RAPID COMMUNICATION

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Regulation of α-galactosidase gene expression in primary foliage leaves of barley (*Hordeum vulgare* L.) during dark-induced senescence

Abstract α-Galactosidase activity (α-D-galactoside galacto-hydrolase, EC 3.2.1.22) increased during dark-induced senescence in primary foliage leaves of barley (*Hordeum vulgare* L. cv. Steffi). The changes in activity were accompanied by parallel changes in expression of the *HvSF11* gene encoding a putative α-galactosidase. The transcript level of *HvSF23* encoding a second putative α-galactosidase stayed constant during leaf senescence. Both α-galactosidase activity and the level of the *HvSF11* transcript decreased after exogenous application of sucrose and glucose to detached dark-incubated leaves. In contrast, the *HvSF23* transcript level was not influenced by sugars. Application of glucose analogs to detached and dark-incubated leaves revealed that phosphorylation of hexose by hexokinase modulates both the α-galactosidase activity and the expression of *HvSF11*. These results indicate that the expression of the genes coding for two α-galactosidase isoenzymes is regulated by different signalling pathways, suggesting different functions for the two gene products.

Keywords α-Galactosidase (enzymes, cDNAs) · *Hordeum* · Leaf senescence (barley) · Primary foliage leaves · Sugar-mediated gene repression (hexokinase)

Abbreviations *Glc* Glucose · *2-d-Glc* 2-Deoxy-glucose · *6-d-Glc* 6-Deoxy-glucose · *3-OMG* 3-O-Methyl-glucose · *Suc* Sucrose · *RFOs* Oligosaccharides of the raffinose family

Introduction

Plant α-galactosidases are important hydrolytic enzymes known to catalyze the hydrolysis of various storage substances in seeds and fruit tissues (Dey and Pridham 1972). Since work on plant α-galactosidases has mostly focused on seed and fruit tissues (Feurtado et al. 2001; Guimaraes et al. 2001), little is known about the nature and function of α-galactosidases in photosynthetically active tissues. However, α-galactosidase activities have also been measured in photosynthetically active tissues (Gatt and Becker 1970; Smart and Pharr 1980). In plant families that synthesize and transport raffinose-family oligosaccharides (RFOs), different isoforms of α-galactosidases occur during the transition of leaves from sink to source tissues (Pharr and Sox 1984; Chrost and Schmitz 2000). In plants not known as synthesizing such RFOs, α-galactosidases are assumed to be involved in galactolipid metabolism of thylakoid membranes, especially in the senescence-associated breakdown of these membranes (Wanner et al. 1991; Thompson et al. 1998). Recent studies, however, indicate that under certain conditions, e.g. freezing, drought and temperature stress, various plants synthesize RFOs (Santarius 1973; Bachmann et al. 1994; Taji et al. 2002; Pennycooke et al. 2003). Investigations on *Arabidopsis*, which was not categorized as an RFO-synthesizing plant, showed that *Arabidopsis* leaves synthesize and translocate raffinose (Hariratos et al. 2000). RFOs seem to play roles during desiccation and cold tolerance, and accumulate also in senescent leaves, as shown for leaves of *Popolus nigra* by Fialho and Bücher (1995).

Leaf senescence is a highly regulated and genetically determined process. Expression of new and senescence-associated genes is necessary for senescence to progress (Quirino et al. 2000). Genes specifically induced during senescence encode enzymes that are involved in the hydrolysis of carbohydrates, proteins and lipids. Senescence is characterized by a significant decrease in photosynthesis and degradation of cellular components like chlorophylls, protein and lipids. One of the most efficient stimuli that accelerate leaf senescence is the exposure to darkness. Since light is essential for photosynthesis, supplying energy and sugars as major respiratory substrates for leaf development, leaves fall easily into sugar

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limitation upon exposure to darkness (Stitt et al. 1985; Brouquisse et al. 1998). Plants are able to survive sugar starvation by switching from sugar catabolism to protein and lipid catabolism. This physiological switch is linked to the expression of various genes related to the degradation of proteins and lipids as well as to gluconeogenesis (Smart et al. 1995; Quirino et al.1999).

Sugars are known as signalling molecules in plant development, including leaf senescence (Rolland et al. 2002). The expression of several senescence-associated genes is induced by sugar depletion and suppressed by sugars (Noh and Amasio 1999; Fujiki et al. 2000, 2001). There is an obvious relationship between senescence-associated gene expression and sugar starvation but the effects of sugars on leaf senescence remain controversial (Yoshida 2003). The mechanism controlling gene expression in response to sugar starvation, e.g. during dark-induced senescence of *Arabidopsis* leaves, involves hexokinase as sugar sensor (Fujiki et al. 2000, 2001).

