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Cold-induced starch degradation in potato leaves – intercultivar differences in the gene expression and activity of key enzymes

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Abstract

Exposure to low temperatures is one of the most important factors that generate abiotic stress in plants, and the rapid accumulation of soluble sugars belongs to significant metabolic responses to cold stress. The accumulation of soluble sugars may be at least partially triggered by an increased rate of starch degradation. The analysis of transcript profiles and starch degrading enzyme activities in leaves of two potato cultivars was performed during a 12-h exposure to 2 °C. An induction of β-amylase expression and activity as well as an accumulation of reducing sugars were observed in cv. Desiree. No accumulation of reducing sugars and no significant changes in the β-amylase activity were initially observed in cv. Russet Burbank. Surprisingly, an increased α-amylase activity was observed in the last hours of the experiment, which was accompanied by an increased amount of reducing sugars. The results indicate that the leaves of Desiree and Russet Burbank potatoes growing under cold stress may degrade starch *via* different pathways.

Additional key words: α-amylases, β-amylases, glucan phosphorylases, low temperature, reducing sugars, *Solanum tuberosum*.

Introduction

During the course of evolution, plants have developed an array of mechanisms that enable them to survive in suboptimal conditions. The ability of plants to acclimatize to cold conditions arises from numerous changes in their metabolism which take place at all cellular levels and involve, for instance, changes in gene expression, enzyme activities, the stabilization of phospholipids that compose cell membranes, and the accumulation of soluble sugars and proteins (Kosová *et al.* 2007, Ruelland *et al.* 2009, Krasensky and Jonak 2012).

Potato is a cold sensitive plant. Sub-zero temperatures after plant emergence may cause damage to the above-ground biomass, delaying development and possibly reducing yield. The increasing demand for food due to the growth of the world population results in a constant expansion of the potato cultivation range, mostly in developing countries. The results of recent studies increase our knowledge on the role of the activated pathways and the significance of the induced gene products, which – when used efficiently – will improve plant tolerance to stress (Jain 2013, Patrick *et al.* 2013).

The accumulation of soluble sugars is one of the first significant metabolic responses observed in cold-treated plants. Soluble sugars play an important role in protecting plant cells against damage caused by cold *via* serving as osmoprotectants, nutrients, or interacting with the lipid bilayer. Additionally, they may act as primary messengers during signal transduction in cells. On the other hand, the sugar accumulation may signal to the cell that the flowering season is approaching, which may negatively impact the productivity of cultivated plants (Yuanyuan *et al.* 2009). Under cold stress, maltose and maltotriose achieve half of their maximum content in *Arabidopsis* leaves already after 10 and 40 min, respectively (Kaplan *et al.* 2007). Maltose produced in a single-step reaction catalysed by β-amylase is transferred to the cytoplasm and undergoes a further reaction catalysed by 4-α-glucanotransferase DPE2 (disproportionating enzyme, EC 2.4.1.25), in which one glucose residue is released to the cytoplasm, whereas the other is incorporated into a soluble heteroglycan (SGH) (Fettke *et al.* 2005, 2006). The released glucose residue is then phosphorylated by hexokinase, whereas heteroglycan undergoes phosphorolysis to glucose 1-phosphate (G1P) which is the source of hexose phosphates during the sucrose synthesis (Lu *et al.* 2006). In addition to maltose, β-amylolytic degradation also generates a smaller amount of maltotriose since the enzyme is unable to hydrolyse

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Abbreviations: DPE1 - disproportionating enzyme 1, DPE2 - disproportionating enzyme 2, G1P - glucose 1-phosphate, LDA - limit dextrinase, SGH - soluble heteroglycan, SPS – sucrose-6-phosphate synthase
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chains of less than four glucosyl residues. Maltotrioses are metabolised by 4-α-glucanotransferase (DPE1) which significantly differs from its cytosol isoform DPE2 (Steichen *et al.* 2008). On the contrary to DPE2, not maltose but maltotriose is used by DPE1 and a maltosyl unit from one maltotriose is transferred to another one. Maltopentaose produced by DPE1 is acted on by β-amylase, and the released glucose is exported from the chloroplast by the glucose transporter (Critchley *et al.* 2001). The increasing content of mono- and di-saccharides form a good protection for plants, providing an additional time for cells to produce proper stress proteins and metabolites to adjust the metabolism and physiology of the plant to cold stress. The rapidly increasing maltose content in *Arabidopsis* leaves caused by exposure to low temperature indicates a considerable role of starch degradation during the initial response to cold (Kaplan *et al.* 2007).

