Chapter 5
pH Signaling During Anoxia

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Abstract Plant cells that are exposed to anoxia run into an energy crisis. As a result of this, compartmental transmembrane gradients break down (sooner or later) leading ultimately to cell death. Regulation of intracellular pH and intercompartmental pH signaling may play a critical role in tolerating anoxia for some time. An early consequence of anoxia is a cytoplasmic pH drop which, according to the Davis-Roberts hypothesis, arises from the lactate formation. H^+ leakage from the vacuole or H^+ arising from nucleotide triphosphate hydrolysis have been also suggested. Research has focused on the assumption that the anoxic cytoplasmic pH change is an "error signal" to which the cell should respond to avoid cell-damaging acidosis. This view is challenged here. It is argued that pH under anoxia represents a new set point required for the anaerobic metabolism and for gene activation. It is concluded that acidosis does not occur because of H^+ leaking through membranes or because of the production of acids, but because of energy shortage which prevents the maintenance of transmembrane gradients; the leveling of the pH gradients and subsequent cytoplasmic acidosis are a consequence thereof.

5.1 Introduction

Higher plants as aerobic organisms occasionally experience situations of reduced oxygen or total anoxia because of environmental factors. Waterlogging blocks the transfer of O_2 and other gases between plant organs and the atmosphere. As a result of this, plants or parts thereof become O_2 deficient and run into an energy crisis (Greenway and Gibbs 2003), primarily caused by a reduced or, in case of anoxia, a complete shutdown of the oxidative respiration. Unlike facultative bacteria, most

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higher plants cannot withstand anoxia for long, but some species have developed strategies to tolerate anaerobiosis to a high degree. There are quite a number of plant responses to anoxia (reviewed by Perata and Alpi 1993; Ratcliffe 1999; Vartapetian 2005; Greenway et al. 2006; Drew 1997) from which mainly the pH response will be focused on in this chapter.

The fast drop in cytoplasmic pH, which occurs within seconds following oxygen shortage, is one of the earliest measurable features of anoxia, the extent of which depends on how well the organism is adapted to oxygen stress. The development of cytoplasmic pH as a consequence of anoxia can shortly be described as follows (Fig. 5.1): upon anoxia, normoxic well-regulated cytoplasmic pH (Smith and Raven 1979; Davies 1986; Felle 1988) drops to a new stable level, which is required by anaerobic metabolism and is mainly set by H^+-consuming and -producing (enzymatic) processes, i.e. by a biochemical pH-stat (Davies 1986; Felle 2005). This relatively stable pH level can be maintained as long as sufficient energy can be provided to maintain transmembrane gradients, depending on adaptation, acclimation, etc. In case of enduring energy shortage, cytoplasmic pH will decrease further eventually leading to cell-damaging acidosis.

With respect to pH development under anoxia, research has focused on major questions: (1) where does the anoxic acidification come from? (2) To avoid acidosis, must cells under anoxia restore the normoxic pH? (3) Is acidosis the cause for anoxia intolerance? (4) Does an improved pH regulation ameliorate anoxic effects? Taking most of the experimental data and observations into consideration, it is attempted in this chapter to judge some of the observations from a

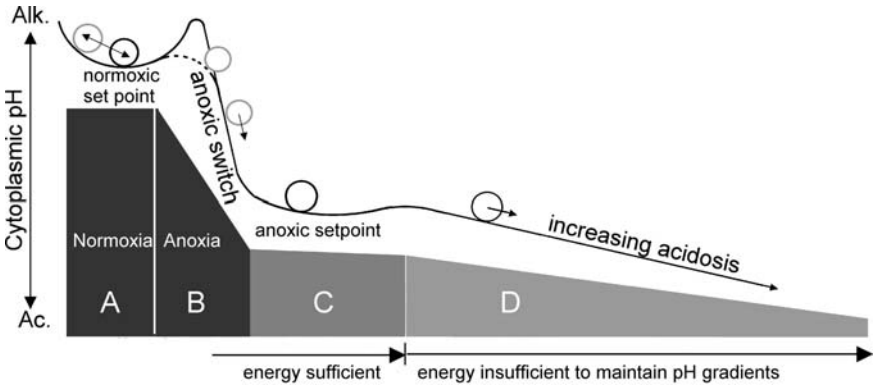

Fig. 5.1 A model to illustrate cytoplasmic pH development with respect to energy availability before and during anoxia. (**a**) Normoxic conditions and stable, well-regulated cytoplasmic pH. (**b**) Switch from normoxia to anoxia which involves a massive drain in energy and a pH drop to another stable value (anoxic set point). (**c**) Phase of relative stability during which energy is harvested from resources or from non-affected photosynthetic organs. The duration of this phase varies strongly, being relatively short for anoxia-intolerant plants. (**d**) Failure to harvest sufficient energy to keep up substrate gradients, including pH. Declining energy availability and subsequent increasing acidosis lead to cell death

slightly different point of view which, however, may result in causalities that are not always totally congruent with the current opinion.

