

Leaf apoplastic alkalinisation promotes transcription of the ABA synthesising enzyme Vp14 and stomatal closure in *Zea mays* L

Christoph-Martin Geilfus^{1*}, Xudong Zhang^{2*}, Axel Mithöfer³, Lisa Burgel^{2,§}, Gyöngyi Bárdos², Christian Zörb²

¹Division of Controlled Environment Horticulture, Albrecht Daniel Thaer-Institute of Agricultural and Horticultural Sciences, Humboldt-University of Berlin, Albrecht-Thaer-Weg 1, D-14195 Berlin, Germany. ²Institute of Crop Science, Quality of Plant Products, University Hohenheim, Schloss, Westhof-West, 118, D-70593 Stuttgart, Germany. ³Max Planck Institute for Chemical Ecology, Research Group Plant Defense Physiology, Hans-Knöll-Straße 8, D-07745 Jena, Germany. [§]Present address: Department of Agronomy, Institute of Crop Science, University Hohenheim, D-70593 Stuttgart, Germany.

*Correspondence: Christoph-Martin Geilfus, E-mail geilfusc@hu-berlin.de, Phone: +49 30 2093 46475, Fax: +49 30 2093 46412 and Xudong Zhang, E-mail xudong.zhang@uni-hohenheim.de, Phone: + 49 049 711 45922372, Fax: +49 711 459-23946.

Christoph-Martin Geilfus: geilfusc@hu-berlin.de

Orcid ID: <https://orcid.org/0000-0002-9820-8111>

Xudong Zhang: xudong.zhang@uni-hohenheim.de

Axel Mithöfer: amithoefer@ice.mpg.de

Orcid ID: <https://orcid.org/0000-0001-5229-6913>

Gyöngyi Bárdos: gyongyi.bardos@uni-hohenheim.de

Lisa Burgel: lisa.burgel@uni-hohenheim.de

Christian Zörb: christian.zoerb@uni-hohenheim.de

Orcid ID: <https://orcid.org/0000-0003-0000-5138>

Highlight

In maize, the transient alkalization of the leaf apoplast acts as a switch that increases transcription of the ABA-synthesizing key gene *viviparous 14* and reduces transpiration.

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Abstract

The chloride component of NaCl-salinity causes the leaf apoplast to transiently alkalinize. This transition in pH reduces stomatal aperture. However, whether this apoplastic pH (pH_{apo}) transient initiates stomatal closure by interacting with other chloride-stress-induced responses or whether the pH transient alone initiates stomatal closure is unknown. To clarify the problem, the transient alkalinization of the leaf apoplast was mimicked in intact maize (*Zea mays* L.) by infiltrating near-neutral pH buffers into the leaf apoplast. Effects of the pH_{apo} transient could thus be investigated independently from other chloride-stress-derived effects. Microscopy-based ratiometric live pH_{apo} -imaging was used to monitor pH_{apo} *in planta*. LC-MS/MS and real-time-qRT-PCR leaf analyses showed that the artificially induced pH_{apo} transient led to an increase in the concentrations of the stomata-regulating plant hormone abscisic acid (ABA) and in transcripts of the key ABA-synthesizing gene *ZmVp14* in the leaf. Since stomatal aperture and stomatal conductance decreased according to pH_{apo} , we conclude that the pH_{apo} transient alone initiates stomatal closure. Therefore, the functionality does not depend on interactions with other compounds induced by chloride-stress. Overall, our data indicate that the pH of the leaf apoplast links chloride-salinity with the control of stomatal aperture via effects exerted on the transcription of ABA.

Key words: Chloride, Salinity, Apoplast, Alkalinization, ABA, Guard Cell, NCED, Stomata, Transpiration

Abbreviations: 2-(N-morpholino) Ethanesulfonic Acid, MES; 2-Hydroxy-3-morpholinopropanesulfonic acid, MOPSO; 9-Cis-Epoxy-carotenoid Dioxygenase, NCED; Abscisic Acid, ABA; Apoplastic pH, pH_{apo}; Atmospheric Water Vapor Pressure Deficit, VPD; Calcium, Ca²⁺; Day, d; Dry Weight, DW; Liquid Chromatography Tandem Mass Spectrometry, LC-MS/MS; Oregon Green, OG; Plasma Membrane, PM; Potassium, K⁺; Proton, H⁺; Real-Time Quantitative Reverse Transcription PCR, Real-Time qRT-PCR; Stomatal Conductance, g_s; Transpiration Rate, E.

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Introduction

During soil water scarcity, the pH of the leaf apoplast (pH_{apo}) is relevant for the regulation of the guard cell pore size. This is because leaf pH_{apo} is implicated in the root-to-shoot communication of progressive soil drying (Sobeih et al., 2004; Wilkinson and Davies, 2008). A drought-stress-induced increase in pH_{apo} links root-zone drying with the regulation of the stomatal aperture by acting on the compartmental distribution of the guard-cell-regulating phytohormone abscisic acid (ABA) between the leaf apoplast and symplast (Davies *et al.*, 2002; Davies et al., 2005; Wilkinson and Davies, 2008).

There is, however, an ongoing debate over the site of synthesis of the ABA that accumulates in the leaf in response to soil water scarcity. Some studies conclude that the root is the major site of ABA biosynthesis during soil drying, with root-derived ABA acting as a long-distance chemical signal regulating stomatal conductance (Wilkinson, 1999; Wilkinson et al, 2007; Boyle *et al.*, 2016). New knowledge gained from experiments on reciprocal- grafts of ABA-deficient mutants and wild- type (WT) tomato (*Solanum lycopersicum*) plants has challenged this view. Reciprocal grafting experiments with ABA-synthesising wild-type and ABA-deficient mutants revealed rootstock-independence of stomatal regulation, irrespective of the ABA concentration delivered via the xylem from root-to-shoot (Holbrook et al. 2002; Dodd et al. 2009; Li et al., 2018). These grafting experiments indicate that rootstock ABA synthesis seems to be less important in regulating leaf ABA concentration and stomatal conductance as the soil dries than originally assumed (Holbrook et al. 2002; McAdam et al., 2016a). Instead, a production of ABA in phloem companion cells of the vasculature (Kuromori et al., 2014) might feed the apoplast of water-stressed leaves with ABA; as shown for water-stressed *Arabidopsis thaliana*. In addition, *A. thaliana* guard cell-autonomous ABA synthesis could allow the leaf to maintain hydrated (Bauer et al., 2013). In maize, the 9-cis-epoxycarotenoid dioxygenase (*NCED*) gene family ortholog *viviparous 14* (*Vp14*) encodes the rate-limiting enzyme in the ABA biosynthesis (Seo and Koshiba, 2002; Xiong and Zhu, 2003). The *ZmVp14* gene was characterized from the ABA-deficient maize mutant *viviparous-14* (*Vp14*) (Tan et al., 1997) and is known to be regulated by salinity and osmotic stress Geilfus *et al.* 2018).

