Role of TaALMT1 malate-GABA transporter in alkaline pH tolerance of wheat

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Abstract

Malate exudation through wheat (*Triticum aestivum* L) Aluminium-activated Malate Transporter 1 (TaALMT1) confers Al^{3+} tolerance at low pH, but is also activated by alkaline pH, and is regulated by and facilitates significant transport of gamma-aminobutyric acid (GABA, a zwitterionic buffer). Therefore, TaALMT1 may facilitate acidification of an alkaline rhizosphere by promoting exudation of both malate and GABA. Here, the performance of wheat near isogenic lines (NILs) ET8 (Al^{+3}-tolerant, high *TaALMT1* expression) and ES8 (Al^{+3}-sensitive, low *TaALMT1* expression) are compared. Root growth (at 5-weeks) was higher for ET8 than ES8 at pH 9. ET8 roots exuded more malate and GABA at high pH and acidified the rhizosphere more rapidly. GABA and malate exudation was enhanced at high pH by the addition of aluminate in both ET8 and transgenic barley expressing *TaALMT1*. *Xenopus laevis* oocytes expressing *TaALMT1* acidified an alkaline media more rapidly than controls corresponding to higher GABA efflux. *TaALMT1* expression did not change under alkaline conditions but key genes involved in GABA turnover changed in accord with a high rate of GABA synthesis. We propose that TaALMT1 plays a role in alkaline tolerance by exuding malate and GABA, possibly coupled to proton efflux.

Key words: Alkaline tolerance, TaALMT1, proton efflux, GABA, rhizosphere acidification, malate

1 Introduction

Soil alkalinity is a major environmental factor limiting agricultural production throughout the world by affecting crop growth through poor soil structure, ion toxicities and nutrient (e.g. iron, phosphorus) deficiencies (Wong, Dalal, & Greene, 2008). About 434 million ha of cultivable land in the world is affected by soil alkalinity (Jin et al., 2006; Xu et al., 2013). Soil alkalinisation is a constraint for crop production in Australia with about 30% of Australian soils classified as sodic and 86% of these classified as alkaline (Rengasamy & Olsson, 1991). Improving alkaline tolerance of plants through molecular breeding is an attractive option to maintain crop productivity on alkaline soils and to achieve this goal alkaline tolerance mechanisms and the genes underlying these mechanisms need to be identified. In this respect, previous research has focused on the ability of plants to acidify the rhizosphere through enhanced activity of plasma membrane localised H^+-ATPases (Fuglsang et al., 2007; Li, Xu, Liu, Zhang, & Lu, 2015; Xu et al., 2012; Xu et al., 2013; Yang et al., 2010), auxin regulation (Yu et al., 2017), and the ability to reduce excess ROS under alkali stress in rice (Guo et al., 2014; Zhang et al., 2017) and wheat (Meng et al., 2017). Here, we

explore another potential tolerance mechanism – the exudation of organic compounds from roots to lower rhizospheric pH.

Plant roots exude organic compounds to keep the rhizosphere environment favourable for plant growth and development. In acid soils (pH < 4.5), Al^{3+} toxicity reduces root growth, and to alleviate Al^{3+} toxicity several plant species exude organic anions (OA, e.g. malate and citrate) (Delhaize & Ryan, 1995; de la Fuente, Ramirez-Rodriguez, Cabrera-Ponce, & Herrera-Estrella, 1997; Ryan et al., 2011). These OA chelate Al^{3+} in the rhizosphere (and/or the apoplast), resulting in the formation of non-toxic Al complexes that prevent phytotoxic Al^{3+} entering the root (Kochian, Hoekenga, & Piñeros, 2004; Ma, Ryan, & Delhaize, 2001; Ryan, Delhaize, & Jones, 2001). This mechanism, first demonstrated in the root tips of Al^{3+}-tolerant varieties of wheat (Delhaize & Ryan, 1995; Ryan, Delhaize, & Randall, 1995; Ryan, Skerrett, Findlay, Delhaize, & Tyerman, 1997), became the basis for the identification and characterization of the first member of the Aluminium-activated Malate Transporter (ALMT) family, TaALMT1 (Sasaki et al., 2004). Transgenic barley overexpressing *TaALMT1* enhanced Al^{3+} tolerance in acid soils by exuding malate (Delhaize et al., 2004). The expression of *TaALMT1* in barley was also shown to improve phosphate nutrition on acid soils and not to impose a yield penalty or difference in shoot biomass in the absence of Al^{3+} (Delhaize et al., 2009). Interestingly, bread wheat genotypes with the *ALMT1* allele have been shown to have a slight but significant yield advantage in alkaline soils which may also be associated with boron tolerance (McDonald, Taylor, Verbyla, & Kuchel, 2013). Also the presence of the Al^{3+} tolerance allele *ALMT1* in wheat was shown to be advantageous in alkaline soils with low rainfall across southern Australia, and suggested to be linked to

tolerance to the aluminate anion or through the efflux of malate to aid the uptake of other nutrients at high pH (Eagles et al., 2014). Calcicoles (plants well adapted to grow in alkaline soils) also release several-fold higher organic acids in alkaline soils than in acid soils (Lee, 1999; Lopez-Bucio, Nieto-Jacobo, Ramirez-Rodriguez, & Herrera-Estrella, 2000).

Two near isogenic lines (NILs) of wheat that have different expression and alleles for *TaALMT1* (ET8 Al^{3+} tolerant and ES8 Al^{3+} sensitive) have been compared for tolerance to alkaline soils and alkaline hydroponic solutions (Silva, Zhang, Habermann, Delhaize, & Ryan, 2018). The ET8 NIL has the Type V allele of *TaALMT1* (TaALMT1-V) associated with high expression of *TaALMT1* in root apices and greater malate and gamma-aminobutyric acid (GABA) efflux, while ES8 NIL possesses the Type I allele of TaALMT1 (TaALMT1-I) associated with low expression of *TaALMT1* and low malate and GABA efflux (Raman et al., 2005; Ramesh et al., 2018; Ryan et al., 1995; Ryan et al., 2010; Sasaki, 2004; Silva et al., 2018). These two NILs have been extensively compared in respect of malate efflux and the channels accounting for this efflux (Ryan et al., 1997; Zhang, Ryan, & Tyerman, 2001) as well as the newly discovered role of TaALMT1 as a GABA transporter (Ramesh et al., 2018). Silva et al. (2018) found no consistent tolerance for alkaline conditions of ET8 over ES8. However, further investigation is warranted in the light of the recent discovery that TaALMT1 and other ALMTs are efficient GABA transporters and that external anion activation at pH 7.5 results in large GABA efflux via TaALMT1 (Ramesh et al., 2018).

Alkaline pH in the absence of Al^{3+} stimulated malate efflux through TaALMT1 expressed in tobacco BY2 cells, reaching a maximum above pH 8 (Ramesh et al., 2015). A

similar response was observed in *Xenopus laevis* oocytes for the ionic current attributed to malate efflux (*ibid*). Malate efflux through TaAMLT1 and other ALMTs was also found to be inhibited by micromolar concentrations of GABA, and its analogue muscimol, although in intact roots higher concentrations of GABA in the millimolar range were required for inhibition (Ramesh et al., 2015). Recently it was found that the single channel currents corresponding to malate efflux through TaALMT1 are inhibited by GABA from the cytosolic face of the channel (Long, Tyerman & Gillham, 2020). Comparing the ET8 and ES8 NILs it was found that ET8 seedlings exuded more malate at pH 9 and that root growth of 3-4 day old seedlings was higher in ET8 than ES8 at pH 9 (Ramesh et al., 2015). Furthermore, both root growth and malate exudation were inhibited by muscimol to a greater degree in ET8 at high pH compared to that of ES8, implicating TaALMT1 in the response.

To summarise, the literature cited above leads to the following hypotheses regarding the possible role of TaALMT1 in alkaline pH tolerance: 1) higher expression of *TaALMT1* imparts an advantage for plant growth under alkaline conditions; 2) TaALMT1 enables acidification of the rhizosphere at alkaline pH; and, 3) aluminate, which becomes accessible at high pH and is toxic to roots (Kopittke, Menzies, & Blamey, 2004), enhances the activation of TaALMT1 at high pH. To test these hypotheses experiments were performed using ET8 and ES8 wheat NILs, transgenic barley expressing TaALMT1 (Ramesh et al. 2018), and *Xenopus laevis* oocytes expressing *TaALMT1*.

2. Materials and Methods

2.1 Chemicals

All chemicals were purchased from Sigma Aldrich.

2.2 Plant material and growth

NILs of wheat (*Triticum aestivum* L) ET8 and ES8 (Sasaki et al., 2004; Delhaize et al., 2004) were surface sterilised in 1% bleach for 1 min, rinsed three times in water and germinated on moist filter paper in the dark at room temperature. For growth experiments three-day-old seedlings were transferred into pots filled with washed sand. The pots were placed on a bench in a growth room (randomly arranged), with average photosynthetic active radiation (PAR) at plant height of 300-350 µmol m^{-2} s^{-1} on a 12-hour photoperiod and with day/night temperature of 21℃/16℃. The plants were supplied with a basal nutrient solution (BNS): 2 mM NH_4NO_3, 3 mM KNO_3, 0.1 mM $CaCl_2$, 2 mM KCL, 2 mM $Ca(NO_3)_2$. $4H_2O$, 2 mM $MgSO_4.7H_2O$, 0.6 mM KH_2PO_4, 50 µM NaFe(III)-EDTA, 50 µM H_3BO_3, 5 µM $MnCl_2.4H_2O$, 10 µM $ZnSO_4.7H_20$, 0.5 µM $CuSO_4.5H_2O$ and 0.1 µM Na_2MoO_4 and pH adjusted to 6 with NaOH. For pH 9 treatment, the same nutrient solution was supplied with 1 mM $NaHCO_3$ and 80 µM Na_2CO_3 (Ma, Rengasamy, & Rathjen, 2003) and pH adjusted to 9 using NaOH. A subset of plants were also treated with the addition of 10 mM GABA to the BNS solution and the pH checked and adjusted to that of the treatment pH with NaOH. The treatment solutions were applied 3-4 times/week allowing pots to drain as their only water

supply and the plants were treated for 5 weeks. At 5 weeks shoots and roots of the wheat ET8 and ES8 plants were harvested. Fresh mass was measured and then roots were scanned with an Epson digital scanner (Expression 10000XL, Epson Inc.). Scans were analysed with the WinRHIZO software (Regent Instruments Inc.).

2.3 Relative chlorophyll content, and leaf gas exchange

Relative chlorophyll content (SPAD values) were measured using a portable chlorophyll content meter (CCM-200 plus, OPTI-SCIENCES). Transpiration (*E*), stomatal conductance (g_s) and assimilation rate (*A*) were measured using a portable infrared data analysis system (LC-pro SD, ADC Bioscientific, Hoddesdon, England). PAR was set at 1000 μmol m^{-2} s^{-1} using the internal chamber LED light system. The CO_2 concentration of the measuring chamber was kept at ambient (between 370–400 μmol mol^{-1}). Measurements once stable for each replicate were made between 4-7 hours post dawn on two fully expanded youngest leaves of each plant after 5 weeks growth under each of the treatments described above. A section of each leaf was placed in the broad leaf chamber of the instrument while still attached to the plant and leaf area adjusted for internal calculations based on measured leaf width.

2.4 Estimates of rhizosphere malate and GABA

A root wash was applied for plants *in situ* after five weeks of growth in the washed sand by adding 120 mL of the appropriate treatment solution over the surface of the sand and collecting the eluate from the base of the pot over 1 hour. The malate and GABA contents

were normalised on a root fresh mass basis. Malate and GABA concentrations were measured as described below.

2.5 Net fluxes of malate and GABA from excised root apices and intact seedlings

Root apices (5 mm) were excised from three-day-old wheat NIL seedlings and washed three times in 3 mM $CaCl_2$, pH 6 adjusted with NaOH. In one experiment five apices were incubated in 0.2 mL per replicate for one hour in treatment solutions at pH 6 and 9 with and without buffer (all with 3 mM $CaCl_2$, 10 mM Na_2SO_4): *pH 6 solution*, 0.01 mM NaOH; *pH 6 + buffer*: 5 mM MES, 2.6 mM NaOH; *pH 9 solution*, 0.05 mM NaOH; *pH 9 + buffer*, 5 mM BTP, 2 mM MES. In another experiment root apices were treated as above for 1 h in the following solutions (all 3 mM $CaCl_2$): *pH 4.5*, 5 mM MES; *pH 4.5+Al*, 5 mM MES, 0.1 mM $AlCl_3$; *pH 9 NaOH*, 0.12 mM NaOH; *pH 9 KOH*, 0.2 mM, KOH; *pH 9 BTP Na_2SO_4*, 20 mM BTP, 10 mM Na_2SO_4, 9 mM MES; *pH 9 Carbonate*, 18 mM $NaHCO_3$, 2 mM Na_2CO_3; *pH 9 Carbonate* Na_2SO_4, 18 mM $NaHCO_3$, 2 mM Na_2CO_3, 10 mM Na_2SO_4. The same solutions were used for intact seedling root experiments where intact roots of one 3-day old seedling per replicate were placed in to 1.8 mL and treated for 21-22 h (Ramesh et al., 2018). A further experiment consisted of exposing three-day-old wheat NIL ET8 and ES8 seedlings and transgenic barley expressing *TaALMT1* (Golden Promise background) (Ramesh et al, 2018) to a basal bathing solution composed of 3 mM $CaCl_2$ pH 9 (NaOH) plus and minus 100 µM aluminate. Fluxes were measured from individual seedlings (per replicate) over a 22 h incubation period

2.6 Measurement of root medium malate and GABA concentrations and external solution pH for seedlings suddenly exposed to pH 9

Wheat NILs ET8 and ES8 were surface sterilized and germinated as described above. Three-day-old seedlings were placed in a microcentrifuge tube with roots immersed initially in a solution consisting of 3 mM $CaCl_2$ and 5 mM MES, pH 5 for 24 h after which 7-8 seedlings/replicate were selected. These were transferred so that their roots were bathed in 200 mL (per replicate) of aerated BNS solution at pH 9 (composition as described above). pH in the root bathing solution was monitored over time using a pH electrode (pH-HI2211, HANNA instruments). For malate and GABA concentration, samples of the solution were collected after the seedlings were transferred to the pH 9 solution and measured as described below. Root fresh mass per replicate was measured at the end of the experiment after 22-24 hours.

