pH regulation in anoxic rice coleoptiles at pH 3.5: biochemical pHstats and net H+ influx in the absence and presence of NO3−

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Abstract

During anoxia, cytoplasmic pH regulation is crucial. Mechanisms of pH regulation were studied in the coleoptile of rice exposed to anoxia and pH 3.5, resulting in H+ influx. Germinating rice seedlings survived a combination of anoxia and exposure to pH 3.5 for at least 4 d, although development was retarded and net K+ efflux was continuous. Further experiments used excised coleoptile tips (7–10 mm) in anoxia at pH 6.5 or 3.5, either without or with 0.2 mM NO3−, which distinguished two processes involved in pH regulation. Net H+ influx (μmol g−1 fresh weight h−1) for coleoptiles with NO3− was ~1.55 over the first 24 h, being about twice that in the absence of NO3−, but then decreased to 0.5–0.9 as net NO3− uptake declined from ~1.3 to 0.5, indicating reduced uptake via H+–NO3− symports. NO3− reduction presumably functioned as a biochemical pHstat. A second biochemical pHstat consisted of malate and succinate, and their concentrations decreased substantially with time after exposure to pH 3.5. In anoxic coleoptiles, K+ balancing the organic anions was effluxed to the medium as organic anions declined, and this efflux rate was independent of NO3− supply. Thus, biochemical pHstats and reduced net H+ influx across the plasma membrane are important features contributing to pH regulation in anoxia-tolerant rice coleoptiles at pH 3.5.

Key words: Acid load, anoxia tolerance, biochemical pHstat, H+ influx, NO3− uptake, pH regulation, H+ permeability, organic acids, ethanol production, rice coleoptile.

Introduction

Acidosis of the cytoplasm has been proposed to lead to the death of anoxic maize root tips (Xia and Roberts, 1996). Although this conclusion has been questioned (Greenway and Gibbs, 2003; Felle, 2005), there is agreement that acidosis is a consequence of an inability to maintain cellular compartmentation during anoxia. In this paper, the mechanisms of pH regulation in anoxia-tolerant rice coleoptiles (Kulichikhin et al., 2009) were further elucidated by exposing excised tips of coleoptiles to a combination of anoxia and pH 3.5. Assessment of functioning of the pHstats (i.e. systems that regulate the pH of the cytoplasm and vacuole to their set points, using biochemical and/or biophysical mechanisms) in rice coleoptiles under the challenging conditions of anoxia and an acid load is relevant to rice seeded directly into some flooded soils (Ismail et al., 2009). An acid load is an environment that tends to decrease the pH of cells such as exposure to a weak acid or a low external pH. Rice grown in acid sulphate soils (de Datta, 1981; Lang et al., 2010) will need to tolerate an acid load, and in other flooded soils an acid load may result from high concentrations of organic acids and/or H2CO3 (Ponnampерuma, 1984; Greenway et al., 2006).

When plant tissues become anoxic, decreases in the pH of the cytoplasm are common (Greenway and Gibbs, 2003;
Felle, 2005). In anoxia-tolerant tissues, although cytoplasmic pH declines, it soon stabilizes well above pH 7.0; examples are (i) stem segments of *Potamogeton* for at least 9 or for 7 h anoxia (Dixon et al., 2006; Koizumi et al., 2011), (ii) rice ‘shoots’ (presumably mainly coleoptiles) for at least 14 h anoxia (Menegus et al., 1991), and (iii) excised tips of rice coleoptiles at pH 6.5, for which a stable cytoplasmic pH has been observed between 60 and 78 h anoxia (Kulichikhin et al., 2009). Such observations on anoxia-tolerant tissues are consistent with the hypothesis that under anoxia the decrease in cytoplasmic pH will not be perceived as an error signal but reflects a decrease in the set point for cytoplasmic pH (i.e. the pH value that is maintained by a pHstat), consistent with the greatly altered metabolism during anoxia (Felle, 2005).

The putative regulation of intracellular pH can be investigated further by imposing acid loads. pH regulation despite an acid load may be achieved by: (i) a biochemical pHstat, (ii) a biophysical pHstat, i.e. H⁺ extrusion across the plasma membrane (Smith and Raven, 1979), and/or (iii) a decrease in H⁺ influx. Rice coleoptile tips are an excellent experimental system for studies of pH regulation during anoxia because these organs can survive at least 90 h anoxia, even when exposed to pH 3.5 for the last 30 h (Kulichikhin et al., 2009). Evidence for the health of these tissues, even after exposure to pH 3.5 during anoxia, consists of the vigorous net uptakes of K⁺ and Cl⁻ following a return to aerated solution (Kulichikhin et al., 2009). When anoxic rice coleoptile tips were transferred from pH 6.5 to pH 3.5, the cytoplasmic pH only dropped from 7.35 to 7.2 with a half-time of 0.5 h and then remained steady for at least 17 h, while vacuolar pH decreased from 5.7 to 5.25 with a half-time of 1–1.5 h (Kulichikhin et al., 2009). During the following 15 h, the vacuolar pH remained steady for 5.3 and 5.4 despite a continuous large net H⁺ intake of 2.8 μmol g⁻¹ fresh weight h⁻¹ for the first 12 h, and then of 1 μmol g⁻¹ fresh weight h⁻¹ during the last 6 h, at pH 3.5 (Kulichikhin et al., 2009).

In the present paper, the participation of a biochemical pHstat in pH regulation of anoxic rice coleoptile tips was explored by measuring changes in cellular concentrations of organic acids, amino acids, putrescine and selected inorganic ions, as well as net fluxes of H⁺, K⁺, NH₄⁺ and NO₃⁻, all when the tissue was anoxic and challenged with an acid load. The acid load was imposed by transfer to pH 3.5. In addition, treatment with or without a NO₃⁻ supply indicated a link between H⁺ and NO₃⁻ uptake, presumably via H⁺–NO₃⁻ symports, and showed a reduction in rate of this putative H⁺–NO₃⁻ co-transport with time after transfer to pH 3.5.

Materials and methods

Rice (*Oryza sativa* L. cv. Amaroo), an anoxia-tolerant cultivar, at least during germination (Huang et al., 2003), was used in experiments to assess acclimation during anoxia to an acid load, consisting of exposure to pH 3.5 (Kulichikhin et al., 2009). One experiment used intact seedlings and all others used excised 7–10 mm tips of coleoptiles from ~3-d-old seedlings. For the excised coleoptile tips, the following were carried out: (i) time courses of ion net fluxes and degradation of organic acids; and (ii) experiments that established to what extent net H⁺ entry was linked to net NO₃⁻ uptake.

Common procedures included treatment in 50 ml conical flasks at 30°C in the dark. The flasks were sealed with rubber bungs and had one inlet and one outlet. All tubing was Tygon, which has a very low permeability to O₂. Continuous flushing ensured anoxia in solutions bubbled with high-purity N₂ gas. O₂ in the outlet of the N₂-flushed system was below the detection limit of 0.01% (gas chromatograph; Huang et al., 2005). The volume of incubation solution depended on the objective. For the intact seedlings it was 50 ml, while for the excised coleoptile tips it varied between 10 and 40 ml, depending on the length of the interval and the requirement to measure appreciable differences over intervals in concentrations of ions and H⁺ to calculate net fluxes. Uniform bubbling rates of gases (see below) were achieved in the flasks by inserting high resistances in the tubes leading to the individual vessels by using hypodermic needles of 0.4 mm diameter (Teruma G 27). The basal nutrient solution contained (in mM): Ca²⁺ 0.5, K⁺ 0.2, NH₄⁺ 0.1, NO₃⁻ 0.2, and SO₄²⁻ 0.45. The basal solution also contained 50 mg l⁻¹ ampicillin and 0.2 mM MES, and the pH was adjusted to 6.5 with Ca(OH)₂. When NO₃⁻ was omitted from the solution, SO₄²⁻ was 0.55 rather than 0.45 mM to maintain ion balance.

Solutions were flushed continuously with high-purity N₂ and refreshed every 12–24 h; the used medium was removed, without interruption of anoxia, using a 150 mm long hypodermic needle (gauge 14) with a blunt tip and re-injecting solutions that had been pre-flushed with high-purity N₂ so that anoxia was continuous. Loss of coleoptiles into the hypodermic syringe was avoided by inserting a thin stainless-steel wire into the tip of the hypodermic needle.

For the intact seedlings, ten seeds in each flask were exposed from the start of imbibition to high-purity N₂-flushed nutrient solution at either pH 3.5 or pH 6.5 in both cases unbuffered. For excised coleoptile tips, the time schedule, including the various treatments, is shown in Fig. 1. Raising seedlings and pre-treatment of excised tips have been described in previous papers (Huang et al., 2005; Kulichikhin et al., 2009). Briefly, after 48 h aerated followed by an 18 h hypoxic pre-treatment at 0.05 mM O₂, 7–10 mm tips were excised from coleoptiles and 0.1–0.13 g fresh weight was each placed in a 50 ml conical flask. After 5 h heating at 0.05 mM O₂, anoxia was imposed. The substrate supply

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**Fig. 1.** Schematic presentation of the O₂ regime, pH treatment, and composition of the medium.
of the seed was replaced by exogenous glucose. Glucose was at 20 mM after excision and was increased to 50 mM during anoxia, because at 20 mM glucose endogenous sugar levels still decrease over several days of anoxia (Huang et al., 2005). All stages were in darkness.

