South African Journal of Botany 118 (2018) 98–104

Contents lists available at ScienceDirect

South African Journal of Botany

journal homepage: www.elsevier.com/locate/sajb

Dark-induced changes in the activity and the expression of tomato hexokinase genes depend on the leaf age

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ARTICLE INFO

Article history:
Received 3 May 2018
Received in revised form 30 May 2018
Accepted 6 June 2018
Available online 10 July 2018

Edited by E Balázs

Keywords:
Dark
Glucose
Hexokinase
Leaf position
Senescence
Tomato

ABSTRACT

Exposure of tomato plants to dark period promotes leaf senescence, which takes place at different speed in young, mature and old leaves of intact plants. Dark-induced senescence is accompanied with decreased glucose levels, chlorophyll content and photosynthetic activity and it induces changes in sugar metabolism, in which hexokinases (HXKs) play a prominent role. The aim of this work was to reveal changes in the expression of various HXK genes and in HXK activity in different leaf positions of tomato kept in darkness. A 24-h-long dark period reduced the expression of the mitochondrial *SlHXK1* and chloroplastic *SlHXK4* in the young and mature leaves, but induced the expression of all *SlHXKs* especially that of *SlHXK3* in the old leaves. In contrast to HXK expression, HXK activity decreased in all leaf positions, however the smallest changes were observed in young, sink leaves. In addition, cessation of CO_2 assimilation in the dark led to low glucose levels, which can also participate in the early induction of dark-induced leaf senescence. With the exception of the mitochondrial *SlHXK3* expression, this decline in the activity and relative transcript abundance of HXKs, as well as in the photosynthetic parameters was more pronounced after a 7-day-long dark treatment. It can be concluded that slower rate of dark-induced chlorophyll loss and senescence was accompanied with higher HXK activities. A single *HXK* gene, *SlHXK3* was up-regulated during dark starvation suggesting that it can play a role in the maintenance of HXK activity and integrity of mitochondrial functions in young and mature leaves.

1. Introduction

Leaf senescence is a part of normal plant life cycle, which is associated with degradation and recycling of macromolecules. It is well characterized by a decline in chlorophyll content and in photosynthetic activity (Van Doorn and Woltering, 2004; Lim et al., 2007). Leaf senescence is regulated by various external and internal factors, such as shortening of the light period in autumn, drought stress, nitrogen deficiency, natural shading or induced darkness. Internal factors, such as the lack of nutrients, changes in sink–source relations or in intracellular sugar levels participate also in the induction of senescence (Van Doorn, 2008; Zhang and Zhou, 2013). Starvation or accumulation of sugars can also induce senescence, which strongly depends on the experimental setup thus there are controversial hypotheses and results in this field (Van Doorn, 2008). It can be concluded that sugars, especially hexoses are not only one, but very important factors in the initiation of senescence. Other senescence-inducing components can also be found in mitochondria, thus they connect sugar metabolism to the initiation of senescence (Bolouri-Moghaddam et al., 2010). Both the mitochondrial and chloroplastic electron transport chains may generate reactive oxygen species (ROS) in plant cells. The imbalance between ROS production and antioxidant defence leads to oxidative stress, which contributes to the initiation of cell death. These processes can be different in light or dark environments (Poór et al., 2017).

Hexokinases (HXKs) are major regulatory enzymes in sugar metabolism and in sugar sensing in plants (Claeyssen and Rivoal, 2007; Granot et al., 2013; Sheen, 2014; Aguilera-Alvarado and Sánchez-Nieto, 2017). HXK can phosphorylate both glucose and fructose to respective hexose-6-phosphates as a first step of glycolysis. Moreover, mitochondria-associated HXKs (mtHXKs) have a key role in the control of cell death and senescence. HXK isoenzymes are integral component of permeability transition (PT) pore through their interaction with voltage-dependent anion channels (VDAC). mtHXK proteins can bind to VDAC, thereby they inhibit the opening of PT pore and cytochrome *c* release from the intermembrane space, which can prevent the induction of cell death. The loss of the integrity of the inner mitochondrial membrane can cause mitochondrial dysfunction such as ROS production and ATP depletion. Increasing glucose phosphorylation activity by mtHXKs may reduce cell death owing to the inhibition of the

Abbreviations: Chl *a*, chlorophyll *a*; CRE, *cis*-regulatory element; HXK, hexokinase; PMP, 1-phenyl-3-methyl-5-pyrazolone; PT pore, permeability transition pore; ROS, reactive oxygen species; VDAC, voltage-dependent anion channel.

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https://doi.org/10.1016/j.sajb.2018.06.006
0254-6299/

opening of mitochondrial PT pore and a more efficient glucose metabolism due to the better access to ATP (Sarowar et al., 2008; Sun et al., 2008; Camacho-Pereira et al., 2009; Godbole et al., 2013). In contrast to active mtHXKs, the loss of mtHXK activity increases ROS production, cyt *c* release and cell death induction in tomato leaf tissues. However, this important function of mtHXKs in the initiation phase of dark-induced senescence has not been investigated at various leaf positions of intact plants.