2.7 Measurement of malate and GABA concentrations

For malate, a sample of solution (usually 0.1 ml) was added to a master mix containing the various components of the KLMAL- assay (Megazyme) kit as per the manufacturer's instructions and the change in NADH was measured using an OMEGA plate-reading spectrophotometer (BMG) from the change in absorbance at 340 nm (details in Ramesh et al. 2018). GABA concentrations were measured in treatment solutions following the GABase enzyme assay (Sigma-Aldrich) (Zhang & Bown, 1997) and the production of NADPH measured as above. This was previously validated for similar experiments by comparison with UPLC assays in Ramesh et al. (2018).

2.8 Gene expression in root apices

Root apices (5 mm) were excised from 5-week old seedlings after gently removing the washed sand from around the seedling roots. Apices were frozen in liquid nitrogen and stored at -80 °C until further analysis by quantitative reverse transcription PCR (RT-qPCR). Root apices were ground to a fine powder in liquid nitrogen using a mortar and pestle and total RNA was extracted using the Spectrum Plant Total RNA extraction Kit (Sigma-Aldrich, St. Louis, MO, USA). DNA contamination was avoided by treatment with the On-Column DNase I Digestion Set (Sigma-Aldrich, St. Louis, MO, USA) during RNA extraction according to manufacturer recommendations. Concentration and purity of total RNA were determined on a NanoDrop™ 1000 Spectrophotometer (Thermo Fisher Scientific Inc., MA, USA). Agarose gels electrophoresis (1.2 % agarose) was carried out to visualize the integrity of RNA. For cDNA synthesis, 1 µg of total RNA was reverse transcribed using iScript™ cDNA Synthesis Kit (Bio-Rad, CA, USA) according to manufacturer instructions. Gene expression analysis was carried out by quantitative reverse transcription PCR (RT-PCR) (QuantStudio™ 12K Flex; ThermoFisher Scientific Inc., MA, USA). Gene-specific primers (*TaALMT1*, *GAD*, *GABA-T*, *tubulin* and *cyclophilin*, Supplemental Table S1) were used to amplify products from control cDNA and used for generation of standard curves. The amplified products were confirmed by sequencing. Briefly 1:10 serial dilutions of purified PCR products were made and used in RT-qPCR. The reaction volume was 10 µL and contained 1 µL of either the serial dilution or undiluted cDNA, 5 µL KAPA SYBR® FAST Master Mix (2X) Universal (Kapa Biosystems Inc., MA, USA), 100 nM of gene-specific

primers, and 0.2 µL of ROX Reference Dye Low (50X). The thermal cycling conditions were: one cycle of 3 min at 95 °C followed by 40 cycles of 16 s at 95 °C and 20 s at 60 °C. Subsequently, melting curves were recorded from 60 to 95 °C at a ramp rate of 0.05°C/s. Normalized relative quantities (NRQ) of gene expression were calculated between the gene of interest and three reference genes taking differences in PCR efficiency into account (Hellemans, Mortier, De Paepe, Speleman, & Vandesompele, 2007; Pfaffl, 2001).

2.9 Ion-selective microelectrode measurements of pH and proton flux

External pH and net H^+ flux from root apices of intact 3-4 day old seedlings of wheat (ET8 and ES8) and transgenic barley expressing TaALMT1 (Golden Promise background), or *Xenopus laevis* oocytes, were measured using ion-selective microelectrodes (Newman, 2001). Microelectrode fabrication, conditioning, and calibration were followed as detailed by Bose, Xie, Shen, and Shabala (2013). The ion sensitive microelectrode was placed near the surface of the root apex or oocyte using a 3D-micromanipulator (MMT-5, Narishige, Tokyo, Japan). A computer-controlled stepper motor moved the electrode between two positions from the surface in 6 s cycles. The CHART software (Newman, 2001) recorded the potential difference between the two positions and converted these into electrochemical potential differences using the calibrated Nernst slope of the electrode. Net ion fluxes were calculated using the MIFEFLUX software for either spherical geometry (*X. laevis* oocytes) or cylindrical geometry (for roots) (Newman, 2001). For rhizosphere pH and net H^+ fluxes 3-4 day-old intact seedling roots were gently immobilized in the measuring chambers as described elsewhere (Bose et al., 2013) and pre-conditioned in 0.2 mM KCl + 0.2 mM $CaCl_2$

+ 3 mM MES + 4 mM TRIS, pH 7.87 for about 30 minutes. After pre-conditioning, steady-state rhizosphere pH and proton fluxes were recorded at the root apex (≈ 1.5 mm from root cap, 50 μm from the surface) over a period of 5 minutes. Then, 10 mM Na_2SO_4 was added and changes in rhizosphere pH and proton fluxes were measured for up to 60 minutes. External pH and proton flux from *Xenopus laevis* oocytes expressing *TaALMT1* or water injected controls were also measured using the MIFE technique. An oocyte (one per replicate) was transferred from calcium Ringers solution (see below) to the test solution containing 0.7 mM $CaCl_2$, 10 mM Na_2SO_4, pH adjusted to 9 using NaOH and the osmolality adjusted to 231 mosmol kg^{-1} using D-mannitol. The electrode was positioned at different distances from the oocyte surface in order to monitor the changes in bulk solution pH and the pH and flux at 100 μm from the surface.

2.10 Heterologous expression of TaALMT1 in Xenopus leavis oocytes

Capped RNA (cRNA) of *TaALMT1* previously cloned from wheat ET8 (Ramesh et al. 2015) was synthesized *in vitro* with mMessage mMachine T7 kit (Ambion, Carlsbad, CA, USA) using linearised plasmid as template. Stage V and VI defolliculated oocytes surgically extracted from *Xenopus laevis* frogs were injected with 46 nl cRNA (32 ng cRNA), or sterile water. Injected oocytes were incubated in calcium Ringers solution (96 mM NaCl, 2 mM KCl, 5 mM $MgCl_2$, 0.6 mM $CaCl_2$, 5 mM HEPES, 5% (v/v) horse serum, 50 μg ml^{-1} tetracycline and 1 x penicillin-streptomycin (Sigma P4333). The water and TaALMT1 cRNA injected oocytes were incubated for 48 h in Ringer's solution prior to experimentation. Randomly selected oocytes were alternated between control and gene injected to limit any

bias caused by time-dependent changes after gene injection. The University of Adelaide Animal Ethics Committee approved the *Xenopus laevis* oocyte experiments; project number S-2014-192.

2.11 Endogenous GABA concentrations and GABA efflux from *Xenopus laevis* oocytes

Oocyte GABA concentrations were measured as described in Ramesh et al (2018). For GABA efflux oocytes injected with TaALMT1 cRNA or water were imaged in groups of 4-5 to obtain cell volumes using a stereo zoom microscope (SMZ800) with a Nikon (cDSS230) camera at 48 h post-injection. The batches of oocytes were incubated in treatment solutions (pH 7: 0.7 mM $CaCl_2$, 10 mM Na_2SO_4, mannitol to 220 mOsm kg^{-1}. pH 9: 0.7 mM $CaCl_2$, 10mM Na_2SO_4, mannitol to 220 mOsm kg^{-1}, pH to 9 with NaOH) for 10 min. After 10 min, the solution was removed for the GABA assay and oocytes snap frozen in liquid nitrogen and stored at -80 °C. The frozen oocytes were ground in liquid nitrogen, added to methanol and incubated at 25 °C for 10 min. The samples were vacuum dried, resuspended in 70 mM $LaCl_3$, pelleted at 500 x g in a desktop microcentrifuge and precipitated with 1M KOH. These samples were re-centrifuged at 500 x g and 45.2 µl of supernatant was assayed for GABA concentrations using the GABase enzyme from Sigma as described above.

2.12 Statistical analysis

All graphs and data analysis were performed in GraphPad Prism 7 (version 7.02 & 8.4.0). Star symbols indicate significance ($P<0.05$) between values as determined by one-way

or two-way analysis of variance. Post tests were performed taking account of total variation in the data.

2.13 Accession numbers

Sequence data from this article can be found in the EMBL/GenBank data libraries under accession number(s) *TaALMT1* (DQ072260); *Tubb4* (U76895.1); *Cyclophilin* (EU035525.1); *GAPDH* (EF592180.1); GABA transaminase *TaGABA-T* (AK333709.1); glutamate decarboxylase *TaGAD* (AK331230.1); *TaGAD1* (AK331897.1).

3. Results

3.1 Comparisons of growth and shoot gas exchange between ET8 and ES8 wheat NILs at acid and alkaline pH and the interaction with applied GABA

To examine the effect of alkaline pH on growth characteristics and shoot gas exchange of wheat NILs (ET8 and ES8), plants were grown in washed sand with nutrient solution pH set at 6 and 9 over 5 weeks. A subset of these plants were also treated with 10 mM GABA at each pH. GABA (10 mM) was used since Ramesh et al (2015) had shown that this concentration could inhibit root growth and malate efflux in ET8 to phenocopy ES8 in the presence of Al^{3+} at pH 4.5. After 5 weeks growth significant differences were observed in shoot and root growth characteristics between the genotypes at each pH (Figure 1a,b). Comparing shoot fresh mass of genotypes at the same pH showed a significantly higher mass for ES8 at pH 6 and pH 9 (Figure 1a) while there was a significantly higher root mass for ET8 at pH 9 (Figure 1b). For both genotypes, GABA treatment significantly decreased shoot and root biomass to between 30-60% of controls at the same pH, but with no differences

between ET8 and ES8 at each pH (Supplemental Figure S1a,b). There was a tendency for ET8 to show greater differences between pHs with GABA treatment (Supplemental Figure S1b) relative to that without GABA treatment (Figure 1b). Analysis of root morphology showed a significantly higher root length and root surface area for ET8 at pH 9.0 compared to ES8 (Figure 1c,d). GABA treatment reduced both root length and root surface area for both genotypes and for each pH. There were no significant differences between genotypes when treated with GABA (Supplemental Figure S1c,d). Figure 1e shows root images illustrating the typical differences in root morphology between genotypes at each pH.

Transpiration rate (*E)* was significantly higher in ET8 than ES8 at pH 9 (Figure 2a) but there were no significant differences between genotypes and pH for g_s (Figure 2b). Relative chlorophyll concentration was significantly reduced in ES8 leaves at pH 9 compared to ET8 (Figure 2c). Net assimilation (*A*) was significantly reduced in ES8 compared to ET8 at pH 9 (Figure 2d). GABA treatment significantly reduced leaf chlorophyll concentration for both genotypes to between 40 and 60% of controls independent of pH (Supplemental Figure S2c), while *A* was unaffected in both genotypes (Supplemental Figure S2d). Both *E* and g_s showed interesting responses to GABA treatment; with stimulation by up to 150% at pH 6 and pH 9 (Supplemental Figure S2a,b), but with no differences between genotypes.

3.2 Estimates of rhizosphere malate and GABA concentrations for ET8 and ES8 wheat NILs

Pots containing the plants used in the experiments described above were flushed with a constant volume of treatment solution and allowed to drain to capture a root-wash eluate. GABA and malate concentrations of the eluate were measured, and an estimate was made of

the rhizosphere malate and GABA concentrations on a root fresh mass basis (Figure 3). Rhizosphere malate concentration was significantly higher from ET8 compared to ES8 at pH 9 (Figure 3a). GABA treatment tended to enhance malate concentration but with no significant difference between genotypes and pH (Figure 3b).

Rhizosphere GABA estimations were also made for the non-GABA treated plants (Figure 3c). Rhizosphere GABA was higher from ET8 roots than for ES8 roots at pH 9 but not significantly different at pH 6.

3.3 Efflux of malate and GABA from root apices and intact seedling roots at high pH and enhancement by aluminate

To elaborate on the results shown in Figure 3, the efflux of malate and GABA was measured from excised root apices from 3-day old seedling roots of ET8 and ES8, and transgenic barley (Golden Promise background) overexpressing *TaALMT1* (TaALMT1-OE) (Ramesh et al. 2018) exposed to solutions at pH 6 and pH 9 (Figure 4). This was performed over the shortest time possible (1h). Malate efflux was significantly higher for ET8 root tips at pH 9 in both unbuffered and buffered media and there was a significant elevation in malate efflux comparing pH 6 and pH 9 for ET8 (Figure 4a). There was no significant effect of pH on malate efflux from ES8 apices. There was also a large increase in GABA efflux from ET8 apices at pH 9 compared to pH 6 (with or without buffer) that was significantly higher in ET8 compared to ES8 (Figure 4c). There was no significant effect of pH on GABA efflux from ES8 apices. Similar to ET8, the barley line over expressing TaALMT1 showed higher malate (Figure 4b) and GABA (Figure 4d) efflux at pH 9 than null.

A comparison was made for ET8 and ES8 between short term malate efflux (1 h) from root apices (Figure 5a) and longer term (21 h) efflux (Figure 5b) from intact 3-day old seedlings. In this experiment the response to Al^{3+} at low pH was added as a positive control, since this has been previously shown for apices and intact seedlings of ET8, ES8 (Ramesh et al., 2018; Ryan et al., 1995; Silva et al., 2018). For root apices the results confirmed those in Figure 4a, but also showed that the increased malate efflux from ET8 apices at high pH over the short term was similar to that triggered by Al^{3+} at low pH, and at high pH, the response of ET8 apices was independent of buffer or the presence of Na_2SO_4 (Figure 5a). The presence of Na_2SO_4 was previously shown to enhance malate efflux at high pH (Ramesh et al. 2015). There was no significant difference between treatments for ES8. For longer term (21h) efflux of malate from intact seedling roots (Figure 5b) the situation changed dramatically, in that the presence of buffer (BTP or carbonate/bicarbonate) and Na_2SO_4 made a large difference to the response. Only with buffer present was there a significant increase in net malate efflux from ET8 roots at high pH and this was also lower than that observed for Al^{3+} activation.

To understand the possible role of TaALMT1 in aluminium tolerance under alkaline conditions, the effect of added aluminate at pH 9 was examined for the wheat NILs (ET8 & ES8) and the transgenic barley (TaALMT1-OE) (Figure 6). There was a higher malate efflux from ET8 roots in basal (pH 9) than ES8 but was not significant probably due to the lack of buffering. The addition of 100 μM sodium aluminate significantly elevated malate efflux compared to pH 9 basal for ET8 but not for ES8 (Figure 6a). There was also a large increase in GABA efflux from ET8 seedlings at pH 9 plus aluminate compared with pH 9 basal, and both were (with or without aluminate) significantly higher in ET8 compared to ES8 (Figure

6c). There was no significant effect of the addition of aluminate on GABA efflux from ES8 seedlings.