Treatment at pH 3.5
In all but one experiment with excised coleoptile tips, the pH was changed from 6.5 to 3.5 after 60 h anoxia. Recently, we found that this long pre-treatment under anoxia at pH 6.5 was not required, as similar responses during anoxia and after return to aeriation were obtained when the pH was changed to pH 3.5 after only 24 h anoxia at pH 6.5.

At pH 6.5, MES was at 0.2 mM, while at pH 3.5 there was no MES and usually no buffer, as the tissue/volume ratio was kept low enough to keep the external pH within 0.3 units or less from the starting pH. However, 0, 0.2 or 2 mM β-alanine was used in one experiment, as a check, as it was necessary to relate the present findings to earlier experiments using 1H-nuclear magnetic resonance (NMR) spectroscopy, which used β-alanine to buffer the solutions at pH 3.5 (Kulichikhin et al., 2009). During preparation of the pH 3.5 solution, the pH adjustment was with H2SO4.

Measurement of H+ net fluxes
Net fluxes of H+ were measured by titration of the medium with 1 mM NaOH or 1–2.5 mM H2SO4, after various periods of exposure of the tissues. For the first sampling period, the collected medium was titrated to the pH of a 10 ml subsample, taken ~3 min after the change from pH 6.5 to 3.5. This procedure avoided variability due to the residual small volumes of solution used prior to the pH change, from the walls of the flasks and the free space of the tissue. Values were further corrected for blanks, which were flushed for the same period as the solutions containing the tissues. Titration of H+ within 1–2 h after collection of the solutions was required, due to a slow drift in pH of these weakly buffered solutions upon storage.

Preparation of extracts and measurements of ions in nutrient solutions and tissues
Selected ions were measured in the external medium and in tissue extracts. As the primary goal of the experiments was to evaluate the biochemical pHstat, tissues were extracted with ice-cold 5% PCA. The recoveries (% SE) were: malate 90 ± 4, citrate 95 ± 7, shikimic acid 87 ± 5, fumarate 94 ± 3, succinate 87 ± 5, alanine 92 ± 6, and putrescine 88 ± 11. Data were corrected for these recoveries.

Organics in tissue extracts and in nutrient solution samples were analysed by HPLC (600E pump, 717 plus autoinjector, 996 photodiode array detector (PDA), Waters, Milford, MA, USA) by slightly adapting the method described by Crawthay (2003). Organic acid standards were: acetic, cis-aconitic, trans-aconitic, citric, iso-citric, fumaric, lactic, malic, maleic, malonic, shikimic, and succinic acids. In brief, separation was achieved at 22 ± 0.5°C on a Prevail C-18 column (250 x 4.6 mm internal diameter (i.d.) with 5 μm packing; Alttech Associates, Deerfield, IL, USA) with a mobile phase consisting of 25 mM KH2PO4 at pH 2.5 at 1 ml min−1. A gradient-elution program using 60% methanol was used every fifth sample to flush the column of the more hydrophobic compounds and reduce carryover. Detection was at 210 nm, with PDA acquisition from 190 to 400 nm to enable positive identification of organic acids by comparing retention time and PDA peak spectral analyses, including peak purity, of standards with the unknowns. Calibration curves for each organic acid were generated from peak area versus the mass of standard organic acid injected. Data acquisition and processing were with Millenium 32 software version 3.05 (Waters, Milford, MA, USA).

Amino acid analysis by GLC-FID for tissue extracts and nutrient solution samples was conducted with a Phenomenex EZ:faast analysis kit (Lane Cove, NSW, Australia), adapted from the method of Nozal et al. (2004). Amino acid standards were: L-alanine, α-aminoacidic acid, γ-aminobutyric acid, α-aminoisobutyric acid, asparagine, aspartic acid, cystine, glutamic acid, glutamine, glycine, histidine, leucine, allo-isoleucine, isoleucine, lysine, methionine, ornithine, phenylalanine, proline, 4-hydroxyproline, sarcosine, serine, threonine, tryptophan, tyrosine, and valine. A GC-17A (Shimadzu, Kyoto, Japan) coupled with an AOC-20i autoinjector (Shimadzu, Kyoto, Japan) and controlled by GC Solution version 2.30 software was used. The column was a Zebro ZB-AAA amino acid GC column of 10 m x 0.25 mm from Phenomenex (Lane Cove, NSW, Australia), with a carrier gas of high-purity He at 1.5 ml min−1. The oven temperature regime was as follows: initial temperature 110°C with a 32°C min−1 increase to 320°C and held at 320°C for 0.50 min. The injection port temperature was 250°C and the detector was held at 320°C. Samples were injected at 1 μl in
split mode (1:15 or 1:5). Solutions of known standards at 200 μM were treated in the same way as samples. The peak areas of standard amino acid derivatives were used to quantify the concentrations of amino acids in samples using the internal standard (norvaline) method (see Nozal et al., 2004).

The above GLC-FID method did not separate serine from γ-amino butyric acid; thus, separation and quantification of these two compounds was achieved using HPLC, with the Waters AccQ.Tag package. The HPLC system consisted of a 600E dual-head pump, 717 plus autosampler, 470 scanning fluorescence detector, and a Nova-Pak C18 column (150 mm length x 3.9 mm i.d.) with 4 μm particle size (all from Waters, Milford, MA, USA), held at 35°C. HPLC data were acquired and processed using Empower chromatography software (Waters, Milford, MA, USA) with a fluorescence detector (excitation: 250 nm; emission: 395 nm; filter: 0.5; gain: 100).

Putrescine analysis involved dansylation (Bartzatt, 2003) and subsequent separation by HPLC (600E pump, 717 plus auto-injector, 996 PDA and 470 fluorescence detectors; Waters, Milford, MA), adapted from the method of Linares et al. (1998). A 200 μl aliquot of standard or sample was vortexed with 200 μl 2 M Na2CO3 and 60 μl dansyl chloride in acetone solution (5 mg ml−1). Sealed samples were then incubated at 40°C, before extraction of the derivitized putrescine into ether (2 x 200 μl), which was then dried down and the residue redissolved in 600 μl acetonitrile for direct HPLC injection. Separation was achieved with an Alltima C-18 column (150 mm length x 4.6 mm i.d.) with 5 μm packing (Alltech Associates, Deerfield, IL, USA), at 22 ± 0.5°C with a flow rate of 1.2 ml min−1 of a mobile phase of 25% Milli-Q water and 75% acetonitrile. Detection was with both the PDA set at 216 nm for peak purity and spectrum matching, and the fluorescence detector (excitation: 320 nm; emission: 523 nm; gain: 100) with outputs used for quantification based on peak area.

Calculations

Data on tissue solutes are presented on a fresh-weight basis. However, for calculations involving relationships between ions and comparison with previously presented pH changes in vacuole and cytoplasm (Kulichikhin et al., 2009), the solute concentrations on a fresh-weight basis were converted to a protoplast-water basis, by multiplication with a factor of 1.17. This factor is based on 9% dry weight (Alpi and Beavers, 1983, and unpublished data) and 5% free space (Pitman, 1963), and allowing for 1% water adhering to the tissue surface.

The amount of H+ that could be accommodated by the organic acid pHstat was calculated as shown in Appendix 1. The permeability of the plasma membrane to H+ (PfH+) was calculated according to Lütge and Higimbotham (1979) and the free energy according to Nobel (1974), taking the previously measured cytoplasmic pH for excised coleoptiles as 3.5 (Kulichikhin et al., 2009) and a plasma membrane potential (Em) of depolarized cells of rice coleoptiles (Zhang and Greenway, 1995), as the cells at 3.5 are likely to be depolarized as indicated by the K+ influxes (see Fig. 5). The values of PfH+ and free energy were therefore only approximate; deviations were, however, unlikely to be large (e.g. even if Em was 20 mV different from the assumed value of ~80 mV, the free energy would only be ~7% different from those given in the text). The PfH+ calculations also used unpublished data on cell dimensions to estimate total cellular surface areas in anoxic rice (cv. Calrose) coleoptiles, provided by BJ Atwell (Macquarie University, Australia). The PfH+ was based on net H+ influxes normalized to pH 3.5; normalization was needed for several reasons: the solutions were not buffered, the pH changed by 0.1–0.3 units during incubation, and starting pH differed by a small amount between different treatments and fresh solutions used at different times (described in Fig. 5).

Statistical analyses of data

Data were analysed by calculation of means, standard error of the mean (SE) and analysis of variance where appropriate, using GenStat version 12.1.

Results

Effects of pH 3.5 during anoxia, imposed on germinating seedlings

This experiment: (i) tested whether the previously observed high tolerance of excised rice coleoptile tips to a combination of anoxia and pH 3.5 (Kulichikhin et al., 2009) also exists in intact, germinating seedlings; and (ii) evaluated the effects on growth and survival.