5.2 pH, Signal and Regulator

The pH of an aqueous compartmental solution represents the basic condition to which many processes therein, e.g. enzymatic reactions as well as membrane transport, have to succumb to enzymatic reactions. Since protons can be at the same time substrate, activator and product, it is mostly difficult to pinpoint the primary effect and to distinguish it from the subsequent processes, and often this may not even be possible or reasonable. For instance, the activity of an H^+ATPase that transports H^+ across a membrane depends chemically on the ambient pH (binding, optimum). This H^+ transport generates or changes a transmembrane pH gradient which in turn will influence the activity of this transporter for thermodynamic reasons. Since in all cellular compartments these principles hold for a variety of processes that involve protons in one way or the other, for a given pH change the term "signal" appears problematic (Felle 2001). Undoubtedly, a pH change may signal a process that has already happened or is just about to take place, but it represents much more. In Fig. 5.2, an attempt is made to shortly outline the problem: a pH change is multifunctional and retroactive with respect to its own cause (a self-regulating aspect). This problem is enhanced by the fact that a pH change need not be the result of an H^+ shift across some barrier (membrane) or the result of a biochemical reaction. pH changes may well occur through net shifts of other (strong) ions like K^+, Ca^{2+}, Cl^- (strong ion difference; Stewart 1983; Ullrich and Novacki 1990) or simply through ion exchange, within cell wall constituents like glucoronic acid. If we consider pH changes as signals, we have to ask how and where information is transferred to. With regard to anoxia, this would be intracellular in the first place, but it is also systemic, i.e. from one organ (e.g. root) to another (e.g. shoot; leaf) or vice versa.

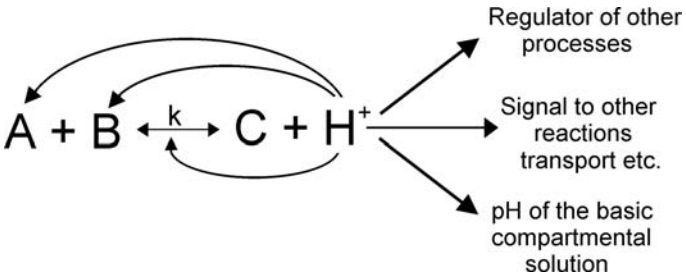

Fig. 5.2 A simplified scheme to illustrate the problem of overlapping and retroactive involvements of H^+ in cellular processes. H^+ produced (or consumed) by cellular reactions have a tendency to shift the basic pH of the respective compartment, influence other reactions and membrane transport, but also have a retroactive effect on the reagents A, B, C and the enzyme (k)

5.2.1 pH as Systemic Signal

In case of flooding, the root is the most likely organ that experiences shortage of oxygen first; a stress event that is signaled to the shoot. There is some evidence that electrical long-distance signals are forerunners which signal "stress" to the shoot or the leaves (Shvetsova et al. 2002; Felle and Zimmermann 2007). There is also evidence that such electrical signals cause a relatively unspecific upregulation of a variety of genes and the production of stress-related proteins (e.g. Wildon et al. 1992; Stanković and Davies 1997). More specific information as to the kind of stress has to take place as mass transfer, both through the xylem and, to some extent (albeit slower), through the phloem. Whereas the xylem pathway appears unproblematic, because it is fuelled by the transpiration (providing it still exists under anoxia), the inverse direction from shoot to root is not so simple, because mass flow within the sieve tubes depends on source-sink principles, which may be disturbed during anoxia. Additionally, since the pH of the sieve tube cytoplasm (as in any cell) is well regulated, it would not permit substantial pH shifts, and therefore is not suited for the transmission of large pH signals (e.g. 1 unit or more). This brings us to the question "how large a pH shift must be to be sensed as a signal?" Physiological normoxic pH changes exceeding 0.3 units are untypical for the cytoplasm. Would this be sufficient to bring across a message? The answer is "yes"! A pH change of 0.3 means a doubling of the H^+ activity which is easily recognized and responded to by enzymes and transporters. Anoxic pH shifts are mostly larger (see Table 5.1). As a rule of thumb, a cytoplasmic acidification of 0.6 units can be expected within the first few minutes of oxygen shortage in a cell that experiences sudden anoxia, albeit

Table 5.1 Selection of measured cytoplasmic pH changes of different plant (organs) responding to anoxia. "Time interval" denotes the period in which the pH change takes place

