TREHALOSE AND TREHALOSE-6-PHOSPHATE INDUCE STOMATAL MOVEMENTS AND INTERFERE WITH ABA-INDUCED STOMATAL CLOSURE IN GRAPEVINE

Magdalena GAMM, Marie-Claire HÉLOIR and Marielle ADRIAN*

Université de Bourgogne, UMR 1347 Agroécologie, BP 86510, 21000 Dijon, France

Abstract

Aims: The effects of trehalose and trehalose-6-phosphate (T6P), among other sugars, were assessed on grapevine stomatal movements.

Methods and results: Epidermal peels were used to assess the effects of sugars. Low concentrations of trehalose and T6P (1 µM) induced an osmotic-independent reduction of the stomatal aperture in light conditions. Furthermore, ABA-induced stomatal closure was reduced by sugar application in association with lower accumulation of reactive oxygen species in guard cells. Similar effects, although weaker, were observed in response to the disaccharides sucrose and maltose, but not in response to the monosaccharides fructose and glucose.

Conclusion: This study clearly highlights the effects of sugars, especially trehalose and T6P, on grapevine stomatal movements.

Significance and impact of the study: This is the first time that such effects are described in grapevine and the results obtained provide new insights about the role of sugars on stomatal regulation at the whole plant level.

Key words: grapevine, stomata, sugars, trehalose, ABA

Résumé

Objectifs: Des sucres, en particulier le tréhalose et le tréhalose-6-phosphate (T6P), ont été étudiés pour leurs effets sur les mouvements stomatiques chez la vigne.

Méthodes et résultats: Des stomates obtenus à partir d'épidermes isolés ont été utilisés pour tester ces sucres. De faibles concentrations en tréhalose et en T6P (1 µM) ont induit une réduction non osmotique de l'ouverture stomatique à la lumière. Ces sucres ont également induit une réduction de la fermeture stomatique induite par l'ABA corrélée à une plus faible accumulation de formes actives de l'oxygène dans les cellules de garde. Des effets similaires, plus faibles, ont été observés avec les disaccharides saccharose et maltose, mais pas avec les monosaccharides fructose et glucose.

Conclusion: Cette étude montre un effet des sucres, en particulier le tréhalose et le T6P, sur les mouvements stomatiques chez la vigne.

Signification et impact de l'étude: Les résultats sont originaux et soulignent le rôle que pourraient jouer les sucres dans la régulation stomatique chez la vigne.

Mots clés: vigne, stomates, sucres, tréhalose, ABA

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*Corresponding author: marielle.adrian@u-bourgogne.fr

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INTRODUCTION

Stomata are natural pores bordered by two guard cells and present in the epidermis of most aerial parts of higher plants. They allow gas exchanges between the plant and its environment, balancing CO_2 uptake for optimal photosynthesis and limiting water losses by transpiration (Assmann 1993). Opening or closure of stomata is achieved by osmotic swelling or shrinking of guard cells driven by K^+, Cl^-, malate^{2-} (for review, see Outlaw, 2003), and probably also sucrose (Tallman and Zeiger, 1988 ; Lawson *et al.,* 2014). Stomatal movements are finely regulated by endogenous and environmental factors such as phytohormones, light, CO_2 level, and atmospheric humidity (Outlaw, 2003). The phytohormone abscisic acid (ABA) is a signal molecule for water stress which induces stomatal closure. This involves a complex cascade of signaling events, including the second messenger H_2O_2 acting on ion channels and leading to guard cell plasmolysis (for review, see Li *et al.,* 2006 ; Sirichandra *et al.,* 2009).