It is now well documented that the initiation of starch degradation requires enzymes that catalyse the phosphorylation of amylopectin polymers on the granule surface. Starch phosphorylation plays a pivotal role in promoting starch digestion by increasing the hydrophilicity of insoluble granules, thereby ensuring a better accessibility of hydrolytic enzymes to starch (Hejazi *et al.* 2008). Hydrolytic enzymes that may further degrade starch granules include α-amylases (EC 3.2.1.1), β-amylases (EC 3.2.1.2), and isoamylases (EC 3.2.1.68) (Zeeman *et al.* 1998b, Yu *et al.* 2005, Orzechowski 2008).

The involvement of α-amylases in transitory starch degradation in potato leaves has not been determined, but their role in starch degradation in cereal grain endosperm (*e.g.*, Smith *et al.* 2005 and references therein) is indisputable. In addition to playing a key role in starch degradation at night, β-amylase also participates in the response to temperature shock. It was previously shown in *Arabidopsis* that the stress caused by low temperatures induces the expression of one of β-amylase isoenzymes – BMY 8 (Kaplan and Guy 2004).

Starch phosphorolysis which involves the chloroplast isoform of phosphorylase PHS1 may be an alternative or addition to the hydrolytic breakdown of starch that occurs in plastids (Zeeman *et al.* 1998a). The analysis of an *Arabidopsis* mutant with a silenced plastidic phosphorylase gene has shown that even without any major changes in starch accumulation during the day and starch degradation at night, this plant shows reduced tolerance to abiotic stresses (Weise *et al.* 2006).

Numerous studies were conducted to identify the enzymes involved in starch breakdown in potato tubers. However, it has not yet been fully clarified which of the potentially involved enzymes participate in starch degradation in these organs. Viksø-Nielsen *et al.* (1997) highlighted the ambiguities and overly controversial differences in the results of previous studies on cold-induced sugar accumulation in potato tubers. They considered that these differences may arise from imperfections in the activity-testing techniques which sometimes fail to differentiate between the activities of various types of enzymes, thus rendering the detection of activity of particular enzyme isoforms impossible. However, these authors suspected that such discrepancies may also result from cultivar-related differences. Therefore, the purpose of our work was to verify results suggesting that leaves of Desiree and Russet Burbank potatoes grown under cold conditions use different starch degradation mechanisms.

Materials and methods

Plants and cold treatments: Leaf samples were collected from 5 to 6 weeks old potato (*Solanum tuberosum* L.) cultivars Desiree and Russet Burbank growing in a growth chamber under control (day/night temperatures of 22/15 °C) and stress conditions (2 °C) with a 16-h photoperiod, a 50 % relative humidity, and a midday photosynthetic photon flux density of approximately 400 μmol m^{-2} s^{-1}. Analyses were performed with the consideration of day and night cycles which regulate the expression of numerous genes encoding enzymes involved in starch degradation (Smith *et al.* 2004, Orzechowski *et al.* 2013). Some of the plants that were initially grown under optimal conditions were transferred to low temperature conditions by the end of the night period, simulating the naturally occurring temperature drops. Except temperature, other conditions in the growth chamber remained unchanged. Samples were collected at three-hour intervals for 12 h from both the stressed and control plants. Control and cold-stressed leaves were collected at the same time to eliminate effects derived from diurnal and circadian rhythms. The samples were immediately frozen in liquid nitrogen and stored in -80 °C until analyzed. Leaves were gathered three times from independent experiments.

Transcript measurements: Control and cold-shocked potato leaf tissues were ground to a fine powder in liquid nitrogen using a mortar and pestle. Total RNA was extracted using a *NucleoSpin* RNA plant kit (*Macherey Nagel*, Haerdt, France), including DNase digestion according to the manufacturer's protocol. RNA concentrations were determined spectrophotometrically by measuring absorbances at 260, 230, and 280 nm. The quality of the RNA was examined by electrophoresis on 1.3 % (m/v) denaturing formaldehyde/agarose gels. Semi-quantitative RT-PCR analysis was carried out using a *Novagen*® one step RT-PCR *Master Mix* kit (*Merck*, Darmstadt, Germany) following the manufacturer's instructions with minor modifications (Grabowska *et al.* 2012). The oligonucleotide primer sequences are displayed in Table 1. The selected reference gene was 18S rRNA. Transcription changes were evaluated to

identify responses of genes encoding starch hydrolases and/or starch phosphorylases to the cold stress.

