-2} s^{-1}, we conclude that PA cultivation lead to improved water balance of the studied plants similar to Sáez et al. (2015) has shown for *Castanea sativa.*

We aimed to contribute to the knowledge on the carbohydrate metabolism-related processes of *in vitro* propagated plants, which may improve their properties during the acclimatization to *ex vitro* conditions. We regard the photosynthetic capacity and readiness of leaves developed *in vitro* as one of the key factors for a successful *ex vitro* transition. In the present study, we demonstrated that all the studied plants grown in PA conditions showed better photosynthetic readiness than their MT grown counterparts in a considerable irradiance interval (100-800 µmol photons m^{-2} s^{-1}). Our gas exchange measurements were conducted after removing the plants from the cultivation vessels, thus, we cannot directly prove their actual photosynthetic performance under *in vitro* conditions. Nevertheless, Hoang et al. 2017 assessed P_n directly in the cultivation vessel and reported stable lower photosynthesis rate in MT cultivated *Wasabia japonica* plants in comparison to the increasing P_n rates in PA conditions with proceeding cultivation. Based on the assumption that our cultivation system was comparable to the Hoang´s one, we infer that photosynthetic activity of our plants during *in vitro* cultivation does not need to be diametrically opposite from the performance in the gas analyser measuring chamber.

We are aware that measuring conditions in leaf chamber are not identical with the growth conditions regarding the CO_2 concentration. Particularly in PA vessels, the CO_2 concentration could be much lower than 600 ppm we used (e.g. up to 400 ppm, Hoang et al. 2017, Martins et al. 2015). However, Martins et al. 2015 declared the CO_2 level fluctuation in very similar MT cultivation system as ours (low ventilation, vessel volume, photoperiod, irradiance) with *Billbergia zebrina* between 250 and 600 ppm CO_2 during the light and dark phase, respectively. Therefore, we assume our conditions for the gas exchange comparable with the situation of MT cultivated plants at the beginning of the light period and thus providing valuable information on the plant photosynthetic readiness. Moreover, the majority of the plant growth parameters (phenotype, shoot biomass, leaf area) approve better assimilate production under PA conditions.

Regarding the two strategies of how leaves of *in vitro* cultivated plants will probably cope with *ex vitro* transition, as mentioned above (Van Huylenbroeck et al. 1998), considering photosynthetic rates, MT planted potato resembled the first strategy with the photosynthetically incompetent leaves, containing high sugar contents in comparison to their PA counterparts. On the other hand, tobacco, strawberry and rapeseed MT cultivated plants proved to be photosynthetically competent.

In vitro plant cultivation is an important method used not only commercially for fast and cheap plant reproduction but also as a widely used experimental system for plant physiology studies. To know each plant behaviour under an excess sucrose is crucial for understanding other important physiological processes. The differences between plants grown under MT and PA cultivation conditions were strongly species-specific (Fig. 9), probably due to the different strategies each plant uses to deal with the high amount of assimilates. Moreover, in a broader sense and context the basic division can be according to the plant phloem loading strategy (Ainsworth and Lemonnier 2018). Symplastic loaders (not using polymer trap loading mechanism) mostly tolerate much higher levels of sucrose in mesophyll cells than apoplastic loaders and thus might react differently to high sucrose supply under *in vitro*. A possible role can play also the plants sugar sensing and signalling systems such as hexokinase or SnRK1 and its interactions, causing a unique metabolic reaction in different plants. Sugars affect central aspects of plant physiology, including photosynthesis, stomatal behaviour and the loss of water through the stomata. For example increased expression of Arabidopsis hexokinases 1 strongly modifies expression of aquaporins (Kelly et al. 2017) leading to coordination of sugar levels to water management. In conclusion, our results, though based on the use of limited number of model plants, clearly show marked and species-dependent reaction to exchange of MT for PA conditions. Therefore, it is obvious that the impact of artificial sugar supply is so complicated, manifested on number of structural and metabolic levels that it is not advisory to use mixotrophic *in vitro* cultivation for basic physiology research.

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Figures:

	Hypothetical limitation of P_n in MT cultivated plants			
PLANT SPECIES	**Stomatal conductance**	**Primary photosynthesis** (chlorophyll content)	**Rubisco** (content, activation)	**High sugar in leaf tissue** (Hex)
Tobacco	+	-	+ Roh and Choi 2004	+ Fig. 4
Potato	-	-	+ Hdider and Desjardins 1994	+ Fig. 4
Strawberry	+	+	+ Hdider and Desjardins 1994	-
Rapeseed	?	?	?	-

Tab. 1 Hypothetical limitation of Pn in MT cultivated plants

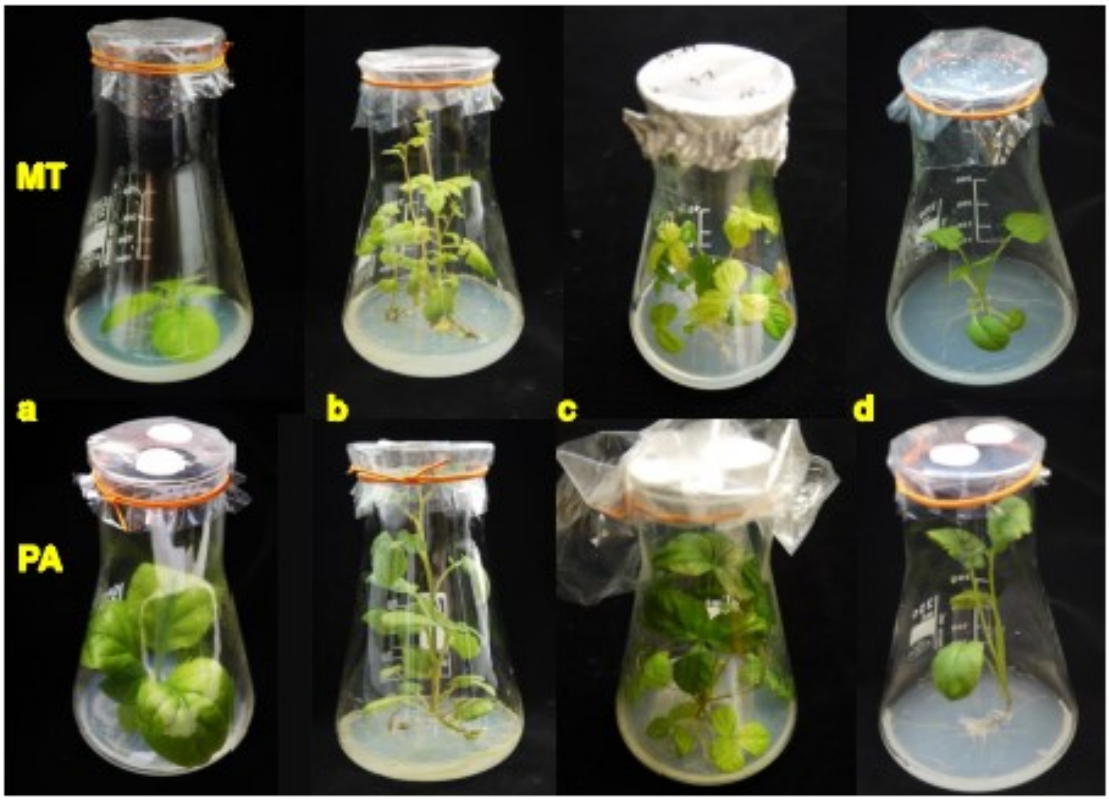

Fig. 1 Phenotypes of mixotrophically (MT) and photoautotrophically (PA) cultivated plants: a) tobacco, 4 weeks old b) potato, 6 weeks old c) strawberry, 4 weeks old d) rapeseed, 3 weeks old

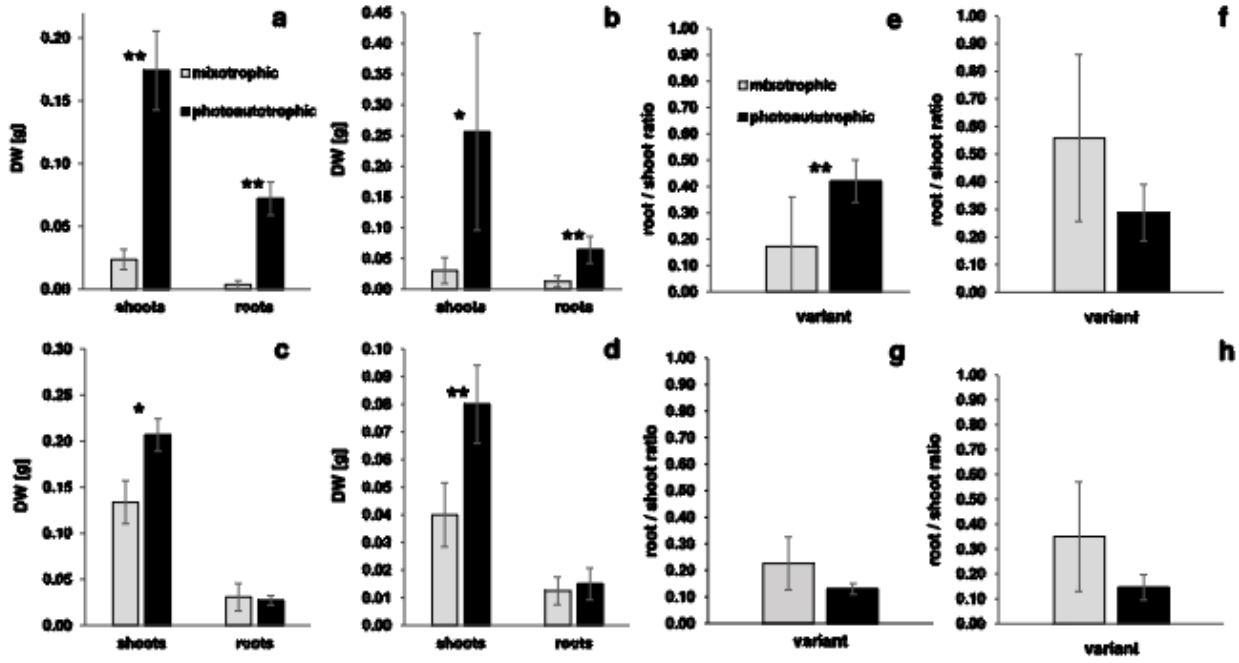

Fig. 2 Shoot and root biomass and root/shoot ratio of MT and PA cultivated plants: a) tobacco b) potato c) strawberry d) rapeseed shoot and root biomass, e) tobacco f) potato g) strawberry h) rapeseed root/shoot ratio, plants cultivated for 4, 6, 4 and 3 weeks, respectively. Bars refer to standard deviations of dry weight, * and ** indicate significant differences at $\alpha = 0.05$ and 0.01 respectively, n = 4 – 8

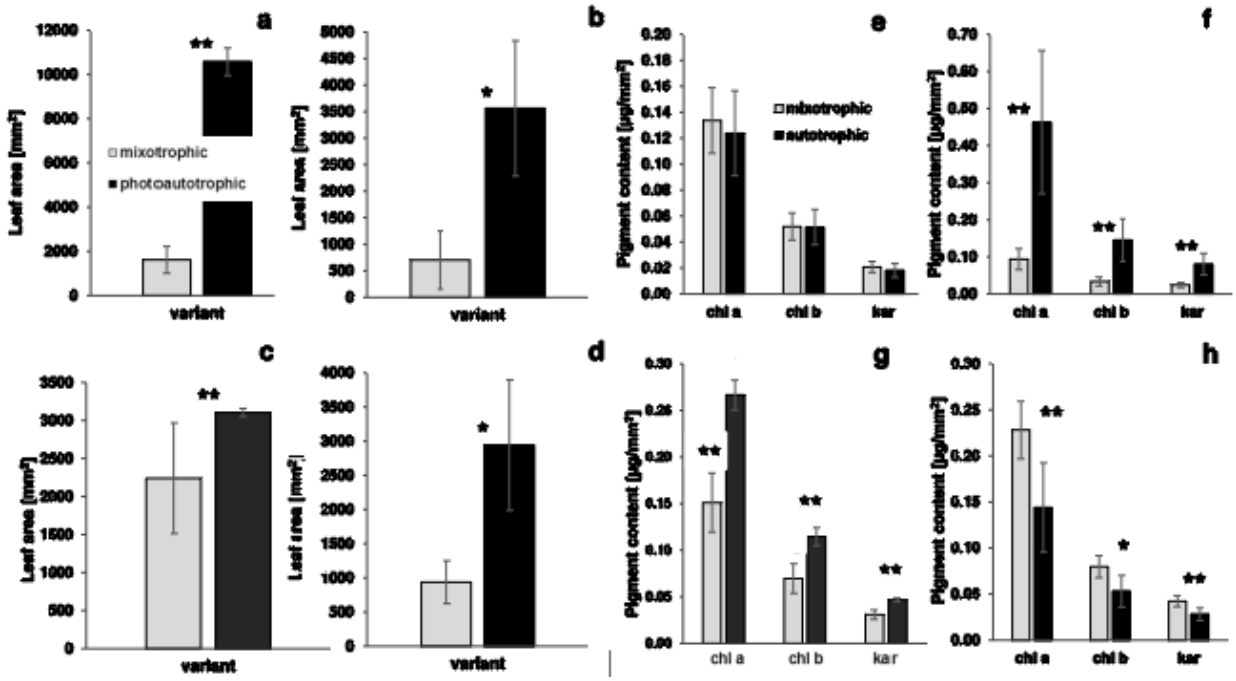

Fig. 3 Total leaf area and photosynthetic pigments contents of MT and PA cultivated plants: a) tobacco b) potato c) strawberry d) rapeseed total leaf area, e) tobacco f) potato g) strawberry h) rapeseed photosynthetic pigments contents, plants cultivated for 4, 6, 4 and 3 weeks, respectively. Bars refer to standard deviations of total leaf area, * and ** indicate significant differences at $\alpha = 0.05$ and 0.01 respectively, n = 2 – 8

