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ARTICLE

Cell wall acidification in growing barley (*Hordeum vulgare* L.) leaves

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ABSTRACT Apoplast acidification associated with growth is well-documented in roots and coleoptiles but not in leaves. In the present study, advantage was taken of high cuticle permeability in the elongation zone of barley leaves to measure apoplast pH and acidification and the role which the plasma membrane H^+ ATPase (PM-ATPase) plays in this process. An in-vitro gel system and pH-microelectrodes were used to monitor pH, and growth was measured with a linear variable differential transformer (LVDT). Test reagents which blocked (vanadate) or stimulated (fusicoccin) PM-ATPase were applied to the leaf elongation zone. In addition, the expression level (qPCR) and activity of PM-ATPase was determined. Apoplast pH was lower in growing compared with non-growing tissue. Growth and apoplast acidification were stimulated by fusicoccin and reduced by vanadate. Expression of PM-ATPase, as judged from C_t-values, was almost identical in the two leaf regions. In contrast, activity of PM-ATPase, expressed per unit plasma membrane protein, was about twice as high in growing tissue. It is concluded that PM-ATPase is required in growing leaf cells to achieve maximum rates of elongation and apoplast acidification and that this is due in part to a higher plasma membrane surface density of PM-ATPase activity.
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KEY WORDS

apoplast pH
cell elongation
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plasma membrane H^+-ATPase
proton pump

Growing leaf cells need to constantly accumulate solutes to maintain the osmotic gradient between inside and outside of cell while expanding many-fold in size (Fricke and Flowers 1998). It is the osmotic gradient, which drives water uptake and enables turgor to build up sufficiently high for walls to yield. The uptake of solutes into cells is directly or indirectly driven by a proton (H^+) gradient across the plasma membrane. The H^+ gradient is established through the activity of the plasma membrane H^+-ATPase (PM-ATPase). In growing tissues, the activity of the PM-ATPase also leads to the growth-dependent acidification of the apoplast, a phenomenon which has been termed in connection with stimulatory effects of auxin on growth as 'acid growth' (Rayle and Cleland 1970; Hager et al. 1971; Rayle and Cleland 1992). The discovery of expansins has provided molecular entities through which wall acidification can be linked to altered chemical interactions and loosening in the wall (McQueen-Mason et al. 1992; Cosgrove 1993, 1997). Changes in wall pH can also be linked to further processes which are relevant to growth, such as signalling and uptake of nutrients, in particular potassium (K^+) (Tode and Lüthen 2001; Felle et al. 2005; Felle 2006).

Studies on apoplast (and cell wall) pH in relation to growth have focused on the grass coleoptile, on hypocotyls, internodes and roots (Moloney et al. 1981; Terry and Jones 1981; Schopfer 1989; Peters and Felle 1999; Peters 2004). Leaves have not been studied in detail, despite being the main photosynthetic organs, and there exist few studies which have attempted to relate apoplast pH to growth (Van Volkenburgh and Cleland 1980; Taylor and Davies 1985; Van Volkenburgh and Boyer 1985; Keller and Van Volkenburgh 1998; Stahlberg and Van Volkenburgh 1999; Neves-Piestun and Bernstein 2001). These studies either did not measure directly pH in the apoplast space or were carried out largely on leaves of dicotyledonous plants and, therefore, suffered from the presence of a fully-developed waxy cuticle which renders apoplast pH measurements and application of reagents difficult. A recent study on cuticle development in barley showed that cuticle permeability in the leaf elongation zone is orders of magnitude higher than in the mature blade (Richardson et al. 2007). In the present work, we took advantage of this observation and measured apoplast pH in dependence of growth using

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an in-vitro gel system, electrophysiology and a linear variable differential transformer (LVDT). Growth and apoplast acidification were analysed in response to the application of reagents which affect the activity of PM-ATPase. Since results of these experiments pointed to PM-ATPase playing a key role in apoplast acidifications, we subsequently analysed the expression level and activity of PM-ATPase using qPCR and isolated plasma membrane fractions, respectively. All experiments were repeated at least three times and representative results are shown.