In addition, chloroplastic HXKs also play a crucial role in the regulation of ROS levels (Bolouri-Moghaddam et al., 2010). Thus, the early changes in the expression and activity of various HXKs can be significant, because this is the first step before alterations in mitochondrial and photosynthetic functions (Zhang and Xing, 2008; Breeze et al., 2011; Liebsch and Keech, 2016). Moreover, it was observed earlier that the expression of photosynthesis-associated nuclear genes (e.g. Chl *a*/*b* binding protein, small subunit of ribulose-1,5-bisphosphate carboxylase oxygenase) was repressed by high glucose concentrations. These changes showed correlation with the increased expression of HXKs and with the rate of glucose-induced leaf yellowing (Xiao et al., 2000; Moore et al., 2003). However, low sugar levels can also induce leaf yellowing and senescence, but the role of mtHXKs is not clear in this process (Van Doorn, 2008).

In tomato, four *HXK* genes (*SlHXK1–4*) were identified (Menu et al., 2001; Dai et al., 2002; Kandel-Kfir et al., 2006), which were expressed in various organs, including leaves and fruits (Damari-Weissler et al., 2006). Based on the use of GFP fusion protein it was shown that tomato *SlHXK1*, *2* and *3* are associated with mitochondrial envelope membrane and *SlHXK4* is localized to plastids (Kandel-Kfir et al., 2006). *SlHXK4* belongs to A type HXKs, having a 30 amino acid-long chloroplast transit peptide on its N-terminal, while *SlHXK1*, *2* and *3* are members of B type HXKs. They share a common, hydrophobic region at the N-terminal, which anchors these proteins to the mitochondrial outer membrane. Based on the analysis of the upstream promoter sequences of tomato *HXK* genes, the most abundant *cis*-regulatory element (CRE) was *GT1CONSENSUS* with 45 duplications, which plays a role in the regulation of many light-dependent genes (Poór et al., 2015). However, there are differences between HXK activities in sink and source leaves. In fully developed, photosynthetically active source leaves HXKs and fructokinases function mainly during dark period, when starch and sucrose degradation yields free glucose and fructose monomers. In sink tissues, however, HXKs and fructokinases might be required during both dark and light periods (Granot et al., 2013). These results suggest that *HXKs* are regulated by light and function differently in light or dark periods in plant metabolism, development and stress responses in the leaves of different maturity levels.

The induction of senescence can be achieved by several experimental methods in the laboratory. This process can be studied in detached leaves kept in darkness. In this case the excised tissues are separated from hormonal and metabolite sources of other plant parts. Leaves at various positions can be covered with aluminium foil, while other parts of the plant are exposed to normal photoperiod. This ensures a constant transport of carbohydrates from the photosynthetically active tissues to the senescing leaf. Specifically, the interactions between the HXKs and glucose metabolism remain unclear in the leaves of various ages if the whole plant is darkened.

In this article, comparative biochemical and molecular analyses of HXKs were carried out in time course experiments after short- and long-term dark treatments of intact plants in order to reveal their connection with the initiation of dark-induced senescence from the apical to basal leaves of plants.

2. Materials and methods

2.1. Plant material

Seeds of tomato plants (*Solanum lycopersicum* L. cv. Ailsa Craig) were germinated at 26 °C for three days in the dark and the seedlings were subsequently transferred to perlite for two weeks. Plants were grown hydroponically afterwards in a controlled environment under 200 μmol m^{-2} s^{-1} photon flux density (F36W/GRO lamps, OSRAM SYLVANIA, Danvers, MA, USA), with a 12/12-h light/dark period, a day/night temperature of 24/22 °C and a relative humidity of 55–60% for eight weeks (Kovács et al., 2016). Half of the plants remained for 7 days in the original light/dark cycle (normal photoperiod) and half of them were put into darkness at the same condition. The experiments were conducted from 9 a.m. and were repeated three times. The samples were prepared from the youngest, emerging leaves (young, sink leaves), the second, fully expanded leaves (mature leaves) and the oldest, senescing leaves (old leaves) at least in three replicates 1; 3; 6; 12; and 24 h and 7 days after the prolonged dark treatments.

2.2. RNA extraction, expression analyses by quantitative real-time PCR

Quantitative real-time reverse transcription-PCR (qRT-PCR; Piko Real-Time qPCR System, Thermo Scientific) was used to detect the expression pattern of the selected tomato *HXK* genes mined from the Sol Genomics Network (SGN; http://solgenomics.net/) database (Poór et al., 2017). Primers were designed using NCBI and Primer 3 software (http://frodo.wi.mit.edu/) and listed in Table 1. The PCR reaction in a total volume of 10 μl consisted of 10 ng cDNA template, 400–400 nM forward and reverse primers, 5 μl of Maxima SYBR Green qPCR Master Mix (2X) (Thermo Scientific) and nuclease-free water. After the PCR (denaturation at 95 °C for 7 min, followed by 40 cycles of denaturation at 95 °C for 15 s and annealing extension at 60 °C for 60 s), a melting curve analysis of the product was performed [by increasing the temperature from 55 to 90 °C (0.2 °C s^{-1})] to determine the specificity of the reaction. Data analysis occurred by PikoReal Software 2.2 (Thermo Scientific). Tomato 18S rRNA and elongation factor-1α subunit genes were used as reference genes. $2^{(-\Delta\Delta Ct)}$ formula was applied to calculate expression data. Each reaction was repeated at least three times.