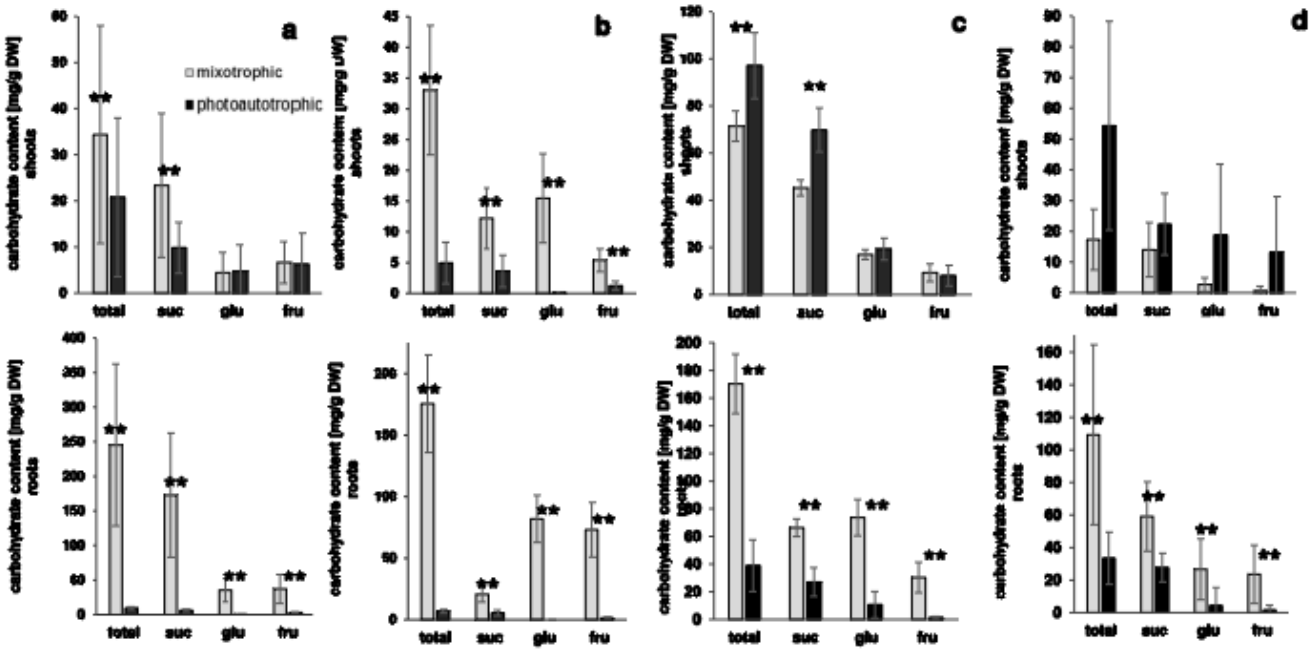

Fig. 4 Sugar contents in leaves and roots of MT and PA cultivated plants: a) tobacco b) potato c) strawberry d) rapeseed (upper line shoots, lower line roots) cultivated for 4, 6, 3 and 4 weeks, respectively. Bars refer to standard deviations of sugar content, ** indicate significant difference at $\alpha = 0.01$, $n = 6 – 10$

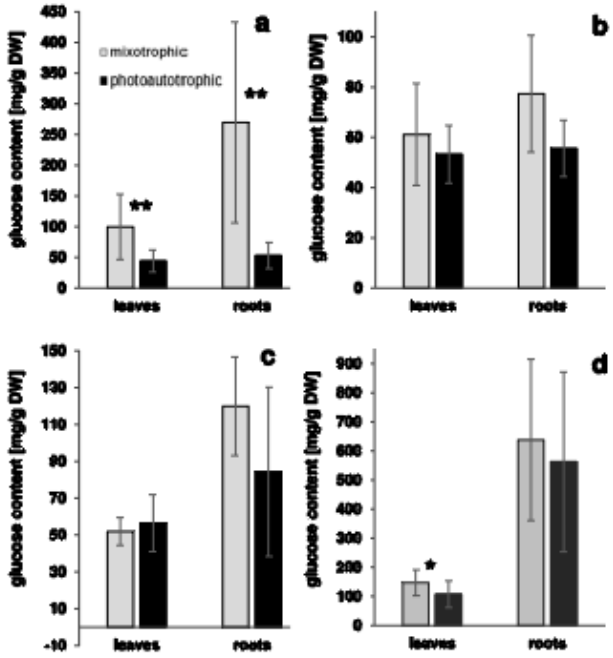

Fig. 5 Starch content in MT and PA cultivated plants: Starch contents are given as glucose levels after starch enzymatic digestion. a) tobacco b) potato c) strawberry d) rapeseed cultivated for 4, 6, 4 and 3 weeks, respectively. Bars refer to standard deviations of glucose content after enzymatic digestion, * and ** indicate significant differences at $\alpha = 0.05$ and 0.01 respectively, $n = 6 – 10$

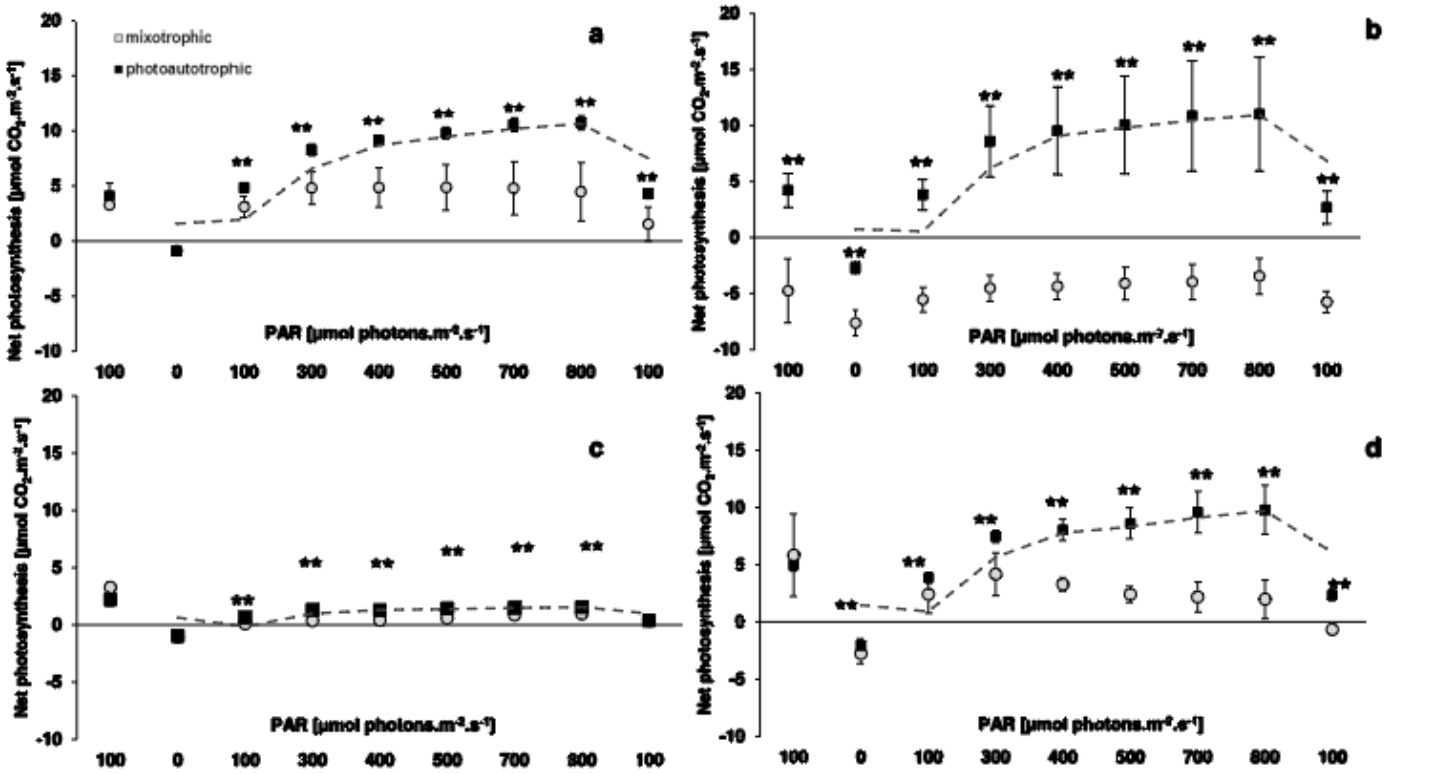

Fig. 6 Net photosynthetic rate in MT and PA cultivated plants: a) tobacco b) potato c) strawberry d) rapeseed cultivated for 4, 6, 4 and 3 weeks, respectively. Bars refer to standard deviations of net photosynthetic rate, * and ** indicate significant differences at α = 0.05 and 0.01 respectively, n = 3 – 4

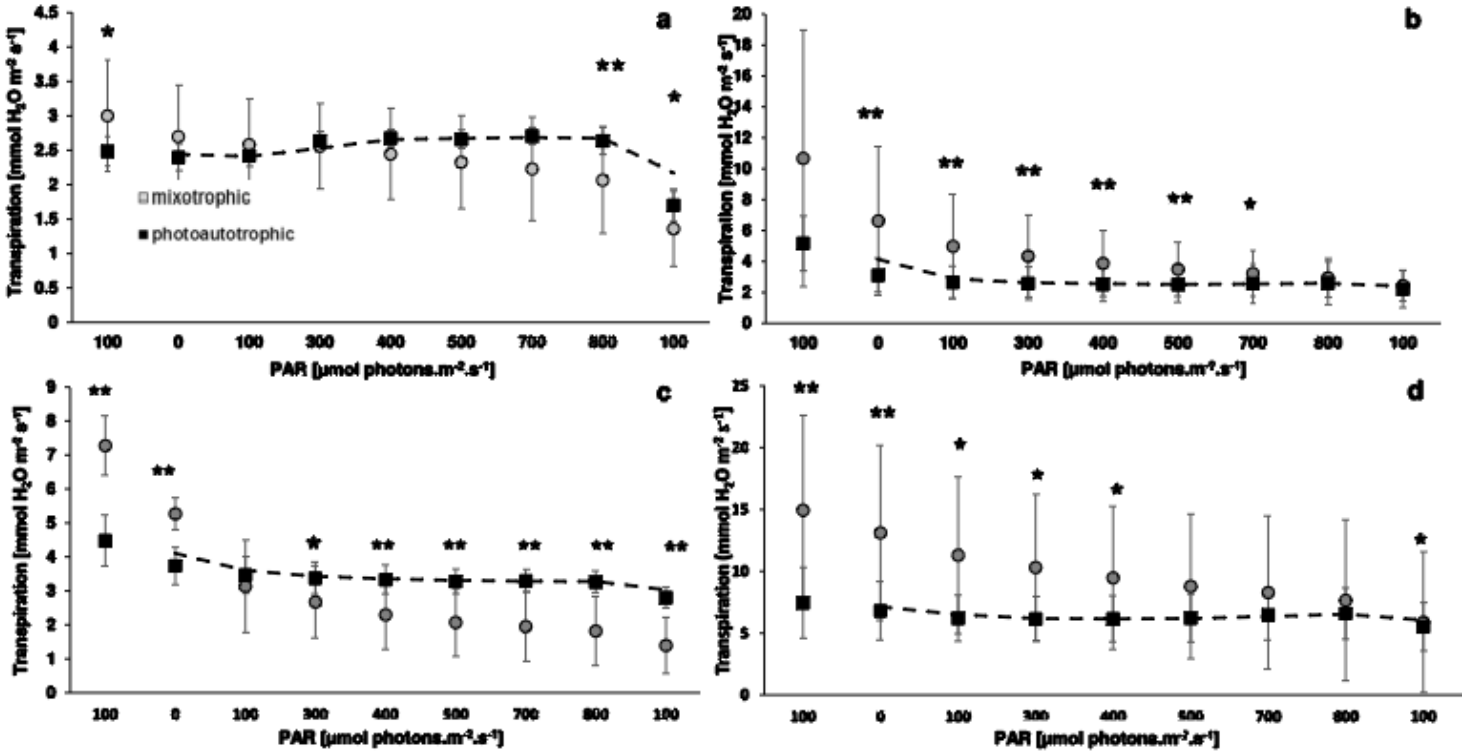

Fig. 7 Transpiration rate in MT and PA cultivated plants: a) tobacco b) potato c) strawberry d) rapeseed cultivated for 4, 6, 4 and 3 weeks, respectively. Bars refer to standard deviations of net transpiration rate, * and ** indicate significant differences at α = 0.05 and 0.01 respectively, n = 3 – 4

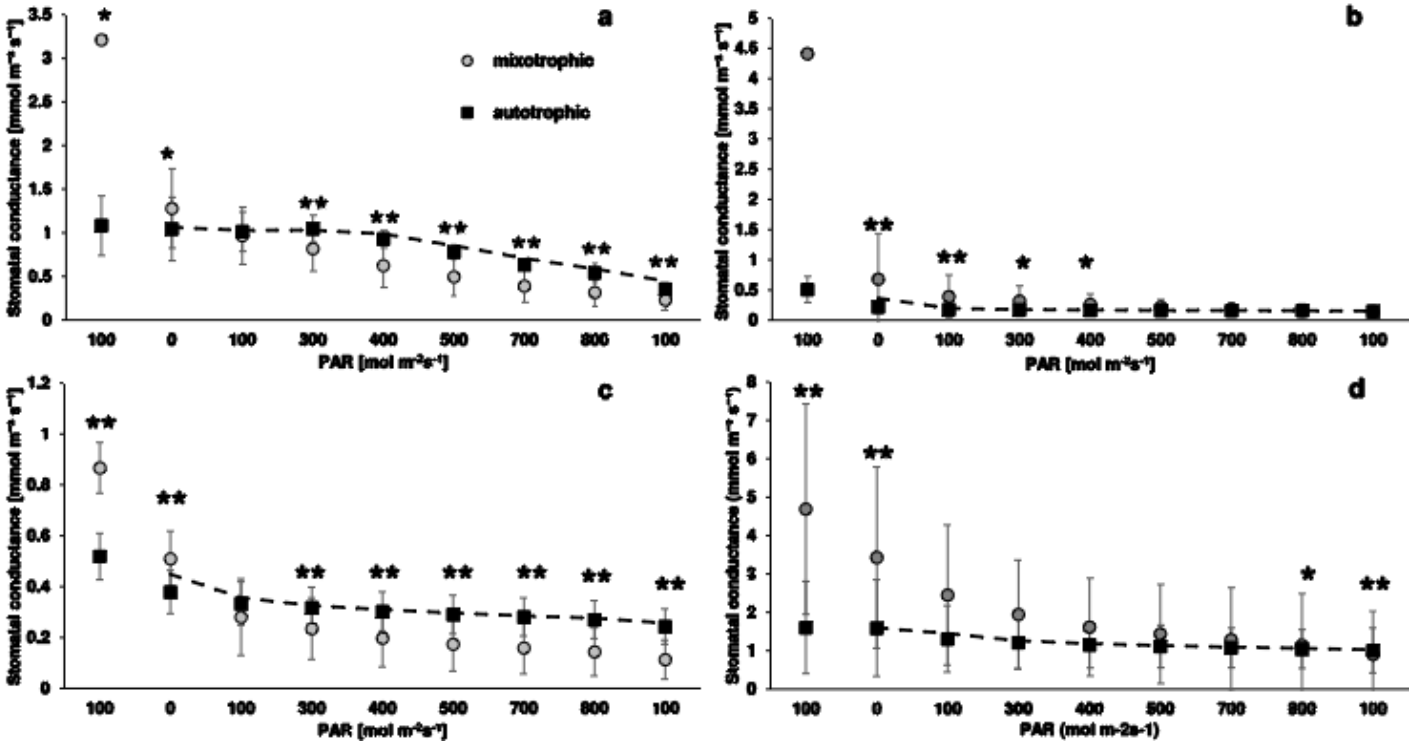

Fig. 8 Stomatal conductance in MT and PA cultivated plants: a) tobacco b) potato c) strawberry d) rapeseed cultivated for 4, 6, 4 and 3 weeks, respectively. Bars refer to standard deviations of stomatal conductance, * and ** indicate significant differences at α = 0.05 and 0.01 respectively, n = 3 – 4

	tobacco	potato	strawberry	rapeseed
shoot biomas	++	+	+	++
root biomas	++	++	0	0
root/shoot ratio	++	(-)	(-)	(-)
leaf area	++	+	++	+
pigment content	0	++	++	- -
leaf sugars	- -	- -	++	(+)
root sugars	- -	- -	- -	- -
leaf starch	- -	(-)	0	-
root starch	- -	(-)	0	0
photosynthetic rate	++	++	++	++
transpiration rate	++	- -	++	-
conductance	++	++	++	++

++	** significant increase
+	* significant increase
(+)	tendency to increase
0	no difference
(-)	tendency lo decrease
-	*significant decrease
- -	** significant decrease

Fig. 9 The difference between PA and MT cultivated plants: blue colour means a positive difference, yellow colour a negative difference in PA plants

5.2.1. Statement of contribution

HL and HS conceived and designed research. JH conducted strawberry experiments, HS conducted potato, tobacco and rapeseed experiments, ZL designed the gas-exchange measurement experiments. HS, HL and ZL wrote the manuscript.