In plants, sugars produced by photosynthesis play multiple roles as vital sources of energy, carbon skeletons, and storage components. Furthermore, their pivotal role as signaling molecules becomes more and more apparent (Sheen *et al.,* 1999 ; Rolland *et al.,* 2006). As sugar signaling interacts with diurnal changes, abiotic and biotic stresses, and hormone signaling (Roitsch, 1999 ; Smeekens, 2000 ; Gazzarrini and McCourt, 2001 ; Finkelstein and Gibson, 2002 ; Trouvelot *et al.,* 2014), sugars form part of a sophisticated communication system necessary for the coordination of metabolism with growth, development, and environmental changes (Tomé *et al.,* 2014). This is particularly true for the disaccharide trehalose (two α,α,1-1-linked glucose units) and its phosphorylated precursor trehalose-6-phosphate (T6P) (Paul *et al.,* 2008 ; Fernandez *et al.,* 2010), which have emerged as novel regulators in carbohydrate metabolism and/or signaling (Goddijn and Smeekens, 1998 ; Paul, 2007). As reviewed by Paul *et al.* (2008), the alteration of trehalose metabolism or signaling pathway has effects on key plant functions including development, photosynthesis, sucrose utilization, starch metabolism, and tolerance to abiotic stresses (drought).

Sugar/ABA metabolism and signaling were shown to be interconnected : hexoses are involved in the regulation of ABA biosynthetic genes, ABA sensing, and the ABA-signaling pathway (Koch, 2004). In turn, ABA participates in the regulation of sugar metabolism and/or transport (Gibson, 2005 ; Ramon *et al.* 2007). This interconnection between sugar and ABA indicates that sugar could affect stomatal movements. Several studies have described the osmotic role of apoplastic sucrose in modulation of stomatal aperture in apoplastic phloem loader plants such as *Vicia faba* (Lu *et al.,* 1995 ; Outlaw *et al.,* 2001 ; Kang *et al.,* 2007). However, little is known about the signaling effects of sugars on stomatal movements (Dittrich and Mayer, 1978 ; Van Houtte *et al.,* 2013). A recent study also showed that the increased expression of the sucrose-phosphorylating enzyme hexokinase in guard cells of tomato lines reduced stomatal aperture. This effect is mediated by ABA and induced by sucrose (Kelly *et al.,* 2013). In grapevine, the stomatal deregulation in *Plasmopara viticola* (downy mildew)-infected leaves (Allègre *et al.,* 2007) is associated to severe changes in sugar metabolism, notably trehalose accumulation, and to a loss of responsiveness to ABA-induced stomatal closure (Allègre *et al.,* 2007 ; Gamm *et al.,* 2011), suggesting that both alterations might be interconnected. Therefore, in this study, we address the effects of exogenously applied sugars, especially trehalose and T6P, upon grapevine stomatal movements.

MATERIALS AND METHODS

1. Plant material

Grapevine (*Vitis vinifera* L. cv. Marselan) herbaceous cuttings were grown in individual pots (10 x 7 x 7 cm) containing a mixture of blond peat and perlite (3:2, vol/vol). They were grown in a glasshouse at a temperature of 24 and 18 °C (day and night, respectively) with a photoperiod of 16 h light and a relative humidity of 70 ± 10 % until they developed six leaves. Plants were sub-irrigated with a fertilizer solution (NPK 10-10-10, Plantin, France).

2. Assays on epidermal peels

Epidermal peels were prepared from leaves harvested at the end of the dark period as previously described (Allègre *et al.,* 2007) and placed under light conditions (PPFR : 350 µmol m^{-2} s^{-1}) in 5 ml buffer (30 mM KCl, 10 mM Mes-KOH, pH 6.5) at 24 °C for 2.5 h to induce stomatal opening. Then, epidermal peels were placed in fresh buffer containing the compounds to be tested : trehalose (0.1, 1, 10 µM or 100 mM), T6P, fructose, glucose, maltose or sucrose (1 µM), ABA (50 µm), ABA (50 µm) combined with trehalose (0.1, 1 or 10 µM), ABA (50 µm) combined with T6P, fructose, glucose, maltose or sucrose (1 µM).

The stomatal aperture was analyzed 2.5 h after incubation with the fluorescein diacetate probe (FDA, 20 µg ml^{-1}) (Allègre *et al.,* 2007). Pictures of 30 stomata per epidermal peel were recorded using a Leica DMLB fluorescence microscope equipped with a digital camera and the stomatal apertures were measured using the NIS-Elements BR (Nikon Instruments) software. The results presented correspond to the mean values of three independent experiments, each performed with two epidermal peels.