For malate efflux in barley, TaALMT1-OE showed a significantly higher malate efflux at pH 9 plus aluminate compared with nulls. However, there was no significant genotype effect between TaALMT1-OE and null malate efflux at pH 9 basal (Figure 6b). Aluminate had a large stimulatory effect on malate efflux from the barley TaALMT1-OE roots (Figure 6b, note difference in scale between a & b). There was a significantly higher GABA efflux for TaALMT1-OE than nulls at pH 9 plus aluminate (Figure 6d) but aluminate did not significantly stimulate the efflux of GABA for TaALMT1-OE.

3.4 Acidification and the release of malate and GABA from ET8 and ES8 seedling roots at high pH

To test the hypothesis that release of malate and/or GABA at high pH may be associated with a reduction in external pH, wheat ET8 and ES8 NILs were treated with a slightly (carbonate) buffered alkaline pH and changes monitored in external concentrations of malate and GABA as well as the external pH over 22-24 h (Figure 7). Over this time the solution pH reduced from about 9.2-9.3 to 7.5 with ET8 showing significantly greater acidification than ES8 (Figure 7a). Control aerated solutions of the same volume and in the same conditions but without plants showed a much smaller drop in external pH (from pH 9.2 to 8.7). Solution concentrations of malate and GABA were normalised to the root fresh mass. The external

malate concentration arising from ET8 seedling roots was initially (at 2-3h) higher compared to that for ES8 and then decreased. The external malate concentration converged to similar values for ET8 and ES8 seedlings after 22-24 hours (Figure 7b). External GABA concentration was also initially much higher from ET8 than ES8 roots and converged to similar values (Figure 7c). Note that where there was a decrease in external malate and GABA, this would indicate that malate/GABA was first released then taken up from the solution or consumed.

In order to examine more closely the capacity of ET8 roots to acidify alkaline solutions the micro-electrode ion flux estimation (MIFE) technique was used to compare ET8 and ES8 wheat NILs and transgenic barley expressing *TaALMT1* (Figure 8). The external pH and apparent proton flux were examined near the root apex since *TaALMT1* is mainly expressed in root apices of ET8 correlating with the high malate and GABA release in response to high pH (Figure 4). Also, it was previously shown that TaALMT1 was activated by anions at alkaline pH (Ramesh et al 2015), therefore SO_4^{2-} (as 10 mM Na_2SO_4) was applied in the external solution about 5 minutes after the alkaline solution was applied. Figure 8a shows the external pH near the root apex over time after 10 mM Na_2SO_4 was added to the alkaline solution. ET8 was able to acidify the immediate rhizosphere (50 μm from the root surface) to a greater extent than ES8 settling to an external pH that was 0.2 units lower than that for ES8. However, both genotypes showed a greater rate of acidification in response to Na_2SO_4. The greater degree of acidification by ET8 apices correlated with a larger apparent H^+ efflux (larger negative flux) that was sustained over time (Figure 8b). The apparent H^+ effluxed is shown in Figure 8c calculated from the integration of the H^+ flux. The response of

the barley TaALMT1-OE and null showed some differences to the ET8-ES8 comparison (Figure 8d,e,f). The rate of acidification was overall faster in barley than in wheat and the pH in the immediate rhizosphere was similar between the TaALMT1-OE and null (Figure 8d), however the net proton efflux was larger in the TaALMT1-OE compared to the null by about the same magnitude as ET8 versus ES8 (Figure 8e). This resulted in a greater net release of H^+ over 60 min. for the TaALMT1-OE compared to the null (Figure 8f). The pH response and net proton release in response to 100 μM aluminate at pH 9 was also examined for the TaALMT1-OE barley and null (Sup. Figure S3). The reduction in external pH was very rapid and to quite low pH (Sup. Figure S3a), to the extent that only the tail end of the response could be captured with the MIFE pH electrode after the solution was changed. The TaALMT1-OE reduced the rhizosphere pH more rapidly and had a higher net proton efflux than the null over the first 7 min. after exposure to aluminate (Sup. Figure S3a,b). After 2 h there was no significant difference in rhizosphere pH or net proton flux between the barley TaALMT1-OE and null (Figure S3 c,d) with a relatively large net proton efflux from both genotypes (TaALMT1-OE 107 nmol m^{-2} s^{-1}, null 125 nmol m^{-2} s^{-1}) and with the rhizosphere pH setlling below 5 while the bulk solution remained at pH 9 (Sup. Figure S3e).

3.5 GABA release and acidification at high pH associated with the expression of TaALMT1 in *Xenopus laevis* oocytes

To confirm that TaALMT1 actually released GABA at high pH and that this may be associated with medium acidification, *Xenopus laevis* expressing *TaALMT1* were exposed to alkaline pH with Na_2SO_4 (Figure 9). The *TaALMT1* cRNA-injected oocytes were compared to water injected controls. The changes in external pH and H^+ fluxes were monitored using

MIFE (Figure 9a,b,c), while GABA efflux over a similar time frame (10 minutes) was estimated by measuring GABA in the oocyte and in the external solution for separate batches of oocytes (Figure 9d). These oocytes were not malate injected therefore malate efflux was not measured. GABA was previously found to be at mM concentrations in *Xenopus* oocytes (Ramesh et al. 2018) so did not need to be injected to measure efflux. *TaALMT1* injected oocytes acidified the external medium much faster than the water injected controls (Figure 9a, Sup. Figure S4). Interestingly, the *TaALMT1* injected oocytes took only 3 minutes to reduce the external pH (100 μm from the oocytes' membrane) from 9 to 7.5. The apparent H^+ efflux from *TaALMT1* injected oocytes was four-to- five fold higher than water-injected oocytes at the peak efflux (4 min) (Figure 9b). This large difference is more clearly indicated from the integral of the apparent proton flux shown in Figure 9c. Concomitant with the acidification associated with the expression of *TaALMT1*, there was a large efflux of GABA to the external medium at pH 9 over 10 minutes. This resulted in a substantial loss of GABA from the oocytes (Figure 9d).

3.6 Expression of *TaALMT1* and genes related to GABA metabolism in wheat root apices exposed to high pH and GABA

It was previously shown that ET8 roots have higher expression of *TaALMT1* compared to ES8 (Yamaguchi et al., 2005) and that *TaALMT1* expression in root apices was further increased in ET8 by external Al^{3+} treatment at pH 4.5 (Ramesh et al., 2018). Therefore, the expression of *TaALMT1* was measured in root apices after 5 weeks growth in pH 6 and pH 9 in the same conditions as those for Figure 1 plus and minus GABA treatment. Not surprisingly there was higher expression of *TaALMT1* in ET8 apices compared to that of

ES8 (Figure 10a); however, there was no change in expression at pH 9 compared to pH 6. Treatment with 10 mM GABA depressed expression of *TaALMT1* in ET8 significantly at pH 9, but there was no overall significance between pHs or genotypes (Figure 10e). Since GABA efflux is stimulated at pH 9 in ET8, expression of genes involved in GABA metabolism were measured: glutamate decarboxylase (*TaGAD* and *TaGAD1*) and GABA transaminase (*TaGABA-T*). Glutamate decarboxylase is the primary enzyme responsible for GABA synthesis, while GABA-T catalyses the conversion of GABA and 2-oxyglutarate in the mitochondria to succinate semialdehyde and glutamate. Of the two GAD genes (Figure 10b,c) the more lowly expressed gene *TaGAD*, showed significantly higher expression in ET8 at pH 9 than ES8 (Figure 10b) but there was no significant change with pH. The ET8 *TaGABA-T* showed significantly lower expression at pH 9 compared to pH 6 while ES8 showed no difference (Figure 10d). Neither *TaGAD*, *TaGAD1* or *TaGABA-T* were significantly altered in expression by GABA treatment (Figure 10f,g,h).

4. Discussion

In alkaline soils, the capacity of plants to acidify the rhizosphere is critical for the uptake of nutrients such as iron (Kakei et al., 2012) and phosphorus (Raghothama, 1999), as well as enabling a proton gradient into root cells for the uptake of anions such as nitrate through proton-coupled transporters (Wang, Lin, Cao, & Wu, 2015). To recap, here, the following hypotheses were tested: 1) that the higher expression of *TaALMT1* imparts an advantage for plant growth under alkaline conditions; 2) that TaALMT1 enables acidification of the rhizosphere at alkaline pH; and, 3) aluminate enhances the activation of TaALMT1 at

high pH. Each of these hypotheses are supported by our data as outlined in more detail below.

4.1 Root growth over 5 weeks is enhanced at high pH by the presence of the TaALMT1-V allele in ET8 plants

Previously it was shown that root growth and malate efflux at pH 9 was higher in young seedlings of ET8 compared to ES8 (Ramesh et al., 2015). However, this result was not confirmed by Silva et al. (2018) using a variety of naturally alkaline soils and hydroponic solutions. Comparing 5 weeks old ET8 and ES8 plants grown in washed sand supplied with nutrient solution at pH 6 and 9, we found that root growth was enhanced in the ET8 plants at pH 9 (Figure 1b) in support of Hypothesis 1. This was also evident in terms of total root length and root surface area (Figure 1c,d). The parameters measured at pH 6 appeared to be typical for wheat of the same age from other studies (Narayanan, Mohan, Gill, & Prasad, 2014). For the younger seedlings (less than 10 days old) used by Silva et al., (2018) these differences in root growth were not observed or there were variable responses in hydroponics at high pH depending on the buffer solution. In our longer-term growth experiments bicarbonate buffer was used (as did Silva et al), but the roots were continuously challenged with high pH, 3-4 times per week over 5 weeks. Considering the rapidity that roots acidify alkaline media over 24 hours (Figure 7), it is possible that in experiments of a few days without replenishment of the solution, the average pH is much less than the initial alkaline pH. One other possible explanation for this contrasting result could be the difference in the period over which root growth was determined.

Interestingly shoot growth of ET8 and ES8 in the different pHs did not reflect differences in root growth, with ES8 showing slightly higher but significant shoot biomass at pH 6 and pH 9 (Figure 1a). A greater depression in root growth relative to that of the shoot was also observed in a comparison between alkali tolerant and intolerant rice cultivars (Wang et al., 2015). The results reported here for wheat may reflect a difference in shoot:root carbon allocation and in this respect, it is relevant that ET8 had higher relative chlorophyll, higher transpiration and higher assimilation at pH 9 (Figure 2). This is also similar to the results for rice cultivars differing in alkali tolerance (Wang et al., 2015).

Previously it was shown that GABA inhibited the efflux of malate at acid pH in the presence of Al^{3+} in ET8 seedlings, causing root growth of ET8 to phenocopy that of ES8 (Ramesh et al., 2015). Therefore, the effect of GABA at 10 mM was tested in a subset of the wheat NILs at each of the pHs over 5 weeks of growth. GABA at 10 mM strongly inhibited both root and shoot growth over this period in both ET8 and ES8 plants and there were no differences observed between the two NILs at each pH in the degree of growth inhibition. However, there was a greater dependency of the inhibition on pH for ET8 (Sup. Figure S1a), which may be explained by GABA having a blocking action on malate exudation in the ET8 NIL. This however could not be confirmed from rhizosphere estimations of malate concentration in response to GABA after 5 weeks growth (Figure 4c).

4.2 Higher root exudation of malate and GABA occurs at alkaline pH in wheat associated with the expression of TaALMT1 and is enhanced by the presence of aluminate

Associated with the greater root growth observed for ET8 over ES8 at pH 9 there was also a greater apparent rhizosphere concentration of malate and GABA for ET8 root systems sampled after 5 weeks of growth compared to that for ES8 (Figure 3). The concentrations of malate and GABA were also higher for ET8 at pH 9 than for pH 6, while ES8 showed no differences. The values obtained cannot be attributed to actual rhizosphere concentrations, which are likely to be higher, but can be used for comparison between the genotypes. Elevated concentrations of organic acids in roots including malate have been observed for alkali tolerant rice grown under alkaline hydroponic conditions (Wang et al., 2015), and especially malate for wheat in combination with mild salinity (Guo et al., 2010), but media/rhizosphere concentrations in these studies were not reported.

There was no inhibitory effect of GABA treatment on the concentration of malate sampled from root systems, which is contrary to the expected effect of GABA as a blocker of malate efflux through TaALMT1 (Ramesh et al. 2015). Perhaps longer-term treatment by GABA has secondary effects as indicated by the alteration of leaf gas exchange (Sup. Figure S2). Interestingly for ES8, there was a significant increase in malate rhizosphere concentration in response to GABA that was not observed for ET8 (Figure 3b). It was previously shown that the expression of *TaALMT1* increased endogenous GABA concentrations by an unknown mechanism (Ramesh et al., 2018). This may account for the

difference in malate exudation in response to GABA associated with TaALMT1 if GABA is a source for malate synthesis through the GABA shunt and tricarboxylate acid (TCA) cycle.

The efflux of malate and GABA from root apices were increased in the short term (1h) in response to pH 9 for the wheat ET8 (Figure 4). This effect was not dependent on solution buffering. These results reflect the situation observed for whole root systems of ET8 and ES8 (Figure 4). In contrast Silva et al (2018) did not observe a significant difference for root apex efflux of malate at pH 9 for the ET8/ES8 comparison using unbuffered solution. Although the malate effluxes reported here for root apices of both ET8 and ES8 at pH 6 and for ES8 at pH 9 are very similar to those reported by Silva et al (2018) they differ substantially for malate efflux from ET8 at pH 9. Values at pH 9 are very similar to those of Silva et al for Al^{3+} activation at pH 4.5, and also to previous values reported for Al^{3+} activation (Ramesh et al. 2018). Despite this similarity in the magnitude of flux, the activation of TaALMT1 by high pH is likely to be very different to the situation for Al^{3+} activation. At pH 9, $[OH^-]$ is initially at 10 μM compared to 100 μM $[Al^{3+}]$ normally used to activate TaALMT1. Since the roots rapidly reduce the external pH, this in turn would reduce the activation of TaALMT1 according to the data presented by Ramesh et al. (2015). The degree of this effect will depend on the proportion of root mass to solution volume in the efflux experiment. In the experiments reported here, the minimum mass of roots compared to solution volume was used that would still allow accurate estimations of malate and GABA concentrations. The presence of buffer, which would tend to maintain a high pH in the media for longer, would be expected to enhance the apparent malate and GABA efflux. This buffer effect is clearly evident when longer term efflux experiments are performed (Figures 5 & 7).