5.3. Publication 3 – Ševčíková et al., submitted

BRIEF COMMUNICATION submitted to Biologia Plantarum (IF_{2017}=1.09)

Root cultures of spontaneously tuberizing potato mutant lacking manganese-stabilizing protein isoform exhibit growth and sugar status changes similar to the roots of intact plants

HANA ŠEVČÍKOVÁ*, PETRA MAŠKOVÁ AND HELENA LIPAVSKÁ

Department of Experimental Plant Biology, Faculty of Science, Charles University, Viničná 5, 12843, Prague, Czech Republic

Abstract

Potato is an important crop and our knowledge of the mechanisms behind the induction of potato tuber formation are still scarce. We used an interesting model, spontaneously tuberizing (ST) potato mutant, derived from *Solanum tuberosum* cv. Lada (WT). Previously we found disturbed sugar distribution within the ST plant body, especially eminent in the roots. We cultivated root organ-cultures of ST plants and observed the same changes in growth and sugar contents when compared to WT as we did in the intact plants. That is remarkable given that the only alteration of ST plants found so far is a lack of one isoform of mangan-stabilizing protein (MSPI) of photosystem II. The MSPI gene expression analysis, however, revealed no MSPI expression in roots in both WT and ST root organ-cultures. Thus, further studies including those checking the possible involvement of other changes in genome must be performed to explain the observed phenotype.

Abbreviations: mangan-stabilizing protein I (MSPI), mixotrophic cultivation (MT), photoautotrophic cultivation (PA), spontaneously tuberizing (ST)

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* *Corresponding author, e-mail:* hana.sevcikova@natur.cuni.cz

Potato is one of the most important crops worldwide therefore the attempts to understand its physiological processes, especially regulation of tuber formation, is of a great interest. In this work we used potato (*Solanum tuberosum*) cv. Lada (WT) and its spontaneously tuberizing mutant (ST) root organ-cultures. Readiness of ST plants to form tubers under wide range of conditions makes them a great model to study tuberization. Previous characterization of ST mutant (Ševčíková *et al.* 2017) revealed changes in carbohydrate content and distribution when compared with WT, along with changes in soluble carbohydrate allocation and starch deposition, favoring basal stem part in ST mutants. Exceptionally large differences in sugars contents were found in roots of intact plants too. ST plants preferably accumulated sucrose in the roots whereas WT plants preferred hexoses such as glucose and fructose.

Small plant phenotype and poor root growth were the noticeable changes observed at ST plants but the exact mechanism causing such profound changes on plant metabolism and phenotype is still elusive. Originally, the WT was transformed with a gene-trap construct for random gene activation. Proteomic analyses revealed the lack of one of three mangan-stabilizing protein I (MSPI) isoforms of photosystem II in ST plants as the only known difference probably responsible for this complex phenotypic changes (Fischer *et al.* 2008). This may seem as a random mutation and not the cause of the extraordinary ST phenotype, bud an independent work of Gururani *et al.* (2012) proved that there is in fact a connection between the regulation of tuberization initiation and MSP caused by changes in the primary photosynthetic phase. Gururani *et al.* worked with potato plants with altered MSP expression (both enhanced and reduced), although they did not report which MSP isoform they used. Plants with reduced MSP expression were photosynthetically more active and showed enhanced tuberization and increased carbohydrate content. Even though we are still lacking some basic information, their results strongly suggest that altered photosynthetic machinery can be possibly connected with early tuberization. The photosynthesis is closely connected with sugar metabolism and sugars may as well be the link we are looking for between tuberization and photosynthesis.

The cultivation of *in vitro* root organ-cultures has a long tradition, since they were firstly developed in the forties of the last century (White 1943, Butcher and Street 1964). However, profound growth of roots with numerous branches of lower order, an essential factor for rapid biomass increase, was observed only in few plant species

such as pea and tomato. The hairy-root cultures, made by stable genetic transformation of plants by ubiquitous soil bacteria *Agrobacterium rhizogenes* (Tepfer *et al.* 1989), are mainly used nowadays, since they have vital growth and high biomass yield. To our knowledge there has been no paper dealing with potato root organ-cultures published yet.

The previous work with intact plants suggested that ST plants may have disturbed /changed distribution of carbohydrates within plant body with the weakened allocation to roots when growing *in vitro* mixotrophically. To learn whether root metabolism or poor assimilate supply from aboveground parts is responsible for observed characteristics of root system, we derived a root organ-culture and cultivated just the roots. As *MSP* codes a protein working in the photosynthetic machinery one would not expect the MSP to be expressed in roots, and thus to have any function in roots. So theoretically there should be no difference between ST and WT root cultures. Surprisingly, we found a big difference in both growth and sugar metabolism between ST and WT roots growing as isolated root culture. Our previous data point towards the possibility of two parallel cross-talking pathways (carbohydrate – and gibberellin-dependent ones) controlling tuberization onset with the power of both to outcompete the other one when its signal is for some reason extraordinary strong. But how is the reduced expression of MSP linked with reduced root system? Does potato express the MSP in roots? The bioinformatical analysis (performed on the genevestigator.com platform) showed, MSPI expression in root organ-cultures of Arabidopsis, so some possibility of finding similar behavior of potato MSPI exists.

In this study we used root organ-cultures of potato plants (*Solanum tuberosum* L.) cv. Lada and its spontaneously tuberizing (ST) potato mutant (Fischer *et al.* 2008). The roots were cultivated *in vitro* from 1 cm long cuttings taken from four-week-old roots, containing the intact root tips. Cultivation took place in 100ml Erlenmeyer flasks with liquid MS medium (Murashige and Skoog 1962) containing 0.5% or 3% sucrose, in dark at the temperature range from 18 to 21°C. After 5 to 6 weeks of cultivation the roots were collected scanned and analysed. Subsequently, the root system area, main root length and number of lateral roots was measured semi-automatically by Smart Root software (ImageJ, Schneider *et al.* 2012). Parallel set of roots was used for biomass determination in the beginning of sub-cultivation period and after 5 to 6 weeks of cultivation. For root primordia visualisation the clearing

method was used. The roots were fixed in acetone, washed in phosphate buffer, mounted in 65% aqueous glycerol and observed with an Olympus BX51 microscope equipped with anvApogee U4000 digital camera. For the carbohydrate content determination, each sample of 50-100 mg fresh weight was immediately frozen in liquid nitrogen than freeze-dried, boiled in 80% methanol at 75°C for 15 min, the solvent vacuum-evaporated and the residue resuspended in ultrapure water. Then, the sonicated samples were purified by centrifugation and filtration. The content of soluble non-structural carbohydrates was determined using high-performance liquid chromatography (HPLC, flow rate 0.5 ml min^{-1}, column temperature 80°C) with refractometric detection (refractive index range 1–1.75; refractometer Shodex RI-71; Spectra Physics – Newport Corporation), columns assembly: Shodex Sugar Column SC-LG 6x50mm pre-column and Shodex sugar SC1011 column (Shodex) according to Vitova *et al.* (2002). The starch in pellets remaining after the extraction of soluble carbohydrates was hydrolyzed by α-amylase (Sigma-Aldrich, 30U) and amyloglucosidase (Sigma-Aldrich, 60U) in Na-acetate buffer (pH 4.5), and the glucose content was measured by the HPLC (for details see (Steinbachova-Vojtiskova et al., 2006). Total RNA was isolated from leaves and root organ-cultures using the TRI reagent (Sigma Aldrich) isolation protocol. Twenty micrograms of total RNA were treated with DNase I using a Turbo DNA-free Kit (Ambion) to eliminate the genomic DNA contamination. Eight micrograms of purified RNA and a mixture of oligodT (0.5 µg/µl)/random hexamers (0.2 µg/µl) primers were employed for cDNA synthesis using 2 µl of RevertAid Reverse Transcriptase (Thermo Scientific) in 40µl reactions. One microlitre of RT reaction was subjected to PCR amplification of *MSPIa-c* templates using allele-specific primers (*MSPIa* forward primer (FP): 5′-ACCAGGCAAATACACTGCCATG-3′; reverse primer (RP): 5′-ACTCCAATGACCTCACCAGAC-3′, *MSPIb* FP: 5′-ACCAGGCAAATACACTGCCATG-3′; RP: 5′-ACTCCAATGACCTCACCAGAG-3′, *MSPIc* FP: 5′-ACCAGGCAAATACACTGCCATT-3′; RP: 5′-ACTCCAATGACCTCACCAGAC-3′). PCR was carried out using Fast Start PCR Master system (Roche). After 35 (leaf samples) or 40 (root samples) cycles of amplification (initial polymerase activation 95 °C 8 min, denaturation 95 °C 30 s, primer annealing, 59 °C 40 s, extension, 72 °C 45 s, final extension, 72 °C, 7 min), aliquots of the PCR samples were run on 1.5% TAE agarose gels and visualised by ethidium bromide staining. For comparison of cDNA synthesis efficiency,

constitutively expressed transcripts of potato actin gene *Pot58* (Ac.Nr. XM_006345899.2) were detected in independent PCR reactions from RT samples (FP: 5′-TGGTCGTACCACCGGTATTG- 3′; RP: 5′-CGAGTTGTATGTGGTCTCGTG-3′) using the same amplification conditions as for *MSPI*. To exclude DNA contamination, every experiment involved control RT-PCR without reverse transcriptase. All data were analyzed with NCSS 9 statistical software (NCSS, LLC. Kaysville, Utah, USA). Analysis of variance (ANOVA) and Tukey-Kramer Test (for normally distributed data) or Kruskal-Wallis Multiple-Comparison Z-Value Test (for data not normally distributed) were used. The differences were examined at $P \leq 0.05$ and $P \leq 0.01$ levels.

We observed striking differences in root organ-cultures phenotype (Fig. 1A) between the WT and ST roots. The results of our analysis show that ST roots have smaller biomass and significantly smaller growth increase (Fig. 1B) and less number of lateral roots caused probably by less primordia forming. The average number of lateral roots formed by WT root cultures was 12 whereas ST roots had on average only one lateral root. Similar situation we observed at root primordia formation at 4 to 5 weeks old roots. In addition to already formed lateral roots, WT plants had on average eight primordia per root visible or in a protruding stage. ST roots on the other hand had only occasionally any primordia visible in the whole root, less than one primordia per root on average. WT primary roots were on average 6 cm long and ST 2,5 cm long. These data are reflecting in the whole root system area (Fig. 1D) with significant difference between the root organ-cultures under study.

ST roots had significantly higher levels of sucrose and similar amount of glucose and fructose as WT (Fig. 2A). This is especially interesting when we look at the sugar spectrum. WT has similar levels of all measured sugars and obviously is using it dynamically to support the roots growth. ST on the other hand has a problem with sucrose cleavage and is just storing it, therefore has very little energy for growth. We can compare this results with data obtained in our previous work, using the same plant material but as intact plants. We cultivated them under traditional mixotrophic (MT) *in vitro* conditions (with sucrose addition to the cultivation media) and under photoautotrophic (PA) *in vitro* conditions (no sugar added and better gas exchange allowed) too. In MT cultivated plants the sugar content is influenced by the for plant artificial sucrose addition to the media, on the other hand the root cultures are

cultivated in dark and cannot survive without the sucrose in cultivation media. In this context is quite surprising that the ST root organ-culture sugar profile has much bigger resemblance to the PA cultivated ST plants (Ševčíková *et al.* 2017).

ST root organ-cultures had significantly more starch than WT roots (Fig. 2B). It is known that plants under some stresses tend to starch accumulation (Kosegarten and Mengel 1998) and the intact ST plants also look like they are reacting to a stress (Ševčíková *et al*. 2017). It is known, that high sucrose supplement to the cultivation media can cause e.g. osmotic stress, so we looked at how the root organ-cultures perform when growing in much lower sucrose concentration. We cultivated them with 0.5% sucrose supplement and we observed the same pattern of limited growth with almost no branching in ST roots. WT was growing very poorly too, but was still able to form lateral branches and to increase biomass (data not shown).

To answer the question whether the observed phenotype can be ascribed to changes of intact plants genome, we analysed *MSPI* expression both, in the leaves of intact plants as a control and root organ-cultures. Leaf expression showed the lack of one allele (*MSPIa*) coding the missing MSPI isoform in ST plants leaves. In the root organ-cultures, however, we were not able to detect expression of any *MSPI* allele in both ST and WT plants (data shown for *MSPIb*) that is in contrast to Arabidopsis published data where *MSPI* expression was documented in the root cultures (Genevestigator platform). Based on this we can speculate that the lack of one MSPI isoform is not the only alteration of ST plants. L. Fischer (personal communication) suspects that larger deletion near the *MSPI* gene region could be also the reason for observed changes. In the chromosome 2 region in question an interesting candidate gene coding for transcription factor BEL22 with possible function in the regulation of potato tuberization initiation is located (PGSC, Michigan State University). The superfamily BEL1-like transcription factors regulate a range of developmental processes (Müller *et al.* 2001); in potato leaves and stolons, thirteen BEL1-like proteins were found so far (Chen 2003, Sharma *et al*. 2014). StBEL5 is already established as one of the main mobile tuberization positive regulators (Banerjee *et al.* 2009, Hannapel 2013). To StBEL22 is usually dedicated a function in modulating the leaf morphology, but its expression was also detected in stolon and root, however in small amounts. It is also known that the StBEL22 expression is under StBEL5 direct regulation (Sharma *et al.* 2014). Therefore, we are considering the BEL22 as a

plausible candidate causing the ST plants distinctive phenotype. However, other studies are needed, especially to discover if there are any discrepancies in StBEL22 expression in ST plants and elucidate the connection with overall mutant phenotype.