Determination of enzyme activities and reducing sugar contents: Control and cold-stressed leaves from three independent experiments were ground to a fine powder in liquid nitrogen using a mortar and pestle. For determination of β-amylase (EC 3.2.1.2) activity, crude extracts were prepared from 500 mg of the powder in 1 cm^3 of an extraction buffer containing 50 mM Tris-HCl (pH 8.0), 1 mM Na_2EDTA, 2 % (m/v) polyvinyl-pyrrolidone (PVP), and 100 mM cysteine hydrochloride. Then the mixture was centrifuged at 16 000 *g* and 4 ºC for 15 min. The supernatant was diluted 10-fold with 100 mM MES (pH 6.2) containing 1 mM Na_2EDTA and used in an enzyme activity assay with *p*-nitrophenyl maltotrioside (PNPG3) as substrate (*Betamyl-3 Reagent*, *Megazyme*, Bray, Ireland) according to the manufacturer's instructions. Activities were determined after 2 and 4 h of incubation at 40 ºC and the absorbance of the samples was measured by monitoring the production of *p*-nitrophenol at 400 nm.

For determination of α-amylase (EC 3.2.1.1) activity, an extraction buffer contained 50 mM sodium malate, 50 mM sodium chloride, 2 mM calcium chloride, 2 % PVP, and 0,0005 % (m/v) sodium azide (pH 5.2). Crude extract prepared from 500 mg of the powder in 1 cm^3 of the extraction buffer was centrifuged at 16000 *g* and 4 ºC for 15 min. The α-amylase activity was measured using a blocked *p*-nitrophenyl maltoheptaoside (PNPG7) as substrate (*Ceralpha*, *Megazyme*) according to the manufacturer's instructions. The activity was measured spectrophotometrically at 400 nm after 20 min of incubation at 40 ºC.

For determination of starch phosphorylase (EC 2.4.1.1) activity, 500 mg of the leaf powder was extracted in 1 cm^3 of 50 mM HEPES (pH 7.0), 1 mM Na_2EDTA, 4 mM 2-mercapto-ethanol, and 0.1 mM phenylmethane-sulfonyl fluoride (PMSF). The phosphorylase activity was carried out according to Steup and Latzko (1979). The activity, expressed as the production of NADPH, was measured by monitoring the absorbance at 340 nm at 5-min intervals at 30 ºC for 30 min.

The content of reducing carbohydrates was assayed using 3,5-dinitrosalicylic acid sodium salt according to the method described by Lindsay (1973). All the crude extracts that were used for enzyme activity determinations were also assayed for reducing sugar content. The total protein content was quantified in all the leaf samples according to Bradford (1976) with bovine serum albumin as standard.

Statistical analyses: All data were analyzed using two-way *ANOVA* and the Tukey's HSD (honestly significant difference) post hoc test to assess main effects for treatments and time as the independent variables (Wessa 2014). The level of significance was set at $\alpha = 0.05$.

Table 1. Primer sequences used for the semi-quantitative RT-PCR analysis.

Gene	Accession No.	Oligonucleotide sequence (5'-3')	Product length [bp]	Product name
StAMY21	M81682	F: TGGCACAGGGAATCCAGACACG R: CTGTTACAGCCCGCCCTGCAT	326	AMY21
StAMY23	M79328	F: GGATTGATGGCGCGGGACAACT R: GGGAAAGGCCAATGCGCCTGA	202	AMY23
PCT-BMYI	AF393847	F: AGATCAGGCCGGAGAAATCCTGAGT R: CTGTTACAGCCCGCCCTGCAT	289	BAM
POTAGPTHI	M69038	F: AGGGAACTCGGGATATGGTCGTGG R: TGTGCTCAAGGCACGCGACA	228	PhoH
POTGP (Pho1a)	D00520	F: AGGCGATTGACGAGGAGCTGGT R: TCTGCAGCTGCTTCTATCTTCACAC	277	Pho1a
STP1 (Pho1b)	X73684	F: GGTGAACTGCTTCCTCGGCACG R: TGCCTTTACAGCCTCAGTTTC	277	Pho1b
18s RNA	X67238	F: CCAGGTCCAGACATAGTAAG R: GTACAAAGGGCAGGGACGTA	427	18s rRNA

