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

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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.

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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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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},

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