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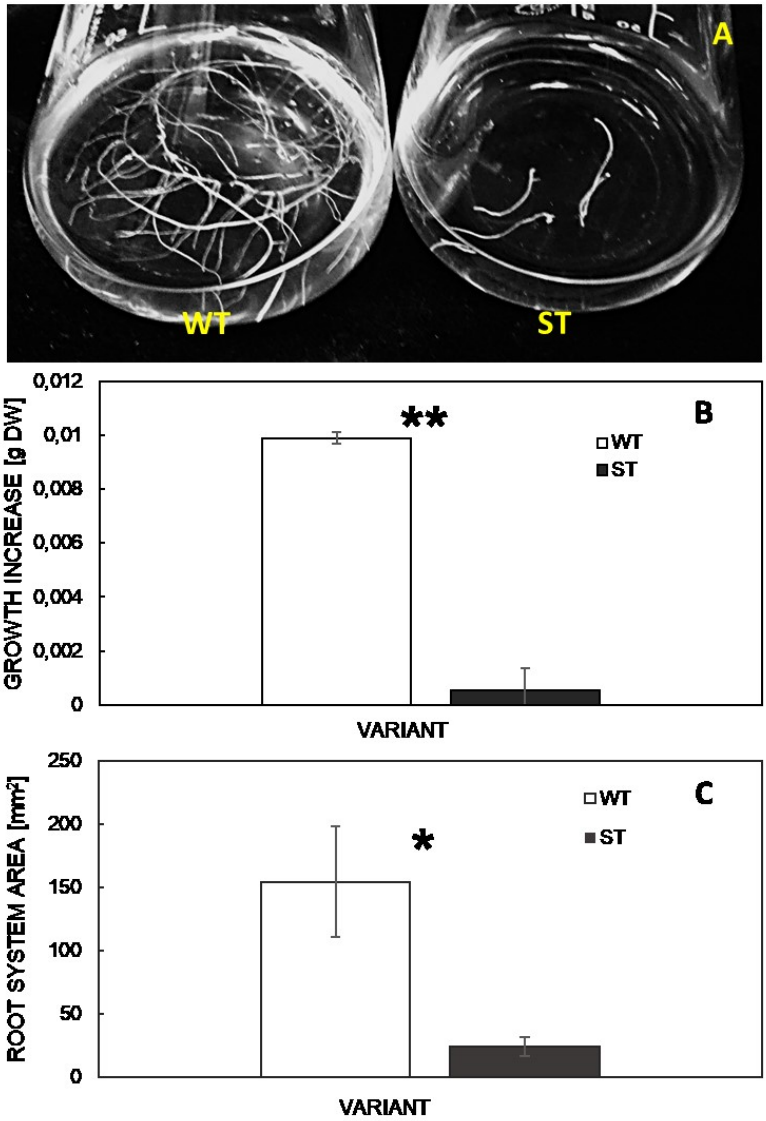

Fig. 1. The phenotype (A), growth increase (B), and root system area (C) of root organ-cultures of WT and ST cultivated for 5 to 6 weeks, * indicate significant difference at $\alpha = 0.01$, ** indicate significant difference at $\alpha = 0.01$, $n = 3 – 8$.

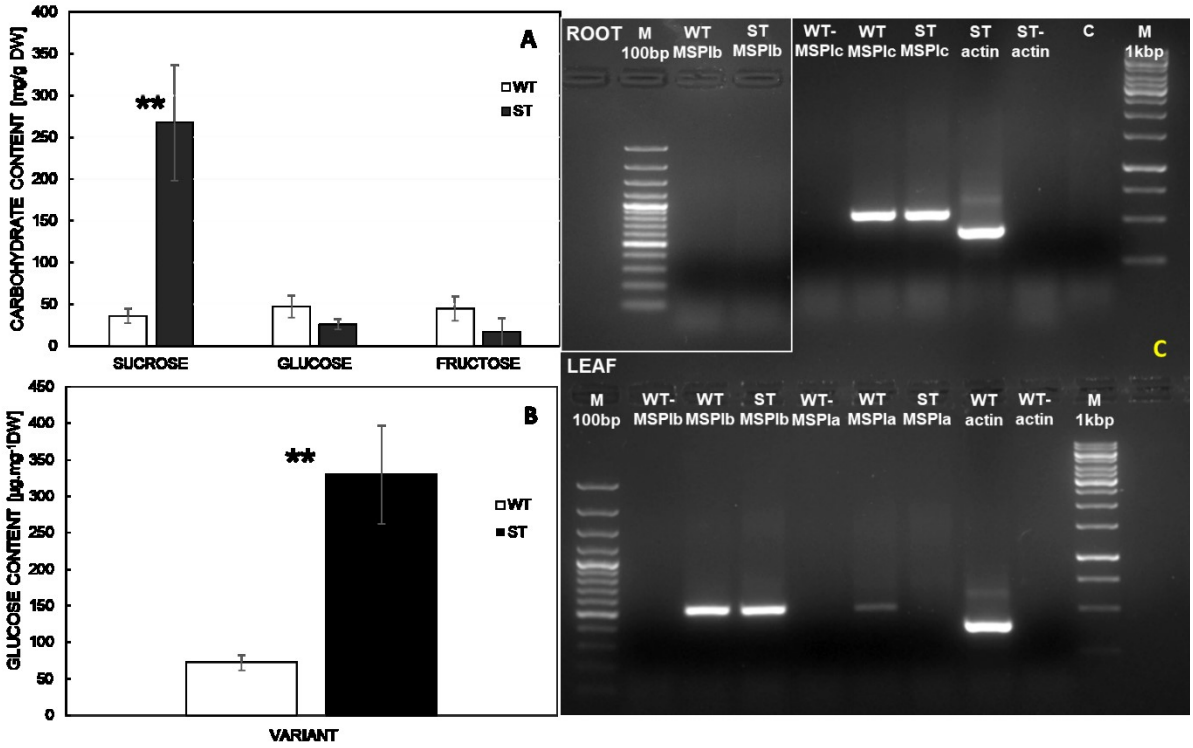

Fig. 2. Sugar (A) and starch (B) contents in root organ-cultures of WT and ST cultivated for 5 to 6 weeks. Starch contents are given as glucose levels after starch enzymatic digestion. Bars refer to standard deviations of sugar content, ** indicate significant difference at $\alpha = 0.01$, n = 2 – 4. MSPI alleles gene expressions (C) in leaves and in root organ-cultures of WT and ST. MSPIa-c, transcripts of individual *MSPI* alleles; WT- or ST-, controls of RT-PCR reactions of WT or ST, respectively; actin, transcript of constitutively expressed actin gene *Pot58*; C, PCR no template control, M 100bp and 1kbp, 100bp and 1kbp gene ruler (Fermentas), respectively.

5.3.1. Statement of contribution

HL and HS conceived and designed research. HS conducted all the experiments, PM performed the gene expression analysis. HS and HL wrote the manuscript.

6. Discussion

Rather than discussing all possible connected subjects of the presented research, I have chosen to discuss in more detail and to wider context few topics of special interest.

- The role of endogenous signalling in the regulation of tuberization in cultured potato cultivars
  - the role of GAs and sugars and their possible interactions
  - similarities between tuberization and flowering regulation

- MSP (mangan-stabilizing protein) and its possible role in tuberization regulation
  - how can we fit this role to the ST plants root organ-culture characteristics

- The effect of exchange of the mixotrophic cultivation for autotrophic one with special focus on the strongly species-specific reaction

Please kindly note that there are references throughout the text linking the information to specific figures and tabs presented in the publication and manuscripts enclosed in the Chapter 5.

6.1. Exogenous regulation of tuberization

As the wild potato place of origin are considered South American high-altitude regions. Till today we can find the wild Andean potatoes (*Solanum tuberosum* spp. andigena) growing there, fully adapted to the short day (SD) and low night temperature conditions. For its predictable behaviour (mainly strictly SD induced tuberization) they are sought-after model plants by researchers studying tuberization. As far as we know, the modern cultivated potato (*Solanum tuberosum* spp. tuberosum) was first domesticated in the southern Chilean lowlands roughly 10 000 years ago (Spooner et al. 2005). These potato subspecies have a predisposition to be cultivated in much warmer regions with much longer summer days. The long centuries of breeding all around the world used this predisposition and practically wiped out the SD

dependence. Therefore, the cultured potatoes are day neutral and must rely on other factors in their tuberization regulatory mechanisms. Despite this fact, day length has been the most intensively studied external factor having impact on the tuberization and the pathways involved are well known nowadays. This knowledge can help us since even though modern-day cultured potatoes are almost photoperiodically insensitive, they still possess the elements of the photoperiodic pathways and there is a high possibility of their involvement in another regulatory pathways either through cross-talk or by gaining a whole new function. In this work, however, I am focusing merely on the endogenous factors regulation tuberization.

6.2. Endogenous regulation of tuberization – the case of sugars and gibberellins

From what was previously said, once the role of photoperiodic dependant pathway has weakened, some of its components, originally without deciding role, are in the position of the control now. We must clarify the actual role of the agents originally playing role in the day-length control of the tuberization in the cultured potatoes and find out what is, or more probably are, the factor(s) actually regulating the tuberization in these plants. From what we already know we can deduce, that some other internal factors are involved. There are several possible candidates, mainly the carbohydrates and phytohormones such as jasmonates, abscisic acid or cytokinins but we focused mainly on two internal factors, namely the sugars and gibberellins (GAs).

The spontaneously tuberizing (ST) potato mutant is an interesting model for studying the involvement of the internal factors. We chose to further investigate the role of sugars, because it had been already known that the ST mutant have different sugar content from WT (Fischer at al. 2008). Fischer at al. (2008) proposed that the strengthened tuberization may be a result of somewhat weakened GAs inhibitory effect on tuberization, probably due to strong inducing effect of the sucrose acting upstream of GAs. Therefore, we measured the GAs content in ST plants and found similar or more surprisingly higher gibberellins (GAs) content (Fig. 5, paper 1) under photoautotrophic (PA) and mixotrophic (MT) *in vitro* conditions, respectively, comparing to WT. Thus, we conclude, that the weakening of the GA signal is probably not responsible for higher tuberization in ST mutant and importantly, that the sugars and GAs are parts of two parallel pathways regulating the tuber onset and that they are

not under each other's control directly. That is in contradiction with an earlier finding by Xu et al. (1998) who presumed that it is in fact the sucrose regulating internal GAs levels during the onset of tuberization, after series of *in vitro* experiments comparing the potato plants growing on various sucrose concentrations and measuring their GAs levels, (Xu et al. 1998). But from our results it is clear that even the ST plants while cultivated on high GAs concentrations, with sufficient sugar supply do not form tubers. This is common phenomenon to all potato cultivars, under various cultivation conditions (Tizio 1971) and of course the concentrations of GAs usually used in these experiments are more than artificial for plants so it can blur the results significantly.

On the other hand, during our research the addition of moderate amounts of sucrose to the media (3%) induced opposite reaction − massive tuber formation even in presence of high endogenous GA contents in the ST plants. This sucrose concentration is considered as non-inducing for the potato tuberization and yet, about 75% of ST plants formed tubers. Is it possible for the ST plants to be more sensitive to the sucrose induction and less sensitive to the GAs inhibition at once? Although it was proposed earlier that the sucrose can be sensed as a signal directly (Chiou and Bush 1998) this was not proved till today. Is some of the sucrose cleavage product serving as a signal about sucrose level in the tuberization pathway? The sugars with already confirmed signalling role are e.g. glucose, fructose or UDP-glucose (Rolland et al. 2002, Price et al. 2004). We can support this idea, because we found that leaves of the ST plants, where the tuberization signal should originate, contained significantly more fructose than in WT plants (Fig. 8, paper 1) although the only known fructose sensor is fructose-1,6-bisphosphate phosphatase (Cho et al. 2011) of which activity at the ST plants we have no information.

Another possibility we must consider is that the ST plants may have somehow modified / disturbed sugar sensing mechanisms. It is known that sugar abundance usually induces some of the typical sink organ activities such as the starch and anthocyanin biosynthesis (Eveland and Jackson 2012). We observed both, the higher amount of starch stored at the basal parts of the plants (Fig. 9, paper 1) and visibly darker colouration (the ST plants were almost purple sometimes, (Fig. 2, paper 1) under both MT and PA conditions. The uneven starch deposition is in fact very typical for the ST plants appearing under both PA and MT cultivations, whereas the WT always distributed starch evenly within the whole plant body. Another possibility causing the higher sensitivity towards sugars can be via mobile signalling elements.

The ST plants have higher *StSP6A* expression comparing to the WT (Fig. 10, paper 1) and StSP6A is known to have the ability to enhance the *SUT1* (SUCROSE TRANSPORTER 1) expression in stolon and strengthen the sink capacity there (Sharma et al. 2016).

There are several possible crosstalk points between the sugar and GAs signalling pathways. One can be via the sucrose transporter SUT4 (SUCROSE TRANSPORTER 4) as the StSUT4RNAi plants showed an inhibition of GA20-oxidase (key enzyme of GAs synthesis) expression (Chincinska et al. 2007). Moreover, both SUT4 and GA20-oxidase are reported to be phloem mobile. *SUT4* is predominantly expressed in the sink tissues and the StSUT4RNAi plants have significant changes in the source-to-sink allocation possibly causing premature tuberization and interestingly also flowering (Chincinska et al. 2007). Another possible crosstalk we can speculate about based on our results can be mediated by *StSP6A* (the potato homologue of FT), which transcript we find in elevated levels in the ST plants (Fig. 10, paper 1). StSP6A is also one of the elements probably controlled by SUT4 (Chinchinska et al. 2007) and moreover it can activate expression of a *GA2ox* gene coding one of the enzymes of GAs degradation machinery (Navarro et al. 2011). Nonetheless more experiments are needed to prove these hypotheses.

6.3. Similarities between tuberization and flowering regulation

For more than ten years an interesting idea exist that the regulation of the tuberization has very much in common with the regulation of the flowering (more on this in Rodriguez-Falcón et al. 2006). Over the following few years several publications confirming this idea emerged and the similarities found are of various nature from the long-distance signalling (e.g. the role of florigen and tuberigen) to the sugar balance and hormonal regulation. Some of the most interesting research outcomes are mentioned bellow and discussed with our results.