3. ROS detection in guard cells

Reactive oxygen species (ROS) production in guard cells was monitored using 2,7-dichlorofluorescein diacetate (H_2DCFH-DA), as previously described (Allègre *et al.,* 2009). In short, epidermal peels were placed in conditions to promote stomatal opening and incubated for 60 min in the buffer alone (control) or combined with ABA (50 µM), trehalose or T6P (1 µM). The fluorescent probe H_2DCFH-DA (50 µM, 0.1 % dimethyl sulfoxide, vol/vol) was added to the medium 10 min prior to the end of the incubation period, and Petri dishes were placed in the dark. Pictures of stomata were recorded using a Leica DMLB fluorescence microscope equipped with a digital camera using the L5 filter (λ_{exc} 480 to 540 nm, λ_{em} 527 to 630 nm). Because the probe is highly photosensitive (Murata *et al.,* 2001), eight pictures of stomata were rapidly recorded for each epidermal peel. The mean intensity of the gray levels was measured using the Image J software.

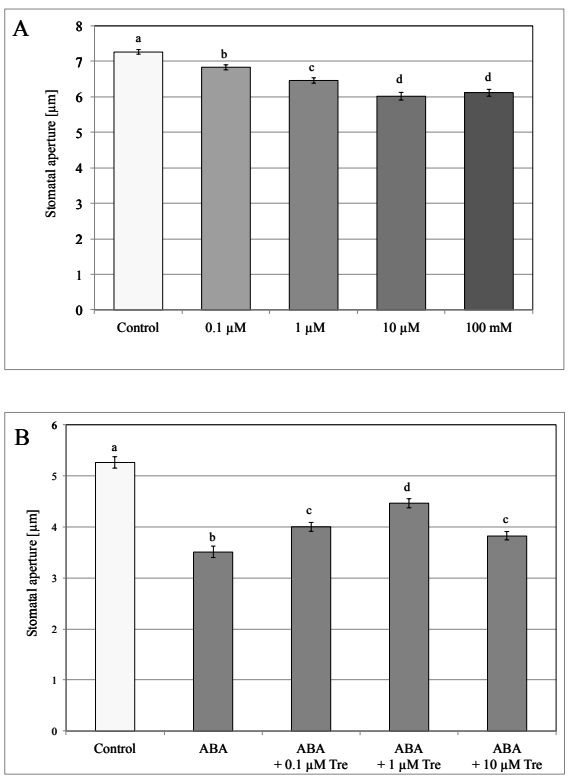

Figure 1. Effect of trehalose on grapevine stomatal aperture in the light (A) and on ABA-induced stomatal closure of grapevine stomata (B). Epidermal peels were prepared and incubated with 0.1 µM, 1 µM, 10 µM, 100 µM trehalose (Tre) or 50 µM abscisic acid (ABA) alone or in combination. Data are the arithmetic means of 3 biological replicates with 60 stomata each, with bars representing the standard error of means. Values with different letters are statistically different ($p < 0.05$).

4. Statistical analysis

Data were analyzed using a one-way analysis of variance (ANOVA) and the means were compared by Fisher's least significant difference (LSD) test.

RESULTS

1. Trehalose and T6P induce closure of grapevine stomata and inhibit their ABA-induced closure

Trehalose applied at low concentrations (0.1, 1, and 10 µM) to grapevine epidermal peels induced a significant closure of stomata in a dose-dependent manner, with a maximum effect at 10 µM (Figure 1A). The differences in mean aperture between trehalose-treated stomata and the control were 0.42, 0.80, and 1.24 µm for 0.1, 1, and 10 µM trehalose, respectively. A higher trehalose concentration (100 mM) induced a stomatal closure of 1.14 µm compared to the control treatment, similar to the response to 10 µM trehalose. To exclude a possible osmotic effect, mannitol was applied but failed to induce closure below 0.5 M (data not shown).