Plant (organ)	pH change (ΔpH)	Time interval	References
Maize (root tips)	7.4–6.8 (0.6)	First few min	Roberts et al. (1984a, 1984b) Fox et al. (1995)
Rice (coleoptiles)	(0.2)		Fan et al. (1992)
Rice (shoots)	7.4–7.1 (0.3)		Menegus et al. (1991)
Wheat (shoots)	7.4–6.5 (0.9)		Menegus et al. (1991)
Medicago (root hairs)	7.32–6.8 (0.52)	2 min	Felle (1996)
Maize (root tips)	7.55–7.0 (0.55)	30 min	Ratcliffe (1992)
Article I. *Potamogeton* (tuber)	7.48–7.32 (0.16)	60 min	Summers et al. (2000)
Pea (internodes)	7.48–6.8 (0.58)	30 min	Summers et al. (2000)
Acer susp. (cultured cells)	7.5–7.1 (0.4)		Gout et al. 2001
Nicotiana			
a. Roots	7.57–6.27 (1.3)	105 min	Stoimenova et al. (2003)
b. Transformant lacking nitrate reductase	7.50–6.48 (1.02)	105 min	
Arabidopsis			
a. WT	8.03 – 7.29 (0.74)	6 h	Mattana et al. (2007)
b. Transgene	7.98 – 7.07 (0.91)	6 h	

smaller as well as larger changes have been reported (see below). The question must be: is the anoxic pH change suffered at the root in any way signaled to the shoot? If yes, what is the nature of the transfer?

5.2.2 The Nature of pH Transmission

As demonstrated recently, flooding of barley roots which caused hypoxia had no effect on the apoplastic pH of shoot tissue within the experimental period of 2 h. On the other hand, anoxia, imposed to the same system (caused by N_2) led to a strong alkalinization within the leaf apoplast right away, the signal traveling at 2–5 cm min^{-1} (Felle 2006). However, whereas the cytoplasmic pH change was an acidification, the pH change measured within the normoxic leaf apoplast was an alkalinization, meaning that the pH shift was transformed into a stress signal to be transmitted within the xylem. This is nothing out of the ordinary as all kinds of stress seem to cause apoplastic pH increase (Wilkinson 1999; Felle et al. 2005).

Since the velocity of pH transmission from root to shoot is related to the aperture of stomata, one would expect that such a pH increase should be transmitted along with mass flow within the xylem, e.g. as protonated/deprotonated weak acids. This does not seem to be the case! pH changes fed directly into the xylem of a dissected leaf petiole, are either not or at best marginally (at about one-tenth of any pH unit altered) transferred to the point of measurement (leaf blade). pH changes of several units, externally applied directly to the roots of an intact plant (barley), were not transferred to the shoot at all! (Felle 2005). This means that other transfer mechanisms must apply. Root-to-shoot studies revealed that apoplastic pH readily changed and was transmitted when the apoplastic activity of inorganic ions, e.g. K^+ or Ca^{2+} was altered (Felle 2005). As a matter of fact, apoplastic changes in pH occurred simultaneously with K^+ (Ca^{2+}) activity, i.e. an increase in apoplastic pH goes along with an increase in K^+ or Ca^{2+} activity. This could explain the conversion of the anoxic cytoplasmic acidification into apoplastic alkalinization. Owing to H^+ pump deactivation under anoxia and the subsequent decrease in inwardly directed driving force for cations, K^+ ions leave the cell and, according to the strong ion principles (Stewart 1983; Ullrich and Novacki 1990; Gerendas and Schurr 1999), alkalize the apoplast. Subsequently, K^+ is transported in the xylem into the shoot and thus transmits the pH change. These principles also hold for anoxic situations. Whereas flooding of roots does not necessarily lead to a rapid change in the apoplastic leaf pH (within the first 2 h), anoxia under N_2 does right away (Felle 2005). This indicates that the stress signal "pH increase" seems only released and transmitted after oxygen availability has fallen short of a critical value.

5.2.3 What is the Information?

What information can there be in a pH shift? Obviously, the direction of the pH shift is different in the apoplast (alkalinization; exceptions see below) and in the