For the fraction of the ABA that is not synthesized directly within the GC, but has to overcome the leaf apoplastic passage towards the GCs, the alkalinization of the leaf apoplast or xylem is believed to be important. Such drought stress-induced increases in leaf pH_{apo} and

shoot xylem sap pH, respectively, have been shown for field bean (*Vicia faba* L.; Karuppanapandian *et al.*, 2017), maize (*Zea mays* L.; Bahrin *et al.*, 2002), sunflower (*Helianthus annuus* L.; Gollan *et al.*, 1992; Schurr *et al.*, 1992) Asiatic dayflower (*Commelina communis* L.; Wilkinson and Davies, 1997), grapevine (*Vitis vinifera* L.; Stoll *et al.*, 2000), tomato (*Solanum lycopersicum* L.; Wilkinson *et al.*, 1998), hops (*Humulus lupulus* L.; Korovetska *et al.*, 2014), and many others.

However, this drought-induced alkalization is not universal in all plant species, which has cast doubt on the assumption that it is a ubiquitous long-distance signal that regulates stomatal responses to soil drying (Sharp and Davies, 2009). Of 22 woody perennial species from different plant families, only four (*Buddleja davidii*, *Dicksonia antarctica*, *Penstemon heterophyllus*, and *Rhododendron obtusum*) evolutionarily-unrelated species alkalised their xylem sap as the soil dried, but these species showed the greatest control of water status during water scarcity (Sharp and Davies 2009). Moreover, within eight herbaceous species from various taxonomic groups the ability to alkalize xylem sap in response to soil drying was not universal, and unrelated to phylogeny (Gloser *et al.* 2016). Overall, alkalization was more likely in annual plant species than in woody perennial plants, but there are few insights as to why this is so.

Nevertheless, for those species that alkalize the apoplast in response to soil drying, the following mode of action is proposed for regulating stomatal aperture. Under well-watered conditions, ABA is released into the leaf apoplast. The apoplastically-located ABA is present in its undissociated form (ABA^H) because it is a weak acid and because the apoplast is slightly acidic (Hartung, 2010). By following its chemical gradient, the uncharged ABA^H diffuses from the leaf apoplast through the plasma membrane (PM) into mesophyll cells. As soon as ABA^H enters the slightly alkaline cytosol of the mesophyll, the weak acid dissociates to ABA⁻ by releasing the H⁺. The phytohormone is now charged (ABA⁻) and is trapped inside the mesophyll cells because charged compounds cannot diffuse across the PM (Hartung *et al.*, 1988). It is then stored or metabolized in the mesophyll (Slovik and Hartung, 2012; Wilkinson and Davies, 1997).

However, during progressive soil drying, the pH of the leaf apoplast increases above the pK_a of ABA (= 4.87) (Karuppanapandian *et al.*, 2017). Thus, under conditions of water scarcity, the ABA that is increasingly delivered from the root or stem dissociates in the leaf apoplast into its charged anionic form, becoming trapped extracellularly (Wilkinson and Davies,

1997). Such a pH_{apo} -based accumulation of ABA^- in the leaf apoplast was recently demonstrated under conditions of NaCl-salinity when the chloride-component of NaCl-salinity provoked the leaf apoplast of the field bean to alkalize over a period of 2-3 hours, before it re-acidified once again (Geilfus *et al.*, 2015). After re-acidification of the leaf apoplast back to the initial steady-state pH, the guard-cell-intrinsic ABA concentration was increased and the stomata were closed. The guard-cell-intrinsic ABA concentration was proposed to rise because the sudden re-acidification of the leaf apoplastic compartment established such a high free proton concentration in the apoplast that the bulk of the apoplastically trapped ABA^- was associated with protons. In other words, a fraction of membrane-permeable ABAH was formed in the leaf apoplast in the vicinity of the guard cells; this ABAH followed its gradient and streamed into the guard cells. This is in good agreement with Goodger *et al.* (2005), who report for maize that the release rates of both ABA and chloride out of mesocotyl xylem sap that is flowing from roots into the shoot correlate with stomatal conductivity. We previously demonstrated that the chloride-induced transient leaf apoplastic alkalization was instrumental in inducing stomatal closure during the beginning of NaCl-salinity through pH_{apo} -based effects on the compartmental distribution of ABA (Geilfus *et al.*, 2015). Experiments with different chloride- or sodium-accompanying counter ions, and with agents that mimicked osmotic stress, revealed that the reduction of the stomatal aperture was linked to the chloride component, i.e. to the chloride-induced transient leaf apoplastic alkalization (Geilfus *et al.*, 2015). Nevertheless, stomatal aperture was not reduced when leaf concentrations of chloride increased during the experimental suppression of the formation of the pH_{apo} transient. This was established by clamping the pH_{apo} in the acid range by the infiltration of an acid pH buffer. Hence, the chloride-induced transient alkalization of the leaf apoplast was inhibited, although chloride was taken up excessively into the leaf. These experiments showed that the pH_{apo} transient mechanistically links chloride-stress with a reduction in stomatal aperture (Geilfus *et al.*, 2015).

Nevertheless, these experiments have not revealed whether the formation of the transient apoplastic alkalization alone, i.e. (i) independently from other chloride-stress induced responses and (ii) in the absence of excessive uptake of chloride, is functional in initiating a reduction in stomatal aperture. Only an experimental setup in which this transient leaf apoplastic alkalization is mimicked but without stressing the plants with chloride is capable of clarifying this point. With this aim, the chloride-inducible alkalization of the leaf

apoplast was mimicked by infiltrating near-neutral pH-buffers in this study. We hypothesized that the formation of this artificially induced pH_{apo} transient reduces stomatal aperture, despite the plants not being salinity-stressed, and that ABA synthesis is under control of the pH_{apo} transient. To elaborate these hypotheses, the apoplastic pH dynamics, the transcript abundance of the ABA-synthesizing key enzyme *ZmVp14*, the abundance of ABA, the stomatal pore size, stomatal conductance and the transpiration rate were quantified in the leaves of hydroponically grown maize.