2.3. Determination of hexokinase (EC 2.7.1.1) activity

HXK activity was determined with glucose substrate according to Whittaker et al. (2001). Firstly, 0.5 g of leaf samples was crushed to a fine powder in a mortar under liquid N_2 and then soluble proteins were extracted by resuspending the powder in 1 ml of cold extraction buffer (20 mM KH_2PO_4, pH 7,5; 0,5 mM NaEDTA, 5 mM dithiothreitol). The homogenate was centrifuged at 12,000*g* for 20 min at 4 °C. HXK activity was measured in a reaction mixture containing 100 mM KH_2PO_4 buffer (pH 7.5), 2 mM $MgCl_2$, 1 mM NaEDTA, 1 mM ATP, 10 mM glucose, 1 U of glucose-6-phosphate dehydrogenase (EC 1.1.1.49, G6PDH), 1 U of phosphoglucose isomerase (EC 5.3.1.9, PGI) from baker's yeast and 100 μl of plant extract. The activity measurements were performed by following the absorbance at 340 nm for 5 min at 25 °C (KONTRON, Milano, Italy). The amount of enzyme producing 1 μmol min^{-1} of phosphorylated glucose was defined as one unit (U) and the enzyme activities were expressed as U mg^{-1} protein. Soluble protein concentration was determined according to Bradford (1976) using bovine serum albumin as a standard.

2.4. Measurement of photosynthetic parameters

Stomatal conductance (g_s, mol H_2O m^{-2} s^{-1}), net CO_2 assimilation rate (A, μmol fixed CO_2 m^{-2} s^{-1}), and chlorophyll *a* (Chl *a*) fluorescence parameter, Fv/Fm, the maximal quantum efficiency of photosystem II (PSII) in dark-adapted leaves, were measured in different leaf positions of tomato plants with a portable photosynthesis system (LI-6400, LI-COR, Inc.; Lincoln, NE) from 9 a.m., as described by Poór et al. (2011). Leaf temperature was maintained at 25 °C, the flow rate of air (containing 400 μmol s^{-1} CO_2 from exogenous source) and photon flux density (PPFD) were set at 200 μmol m^{-2} s^{-1},

Table 1
Primer pairs used for qRT-PCR.

Name	Gene ID	Chromosome	Gene product localization		Primer pair sequences (5′–3′)
SlHXK1	Solyc03g121070	3	Mitochondrial	F:	TCATCAACCTCCTGGTAAGCA
				R:	CCTTTTGTCCACCGCATAAT
SlHXK2	Solyc06g066440	6	Mitochondrial	F:	TCATCCACCTCCTGGTAAGC
				R:	TGCCAACCGTGTCATCAAT
SlHXK3	Solyc12g008510	12	Mitochondrial	F:	TAATGATGGTTCAGGCGTTG
				R:	CAGGCACTTTTGGTTGTGTC
SlHXK4	Solyc04g081400	4	Plastidic	F:	GCTGGCAAAAAGGATGTCTAA
				R:	CTCCCCATTCGGTATTCACA

respectively. F_0 was determined after 15 min of dark adaptation and the maximal fluorescence (Fm) was measured by applying 14,000 μmol m^{-2} s^{-1} pulse of saturating light. Each measurement was repeated three times on three different plants. The whole experiment was also repeated three times. Samples growing under normal photoperiod and prolonged darkness were exposed to the same experimental conditions during measurements.

2.5. Determination of photosynthetic pigment contents

Leaf pigments were extracted from 30 mg tissues prepared from young, mature and old leaves of tomato by grinding in 1.5 ml ice-cold 100% (v/v) acetone. Samples were then centrifuged (12,000*g*, 20 min, 4 °C) and the pellet was extracted again with 80% (v/v) acetone (1.5 ml) for 24 h. After a second centrifuge (12,000*g*, 20 min, 4 °C), the supernatants were collected and the pigment composition was measured by a spectrophotometer according to Wellburn and Lichtenthaler (1984).

2.6. Determination of glucose contents

For glucose analysis, 0.5 g of leaf tissues was homogenized and boiled for 30 min in ethanol and the homogenate was centrifuged twice at 12,000*g* for 20 min at 4 °C. The supernatant was evaporated to dryness in 1.5 ml HPLC vials and was resolved in 100 μl ethanol. One hundred μl volumes of 0.3 M NaOH and 0.5 M methanolic 1-phenyl-3-methyl-5-pyrazolone (PMP) were added to the samples, which were incubated for 30 min at 70 °C. After cooling down to room temperature, 100 μl of 0.3 M HCl was added to reaction mixture and then samples were extracted three times with 200 μl chloroform (Li et al., 2013). The aqueous phase was centrifuged (16,200*g*, 10 min) and a 5 μl aliquot was injected into a Shimadzu HPLC system (Shimadzu Corp., Kyoto, Japan) equipped with two LC20-AD pumps, DGU-14A degasser, SIL-20A autosampler, CTO-10ASVP column oven, SPD-10AVP UV–VIS detector and CBM-20A system controller. The isocratic elution was achieved on a Prodigy C18 (150 × 4.6 mm 5 μm, Phenomenex, USA) column via sodium phosphate buffer (40 mM, pH 8.0)/acetonitrile (79:21, V/V) at 40 °C with 1 ml/min flow rate. The PMP derivative of glucose was analysed at 245 nm.