It is also evident in the results of Figure 7 that there may have been re-uptake of initially released GABA and malate from ET8 seedlings. It was previously shown that GABA released by wheat roots is recycled back into roots (Warren, 2015). Extending this discussion to the actual situation in the rhizosphere around a growing root would suggest that the amount of malate and GABA released could be quite small in response to high pH, relative to what happens in a large volume of solution in the experiments reported here.

The aluminate anion becomes available at high pH and given that TaALMT1 is activated by the Al^{3+} cation at low pH and also by anions at high pH it may not be surprising that aluminate enhances malate and GABA efflux at high pH (Figure 6). The response was observed in both the ET8 NIL wheat and golden promise barley overexpressing TaALMT1. This response though significant seems not to be the primary stimulus activating TaALMT1 and it is also not clear how the exudation of GABA and malate may reduce the availability of aluminate in the rhizosphere other than to reduce the external pH.

4.3 GABA release and acidification at high pH associated with the expression of TaALMT1

ET8 roots and root apices showed a greater capacity to acidify alkaline media in comparison with ES8 (Figures 7,8). This was associated with the expression of TaALMT1 as indicated from the comparison between the barley TaALMT1-OE and null (Figure 8), though in barley there appears to be a greater intrinsic capacity to acidify the rhizosphere under alkaline conditions compared to wheat, reducing the impact of the expression of TaALMT1

(Fgiure 8). This difference for whole root systems was evident after only 10 minutes for the rhizosphere within 50 μm from the root apices. In parallel there was higher malate and GABA released from the root apices of ET8 and TaALMT1-OE barely (Figure 4 & 5). GABA can act as an effective buffer at both low and high pH, and at high pH GABA released from the root cytosol (assumed to be near neutrality) would be detected as an apparent H^+ efflux. GABA is a zwitterion with an amine group (pKa = 10.43) and a carboxyl group (pKa = 4.23) (Thwaites, Basterfield, McCleave, Carter, & Simmons, 2000). At neutral pH the amine group is protonated, and the carboxyl group is deprotonated to give a net neutral charge. At high pH the amine group will lose its proton, thus GABA becomes an anion at alkaline pH. Simulations of change in pH with addition of GABA using the concentrations of nutrients that have buffering capacity (e.g. NH_4^+, $H_2PO_4^-$ H_3BO_3, HCO_3^-, CO_3^{2-}) would indicate that high millimolar local concentrations of GABA are required to lower pH to near 7.0 (Gutz, 2018). This was confirmed by a simple experiment of adding GABA to a pH 9 unbuffered solution (Sup. Figure S5) where about 2 mM GABA was required to reduce the pH from 9 to 8. Further reduction in pH required high GABA concentrations (20 mM for pH 7). It is therefore unlikely that the release of GABA alone can accont for the entirity of the acidification unless there is a high mM GABA concentration adjacent to the root or that a proton is also released with GABA. Malate efflux could allow the H^+-ATPase in roots to operate at higher rates by balancing charge across the plasma membrane (summarised in Figure 11).

The expression of *TaALMT1* in *X. laevis* oocytes demonstrated a strong apparent effect of GABA release via TaALMT1 in lowering external pH (Figure 9). Since *X. laevis*

does not have an H^+-ATPase it is unlikely that anion release through TaALMT1 in this case could stimulate H^+ release from oocytes. There was a large release of GABA over 10 minutes from the oocytes expressing *TaALMT1* resulting in a substantial reduction in internal GABA concentration (Figure 9d). This corresponded to a rapid reduction in pH close to the oocyte membrane (Figure 9a). The external pH (100 μm from membrane) dropped to around 7.6 within a few minutes in *TaALMT1* expressing eggs after imersion in high pH (Figure 9a). The concentration of GABA at a distance of 100 μm from the oocyte surface required to reduce pH from 8.7 to 7.6 is approximately 5 mM. For the controls the pH dropped to about 8.1 after 10 minutes. This would require a GABA concentration at 100 μm of about 1 mM, more slowly accumulated over 10 minutes. There are two issues arising from this simple calculation. First the measured concentration of GABA in the oocytes for controls and *TaALMT1* expressing eggs was approximately 1.2 mM (Figure 8d), thus for the GABA concentration to be higher than this (5 mM) adjacent to the external surface requires that GABA be effluxed against a concentration gradient for *TaALMT1* expressing oocytes. Second, is it even possible for the measured rate of GABA efflux to account for such high concentrations of GABA adjacent to the external face of the membrane? Taking the volume of a thin shell 200 μm thick around the exterior of an oocyte it is possible to calculate the time it would take for this shell to fill with GABA using the measured efflux rates. For TaALMT1 expressing eggs the shell would fill in less than 100 ms based on the fluxes normalised to oocyte surface area (mean for TaALMT1 = 293 nmol m^{-2} s^{-1}). For the controls a concentration of about 1 mM would be reached within about the same time (mean flux = 172 nmol m^{-2} s^{-1}), but if passively distributed it would not increase beyond the concentration

of GABA within the oocyte. This approximate calculation serves to illustrate that it is likely that GABA efflux is against a concentration gradient in *TaALMT1* expressing oocytes, and that the fluxes measured are likely to be able to account for the concentration gradient adjacent to the egg membrane. A more complex mathematical model of diffusion and simultaneous reaction would be required to properly model this. It is possible that GABA efflux is coupled to the release of protons for which in the conditions of the experiment there would be an initial gradient of 1.5 pH units (pH of oocyte cytoplasm of 7.2, initial pH outside of 8.7) however, the gradient rapidly diminished. The net proton flux from the oocyte (Figure 9b) may be a combination of the actual proton flux and the consequences of GABA dissociation. GABA influx through TaALMT1 was also stimulated by low external pH (Ramesh et al., 2018) pointing to the possibility of reversible proton coupling of GABA transport. The possibility that GABA efflux is coupled to proton release is illustrated in Figure 11.

4.4 Expression of genes associated with GABA metabolism change in response to growth at high pH, but *TaALMT1* expression is not altered.

Here it was confirmed that ET8 plants had higher root apex expression of *TaALMT1* than that of ES8, but unlike the increase in *TaALMT1* expression shown for ET8 in response to Al^{3+} at low pH (Ramesh et al., 2018) there was not a significant increase in expression in response to pH 9 (Figure 10a). A *GAD-like* gene had increased expression at pH 9 in ET8 roots and this was significantly higher than that for ES8 (Figure 10b). This would correspond to the increase in GABA efflux that may require increased GAD to support GABA synthesis.

However, the more highly expressed *GAD1* gene did not show a significant difference (Figure 10c) and we note that for wheat in response to salinity the increase in GAD activity did not correspond to any change in transcript or protein abundance of this same *GAD* gene (Che-Othman et al, 2020). In support of a "supply and demand" interpretation is the depression of *GABA-T* expression at pH 9 in ET8, but not in ES8 roots (Figure 10d). GABA-T converts GABA to succinate semialdehyde so a reduced enzyme activity may result in higher GABA accumulation in ET8. These possibilities need confirmation from enzyme activity measurements and protein levels (Che-Othman et al, 2020).

GABA treatment of roots did not result in significant changes in expression, although the *GAD*-like transcript that showed a pH effect was depressed in both ET8 and ES8. There was a significant reduction in *TaALMT1* expression at pH 9 in response to GABA, which further supports the observations by Ramesh et al (2018) of a close link between *TaALMT1* expression and GABA concentrations in roots.

4.5 A new mechanism for rhizosphere control of pH via TaALMT1 under alkaline conditions

Several studies have demonstrated the importance of H^+ exudation as a mechanism of alkaline soil tolerance (Fuglsang et al., 2007; Yang et al., 2010; Xu et al., 2012; Xu et al., 2013; Li et al., 2015). Here it is shown how TaALMT1 can enhance rhizosphere acidification in alkaline soils. TaALMT1 and possibly other ALMTs in other plants associated with Al^{3+} tolerance are likely to have broader functions than the well-established Al^{3+} tolerance at low

pH and may be associated with both acid and alkaline soil pH tolerance and metabolic signalling (Ramesh et al. 2018). The results reported here explain previous associations between higher grain yield in alkaline soil and the presence of the TaALMT1 allele (Eagles et al., 2014; McDonald et al., 2013). Elevated malate exudation in alkaline soils, will improve root growth in two ways: (1) providing charge balance for enhanced H^+ efflux mediated by the H^+-ATPase thereby lowering the apoplastic pH to a more conducive level to allow root elongation (Landsberg, 1981); and, (2) improving iron and phosphorus availability (Delhaize et al., 2009; Landsberg, 1981; Lopez-Bucio, de la Vega, Guevara-Garcia, & Herrera-Estrella, 2000). In addition, the concomitant release of GABA by TaALMT1 and possibly protons at high pH would further enhance acidification of the rhizosphere (Figure 11). GABA released into the rhizosphere is likely to be taken back up by roots either via a high affinity GABA transporter (Meyer, Eskandari, Grallath, & Rentsch, 2006) or back through TaALMT1 once the pH has been lowered (Ramesh et al., 2018). The role of the GABA shunt in regulating the supply of GABA (Bown and Shelp, 2020) for efflux via TaALMT1 and their possible interactions with GABA signalling and pH under alkaline conditions remain to be fully explored.

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References

Bown, A. W., & Shelp, B. J. (2020). Does the GABA Shunt Regulate Cytosolic GABA? Trends in Plant Science, 25(5), 422-424. doi:10.1016/j.tplants.2020.03.001

Bose, J., Xie, Y., Shen, W., & Shabala, S. (2013). Haem oxygenase modifies salinity tolerance in Arabidopsis by controlling K^+ retention via regulation of the plasma membrane H^+-ATPase and by altering SOS1 transcript levels in roots. *Journal of Experimental Botany, 64*(2), 471-481. doi:10.1093/jxb/ers343

Che-Othman, M.H., Jacoby, R.P., Millar, A.H., & Taylor, N.L. (2020) Wheat mitochondrial respiration shifts from TCA cycle to the GABA shunt under salt stress. *New Phytologist*, *225(3)*, 1166-1180. doi: 10.1111/nph.15713

De La Fuente, J. M., Ramirez-Rodriguez, V., Cabrera-Ponce, J. L., & Herrera-Estrella, L. (1997). Aluminum tolerance in transgenic plants by alteration of citrate synthesis. *Science, 276*(5318), 1566-1568. doi:10.1126/science.276.5318.1566

Delhaize, E., & Ryan, P. R. (1995). Aluminum Toxicity and Tolerance in Plants. *Plant Physiology*, *107*(2), 315-321.

Delhaize, E., Ryan, P. R., Hebb, D. M., Yamamoto, Y., Sasaki, T., & Matsumoto, H. (2004). Engineering high-level aluminum tolerance in barley with the ALMT1 gene. *Proceedings of the National Academy of Sciences of the United States of America, 101*(42), 15249-15254. doi:10.1073/pnas.0406258101

Delhaize, E., Taylor, P., Hocking, P. J., Simpson, R. J., Ryan, P. R., & Richardson, A. E. (2009). Transgenic barley (Hordeum vulgare L.) expressing the wheat aluminium resistance gene (TaALMT1) shows enhanced phosphorus nutrition and grain production when grown on an acid soil. *Plant Biotechnology Journal, 7*(5), 391-400. doi:10.1111/j.1467-7652.2009.00403.x

Eagles, H., Cane, K., Trevaskis, B., Vallance, N., Eastwood, R., Gororo, N., . . . Martin, P. (2014). Ppd1, Vrn1, ALMT1 and Rht genes and their effects on grain yield in lower rainfall environments in southern Australia. *Crop and Pasture Science, 65*(2), 159-170.

Fuglsang, A. T., Guo, Y., Cuin, T. A., Qiu, Q., Song, C., Kristiansen, K. A., . . . Schumaker, K. S. (2007). Arabidopsis protein kinase PKS5 inhibits the plasma membrane H+-ATPase by preventing interaction with 14-3-3 protein. *The Plant Cell, 19*(5), 1617-1634.

Guo, M., Wang, R., Wang, J., Hua, K., Wang, Y., Liu, X., & Yao, S. (2014). ALT1, a Snf2 Family Chromatin Remodeling ATPase, Negatively Regulates Alkaline Tolerance through Enhanced Defense against Oxidative Stress in Rice. *PLOS ONE*, *9*(12), e112515. doi:10.1371/journal.pone.0112515

Guo, R., Shi, L. X., Ding, X. M., Hu, Y. J., Tian, S. Y., Yan, D. F., . . . Yang, Y. F. (2010). Effects of Saline and Alkaline Stress on Germination, Seedling Growth, and Ion Balance in Wheat. *Agronomy Journal*, *102*(4), 1252-1260. doi:10.2134/agronj2010.0022

Gutz, I. G. R. (2018). CurTiPot – pH and Acid–Base Titration Curves: Analysis and Simulation freeware, version 4.2 (Version 4.3.0). Retrieved from http://www.iq.usp.br/gutz/Curtipot_.html

Hellemans, J., Mortier, G., De Paepe, A., Speleman, F., & Vandesompele, J. (2007). qBase relative quantification framework and software for management and automated analysis of real-time quantitative PCR data. *Genome biology*, *8*(2), R19.

Jin, H., Plaha, P., Park, J., Hong, C., Lee, I., Yang, Z., . . . Lee, J. (2006). Comparative EST profiles of leaf and root of Leymus chinensis, a xerophilous grass adapted to high pH sodic soil. *Plant Science*, *170*(6), 1081-1086.

Kakei, Y., Ishimaru, Y., Kobayashi, T., Yamakawa, T., Nakanishi, H., & Nishizawa, N. K. (2012). OsYSL16 plays a role in the allocation of iron. *Plant Molecular Biology*, *79*(6), 583-594.

Kochian, L. V., Hoekenga, O. A., & Piñeros, M. A. (2004). How do crop plants tolerate acid soils? mechanisms of aluminum tolerance and phosphorous efficiency. *Annual review of plant biology, 55*(1), 459-493. doi:10.1146/annurev.arplant.55.031903.141655

Kopittke, P. M., Menzies, N. W., & Blamey, F. P. C. (2004). Rhizotoxicity of aluminate and polycationic ahnninium at high pH. *Plant and Soil, 266*(1-2), 177-186.

Landsberg, E. C. (1981). Organic-acid synthesis and release of hydrogen-ions in response to fe deficiency stress of mono-ledonous and dicoty-ledonous plant-species. *Journal of Plant Nutrition, 3*(1-4), 579-591. doi:10.1080/01904168109362862

Lee, J. A. (1999). The calcicole-calcifuge problem revisited. In J. A. Callow (Ed.), *Advances in Botanical Research Incorporating Advances in Plant Pathology, Vol 29* (Vol. 29, pp. 1-30). London: Academic Press Ltd-Elsevier Science Ltd.