Material and methods

Experimental design

Maize (*Zea mays* L.) was cultivated hydroponically in nutrient solution (years 2019 & 2020). To mimic the formation of the transient alkalization of the leaf apoplast in a way comparable (regarding duration and magnitude) to the chloride-induced alkalization of the leaf apoplast (see Geilfus *et al.*, 2015), the pH buffer 2-(N-morpholino)ethanesulfonic acid (MES) (5 mM, pH 6.5) was infiltrated into the leaf apoplast. Ratiometric real-time pH_{apo} imaging was applied to monitor the pH_{apo} transient *in planta* (see below). The pH buffer was mixed with the pH indicator dye Oregon Green (OG) 488-dextran (25 μM) before infiltration. A volume of 0.4 ml of the pH-buffer/dye mixture was infiltrated using a needleless syringe. A second experimental group was set up to rule out unspecific effects that may have arisen from the buffering agent (i.e. effects that did not arise from the pH_{apo}). For this purpose, 0.4 ml of a mixture of the pH buffer 2-hydroxy-3-morpholinopropanesulfonic acid (MOPSO) (7.5 mM, pH 6.5) and OG dye were infiltrated into the leaf. To guard against effects possibly arising from the infiltration procedure or the infiltration of water, control plants were infiltrated with 0.4 ml of a mixture of water and the OG dye. Osmotic controls were used to test for osmotic effects possibly arising from the pH buffer agents. For this, 0.4 ml of a mixture of the pH buffer MES (7.5 mM, pH 4.7) and OG dye were infiltrated into the leaf. In this fourth experimental group, the pH was set to 4.7 because this proton concentration prevails in the leaf apoplast.

In total, four experimental groups were tested:

- 1) Control: Infiltration of 0.4 ml of an aqueous 25 μM OG solution.

- 2) Osmotic control: Infiltration of 0.4 ml of a mixture of 7.5 mM MES (pH 4.7) and 25 μ M OG.
- 3) MES buffer: Infiltration of 0.4 ml of a mixture of 5 mM MES (pH 6.5) and 25 μ M OG.
- 4) MOPSO buffer: Infiltration of 0.4 ml of a mixture of 7.5 mM MOPSO (pH 6.5) and 25 μ M OG.

To investigate effects of the formation of the artificially induced pH_{apo} transient on the transcription of the ABA-synthesizing maize gene *9-cis-epoxycarotenoid dioxygenase 1* (*ZmVp14*; Tan *et al.*, 2003) and on the abundance of ABA, leaves were sampled at eight key time points to reflect the situation before, during and after the alkalization. Microscopy-based live pH_{apo} imaging allowed the identification of these eight time points. A separate batch of plants was necessary for sampling leaf material at each time point. Sampled leaf material was immediately frozen in liquid nitrogen. Since pH_{apo} imaging was performed *in planta* by using a size-calibrated microscope, the size of the stomatal aperture could be quantified concurrently. Transpiration was measured in parallel on separate batches of plants that were treated identically. To confirm that one-time infiltration of 0.4 ml MES or the MOPSO buffers does not disturb the leaf in the long term, we checked rates of photosynthesis and transpiration at 24, 48 and 72 h after infiltration (Supplementary Table S1). For all measurements, the number of biological replicates varied between four to six, as indicated in the figure legends or table captions. Biological replicates represented individual plants taken from different cultivations.

Plant cultivation

Zea mays (cv. Susann, Nordsaat Saatzucht GmbH, Langenstein, Germany) was cultivated in a hydroponic system using a controlled environment chamber. Seeds were soaked for 1 d before being placed in moistened quartz sand for germination. Four days (d) later, seedlings were transferred into 5-L plastic pots containing 1/4-strength nutrient solution. After 2 d, the nutrient concentration was increased to 1/2-strength and, after 4 d, to full-strength. The solution was changed every 3.5 days to avoid nutrient depletion. The nutrient solution had the following composition: 1.0 mM K_2SO_4 , 2.5 mM $\text{Ca}(\text{NO}_3)_2$, 0.6 mM MgSO_4 , 0.2 mM KH_2PO_4 , 1.0 mM CaCl_2 , 0.01 mM NaCl , 1.0 μ M H_3BO_4 , 2.0 μ M MnSO_4 , 0.3 μ M CuSO_4 , 0.5 μ M ZnSO_4 , 200 μ M Fe-EDTA, 0.005 μ M $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24}$. Plants were cultivated under a 14 h (20°C): 10 h (18°C) light: dark cycle (photoperiod 08:00-22:00 h) with an atmospheric

water vapour pressure deficit (VPD) of 0.58 kPa (75% RH) during the photoperiod. Light intensity was $500 \mu\text{mol s}^{-1} \text{m}^{-2}$ above leaf canopy. Plants grew for 10 d in full-strength nutrient solution before the pH_{apo} transient was induced by infiltration of the pH buffer.

Quantification of leaf apoplastic pH and determination of stomatal aperture

Leaf pH_{apo} and stomatal aperture were quantified using a calibrated Leica microscope (Leica DMI8; Leica Microsystems, Wetzlar, Germany). The entire plant was located in a cage incubator system, which allowed the precise control of the VDP (0.58 kPa; 75% relative humidity at 20°C) and white light illumination via a LED over the entire experiment (light intensity was $400 \mu\text{mol m}^{-2} \text{s}^{-1}$ of PPFD). LED illumination was automatically switched off during fluorescence image acquisition for pH_{apo} quantification. For the *in vivo* quantification of leaf pH_{apo} , a 25 μM solution of the fluorescent pH indicator dye OG 488-dextran (Thermo Fisher Scientific, Darmstadt, Germany) was infiltrated into the leaf apoplast of intact plants by using a needleless syringe (Geilfus and Mühling, 2011), together with a pH buffer where applicable. *OG 488-dextran is a sensor for pH_{apo} because it does not enter the symplast, as shown by confocal imaging.* Fluorescence images for pH_{apo} calculation were collected as a time series with the Leica DMI8 microscope connected to a cooled sCMOS camera (Leica DFC9000 GT; Leica Microsystems). An HXP lamp (HXP Short Arc Lamp; Osram) was used for illumination at the excitation wavelengths of 440/10 and 490/10 nm. The exposure time was 25 ms for both channels. Emission was collected at 535/25 nm by using a band-pass filter in combination with a dichromatic mirror. The fluorescence ratio F_{490}/F_{440} was obtained as measure of pH_{apo} on a pixel-by-pixel basis. For conversion of the fluorescence ratio data into pH_{apo} values, an *in vivo* calibration was conducted as described elsewhere (Geilfus and Mühling, 2014). Stomatal aperture was quantified off-line on captured images by using the calibrated microscope.