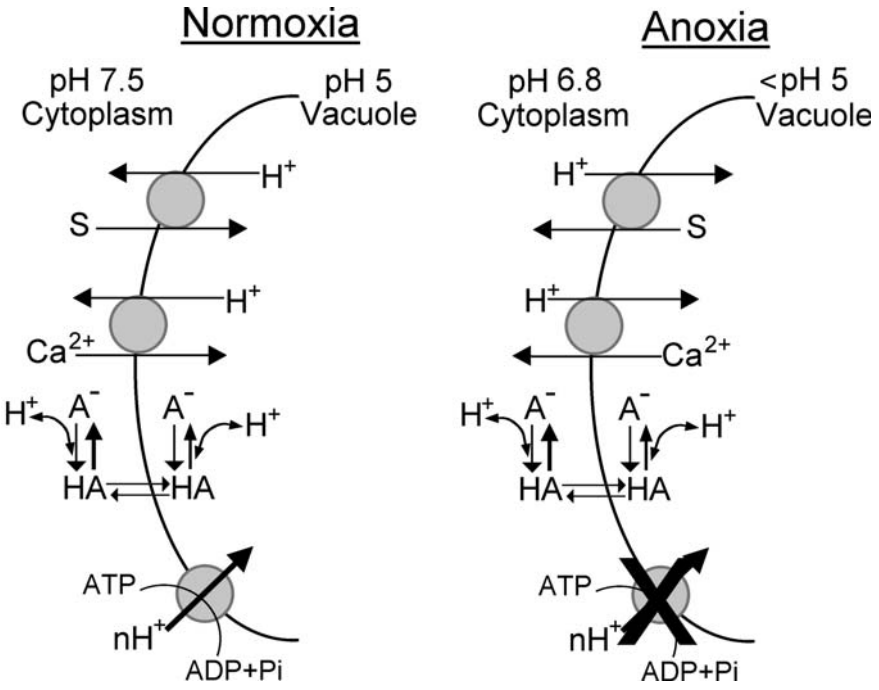

Fig. 5.3 Vacuolar pH and the switch from normoxia to anoxia. (1) Under anoxia, the activity of the Tp-ATPase is negligible (X). As a consequence thereof, merely passive processes prevail. (2) Cotransported (H^+ antiport) substrates (S) and ions (Ca^{2+}) that were accumulated in the vacuole flow back into the cytoplasm. Because of the antiport, H^+ will be translocated into the vacuole. (3) Weak acids (organic acids) dissociate according to their pKs and the existing pH value. Anoxia causes a cytoplasmic acidification, i.e. an increase in protonated weak acid(s). Since HA is better permeable than A^-, HA will flow into the vacuole, dissociate there into A^- and H^+. Thus, as long as transmembrane gradients exist, the vacuolar pH will have the tendency to acidify under anoxia (see text)

cytoplasm (acidification). The latter means "activation" but this holds primarily for cells that are not directly affected by anoxia. Activation also means the upregulation of a broad array of genes, the products of which will ameliorate the immediate effects of the energy shortage in the affected organs. Activation also means the upregulation of transport and enzyme activities that are involved in gaining and transporting energy-rich compounds. It is difficult, however, to explain how the apoplastic alkalinization that arrives in the shoot (leaf) can be transformed into a cytoplasmic acidification. One possibility is anion channel activation. As discussed above, pH transmission is not carried by compounds with dissociable groups like weak acids, but indirectly through the mass transport of K^+, Na^+ or especially Ca^{2+} within the xylem. Apart from the resulting alkalinization, the increase in the apoplastic Ca^{2+} activity could well lead to an activation of Ca^{2+}channels: the subsequent influx of Ca^{2+} elevates cytoplasmic Ca^{2+} and activates anion channels, as frequently shown (e.g. Lewis et al 1997; Felle and Zimmermann 2007). These

relatively unspecific anion channels do not only release Cl^- and NO_3^-, but also organic acid anions, which due to their weak acid properties, alkalize the apoplast and at the same time acidify the cytoplasm. Obviously, pH signaling cannot be discussed without taking into account changes in ion channel activity, a realization that holds for aspects of pH regulation under anoxia as well.

5.3 Anoxic Energy Crisis and pH Regulation

Depending on their specialization and acclimation to oxygen shortage, plants are able to survive anoxia without damage for a few hours at least, but in many cases much longer. Since under anoxia or inhibition of the oxidative phosphorylation with cyanide cytoplasmic ATP drops to about 20% or less within seconds or a minute at the most (Felle 1981), survival is obviously not linked to normoxic ATP concentrations, but to much lower levels, which will have to be generated and maintained by anaerobic processes. Energywise, there are three main problems during anoxia. Firstly, owing to the elimination of oxidative phosphorylation only a fraction of the energy stored chemically within glucose can be harvested. Secondly, owing to the energy shortage and partial deactivation of the H^+ ATPases, the H^+ circulation across membranes which drives cotransport is massively impaired. Thirdly, the driving force for H^+- cotransportable substrates is reduced. Therefore, extrusion of H^+ by the H^+ pumps does not serve mitigation of cytoplasmic acidosis, but to keep up H^+ cotransport, which is the only linkage to any of the scarce energy sources left.

5.3.1 The Davis-Roberts-Hypothesis: Aspects of pH Signaling

In short, this hypothesis says: the anoxia-induced cytoplasmic acidification is due to lactic acid formation/accumulation; the stabilization of the cytoplasmic pH is due to a metabolic shift to ethanolic fermentation, which is mediated and pH stimulated by the pyruvate decarboxylase, having its pH optimum in a more acid range than the lactate dehydrogenase; this will result in a redirection of carbon flow to ethanol synthesis with no further H^+ production (Davies et al. 1974; Davies 1986; Roberts et al. 1984a, b). Although this hypothesis has been challenged, more or less successfully, it is not the issue of this article to prove or disprove the validity of this hypothesis nor its critics. It is rather the question "whether pH signaling is a component therein?" If we take the cytoplasmic acidification to be the result of the lactic acid production, altered pH would have a retroactive effect on the lactate dehydrogenase (to reduce activity), but would also affect the pyruvate decarboxylase which responds accordingly with the activity increase to stabilize the anoxic pH on a new level. This is a typical feature of the biochemical pH regulation: the pH shift from one enzyme is a result, but at the same time also an error signal to the

other enzyme, which responds and produces/consumes H^+ because of its pH optimum.