Li, J., Xu, H.-H., Liu, W.-C., Zhang, X.-W., & Lu, Y.-T. (2015). Ethylene Inhibits Root Elongation during Alkaline Stress through AUXIN1 and Associated Changes in Auxin Accumulation. *Plant physiology, 168*(4), 1777-1791. doi:10.1104/pp.15.00523

Long, Y., Tyerman, S.D., & Gilliham M. (2020). Cytosolic GABA inhibits anion transport by wheat ALMT1. *New Phytologist, 225*, 671-678.

Lopez-Bucio, J., de la Vega, O. M., Guevara-Garcia, A., & Herrera-Estrella, L. (2000). Enhanced phosphorus uptake in transgenic tobacco plants that overproduce citrate. *Nature Biotechnology, 18*(4), 450-453.

Lopez-Bucio, J., Nieto-Jacobo, M. F., Ramirez-Rodriguez, V., & Herrera-Estrella, L. (2000). Organic acid metabolism in plants: from adaptive physiology to transgenic varieties

for cultivation in extreme soils. *Plant Science, 160*(1), 1-13. doi:10.1016/s0168-9452(00)00347-2

Ma, G., Rengasamy, P., & Rathjen, A. (2003). Phytotoxicity of aluminium to wheat plants in high-pH solutions. *Australian Journal of Experimental Agriculture, 43*(5), 497-501.

Ma, J. F., Ryan, P. R., & Delhaize, E. (2001). Aluminium tolerance in plants and the complexing role of organic acids. *Trends in Plant Science, 6*(6), 273-278. doi:http://dx.doi.org/10.1016/S1360-1385(01)01961-6

McDonald, G. K., Taylor, J., Verbyla, A., & Kuchel, H. (2013). Assessing the importance of subsoil constraints to yield of wheat and its implications for yield improvement. *Crop and Pasture Science, 63*(12), 1043-1065.

Meng, C., Quan, T. Y., Li, Z. Y., Cui, K. L., Yan, L., Liang, Y., . . . Liu, S. W. (2017). Transcriptome profiling reveals the genetic basis of alkalinity tolerance in wheat. *Bmc Genomics, 18*, 14. doi:10.1186/s12864-016-3421-8

Meyer, A., Eskandari, S., Grallath, S., & Rentsch, D. (2006). AtGAT1, a high affinity transporter for gamma-aminobutyric acid in Arabidopsis thaliana. *Journal of Biological Chemistry, 281*(11), 7197-7204. doi:10.1074/jbc.M510766200

Narayanan, S., Mohan, A., Gill, K. S., & Prasad, P. V. V. (2014). Variability of Root Traits in Spring Wheat Germplasm. *PLOS ONE, 9*(6), 15. doi:10.1371/journal.pone.0100317

Newman, I. (2001). Ion transport in roots: measurement of fluxes using ion-selective microelectrodes to characterize transporter function. *Plant, cell & environment, 24*(1), 1-14.

Pfaffl, M. W. (2001). A new mathematical model for relative quantification in real-time RT–PCR. *Nucleic Acids Research, 29*(9), e45-e45. doi:10.1093/nar/29.9.e45

Raghothama, K. (1999). Phosphate acquisition. *Annual review of plant biology, 50*(1), 665-693.

Raman, H., Zhang, K. R., Cakir, M., Appels, R., Garvin, D. F., Maron, L. G., . . . Ryan, P. R. (2005). Molecular characterization and mapping of ALMT1, the aluminium-tolerance gene of bread wheat (Triticum aestivum L.). *Genome, 48*(5), 781-791. doi:10.1139/g05-054

Ramesh, S. A., Kamran, M., Sullivan, W., Chirkova, L., Okamoto, M., Degryse, F., . . . Tyerman, S. D. (2018). Aluminium-Activated Malate Transporters Can Facilitate GABA Transport. *The Plant Cell*. doi:10.1105/tpc.17.00864

Ramesh, S. A., Tyerman, S. D., Xu, B., Bose, J., Kaur, S., Conn, V., . . . Gilliham, M. (2015). GABA signalling modulates plant growth by directly regulating the activity of plant-specific anion transporters. *Nature Communications, 6*. doi:10.1038/ncomms8879

Rengasamy, P., & Olsson, K. (1991). Sodicity and soil structure. *Soil Research, 29*(6), 935-952.

Ryan, P., Delhaize, E., & Jones, D. (2001). Function and mechanism of organic anion exudation from plant roots. *Annual review of plant biology, 52*(1), 527-560.

Ryan, P., Delhaize, E., & Randall, P. (1995). Characterisation of Al-stimulated efflux of malate from the apices of Al-tolerant wheat roots. *Planta, 196*(1), 103-110. doi:10.1007/BF00193223

Ryan, P., Tyerman, S., Sasaki, T., Furuichi, T., Yamamoto, Y., Zhang, W., & Delhaize, E. (2011). The identification of aluminium-resistance genes provides opportunities for enhancing crop production on acid soils. *Journal of Experimental Botany, 62*(1), 9-20.

Ryan, P. R., Raman, H., Gupta, S., Sasaki, T., Yamamoto, Y., & Delhaize, E. (2010). The multiple origins of aluminium resistance in hexaploid wheat include Aegilops tauschii and more recent cis mutations to TaALMT1. *The Plant Journal, 64*(3), 446-455.

Ryan, P. R., Skerrett, M., Findlay, G. P., Delhaize, E., & Tyerman, S. D. (1997). Aluminum activates an anion channel in the apical cells of wheat roots. *Proceedings of the National Academy of Sciences of the United States of America, 94*(12), 6547-6552. doi:10.1073/pnas.94.12.6547

Sasaki, T., Yamamoto, Y., Ezaki, B., Katsuhara, M., Ahn, S. J., Ryan, P. R., . . . Matsumoto, H. (2004). A wheat gene encoding an aluminum-activated malate transporter. *The Plant Journal, 37*(5), 645-653. doi:10.1111/j.1365-313X.2003.01991.x

Sasaki T, Y. Y., Ezaki B, Katsuhara M, Ahn SJ, Ryan PR, Delhaize E, Matsumoto H. . (2004). A wheat gene encoding an aluminium-activated malate transporter. *The Plant Journal, 37*,, 645–653.

Silva, C. M. S., Zhang, C. Y., Habermann, G., Delhaize, E., & Ryan, P. R. (2018). Does the major aluminium-resistance gene in wheat, TaALMT1, also confer tolerance to alkaline soils? *Plant and Soil, 424*(1-2), 451-462. doi:10.1007/s11104-017-3549-6

Thwaites, D. T., Basterfield, L., McCleave, P. M. J., Carter, S. M., & Simmons, N. L. (2000). Gamma-aminobutyric acid (GABA) transport across human intestinal epithelial

(Caco-2) cell monolayers. *British Journal of Pharmacology, 129*(3), 457-464. doi:10.1038/sj.bjp.0703069

Wang, H., Lin, X., Cao, S., & Wu, Z. (2015). Alkali tolerance in rice (Oryza sativa L.): growth, photosynthesis, nitrogen metabolism, and ion homeostasis. *Photosynthetica, 53*(1), 55-65. doi:10.1007/s11099-015-0079-4

Warren, C. (2015). Wheat roots efflux a diverse array of organic N compounds and are highly proficient at their recapture. *Plant and Soil*, 1-16. doi:10.1007/s11104-015-2612-4

Wong, V. N., Dalal, R. C., & Greene, R. S. (2008). Salinity and sodicity effects on respiration and microbial biomass of soil. *Biology and fertility of soils, 44*(7), 943-953.

Xu, W., Jia, L., Baluška, F., Ding, G., Shi, W., Ye, N., & Zhang, J. (2012). PIN2 is required for the adaptation of Arabidopsis roots to alkaline stress by modulating proton secretion. *Journal of Experimental Botany, 63*(17), 6105-6114. doi:10.1093/jxb/ers259

Xu, W., Jia, L., Shi, W., Baluška, F., Kronzucker, H. J., Liang, J., & Zhang, J. (2013). The tomato 14-3-3 protein TFT4 modulates H+ efflux, basipetal auxin transport, and the PKS5-J3 pathway in the root growth response to alkaline stress. *Plant Physiology, 163*(4), 1817-1828.

Yamaguchi, M., Sasaki, T., Sivaguru, M., Yamamoto, Y., Osawa, H., Ahn, S. J., & Matsumoto, H. (2005). Evidence for the Plasma Membrane Localization of Al-

activated Malate Transporter (ALMT1). *Plant and Cell Physiology*, *46*(5), 812-816. doi:10.1093/pcp/pci083

Yang, Y., Qin, Y., Xie, C., Zhao, F., Zhao, J., Liu, D., . . . Schumaker, K. S. (2010). The Arabidopsis chaperone J3 regulates the plasma membrane H+-ATPase through interaction with the PKS5 kinase. *The Plant Cell*, *22*(4), 1313-1332.

Yu, Y., Duan, X. B., Ding, X. D., Chen, C., Zhu, D., Yin, K. D., . . . Zhu, Y. M. (2017). A novel AP2/ERF family transcription factor from Glycine soja, GsERF71, is a DNA binding protein that positively regulates alkaline stress tolerance in Arabidopsis. *Plant Molecular Biology*, *94*(4-5), 509-530. doi:10.1007/s11103-017-0623-7

Zhang, G., & Bown, A. W. (1997). The rapid determination of γ-aminobutyric acid. *Phytochemistry*, *44*(6), 1007-1009.

Zhang, H., Liu, X. L., Zhang, R. X., Yuan, H. Y., Wang, M. M., Yang, H. Y., . . . Liang, Z. W. (2017). Root Damage under Alkaline Stress Is Associated with Reactive Oxygen Species Accumulation in Rice (Oryza sativa L.). *Frontiers in Plant Science, 8*, 12. doi:10.3389/fpls.2017.01580

Zhang, W. H., Ryan, P. R., & Tyerman, S. D. (2001). Malate-permeable channels and cation channels activated by aluminum in the apical cells of wheat roots. *Plant physiology*, *125*(3), 1459-1472. doi:10.1104/pp.125.3.1459

Figure Legends

Figure 1. Effect of nutrient solution pH on root and shoot growth of ET8 and ES8 wheat plants. Near-isogenic wheat lines ET8 (Al^{3+} tolerant, black bars) and ES8 (Al^{3+} sensitive, grey bars) were grown in pots for 5 weeks (see methods). Nutrient solution at pH 6 and pH 9 were applied to each pot 3-4 times a week. (a) Shoot fresh mass (n=10), (b) Root fresh mass (n=10), (c) Root length (n=5), (d) Root surface area (n=5). (e) Examples of root systems harvested at 5 weeks growth for both genotypes at each pH (scale bar = 5 cm). ** and *** indicate significant difference between genotypes at a given pH where $P < 0.01$ and 0.001 respectively, error bars =SEM. Different letter combination for a genotype indicate significant difference ($P < 0.05$) between pHs (lower case = ET8, upper case = ES8) using two-way ANOVA and post tests.

Figure 2. Effect of nutrient solution pH on leaf gas exchange of ET8 and ES8 plants. (a) Transpiration rate (E) (n=5), (b) Stomatal conductance (g_s) (n=5), (c) Relative chlorophyll concentration measured using SPAD (n=10), (d) Net assimilation rate (A) (n=5). All parameters were measured after 5 weeks. * and ** indicates significant difference between genotypes at a particular pH, $P < 0.05$ and $P < 0.01$ respectively, error bars = SEM. Different letter combination for a genotype indicate significant difference ($P < 0.05$) between pHs (lower case = ET8, upper case = ES8) using two-way ANOVA and post tests. ns = no significant differences.

Figure 3. Estimates of rhizosphere malate and GABA for ET8 and ES8 plants in response to nutrient solution pH and the effect of 10 mM GABA on malate concentration. (a) Malate concentration. (b) Effect of 10 mM GABA on the estimated rhizosphere malate concentration (% of the control without GABA). (c) GABA concentration. Root washes were measured for plants *in situ* after five weeks of growth. For (a) and (c) values are normalised to the root fresh mass of each replicate plant. In each case different letter combination for a genotype indicates significant difference ($P < 0.05$) between pHs (lower case = ET8, upper case = ES8) and ** indicates significant difference between genotypes at a given pH $P < 0.01$ (two-way ANOVA with post tests). ns = no significant differences. # = significantly different from 100% (one-tailed t-test). All n=5 ± SEM.

Figure 4. Root apex exudation of malate (a,b) and GABA (c,d) at pH 6 and 9 (+/- buffer) comparing wheat ET8 and ES8 (a,c) and barley transgenic line over-expressing TaALMT1 (b,d). Net flux was measured over 1 h after placing excised root apices (5 mm) in different pH +/- buffer (all 3 mM $CaCl_2$, 10 mM Na_2SO_4): pH 6, 0.01 mM NaOH; pH 6 buffer, 5 mM MES, 2.6 mM NaOH; pH 9*:* 0.05 mM NaOH, pH 9 + buffer, 5 mM BTP, 2 mM MES. In each case different letter combination for a genotype indicates significant difference ($P < 0.05$) between pHs (lower case = ET8, ALMT1 OE, upper case = ES8, null) and *, **** indicate significant difference between genotypes at a given pH $P < 0.05$, 0.0001 respectively (two-way ANOVA and post tests), n=5 ± SEM.

Figure 5. Exudation of malate comparing low pH and effect of Al^{3+}, and high pH with +/- buffer for excised root apices and intact seedling roots measured over different time scales. (a) Net flux from excised root apices measured over 1 h after placing apices in the respective pH solution (n=8). (b) Net flux measured over 21 h from intact seedlings roots in the respective pH solution (n=5). Solutions (all 3 mM $CaCl_2$) from left to right were: pH 4.5, 5 mM MES; pH 4.5, 5 mM MES, 0.1 mM $AlCl_3$; pH 9, 0.12 mM NaOH; pH 9, 0.2 mM, KOH; pH 9, 20 mM BTP, 10 mM Na_2SO_4, 9 mM MES; pH 9, 18 mM $NaHCO_3$, 2 mM Na_2CO_3; pH 9, 18 mM $NaHCO_3$, 2 mM Na_2CO_3, 10 mM Na_2SO_4. *, **,***, **** indicate significant difference between genotypes at a given pH $P < 0.05$, 0.01,0.001, 0.0001, respectively (two-way ANOVA and post tests), error bars = SEM.