At the end of 4 d anoxia, the length of the coleoptile was 3.5-fold smaller at pH 3.5 than at pH 6.5; hence, growth was reduced at the lower pH. Nevertheless, the seedlings survived the challenging combination of anoxia and pH 3.5, as shown by rapid elongation of the first leaf after a shift to an aerated solution at pH 6.5 (Table 1A). The shorter leaf length of the seedlings that had been at pH 3.5 during anoxia (Table 1A) was presumably mainly due to the delayed development during anoxia rather than to reduced elongation after the return to aeration at pH 6.5 (Table 1A).

Table 1. Effects of pH 3.5 or 6.5 on germinating, intact rice seedlings during anoxia started at imbibition

| Treatment | pH 6.5 | pH 3.5 |
|-----------|--------|--------|
| Length coleoptile (mm) after 4 d in anoxia | 7.3 ± 0.5 | 2.0 ± 0.1 |
| Aeration starting at d 5 | Returned from pH 3.5 to pH 6.5 |
| Length leaf (mm) after 2 d in aeration following 4 d of anoxia | 19.5 ± 1.1 | 9.6 ± 0.9 |

B. K+ net fluxes under anoxia

Table 1. Effects of pH 3.5 or 6.5 on germinating, intact rice seedlings during anoxia started at imbibition

| Days after start of imbibition | K+ net uptake or loss (nmol per seeding h−1)* |
|-------------------------------|-------------------------------------------|
| pH 6.5 | pH 3.5 |
| 1 | −4.8 ± 2.8 | −5.5 ± 0.3 |
| 2 | −1.6 ± 0.9 | −5.1 ± 0.6 |
| 3 | 1.5 ± 0.8 | −5.5 ± 0.3 |
| 4 | 1.0 ± 1.6 | −4.0 ± 1.6 |
| Total K+ loss over 4 d (nmol per seeding) | 94 | 482 |

* Negative values represent loss to the solution.
The seedlings lost substantial K\(^+\) during the first day of anoxia, even at pH 6.5; however, at this pH these K\(^+\) losses declined on the second day and subsequently there was a small net K\(^+\) uptake during the last 2 days of the experiment (Table 1B). In contrast, at pH 3.5, the net K\(^+\) losses continued at the same rate throughout the 4 days of anoxia (Table 1B). The responses for net K\(^+\) fluxes in these intact seedlings were similar to those for excised coleoptile tips (Kulichikhin et al., 2009). The severe decrease in elongation of the coleoptile at pH 3.5 under anoxia contrasts with the stimulatory effects of acidity on extension growth in aerobic oat coleoptiles (Cosgrove, 2001).

Having shown that an acid load can impair the growth of intact coleoptiles of seedlings in anoxia, the further investigations reported in this paper aimed at elucidating the mechanisms of intracellular pH regulation under anoxia, and hence excised coleoptile tips were used. Excised coleoptile tips provide a simpler system than intact coleoptiles and the data also assisted in elucidating the mechanisms involved in the regulation of cytoplasmic and vacuolar pH in coleoptile tips measured in our previous \[^{31}P\]NMR spectroscopy experiments (Kulichikhin et al., 2009).

**Protein synthesis**

The large effects on growth of the intact coleoptiles at pH 3.5 raised the question of whether in the excised system net protein synthesis was similarly affected. Net protein synthesis was never found at pH 3.5 (four separate experiments). At pH 6.5, there was net synthesis in two experiments, e.g. of \(2.3\pm0.9\) mg g\(^{-1}\) fresh weight between 0 and 90 h (Table 2), but no measurable net protein synthesis in two other experiments (data not shown). However, there was turnover of proteins, even in anoxia at pH 3.5, between 24 and 28 h after start of the pH 3.5 treatment: \[^{14}C\]leucine incorporation, expressed as the percentage of total leucine incorporation, was 7.4% at pH 3.5 and 7.2% at pH 6.5 (only assessed in one experiment). Thus, overall there was little evidence of any substantial differences in protein synthesis between anoxic coleoptile tips at pH 3.5 and 6.5.

**Table 2.** Total protein (mg g\(^{-1}\) fresh weight) in excised rice coleoptile tips before and after exposure to anoxia at pH 6.5 or 3.5.

| pH treatment | 60 h | 78 h | 90 h |
|-------------|------|------|------|
| pH 6.5      | 8.0±0.9 | –    | 9.2±0.5 |
| pH 3.5 starting at 60 h | – | 8.4±0.8 | 7.6±0.2 |
| pH 3.5 between 60 and 78 h, then pH 6.5 | – | – | 7.1±0.6 |

**Organic and amino acids**

Our first priority was to ascertain to what extent the biochemical pHstat contributed to pH regulation. As mentioned before in an earlier paper, changes in cytoplasmic and vacuolar pH were evaluated in anoxic excised coleoptile tips at pH 3.5, and a substantial net influx of H\(^+\) was apparent at pH 3.5 (Kulichikhin et al., 2009). The present experiments investigated possible changes in the concentrations of organic and amino acids with time of exposure to pH 3.5. The earlier experiments used \(\beta\)-alanine as a buffer at pH 3.5 (Kulichikhin et al., 2009), while in the present experiments the pH of the external solution was retained within 0.3 units of the starting value by using suitable tissue-to-volume ratios. To facilitate linking of the results of these two investigations, it was therefore first established that \(\beta\)-alanine had no effect on the changes in endogenous organic and amino acids (data in Appendix 2).

We then measured endogenous organic solutes involved in a possible biochemical pHstat for coleoptile tips at pH 6.5 or 3.5 without \(\beta\)-alanine. During the first 60 h anoxia at pH 6.5, there were no consistent changes in malate concentration, which ranged between 9 and 10 \(\mu\)mol g\(^{-1}\) fresh weight, while there was net succinate formation of \(~2.7\) \(\mu\)mol g\(^{-1}\) fresh weight (Fig. 2). Exposure to pH 3.5 during anoxia decreased the concentrations of malate from 9–10 to \(~3.8\) \(\mu\)mol g\(^{-1}\) fresh weight during the first 18 h, with further decreases to \(~2\) \(\mu\)mol g\(^{-1}\) fresh weight during
the next 18 h at pH 3.5 (Fig. 2). Succinate also decreased at pH 3.5 although more gradually than malate (Fig. 2). Fumarate was at very low levels but followed a similar trend to malate and succinate (Fig. 2). Anoxia at pH 6.5 greatly increased the L-alanine concentration from 2 to ~30 µmol g⁻¹ fresh weight over the first 60 h of anoxia and then to ~43 µmol g⁻¹ fresh weight over the next 40 h (Fig. 3A). After transfer of coleoptile tips to pH 3.5 at 60 h anoxia, the means of three experiments showed no appreciable changes in L-alanine (Fig. 3A), while there was an appreciable increase in a fourth experiment (although this was only about half of the increase at pH 6.5; Fig. 4B). At pH 6.5, γ-aminobutyric acid increased from ~1.5 to 2.8 µmol g⁻¹ fresh weight during the first 60 h in anoxia; after 60 h the increase at pH 6.5 slowed but accelerated at pH 3.5, and this increase after 60 h was ~8-fold greater at pH 3.5 than at pH 6.5 (Fig. 3B). Furthermore, the analyses showed that serine increased from 1.6 µmol g⁻¹ fresh weight at the end of hypoxia to 3.4–6 µmol g⁻¹ fresh weight during anoxia, with little difference between pH treatments.

Fig. 3. Amino acids (µmol g⁻¹ fresh weight) in excised tips of rice coleoptiles under anoxia in the presence of 0.2 mM NO₃⁻ at pH 6.5 and after 60 h at either pH 6.5 or 3.5. (A) L-alanine, (B) γ-aminobutyric acid (GABA). The start of anoxia is at 0 h. pH 6.5, open squares, thin line; pH 3.5, closed squares, bold line. Data are the means of three experiments, each with three replicates. Values at 0 h are tissues sampled at the end of an 18 h pre-treatment at 0.05 mM O₂. In one experiment, coleoptile tips were returned at 78 h from pH 3.5 to 6.5 while maintaining anoxia, and after another 18 h L-alanine had decreased from 30 to 21 (µmol g⁻¹ fresh weight); no data for γ-aminobutyric acid are available after this shift.

The changes in organic and amino acids at pH 6.5 were consistent with earlier experiments with rice coleoptiles (Fan et al., 1997). Rates of L-alanine net synthesis observed here were of the same magnitude as in young shoots of intact rice seedlings exposed to anoxia at 3 d after imbibition, which synthesized 35 µmol g⁻¹ fresh weight L-alanine over 48 h anoxia (calculated from Menegus et al., 1993). The L-alanine accumulation would contribute ~0.1 MPa to the osmotic pressure compared with the observed osmotic pressures of 0.4–0.6 MPa in anoxic rice coleoptiles (Atwell et al., 1982; Menegus et al., 1984). This contribution gains in importance when the tissues are transferred to pH 3.5, as the L-alanine accumulated previously assists in keeping the osmotic pressure at a tolerable level despite losses of other solutes, for example K⁺ by 0.125 MPa and organic acids by 0.065 MPa (estimated from the present data). Following the transfer of tips from pH 3.5 back to pH 6.5 with continuous anoxia, the organic acids remained at the levels attained at pH 3.5 (Appendix 3), while there was a possible decrease in L-alanine (see caption of Fig. 3).