In spite of the reports which show that the Davies–Roberts hypothesis may not be universal, it should be stressed that pretreatments with acetic acid which decreased the cytoplasmic pH of maize root tips (1) prevented the transient formation of lactate, and (2) eliminated the lag in the production of ethanol (Roberts et al. 1984a). Fox et al. (1995) extended this work using methylamine to increase cytoplasmic pH and found that ethanol production ceased as cytoplasmic pH increased. Both studies showed that pH was a signaling component in the pathway to activate or deactivate the respective enzymes.

There are a number of examples indicating that there is no single or universal mechanism for the induction of lactic/ethanol fermentation and the pH development do not always follow lactate formation. Sakano et al. (1997) demonstrated in suspension-cultured *Catharanthus* cells that accumulation of lactate was not related to cytoplasmic acidification. Tadege et al. (1998) showed that in transgenic tobacco concentration of lactate remains at the limit of detection under anoxia while ethanol increases dramatically, indicating that no obvious interrelation between lactate formation and cytoplasmic pH need to exist. Kato-Noguchi (2000) found in lettuce that ethanolic fermentation was activated without preceding activation of lactate fermentation. Saint-Ges et al. (1991) argued that hydrolysis of nucleotide triphosphates was correlated to pH development under anoxia rather than lactate formation, which continued in spite of a stabilized pH. These are only some aspects of the observation that pH development under anoxia is the result of several metabolic steps that work differently under oxygen shortage than under normoxia. The question whether pH or the pH change is a signal therein must be investigated for each plant and enzyme individually, taking the state of respective adaptation to anoxic situations into account. Whereas one can assume that the basic enzyme pattern is essentially similar in higher plants, already minor shifts in the pH optima of the involved enzymes may result in set points that differ in a few tenths of a pH unit. It might be an error, however, to assume that the new pH level reached under anoxia is the result of an active process, such as membrane transport. Once the mitochondrial energy production has ceased, energy gets scarce and ions and molecules are transported because of their transmembrane gradients until a new more or less stable dynamic equilibrium is established; protons are a part thereof and cytoplasmic pH will rest (for some time) at a new set point.

5.3.2 Cytoplasmic Acidification, ATP and Membrane Potential

It has been suggested that cytoplasmic acidification was causally linked to the cytoplasmic nucleotide triphosphate potential rather than to lactate formation (Saint-Ges et al. 1991; Gout et al. 2001). The problem with the kinetics given in these studies is that there is just about 1 measuring point per min. As shown here in Fig. 5.4a, the ATP decay can be very fast and levels off already 1 min after

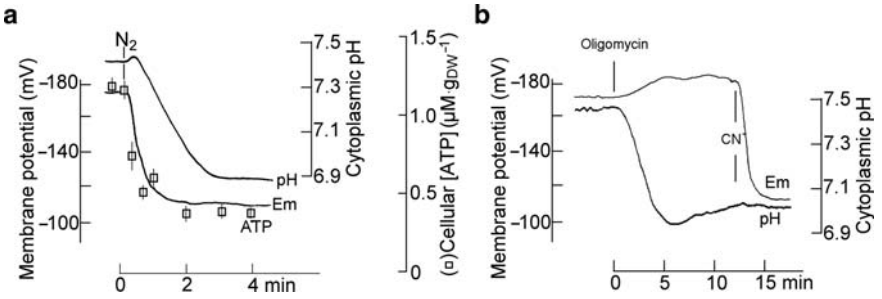

Fig. 5.4 Kinetics of cytoplasmic pH, [ATP] and membrane depolarization following anoxia (**a**; N_2) or inhibition of the oxidative phosphorylation by oligomycin (**b**) (see text)

imposing anoxia (N_2). Whereas kinetics of membrane depolarization seems to follow the ATP-decay (indicating H^+ pumps as major ATP consumers), there is no correlation with the pH kinetics. The deviation from the results given by Saint-Ges et al. (1991) and Gout et al. (2001) probably arises from the resolution of the pH measurement. Anoxia, suddenly imposed to cells, may yield pH and membrane potential kinetics that apparently correspond with each other. The problem with such an approach is that potentially different onsets of the kinetics become indistinct. One way out of this would be to impose a gradual O_2 decrease or use subthreshold respiratory chain inhibitors, which then will show up whether pH, ATP, and membrane depolarization start to change, either simultaneously or not. Fig. 5.4b in fact reveals that cytoplasmic pH already starts to decrease well before the membrane potential started to depolarize (Fig. 5.4b; Felle 1996), indicating that the level of ATP available or pump activity were in no causal relationship with the cytoplasmic pH or its anoxic shift.