Figure 6. Efflux of malate (a,b) and GABA (c,d) in 3-day old seedlings of ET8 and ES8 wheat (a,c) and TaALMT1-OE and Null barley (b,d) in response to high pH (initially 9, no buffer) and high pH plus 100 μM aluminate. Fluxes were measured from individual seedlings (per replicate) over a 22 h incubation period (n=5). Note different scale in (b) compared to (a). In each case different letter combination for a genotype indicates significant difference ($P < 0.05$) between treatments (lower case = ET8 or TaALMT1-OE, upper case = ES8 or GP) *, **, ***, **** indicate significant difference between genotypes at a given pH $P < 0.05$, 0.01, 0.001, 0.0001, respectively (two-way ANOVA and post-tests), error bars = SEM.

Figure 7. Malate and GABA release from roots and change in external pH for ET8 and ES8 seedlings in hydroponic solution during alkaline pH treatment. Four days old seedlings were bathed in aerated basal nutrient solution (pH 6) for 3 days then transferred to aerated nutrient solution (200 ml) initially at pH 9.2-9.3 for 24 hours. Two independent experiments are combined. External medium pH (a) malate concentration (b) and GABA concentration (c) at 2h (Initial) and 22 hours (Final) **, and *** indicate significant difference between genotypes $P < 00.01$ and 0.001 (2-way repeated measures ANOVA with post test). All data n = 10 biological replicates consisting of approximately 7-8 seedlings per replicate 0.36-0.55 gm_{fwt} of roots, error bars = SEM.

Figure 8. Comparison of ET8 and ES8 (a,b,c), and barley TaALMT1 OE and null (cv golden promise) (d,e,f) rhizosphere acidification in response to alkaline pH and 10 mM Na_2SO_4. (a,d) External pH (pH_o) measured at the root apex (≈ 1.5 mm from root cap) of three-to-four days old seedlings. (b,e) Apparent net H^+ flux. (c,f) total H^+ effluxed (calculated as the integral of apparent H^+ fluxes). Micro Electrode Ion Flux Estimation (MIFE) technique was used to measure changes in rhizosphere pH and proton fluxes 50 µm from the root surface. The roots of intact seedlings were pre-conditioned in 0.2 mM KCl + 0.2 mM $CaCl_2$ + 3 mM MES + 4 mM TRIS, pH 7.87 for 30 minutes. After pre-conditioning, rhizosphere pH and proton fluxes were measured over time when the above solution plus 10 mM Na_2SO_4 was introduced in to the chamber. Note the solution remains unstirred after the introduction of the new solution and it takes about 2 minutes before measurements can start. Wheat n=6 seedlings, barley n=8 seedlings. For a,b,d,e data is fit to an exponential decay and 95%

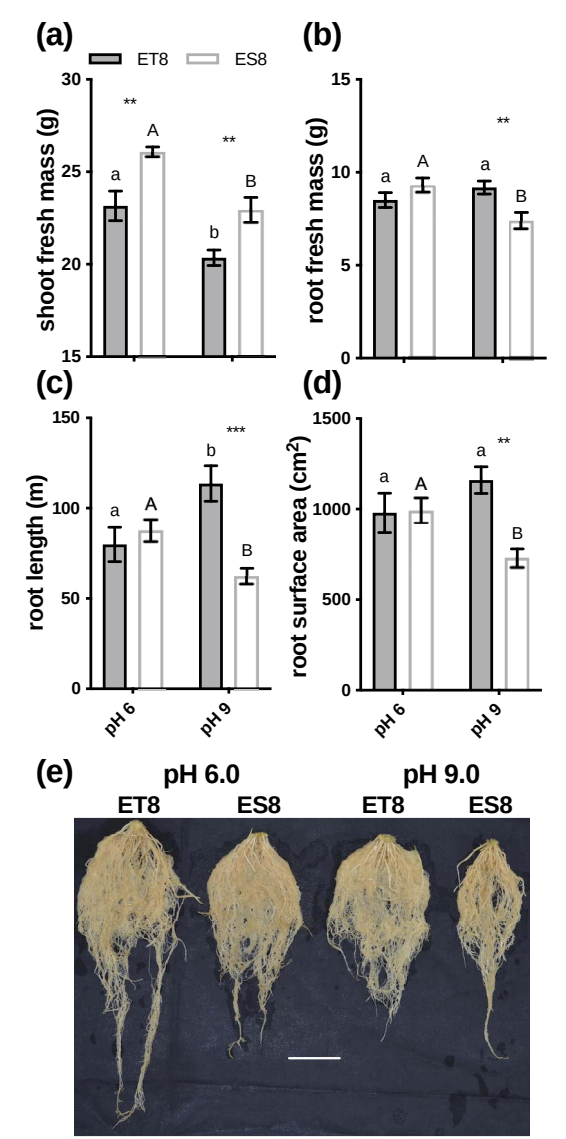
confidence intervals are shown for the fit to all data. For c & f the data was best fit by a 2nd order polynomial and 95% confidence intervals are shown.

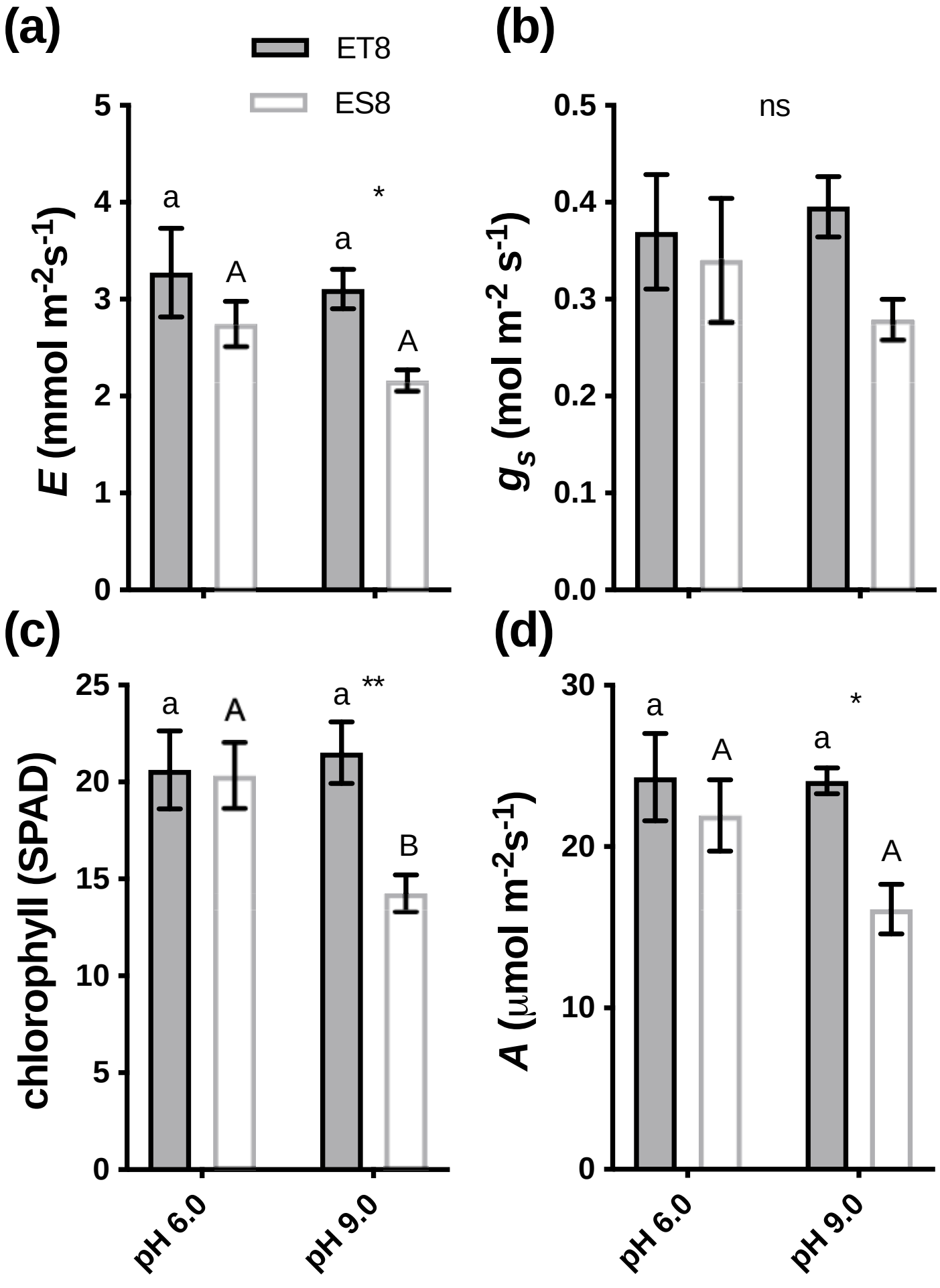
Figure 9. The effect of alkaline pH on efflux of protons and GABA from *Xenopus laevis* oocytes expressing TaALMT1. TaALMT1 and water injected (Control) *X. laevis* oocytes were used in these experiments. (a) External pH (pH_o), (b) Apparent H^+ fluxes, (c) Total H^+ effluxed (calculated as the integral of apparent H^+ fluxes), were measured at 100 μM from the oocyte membrane (or as indicated) using MIFE. (d) The $[GABA]_i$ of separate oocytes was measured after a 10 minute efflux period in either pH 7 or pH 9 and the GABA effluxed was measured from GABA concentrations in the external medium. The initial $[GABA]_i$ for each replicate was calculated from the sum of that effluxed and 10 minute $[GABA]_i$. All values are normalised to the geometric volume of each oocyte. *, ***, indicate significant difference between genotypes at the different times where $P < 0.05$, 0.001, respectively. For A,B,C n =6 oocytes, for D n=5 batches of 4-5 oocytes, error bars = SEM.

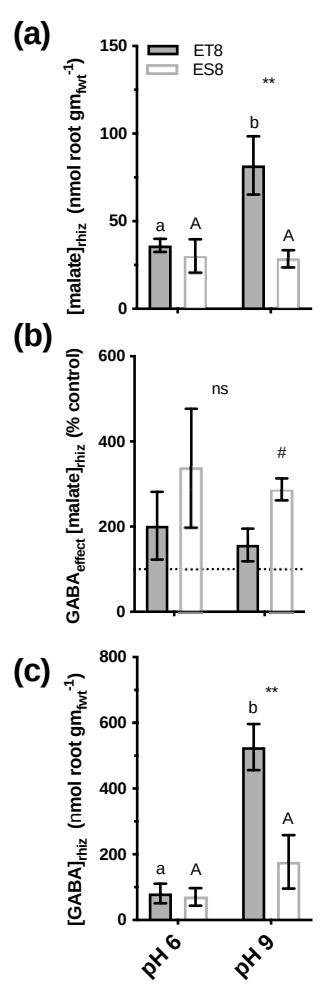
Figure 10. Expression of *TaALMT1* (a), *GAD* (b), *GAD1* (c) and *GABAT* (d) relative to three housekeeping genes *(GAPDH, Cyclophilin and Tubulin)* in root tips and the effect of GABA treatment on expression (e,f,g,h respectively). Wheat NILs ET8 (black) and ES8 (grey) were grown in pots for 6 weeks. Nutrient solution (see material and methods) at pH 6 and pH 9 ± 10 mM GABA were applied to each pot 2-3 times a week. Housekeeping genes were not affected by the treatments. Primers used and Genbank accessions numbers are listed in Supplementary Table 1. In each case different letter combination for a genotype indicates

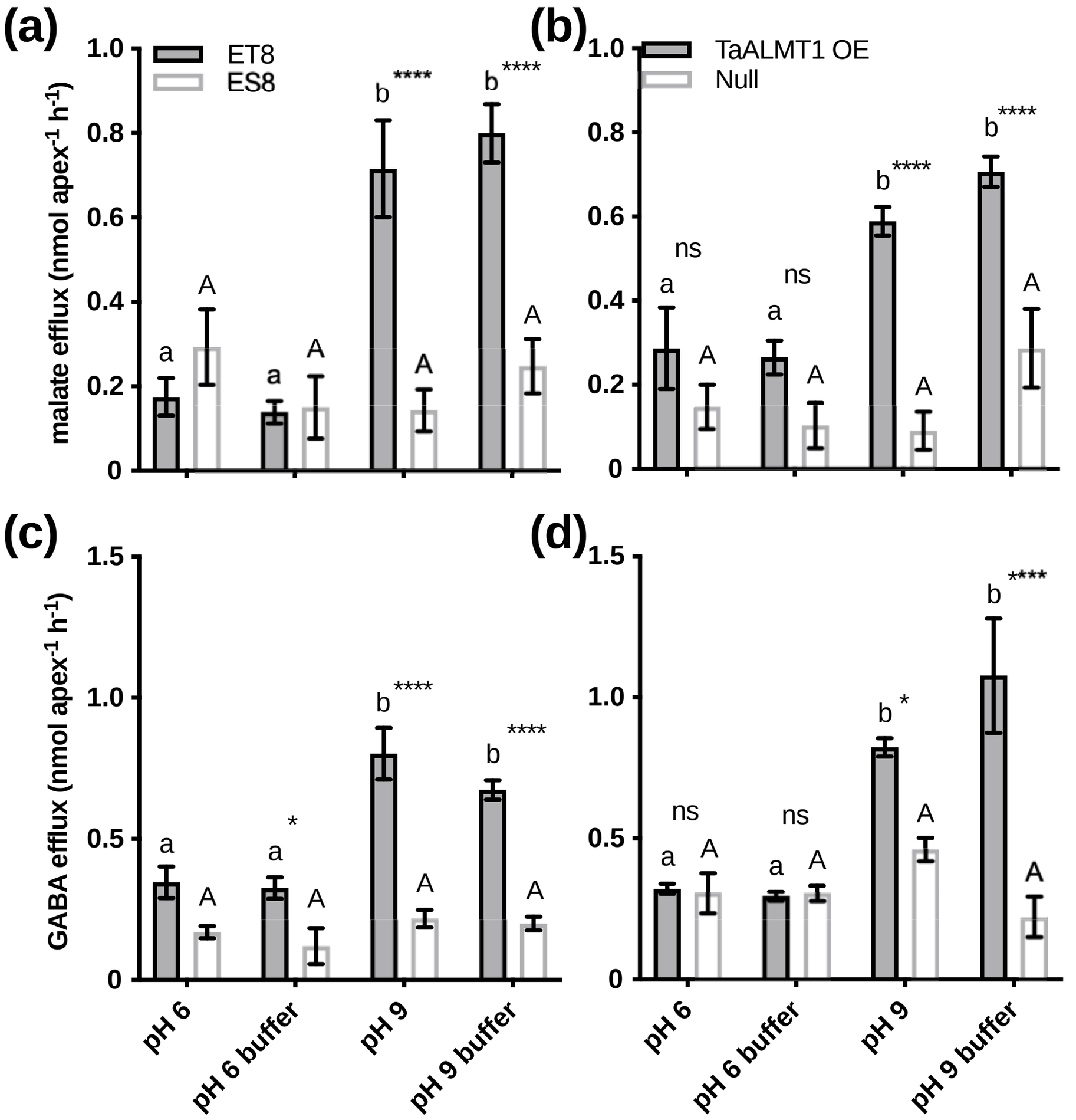
significant difference (P<0.05) between pHs (lower case = ET8, upper case = ES8) and *, ***, indicate significant difference between genotypes at a given pH $P < 0.05$, 0.001, (2-way ANOVA and post tests, n= 3-4, error bars = SEM.