Surely, an attentive reader noted a mention of potato *FT* (FLOWERING LOCUS T) orthologue *StSP6A* on several occasions and its link to the tuberization regulation. Navarro et al. (2011) demonstrated that expression of the gene *Hd3a*, *FT* homologue from rice, induces tuberization in strictly short-day-dependent potato spp. andigena even under long day. The Hd3a-mediated induction of tuberization is graft-

transmissible and the Hd3a-GFP protein was detected in the stems of grafted plants. The authors showed that the tuberization and flowering in potato are controlled by two different FT paralogs (*StSP6A* and *StSP3D,* respectively) responsive to independent environmental cues (Navarro et al. 2011). In our paper we determined the *StSP6A* expression in the leaves of the PA cultivated ST and WT plants and found significantly higher *StSP6A* transcript level in the ST plants (Fig. 10, paper 1). Is it possible that the lack of one isoform of the mangan-stabilizing protein is causing intervention to the primary photosynthetic phase of ST plants big enough to cause positive signalisation to enhance the *StSP6A* expression? Or is it the disrupted carbohydrate balance, caused by lack of the MSP, being responsible for enhanced *StSP6A* expression? In Arabidopsis, *StSP6A* can partially replace function in plants with loss of function mutation in genes *FT* and *CO (CONSTANS)*, so it seems to have residual florigenic properties. Expression of *StSP6A* is controlled by an autoregulatory loop which stimulates the synthesis of the signal in the stolon. This maintenance mechanism is partly mediated via the StCO (Navarro et al. 2011). Moreover, the*StSP6A* is known to be subjected to similar upstream regulatory pathways as the *FT*.

The situation, however, is not clear at all, since there are also other players and there is more than one long-distance signalling agent capable of transfering the information from the leaves to the stolon or newly formed tuber in the case of the tuberization and to the shoot apical meristem in the case of flowering. In Arabidopsis, besides FT, miR172 was suggested to participate in the flowering control (Aukerman and Sakai 2003). And according to Hannapel et al. (2013) it is more than possible that miR172 is one of the main regulators of both flowering and tuberization in potatoes, according to the specific exogenous conditions (Hannapel et al. 2013). Another important player is previously mentioned *StSUT4* which is strongly expressed in both tubers and flowers and StSUT4-RNAi plants are prematurely tuberizing and flowering under non-inducible long day conditions which suggests StSUT4 role in the regulation of both processes (Chinchinska et al. 2007). Even more convincing piece of evidence showing the significance of StSUT4 is that its effect on both processes is graft transmissible and depends on the presence or lack of the source leaves, which can mean that StSUT4 has an important role in the source leaves also. Other possible phloem-mobile molecules with signalling function are considered, such as the sucrose itself (Smeekens 2000), even though we are still waiting for the definite proof of it. Another possibility are assimilates serving as a part of the florigenic signal (Bernier

and Perilleux 2005) or at least they can be the driving force assuring the FT mobility through the phloem when there is a sufficient mass flow of the assimilates (Thomas 2006). This mechanism may work in the tuberization regulation too, since the developing tuber is a very strong sink. Moreover, the sucrose synthase (SuSy) has been suggested as important component of the sucrose signalisation by Nguyen et al. (2016) who proposed the existence of a novel sucrose signalling pathway. They demonstrated that the increased sucrose levels found in the tobacco plants overexpressing *SuSy* were a direct result of enhanced photosynthesis and photosynthetic sucrose synthesis triggered at first by increased chlorophyll synthesis. The ST plants had also significantly higher total chlorophyll content (Fig. 4, paper 1) and higher sucrose content than WT in almost all organs (Fig. 8, paper 1) therefore we can assume possible engagement of this newly discovered pathway.

Another striking resemblance between the tuberization and flowering is that not only the sucrose but also GAs (specifically GA_4) are considered to be a part of the florigenic signal in Arabidopsis. The plant can flower even in the non-inductive short-day conditions and the flowering can be started by a sharp increase of the GAs and sucrose in the shoot apical meristem (Eriksson et al. 2006). Obviously, the spatial and temporal fine tuning of the GAs and sucrose concentration is crucial for running the desired developmental program be it tuberization or flowering.

6.4. MSP and its role in tuberization

ST plants are used as one of the main models in the work presented but what is the possible connection between the lack of one of three isoforms of mangan-stabilizing protein (MSP) of photosystem II (PSII) and the tuberization induction still remains a mystery. I would like to discuss here some of the possible explanations.

Firstly, the previously published work by Fischer et al. (2008), showing stronger tuberization induction and higher sugar content in the ST plants, was fully supported by results of Gururani et al. (2012). Fischer et al. (2008) used a day-neutral potato cv. Lada and its ST mutant, described earlier, while Gururani et al. worked with a short-day (SD) potato cv. Taedong valley with reduced and increased MSP (however, the information about specific MSP isoform is missing) which may indicate that MSP involvement in the tuberization regulation is not photoperiod dependent. Their theory of how the tuberization and MSP are connected goes for the obvious connection via

the altered photosynthesis. Their findings of altered PSII activity (measured via. chlorophyll a fluorescence) are consistent with our findings (Fig. 1, paper 1). The ST plants had significantly lower quantum yield of chlorophyll a fluorescence, which may indicate affected primary photosynthetic reactions and lower numbers of open reaction centres of PSII. Surely, other studies are needed to strengthen our knowledge about the relationship between mechanisms regulating the potato tuberization and photosynthesis.

The photosynthesis is closely connected with the sugar metabolism and sugars can be the link we are looking for between the tuberization and photosynthesis. The ST plants (Fig. 8, paper 1), and Gururani et al. (2012) MSP-antisense plants as well, had higher sugar content. There is a question whether the higher sugar content is a cause or a consequence. Do the ST plants have higher sugar demand due to being induced to tuberize thus having more sinks in the form of newly formed tubers, therefore bigger need for assimilates and bigger photosynthetic activity? Or is the lack of one of three MSP isoforms enough to alter the photosynthesis into bigger yields causing induction of the tuberization?

Of course, there can be other more complicated connections, for example with some phytohormonal regulation, e.g. cytokinins which play a role in the source-to-sink relations (Roitsch and Ehness 2000). Another factor which may complicate the obtaining of precise knowledge of connection between the photosynthesis and tuberization is the signalling role of the sugars.

The changed sugar distribution observed in the ST plants led us to an idea to investigate the ST roots more precisely. Surprisingly, when we cultivated just roots of the ST plants, the results were similar to the ones obtained with the intact plants. For example, the sugar content determination shoved that ST plants tend to accumulate the sucrose, whereas in the WT the hexoses (glucose and fructose, namely) prevailed (Fig. 2, manuscript 3). This indicates the MSP being included in the regulation of more than one process because how possibly can be connected PSII to what is happening in the roots?

At the end, even though some of above mentioned ideas seem plausible, we cannot forget the role of the epigenetical changes that may be a result of a long-term *in vitro* cultivation of the plant material and can be also the source of ST plants abnormal behaviour. Another simple, but not very desirable, explanation can be hidden in so far not perfectly searched ST potato genome. Although after thorough

examination by Fischer et al. (2008) the only one difference in the plant proteome was found, there are also some deletions in the non-coding area near the MSP gene on chromosome 2 (Fischer, personal communication) that may have some unknown but important regulatory role in the tuberization regulation. But the results obtained by Gururani et al. (2012) speak against these two notions and in favour of the MSP having a role in the tuberization regulation.

6.5. Plants do surprisingly vary in their reaction to the mixotrophic *in vitro* conditions

The main message of the second manuscript presented is that it is very hard to predict how certain plant would react to the artificial conditions of the widely used mixotrophic (MT) *in vitro* cultivation. Of course, we should not just stop using this important approach, but rather proceed cautiously, especially if we are studying the sugar metabolism and related topics, and gain as much knowledge as is possible about the reaction of certain plants to these conditions before we make any conclusions. We studied several selected characteristics (growth, sugar-metabolism related and photosynthetic) of four plants commonly used as a models in plant physiology research – potato, tobacco, strawberry and rapeseed and compare them to their photoautotrophically (PA) grown counterparts.

The fact that plants / explants cultivated under the MT conditions usually photosynthesize very badly due to changes in their anatomy and physiology is nothing new (e.g. Arigita et al. 2002), but what is shocking is the variety of different reactions of the plants under our study. The only similarities are confirming the Arigita et al. 2002 results shoving that under the PA cultivation all plants were bigger and more developed with larger leaf area (Fig. 1, manuscript 2). We also observed a significant increase in the photosynthetic rate (Pn) and conductance of all studied plants under PA in comparison with MT cultivation (Fig. 6 and 8, manuscript 2) which is only logical, because otherwise the plants could not survive without any external energy source. Although, there are publications countering this simple logic, e.g. Badr et al. 2015 studied growth and rooting of a potato *in vitro* on a media with or without the sucrose and found no difference at all between the two cultivations. They also find no difference in various photosynthetic parameters. Our potato on the other hand was the plant with biggest difference in the Pn (Fig. 6, manuscript 2) between the PA and MT conditions. Especially interesting is that potato was the only plant we studied with negative Pn under the MT conditions. However, the explanation of this conundrum may be simple if we look at the cultivation method used by Badr et al. 2015 more closely – they used the exact same vessel without enough ventilation for both cultivation conditions. Since it is well known that potato very strongly negatively reacts on the presence of the ethylene in the cultivation vessel, this can be our solution

to this discrepancy. Indeed, one should be always careful when choosing the cultivation vessel and especially the type of closure he uses.

One of the most surprising parameters in which the studied plants differ among each other is photosynthetic pigment content (Fig. 3, manuscript 2). Potato and strawberry reacted similarly and in the most obvious way – they increased all their photosynthetic pigments content significantly under the PA cultivation compared to the MT. Since it is known that external sucrose added to the cultivation medium can reduce the chlorophyll synthesis (Bender et al. 1987; Kirdmanee et al. 1992) it was the anticipated reaction. The rapeseed on the other hand reacted in completely opposite way and had all the photosynthetic pigments levels increased under the MT cultivation. Similar reaction was observed for example in the citrus plantlets (Hazarika et al. 2000). The third unique reaction we observed in the tobacco, which had the photosynthetic pigments levels still more or less the same, no matter the cultivation conditions. Interesting recent research can shed some light onto the issue of the chlorophyll contents and chloroplasts biogenesis regulation. The chloroplasts biogenesis needs precisely coordinated regulation of the nuclear and proplastid genomes consisting of both the anterograde (nucleus to plastid) and retrograde (plastid to nucleus) signalling (de Souza et al. 2017). A novel research by Debruil et al. (2018), with use of an Arabidopsis single-cell system in which they could track dynamics changes in the transcripts and metabolites, revealed that there are two distinct phases in the chloroplast development. The initial light-induced anterograde phase (in this phase chlorophyll synthesis takes place) and the second phase that is characteristic by retrograde signalling that causes dramatic changes in the expression of the nucleus-encoded photosynthetic genes and is important for a transition to the photosynthetic competence. Interestingly, the retrograde phase can be inhibited by high levels of sucrose. Thus, the excess in the sucrose can restricts the maturation of chloroplasts (Debruil et al. 2018).

The sugar and starch content in the leaves (Fig. 4 and 5, manuscript 2) of the plants under study is another example of interspecies variability we observed. In this case the potato and the tobacco shared similar reaction to the MT conditions and accumulated more sugars and starch under MT conditions consistently with observations Hdider and Desjardins 1994 made with the strawberry. Surprisingly our strawberry plants had completely opposite reaction and accumulated significantly more soluble sugars under the PA conditions similarly to the rapeseed plants. The

starch content on the other hand varied in these two plants, but tended to by higher under the MT conditions.

The photoautotrophic *in vitro* cultivation has clear advantages over the mixotrophic one. For example, the elimination of the sucrose addition to the cultivation media significantly reduces the risk of microbial contamination and is causing an improved plant photosynthetic capacity leading to easier *ex vitro* acclimation of such plants (Zobayed et al. 2000, Xiao et al, 2011). Unfortunately, it is not always possible to cultivate the plants photoautotrophically, thus we should broaden our knowledge about the alteration in the growth and metabolism of specific species (maybe even cultivars) caused by the MT cultivation and study of the sugar metabolism is a good starting point.

7. Závěry (in Czech)

Abychom rozšířili naše poznatky o úloze sacharidového metabolismu při regulaci morfogenních procesů, zvláště tuberizace, studovala jsem spontánně tuberizujícího (ST) mutanta bramboru. Rostliny byly kultivovány jak fotoautotrofně (PA), tak mixotrofně (MT) za podmínek *in vitro*. Výsledky ukazují na existenci dvou paralelních drah, kterými sacharóza a gibereliny regulují nástup tvorby hlíz. ST rostliny mají narušenou sacharidovou signalizační dráhu do které pravděpodobně zasahuje i bramborový ortholog *FT StSP6A*.

Podrobná studie čtyř druhů rostlin (brambor, tabák, jahoda a řepka) kultivovaných v podmínkách MT a PA *in vitro* s uzávěry kultivačních nádob našeho zdokonaleného designu odhalila výrazné druhově-specifické reakce růstu, sacharidového metabolismu a fotosyntézy na tyto podmínky. Proto bych důrazně doporučovala vzít v úvahu tyto znalosti při navrhování *in vitro* experimentálního uspořádání.

Kořenové orgánové kultury ST rostlin sdílejí mnoho zvláštních znaků s intaktními ST rostlinami, což je překvapující, protože jediná známá změna těchto rostlin je v absenci proteinu stabilizujícího fotosystém II. Analýza genové exprese však ukázala, že gen kódující tento protein v kořenových kulturách není exprimovaný. Další studie na toto téma jsou tedy nezbytné.

8. Conclusions

To broaden our knowledge about the role of sugar metabolism in the regulation of the morphogenic processes, especially the tuberization, I studied spontaneously tuberizing potato plant mutant cultivated under both photoautotrophic (PA) and mixotrophic (MT) *in vitro* conditions. The results are pointing to the existence of two parallel pathways by which the sucrose and gibberellins regulate tuber onset. The ST plants have disturbed the sugar pathway with a possible involvement of potato *FT* orthologue *StSP6A* in this patway.

A detailed study of four plants species (potato, tobacco, strawberry and rapeseed) cultivated under MT and PA *in vitro* conditions, with the cultivation vessels covers of our improved design, revealed strongly species-specific reactions of the growth, sugar metabolism and photosynthesis to these conditions. Therefore, it is advisory, to take this knowledge under consideration when designing an *in vitro* experimental layout.

ST plants root organ-cultures are sharing many distinctive features with the intact ST plants which is surprising, because the only known alteration of these plants is in the absence of a protein stabilizing the photosystem II. Gene expression analysis revealed, that the gene coding this protein is not expressed in root organ-cultures. Therefore, other studies are needed.

9. Intermezzo

Expression of Arabidopsis *WEE1* in tobacco, induces unexpected morphological and developmental changes

Due to unexpected circumstances caused by personal changes in our team and in order to continue in the research supported by university funding I worked for some time of my PhD studies on a topic somewhat unrelated to my thesis aims. Since this work took significant amount of my time and the project was successfully finished with a publication and there is another manuscript under preparation with a colleagues from Cardiff University UK containing my results, I decided to write a brief overview of my results in this topic and include them into my doctoral thesis.