We have previously described the isolation and characterization of two different cDNA clones, *HvSF23* and *HvSF11*, from senescent barley flag leaves. The corresponding genes are also expressed in primary foliage leaves induced to senesce by darkness. Sequence analyses revealed that both genes encode putative α-galactosidase enzymes (Chrost and Krupinska 2000). In the present study we have investigated the expression of the two genes in primary foliage leaves of barley in response to sugar depletion in the course of dark-induced senescence and after application of sugars. Although both genes encode α-galactosidases, only the expression of *HvSF11* is modulated by sugars, implying that different mechanisms control the expression of *HvSF23* and *HvSF11*.

Materials and methods

Plant material

Barley (*Hordeum vulgare* L. cv. Steffi) plants were grown under controlled growth chamber conditions on vermiculite under a daily light/dark rhythm of 16 h light (120 μmol photons m^{-2} s^{-1}) and 8 h darkness. The temperature changed from 21°C during the day to 16°C at night. Plants were grown for 7 days until the primary foliage leaves were fully expanded. To study dark-induced senescence, primary foliage leaves were cut from the plants after 7 days growth, incubated in tap water and transferred into darkness for a period of 7 days.

Sugars and glucose (Glc) analogs were applied at concentrations of 3% (w/v) in tap water to detached primary foliage leaves of the plants. The leaves were detached from the plants after 7 days growth, transferred into the sugar solutions and then incubated for 7 days in the dark.

Leaf blades were sampled, frozen in liquid nitrogen and stored at −80°C for later analysis.

Extraction and assay of α-galactosidase activity

α-Galactosidase activity was extracted as described by Chrost and Schmitz (1997), and was measured with *p*-nitrophenyl-α-D-galactopyranoside as artificial substrate. Formation of the product, *p*-nitrophenol, was measured by its absorbance at 400 nm.

Isolation of RNA and Northern blot analysis

RNA of primary foliage leaves was isolated with Trizol (Invitrogene, Freiburg, Germany) reagent following the procedure given by the manufacturer. Equal amounts of each RNA sample were separated electrophoretically on 1% (w/v) agarose gels containing formaldehyde. The RNA was transferred onto positively charged nylon membranes (Hybond N^+; Amersham, Freiburg, Germany) by capillary blotting. To control loading of RNA the membrane was stained with methylene blue. Hybridizations were done with radiolabelled cDNA fragments of *HvSF23* and *HvSF11* fragments, as described by Chrost and Krupinska (2000).

Results and discussion

α-Galactosidase activity increased in primary foliage leaves of barley during senescence of detached dark-incubated leaves (Table 1). To investigate whether the senescence-related changes in α-galactosidase activity are linked to one or both of the two α-galactosidase genes represented by the cDNA clones *HvSF11* and *HvSF23* (Chrost and Krupinska 2000), the expression of both genes was examined in parallel (Fig. 1). Our data show that the changes in α-galactosidase activity during senescence are closely followed by changes in the expression of the *HvSF11* gene. In contrast, the *HvSF23* transcript level stays rather constant during leaf senescence, and therefore this gene may be categorized as a housekeeping gene (Buchanan-Wollaston 1997). The *HvSF11* gene belongs to the group of genes that are expressed during dark-induced senescence of leaves. It is well known that chlorophyll degradation and reduced photosynthesis rates are features of dark-induced senescence (Buchanan-Wollaston 1997). The rapid decline in photosynthesis leads to sugar starvation, which might be involved in regulating the expression of dark-inducible genes. Accordingly, alterations in carbohydrate levels have been shown to influence the expression of dark-inducible genes like *sen1*, *ASN1* and *din* (Fujiki

Table 1 Effects of sucrose, glucose and glucose analogs on the α-galactosidase activity in primary foliage leaves of barley (*Hordeum vulgare*). Seven-day-old primary foliage leaves of barley plants grown in a light/dark regime (*Control*) were detached from the plants and incubated in darkness for a further 7 days in tap water or in tap water containing 3% (w/v) sucrose, glucose or glucose analogs. As an osmotic control, 3% (w/v) sorbitol was applied. Extraction and assay of α-galactosidase activity were performed as described in Materials and methods. Data are mean values ± SD from at least four independent measurements

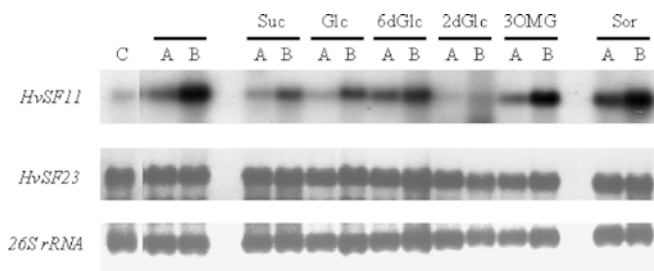| Primary foliage leaves	α-Galactosidase-activity (μUnit g FW^{-1})
Control	247.0 ± 31
Dark-incubated	624.5 ± 35
Dark-incubated plus sucrose	511.0 ± 16
Dark-incubated plus glucose	499.5 ± 35
Dark-incubated plus 6-d-Glc	626.0 ± 32
Dark-incubated plus 2-d-Glc	241.0 ± 15
Dark-incubated plus 3-OMG	481.0 ± 26
Dark-incubated plus sorbitol	614.0 ± 25