2.7. Statistical analysis

The results are expressed as means ± S.E. After analyses of variance (ANOVA) a multiple comparison followed by the Tukey test was performed with SigmaPlot version 11 software (SYSTAT Software Inc. SPSS). The means were treated significant if $P \leq 0.05$.

3. Results

To examine the time and organ specific effect of normal photoperiod and dark environment on the expression of different *SlHXK*s and on the enzyme activities, these parameters were determined in different leaf positions of tomato plants. Under control conditions, transcript levels of HXK coding genes, especially those of *SlHXK1* and *SlHXK4* exhibited diurnal fluctuations in the mature leaves of tomato with a maximum in the late light period and a subsequent decrease in the dark (Fig. 1A, G). Similar tendency can be found in the case of *SlHXK1* in the old leaves (Fig. 1A). Moreover, the expression of *SlHXK3* was the highest in the early dark period in the mature leaves (Fig. 1E). *SlHXK1* expression showed similar tendency but lower transcript abundance during the first 24 h of dark exposure in the mature leaves, however transcript levels of *SlHXK1* exhibited a transient peak in the old leaves after 6 h followed by a second maximum at 24 h in the dark (Fig. 1B). Similar tendencies can be detected in the expression pattern of *SlHXK2* in the old leaves while the transcript abundance of *SlHXK2* increased only in the mature leaves after 6 h. The mitochondrial *SlHXK3* responded very early to the absence of light, because the transcripts of *SlHXK3* accumulated 8-fold from 3 to 12 h upon the dark treatment in the mature and old leaves (Fig. 1F).

Surprisingly, the expression of *SlHXK3* increased significantly at all leaf positions under long-term darkness and the highest expression was found in the old leaves, while the expression of the other genes was inhibited after 7 days of dark treatment (Fig. 2).

The specific activity of HXK with glucose substrate showed also leaf age-specific and diurnal regulation in the leaves of tomato plants (Fig. 3). During the normal photoperiod, the most characteristic changes were observed in the mature leaves, where HXK activity showed the highest levels compared to other leaf stages (Fig. 3A). Similar but not so pronounced tendencies can be found in the other leaf positions during the normal photoperiod. Interestingly, HXK activity increased after 3 h in the mature leaves, but then decreased continuously in all leaves of tomato under prolonged darkness (Fig. 3B).

After 7 days of dark treatment HXK activities decreased significantly at all leaf positions of plants and the most significant decrease was found in the old leaves (Fig. 4).

The activity of HXKs depends on a number of physiological processes during dark-induced senescence, which determine the substrate concentration available for enzymes. After all, it is photosynthetic activity that is responsible for the optimal glucose concentration for HXKs. The stomatal conductance can determine the net CO_2 assimilation rate of leaves. Under normal photoperiod the lowest stomatal conductance was observed in the old leaves and the highest in the mature leaves (Table 2). The stomatal opening was inhibited and stomatal conductance decreased if the plants were kept in darkness for 24 h or 7 days, but there were no significant differences between the two time points. In parallel, CO_2 assimilation showed similar tendencies as the changes in stomatal conductance in the different leaf positions under normal photoperiod, while photosynthetic activity ceased in the dark (Table 2). Fv/Fm, the maximal quantum efficiency of PSII, is an important stress marker of PSII damage. This parameter was lower in the old leaves but did not change after 24 h in the dark. However, Fv/Fm decreased significantly after 7 days under darkness in all leaf positions (Table 2). Similar changes were observed in chlorophyll $a + b$ contents. The amount of these photosynthetic pigments was lower in the old leaves and it was maintained at constant level after a 24 h dark period. Chlorophyll $a + b$ content decreased further in all leaves of tomato plants, which proved to be significant in old leaves after a 7-day-long dark treatment (Table 2). Glucose content, a product of photosynthesis was the highest in the young leaves and lower in the other leaf