Abbreviations:
cyclin (Cyc), cyclin-dependent kinase (CDK), ICK (inhibitor of CDK), *S. pombe* gene *cdc25* (*Spcdc25*), tobacco line expressing *WEE1* from *arabidopsis* (NT-Arath;Wee1)

9.1. Introduction

In order for the cells to divide accurately it is substantial to build a carefully organized sequence of events during which a cell doubles its content, copies its genetic information, and passes it to the next generation – the cell cycle.

A sessile lifestyle has led plants to create various adaptations that are related with cell cycle and cell division. The most important of these include unfinished organogenesis and related pluripotency of plant cells. Another plant specificity is very limited ability of cell migration both in embryogenesis and organogenesis. Daughter cells remain at the site of their origin and still have the same neighbouring cells. An important role in the process of organogenesis and embryogenesis has in particular the change of the polarity of cell

division. There is no doubt that precise regulation of cell division is indispensable for proper development of the plant body.

The ability of cell division is one of the trademarks of all living organisms, be it a unicellular bacterium, a mammal or a plant. Eukaryotic organisms have a much larger and more complex genome than prokaryotic therefore to secure the flawless replication of the genome, it was necessary for eukaryotic cells to separate the replication process from the cytokinesis. The cell cycle thus formed is divided into two basic parts, mitosis and interphase. During mitosis (M phase) segregation of chromosomes into nuclei of emerging daughter cells occurs. Mitosis is made up of four consecutive steps – prophase, metaphase, anaphase, telophase. After an even distribution of genetic information into the newly formed nuclei the separation of the two daughter cells, the so-called cytokinesis, occurs. Subsequently, the daughter cells can begin to prepare for new cell division during the consecutive phases of interphase – G1 phase (postmitotic), S phase (synthetic) and G2 phase (premitotic). During the G1 phase, the cell increases its volume and exhibits a high metabolic activity, because it synthesizes the components necessary for a successful replication such as basic constituents of nucleic acids and replication apparatus. These molecules are used in the S phase for DNA replication. The next stage is G2, during which the cell further increases its volume and synthesizes the proteins necessary for mitosis (Nurse 2001, Francis 2007).

Using yeast mutants, the checkpoints in which the cell decides whether it can go into the next phase of the cell cycle have been discovered. One of them is at the end of G1 phase and the cell verifies in it whether it has reached the limiting size and has enough protein stock to replicate its DNA and divide it into two daughter cells (Hartwell 1974). The second checkpoint is located at the transition point in between the G2 and M phase. G2 / M transition regulation is necessary to avoid premature entry into mitosis before replication is complete (Nurse and Bissett 1981). Important controlling components of the checkpoints are serine-threonine protein kinases that require binding to regulatory proteins the so-called cyclins to perform their function. For this reason, they are called cyclin-dependent kinases (CDKs). In addition to cyclins, CDKs activity can be regulated by binding of inhibitors called ICK (inhibitor of CDK) and KRP (Kip-related proteins. The latter way of regulating CDK activity is specific phosphorylation (which occurs on the threonine residue 160) and dephosphorylation (on the threonine residue 14 and the tyrosine residue 15, Dewitte and Murray, 2003) serving as the final regulatory step in all-or-nothing manner.

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In higher plants, two families of protein kinases involved in the regulation of cell entry into mitosis, CDKA and CDKB, which is typical for plants only (Dewitte and Murray 2003), have been found. These kinases can be associated with class A, B, D and H cyclins (Cyc). CycAs are active primarily in the period between the S and M phases, CycBs are actively expressed at the G2 / M transition, and their expression remains high during mitosis (Breyne and Zabeau 2001, Potuschak and Doerner 2001) and CycDs are expressed according to their type throughout the whole cell cycle (Menges et al., 2005). In eukaryotes, except plants where this mode of control has not yet been experimentally verified, plays an important role in the regulation of CDK activity their phosphorylation and dephosphorylation mediated by WEE1 kinase and CDC25 phosphatase. The function of WEE1 kinase in fission yeast (*Schizosaccharomyces pombe*) is phosphorylation of cdc2 (a yeast homologue of CDK), which leads to its inactivation, thus preventing entry into mitosis. The first plant homologue of WEE1 kinase was discovered in maize (*Zea mays*, Sun et al. 1999), and early after that detection of this kinase also occurred in a dicotyledonous plant (*A. thaliana*, Sorrell et al. 2002). WEE1 kinase together with ICK1 / 2 appears to be the major negative regulators acting on the CDK family A and B. CDC25, which activates CDK by dephosphorylation of Tyr-15 and Thr-14 (Millar et al. 1991, Norbury and Nurse 1992) serves as a WEE1 kinase antagonist in animals. The role of dephosphorylation in the plant cell cycle is less clear since only abridged version of the yeast *CDC25* gene containing only the catalytic domain was found in the Arabidopsis genome (Dissmeyer et al. 2009).

The fact that CDK activatory dephosphorylation also occurs in plants show, for example results obtained from experiments with tobacco (*Nicotiana tabacum* cv. Samsun) transformed with S. pombe gene *cdc25* (*Spcdc25*). In these plants, it has been observed that cells have been stopped in the G2 phase. Expression of the fission yeast CDC25 gene in both tobacco (Lipavská et al. 2011) and Arabidopsis (Spadafora et al. 2012a), resulted in phenotypes that are consistent with its action in dephosphorylating and activating CDK. Moreover, in these cells cytokinin levels were greatly reduced and the cells were insensitive to the cytokinin biosynthetic inhibitor, lovastatin, indicating a link between CDK dephosphorylation and cytokinin signalling. In whole plants, this cytokinin-independent phenotype was supported by an ability of *Spcdc25* expressing stem explants to produce shoots in the absence of exogenous cytokinin (Orchard et al. 2005). Another effect of *Spcdc25* expression in tobacco was a precocious flowering with a dramatic reduction both in the time to flowering, and the number of leaves formed prior to flowering

(Vojvodová et al. 2013). Orchard et al. (2005) also investigated CDKB1 activity in *Spcdc25* transformed cells and observed higher CDKB1 activity during S / G2 and early M phases compared to control cells. So far, it is not at all certain whether dephosphorylation plays an equally important role in the G2 / M transition in plants as in yeasts and animals.

In fact, the importance of the inhibitory phosphorylation can be somehow weakened in plants also, since Arabidopsis mutants deficient in WEE1 kinase grow and develop normally although they are hypersensitive to DNA replication inhibitors such as hydroxyurea (De Schutter et al. 2007, Dissmeyer et al. 2009). However, the role for WEE1 in plants is not restricted to the DNA replication checkpoint. WEE1 regulates CDK activity in a cell cycle dependent manner with a drop in WEE1 activity at the G2/M transition (Cook et al., 2013).

Cultured hypocotyls of *Arabidopsis wee1-1* mutants showed increased morphogenetic capacity, and *wee1-1* seedlings produced more lateral roots per millimetre of primary root (Spadafora et al. 2012b). In accordance with the mutants behaviour over-expression of *Arath;WEE1* in *Arabidopsis* repressed the morphogenetic capacity of hypocotyls in culture and primary roots of these transgenic plants were shorter with less lateral roots than in the wild type.

9.2. Materials and methods

9.2.1. Plant material

Tobacco (*Nicotiana tabaccum*) cv. Samsun plans were used as a wild type. Into this plants *Arath;WEE1* was cloned to pkanII-SPYCE(M) as described in Cook et al. (2013). Transgenic plants were prepared by the Cardiff University team and seeds of two transgenic lines (NT-Arath;Wee1#8 and #2) were shipped to Prague.

9.2.2. Analysis of tobacco plants growth and flowering onset

Wild type and transgenic tobacco plants were grown from seeds in a growth chamber at 22/18 ºC day/night thermoperiod with 16 hrs illumination (irradiance 435 W m^{-2}), relative humidity 50–75%. Plants were grown in pots (30 cm in diameter) in soil. The leaves were numbered from the base (1 oldest) and when first flower bud emerged the length of leaves without petiole was measured and the leaves above 10 cm were counted. The age of the plants is given as days of growth after sowing.

9.2.3. Tobacco roots analysis

Sterilized tobacco seeds were sown in a square Petri dish containing MS medium (Murashige and Skoog Basal Salt Mixture, plant cell culture tested, Sigma-Aldrich) containing 3% sucrose, 2 cm apart. After 21 days of cultivation at 25ºC under a 16 h photoperiod with PPFD (photosynthetic photon flux density) approximately 100 µmol m^{-2} s^{-1} (daylight fluorescent tubes; Osram). The length of the main root was measured and lateral roots counted semi-automatically with Smart Root software (ImageJ, Schneider et al. 2012). Following clearing method was used for root primordia visualisation. The roots were fixated in acetone overnight and then fixated in phosphate buffer and mounted in 65% aqueous glycerol and observed with an Olympus BX51 microscope equipped with anvApogee U4000 digital camera.

9.2.4. Organogenesis

Tobacco stem segments, 1 cm long, were put onto MS medium containing 3% sucrose, or SIM (shoot inducing medium) consisting of MS medium with 3% sucrose, 0.1mg/l NAA (naphthaleneacetic acid, Sigma-Aldrich), and 2mg/l BAP (benzylaminopurine, Sigma-Aldrich). After 21 days of cultivation the number of shoots and protruded shoot primordia was counted.

9.3. Results

9.3.1. *Arath;WEE1* expression in tobacco plants results in premature flowering, altered root system morphology and spontaneous shoot formation in culture.

Constitutive *Arath;WEE1* expression in tobacco caused significant changes in plant development and led to premature flowering (Fig. 1A). WT plants grown in a growth chamber took approximately 150 days to flower from day of sowing, whereas the *Arath;WEE1* –expressing transgenic plants (lines NT-Arath;Wee1#8 and #2) flowered significantly earlier, after about 100 days (Fig. 1B). Moreover, WT plants flowered when they had produced more than 20 leaves longer than 10 cm, while transgenic plants expressing *Arath;WEE1* formed only around seven leaves before they started to flower (Fig. 1C).

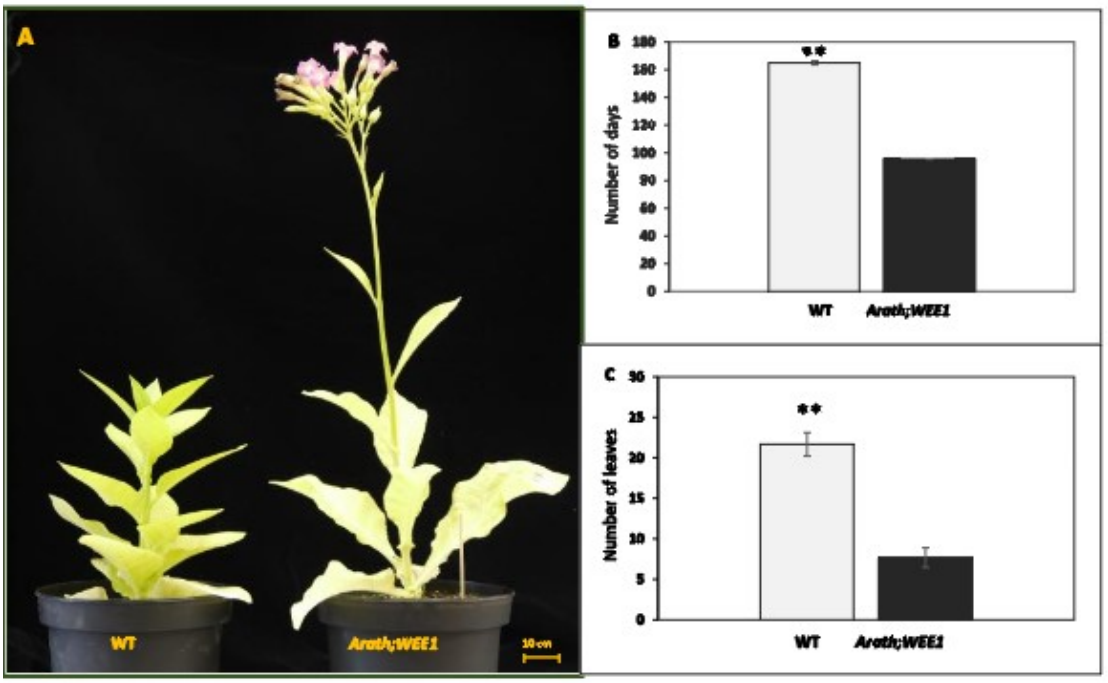

Fig. 1 Expression of *Arath;WEE1* in tobacco changed the onset of flowering: (A) Tobacco plants (WT) and *NT-Arath;Wee1#8* after 100 days of growth; (B) number of days and (C) number of leaves over 10 cm in length, at flowering (n=6 + SE; ** $P < 0.01$).

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Expression of *Arath;WEE1* in tobacco plants also affected root morphology: NT-Arath;Wee1#8 plants had a significantly shorter primary root and the whole root system appeared to be less developed (Fig. 2A). However the number of lateral roots showed no difference between WT and *Arath;WEE1*-expressing plants (Fig. 2B). Indeed the number of primordia formed on the root revealed that *Arath;WEE1*-expressing plants form less primordia but probably with better capacity to outgrow into a fully grown root (Fig. 2C).

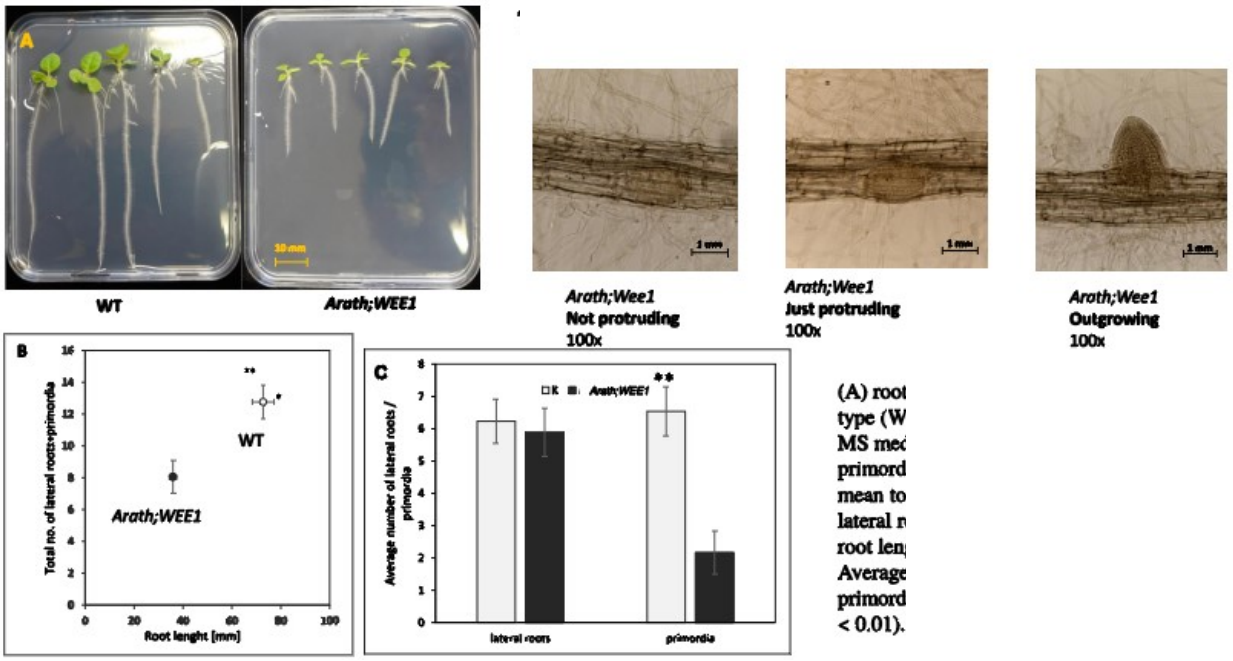

Fig. 2 Tobacco root development is affected by expression of *Arath;WEE1*: (A) Root growth after 21 days of WT and *NT-Arath;Wee1#8* on MS medium and morphology of the root primordia (B) The relationship between mean total number of lateral roots and lateral root primordia and mean primary root length for 21-d-old seedlings (C) Average number of lateral roots and primordia (n=18 + SE; * $P < 0.05$; ** $P < 0.01$).