RNA extraction, cDNA synthesis and real-time quantitative RT-PCR

Forward (f 5'–3': TTCTCGGAGGAGGAACAGAGGA) and reverse (r 5'–3': CCAACTGTA ACTCTGGTGTGCG) primers for amplifying *Zea mays viviparous 14* (*ZmVp14*) mRNA were taken from Geilfus *et al.*, 2018. For cDNA synthesis, RNA was isolated from 100 mg ground lyophilized leaf material by using phenol-chloroform extraction according to the method of Cox and Goldberg, 1988. The quality of RNA was checked by

OD₂₆₀ and OD₂₈₀. One µg of total RNA was digested with PerfeCTa DNaseI (Quanta Biosciences, Beverly, USA) to eliminate residual genomic DNA and reverse transcribed to single-stranded cDNA with the SuperScript VILO cDNA Synthesis Kit (Invitrogen by Life technologies, Karlsruhe, Germany) according to the manufacturer's instructions. The SYBR-Green-based real-time quantitative RT-PCR (real-time qRT-PCR) technique was performed on a BioRad CFX96 real-time PCR system by using iTaq Universal SYBR Green Supermix (BioRad Laboratories, Inc.). For each reaction, 0.8 µL diluted single-stranded cDNA was used in a total volume of 10 µL (0.5 µM forward and reverse primer, 5 µl iTaq Universal SYBR Green Supermix, topped up with sterilized autoclaved H₂O_{bidest}). Cycle information with regard to temperature, time and cycle number is given in Geilfus *et al.*, 2018. The specificity of the annealing was checked by dissociation kinetics performed at the end of the experiment (65–95°C). The comparative $\Delta\Delta C_t$ (threshold cycles) method for relative quantification was used to analyse the data according to Pfaffl, 2001. C_t values were normalized by comparison with the two endogenous reference genes actin 1 and ubiquitin-conjugating enzyme. Data are shown as the relative fold-changes in transcript expression. Negative controls were carried out without templates. The specificity of the primer/template interactions was demonstrated by sequencing the real-time qRT-PCR products (GATC Biotech, Konstanz, Germany) (Supplementary Table S2). Moreover, agarose gels were run after real-time qRT-PCR to ensure that only a single PCR product was generated and to confirm the predicted PCR product size on the gel (Supplementary Fig. S1).

ABA quantification

Extraction of ABA from the maize leaf was performed by adding 1 ml extraction solution (5 ng [²H₆] (+) –cis, trans ABA internal standard (Olomouc, Czech Republic) in 7:3 methanol-to-water) to 10 mg dried ground material. After being shaken for 30 min, samples were centrifuged at 16,000 x g at 4°C for 5 min. The supernatant was collected and dried under reduced pressure, re-dissolved in methanol (10 µL per 1 mg dry weight), mixed and centrifuged at 16,000 g and 4°C for 10 min. The supernatant was used for the liquid chromatography tandem mass spectrometry (LC-MS/MS)-based analysis of ABA (Almeida Trapp *et al.*, 2014).

Gas exchange measurements

A portable gas-exchange system (LI-COR 6400 XT; LI-COR) was used to measure stomatal conductance ($\text{mmol H}_2\text{O m}^{-2} \text{s}^{-1}$, abbreviated ' g_s ') and to obtain rates for transpiration ($\text{mmol H}_2\text{O m}^{-2} \text{s}^{-1}$, abbreviated 'E') and photosynthesis ($\mu\text{mol CO}_2 \text{m}^{-2} \text{s}^{-1}$). Photon flux density was $500 \mu\text{mol m}^{-2} \text{s}^{-1}$ as provided by the red, green and blue LEDs of the integrated fluorescence chamber head (6400-02B LED light source; LI-COR). The leaf area included in the chamber was recorded for each leaf. VPD was 0.58 kPa (75% relative humidity; 20°C). CO_2 at a flow rate of $300 \mu\text{mol mol}^{-1} \text{CO}_2$ was controlled by a CO_2 injection system.

Results

Infiltrating pH-buffers set to pH 6.5 mimic a transient alkalization of the leaf apoplast

Infiltrating 0.4 ml of a 5 mM MES solution that was set to a pH of 6.5 into the leaf apoplast immediately increased the leaf pH_{apo} from 4.7 to 6.5 (Fig. 1, green circles). After remaining at pH 6.5 for a period of 80 min, the pH_{apo} started to re-acidify back to the initial steady-state pH of 4.7 over a period of 60 min. A similar transient alkalization of the leaf apoplast was seen when 0.4 ml of a 7.5 mM MOPSO solution set to a pH of 6.5 was infiltrated into the leaf apoplast (blue diamonds). Control plants that were infiltrated with 0.4 ml water showed a stable pH_{apo} of 4.7 over the entire experiment (black triangles). The pH_{apo} was also stable at 4.7 when 0.4 ml of a 7.5 mM MES solution set to a pH of 4.7 were infiltrated into the leaf (grey squares). The latter experimental group served as a control to exclude osmotic effects possibly arising from the infiltration of buffering agents (Fig. 1).

Transient alkalization of leaf apoplast increases abundance of *Vp14* mRNA

Infiltration of either MES or MOPSO buffer solutions each set to a pH of 6.5 into the leaf apoplast resulted in a steady increase of the abundance of *Vp14* mRNA, relative to control leaves (Fig. 2). At 210 minutes after buffer infiltrations (this time point is shown at minute 250 on the X-axis), the increases were the highest (52-fold in response to infiltration with MES at pH 6.5 and 68-fold in response to infiltration with MOPSO at pH 6.5). Subsequently, the alkalization-induced increase in *Vp14* mRNA abundance decreased. Infiltration of the MES buffer set to a pH of 4.7 (osmotic control) resulted in a slight increase of *Vp14* mRNA

abundance. In contrast to the alkalinization-induced increase, the osmotically caused increase was detected 120 minutes later reaching a much lower maximum that occurred at 280 minutes after buffer infiltrations (this time point is shown at minute 320 on the X-axis) (Fig. 2).

Leaf pH_{apo} transient causes leaf ABA concentrations to increase

Infiltration of either MES or MOPSO buffer solutions each set to a pH of 6.5 into the leaf apoplast resulted in a steady increase of the leaf ABA concentrations, whereas the ABA concentration in the control leaves that were only infiltrated with water remained stable at 20±2 (mean ± standard error) ng ABA g⁻¹ DW over the entire experiment (Fig. 3A). At 280 minutes after buffer infiltrations (this time point is shown at minute 320 on the X-axis), the increases were at their highest (943±86 ng ABA g⁻¹ DW in response to infiltration with MES at pH 6.5 and 1481±59 in response to infiltration with MOPSO at pH 6.5). Infiltration of MES buffer set to a pH of 4.7 (osmotic control) did not cause a significant increase in ABA concentration (Fig. 3).