Some other solutes also changed in concentration during anoxia, although the levels were rather low. Valine and putrescine, present at ~0.3 µmol g⁻¹ fresh weight at the start of anoxia, increased by 4- and 2-fold, respectively, over the first 60 h anoxia. The value for putrescine was approximately half of the value reported for coleoptiles of intact anoxic seedlings (calculated from Menegus et al., 1993), but the role of putrescine in the pHstat suggested by Menegus et al. (1993) was not evident in the present experiment, i.e. there was no appreciable net putrescine synthesis after transfer to pH 3.5. There were no appreciable changes in asparagine during anoxia or exposure to pH 3.5 (data not shown).

The three experiments on organic and amino acids (Figs 2 and 3) were with tissues supplied with 0.2 mM NO₃⁻. In a subsequent experiment, treatments with or without NO₃⁻ were started after 60 h anoxia, and after the next 54 h at pH 3.5 the malate and succinate pools were not detectable with or without NO₃⁻ in the medium (data not shown; the detection limits were 8 and 17 nmol g⁻¹ fresh weight for malate and succinate, respectively). The amino acid data for coleoptile tips with or without NO₃⁻ (Fig. 4) are relevant to the fate of the absorbed NO₃⁻ (rates of NO₃⁻ uptake are shown in Fig. 5 and discussed below). Total N in the PCA-soluble extract was higher in the presence of NO₃⁻ than in its absence, mainly due to higher levels of L-alanine and serine in the treatment with NO₃⁻ (Fig. 4). However, changes in γ-aminobutyric acid were more dependent on the external pH than on the NO₃⁻ supply, i.e. exposure for 54 h to pH 3.5 increased γ-aminobutyric acid levels by ~85 and ~120% in the absence and presence of NO₃⁻, respectively; in contrast, there was little change in γ-aminobutyric acid at pH 6.5 (Fig. 4).

Importantly, absorbed N did not flow to PCA-insoluble compounds; analysis of the washed and dried pellet remaining after PCA extraction of the coleoptile tips, using a Macro Elementar Analyser, showed that both the amount of N (by dry weight) at ~3.9% and the C:N ratio of 10.9–11.2 were similar in all treatments. Hydrolysis of some PCA-insoluble
compounds is a probable contributor to the increases in PCA-soluble total N after 60 h, as such increases occurred even in the pH 3.5 treatment without NO$_3$/C0$_3$ (Fig. 4), for which there was no net N uptake (i.e. net NH$_4$ uptake was close to zero) (Fig. 5). However, whether hydrolysis occurred could not be ascertained from the present data, as there were only values for the percentagage of N, while no assessments were made for the total amount of N in the PCA-insoluble fraction.

Finally, experiments were carried out to establish what role the organic acid pHstat and the associated K$^+$ fluxes played in the previously observed increase in vacuolar pH following the return from pH 3.5 to 6.5 (Kulichikhin et al., 2009). Overall, there was remarkably little change in the levels of malate and succinate and K$^+$ fluxes, as shown in Appendix 3.

Net fluxes of H$^+$, K$^+$, NH$_4$ and NO$_3$ during anoxia at pH 6.5 or 3.5

Net H$^+$ fluxes were required to assess to what extent the organic-acid-based pHstat could cope with an acid load. These experiments included treatments of 0 or 0.2 mM NO$_3$$_-$, as net NO$_3$$_-$ uptake by anoxic coleoptile tips supplied with 0.2 mM NO$_3$$_-$, at pH 3.5 or 6.5, can be substantial (Fig. 5B). H$^+$ influx into the cells at pH 3.5 (discussed below) would clearly be driven by the large electrochemical potential gradient across the plasma membrane, assessed at 30 kJ mol$^{-1}$. Furthermore, no organic solutes leaked to the medium (solutions sampled, freeze dried to concentrate and assayed as described in Materials and methods for tissue extracts), indicating that the net H$^+$ decrease in the medium was associated with net H$^+$ influx into the cells, rather than with exchange of weak acids or bases.

Before describing the net ion fluxes during anoxia, we first considered possible injury of the coleoptile tips when challenged by anoxia in combination with pH 3.5, either with or without NO$_3$. Possible injury was tested by return to an aerated solution after anoxia and the two pH treatments. Based on rates of net uptake of K$^+$ and Cl$^-$, there was no indication of serious injury for either treatment; as in earlier experiments, which only had solutions with 0.2 mM NO$_3$, net K$^+$ uptake by coleoptile tips with or without NO$_3$ in the incubation medium was rapid after the return to aerated conditions, while net Cl$^-$ uptake was ~30% lower in the tissues that had been exposed to pH 3.5 compared with pH 6.5 but only for the first 4 h after re-aeration (data not presented).

Ion net fluxes at pH 6.5 will be only briefly mentioned, as the central theme of this paper is the response to exposure at pH 3.5. At pH 6.5, net fluxes of H$^+$ and K$^+$ were

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**Fig. 4.** Amino acids and total N in PCA-soluble extracts from anoxic coleoptiles, in the presence or absence of 0.2 mM NO$_3$ and at pH 3.5 or 6.5. From 0 to 60 h, coleoptiles were at pH 6.5 and with 0.2 mM NO$_3$, and treatments were imposed after the 60 h anoxia. Open bars indicate incubation without NO$_3$ and closed bars indicate with NO$_3$. Results on ion fluxes from the same experiment are presented in Fig. 5. pH treatments commenced at 60 h after starting anoxia, and the final sampling was 54 h later.
variable, with net uptakes or losses at different time periods (Fig. 5A, C). There was some net K⁺ uptake at certain periods: these were between 0.1 and 0.2 μmol g⁻¹ fresh weight h⁻¹ (Fig. 5C), as has been observed previously (Colmer et al., 2001; Kulichikhin et al., 2009). Net NO₃⁻ uptake was ~0.5 μmol g⁻¹ fresh weight h⁻¹ (Fig. 5B). Anion uptake under anoxia is not unique to NO₃⁻, as the coleoptile tips in the present experiments showed a net Cl⁻ uptake of ~0.5 μmol g⁻¹ fresh weight h⁻¹ between 60 and 84 h after the start of anoxia (data not shown; Cl⁻ was supplied in the absence of NO₃⁻, and no measurements were taken after 84 h). Despite the substantial net uptake of NO₃⁻, and for the pH 6.5 treatment also some net uptake of NH₄⁺, tissue concentrations of these ions remained low and independent of pH, being ~0.2 and ~0.3 μmol g⁻¹ fresh weight for NH₄⁺ and NO₃⁻, respectively.

At pH 3.5 with 0.2 mM NO₃⁻, the net H⁺ influx between 0 and 24.5 h was ~1.55 μmol g⁻¹ fresh weight h⁻¹, and then dropped to ~0.5–0.7 μmol g⁻¹ fresh weight h⁻¹ between 24.5 and 54 h at pH 3.5; these time trends were similar to those in two other experiments in the present study and in an earlier investigation (Kulichikhin et al., 2009). In contrast, without NO₃⁻, the net H⁺ influx for the first 5 h at pH 3.5 was only half of that in 0.2 mM NO₃⁻, and this net H⁺ influx did not appreciably slow with time (Fig. 5A). Rates of net NO₃⁻ uptake during the first 24 h at pH 3.5 were as high as ~1.3 μmol g⁻¹ fresh weight h⁻¹, being ~2.5 times higher than at pH 6.5. Similarly, high rates of net Cl⁻ uptake during the first 8 h after exposure to pH 3.5 were observed (measured in the absence of NO₃⁻, data not shown). At pH 3.5 the net NO₃⁻ uptake decreased substantially with time, reaching the rate of the tissue at pH 6.5 between 46 and 54 h after the start of exposure to pH 3.5 (Fig. 5B). Net losses of NH₄⁺ were 0.24–0.35 μmol g⁻¹ fresh weight h⁻¹ when NO₃⁻ was supplied, with little net loss of NH₄⁺ from tissues not supplied with NO₃⁻ (Fig. 5D).

Net K⁺ loss from the coleoptiles at pH 3.5 was higher in the presence than in the absence of NO₃⁻ during the first 5 h, but then became similar during the next 41 h of exposure to pH 3.5 (Fig. 5C). Rates of net K⁺ loss increased during the last 7 h of the experiment (i.e. between 107 and 114 h after the start of anoxia; in contrast, no such increases were observed in previous experiments).