Results

This analysis focused on the expression of six genes that are associated with starch metabolism in leaves of two potato cultivars Desiree and Russet Burbank (Fig. 1). In the Desiree leaves, the β-amylase gene (*BAM*) transcription increased when compared to the control plants as soon as the plants were exposed to the low temperature for 3 h. Its high mRNA content persisted up to the 12th hour of the cold treatment. No differences in the transcriptions of the α-amylase-encoding genes (*AMY21*, *AMY23*) were observed, and in the case of a gene encoding one of the plastidial isoforms of glucan phosphorylase (*Pho1b*), the transcription declined after 9 and 12 h of the cold stress when compared to the control plants. In the Russet Burbank leaves, the

transcription of *Pho1b* increased under the cold stress when compared to the control plants. This increase occurred after 3 h of cold exposure and persisted until 12 h. The Russet Burbank leaves showed no changes in the transcription of *BAM* or the other amylase- and phosphorylase-encoding genes (*AMY21*, *AMY23* and *Pho1a*, *PhoH*).

In the Desiree leaves, the β-amylase activity increased considerably after 3 h of cold exposure, reaching a level that was 2.5-fold higher than the activity measured in the control leaves. After 3 h, the β-amylase activity was 40 - 65 % higher than that in the control plants until 12 h of the cold treatment (Fig. 2*A*). In turn, the β-amylase activity in the Russet Burbank leaves doubled only at 3 h of cold exposure and remained unchanged thereafter (Fig. 2*E*).

The α-amylase activity in the Desiree leaves fluctuated slightly. Initially, it did not change under the stress conditions when compared to the control, only increased insignificantly, by *ca*. 20 %, after 9 and 12 h (Fig. 2*B*). In the Russet Burbank leaves, the α-amylase activity initially (until 6 h of the experiment) remained at the level observed in the control plants. After 9 and 12 h, an increase was observed (30 and 60 %, respectively) when compared to the control (Fig. 2*F*).

The phosphorylase activity in the Desiree and Russet Burbank leaves increased, but only after 3 and 12 h of cold, by 40 and 15 %, and by 10 and 20 %, respectively (Fig. 2*C,G*).

In the Desiree leaves, the content of reducing sugars was three times higher than that in the control plants after 3 h of cold exposure (Fig. 2*D*). Afterwards, the reducing sugar content dropped to a level that was about 30 % higher than that measured in the control plants, which is consistent with the increase in the β-amylase activity observed for this cultivar. Throughout the entire cold exposure treatment, the leaves of cv. Russet Burbank exhibited the content of reducing sugars similar to that observed in the control plants, except a slight increase after 12 h (Fig. 2*H*).