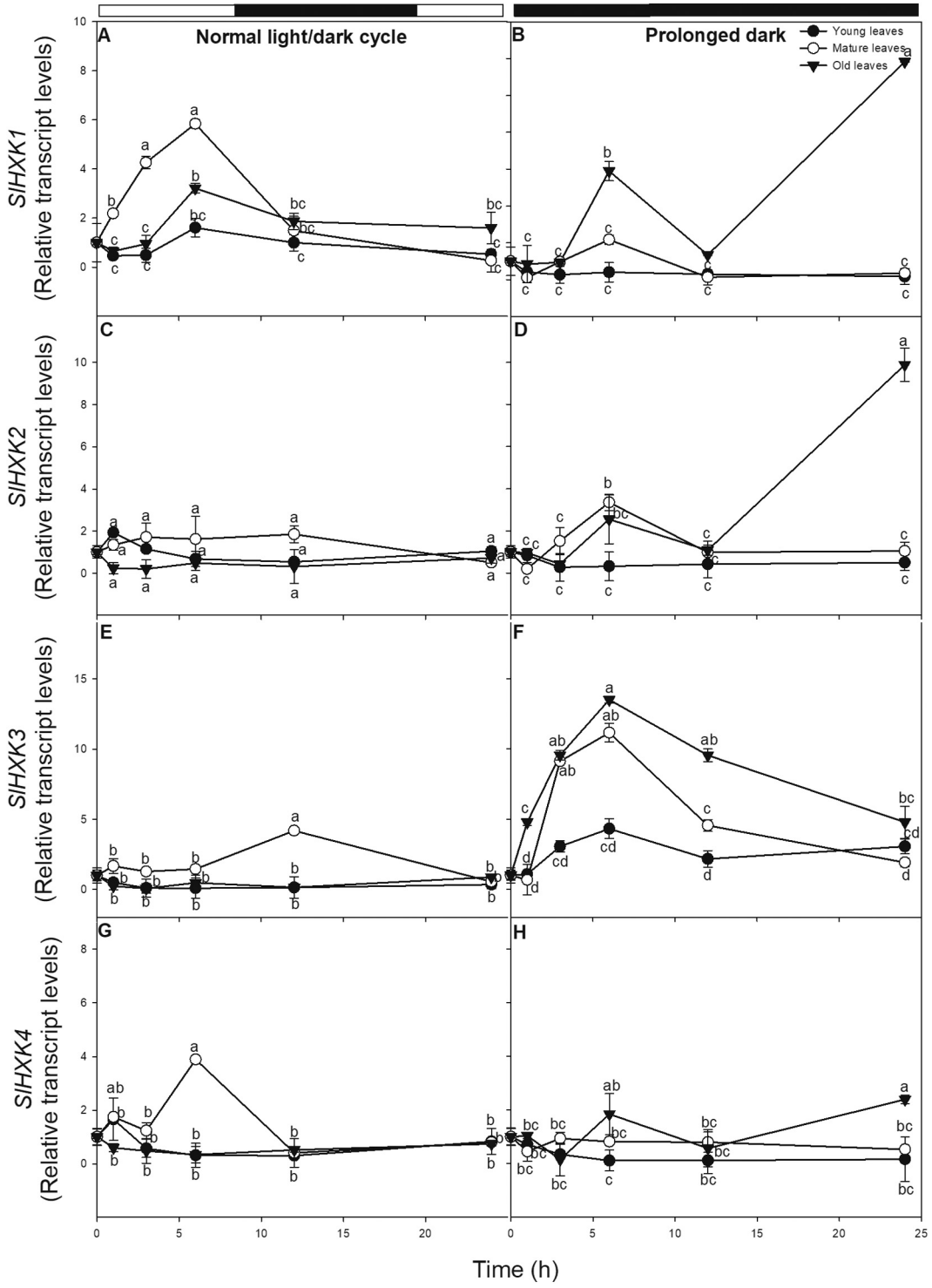

Fig. 1. Changes in the relative transcript levels of *SlHXK1*, *SlHXK2*, *SlHXK3* and *SlHXK4* at different leaf positions of tomato plants during 24 h of normal light/dark cycle or continuous darkness. Means ± SE, n = 3. Data with different letters indicate significant differences at $P \leq 0.05$ level (Tukey test) in each time point.

positions. Interestingly, the 24-hour-long dark treatment already decreased the glucose content significantly in all leaves of tomato and this tendency was much more pronounced after 7 days (Table 2.).

4. Discussion

Light plays a central role in plant growth, development and defence responses. It is required for photosynthesis and other metabolic or transcriptional processes (Kangasjärvi et al., 2012; Zhang and Zhou, 2013; Ballaré, 2014). It is well known that darkness promotes leaf senescence, which is different in the case of detached leaf, in shaded leaves of intact plants or in whole plant exposed to darkness (Weaver and Amasino, 2001; Van Doorn, 2008). Dark-induced senescence results in a significant chlorophyll loss, decline in the photosynthetic activity and changes in sugar metabolism, which is regulated by HXKs (Bolouri-Moghaddam et al., 2010). In addition, HXKs play a key role in the control of leaf senescence by inhibiting mitochondrial PT pore (Sarowar et al., 2008; Godbole et al., 2013). However, the expression pattern and activity of