Materials and Methods

Plant material

Barley (Hordeum vulgare L. cv. Golf; Svalöf Weibull AB, Svalöv, Sweden) was grown hydroponically under controlled environmental conditions as described previously (Fricke and Peters 2002; Knipfer and Fricke 2010). Plants were analysed when they were 14-15d old, 3-12 h into the 16 h photoperiod. At this developmental stage, the main growing leaf of plants was leaf three. The base 40 mm of leaf three contained the leaf elongation zone and was enclosed by the sheath of older leaves one and two (Fricke and Flowers 1998; Fricke 2002). The portion of the leaf three which was emerged from the sheath of leaf two and exposed to ambient environmental conditions (light, wind, air humidity) was the 'emerged blade'. Cells in this region were not growing. All analyses were carried out on leaf three.

Methods of pH measurements

Microelectrode measurements: Apoplast pH was measured with the aid of pH-sensitive microlectrodes (Walker et al. 1998) using the same setup and microelectrode cocktail as described in Dennis et al. (2009). The older leaves one and two were peeled back to expose the abaxial surface of the basal elongation zone of leaf three (see also Volkov et al. (2009). The elongation zone was covered with moist tissue paper which had been soaked in the respective bath solution, as specified in figure legends, to alter the apoplast chemical environment of the leaf elongation zone. In the fully-cutinised emerged blade portion, apoplast pH was measured by inserting the microelectrode through stomatal pores. The rate of leaf elongation during the 4-6h measurement period was determined with a ruler.

In-vitro gel system: The base 60 mm of leaf three was placed into Petri dishes which had been filled with agarose (gelling temperature 38.3°C, Bioline) medium containing the pH indicator bromocresol purple (Tang et al. 2004; Li et al. 2007). Any test reagents were added to the medium while it was fluid and the pH was adjusted to pH 7.0 using 3 mM KOH. Petri dishes were incubated in the growth chamber and photographed with a Cannon EOS 350D digital camera at the times specified.

LVDT measurements

The rate of leaf elongation was determined with the aid of a LVDT to follow rapid and high-resolution (micrometer) responses of growth to application of test reagents (Fricke et al. 2004). Experiments were carried out in a laboratory environment, at ambient temperature, relative humidity and light. Plants were prepared in the same way as for electrophysiological analyses to relate results from both types of experiments to each other.

Expression analyses

Leaf tissues were frozen in liquid nitrogen and stored at -80 ºC until further use. Total RNA was extracted using a QIAGEN RNeasy kit, DNase treatment and cDNA synthesis was carried out as described in Besse et al. (2011) using an anchor oligo$_d$T$_{16}$ primer (NVTTTTTTTTTTTTTTT). Expression analysis was performed on a real time thermal cycler (STRATAGENE Mx3000P, Agilent Technologies) using a SYBR green mastermix (SYBR®Premix Ex Taq™, Takara Bio Inc.) following the manufacturer's instructions. Conditions for qPCR were as follows: (i) initial denaturation of cDNA and activation of the hot start enzyme through 10 s at 95°C; (ii) 45 cycles of denaturation for 5 s at 95°C followed by annealing and extension for 30 s at 60°C.

Plasma membrane isolation and ATPase assay

Plasma membrane vesicles were isolated from barley seedlings based on the two-phase procedure pioneered by Kjellbom and Larsson (1984) see also Yan et al. (1998) and Wei et al. (2007) using 1.5-6 g plant material. Protein content of purified plasma membrane fraction was measured with the method of Bradford (Kruger 2002).

The activity of PM-ATPase was measured by determining that portion of ATP-dependent phosphate release which could be inhibited by vanadate (Sarkadi et al. 1992; Pitann et al. 2009).

Results

Apoplast pH, acidification and growth

Microelectrode measurements revealed that the pH of the leaf apoplast was about 4.8 in the elongation zone and by about 1 pH unit more acidic than the pH in the non-growing emerged leaf region (Fig. 1). The magnitude of pH difference between the two leaf regions depended on the potassium concentration in the apoplast bathing medium (not shown).

The in vitro-gel system confirmed results obtained through electrophysiological analyses in that the elongation zone but not older leaf tissue showed an acidification of the agarose medium which was in direct contact with the leaf apoplast (Fig. 2). The extent of medium acidification depended on the activity of PM-ATPase. Vanadate, which inhibits PM-ATPase

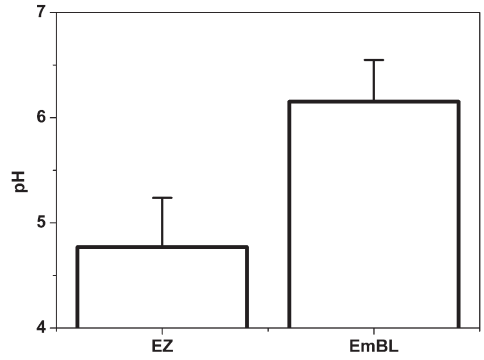

Figure 1. Apoplast pH in the elongation zone (EZ) and emerged leaf blade (EmBL) of leaf three of barley. The electrode bath solution which was in direct contact with the apoplast of the leaf tissue contained 0.1 mM KCl. Values are averages and standard deviations (error bars) of three measurements, obtained through the analysis of 2-3 plants. The difference in apoplast pH between EZ and EmBL was statistically significant ($p < 0.05$; t-test).