Transient leaf apoplastic alkalinization reduces stomatal aperture, stomatal conductance, transpiration and photosynthesis

At 60 minutes after infiltration of the pH buffers (this time point is shown at minute 100 on the X-axis of Fig. 1) or immediately after the re-acidification (this time point is shown at minute 200 on the X-axis of Fig. 1), neither the stomatal aperture (Fig. 3B), nor the stomatal conductance or the transpiration rate (Supplementary Table S3) were influenced by the infiltration of the MES and MOPSO buffer solutions set to a pH of 6.5 (when compared with the control that was infiltrated with water). At 2 hours after the apoplast had re-acidified to a pH of 4.7 in response to the infiltration of the MES buffer at pH 6.5 (this time point is shown at minute 320 on the X-axis of Fig. 1), however, both the stomatal conductance (128±25 mmol H₂O m⁻² s⁻¹) and the transpiration rate (2.3±0.4 mmol H₂O m⁻² s⁻¹) were significantly lower compared to plants from the control group. In controls, g_s was 283±45 and E was 5.8±1.1 mmol H₂O m⁻² s⁻¹, (Supplementary Table S3). At the same time point, the stomatal aperture significantly decreased from 108.2±12.8 to 34.8±8.1 μm² in response to the MES-induced apoplastic alkalinization (Fig. 3B). Thus, it was significantly lower compared to the

control. At 4 hours after re-acidification (this time point is shown at minute 440 on the X-axis of Fig. 1), the transpiration rate ($1.8 \pm 0.3 \text{ mmol H}_2\text{O m}^{-2} \text{ s}^{-1}$), the stomatal conductance ($97 \pm 26 \text{ mmol H}_2\text{O m}^{-2} \text{ s}^{-1}$), and the stomatal aperture ($26.0 \pm 5.3 \text{ }\mu\text{m}^2$) were still significantly lower in plants from the group 'MES $\text{pH}_{\text{apo}} 6.5$ ' when compared to plants from the control (in the control, E was $6.1 \pm 0.9 \text{ mmol H}_2\text{O m}^{-2} \text{ s}^{-1}$, g_s was $294 \pm 48 \text{ mmol H}_2\text{O m}^{-2} \text{ s}^{-1}$, and stomatal aperture was $123.4 \pm 14.2 \text{ }\mu\text{m}^2$). Similar responses were observed when the leaf apoplast was transiently alkalinized by using the MOPSO buffer set to pH 6.5, when compared with the control. The transpiration rate significantly decreased to 1.7 ± 0.1 or $1.9 \pm 0.3 \text{ mmol H}_2\text{O m}^{-2} \text{ s}^{-1}$ at 2 or 4 h after re-acidification. Stomatal conductance significantly decreased to 85.3 ± 14 or $102.26 \pm 26 \text{ mmol H}_2\text{O m}^{-2} \text{ s}^{-1}$ at 2 or 4 h after re-acidification (Supplementary Table S3). Stomatal aperture significantly decreased to 24.3 ± 6.7 or $23.1 \pm 4.4 \text{ }\mu\text{m}^2$ at 2 or 4 h after re-acidification (Fig. 3B). In good agreement with the stomatal closure that was elicited by the apoplastic alkalinisation, the rate of photosynthesis also decreased in response to the alkalinisation at the same time points (Supplementary Table S3). Use of osmotic controls revealed that all these effects were not related to the osmotic properties of the buffer solutions: infiltration of a MES buffer set to a pH of 4.5 did not significantly affect stomatal aperture (Fig. 3B) or stomatal conductance and rate of transpiration (Supplementary Table S3).

The pH_{apo} -induced increase in ABA correlates positively with the decrease in g_s

The Pearson correlation coefficient shows a strong statistical linear relationship between the increasing leaf ABA concentration and the decreasing stomatal conductance only when the apoplast was alkalinized (Pearson $r = -82$ for 'MES $\text{pH}_{\text{apo}} 6.5$ '; Pearson $r = -95$ for 'MOPSO $\text{pH}_{\text{apo}} 6.5$ '; data not shown). There was a much weaker (Pearson $r = -59$) or no (Pearson $r = 0.06$) statistical relationship between both parameters in plants of the osmotic control or the water control, respectively (data not shown). The pH_{apo} effect on both stomatal conductance and ABA concentration becomes evident when fitting a single relationship between data points from all four experimental groups: Pearson r is -97 (Fig. 4) whereby plants from experimental groups with a re-acidified leaf apoplast ('MES $\text{pH}_{\text{apo}} 6.5$ ', 'MOPSO $\text{pH}_{\text{apo}} 6.5$ ') clearly clustered away from groups where pH_{apo} did not change over the experiment ('control' or 'osmotic control').

Discussion

Soil salinization is a big constraint for plants because, among other mechanisms, it reduces availability of water in the soil. Thus, salt and water stress have much in common (Munns, 2002). To maintain turgor and tissue hydration, the plant adjusts its transpiration. With regard to this, the pH of the apoplast is considered to act as a long-distance signal that transmits information about the decreasing soil water availability from root to shoot (Wilkinson, 1999; Wilkinson and Davies, 2008; Geilfus et al., 2015). Upon arrival in the shoot, it regulates stomatal aperture by acting on the compartmental distribution of ABA between the shoot apoplast and symplast (Wilkinson, 1999; Davies *et al.*, 2002). In addition to this Henderson–Hasselbalch-regulated partitioning of ABA (Sharp and Davies, 2009), the present study demonstrates for the first time that the stomatal closure, elicited by the apoplastic alkalisation, is also due to an pH_{apo} -mediated increase in the transcription of the 9-cis-epoxycarotenoid dioxygenase (NCED) gene *viviparous 14 (Vp14)* in the leaf. *ZmVp14* encodes the rate-limiting enzyme in the ABA biosynthesis (Seo and Koshiba, 2002).

First of all, the study aimed at clarifying whether this leaf pH_{apo} transient is only functional with regard to the initiation of stomatal closure when interacting in concert with chloride ions or other chloride-stress-related responses or variables that are induced by chloride-salinity. These could include changes in $[\text{Ca}^{2+}]$, $[\text{H}_2\text{O}_2]$, [ATP] or membrane potential. Following the concept of network signalling (Jordan, et al., 2000; Plieth, 2016), all these variables (signals) would be integrated at downstream junctions to modulate the output (i.e. stomatal closure). Alternatively, the pH_{apo} transient alone might initiate a cascade of events that regulates aperture following the concept of single-file signalling (see Plieth, 2016). The latter proposition suggests that the pH_{apo} transient acts independently from other chloride-stress associated responses that are induced by chloride-salinity.