Fig. 5. Net fluxes of H⁺ (A), NO₃⁻ (B), K⁺ (C) and NH₄⁺ (D) in the presence or absence of 0.2 mM NO₃ during anoxia at pH 3.5 or 6.5. Treatments were imposed after 60 h anoxia, before which time coleoptiles were at pH 6.5 and 0.2 mM NO₃. pH 6.5 with NO₃, open squares; pH 6.5 without NO₃, open circles; pH 3.5 with NO₃, closed squares; pH 3.5 without NO₃, closed circles. The y-axis presents the mean rate of net influx or efflux over the interval. The pH of the solutions was 3.54–3.68 for 0.2 mM NO₃ and 3.4–3.5 for solutions without NO₃, except for the last period when the pH was 3.3–3.5. A second experiment gave similar results. For H⁺, the least significant difference (l.s.d.) (5%) was 0.124 for pH treatments. For K⁺, the l.s.d. (5%) was 0.19 for differences between pH and NO₃ comparisons. For NO₃ and NH₄, the l.s.d. (5%) was 0.114 and 0.087, respectively, for comparisons between pH treatments.
Ethanol formation

The large differences in solute fluxes between treatments raised the question of whether ethanol formation, which is an indicator of energy production linked to glycolysis (Pradet et al., 1985; Greenway and Gibbs, 2003), was increased in the anoxic coleoptile tips during an acid load. No substantial effects were found between coleoptile tips in treatments of pH 3.5 and 6.5 (Table 3).

Discussion

This discussion will argue that pH regulation in anoxic rice coleoptiles involves a pHstat based on organic acids as well as a second pHstat based on NO$_3^-$ reduction to NH$_4^+$. Although anoxic soils typically lack NO$_3^-$ (Ponnampерuma, 1984), NO$_3^-$ reduction might be relevant, provided tissues contain substantial endogenous NO$_3^-$ at the start of anoxia.

For the present metabolic studies, it was crucial to establish that the tissues did not suffer appreciable injury during anoxia lasting up to 120 h, particularly when also exposed to pH 3.5 in the medium. Increases in net K$^+$ loss during the last period in the experiment with or without NO$_3^-$ (Fig. 5C), in all but the pH 6.5 treatment without NO$_3^-$, may reflect some deterioration of membrane semi-permeability. However, a more sensitive indicator of injury is inorganic phosphate (P$_i$) loss to the medium (Menegus et al., 1991), and in the present case the P$_i$ net loss during the last 7 h of the experiment was between 0.04 and 0.065 mmol g$^{-1}$ fresh weight h$^{-1}$, which was only 0.9–1.4% of the P$_i$ in the tissues. Thus, there was only minor injury, which is further supported by the rapid resumption of K$^+$ and Cl$^-$ net uptakes after the resumption of aeration.

Upon exposure of the anoxic coleoptiles to pH 3.5 in the medium, cellular pH decreased during the first 3–4 h (Kulichikhin et al., 2009), i.e. part of the net H$^+$ intake was absorbed by cellular pH buffering. Cytoplasmic buffering would cope with ~55% of the net H$^+$ entry for the first 1.5 h, the time over which cytoplasmic pH dropped from 7.35 to 7.2 (Kulichikhin et al., 2009; for calculation, see Appendix 1). This cytoplasmic buffering may arise for ~60% from P$_i$ (based on 9 mM P$_i$ in the cytoplasm of anoxic sycamore cells; Gout et al., 2001), while the other half of the cytoplasmic buffering was presumably associated with cytoplasmic proteins. Furthermore, net H$^+$ influx would also result in H$^+$ being transported across the tonoplast. Vacuolar P$_i$ would not, however, participate in buffering in the vacuole, because at the vacuolar pH of 5.35–5.7 (Kulichikhin et al., 2009) P$_i$ is nearly entirely in the H$_2$PO$_4^-$ form (pK1 is 2.1 and pK2 is 7.2; Conn and Stumpf, 1972). We also estimated that, after 18 h exposure to pH 3.5, buffering involving organic acids would only have coped with 1.2–2.5% of the net H$^+$ intake into the vacuole (assessment based on a reduction in vacuolar pH from 5.7 to 5.35 and organic acids remaining after 18 h exposure to pH 3.5; see Appendix 1). This small contribution to buffering was due to depletion of the organic acid pool by the biochemical pHstat over the first 18 h of anoxia (Fig. 2). Depending, however, on the rate of decrease in organic acids, their contribution to buffering may have been greater during the first 3 h of exposure to pH 3.5, i.e. before the bulk of the organic acids had been catabolized. This potential contribution was assessed at maximally 43% of the net H$^+$ intake over the first 3 h of exposure to pH 3.5.

Thus, summing up: (i) internal pH decreases were mitigated by buffering during the first few hours after transfer to pH 3.5, but (ii) the prolonged net H$^+$ intake must have been accommodated by biochemical pHstats, as both cytoplasmic and vacuolar pH remained stable between 3 and 18 h after transfer to pH 3.5 (Kulichikhin et al., 2009).

Biochemical pHstat involving organic acid metabolism

The biochemical pHstat involving organic acid metabolism is discussed here first, while that based on NO$_3^-$ reduction will be considered in the next section.

After 18 h exposure to pH 3.5, 25% of the net H$^+$ entry had been neutralized by a pHstat, consisting of organic acids, mainly malate and to a lesser extent succinate. It has been known for decades that, in aerated tissues, malate metabolism is part of a pHstat (Davies, 1980); however, for anoxic plant cells only the switch between lactate and ethanol synthesis has been well established (Davies, 1980). No endogenous products of organic acid catabolism were detected in the tissues (see Results), so the likely fate of the catabolized organic acids is decarboxylation to pyruvate.

Table 3. Ethanol production (mmol g$^{-1}$ fresh weight h$^{-1}$) by anoxic coleoptile tips at pH 6.5 or 3.5, and with or without 0.2 mM NO$_3^-$ in the incubation medium

| Treatment | pH 6.5 without NO$_3^-$ | pH 6.5 with 0.2 mM NO$_3^-$ | pH 3.5 without NO$_3^-$ | pH 3.5 with 0.2 mM NO$_3^-$ |
|-----------|-------------------------|---------------------------|-------------------------|---------------------------|
| Ethanol production rate (mmol g$^{-1}$ fresh weight h$^{-1}$) | 5.7±0.7 | 6.8±1.1 | 6.1±0.5 | 5.8±0.3 |
Under anoxia at pH 3.5, in three out of four experiments most of the pyruvate would have been converted to ethanol, as there was no net synthesis of other end products of anaerobic catabolism such as alanine (Fig. 3). However, in one of the four experiments there was an increase of 4.5 μmol g⁻¹ fresh weight in L-alanine, equivalent to approximately half of the decrease in carbon that had been contained in the catabolized organic acids (Fig. 4B).

The second important difference between the pHstat in anoxic (the present study) and aerated (Hiatt, 1967) cells concerns the fate of the K⁺. In aerated cells, K⁺, which balances the organic anions, remains in the cell, contributing to the electrical balance for Cl⁻ (Hiatt, 1967). In contrast, the principal purpose of the pHstat under anoxia at pH 3.5 is to neutralize H⁺, and catabolism of malate would leave no anion balancing the excess K⁺. Thus, the plasma membrane would depolarize, leading to opening of K⁺ channels and efflux of the K⁺ no longer required to balance the organic anions. This view is supported by the decreases in organic acids and K⁺ in tissues at the end of 48 h at pH 3.5 (means of three different experiments; Fig. 6), although the decrease in charge of organic anions was usually greater than that of K⁺, indicating that another cation must also have been involved in balancing the organic anions. Organic acid pools had nearly been depleted by the end of the experiment (Fig. 2) and K⁺ effluxes had also slowed down (Fig. 5). Further discussion on the ion balance in reference to K⁺, organic anions and Pi is given in Appendix 4. In keeping with previous findings with maize root tips (Roberts et al., 1992), γ-aminobutyric acid also contributed to pH regulation in rice coleoptiles, although to a much lesser extent than the organic acids (Figs 3B and 4D).

The organic acid pHstat also probably operates in tissues exposed to pH 3.5 in the absence of NO₃⁻. A strong indication of pHstat involvement was that, at 48 h after the start of the pH 3.5 treatment, malate was not detectable and succinate was greatly decreased in coleoptiles both with and without NO₃⁻. Moreover, K⁺ net losses from the coleoptiles with and without NO₃⁻ were, respectively, 31 and 35 μmol g⁻¹ fresh weight during 54 h of anoxia at pH 3.5. This similarity and the pronounced link between catabolism of organic acids and K⁺ net losses provide strong evidence for the operation of the organic acid pHstat, in tissues without and with NO₃⁻. Whether the kinetic trends of the decreases of organic acids following exposure to pH 3.5 is also similar with and without NO₃⁻ still needs to be established.

Finally, measurements of organic acids and ion fluxes following a change of pH from 3.5 to 6.5 at 78 h with continuous anoxia, i.e. when the organic acid pHstat was nearly depleted, showed that there was no substantial regeneration of organic acids during anoxia (see Appendix 3).