5.3.3 Cytoplasmic pH (Change), An Error Signal?

Under normoxic conditions, any aberration from the pH set point is experienced as "error" to which it is reacted to, whether biophysically (membrane transport) or biochemically. For instance, H^+ ATPases will respond to cytoplasmic acidification with an increased activity, because H^+ is transport substrate or because the enzyme optimum requires it (Felle 1991). Additionally, the biochemical pH-stat gets active and consumes H^+ by converting malate into pyruvate. Although one could debate which of the two mechanisms are the more effective, this, however, is of no consequence to the question raised here. The same holds for anoxia, although under quite different proportions for all those processes that consume metabolic energy like H^+ATPases. Under anoxia, the activity of the H^+ ATPase is rather low, which is indicated by the fact that the membrane potential drops to the level of the so-called diffusion potential. Therefore, the H^+ATPase activity would not contribute much to

restore the pH. But even so, the basic question must be: is the pH drop following anoxia (Table 5.1) experienced as error signal at all? Most authors who have dealt with this problem in the past appear to be affirmative of the "error" argument, and try to prove that the affected cells attempt to restore the pH to its original (normoxic) level (to mitigate acid loads). This view ought to be reconsidered! A partial pH recovery following the initial pH decrease is often taken as an indication of a back regulation. Apart from the possibility that the system investigated may have experienced hypoxia only (under which partial recovery is quite the rule) rather than anoxia, this transient behavior is nothing else but an adjustment to a new set point, whereby the system recovers from the rapid pH change (overshoot). Following anoxia, cytoplasmic pH drops because of a shift in chemical equilibria, whereby lactose formation may be one, but not the only factor, as the different investigations show. Upon oxygen shortage, pH drops to that level which is set by all H^+-consuming and H^+-producing processes that contribute to establish a new equilibrium under anaerobic conditions. Thus, the anoxic pH is not an error signal therein but the optimal value for the given metabolic conditions to which the cell has to succumb. Differences in this set point between different plants experiencing anoxia may arise from enzyme patterns that might work at slightly different optima as well as with different efficiencies and largely represent different grades of adaptation to anoxic situations.

5.4 pH Interactions Between the (Major) Compartments During Anoxia

Major cellular compartments with respect to H^+ are cytoplasm, vacuole and apoplast. It should be noted, however, that during normoxia chloroplasts can temporarily accumulate rather high amounts of H^+, a fact that has been demonstrated with pH-sensitive microelectrodes. Following light-off or even a reduction of light intensity, the cytoplasmic pH decreases transiently up to 0.3 units, to which the H^+ ATPase responds with a temporary accelerated H^+ extrusion and hyperpolarization, whereas light-on is responded to with the opposite. Taking the cytoplasmic buffer capacity of 50 μeq H^+/pH into account (Felle 1987; Guern et al. 1991), this means that chloroplasts are able to store a substantial amount of H^+. During anoxia, there is no pH reaction to light/dark changes, which means that pH signaling between cytoplasm and chloroplasts need not be considered under these conditions.

5.4.1 The pH Trans-Tonoplast pH Gradient

The vacuole is a more or less acidic compartment, and it is interconnected with the cytoplasm by a variety of H^+ cotransporters which mediate substrate translocation

between the two compartments. Since there is very little voltage across the tonoplast (10–20 mV), any change in the pH gradient across the tonoplast will largely result in a likewise change in H^+ driving force (pmf). Thus, the more acidic the vacuole the steeper the H^+ gradient (which in citrus fruits can reach easily a factor of 10^5, e.g. $pH_{Vac} \approx 2$; Echeverria et al. 1992), the more energy may be needed to hold the trans-tonoplast H^+ gradient. The question is: to what extent does the anoxic drop in cytoplasmic pH affect the tonoplast/vacuole in the short-term and to what extent is the energy shortage responsible for the long-term acidosis? A priori, the anoxic drop in cytoplasmic pH will reduce the H^+ gradient from the vacuole to the cytoplasm, which will reduce the H^+ pressure toward the cytoplasm to some extent. However, despite the remaining steep H^+ gradient, a passive "H^+ leak" from the vacuole to the cytoplasm may be rather small at first. One reason is that biomembranes are not very H^+ permeable (Deamer 1987), i.e. there are no H^+ channels that would permit such a leak as would be for other ions. It is a chemiosmotic principle that protons crossing a membrane do this either via a primary active system (a pump), through cotransport or as weak acids (or bases). Figure 5.3 explains this and one comes to the conclusion that (at least for some time) after anoxic conditions have set in, vacuolar pH should actually become more acidic. Indeed, upon anoxia and shortage of energy substrates and ions that have been cotransported (H^+ antiport) under normoxia and were accumulated within the vacuole will now flow down their gradient (leak back into the cytoplasm) and thus drive H^+ into the vacuole until gradients of substrate and H^+ inversely match. Clearly, the substances involved would be very different from plant to plant and from cell to cell, but since the vacuole is a storage compartment holding energy-rich compounds, for some cells the reactivation of those molecules would be a primary task. Therefore, the assumption that anoxia should lead to an increase in the vacuolar pH right away is – at least from the transport physiological point of view – not necessarily straightforward. In this context, it is also most important to realize that the H^+ circulation through any membrane is heavily impaired under anoxia (Felle 2005).The reason is that the pump works as a current source, i.e., a substantial amount of pump-exported protons are recycled providing a cotransportable substrate/ion is present. As soon as the pump stalls because of shortage of ATP, the H^+ circulation ceases which drastically reduces the amount of H^+ reentry. All in all, pH signaling between these two compartments would probably not play a major role during phase B (Fig. 5.1) of anoxia. There are reports which indeed show vacuolar acidification (Libourel et al. 2006; Kulichikhin et al. 2007), but there are also reports that show the opposite (Roberts et al. 1984a; Menegus et al. 1991; Dixon 2001; Summers et al. 2000). Since for transport physiological reasons vacuolar pH cannot increase in short-term anoxia, the vacuolar pH increases reported may have metabolic reasons. Kulichikhin et al. (2007) compared the pH development in root tips of wheat and rice and demonstrated that in rice (not in wheat) the vacuolar pH had decreased after 3 h of anoxia but had increased after 6 h of anoxia. Obviously, as soon as the substrate and ion gradients across the tonoplast have collapsed, also H^+ will start leaking into the cytoplasm and cause cellular acidosis.