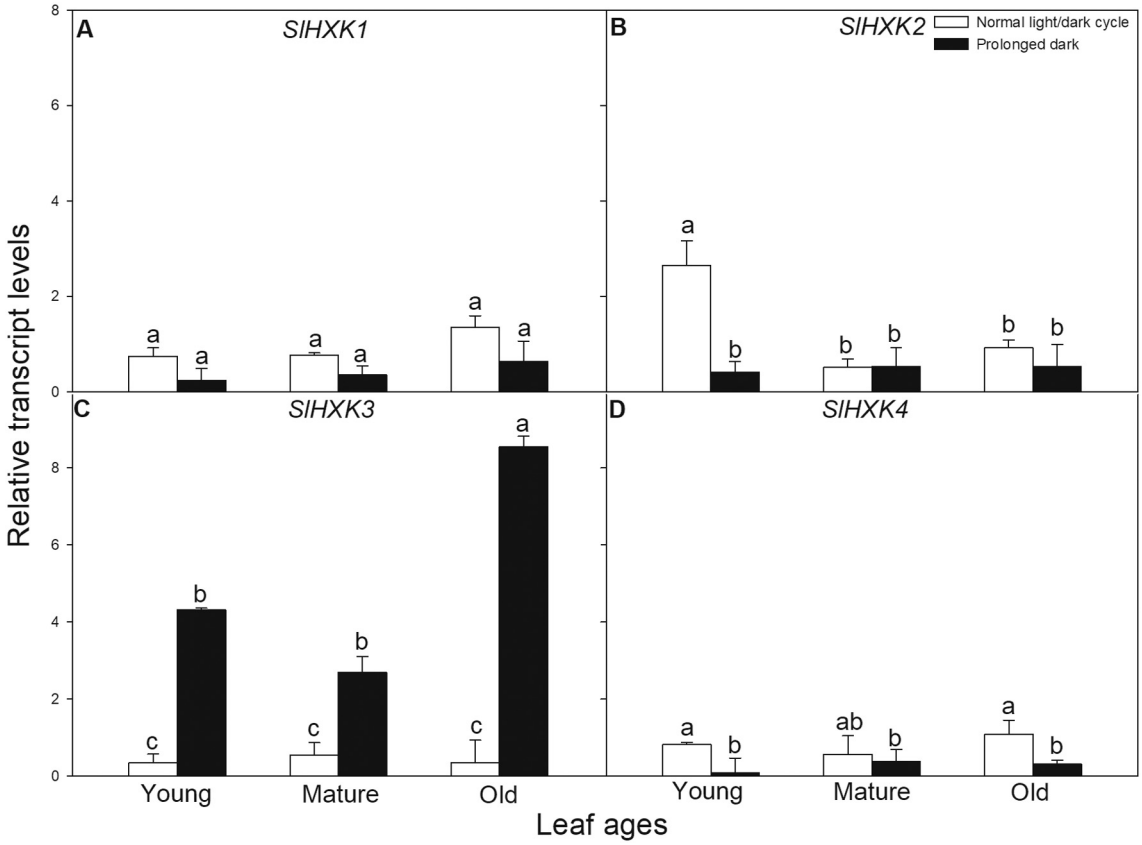

Fig. 2. Changes in the relative transcript levels of *SlHXK1*, *SlHXK2*, *SlHXK3* and *SlHXK4* at different leaf positions of tomato plants under normal light/dark cycle or under 7-day-long darkness. Means ± SE, n = 3. Data with different letters indicate significant differences at $P \leq 0.05$ level (Tukey test).

HXKs in different leaf positions under dark-induced senescence remain unclear if whole plants are exposed to prolonged darkness. Our results demonstrated that the four identified tomato *HXK* genes displayed time and leaf age-specific expression under normal photoperiod. The highest expression was found in the mature leaves especially in the case of the mitochondrial *SlHXK1* and chloroplastic *SlHXK4* transcripts in the late phase of the light period. CREs, which may be responsible for diurnal and circadian rhythm regulation of *HXK*s, were described not only in tomato but also in maize *HXK* promoter sequences (Zhang et al., 2014; Poór et al., 2015). The observed expression patterns in the leaves of various ages may determine the activity of HXKs in source and sink organs.

Earlier, it was found that dark treatment induced the expression of *AtHXK1* in *Arabidopsis* leaves (Buchanan-Wollaston et al., 2005). Our results demonstrated that the expression of *SlHXK*s also altered significantly within 6 h after the dark treatment and showed significant changes in a leaf specific manner. The expression of *SlHXK1* and *SlHXK4* decreased in the mature tomato leaves and was inhibited compared to normal photoperiod in continuous darkness, but transcript abundance of these *HXK* genes was significantly elevated after 6 and 24 h in the