**Fig. 6.** Relationship between K⁺ and charge of organic anions (mEq l⁻¹ tissue water) in excised rice coleoptile tips. The data combine results from treatments at pH 6.5 and 3.5 of three different experiments, which were all part of this investigation. Treatments were imposed after 60 h anoxia, before which time coleoptiles were at pH 6.5. All treatments were carried out in 0.2 mM NO₃⁻. The assays were for samples taken between 18 and 48 h after transfer to pH 3.5. The charge of organic acids was calculated at the pH of the vacuole as measured using the method of Kulichikhin et al. (2009), using the concentrations in Fig. 2 and published pK₅ values.
\[
\text{NO}_3^- + 2H^+ + 2e^- \rightarrow \text{NO}_2^- + \text{H}_2\text{O} \quad (1)
\]

and

\[
\text{NO}_2^- + 8H^+ + 6e^- \rightarrow \text{NH}_4^+ + 2\text{H}_2\text{O} \quad (2)
\]

Part of the absorbed \( \text{NO}_3^- \) reduced to \( \text{NH}_4^+ \) was presumably effluxed as \( \text{NH}_4^+ \) to the medium between 5 and 54 h anoxia at pH 3.5; this efflux over the whole period was 17 \( \mu \text{mol g}^{-1} \text{ fresh weight} \) (Fig. 5D), which could account for 32\% of the \( \text{NO}_3^- \) influx (calculated from Fig. 5B, D). Another 20\% of the absorbed \( \text{NO}_3^- \) was presumably incorporated into soluble N compounds retained in the tissues (this percentage was calculated from Figs 5B and 4 by taking the difference in total soluble N in the absence and presence of \( \text{NO}_3^- \)). In the present experiments, the N flowed for a large part to \( \text{L-alanine} \) and serine, although at pH 3.5 in other experiments there were no large increases in these compounds (Fig. 3A). The flow of the remainder of the N from the absorbed \( \text{NO}_3^- \) might only proceed to \( \text{NO}_2^- \), which may leak from the tissues, as observed for anoxic cereal roots in which ~95\% of the net production was in the external solution (Lee, 1979). At pH 3.5 in the medium, ~50\% of the \( \text{NO}_3^- \) would be in the HNO\(_2\) form (pK\(_a\)=3.4) and hence lost during \( \text{N}_2 \) flushing. There may also be loss of other volatile compounds such as \( \text{NO} \) (based on Igamberdiev and Hill, 2004). These assessments indicate a requirement for 132 \( \mu \text{mol reduced nucleotides g}^{-1} \) fresh weight (calculated from Appendix 5). Our data did not permit establishment of the source of these reduced nucleotides, but a substantial amount may be derived from partial operation of the tricarboxylic acid cycle in anoxic rice coleoptiles (Fan et al., 2003; also discussed by Ratcliffe, 1997).

**Reductions in \( H^+\)–\( \text{NO}_3^- \) symport activity:** \( H^+\)–\( \text{NO}_3^- \) symport presumably has reduced activity after long-term exposure to pH 3.5, as indicated by the concurrent decreases in net \( H^+ \) and \( \text{NO}_3^- \) uptake with time (Fig. 4A, B). Such decreased transporter activity would be consistent with the observed closure of ion channels in anoxic animal cells (Hochachka, 1991) and the inferred closure of \( K^+ \) channels in excised, anoxic rice coleoptiles (Colmer et al., 2001). A remaining enigma is the \( H^+ \) influx in treatments with and without \( \text{NO}_3^- \) during the final hours at pH 3.5; these were similar in value even though there was still a substantial net \( \text{NO}_3^- \) uptake (Fig. 5A, B). The easiest suggestion we can offer is that, even in the absence of \( \text{NO}_3^- \), anion–\( H^+ \) symport(s) conducts some \( H^+ \) (the less readily absorbed anion, \( \text{SO}_4^2^- \), was the major anion present in the incubation solution).

The assessed \( P_{H^+} \) of the plasma membrane of the anoxic rice coleoptile tips during the first 5 h after transfer to pH 3.5 ranged between 0.3 \( \times 10^{-8} \) and 0.43 \( \times 10^{-8} \) m s\(^{-1} \) (for calculation, see Materials and methods). Part of the net entry was presumably associated with an \( H^+\)–\( \text{NO}_3^- \) symport; this influx would account for part of the increase in external pH, as the pH will be determined by the difference between the concentrations of strong cations and anions, i.e. the strong ion difference (Gerendás and Schurr, 1999; Greenway and Gibbs, 2003). \( \text{NO}_3^- \) influx through a channel is unlikely, as at the external concentration of 0.2 mM \( \text{NO}_3^- \) the influx would be against a steep free-energy gradient (McClure et al., 1990), because in anoxic rice coleoptiles the steady-state plasma membrane potential is ~120 to ~130 mV (Zhang and Greenway, 1995). Furthermore, if transport occurred via a channel there would be no apparent reason why \( \text{NO}_3^- \) influx during the first period of exposure to pH 3.5 was 2–3-fold larger than at pH 6.5 (Fig. 5).

\( P_{H^+} \) values in the absence of \( \text{NO}_3^- \) are most relevant to anoxic soil, as anaerobic soils typically lack \( \text{NO}_3^- \) (Ponnampерuma, 1984). There was no decrease over time of net intake of \( H^+ \) following transfer to pH 3.5 when exogenous \( \text{NO}_3^- \) was absent, and the \( P_{H^+} \) of the coleoptiles of 0.06 \( \times 10^{-6} \)–0.09 \( \times 10^{-6} \) m s\(^{-1} \) compares with 0.65 \( \times 10^{-6} \) m s\(^{-1} \) for plasma membrane vesicles of Elodea nuttallii (Miedema et al., 1996) and 1.4 \( \times 10^{-6} \) m s\(^{-1} \) for liposomes (Nichols and Deamer, 1980). Thus, the very low \( P_{H^+} \) of membranes of the anoxic coleoptiles may be a composite of \( H^+ \) fluxes through the lipid bilayer and embedded proteins in the membrane. The caveat of this suggestion is that \( P_{H^+} \) for the coleoptiles was based on the net \( H^+ \) flux and therefore would be underestimated if there was substantial \( H^+ \) extrusion. Nevertheless, even a \( H^+ \) influx three times higher than the measured net uptake would still give a value for the rice coleoptiles of the same order as the \( P_{H^+} \) determined for the vesicles and liposomes (Miedema et al., 1996; Nichols and Deamer, 1980).

**Is there a contribution from a biophysical pH-stat as well as from the biochemical pH-stat?**

Under aeration, \( H^+ \) extrusion, i.e. the biophysical pH-stat, is the main mechanism for coping with a long-term acid load, while the biochemical pH-stat is considered a fine-tuning device (Smith and Raven, 1979). However, it is usually assumed that the plasma membrane \( H^+\)-ATPase does not function under severe energy deficits, while residual maintenance of the transmembrane \( H^+ \) gradient is likely to be associated with biochemical pHstats, coined the battery (Felle, 2005), as the organic acids would have accumulated in aerobic tissues prior to anoxia.

The discussion in the previous section argued that the net \( H^+ \) intake at pH 3.5 was accommodated by biochemical pHstats. Of course, it cannot be excluded that the total \( H^+ \) influx was greater than the net \( H^+ \) influx, i.e. that there was some \( H^+ \) extrusion. For example, \( H^+ \) extrusion (i.e. biophysical pH-stat) may come into play once the organic-acids-based pH-stat is exhausted. This paper has shown that \( \text{Cl}^- \) can be taken up against a pronounced electrochemical gradient but that this can also be associated with ‘the battery-like system’ discussed by Felle (2005). Thus, testing whether the \( H^+\)-ATPase at the plasma membrane can become operative would be best when the biochemical pHstat is exhausted.

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*References cited in the text are available in the original source.*
Perspectives

Mechanisms of adaptation to anoxia: The acclimation to the combination of pH 3.5 and anoxia by rice coleoptile tips in the present study contrasts with early death of root tips of intact maize and wheat plants, which are injured more rapidly when exposed to anoxia at pH 4.0 rather than pH 5–6 (maize, Xia and Roberts, 1996; wheat, Waters et al., 1991).

For anoxic coleoptile tips at pH 3.5, the very steep electrochemical gradient for H\(^+\) entry across the plasma membrane enabled a surprisingly large net influx of NO\(_3\)^-.

The ecological relevance of the pHstat depending on NO\(_3\)^- entry and reduction is not, however, immediately clear, as in anoxic soils NO\(_3\)^- is rapidly reduced and so is scarce or even absent (Ponnamperuma, 1984). Nevertheless, any NO\(_3\)^- in seeds or accumulated in tissues prior to anoxia would be balanced by cations and would provide a powerful pHstat, as indicated in this study by the large H\(^+\) consumption provided the NO\(_3\)^- was converted to NH\(_4\)^+ rather than only to NO\(_2\), as happens in anoxia-intolerant species (Botrel and Kaiser, 1997). For anoxia-tolerant species such as rice, such a pool of NO\(_3\) may well substantially improve growth under anoxia, as the NO\(_3\)^- reduction would facilitate removal of reducing power, generated by some tricarboxylic acid cycle activity (Fan et al., 2003) required for net protein synthesis, which is substantial in anoxic rice coleoptiles (Alpi and Bevers, 1983).

One of our initial objectives was to use the exposure to pH 3.5 as a means to impose extra demands for energy to test whether glycolysis linked to ethanol formation increased, or whether survival was compromised. Neither was the case, presumably because the observed increase in net influx of H\(^+\) at pH 3.5 was accomodated by a combination of the organic acid pHstat, a pHstat based on reduction of NO\(_3\)^-, and reduced P\(_H^+\) at the plasma membrane. Hence, other treatments, likely to require substantial energy, are required to test whether ethanol formation in anoxic coleoptiles at pH 6.5 is below the maximum possible (as suggested by Huang et al., 2005).