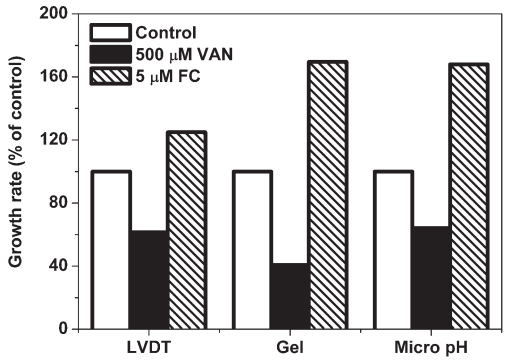

Figure 2. Growth rate of leaf three of barley in response to treatments which affect PM-ATPase activity. The elongation zone of leaf three was exposed to solution which contained no added test reagents (control) or 500 µM vanadate (VAN) or 5 µM fusicoccin (FC). Growth rate of leaf three was analysed through three different approaches (linear variable differential transformer [LVDT], agarose gel and microelectrode pH setup). The growth rate of control leaves was set to 100 % for each approach. Values for treatments are expressed as percent of the respective control. Averages of between 3-15 replicate plant analyses (per approach and treatment) are shown.

abolished almost entirely the acidification whereas fusicoccin, which stimulates PM-ATPase, increased the rate or level of acidification.

Similarly, leaf growth increased in response to stimulation (fusicoccin) and decreased in response to inhibition (vanadate) of PM-ATPase (Fig. 3).

PM-ATPase expression and activity

The level of expression of PM-ATPase was almost identical, as judged from C_t-values, in growing and non-growing leaf regions (Fig. 4A). In contrast, PM-ATPase activity expressed

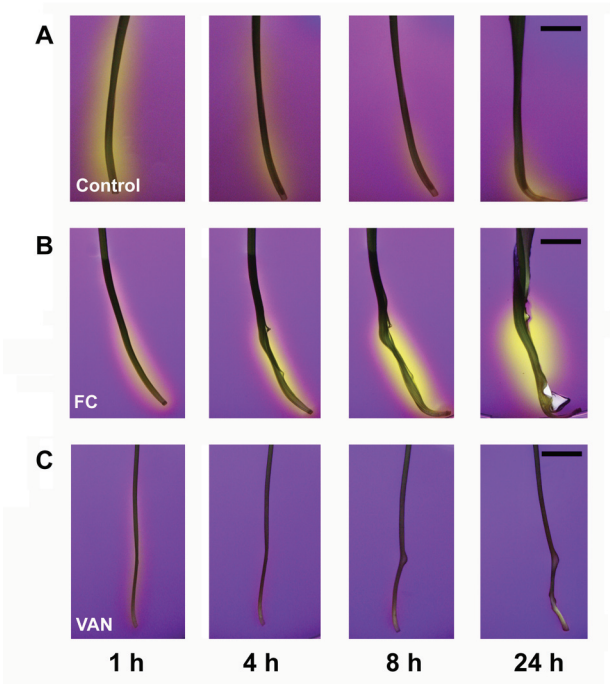

Figure 3. Apoplast acidification in excised barley leaves which were placed in agarose gel medium containing the pH indicator bromocresolpurple. Leaf three of barley was removed from plants, freed of the surrounding sheaths of older leaves one and two and placed in agarose medium containing (A) no added test reagent (control), or (B) 5 µM fusicoccin (FC), or (C) 500 µM vanadate (VAN). Photographs were taken at the times indicated. Excised leaf segments were 6 cm long and contained at their base a 4-cm long elongation zone. Medium (and by implication apoplast) acidification next to leaf tissue is visible as yellowish colour. Scale bars represent 1 cm.

per unit total plasma membrane protein was about twice as high in growing compared with non-growing leaf tissue (Fig. 4B).