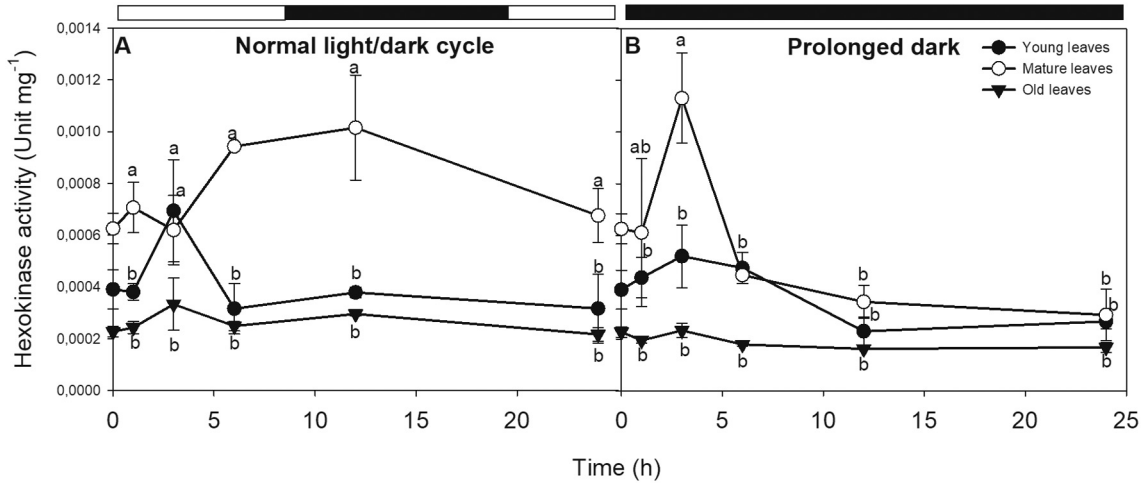

Fig. 3. Changes in the activity of hexokinase (HXK) in leaves at different leaf positions of tomato plants in the presence of glucose substrate during 24 h of normal light/dark cycle or continuous darkness. Means ± SE, n = 3. Data with different letters indicate significant differences at $P \leq 0.05$ level (Tukey test) in each time point.

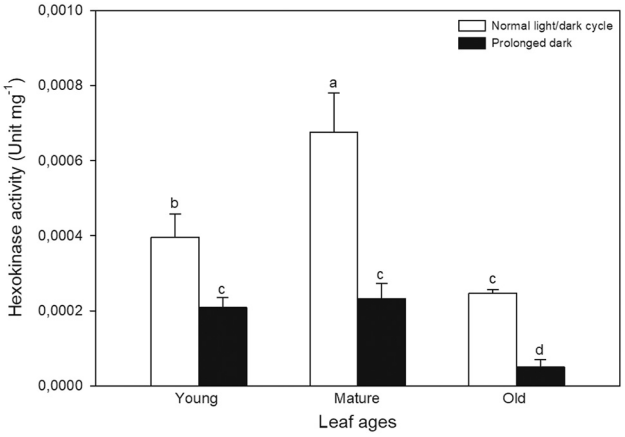

Fig. 4. Changes in the activity of hexokinase (HXK) in leaves at different leaf positions of tomato plants in the presence of glucose substrate under normal light/dark cycle or under 7-day-long darkness. Means ± SE, n = 3. Data with different letters indicate significant differences at $P \leq 0.05$ level (Tukey test).

old leaves. In contrast to the 24-h samples, the expression of *SlHXK1* and *SlHXK4* was suppressed by the 7 day-long dark treatment in the old leaves. However, transcripts of *SlHXK3* accumulated significantly in all leaf positions after dark exposure. Different expression patterns in various leaf positions may suggest different hormonal and metabolic control of the expression of HXK genes during the dark-induced senescence process in intact plants.

These experiments revealed a time and leaf age-specific expression pattern for HXKs and similar changes were observed in the enzyme activity. Earlier it was found that the HXK activity is regulated by diurnal rhythm, it is slightly increased during the light period and decreased during the night in tobacco leaf (Häusler et al., 2000). Our results not only confirmed this tendency in the case of HXK activity but also demonstrated the differences in the enzyme activities between the leaf maturity stages of intact tomato plant. It was found that the highest HXK activity was detected in the mature leaves and the lowest in the old leaves of tomato under control condition. In mature source leaves, the enzyme activity reached the maximum for the 6th hour of the light period and remained high during the early phase of the dark period. In young, sink leaves the maximum of enzyme activity was found in the middle of the light period suggesting a better carbohydrate supply from source leaves at this period. In contrast, HXK activities were inhibited after a 6-hour-long darkness in all leaf positions. Not only early decline but also long-term decrease was observed in HXK activities in all leaf position after 7 days under continuous dark, but this decline was the smallest in sink leaves.

Long-term effects of prolonged darkness on specific physiological activities were also monitored at different leaf positions. Basically, stomatal conductance and CO_2 assimilation were different at various leaf stages and were decreased or inhibited significantly within 24 h in all leaf positions upon prolonged dark treatment. Similar effect of long-term darkness on these parameters was observed after 7 days. In addition, prolonged dark treatment caused a significant decline in Fv/Fm and chlorophyll $a + b$ content, especially in the old leaves of plants suggesting that the functional and morphological disorganisation of chloroplasts was in progressed stage by this time. Based on these results, old leaves were the most sensitive to long-term effect of darkness and exhibited the most pronounced symptoms of senescence. Similar results were found in *Arabidopsis*, where stomatal conductance, Fv/Fm and chlorophyll content decreased differently during senescence of younger or older leaves (Mohapatra et al., 2010). Moreover, Fv/Fm and chlorophyll content decreased also significantly after 24 h in darkened *Arabidopsis* (Buchanan-Wollaston et al., 2005). Since the absence of light inhibited CO_2 fixation and synthesis of carbohydrates, the major source of chemical energy for plants, the leaves had to consume stored organic molecules such as polysaccharides. Sugar starvation, which can be observed in our experiments, can also modulate the activity of HXKs (Cho et al., 2010; Granot et al., 2013; Sheen, 2014).