To clarify this, we mimicked the transient alkalization of the leaf apoplast, as occurs under conditions of chloride-stress (of course, without adding chloride-stress), by infiltrating near-neutral pH buffers (pH 6.5) into the leaf apoplast. By these means, we investigated the effect of the pH_{apo} transient independently from any chloride-stress-derived responses, i.e. unrelated to the excessive accumulation of chloride (or its accompanying cation). If the hypothesis that the pH_{apo} transient alone is able to initiate stomatal closure without interacting with other chloride-stress-related responses is true, then we would expect that guard cell aperture and

stomatal conductance will decrease in response to mimicking the chloride-induced pH_{apo} -transient.

pH_{apo} transient increases transcript abundance of *Vp14* gene in maize leaves

To stimulate transient leaf apoplastic alkalisation in a similar way to that observed for chloride-salinity, a MES-based pH-buffer (pH 6.5) was infiltrated into the maize leaf apoplast (Fig. 1). To link this mimicked pH_{apo} dynamic with the synthesis of ABA, the transcript abundance of the ABA-synthesizing gene *9-cis-epoxycarotenoid dioxygenase 1* (*ZmVp14*) (Tan *et al.*, 2003) was analysed. Relative to the control, the *ZmVp14* transcript abundance increased by up to 52-fold (Fig. 2). To demonstrate that this effect was due to changes in the pH_{apo} and not from the buffering agent MES, a different buffer (MOPSO set to pH 6.5) was infiltrated in a repeat experiment. Relative to the control, *ZmVp14* transcript abundance increased by up to 68-fold (Fig. 2). Infiltration of an osmotic control (i.e. 7.5 mM MES solution that was set to a pH of 4.7) showed that it is the apoplastic pH transient, that is responsible for the fast and steep increase in the transcription of the *Vp14* gene in maize, being unrelated to the osmotic properties of the buffer agents (Fig. 2). Any mechanical effects arising from the infiltration procedure or from flooding the apoplast with water can be excluded because controls were infiltrated with water and the above-mentioned fold changes were expressed relative to these controls. It has long been assumed that xylem-transported ABA, which was synthesized in the roots, is of major relevance for the shoot ABA pool when the plants experiences salt stress at the roots. The presented data on *Vp14* gene transcription demonstrating the leaf is the key site of ABA synthesis, challenging the paradigm that the vast amount of the foliar-located ABA is produced in the roots. Reciprocal grafting experiments with ABA biosynthetic mutant and wild-type plants suggest that there is a signal that carries the information of a declining root water potential through the xylem to the leaves (Holbrook *et al.* 2002; Christmann *et al.* 2007). Upon arrival, leaf hydration, leaf ABA status and stomatal aperture are adjusted (Manzi *et al.*, 2015; McAdam *et al.*, 2016a; McAdam *et al.*, 2016b; McAdam *et al.*, 2016c). The leaf apoplastic alkalization, that travels systemically and acropetally through the shoot (Geilfus *et al.*, 2015), appears to be a candidate for transmitting information about the onset of water scarcity because it induces leaf ABA synthesis (Fig.3, Fig. 4) and elicits stomatal closure (Supplementary Table S3).

Transient apoplastic alkalization modulates ABA and increases it in maize leaves

Next, we investigated whether the artificially induced leaf apoplastic alkalization was associated with a rise in leaf ABA concentration. A shift in leaf apoplastic pH to 6.5 by means of MES and MOPSO infiltration caused the ABA concentrations steadily to increase over the entire experiment relative to the water control and the osmotic control (Fig. 3). A temporal comparison (compare Fig 2 vs. Fig. 3A) reveals that the increase of the *Vp14* mRNA abundance preceded the increase of the ABA concentration. This indicates that the increase in leaf ABA was caused by increased biosynthesis. However, the means by which the pH_{apo} transient is associated within *Vp14* expression await clarification. Is a pH sensor involved? Such a putative sensor would need a pH-responsive (apoplastic-located) domain as part of an integral protein that spans the entirety of the PM. It would sense changes in the pH_{apo} or the transmembrane pH gradient, passing on the information to start signal transduction via intermediate cellular transmitters that finally involve *Vp14* promoter elements. Because the existence of such a sensor is unclear, the elucidation of this problem is extremely difficult. Thus, other explanations have to be taken into consideration. For instance, large changes in the pH_{apo} are known to be able to influence the cytosolic pH (Monshausen *et al.*, 2007). Since cytosolic pH dynamics are accompanied by cellular Ca^{2+} transients (Dindas *et al.*, 2018; Michard *et al.*, 2016; Monshausen *et al.*, 2009; Monshausen *et al.*, 2008) and have been implicated in the specificity of Ca^{2+} -signalling (Behera *et al.*, 2018), large transients in the pH_{apo} might affect ABA transcription via cellular signal transduction. However, such a sequence of events remains speculative unless a clear link between pH_{apo} transients, Ca^{2+} transients and the induction of *Vp14* gene expression is established. Currently, the transcription of the *9-cis-epoxycarotenoid dioxygenase* gene is known to be regulated by light (study on tomato by Thompson *et al.* 2000), salinity and osmotic stress (study on maize by Geilfus *et al.* 2018), water-deficit (study on maize by Tan *et al.* 1997) and drought (study on *Citrus* by Agustí *et al.* 2007).

Apoplastic alkalization reduces stomatal aperture and transpiration rate

Wilkinson and Davies (2008) treated leaves of an ABA-deficient tomato mutant with alkaline-buffered foliar sprays and demonstrated that an increase in the leaf pH_{apo} reduced stomatal conductance only when ABA was simultaneously sprayed onto the leaves of the ABA-deficient tomato. They and others postulated that the apoplastic alkalization required ABA to act on stomatal aperture (Else *et al.*, 2006; Jia and Davies, 2007; Sobeih *et al.*, 2004;

Wilkinson and Davies, 1997, 2008) and suggested a pH_{apo} -based mechanism whereby the rise in pH_{apo} increased the amount of ABA that penetrated into the guard cells by changing the compartmental distribution of ABA between the apoplast, the symplast and various symplastic components.

The presented study adds knowledge about this system as it demonstrates that it is indeed the leaf apoplastic alkalization that induces *Vp14* gene expression (Fig. 2), presenting a further mechanism that causes foliar ABA level to rise (Fig. 3A) and stomatal conductance to decline (Fig. 4). This novel finding provides a compelling explanation for the observed stomatal closure and the decline in photosynthetic rate (Supplementary Table S3), elicited by the apoplastic alkalisation.

Apoplastic alkalization can also be argued to induce stomatal closure directly via pH_{apo} -based effects on guard cell K^+ fluxes. A rising pH in the stomatal cavity decreases the activity of guard-cell-localized inwardly rectified K^+ channels and increases the activity of guard-cell-localized outwardly rectified K^+ channels (Ache *et al.*, 2000; Hedrich *et al.*, 1995). However, two arguments oppose the assumption that stomata close because of the direct pH_{apo} -based stimulation of outwardly rectified guard cell K^+ channels, highlighting the role of a pH_{apo} -based effect of ABA biosynthesis. First, both the stomatal aperture and the transpiration rate remained reduced at 4 hours after re-acidification (Supplementary Table S3). During these 4 hours, the plants were continuously illuminated with white light. As light increases the activity of inwardly rectified K^+ channels (Marten *et al.*, 2007; Roelfsema and Hedrich, 2005), enough time would have been available for a light-induced K^+ influx to mediate the re-opening of the stomata. However, the stomatal aperture remained reduced. Second, the stomatal aperture and transpiration remained reduced, although the apoplast had become acidified once again, over the 4 hours, to a pH_{apo} range that favoured K^+ influx into guard cells via inwardly rectified K^+ channels; such changes could ultimately result in guard cell re-opening, if not blocked by ABA.