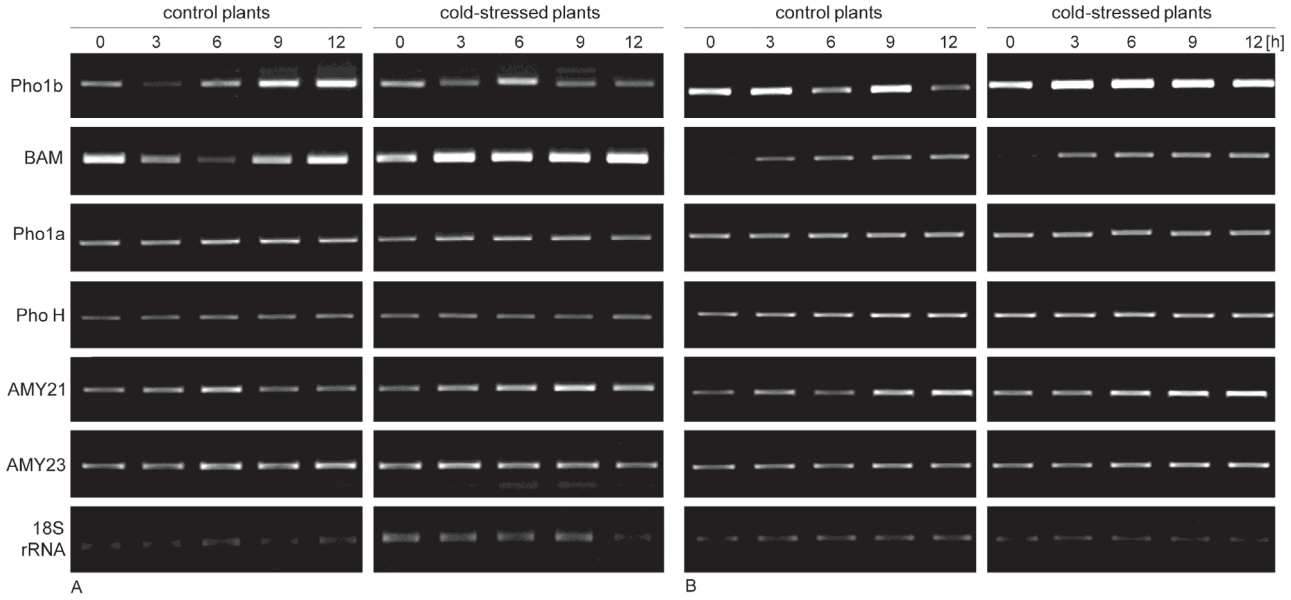

Fig. 1. Changes in mRNA amounts of starch metabolism enzymes [α-amylases (AMY21, AMY23), β-amylase (BAM), glucan phosphorylases (Pho1a, Pho1b, and PhoH)] in leaves of cvs. Desiree (*A*) and Russet Burbank (*B*) under a low temperature and normal growth conditions. RT-PCR analyses were performed with 20 ng of total RNA. The amplification of 18S rRNA was used as control to ensure that equal amounts of templates were added. Representative gels from one of three experiments are shown.

Discussion

In this study, we analyzed two potato cultivars that differ considerably with respect to tuber yield and tuber starch content. Russet Burbank is a cultivar with a high tuber yield (Stark *et al.* 2007) and a high tuber starch content. Desiree is a model cultivar that is characterized by a quite low tuber yield and low tuber starch content (Kaminski *et al.* 2012). It has been repeatedly shown that plant cultivars exhibit certain differences, which justifies studying more than one cultivar of a particular plant (*e.g.*, Saropulus and Drennan 2007, Wang *et al.* 2009).

Our analysis of the expression of starch metabolism genes revealed an increased amount of β-amylase transcripts in the Desiree leaves as result of cold exposure. The induction of a gene encoding β-amylase during a response to low temperatures has been documented for other plant species, such as *Vaccinium corymbosum* (Lee *et al.* 2012) and *Arabidopsis thaliana* (Espinoza *et al.* 2010). A cold-induced transcription of the gene encoding β-amylase is not reflected in an increased activity of this enzyme in *Arabidopsis* leaves.

However, this is caused by much higher activities of extraplastidial isoforms, which disturb the detection of any changes in the activity of the plastidial isoform during the determination of total activity. Nevertheless, maltose accumulation which is formed in a β-amylase-catalysed reaction (Kaplan and Guy 2004) was observed in *Arabidopsis* in response to cold exposure. The induction of the transcription of the gene encoding β-amylase in the Desiree leaves led to a higher enzyme activity and a 3-fold increase in the content of reducing sugars, which was observed 3 h after exposure to the low temperature. During the entire experiment, the activity of β-amylase remained high, which supplied the plant cells with sugars to defend against the effects of long-term cold exposure and enabled the plant to activate other defense mechanisms (Yuanyuan *et al.* 2009). Changes in