Glucose is not only the main reduced carbon and energy source but also a signalling molecule, which regulates more than 2000 plant genes. Thus glucose and its derivatives may interact with phytohormones through the glycolytic and metabolite sensing pathways that rely on the hexose-phosphorylating function of HXK (Li and Sheen, 2016; Aguilera-Alvarado and Sánchez-Nieto, 2017). Basically, the accumulation of glucose, the potential substrate for HXK was the highest at the end of the light period and the lowest at the end of the dark period similarly to HXK activity in the leaves of *Arabidopsis* (Kunz et al., 2015) and tobacco plants (Häusler et al., 2000).

Based on our results, the glucose content was the highest in the young developing leaves and lower in the mature and/or old leaves, but HXK activity and gene expression were the most pronounced in the mature leaves. Significant decrease was found in glucose content and HXK activity in all intact leaves within 24 h under darkness. These changes were detected in parallel with the inhibition of CO_2 assimilation but it occurred before the damage of photosynthetic apparatus or decrease in photosynthetic pigments.

Except for the mitochondrial *SlHXK3*, the expression of all other *HXK* genes in tomato was down-regulated and the HXK activity decreased significantly in all leaf positions under dark starvation, which led to the initiation of chlorophyll breakdown and senescence. Thus it can be supposed, that increased expression of chloroplastic (*SlHXK4*) and mitochondrial *HXK* genes (*SlHXK1* and *SlHXK2*) contributes to the maintenance of enzyme activity under natural day–night cycles, and the hexokinase activity is necessary for the maintenance of steady state status of primary metabolic processes. The most interesting change is the increased expression of *SlHXK3* in both sink and source leaves after 7 days of dark exposure. In contrast to other *HXK*s, this gene was highly up-regulated by starvation and can be considered as a famine gene. We can also speculate that these changes are the last efforts of tissues to maintain minimal metabolic activity and mitochondrial integrity or this enzyme, similarly to plants overexpressing *AtSLHXK1* under the control of 35S promoter, enhances senescence (Granot et al., 2013). However, it can be concluded that higher total HXK activity in younger leaves is connected with slower rate of dark-induced senescence and chlorophyll loss, in which *SlHXK3* may have a central role.

Table 2
Changes in physiological activities in young, mature and old leaves of tomato plants grown on normal light/dark cycle or in 1- or 7-day-long darkness.

Time (day)	Leaf position	Stomatal conductance (mol H_2O m^{-2} s^{-1})		CO_2 assimilation (μmol CO_2 m^{-2} s^{-1})		Fv/Fm		Chlorophyll $a + b$ content (mg gFW^{-1})		Glucose content (μmol gFW^{-1})	
		Light	Dark	Light	Dark	Light	Dark	Light	Dark	Light	Dark
1	Young	0.28 ± 0.03^{a}	0.03 ± 0.01^{c}	11.41 ± 0.71^{a}	n.d.	0.80 ± 0.00^{a}	0.80 ± 0.00^{a}	1.37 ± 0.11^{a}	1.34 ± 0.08^{a}	13.8 ± 0.31^{a}	0.55 ± 0.18^{c}
	Mature	0.44 ± 0.10^{a}	0.02 ± 0.01^{b}	13.36 ± 1.26^{a}	n.d.	0.80 ± 0.00^{a}	0.80 ± 0.00^{a}	1.49 ± 0.13^{a}	1.47 ± 0.08^{a}	8.13 ± 0.89^{b}	1.13 ± 0.40^{c}
	Old	0.04 ± 0.01^{b}	0.01 ± 0.01^{b}	4.29 ± 0.50^{b}	n.d.	0.78 ± 0.01^{b}	$0.78 + 0.0^{b}$	0.93 ± 0.12^{b}	1.00 ± 0.13^{b}	8.01 ± 1.61^{b}	0.89 ± 0.08^{c}
7	Young	0.22 ± 0.02^{a}	0.02 ± 0.01^{b}	12.17 ± 1.96^{a}	n.d.	0.80 ± 0.00^{a}	0.72 ± 0.02^{c}	1.57 ± 0.12^{b}	1.17 ± 0.18^{b}	9.27 ± 1.01^{a}	0.15 ± 0.01^{c}
	Mature	0.33 ± 0.07^{a}	0.01 ± 0.01^{b}	13.03 ± 0.30^{a}	n.d.	0.80 ± 0.00^{a}	0.71 ± 0.01^{c}	1.82 ± 0.06^{a}	1.49 ± 0.06^{b}	7.12 ± 0.92^{b}	0.14 ± 0.03^{c}
	Old	0.05 ± 0.02^{b}	0.01 ± 0.00^{b}	5.51 ± 0.61^{b}	n.d.	0.78 ± 0.01^{b}	0.72 ± 0.01^{c}	1.14 ± 0.12^{b}	0.84 ± 0.02^{c}	5.62 ± 1.08^{b}	0.11 ± 0.01^{c}

Means ± SE, n = 6. Data with different letters indicate significant differences at $P \leq 0.05$ level (Tukey test) in each time point (n.s.: not significant; n.d.: not detected).

Acknowledgements

We thank Bécs Attiláné for her excellent technical assistance. This work was funded by a grant from the National Research, Development and Innovation Office (OTKA PD112855), Hungary. P.P. and A. S. were supported by the New National Excellence Program of the Ministry of Human Capacities (UNKP-17-4).

Conflict of interest

The authors declare that they have no conflict of interests.

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