Summary

We have shown that an artificially induced transient of the pH of the leaf apoplast initiates stomatal closure and reduces the rate of leaf transpiration (Supplementary Table S3). A pH_{apo} -based *de-novo* synthesis of the guard-cell-regulating hormone ABA in leaves (Fig. 2) and a pH_{apo} -based increase of leaf ABA concentration (Fig. 3) may be the reason for the reduced leaf transpiration rate, viz. the reduction in stomatal conductance (Fig. 4). Our first novel finding is that the pH of the apoplast increases the transcription of *viviparous 14*, a gene that is key for ABA synthesis in maize. The second novelty is that the functionality of the pH_{apo} transient with respect to reducing the rate of transpiration does not depend on interactions with compounds that are induced by chloride-stress. These findings are relevant, since the transient alkalization of the leaf apoplast is a widely spread phenomenon that occurs not only under chloride-salinity, but also in response to drought (Bacon et al., 1998) or leaf infections with *Blumeria graminis* (Felle et al., 2008) or *Piriformospora indica* (Felle et al., 2009). Future studies should compare plant species that show the apoplastic alkalization with plant species that do not, to understand the underlying mechanism(s) of the transient shift in the apoplastic pH.

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Author contributions

C.M.G. conceived the project and designed the experiments. C.M.G., X.Z., A.M., L.B., G.B. carried out the experiments, C.M.G. and X.Z. analysed the data. C.M.G. wrote the manuscript with valuable inputs from A.M., C.Z. and X.Z. All authors read and approved the final manuscript.

Data Availability Statement

All data supporting the findings of this study are available within the paper and within its supplementary materials published online.

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Figure Legends

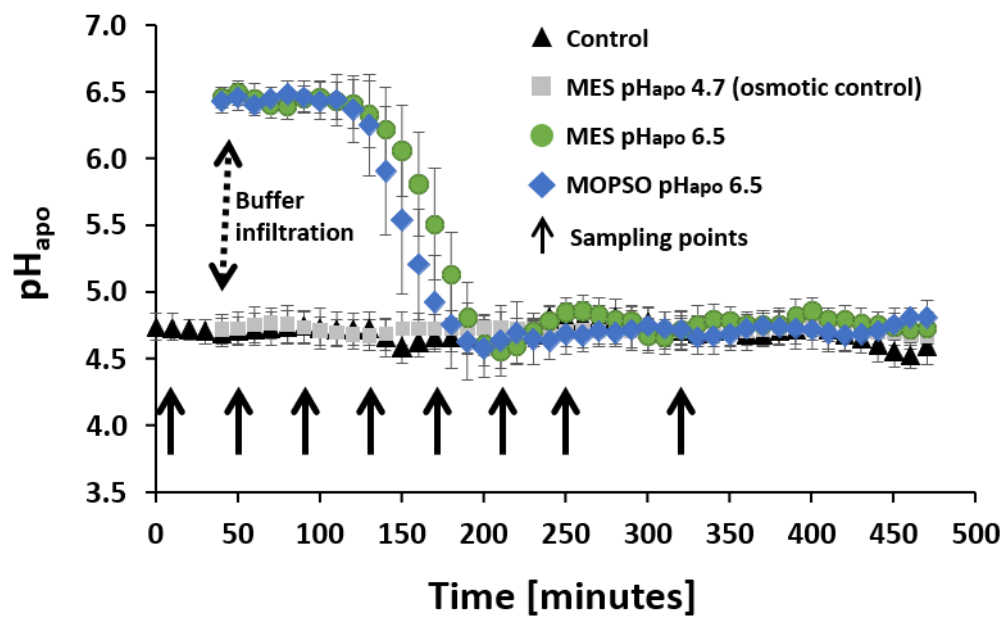
Fig. 1. Infiltration of near-neutral pH-buffers into the leaf apoplast results in a transient apoplastic alkalinization. Apoplastic pH as plotted over time. Green circles, infiltration of 0.4 ml of a mixture of 5 mM MES at pH 6.5 and 25 μ M OG; blue diamonds, infiltration of 0.4 ml of a mixture of 7.5 mM MOPSO at pH 6.5 and 25 μ M OG; black triangles, infiltration of 0.4 ml of an aqueous 25 μ M OG solution (controls); grey squares, infiltration of 0.4 ml of a mixture of 7.5 mM MES at pH 4.7 and 25 μ M OG (osmotic controls). Dashed black arrow, time point of infiltration; solid arrows, time points of the sampling of leaf material for subsequent *vp14* mRNA and ABA quantification. Representative pH kinetics of six equivalent recordings of leaves gained from independent experiments ($n = 6$ biological replicates). Quantitation was technically averaged with $n = 4$ regions of interest per ratio image and time point (shown as \pm SD).

Fig. 2. Transient alkalinization of the leaf apoplast increases mRNA abundance of *viviparous 14* (*Vp14*). Increases in transcript abundance shown as fold-changes, relative to the control group (being set to 1). Green circles, infiltration of 0.4 ml of a mixture of 5 mM MES at pH 6.5 and 25 μ M OG; blue diamonds, infiltration of 0.4 ml of a mixture of 7.5 mM MOPSO at pH 6.5 and 25 μ M OG; black triangles, infiltration of 0.4 ml of an aqueous 25 μ M OG solution (controls); grey squares, infiltration of 0.4 ml of a mixture of 7.5 mM MES at pH 4.7 and 25 μ M OG (osmotic controls). Mean \pm SE of four independent ($n = 4$) biological replications (technically replicated in triplicate).

Fig. 3. Artificially induced pH_{apop} transient increases leaf ABA concentration and reduces stomatal aperture. A, leaf ABA concentration; B, stomatal aperture. Green circles, infiltration of 0.4 ml of a mixture of 5 mM MES at pH 6.5 and 25 μ M OG; blue diamonds, infiltration of 0.4 ml of a mixture of 5 mM MOPSO at pH 6.5 and 25 μ M OG; black triangles, infiltration of 0.4 ml of an aqueous 25 μ M OG solution (controls); grey squares, infiltration of 0.4 ml of a mixture of 7.5 mM MES at pH 4.7 and 25 μ M OG (osmotic controls). For ABA data: mean \pm SE of four independent ($n = 4$) biological replications (technically replicated in triplicate). Statistical significance ($P \leq 0.05$) between groups per time point as indicated by Tukey-HSD test is indicated by letters. For stomatal aperture data: mean \pm SD of six independent ($n = 6$) biological replications. Statistical significance ($P \leq 0.05$) between groups per time point as indicated by Tukey-HSD test is indicated by letters.