Furthermore, trehalose interfered with the ABA-induced stomatal closure (Figure 1B). In our conditions, ABA (50 µm) induced a stomatal closure of 1.75 µm compared to the control. However, the addition of trehalose to the ABA treatment resulted in a significant reduction of the ABA-induced stomatal closure with stomatal aperture values of only 1.26, 0.8, and 1.44 µm compared to the control for 0.1, 1, and 10 µM trehalose added to ABA, respectively. As the concentration of 1 µM trehalose induced a significant closure of pre-opened stomata in the light and had the most pronounced effect on the ABA-induced stomatal closure, this concentration was used for the following experiments.

The effect of T6P, also reported as important in signaling, was compared to that of trehalose. T6P at

1 µM induced a more pronounced stomatal closure than trehalose, with differences of 1.16 and 0.59 µm compared to the control, respectively (Figure 2A). However, T6P affected the ABA-induced stomatal closure to a lesser extent than trehalose with differences in aperture of 1.13 and 1.70 µm for the ABA + T6P and ABA + trehalose co-treatments, compared to the ABA treatment, respectively (Figure 2A).

2. Monosaccharides and other disaccharides have none or weaker effects on the grapevine stomata aperture and ABA-induced closure compared to trehalose and T6P

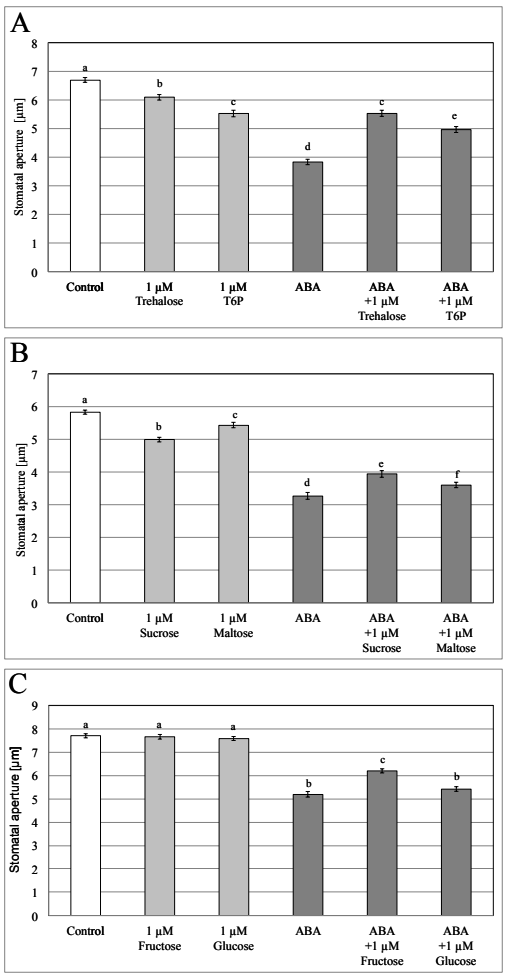

Figure 2. Effect of trehalose and T6P (A), sucrose and maltose (B), and fructose and glucose (C) on grapevine stomatal aperture in the light and on ABA-induced stomatal closure. Epidermal peels were prepared and incubated with 1 µM saccharides and 50 µM abscisic acid (ABA) alone or in combination for 2.5 h in the light. Data are the arithmetic means of 3 biological replicates with 60 stomata each, with bars representing the standard error of means. Values with different letters are statistically different ($p < 0.05$).

In order to investigate the specificity of the effects of trehalose and T6P on stomatal movements, other disaccharides (sucrose, maltose) and monosaccharides (glucose, fructose) were applied to epidermal peels. Both disaccharides were applied at 1 µM, alone or combined with 50 µm ABA, on grapevine epidermal peels (Figure 2B). Sucrose treatment reduced the aperture of stomata in the light by 0.83 µm compared to the control, whereas maltose had a less pronounced effect with a difference of 0.4 µm. Both sugars reduced the ABA-induced stomatal closure by 0.68 and 0.36 µm, respectively. These results highlight a general effect of the disaccharides assessed on stomatal aperture, with trehalose and T6P identified as the most efficient. The monosaccharides glucose and fructose (1 µM) were applied in the same conditions, but had no significant effect on the stomatal aperture (Figure 2C). Fructose induced a statistically significant reduction of the ABA-induced stomatal closure, whereas glucose showed no significant effect.