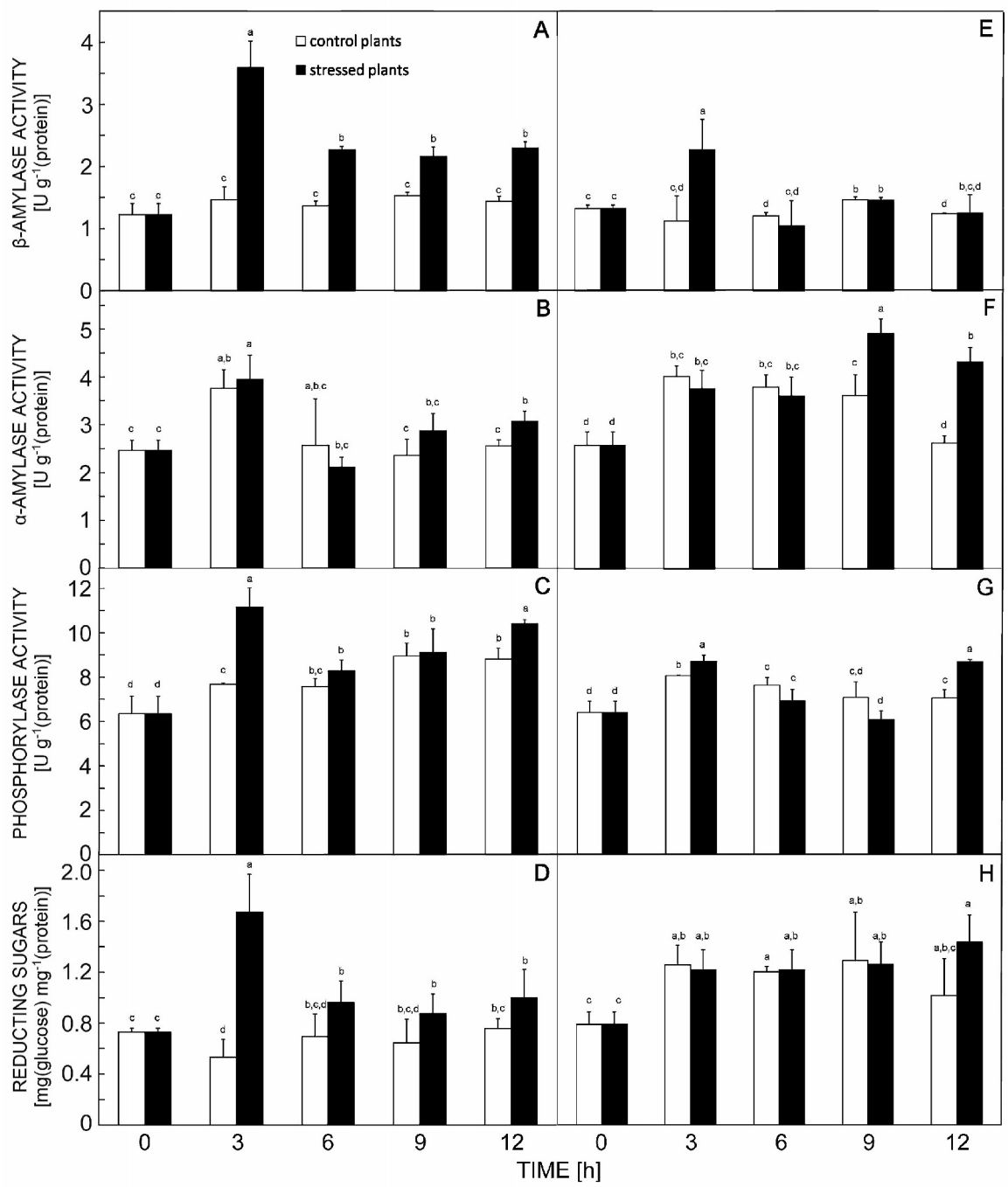

Fig. 2. The activities of β-amylase (*A*, *E*), α-amylase (*B*, *F*), phosphorylase (*C*, *G*), and the content of reducing sugars (*D*, *H*) in leaves of Desiree (*A-D*) and Russet Burbank (*E-F*) growing under optimum conditions and exposed to low temperature. The results are means of three or more replicates ± SD. Different letters at the top of the *bars* mean significant differences ($P \leq 0.05$).

the β-amylase activity in the potato leaves exposed to the low temperature arose from the increased activity of the plastidial isoform. Viksø-Nielsen *et al.* (1997) have demonstrated the presence of only one β-amylase isoform in Desiree potato leaves. Therefore, it can be assumed that the total β-amylase activity measured in our study applied just to the plastidial isoform. Research performed by Viksø-Nielsen *et al.* (1997) on Desiree potato tubers suggests the existence of a mechanism of cold-induced starch degradation that is similar to the one present in leaves. They also observed an increase in β-amylase activity and a maltose accumulation in the tubers.