Fig. 4. Correlation analysis of stomatal conductance and leaf ABA concentration . Circles, infiltration of 0.4 ml of a mixture of 5 mM MES at pH 6.5 and 25 μ M OG; diamonds, infiltration of 0.4 ml of a mixture of 5 mM MOPSO at pH 6.5 and 25 μ M OG; triangles, infiltration of 0.4 ml of an aqueous 25 μ M OG solution (controls); squares, infiltration of 0.4 ml of a mixture of 7.5 mM MES at pH 4.7 and 25 μ M OG (osmotic controls). Filled symbols, data were collected and pooled from the first 100 minutes of the experiment (please see X-axis in figure 1 to relate this phase to the leaf apoplastic pH); open symbols, data were collected and pooled from minutes 101-220; dashed symbols, data were collected and pooled from minute 221. Results of correlation analysis were indicated by the Pearson correlation coefficient r .

Figure 1



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Figure 2

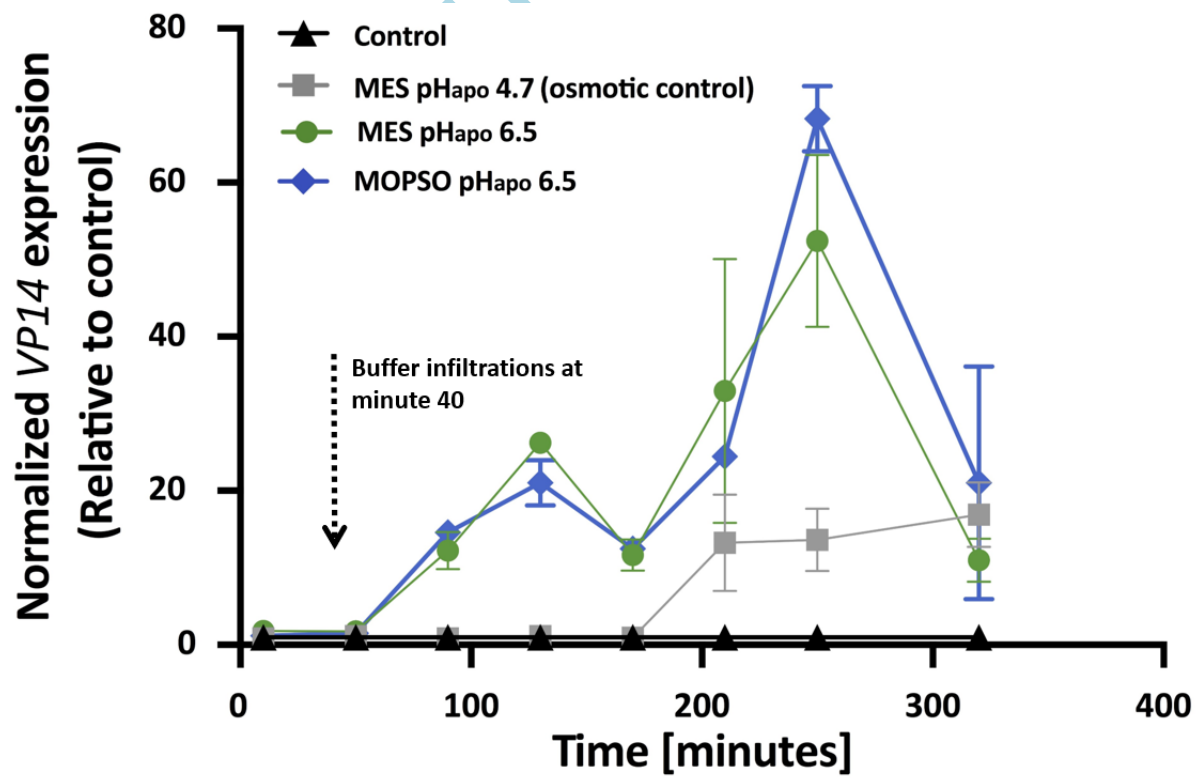
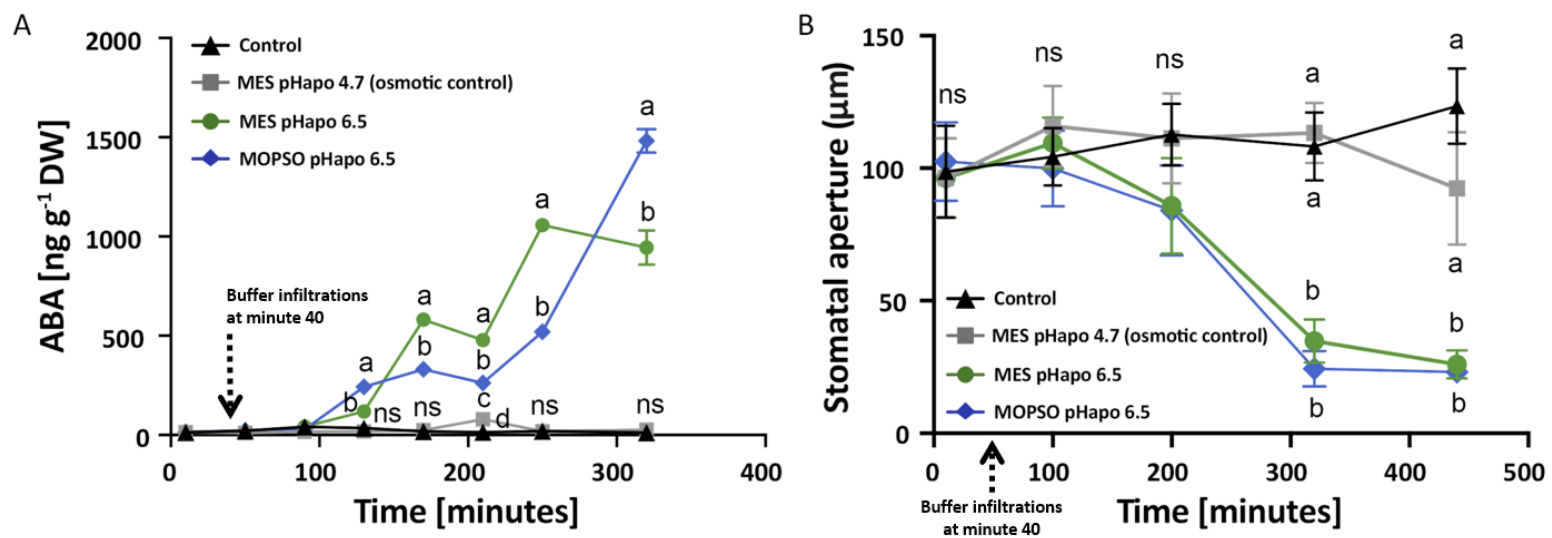


Figure 3



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Figure 4