3. Trehalose and T6P inhibit the ABA-induced ROS production in grapevine stomata

Trehalose and T6P, being the most effective in interfering with the ABA-induced stomatal closure in grapevine, were analyzed for their effect on the ABA-induced ROS production in guard cells (Figure 3). ABA alone induced a significant production of ROS (247.6 % of the level of gray measured in the control), whereas trehalose (1 µM) reduced the ABA-induced ROS production close to the background level of the control treatment. T6P also decreased the ABA-induced ROS production, but to a lesser extent.

DISCUSSION AND CONCLUSION

The effects of trehalose and T6P on grapevine stomatal movements in light conditions were studied using epidermal peels. Two mono- and disaccharides were also assessed for comparison: sucrose as a non-reducing sugar, maltose as a disaccharide formed by two glucose subunits, and glucose and fructose as the constituents of the assessed disaccharides. All the disaccharides used induced a reduction of the aperture of stomata in the light, with T6P showing the strongest effect, whereas the monosaccharides had no effect. Altogether, our results suggest that disaccharides, but not monosaccharides, applied at low concentrations, interfere with the mechanisms regulating the "open state" of grapevine stomata. Dittrich and Mayer (1978) assessed the effects of a set of carbohydrates (used at 100 mM) on stomatal

movements of *Commelina communis* epidermal peels. They reported an effect of hexoses and some disaccharides on stomatal aperture. In particular, trehalose inhibited the opening of stomata, but not sucrose nor maltose. However, as these results were obtained by studying the opening process of closed stomata, it makes it difficult to compare with our conditions.

An increasing number of studies investigates the function of trehalose and T6P in plants. Trehalose was first associated to desiccation tolerance of resurrection plants (Adams *et al.,* 1990). Further studies showed that it is present in trace amounts in plants (Goddijn and Smeekens, 1998 ; Paul *et al.,* 2008) and is accumulated to allow resistance to abiotic stresses, such as drought, heat, chilling, salinity or UV, and also resistance to a range of biotic stresses (for review, see Fernandez *et al.,* 2010). Transgenic plants overexpressing trehalose phosphate synthase genes hence show an improved tolerance to abiotic stresses correlated with the accumulation of trehalose and/or T6P (Romero *et al.,* 1997 ; Avonce *et al.,* 2004). In grapevine, T6P accumulates in stems and leaves of Chardonnay *in vitro* plantlets in response to chilling and could contribute to the chilling resistance conferred by the plant growth-promoting rhizobacterium *Burkholderia phytofirmans* strain PsJN (Fernandez *et al.,* 2012).

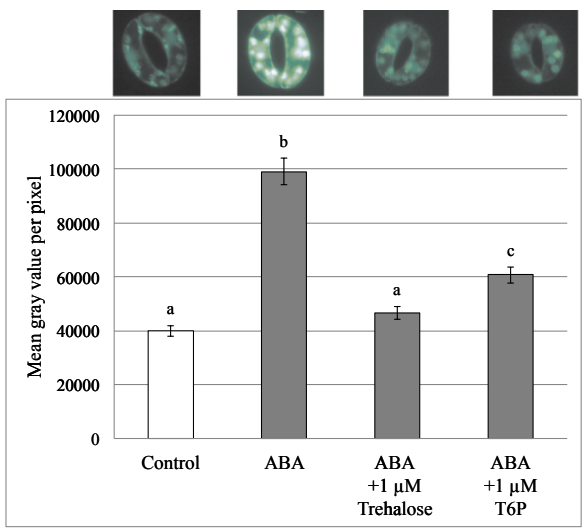

Figure 3. ROS production in grapevine epidermal peels treated with abscisic acid (ABA) alone or in combination with trehalose or T6P. Values correspond to the arithmetic mean of the mean gray value per pixel of 100 guard cells, with bars representing the standard error of means. Data represent the results of one representative experiment of three biological replicates using 50 µM 2'7'-dihydrofluorescein diacetate to assess the production of ROS in guard cells. Values with different letters are statistically different ($p < 0.05$). Images correspond to representative guard cell fluorescence observed using microscopy.