An analogical analysis performed on the potato leaves of the Russet Burbank cultivar shows that the lack of changes in the expression of the gene encoding β-amylase, which was assayed at transcriptional level, was also reflected in the absence of changes in the activity of this enzyme. In contrast to Desiree, the β-amylase activity in the Russet Burbank leaves did not increase under the low temperature when compared to the control plants. However, this cultivar is characterised by an increased transcription of one of the genes encoding starch phosphorylase (*Pho1b*). Still, the phosphorylase activity did not constantly increase under cold stress conditions. Results of studies by Albrecht *et al.* (2001) have suggested that the activity of plastidial phosphorylase is involved in starch biosynthesis rather than degradation. In the Russet Burbank leaves, the α-amylase activity increased after 9-h low temperature exposure, and this increase persisted until the end of the experiment. Knowledge on the number of α-amylase isoforms in the potato is incomplete. Only two genes that encode two isoenzymes, secretory and cytosolic α-amylases, have been identified to date (Stanley *et al.* 2005). In our work, both of the α-amylase genes characterized in potato were analysed but no changes in expression were observed at transcriptional level. It remains unknown if a plastidial isoform exists, as it does in *Arabidopsis* and probably in apple. The sequences of both the mentioned α-amylase isoenzymes have a chloroplast transit peptide and they have been grouped into the third plant α-amylase family. The significance ascribed to the third family of α-amylases is related to transitory and storage starch degradation in plastids (Stanley *et al.* 2002). The importance of α-amylase in starch degradation in the chloroplast is not yet fully understood. It is very likely that α-amylase plays a significant role in starch degradation in rice leaves (Asatsuma *et al.* 2005). Seung *et al.* (2013) suggested that because enzyme activity depends on the redox potential, as has been observed for *Arabidopsis*, this enzyme is most probably involved in the response to cold stress.

The first sugar that accumulates in Russet Burbank tubers exposed to low temperatures is sucrose, and afterwards, the glucose and fructose content also increase (Zhou and Solomos 1998). It has also been demonstrated that under low temperatures, an α-amylase activity increases, becoming considerably higher than a β-amylase activity (Doehlert and Duke 1983). Our results show that the content of reducing sugars in the leaves of the Russet Burbank control plants was *ca*. 60 % higher than in the Desiree leaves. The availability of reducing sugars in the leaves of Russet Burbank together with an increased activity of sucrose-6-phosphate synthase (SPS) (Illeperuma *et al.* 1998) and invertase (Kumar *et al.* 2004) which were observed in cold-treated plants may delay a response to cold involving starch degradation. A considerable amount of sugars in cells may serve as osmoprotectant under short-term stress conditions. The second stage of response to cold involves starch degradation with the participation of α-amylase and probably DPE1 and limit dextrinase-type debranching enzyme (LDA), which may lead to increased content of reducing sugars. In a previous study in potato, StDPE1 and StDPE2 were repressed and their role in leaves of Desiree transgenic lines was studied. This study indicates that the enzymes are active in parallel pathways of starch degradation (Lütken *et al.* 2010). In the future, it might be worth determining the gene expressions and activities of these enzymes in plants exposed to low temperature to verify their possible participation in cold induced starch degradation.

For both the cultivars, the activities of α- and β-amylases increased at the same time during cold exposure, although the intensity of each of these changes differed. This finding suggests the existence of common mechanisms that lead to an increased activity of enzymes that break down starch under cold stress. On the other hand, the different intensities of the observed changes in the activity of the starch degradation enzymes might arise from intercultivar differences. For the Russet Burbank cultivar, the β-amylase activity increased considerably after 3 h of cold exposure but not in the following hours unlike to the cv. Desiree. Moreover, this increase was not correlated to an increase in the reducing sugar content, as it was for Desiree. The activity of α-amylase in both the cultivars also increased after 9 and 12 h of cold exposure when compared to the activity in the control plants, but the differences were much greater in Russet Burbank. A similar tendency was observed in the activity of phosphorylase which increased after 3 and 12 h of the cold treatment when compared to the control plants in both the studied cultivars.

To conclude, the results presented here demonstrate the differences in the gene expression patterns and activities of the hydrolytic enzymes involved in cold-induced starch degradation between two potato cultivars. Verification, which enzyme, α- or β-amylase, more efficiently enhances the tolerance of potato plants to the stress caused by exposure to a low temperature would be valuable, and transgenic lines with altered expression of the mentioned enzymes would support such studies. A few studies have employed mutants with silenced starch metabolism genes in systems other than the one that has been extensively studied in *Arabidopsis*. The efficiency of the above-mentioned starch degradation pathways under cold conditions has yet to be verified. The obtained results offer new perspectives for future studies which

will complement the current knowledge about plant tolerance to unfavourable environmental conditions, such as cold stress.

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