Possible relevance to rice in the field: This study on pH regulation under anoxia is relevant to rice seeded directly into flooded soils but also to situations other than low soil pH, when an acid load may result from high concentrations of organic acids in the soil, including H\(_2\)CO\(_3\) (Ponnamperuma, 1984; Greenway et al., 2006). The sharp reduction in development of germinating seedlings in anoxia at pH 3.5 is relevant to the previously mentioned direct seeding of rice in flooded soils (Ismail et al., 2009), particularly acid sulphate soils, which have a pH between 4.5 and 6.2 during the first 14 d of flooding (de Datta, 1981). These soils occur in many rice-growing areas in South-east Asia, for example in 40% of the rice fields in the Mekong Delta (Lang et al., 2010).

The results presented here on the importance of the biochemical pHstat indicate that anoxia tolerance may depend on conditions prior to the imposition of anoxia, by increasing the capacity of the pHstat. There may be substantial potential for increasing this capacity; for example, organic acid accumulation in excised barley roots was favoured in the presence of K\(_2\)SO\(_4\) rather than KCl (Hiatt, 1967). A high level of organic acids and/or NO\(_3\)^- would provide a substantial biochemical pHstat, so it would be of interest to establish whether conditions before anoxia, which increase the endogenous concentration of organic acids, would increase the tolerance to acid loads under anoxia.

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Appendices

Appendix 1. Assessment of participation of contributions of organic acids to buffering and to the biochemical pHstat

Contributions of organic acids to cellular buffering and the biochemical pH stat were based on the measured pH of the vacuole (Kulichikhin et al., 2009) and the pH(a) of each organic acid, using the Henderson–Hasselbach equation (both based on Conn and Stumpf, 1972).

The assumptions were: (i) ~85% of the fresh weight of the coleoptile tips is in the vacuole and the organic acids are located in that compartment; (ii) the cytoplasm had a buffering capacity of 45 mM in the pH range 7.0–7.5 (mean value for six species in Kurkdjian and Guern, 1989); and (iii) the cytoplasm comprised 10% of the cell volume and the vacuoles 90%.

Appendix 2. Comparisons between changes in organic and amino acids in anoxic rice coleoptile tips with or without β-alanine as a buffer at pH 3.5

As stated in the Materials and methods, comparisons between rice coleoptile tips in solutions containing 0, 0.2 or 2 mM β-alanine were required to provide a link between the present experiments and our earlier in vivo [\(^{31}\)P]NMR spectroscopy experiments, in which β-alanine was used as a buffer at low pH (Kulichikhin et al., 2009). Furthermore, buffering may be required in future experiments at low pH and which require a high tissue-to-volume ratio.

β-Alanine has only a few metabolic functions and its pH(a) is 3.55 (Dawson et al., 1969). There were no substantial effects of exogenous β-alanine on endogenous concentrations of organic acids, or amino acids (see Appendix Table A1). There were also no detectable effects by β-alanine on net H\(^+\) and K\(^+\) fluxes (data not shown). Nevertheless, β-alanine reached tissue concentrations of 9...
and 10 μmol g⁻¹ fresh weight at external concentrations of 0.2 and 2 mM l-alanine, respectively, compared with 0.2−0.3 μmol g⁻¹ fresh weight in tissues without l-alanine in the external medium (see Appendix Table A1).

The data on l-alanine (pKₐ of 3.55) in tissues are of relevance both to its use as a buffer at low pH for which no other biological buffers are available, and also to possible amino acid transport in anoxic rice coleoptiles. Once accumulated, the l-alanine was retained, even when the coleoptiles were transferred to a medium without this solute (data not presented), so the measured influx of l-alanine represented a unidirectional influx. Influx of l-alanine increased only slightly between 0.2 and 2 mM external concentration, so the influx is presumably via a carrier that reaches saturation at low external concentration. Influx of l-alanine could be either as a cation or as the neutral species. Even at the measured vacuolar pH of 5.2 (Kulichikhin et al., 2009), nearly all of the l-alanine taken up would be rapidly converted to the zwitterion by the dissociation of the COOH group, which would produce H⁺, i.e. l-alanine⁻ would act as a H⁺ carrier and, with an influx of 0.55 μmol g⁻¹ fresh weight h⁻¹ of l-alanine, it would considerably intensify the acid load. On the other hand, the influx of the neutral species would be against a free energy gradient, but, provided the alanine−H⁺ symport had a stoichiometry of l-alanine− to H⁺ of 2 (or higher), the energy requirement to cope with this H⁺ entry would then be lower than in the case of influx of the cation (i.e. l-alanine⁻). Such a high stoichiometry would be energetically possible; e.g. even at 0.2 mM external l-alanine and an internal concentration of 9 mM, the free energy gradient for the undissociated species would be ~11 kJ compared with the free energy gradient for H⁺ of 30 kJ. However, the assessed amino acid: H⁺ stoichiometry of amino acid transporters is 1 (Dreyer et al., 1999). Even so, it would be of interest to establish whether more energetically efficient transport is feasible during energy deficits in tissues tolerant to these conditions. Interestingly, there was no evidence that the use of l-alanine intensified the acid load (Kulichikhin et al., 2009), so influx of the neutral species seems most likely.

Finally, the l-alanine was only applied when substantial amounts of l-alanine had already accumulated, so a specific effect by l-alanine on l-alanine synthesis cannot be excluded.

Appendix 3. Consideration of the role of the biochemical pHstat in pH regulation upon return from pH 3.5 to 6.5 with continuous anoxia, as carried out previously by in vivo [³¹P]NMRI spectroscopy experiments on rice coleoptile tips by Kulichikhin et al. (2009)

The substantial contribution of the biochemical pHstat (utilizing organic acids) to regulation of cellular pH in anoxic rice coleoptile tips, after transfer to pH 3.5, leads to the expectation that this pHstat would also contribute to pH regulation during the rise in vacuolar pH from 5.3 to 5.8 upon return of the coleoptiles from pH 3.5 to 6.5 under continued anoxia, as observed by Kulichikhin et al. (2009). However, after 18 h at pH 3.5 in anoxia, the organic acid pool was severely depleted; assuming a vacuolar pH range between 5.2 and 5.8 and that the organic acid pool is the only functioning biochemical pHstat, then an increase from pH 5.2 to 5.8 would require an increase in strong cations of only ~0.15 μmol g⁻¹ fresh weight h⁻¹ to account for the observed rise in vacuolar pH, as can be assessed from the strong ion difference (Stewart, 1983). Such a change would not be detected by the present analysis, as the fluxes observed have larger standard errors (Fig. 5). Upon return from pH 3.5 to 6.5 with continuous anoxia, net K⁺ losses became less, but there were no appreciable influxes to restore K⁺ concentrations, consistent with the failure of the organic acid pHstat to regenerate.

Appendix 4. Balance between decreases in electrical charge due to decarboxylation of organic acids and concentration of K⁺ in anoxic rice coleoptile tips

The intersec of the y-axis in Fig. 6 of 27 mM indicates the amount of K⁺ not balanced by organic anions in anoxic rice coleoptile tips. The major balancing anion to this portion of the K⁺ is likely to be P₄, which was at ~25 mM in 3-d-old aerated rice coleoptiles (Menegus et al., 1984). In the present coleoptile tips, P₄ concentrations (data not shown) exceeded the K⁺ not associated with organic anions, being ~45 μmol g⁻¹ fresh weight without substantial changes with time, at either pH 3.5 or 6.5. Another balancing cation for P₄ would presumably be Mg²⁺, which was at 6.6 μmol g⁻¹ fresh weight in rice coleoptiles at a similar growth stage (Menegus et al., 1984). Importantly, there was almost no detectable P₄ losses to the medium (<2% of tissue P₄), as substantial P₄ losses are a good indicator of cell injury (cf. Menegus et al., 1991).

Appendix 5. Assessments of H⁺ consumption in the NO₃⁻ reductase pHstat at pH 3.5 in anoxic rice coleoptile tips

Uptake of NO₃⁻, if via a 2H⁺−NO₃⁻ symport, would have added 2×52÷104 μmol H⁺ g⁻¹ fresh weight during 54 h exposure to pH 3.5 (Fig. 5B). Disposal of H⁺ via reduction of NO₃⁻ is assessed as follows:

(i) Conversion of NO₃⁻ to NH₄⁺ lost to the medium of 17 μmol g⁻¹ fresh weight would consume 10 mol H⁺ mol⁻¹

| Tissue solutes | pH 6.5 | pH 3.5 started at 60 h and sampled at 78 h |
|---------------|--------|---------------------------------------|
|               | 0 h    | 60 h        | 78 h        | 0 mM β-alanine | 0.2 mM β-alanine | 2 mM β-alanine |
| Malate        | 6.8±0.9 | 7.6±0.35   | 8.1±0.2    | 1.6±0.35      | 2.3±0.3      | 2.3±0.3      |
| Succinate     | ND     | 3.2±0.4    | 2.7±0.11   | 1.5±0.35      | 2.3±0.3      | 1.6±0.2      |
| L-Alanine     | 2.2±0.2 | 3.1±2.0    | 33±1.3     | 27±6.2        | 35±1.4       | 28±2.5       |
| Serine        | 2.5±0.5 | 5.3±0.8    | 5.3±0.1    | 6.7±2.2       | 8.5±0.5      | 6.1±0.2      |
| β-Alanine     | 0.23±0.05 | 0.29±0.07 | 0.32±0.1  | 0.27±0.04     | 8.9±0.26     | 10.2±0.3     |
of NH$_4^+$ produced, i.e. 17×10=170 μmol H$^+$ g$^{-1}$ fresh weight.