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Further effects of expressing *Arath;WEE1* in tobacco plants were seen in stem cuttings culture. When growing on standard cultivation media without any growth regulators, 1 cm long stem cuttings from *Arath;WEE1*-expressing plants formed on average 15 new shoots compared to WT cuttings that formed only callus (Fig. 3). NT-Arath;Wee1#8 tobacco stem segments cultivated on shoot induction medium also showed significantly greater capacity to form new shoots, producing 30, on average, from each stem cutting, while WT cuttings formed on average only 14.

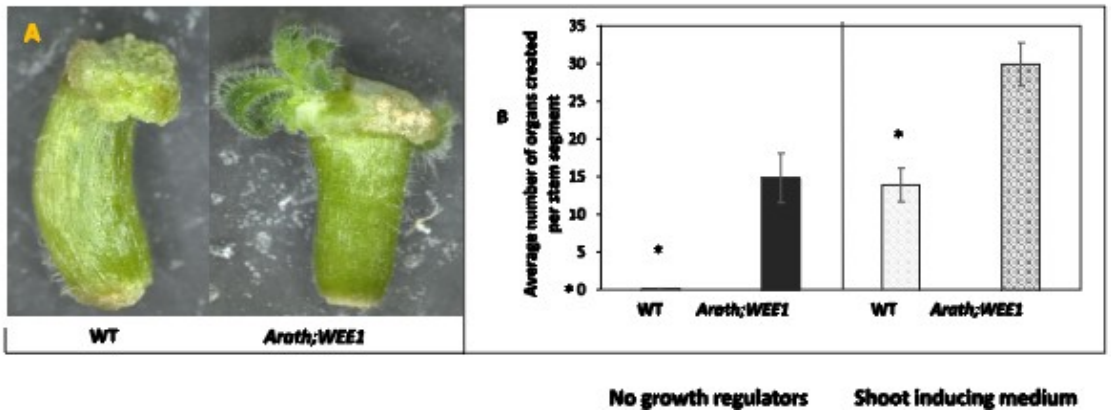

Fig. 3 *De novo* shoot formation from tobacco stem explants is stimulated by expression of *Arath;WEE1*: (A) shoot formation after growth of WT and *NT-Arath;Wee1#8* 21 days on MS medium; (B) organogenesis rate on medium without addition of plant growth regulators and shoot inducing medium (n=12 + SE; * $P < 0.05$).

9.4. Discussion with literature and Cardiff colleague's results

Surprisingly, the precocious flowering phenotype observed in the *Arath;WEE1* tobacco plants shows strong similarities to the phenotype seen when *Spcdc25* was expressed in tobacco (Bell et al., 1993; Teichmanová et al. 2007, Vojvodová et al. 2013). The reduction in time to flowering (a 1.5-fold reduction) and number of leaves produced before flowering (a 2.8 fold reduction) was almost identical. Based on grafting experiments (Vojvodová et al. 2013) it was hypothesised that the premature flowering in the *Spcdc25* plants may be result from an earlier competence of the shoot apical meristem to respond to the floral stimulus (Lipavská et al. 2011). A similar mechanism may be operating in the tobacco plants expressing *Arath;WEE1* although we don't know how to explain fully this behaviour which is in contradiction to what was expected.

The reduction in primary root length and lateral root production in tobacco plants expressing *Arath;WEE1* contrasts with the effect of *Spcdc25* in increasing lateral root production noted by McKibbin et al. (1998) but is consistent with later reports of a restriction in root growth elicited by *Spcdc25* expression in tobacco and attributed to the cytokinin-like effect observed in these plants (Lipavska et al. 2011). However, shorter primary roots were also found when *Arath;WEE1* was over-expressed in Arabidopsis (Spadafora et al. 2012b) and is consistent with a negative effect of increased *WEE1* on root meristematic cell division. In general, there is still much discrepancy to be found in the results concerning root growth and *cdc25* or *WEE1* overexpression.

The spontaneous formation of shoots in the absence of added cytokinins was also seen both in tobacco expressing *Spcdc25* and *Arath;WEE1*. However it contrasts with the phenotype seen in Arabidopsis plants over-expressing *Arath;WEE1* where cultured hypocotyls from the *Arath;WEE1* over-expressors produced less shoots than WT (Spadafora et al. 2012b). In fact the phenotype of the tobacco plants expressing *Arath;WEE1* in this respect is more similar to the *Arath;WEE1* knockout mutant lines, which produced more shoots from cultured hypocotyls than WT (Spadafora et al. 2012b).

Thus at a plant and organ level there are strong similarities between the effects of expressing *Spcdc25* and *Arath;WEE1* in tobacco. This is surprising given the opposing functions of the enzymes encoded. The difference between the expression of *Arath;WEE1* in tobacco and Arabidopsis confirms that *Arath;WEE1* does indeed induce the expected phenotype when expressed in its native environment. However the effects of its expression in tobacco are more consistent with a dominant negative effect, somehow repressing the action of the native tobacco *Nicta;WEE1*.

In an effort to shed a light on these results contradicting expected opposing reactions our colleagues in Cardiff observed an apparent shift in the expression of the native *Nicta;WEE1* (WEE1 protein levels increased during S/G2 and again fell rapidly as cells entered mitosis) when compared to the WT untransformed plant, which may form the underlying mechanism for the activation of a premature mitosis with the resulting phenotypic effects seen at a cellular, organ and whole plant level. One possible mechanism is that *Arath;WEE1* transcript production and translation into protein during S+G2 results in a feedback to *Nicta;WEE1* transcription causing its downregulation, delaying the accumulation of native WEE1 transcripts. This could be mediated through the large number of transcription factors that regulate *WEE1* expression. An alternative mechanism may act at the protein level. The accumulation of Arath;WEE1 protein in S/G2 may

activate the proteasome machinery prematurely due to differences in Arath;WEE1 sequence and conformation and subsequently trigger an early mitosis.

For premature cell division to occur, early increases in CDK activity are expected, which would drive cells into early mitoses. This hypothesis was tested by measuring kinase activity of both Nicta;CDKA;1 (referred to here, as CDKA) and Nicta;CDKB;1 (referred to here as CDKB). CDKA activity was relatively constant. However significant increase in CDKB activity was observed, at 5-7 h following release of the cells from aphidicolin. At this point the *Arath;WEE1* cells were at G2/M phase, while the WT cells were at S/G2. Thus the expression of *Arath;WEE1* resulted in an earlier increase in CDKB consistent with the earlier mitotic peak.

9.5. Conclusion

In conclusion the key finding is that expression of *Arath;WEE1* in tobacco causes an anomalous phenotype consistent with a dominant negative effect and a phenotype that strongly resembles expression of the positive regulator of G2/M progression, *Spcdc25*. Thus, we can conclude that the phosphorylation step, that is necessary for mitosis entry in animals and yeast, probably does exist in plants too ant that WEE1 is part of it.

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11. Appendix

11.1. Certified methodology for photoautotrophic *in vitro* cultivation (in Czech)

Univerzita Karlova v Praze, Přírodovědecká fakulta, Katedra experimentální biologie rostlin

Metodika fotoautotrofní kultivace rostlin za podmínek *in vitro*

Hana Ševčíková, Helena Lipavská, Petra Mašková

duben 2016

Metodika fotoautotrofní kultivace rostlin za podmínek *in vitro*

Certifikovaná metodika vypracovaná jako výstup projektu MŠMT - Centrum experimentální biologie rostlin UK, LO1417

Autoři:

Mgr. Hana Ševčíková

hana.sevcikova@natur.cuni.cz

RNDr. Helena Lipavská, Ph.D.

lipavska@natur.cuni.cz

RNDr. Petra Mašková, Ph.D.

peta_maskova@volny.cz

Oponenti:

Ing. Lenka Langhansová, Ph.D.

Ústav experimentální botaniky AVČR v.v.i.

langhansova@ueb.cas.cz

Obsah

1. Úvod

Udržování a množení rostlin *in vitro* je široce využívaná technika jak pro výzkumné, tak pro komerční účely. Tento způsob kultivace rostlin má své nesporné výhody, například poměrně malé nároky na prostor, nezávislost na ročním období, vysokou rychlost množení, u endemických či ohrožených druhů možnost pěstování mimo původní lokalitu, kultivaci nezávislou na symbiontech či možnost vegetativního množení u rostlin, které se v přírodě tímto způsobem nemnoží. Navíc v současnosti již existují kultivační protokoly pro širokou škálu rostlin, což dále usnadňuje využití tohoto způsobu kultivace. Nepříliš akcentovaným problémem je výrazné omezení fotosyntézy rostlin kultivovaných *in vitro* při běžně používaném postupu, a to z důvodu nedostatečné výměny plynů mezi kultivační nádobou a okolím. Rostliny proto vyžadují pro svůj růst externí zdroj uhlíku (heterotrofní způsob kultivace). Tento přístup nemusí být příliš vhodný z hlediska vědeckého, zejména pokud chceme zkoumat fotosyntetické procesy či sacharidový metabolismus obecně. S ohledem na praktické využití je významná také skutečnost, že heterotrofně kultivované rostliny mají problém s adaptací po přenosu z podmínek *in vitro* do podmínek *in vivo* (nutnost adaptace fotosyntetického aparátu, náchylnost k houbovým a bakteriálním patogenům a také nutnost vypořádat se se snížením relativní vzdušné vlhkosti po přenosu do *ex vitro* podmínek).

2. Cíl metodiky

Cílem metodiky je vytvoření snadno dostupného, efektivního a ekonomicky výhodného systému umožňujícího fotoautotrofní kultivaci *in vitro* u hospodářsky i výzkumně významných druhů rostlin, lilku bramboru (*Solanum tuberosum* L.), tabáku viržinského (*Nicotiana tabacum* L.) a řepky olejky (*Brassica napus* L.) s potenciálem k využití u dalších rostlinných druhů.

3. Dedikace

Tato certifikovaná metodika vznikla za podpory MŠMT jako součást řešení projektu NPU: Centrum experimentální biologie rostlin UK (LO 1417).

4. Stručný popis metodiky

Rostliny jsou kultivovány *in vitro* na standardním kultivačním médiu doporučovaném pro daný rostlinný druh, bez přidaných sacharidů v kultivačním médiu. Kultivační nádoby jsou uzavřené polypropylenovou (PP) autoklávovatelnou fólií s dvěma PP filtračními terčíky zajišťujícími výměnu plynů bez rizika proniknutí patogenů. PP fólie zároveň zaručuje dostatečnou ozářenost umožňující rostlinám fotosyntetizovat při kultivaci za standardních podmínek.

5. Detailní popis metodiky

5.1. Použitý rostlinný materiál a jeho kultivace

Při vývoji a ověřování metodiky byly použity rostliny bramboru *Solanum tuberosum* L. cv. Lada, rostliny tabáku *Nicotiana tabacum* L. cv. Samsun a řepky olejky *Brassica napus* L. cv. Asgard. Kultivace rostlin probíhala v podmínkách *in vitro* na MS médiu (Murashige & Skoog, 1962). Složení média viz Tabulka č. 1. Se stejnými výsledky bylo používáno jak médium míchané ze zásobních roztoků jednotlivých sloučenin, tak z komerčně dodávané směsi MS solí (Murashige and Skoog Basal Salt Mixture, plant cell culture tested, Sigma-Aldrich, St. Louis, USA), v obou případech s přídavkem roztoku vitamínů (roztok D v Tab. 1). pH kultivačního média bylo upravováno na hodnotu 5,75 za použití 10mM KOH.

5.2. Experimentální design

Rostliny bramboru byly množeny na MS médiu s 2,5 % sacharózy pomocí nodálních segmentů, které byly kultivovány ve 100ml Erlenmayerových baňkách (3 segmenty na baňku). Nodální segmenty 3-4 týdny starých rostlin byly přenášeny do 250 ml Erlenmayerových baněk na 50 ml MS média bez sacharózy zakrytých víčky pro fotoautotrofní kultivaci a do 250 ml Erlenmayerových baněk na 50 ml MS média s 3 % sacharózy zakrytých PP fólií (vždy 3 segmenty na baňku). Kultivace probíhala za teploty 21-24 °C při dlouhodenní fotoperiodě - 16 hod světla a 8 hod tmy, ozářenost 400-500 µmol m^{-2} s^{-1} (použité zářivky - daylight fluorescent tubes; Osram, Wintherthur, Switzerland).

Sterilizovaná semena tabáku byla vyseta na MS médium s 3 % sacharózy. Získané 1 týdenní klíční rostliny byly sterilně přeneseny do 250 ml Erlenmayerových baněk na 50 ml MS média bez sacharózy zakrytých víčky pro fotoautotrofní kultivaci a do 250 ml Erlenmayerových baněk na 50 ml MS média s 3 % sacharózy zakrytých PP fólií (vždy 1 rostlina na baňku). Rostliny byly kultivovány za teploty 25-27 °C při dlouhodenní fotoperiodě - 16 hod světla a 8 hod tmy, ozářenost 400-500 µmol m^{-2} s^{-1} (použité zářivky - daylight fluorescent tubes; Osram, Wintherthur, Switzerland).