Discussion

In the present study, leaf growth in relation to apoplast pH could be analysed in detail without having to expose tissues to various forms of mechanical abrasion of the leaf cuticle, and thus avoiding possible artefacts. Neves-Piestun and Bernstein (2001) and Van Volkenburgh and Boyer (1985) measured pH in microlitre-sized droplets applied to the elongation zone of maize leaves yet did not record any stable pH, nor did the authors measure pH directly in the apoplast/wall space of tissue. In addition, the present data provide conclusive and quantitative evidence that the PM-ATPase is required to attain maximum cell elongation in grass leaves. The data also suggest that the pH in the apoplast of elongating leaf tissue is lower than that in mature tissue.

The activity of PM-ATPase per unit total plasma membrane protein was about twice as high in growing compared with non-growing leaf tissue. We do not know whether and

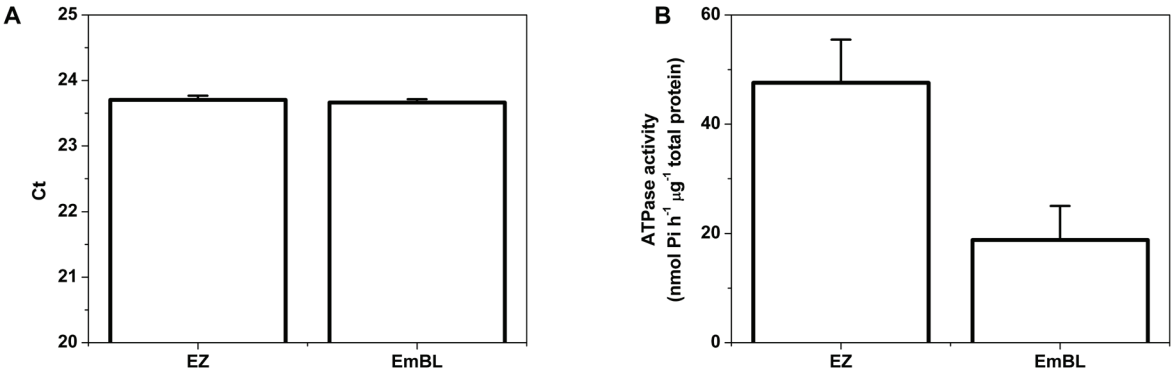

Figure 4. Expression and activity of PM-ATPase in the elongation zone (EZ) and emerged blade portion (EmBL) of the developing leaf three of barley. (A) Expression of PM-ATPase is shown as Ct values obtained through qPCR analyses. (B) PM-ATPase activity per total plasma membrane protein as determined for purified plasma membrane preparations. Results of a typical experiment, involving three technical replicates, are shown. Membrane protein was quantified with a modified Bradford method.

by how much the protein content per unit plasma membrane surface differs between the two leaf regions. If anything, one might expect a higher protein density in growing tissue. Therefore, the results suggest that the activity of PM-ATPase per unit plasma membrane surface area is by at least factor two higher in growing barley leaf tissue. The absence of any difference in expression level of PM-ATPase mRNA between the two leaf regions shows that growing leaf cells do not regulate PM-ATPase at the transcriptional level but through the quantity or efficiency of translated protein.

The higher density of PM-ATPase aids the energisation of continuous solute uptake, in particular uptake of K^+. Continuous solute uptake is required in growing barley leaf cells to maintain cell osmotic pressure and the force driving water uptake in the face of growth dilution (Fricke et al. 1997; Fricke and Flowers 1998). A higher density of proton pump activity aids acidification of the apoplast. As the present data show, apoplast pH in the elongation zone of barley leaves is by about one pH unit more acidic. This corresponds to a 10-fold difference in apoplast H^+ concentration between growing and emerged leaf tissue. It is possible that there exist post-translational modifications which further increase the PM-H^+ATPase pump activity in growing barley leaf cells and that these modifications become non-efficient in isolated plasma membrane fractions. In addition, apoplast acidification in vivo depends on factors which are not related directly to PM-ATPase protein level or activity such as apoplast K^+ concentration (Claussen et al. 1997; Tode and Lüthen 2001; Visnovitz, Touati, Miller and Fricke, unpublished results), temperature (Stoddart and Lloyd 1986) hormones (especially auxin, Rayle and Cleland 1970; Hager et al. 1971; Rayle and Cleland 1992; Claussen et al. 1997; Tode and Lüthen 2001; Hager 2003; Grebe 2005; Kutschera 2006) and light (Stahlberg and Van Volkenburgh 1999).

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