(ii) Flow of NO$_3^-$ to soluble N compounds retained in the tissues, based on the difference in increase of total PCA-soluble N between tissues with or without NO$_3^-$, of 11 μmol g$^{-1}$ fresh weight. This process would remove 11×8=88 μmol H$^+$ g$^{-1}$ fresh weight. In this case, there are eight rather ten H$^+$ consumed, as the N is incorporated as NH$_3$ rather than NH$_4^+$ (see Equation 2 in Discussion).

(iii) Similarly, the difference in increase in L-alanine between tissues with or without NO$_3^-$ of 10 μmol g$^{-1}$ fresh weight would remove another 10×8=80 μmol H$^+$ g$^{-1}$ fresh weight.

(iv) The fate of the remaining of the absorbed NO$_3^-$ of 53–38=15 μmol g$^{-1}$ fresh weight is unknown. If this fraction was lost as NO$_3^-$, the H$^+$ consumption would be another 30 μmol g$^{-1}$ fresh weight (see Equation 1 in Discussion).

Thus, the total H$^+$ consumed in the putative NO$_3^-$ reductase pHstat would be 170+88+80+30=368 μmol g$^{-1}$ fresh weight.

References

Alpi A, Beever H. 1983. Effects of O$_2$ concentrations on rice seedlings. Plant Physiology 71, 30–34.

Atwell BJ, Waters I, Greenway H. 1982. The effect of oxygen and turbulence on elongation of coleoptiles of submergence-tolerant and -intolerant rice cultivars. Journal of Experimental Botany 33, 1030–1044.

Bartzatt R. 2003. Dansylation of aromatic, aliphatic, and medicinal carboxylic acid compounds in 1 M Na$_2$CO$_3$ buffer. Analytica Chimica Acta 488, 203–209.

Beever H. 1960. Respiratory metabolism in plants. New York, London, Tokyo: Harper Row.

Botrel A, Kaiser WM. 1997. Nitrate reductase activity in barley roots in relation to the energy and carbohydrate status. Planta 201, 496–501.

Cawthray GR. 2003. An improved reversed-phase liquid chromatographic method for the analysis of low-molecular mass organic acids in plant root exudates. Journal of Chromatography A 1011, 233–240.

Colmer TD, Huang S, Greenway H. 2001. Evidence for down-regulation of ethanolic fermentation and K$^+$ effluxes in the coleoptile of rice seedlings during prolonged anoxia. Journal of Experimental Botany 52, 1507–1517.

Conn EE, Stumpf PK. 1972. Outlines of biochemistry, 3rd edn. New York: John Wiley & Sons.

Cosgrove DJ. 2001. Wall structures and wall loosening. A look backwards and forwards. Plant Physiology 125, 131–134.

Davies DD. 1980. Anaerobic metabolism and the production of organic acids. In: Davies DD, ed. The biochemistry of plants, Vol. 2. London: Academic Press, 581–611.
Involvement of plasma membrane H+-ATPase in anoxic elongation of potamogeton stems in pond weed Potamogeton distinctus. New Phytologist 190, 421–430.

Kulichikhin KY, Greenway H, Byrne L, Colmer TD. 2009. Regulation of intracellular pH during anoxia in rice coleoptiles in acidic and near neutral conditions. Journal of Experimental Botany 60, 2119–2128.

Kurkdjian A, Guern J. 1989. Intracellular pH: Measurement and importance in cellular activity. Annual Review of Plant Physiology and Plant Molecular Biology 40, 271–303.

Lang NT, Buu BC, Viet NV, Ismail AM. 2010. Strategies for improving and stabilizing rice productivity in the coastal zones of the Mekong Delta, Vietnam. In: Hoanh CT, Szuster BW, Swan-Pheng K, Ismail AM, Nobel AD, eds. Tropical deltas and coastal zones: food production, communities and environment at the land-water interface. Wallingford, UK: CAB International, 209–222.

Lee RB. 1979. The release of nitrite from barley roots in response to metabolic inhibitors, uncoupling agents and anoxia. Journal of Experimental Botany 30, 119–113.

Linares RM, Ayala JH, Afonso AM, Diaz VG. 1998. Rapid microwave-assisted dansylation of biogenic amines: analysis by high-performance liquid chromatography. Journal of Chromatography A 1011, 233–240.

Lowry OH, Rosebrough NJ, Farr AW, Randall RG. 1951. Protein measurement with the Folin phenol reagent. Journal of Biological Chemistry 153, 265–275.

Lüttge U, Higinbotham N. 1979. Transport in plants. New York: Springer-Verlag.

McClure PR, Kochian LV, Spanswick RM, Shaff JE. 1990. Evidence for cotransport of nitrate and protons in maize roots. II. Measurement of NO3 and H+ fluxes with ion selective electrodes. Plant Physiology 93, 290–294.

Menegus F, Brambilla I, Bertani A. 1984. Nutrient translocation patterns and accumulation of free amino acids in rice coleoptile elongation under anoxia. Physiologia Plantarum 61, 203–208.

Menegus F, Cattaruzza L, Mattana M, Beffagna N, Ragg E. 1991. Response to anoxia in rice and wheat seedlings. Changes in the pH of intracellular compartments, glucose-6-phosphate level and metabolic rate. Plant Physiology 95, 760–767.

Menegus F, Cattaruzza L, Molnari H, Ragg E. 1993. Rice and wheat seedlings as plant models of high and low tolerance to anoxia. In: Hochachka PW, Lutz PL, Sick T, Rosenthal M, van den Thillart C, eds. Surviving hypoxia. Mechanisms of adaptation and control. Boca Raton, FL: CRC Press, 53–64.

Miedema H, Staal M, Prins HBA. 1996. pH induced proton permeability changes of plasma membrane vesicles. Journal of Membrane Physiology 152, 159–167.

Motomizu S, Wakimoto T, Toei K. 1983. Spectrophotometric determination of phosphate in river waters with molybdate blue and malachite green. Analyst 108, 361–367.

Nichols JW, Deamer DW. 1980. Net-proton-hydroxyl permeability of large unilamellar liposomes measured by an acid-base titration technique. Proceedings of the National Academy Sciences of USA 77, 2038–2042.

Nobel PS. 1974. Introduction to biophysical and biochemical plant physiology. San Francisco, CA: WH Freeman and Company.

Nozal MJ, Bernal J, Toribio ML, Diego JC, Ruiz A. 2004. Rapid and sensitive method for determining free amino acids in honey by gas chromatography with flame ionisation or mass spectrometric detection. Journal of Chromatography A 1047, 137–146.

Pittman M. 1963. The determination of the salt relations of the cytoplasmic phase in the cells of beet root tissue. Australian Journal of Biological Sciences 16, 947–968.

Ponnamperruma FN. 1984. Effects of flooding on soils. In: Kozlowski TT, ed. Flooding and plant growth. New York: Academic Press, 9–45.

Pradet A, Mocquot B, Raymond P, Morisset C, Aspart L, Delseny M. 1985. Energy metabolism and synthesis of nucleic acids and proteins under anoxic stress. In: Key JL, Kosuge T, eds. Cellular and molecular biology of plant stress. New York: Alan R Liss, 227–245.

Ratcliffe RG. 1997. In vivo NMR studies of the metabolic response of plant tissues to anoxia. Annals of Botany 79, 39–48.

Reggiani R, Mattana M, Aurisano N, Bertani A. 1993. Utilisation of stored nitrate during the anaerobic germination of rice seeds. Plant and Cell Physiology 34, 379–383.

Roberts JKM, Hooks MA, Miaullis AP, Edwards S, Webster C. 1992. Contribution of malate and amino acid metabolism to cytoplasmic pH regulation in hypoxic maize root tips studied using nuclear magnetic resonance spectroscopy. Plant Physiology 98, 480–487.

Smith AM, Raven JA. 1979. Intracellular pH and its regulation. Annual Review of Plant Physiology 30, 289–311.

Stewart PA. 1983. Modern quantitative acid-base chemistry. Canadian Journal of Physiological Pharmacology 61, 1444–1461.

Waters I, Kuiper PJC, Watkin E, Greenway H. 1991. Effects of anoxia on wheat seedlings I. Interaction between anoxia and other environmental factors. Journal of Experimental Botany 42, 1427–1435.

Xia JH, Roberts JKM. 1996. Regulation of H+ extrusion and cytoplasmic pH in maize root tips acclimated to a low-oxygen environment. Plant Physiology 111, 227–233.

Zhang Q, Greenway H. 1995. Membrane transport in anoxic rice coleoptiles and storage tissues of beet root. Australian Journal of Plant Physiology 22, 965–975.