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5.3. Používané laboratorní pomůcky, sklo a chemikálie

- Automatická pipeta
- Kádinky různých velikostí
- Erlenmeyerovy baňky – objem 100 ml, 250 ml
- Alobal
- Víčka pro fotoautotrofní kultivaci (viz Kapitola č. 5.6. Příprava víček pro fotoautotrofní kultivaci) a materiál pro upevnění víček
- Pinzety, skalpel, Petriho misky
- Odměrný válec
- Roztoky pro přípravu MS média připravené podle Tab. 1 nebo komerčně dostupné MS soli
- Agar, sacharóza

5.4. Přístroje a ostatní vybavení

- Elektromagnetické míchadlo
- pH metr
- Autokláv, sušárna
- Mikrovlnná trouba
- Flow box, kahan
- Kultivační boxy s vhodnými podmínkami pro kultivaci vybraných rostlin

5.5. Ochranné pomůcky

- Laboratorní plášť

5.6. Příprava kultivačních médií

Příprava MS média:

A) Ze zásobních roztoků namíchaných v laboratoři podle rozpisu v Tab. 1 a uchovávaných v lednici při 4 °C (roztok D je uchováván v mrazáku při -20 °C). Do kádinky o odpovídajícím objemu bylo napipetováno množství zásobních roztoků dle Tab. 1, přidán agar v množství 8 g/l a sacharóza v množství 30 g/l (do média bez sacharózy nebyla přidávána sacharóza ani jiný cukr). Roztok byl míchán na elektromagnetickém míchadle, po rozpuštění všech složek doplněn na požadovaný objem v odměrném válci pomocí destilované vody. Následně bylo upraveno pH na hodnotu 5,75 a obsah nádoby přiveden k varu v mikrovlnné troubě. Po převaření bylo médium rozlito do připravených nesterilních Erlenmayerových baněk, které byly následně zakryty alobalovým víčkem a sterilizovány v autoklávu (20 min při teplotě 121 °C a tlaku 121 kPa). Sterilní média mohou být uchovávána po dobu až jednoho měsíce za pokojové teploty.

B) Z komerčně dodávané směsi solí pro MS média. Do kádinky o odpovídajícím objemu bylo naváženo množství solí stanovené výrobcem a napipetováno odpovídají množství roztoku vitamínů (roztok D viz Tab. 1). Poté byl přidán agar v množství 8 g/l a sacharóza v množství 30 g/l (do média bez sacharózy nebyla přidávána sacharóza ani jiný cukr). Dále bylo postupováno jako v případě A.

zásobní roztok	složka	zásobní roztok (500 ml) [g]	1 l média [ml]
A	NH_4NO_3	16,50	50
	KNO_3	19,00	
	$CaCl_2$	3,31	
	$MgSO_4$. 7 H_2O	3,70	
	KH_2PO_4	1,70	
B	KI	0,08	5
	H_3BO_3	0,62	
	$MnSO_4.4H_2O$ (H_2O)	2,23 (1,69)	
	$ZnSO_4.7H_2O$	0,86	
	$Na_2MoO_4.2H_2O$	0,03	
	$CuSO_4.5H_2O$	0,00	
	$CoCl_2.6H_2O$ (bezv.)	0,0025 (0,0013)	
C	$FeSO_4.7H_2O$	2,78	5
	$Na_2EDTA.2H_2O$	3,73	
D	inozitol	10,00	5
	kys. nikotinová	0,05	
	pyridoxin - HCl	0,05	
	thiamin - HCl	0,05	
	glycin	0,20	

Tab. 1: Roztoky solí používané při přípravě MS média. V tabulce je uvedeno složení a objem zásobních roztoků pipetovaných pro přípravu 1 litru média.

5.7. Příprava víček pro fotoautotrofní kultivaci

5.7.1. Používané nástroje a pomůcky

- Autoklávovatelná polypropylenová fólie (autoklávovatelný sáček na kontaminovaný materiál

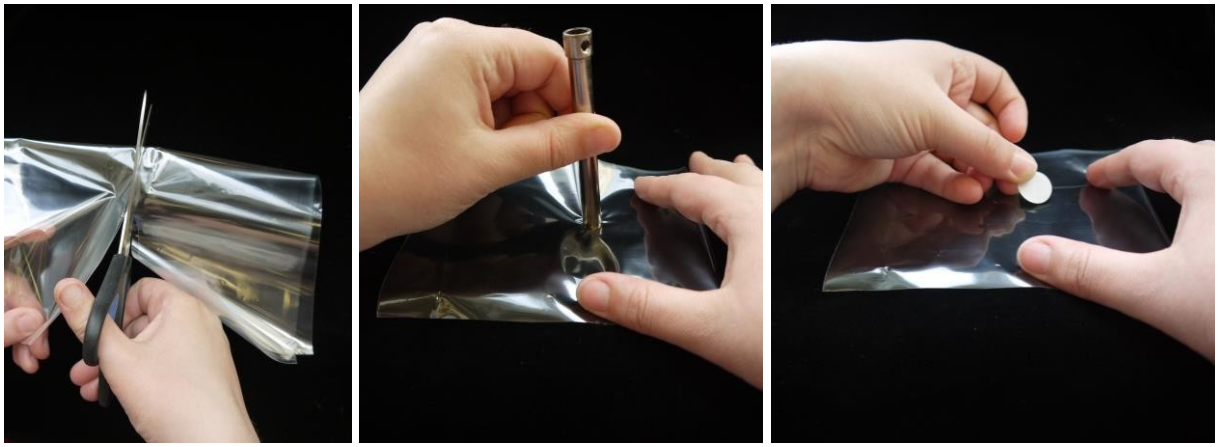
  – Merci, Brno, Česká republika)

- Samolepicí polypropylenové filtry o průměru 8 mm a porozitě 0.04 μm (Sigma-Aldrich, St. Louis, USA, kat. č. S5939-500EA)

- Nůžky
- Korkovrt o průměru 8 mm
- Filtrační papír – kulaté výřezy o průměru 10 cm
- Střička s destilovanou vodou
- Skleněné Petriho misky o průměru 12 cm

5.7.2. Postup přípravy víček pro fotoautotrofní kultivaci

PP fólie byla nastříhána na čtverce o hraně 15 cm, do kterých byly následně pomocí korkovrtu vyraženy dva otvory cca 2 cm vzdálené. Otvory byly přelepeny samolepicími filtry. Takto připravená víčka byla nechána alespoň 2 hodiny zatížená, aby filtry dobře přilnuly a lepidlo dostatečně ztuhlo. Poté bylo nutné víčka vysterilizovat. Do skleněných Petriho misek byly skládány na sebe vždy navlhčený filtrační papír – PP víčko – navlhčený filtrační papír. V jedné Petriho misce lze sterilizovat max. 12 víček. Připravené misky byly sterilizovány autoklávováním (20 min při teplotě 121 °C a tlaku 121 kPa) a víčka uchovávána za pokojové teploty a použita do 48 hodin po sterilizaci.



Obr. 1: Postup přípravy víček pro fotoautotrofní kultivaci

5.8. Zakládání fotoautotrofních kultur a péče o ně

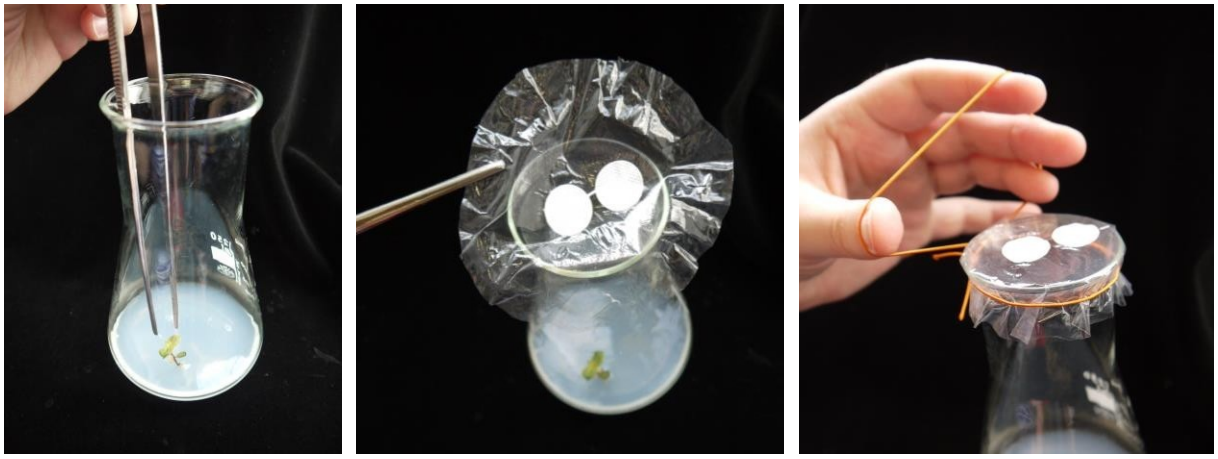
5.8.1. Používaný rostlinný materiál, nástroje a další vybavení

- Napěstované rostlinné kultury / vyklíčené semenáče
- 250 ml Erlenmeyerovy baňky se sterilním MS médiem bez sacharózy
- Sterilní víčka pro fotoautotrofní kultivaci (viz Kapitola 5.6. Příprava víček pro fotoautotrofní kultivaci)
- Gumičky pro připevnění víček pro fotoautotrofní kultivaci
- Sterilní pinzety a skalpel
- Flow box

5.8.2. Postup zakládání fotoautotrofních kultur

Práce probíhala sterilně ve flow boxu. Nodální segment / klíční rostlina byl/a pomocí sterilní pinzety přemístěn/a na MS médium bez sacharózy a baňka zakryta sterilním víčkem pro fotoautotrofní kultivaci. Víčko bylo připevněno gumičkou (viz poznámka) a rostliny umístěny do kultivační místnosti s odpovídajícími kultivačními podmínkami. Rostliny byly pravidelně kontrolovány a v závislosti na relativní vzdušné vlhkosti v kultivační místnosti po cca 3 týdnech kultivace byla provedena sterilní zálivka. Ve flow boxu byla sejmuta víčka pro fotoautotrofní kultivaci a pomocí automatické pipety se sterilní špičkou bylo do baňky opatrně naneseno 10 ml sterilní vody tak, aby nedošlo k mechanickému rozrušení média. Poté byla baňka opět zakryta víčkem pro fotoautotrofní kultivaci.

Pozn.: Testovali jsme různé způsoby upevnění víček pro fotoautotrofní kultivaci – např. pomocí laboratorního parafilmu či klasických kancelářských kaučukových gumiček. Nejlépe se ale osvědčila gumička klobouková nastříhaná na cca 20 cm pásky a následně svázaná. Tento typ gumičky v podmínkách kultivačních boxů nepuchří tak jako kaučukové gumičky a na rozdíl od parafilmu ho lze použít opakovaně.



Obr. 2: Postup zakládáni fotoautotrofních kultur

6. Rozdíly mezi heterotrofně a fotoautotrofně kultivovanými rostlinami

V průběhu vývoje této metodiky byly pozorovány a vyhodnocovány rozdíly mezi rostlinami kultivovanými za standardních (heterotrofních) podmínek (3% sacharózy v médiu, průsvitné PP víčko bez filtru zajišťujícího ventilaci) a za fotoautotrofních podmínek. Fotoautotrofní rostliny vykazovaly větší listovou plochu a větší čerstvou hmotnost než heterotrofně kultivované rostliny (viz Obr. 3 Rozdíly mezi heterotrofně a fotoautotrofně kultivovanými rostlinami a Tab. 2 Srovnání základních charakteristik rostlin).

A)

B)

C)

Obr. 3: Rozdíly mezi heterotrofně a fotoautotrofně kultivovanými rostlinami. A) Rostliny řepky po 3 týdnech kultivace, vlevo heterotrofní kontrola, vpravo fotoautotrofní rostlina. B) Rostliny tabáku po 4 týdnech kultivace, vlevo heterotrofní kontrola, vpravo fotoautotrofní rostlina. C) Rostliny bramboru po 5 týdnech kultivace, vlevo heterotrofní kontrola, vpravo fotoautotrofní rostlina.

7. Souhrnná tabulka – základní charakteristiky rostlin

Rostlina	Způsob kultivace	Průměrná čerstvá hmotnost prýtu / g	Průměrná čerstvá hmotnost kořene / g
Brambor	**heterotrofní**	1,02	0,223
	fotoautotrofní	1,175	0,518
Tabák	**heterotrofní**	1	0,931
	fotoautotrofní	3,186	5,623
Řepka	**heterotrofní**	0,582	0,097
	fotoautotrofní	1,19	0,155

Tab. 2: Srovnání základních charakteristik rostlin používaných při vývoji metodiky. Váženo: řepka po 3 týdnech kultivace, tabák po 4 týdnech kultivace, brambor po 5 týdnech kultivace.

8. Popis uplatnění certifikované metodiky

Metodika je optimalizací a vylepšením stávajících metod fotoautotrofní kultivace rostlin v podmínkách *in vitro*. Dříve používaná komerčně prodávaná víčka (Sigma-Aldrich) pro fotoautotrofní kultivaci již nejsou dostupná a tato metodika je plně nahrazuje a dokonce vylepšuje tím, že dává možnost upravit počet filtrů ve víčku s ohledem na velikost kultivační nádoby a požadavky dané rostliny na výměnu plynů.

Metodika je určena jak pro pracoviště zabývající se teoretickým výzkumem fyziologie rostlin, tak pro aplikovaný zemědělský výzkum či komerční množení.

9. Srovnání novosti postupů

Publikací zabývajících se fotoautotrofní kultivací rostlin v podmínkách *in vitro* je velmi málo a vždy využívají některou z komerčně dostupných variant víček umožňujících fotoautotrofní kultivaci (dnes již často nevyráběných a nedostupných) (Ticha *et al.*, 1998) nebo pracují s komplikovanějšími systémy umožňujícími vyšší výměnu plynů v kultivační nádobě (Schmildt *et al.*, 2015). Tato metodika nabízí jednoduchý protokol k výrobě vlastních víček umožňujících fotoautotrofní kultivaci za podmínek *in vitro* jak u hospodářsky významných rostlin, tak u dalších rostlinných druhů, např. u rostlin s potenciálem využití v komerční sféře. Pomocí drobných úprav lze vyrobit víčka na různé typy používaných kultivačních nádob. Touto metodou vyrobená víčka lze použít opakovaně (vydrží v průměru 5 sterilizací v autoklávu).

10. Ekonomické aspekty

Metoda předpokládá zavedenou laboratoř pro práci s kulturami *in vitro* s běžným vybavením. Je třeba zakoupit pouze samolepicí filtry na výrobu víček, např. Sigma-Aldrich Sun cap closures Biofilter,

O.D. 18 mm, 500 filtrů v ceně 2700 Kč + DPH (ceník Sigma Aldrich 2016). Na Erlenmeyerovu baňku o objemu 250 ml jsou třeba dva filtry, jedno víčko je možné použít přibližně 5x. Autoklávovatelný sáček na kontaminovaný materiál bývá v laboratořích běžně k dispozici, z jednoho sáčku lze udělat několik víček (v závislosti na velikosti), cena 500 sáčků o objemu 3l je 1359 Kč + DPH (ceník – Merci 2016). Cena jednoho víčka tedy vychází na cca 2 Kč (bez DPH) / použití.

11. Seznam použité literatury

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Ticha I, Cap F, Pacovska D, Hofman P, Haisel D, Capkova V, Schafer C. 1998. Culture on sugar medium enhances photosynthetic capacity and high light resistance of plantlets grown in vitro. *Physiologia Plantarum* **102**(2): 155-162.