5.4.2 Cytoplasm and Apoplast

Apart from the energy-rich compounds that are stored within the vacuole, energy has to be provided from other stores; the transport of these compounds will, at least in the last instance before uptake into the cell to be broken down, be apoplastic. Whereas the vacuole is an inner compartment that permits mass storage, the storage capacity of the apoplast is limited; but, since it is linked to other organs, its importance as a transfer organelle for energy-rich compounds during anoxia is high. With respect to the pH, the apoplastic fluid has a buffer capacity of roughly one-tenth of the cytoplasm (Hanstein and Felle 1999; Oja et al. 1999), so apoplastic pH changes can become large (1 pH unit or more) and thus will have signaling characteristics. Although the apoplast is an acidic compartment like the vacuole, the signaling situation at the plasma membrane is different: the transmembrane voltage (membrane potential difference) contributes considerably to the proton motive force (pmf), which is affected much stronger by anoxia than the transtonoplast pmf which is largely a pH-pmf. Therefore, H^+ cotransport at the plasma membrane with energy-rich compounds is usually mediated as a symport, i.e. substrate and H^+ move into the same direction. So, any import of energy-rich compounds from the apoplast into the cytoplasm will always have the tendency to further acidify the cytoplasm. However, since under anoxia the cytoplasmic pH is lower than under normoxia and owing to the decreased pmf the symported substrate import is relatively low, this should be of no further consequence to the cytoplasmic pH.

5.4.3 The Apoplast Under Anoxia

As a typical stress response, the pH of the apoplast increases under anoxia, but not under hypoxia (Felle 2006). Whereas this may be the "normal" response of plants to anoxia that are not tolerant to oxygen deprivation, some plant species like *Oryza sativa* L., *Rumex palustris* and others (possessing highly anoxia tolerant organs) display fast shoot elongation when flooded. This elongation growth is due to acid-induced cell wall expansion and under the control of expansins (Cosgrove 1998), which requires an apoplastic acidification. A strong increase in *EXPA2* and *EXPA4* mRNA levels in rice coleoptiles has been reported (Huang et al. 2000). Considering that the organ really experiences anoxia, it is difficult to envisage how a largely deactivated H^+ pump could be responsible for the cell wall acidification,that is required for acid (elongation) growth. Along with this goes the observation that the cytoplasmic pH apparently drops only marginally. It almost appears that, from the pH point of view, the tissue involved did not make the anoxic switch but remained in a quasi-normoxic state. How is this possible? As to the elongation growth, it is well known that acid growth can be mimicked in the laboratory by acidification of the cell wall, which means pump activity is not required. Therefore, other processes

contributing to cell wall acidification could be involved, arising, for instance, from a net anion (Cl^-) efflux or cation/H^+ exchange at glucuronic acid chains.

In some anoxia-tolerant plants, the so-called "Pasteur effect" has been demonstrated (Summers et al. 2000) which is characterized by an acceleration of carbohydrate consumption to increase energy production. Although the ATP production is still 4.4–9 times lower than in air (Gibbs and Greenway 2003), it may be just enough to provide the energy necessary to drive the elongation growth thus enabling the plant a chance to quickly reach areas of higher oxygen. The acidification of the apoplast and the almost constant cytoplasmic pH would not only favor accelerated elongation growth but also increase the pmf to take up carbohydrates more efficiently. The duration of such a scenario would of course depend on the amount of carbohydrates available, but because of the energy used, it could diminish the survival time under anoxia in case higher oxygen was not reached.