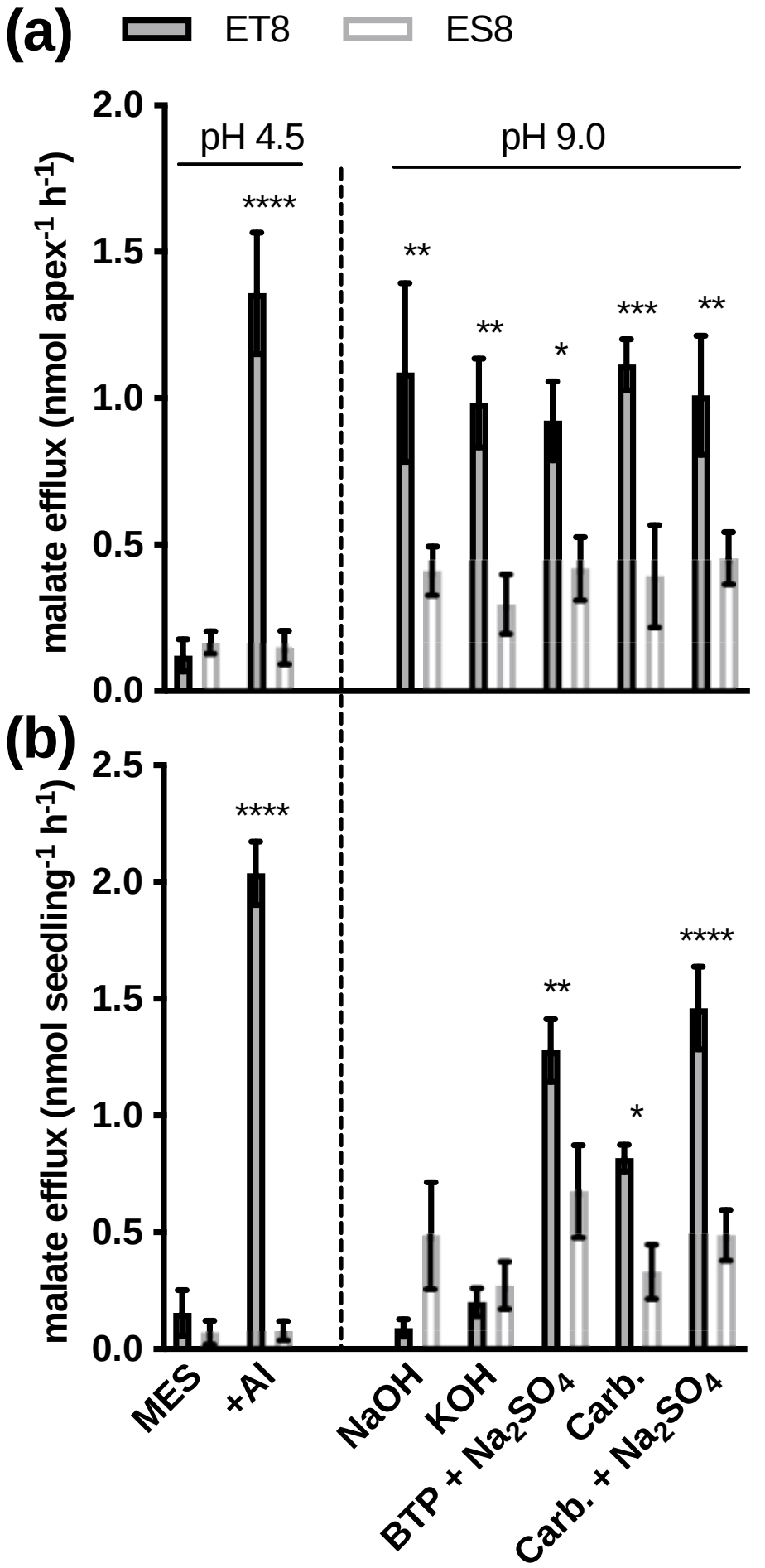
Figure 11. Diagram of the hypothesis of how TaALMT1 may reduce external pH under alkaline conditions. TaALMT1 allows the efflux of both malate anions and neutral GABA (zwitterion at pH 7.4) into the apoplast when activated by high external pH and aluminate. (1) The release of malate anions can balance charge required for the operation of the H^+-ATPase. (2) The release of GABA can lower the external pH by virtue of its pKa of the ammonium group and possible by the coupling to proton efflux. Mitochondria are central to the supply of malate and GABA and key enzymes and transporters on the mitochondrial membrane proposed to be involved in the GABA shunt include 2-oxoglutarate (2-OG)/malate transporter (OMT), unknown transaminases (AT?), glutamate decarboxylase (GAD), GABA permease (GBP) and GABA transaminase (GABA-T) (Bown and Shelp, 2020).

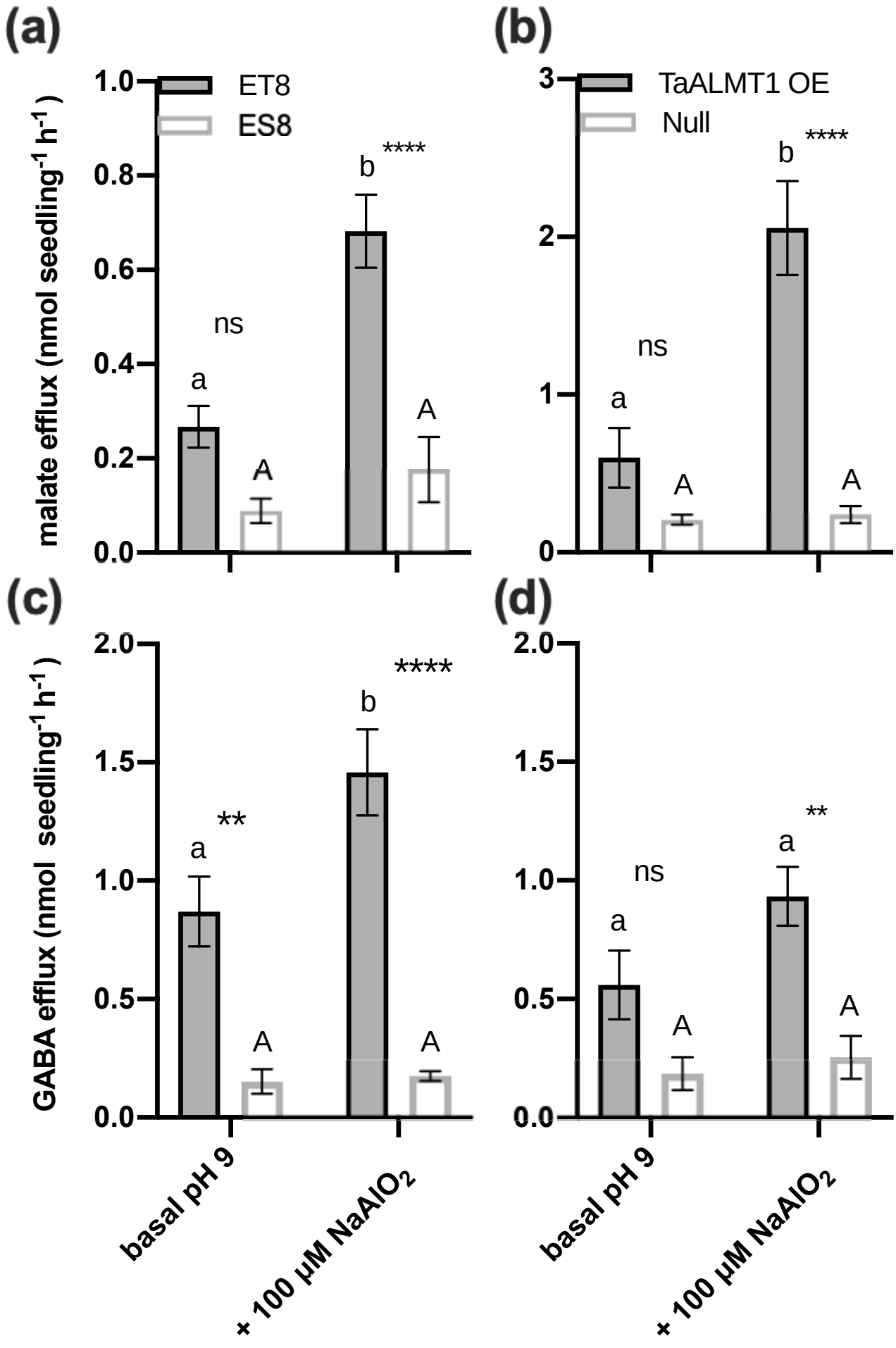

(a)
ET8
ES8
shoot fresh mass (g)
30
25
20
15
**
**
a
A
b
B
(b)
root fresh mass (g)
15
10
5
0
**
a
A
a
B
(c)
root length (m)
150
100
50
0
***
a
A
b
B
pH 6
pH 9
(d)
root surface area (cm²)
1500
1000
500
0
**
a
A
a
B
pH 6
pH 9
(e)
pH 6.0
pH 9.0
ET8
ES8
ET8
ES8

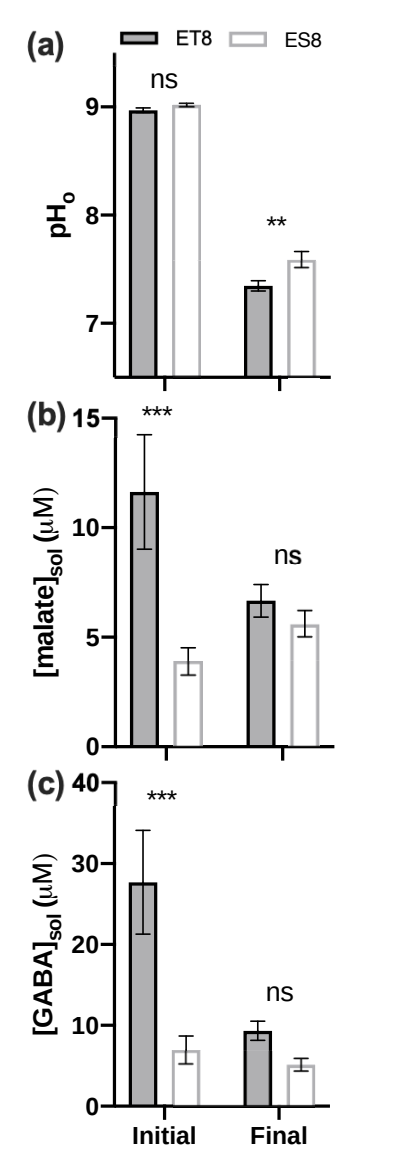

(a)
ET8
ES8
E (mmol m-2s-1)
5
4
3
2
1
0
a
A
a
A
*
(b)
ns
gs (mol m-2 s-1)
0.5
0.4
0.3
0.2
0.1
0.0
(c)
chlorophyll (SPAD)
25
20
15
10
5
0
a
A
a
**
B
pH 6.0
pH 9.0
(d)
A (μmol m-2s-1)
30
20
10
0
a
A
a
*
A
pH 6.0
pH 9.0

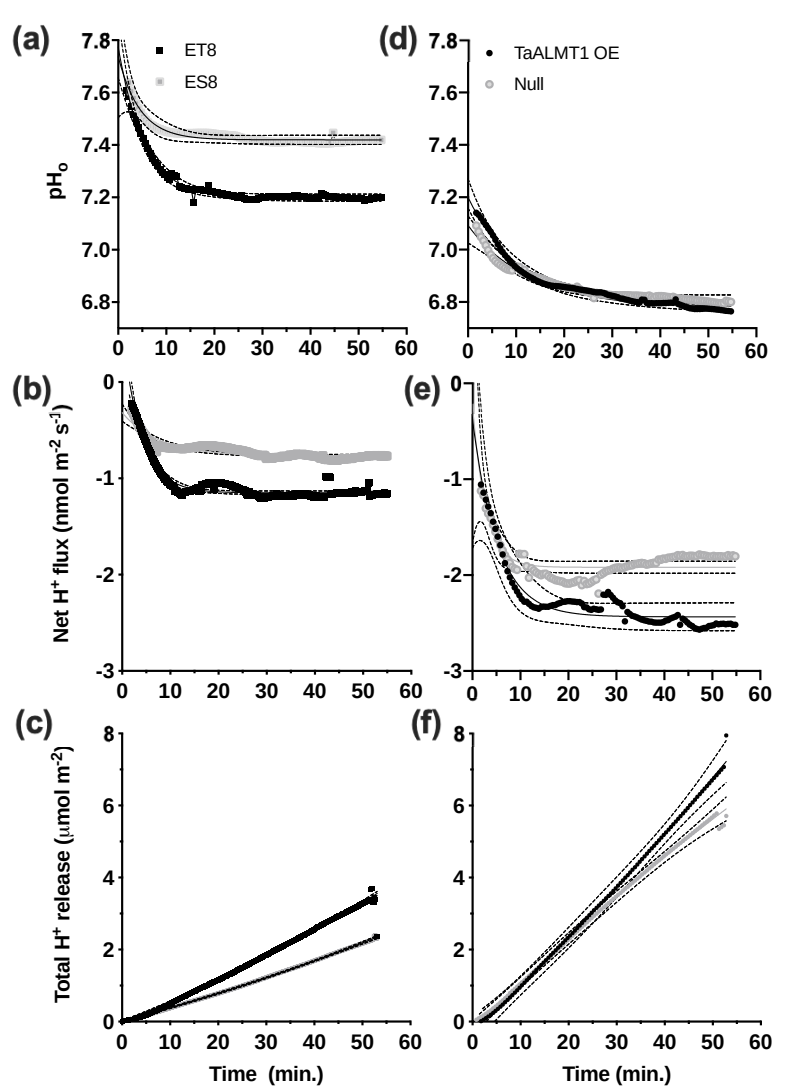

(a)
ET8
ES8
[malate]rhiz (nmol root gmwt-1)
150
100
50
0
**
b
a
A
A
(b)
GABAeffect [malate]rhiz (% control)
600
400
200
0
ns
#
(c)
[GABA]rhiz (nmol root gmwt-1)
800
600
400
200
0
**
b
a
A
A
pH 6
pH 9