Fig. 1 Analysis of the expression of the *HvSF23* and *HvSF11* genes in primary foliage leaves of barley (*Hordeum vulgare*) after different incubation treatments. Seven-day-old primary foliage leaves were detached from the plants after growing in a growth cabinet under a light/dark regime (*C*). For induction of senescence, the detached leaves were incubated for 5 (*A*) and 7 days (*B*) in darkness prior to collecting. In addition, sucrose (*Suc*), glucose (*Glc*) and the glucose analogs 2-deoxy-glucose (*2dGlc*), 6-deoxy-glucose (*6dGlc*) and 3-O-methyl-glucose (*3OMG*) were applied at concentrations of 3% (w/v) to dark-incubated leaves. Sorbitol (*Sor*) was applied as an osmotic control. Total-RNA isolation and hybridization were done as described in Materials and methods. 15 µg of total RNA was loaded onto each lane and analyzed by northern blot hybridization with the cDNA probes *HvSF23* and *HvSF11*. To demonstrate uniform loading and transfer, 26S rRNA is shown after methylene blue staining of the membrane

et al. 1997; Shimada et al. 1998; Nozawa et al. 1999). Since the *HvSF11* gene is expressed in dark-incubated senescing leaves, we hypothesize that a higher *HvSF11* transcript level and a high α-galactosidase activity in dark-incubated leaves are related to sugar starvation. Accordingly, *HvSF11* gene expression is also enhanced when detached leaves are treated with the photosynthesis inhibitor 3-(3,4-dichlorophenyl)-1,1-dimethyl urea (DCMU; data not shown).

To investigate the effect of sugar application on the transcript levels of *HvSF23* and *HvSF11* genes as well as on α-galactosidase activity, detached leaves that contain low sugar levels during dark-incubation were employed. Both, Glc and sucrose (Suc) were able to reduce substantially the transcript levels of *HvSF11* and the α-galactosidase activity of the leaves in comparison to dark-incubated leaves without sugar supplement. Sorbitol as a control for osmotic stress had no effect on gene expression or on α-galactosidase activity (Fig. 1, Table 1). These results indicate that the expression of the *HvSF11* gene in barley leaves is indeed repressed by Suc and Glc and not by osmotic effects. Similar results have been obtained before for *din* genes with *Arabidopsis* leaves and suspension cell cultures (Fujiki et al. 2000, 2001).

The sugar-sensing system for suppression of *Arabidopsis din* genes by sugars was shown to involve phosphorylation of hexoses by hexokinase (Fujiki et al. 2000). To address the question of whether the phosphorylation of hexoses by hexokinase is involved in the regulation of *HvSF11* gene expression, Glc analogs were applied to detached leaves. The Glc analogs 6-deoxy-glucose (6-d-Glc) and 3-O-methyl-glucose (3-OMG), which cannot be phosphorylated by hexokinase did not

affect the *HvSF11* transcript level. In contrast, 2-deoxy-glucose (2-d-Glc), which can be phosphorylated by hexokinase and thus can initiate hexokinase-mediated sugar signalling, completely suppressed the accumulation of *HvSF11* transcripts. Like Suc and Glc, the Glc analogs did not influence the transcript levels of *HvSF23*. Interestingly, also under these conditions the decrease in *HvSF11* transcript level was accompanied by a simultaneous decrease in α-galactosidase activity. These results suggest that the phosphorylation of hexoses by hexokinase generates the signal for the sugar-mediated repression of *HvSF11* gene expression. According to our data the α-galactosidase enzymes encoded by the genes *HvSF23* and *HvSF11* are differentially expressed and regulated in leaves of barley. The sugar-mediated mechanism by which hexose is phosphorylated by hexokinase is involved in the regulation of *HvSF11*, but certainly not in the regulation of *HvSF23* gene expression.

Since they are capable of hydrolyzing terminal α-1,6-linked α-galactose residues from glycolipids, glycoproteins or oligosaccharides, α-galactosidases might provide alternative energy sources, replacing photosynthetic assimilates. It is well known that sugar starvation induces changes in the metabolism of leaves. Since, during darkness and senescence leaves undergo sugar starvation as a direct consequence of the decline in photosynthesis, the presence or induction of α-galactosidases as hydrolytic enzymes might contribute to leaf survival. Future studies aim at unravelling the different localization and different functions of the two differentially regulated α-galactosidases.

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