In downy mildew-infected grapevine leaves, we observed a stomatal dysfunction characterized by stomata abnormally opened in the dark and unresponsive to ABA. This deregulation occurs in the oil spot symptom area together with a source to sink transition characterized by higher levels of soluble sugars, among which trehalose (Gamm *et al.,* 2011). Trehalose and/or its precursor T6P were good candidates to explain the stomatal deregulation. As stomata have no plasmodesmata, the putative soluble sugar signal was likely to occur at the apoplastic level. Interestingly, trehalose was only detected in apoplastic fluids of infected leaves (0.98 ± 0.04 mM) (unpublished data). However, at this concentration trehalose induces a lower (27 %) stomatal opening than that obtained with apoplastic fluids extracted from infected leaves. The role of trehalose and T6P in downy mildew-infected leaves thus remains unclear.

Few studies have reported the concentrations of both trehalose and T6P in grapevine leaves. In our previous study (Gamm *et al.,* 2011), only trehalose was quantified in leaves of cv. Marselan using HPAEC-PAD method. However, as reported by Fernandez *et al.* (2012), this method overestimates the actual concentration in some cases. The same authors therefore used fluorescence spectrometry and HPLC methods allowing the determination of the actual concentrations of trehalose and T6P, respectively, in grapevine organs. In leaves of Chardonnay *in vitro* plantlets, they found higher levels of trehalose than those of T6P (in the range of 5 and 0.3 nmol g^{-1} FW, respectively). These levels increase significantly in response to chilling to reach values of about 14 and 2 nmol g^{-1} FW, respectively. It is difficult to compare trehalose concentrations with those reported by Gamm *et al.* (2011) as they were obtained with different plant materials (*in vitro* plantlets/herbaceous cuttings) of different varieties (Chardonnay/Marselan) and expressed differently (fresh weight/dry weight). It would be interesting to quantify trehalose and T6P in grapevine leaves in response to abiotic and biotic stresses to compare their respective accumulation profile and to progress in the understanding of their respective role.

Like some other sugars, trehalose and T6P are also considered as signaling molecules (Rolland *et al.,* 2006 ; Paul *et al.,* 2008) that could interfere with other signaling pathways. In this study, all the sugars assessed, except glucose, partially inhibited the

ABA-induced stomatal closure, with trehalose being the most effective. This effect appears to be less specific since both mono- and disaccharides (except glucose) were effective to induce it. ABA-induced stomatal closure is known to be mediated by ROS production in guard cells (Pei *et al.,* 2000; Murata *et al.,* 2001; Kwak *et al.,* 2003). In this study, we showed that ROS production is affected in the presence of trehalose and T6P, suggesting that both sugars act upstream of ABA-signaling events associated to stomatal closure. Studies have reported the impact of trehalose/T6P on ABA signaling in association with plant development mechanisms (Avonce *et al.,* 2004; Gomez *et al.,* 2010). However, only few papers have reported an impact of the modulation of trehalose/T6P levels on ABA-induced stomatal closure. In the Arabidopsis mutants *Attre1* (affected in trehalase synthesis), Van Houtte *et al.* (2013) reported a higher trehalose concentration correlated with an impaired ABA-induced stomatal closure and drought susceptibility. Curiously, stomata of the Arabidopsis mutant *tps1-12* (affected in T6P synthesis) are more closed in the dark than those of the wild type and have also an impaired responsiveness to ABA (Gomez *et al.,* 2010). These studies show the high complexity of ABA/trehalose signaling crosstalk.

Using a simplified stomatal model we thus observed that trehalose and T6P induce a reduction of stomatal opening in the light and partially inhibit the ABA-induced stomatal closure. At the leaf level, it is tempting to suggest that variations in their concentrations in the guard cell apoplast could serve as signal for grapevine stomatal movements and the subsequent regulation of photosynthesis/transpiration.

CONCLUSION

Altogether, these results highlight the role of exogenously applied carbohydrates as signaling molecules on grapevine stomata. The characterization and significance of the dynamics of trehalose and T6P at the scale of grapevine plants and their respective role in response to stress is one crucial question that will require further investigation.

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