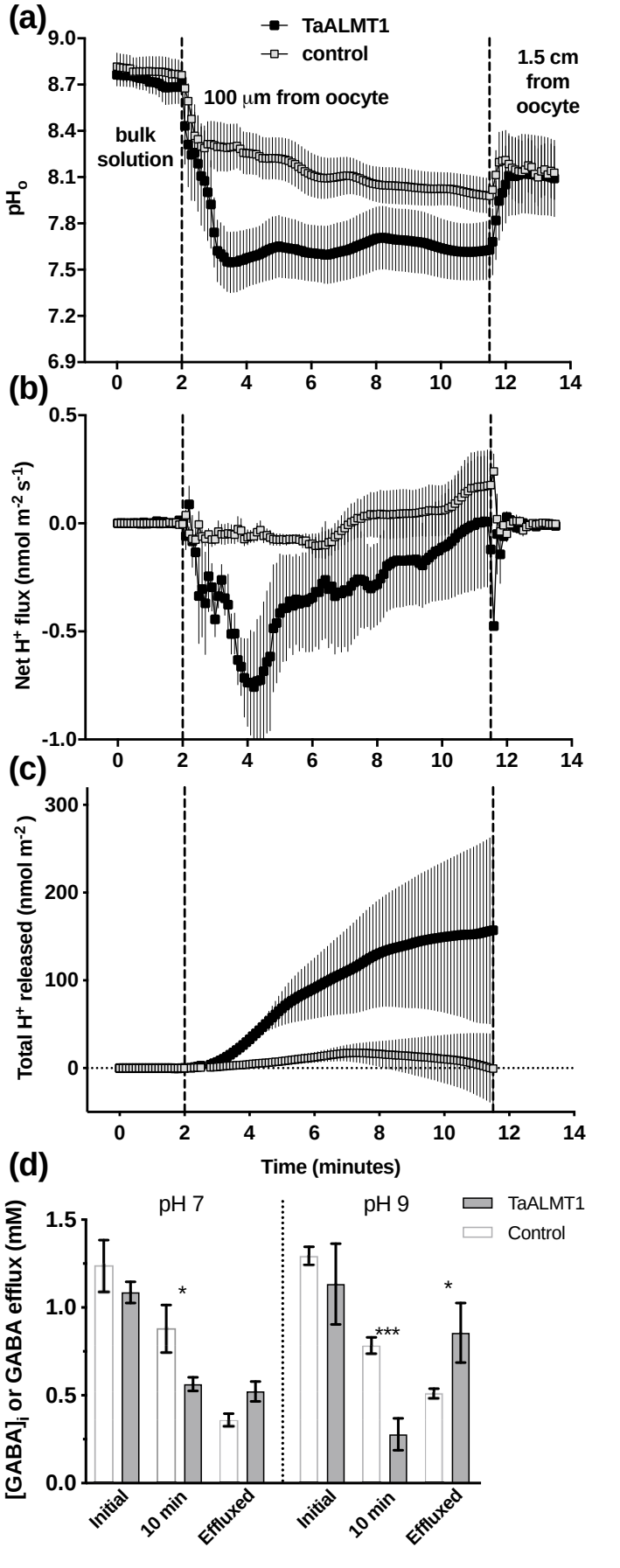

(a)
(b)
(c)
(d)
ET8
ES8
TaALMT1 OE
Null
malate efflux (nmol apex-1 h-1)
GABA efflux (nmol apex-1 h-1)
pH 6
pH 6 buffer
pH 9
pH 9 buffer
ns
*
****

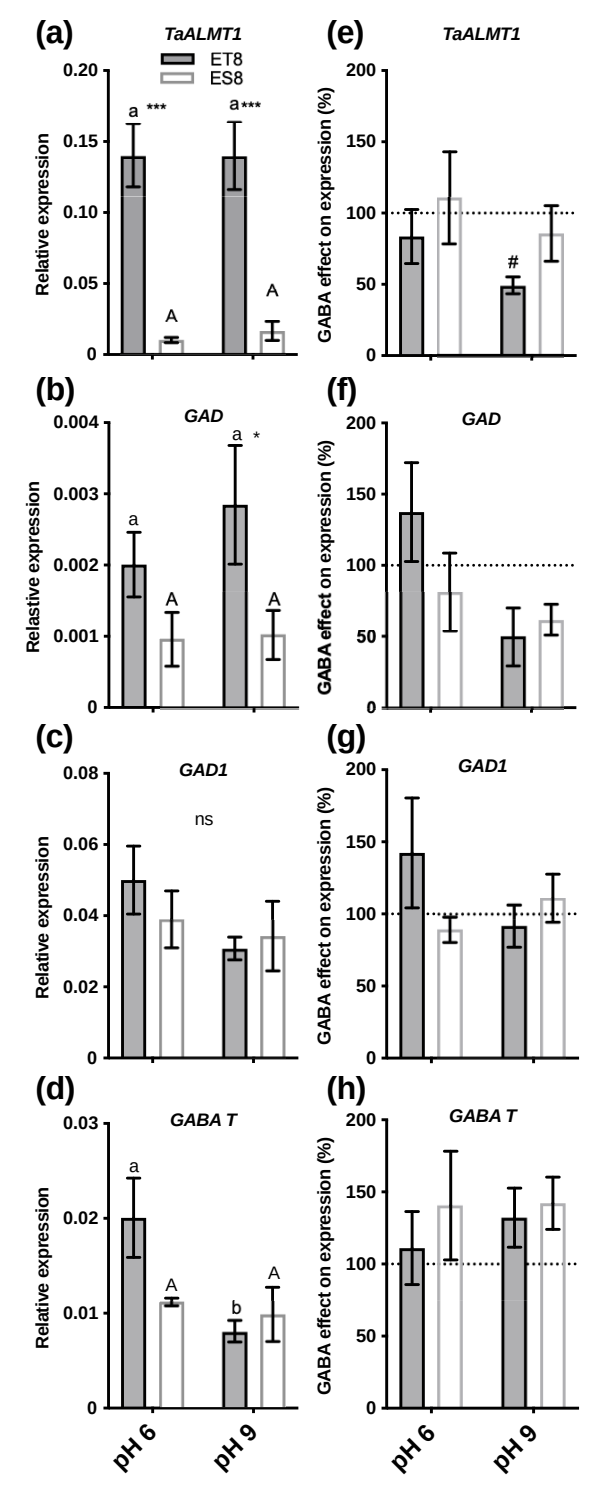

(a)
ET8
ES8
pH 4.5
pH 9.0
malate efflux (nmol apex-1 h-1)
2.0
1.5
1.0
0.5
0.0
****
**
**
*
***
**
(b)
malate efflux (nmol seedling-1 h-1)
2.5
2.0
1.5
1.0
0.5
0.0
****
**
*
****
MES
+Al
NaOH
KOH
BTP + Na2SO4
Carb.
Carb. + Na2SO4

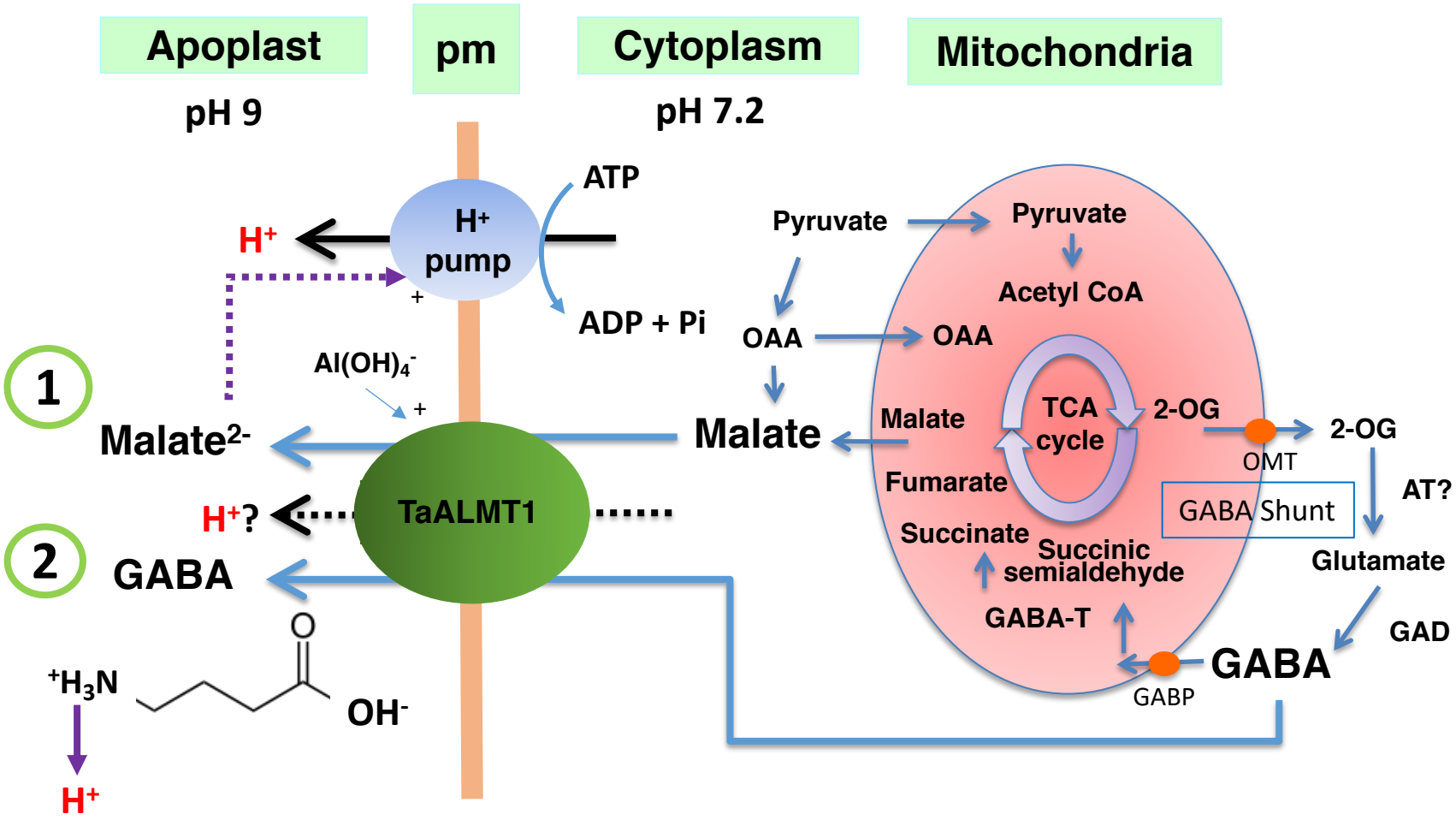

(a)
(b)
(c)
(d)
ET8
ES8
TaALMT1 OE
Null
malate efflux (nmol seedling-1 h-1)
GABA efflux (nmol seedling-1 h-1)
ns
**
****
a
b
A
basal pH 9
+ 100 µM NaAlO2


(a)
ET8
ES8
ns
**
pH$_o$
9
8
7
(b)
***
ns
[malate]$_{sol}$ (μM)
15
10
5
0
(c)
***
ns
[GABA]$_{sol}$ (μM)
40
30
20
10
0
Initial
Final


(a)
ET8
ES8
pH$_o$
7.8
7.6
7.4
7.2
7.0
6.8
0 10 20 30 40 50 60
(d)
TaALMT1 OE
Null
7.8
7.6
7.4
7.2
7.0
6.8
0 10 20 30 40 50 60
(b)
Net H$^+$ flux (nmol m^{-2} s^{-1})
0
-1
-2
-3
0 10 20 30 40 50 60
(e)
0
-1
-2
-3
0 10 20 30 40 50 60
(c)
Total H$^+$ release (μmol m^{-2})
8
6
4
2
0
0 10 20 30 40 50 60
Time (min.)
(f)
8
6
4
2
0
0 10 20 30 40 50 60
Time (min.)


(a)
TaALMT1
control
1.5 cm from oocyte
100 μm from oocyte
bulk solution
pH$_o$
9.0
8.7
8.4
8.1
7.8
7.5
7.2
6.9
0
2
4
6
8
10
12
14
(b)
Net H$^+$ flux (nmol m^{-2} s^{-1})
0.5
0.0
-0.5
-1.0
(c)
Total H$^+$ released (nmol m^{-2})
300
200
100
0
Time (minutes)
(d)
[GABA]$_i$ or GABA efflux (mM)
pH 7
pH 9
TaALMT1
Control
1.5
1.0
0.5
0.0
*
***
*
Initial
10 min
Effluxed
Initial
10 min
Effluxed


(a)
TaALMT1
ET8
ES8
Relative expression
0.20
0.15
0.10
0.05
0
a ***
a***
A
A
(e)
TaALMT1
GABA effect on expression (%)
200
150
100
50
0
#
(b)
GAD
Relastive expression
0.004
0.003
0.002
0.001
0
a
a *
A
A
(f)
GAD
GABA effect on expression (%)
200
150
100
50
0
(c)
GAD1
Relative expression
0.08
0.06
0.04
0.02
0
ns
(g)
GAD1
GABA effect on expression (%)
200
150
100
50
0
(d)
GABA T
Relative expression
0.03
0.02
0.01
0
a
A
b
A
(h)
GABA T
GABA effect on expression (%)
200
150
100
50
0
pH 6
pH 9
pH 6
pH 9


Apoplast
pm
Cytoplasm
Mitochondria
pH 9
pH 7.2
ATP
H+ pump
H+
ADP + Pi
Al(OH)4-
1
Malate2-
TaALMT1
H+?
2
GABA
+H3N
OH-
H+
Pyruvate
Pyruvate
Acetyl CoA
OAA
OAA
Malate
Malate
TCA cycle
2-OG
2-OG
OMT
Fumarate
GABA Shunt
AT?
Succinate
Succinic semialdehyde
Glutamate
GABA-T
GAD
GABA
GABP