5.5 Anoxia Tolerance and pH

"Anoxia tolerance" is generally understood as the ability of plant cells to overcome anoxia for an extended but undefined period of time. Basically, all plant cells can survive anoxia for at least a few hours, but metabolically specialized wetland plants are able to withstand anoxia for much longer. So the terms "short-term tolerance" and "long-term tolerance" would appear to describe the situation more accurately. For instance, emerging leaves of *Scheonoplectus lacustris, Scirpus maritimus, Typha anguistifolia* (Armstrong et al. 1994; Barclay and Crawford 1982) or tubers of *Potamogeton pectinatus* (Summers and Jackson 1996), just to name a few, have been demonstrated to withstand anoxia for weeks or even months. Still, no higher plant as a whole will tolerate total anoxia indefinitely, and even among the given examples long-term tolerance is organ-specific: whereas roots of *Oryza sativa* are as oxygen-sensitive as those of maize, shoots of rice display a remarkable anoxia tolerance.

Frequently, anoxia tolerance is brought into connection with pH, whereby cytoplasmic pH is found not to drop as much as in anoxia-intolerant plants (or organs). From these reports, it is difficult to decide whether this pH behavior is one of the key aspects of anoxia tolerance i.e., a requirement or just a consequence of the different conditions, e.g., metabolic adaptation. Other aspects of this problem are: (1) can one be sure that the plants really experienced full anoxia and not just hypoxia? In fact, hypoxia causes cytoplasmic pH first to drop in a manner similar to anoxia but then recovers to some extent (e.g. Kulichikhin et al. 2007). (2) Are the laboratory tests with the respective plants (or parts thereof) really comparable to field conditions? For instance, root tips in a solution experience anoxia differently than in the soil, as in the latter case the root will not be able to extrude substances to the same extent as in the test tube. A problem may become that excised root tips are without connection to the shoot from which energy-rich compounds could be

transported into the root. This may not be so important for tests of short-term tolerance, but in the long run it could yield an entirely different result.

5.5.1 pH as a Stress Signal – Avoidance of Cytoplasmic Acidosis

No doubt, stress of all kind acidifies the cytoplasm and alkalizes the apoplast (Wilkinson 1999; Felle et al. 2004; Felle et al. 2005), which characterizes the pH response as a very general signal that causes the upregulation of a broad array of genes. Despite the apparent concurrence in the pH response, anoxia is not just stress that can be responded to appropriately, but has far-reaching consequences with the potential death of the entire organism. Thus, the avoidance of cytoplasmic acidosis is generally assumed to be one of the main goals of an organism dealing with oxygen stress. For the short term (Fig. 5.1), this view may be questioned. Clearly, acidosis is fatal to a cell; however, pH 6.8 ($\pm$0.1), a value frequently reported as anaerobic in the cytoplasm, is not acidosis, but a pH very close to neutrality. Thus, tolerance of organisms toward oxygen stress may primarily be related not to acidosis *per se*, but rather to what extent the organism has sufficient energy to keep up the dynamic equilibria between the enzymatic reactions, as well as across membranes: as long as sufficient energy is available, pH will be kept stable at a value not harmful to cells, not by actively removing H^+ but by keeping up transmembrane gradients (ions, energy-rich substrates). Although acidosis and energy shortage appear closely interrelated, which makes it difficult under most circumstances to distinguish them temporarily, it seems important to accept that acidosis within any given compartment is nothing else but the breakdown of transmembrane (ion) gradients, where H^+ may not even be involved primarily (strong ion difference). Anoxic cytoplasmic acidification is also brought into connection with the deactivation of the H^+ ATPases across membranes because of apparently coinciding ATP decay and pH decrease. This view is logical, but in most instances likely to be incorrect as the inhibition of the H^+ ATPase(s) under normoxia proves. There is no reason to conclude that upon H^+ ATPase deactivation protons should leak through that membrane in amounts that would overcome the strong pH buffer capacity and acidify the cytoplasm, as measured. As pointed out above, there are no H^+ channels in biomembranes reported at molecular levels, and the amount of H^+ cotransported is small because of the strongly reduced H^+ circulation (Felle 2005).

Amongst the suggested means of the anoxic cell to ameliorate acidosis are: (1) H^+ consumption by succinate synthesis; this idea is based on the observation that long-term anoxia-tolerant rice had a greater succinate synthesis than the short-term tolerant maize leaves (Menegus et al. 1989). Since the pH data were collected from cell sap of squeezed tissues, an interpretation is difficult because, in such samples, the contents of all cellular compartments with different volumes and buffering capacities contribute to the final value. (2) Alanine as an end product of anaerobic fermentation in roots (Good and Muench 1993; Reggiani et al. 1985; Thomson et al.

1989). The ameliorating significance is unclear. In maize root tips, Roberts et al. (1992) observed that enhanced synthesis of alanine did not modify cytoplasmic pH. (3) Pyrophosphate substitutes for ATP at the tonoplast as an energy source. In fact, a 75-fold increase in pyrophosphatase (PPase) activity was observed in anoxic rice seedlings (Carystinos et al. 1995). A decreasing cytoplasmic pH would also favor pyrophosphate as energy source (Davies et al. 1993). In spite of these observations, an effect on cytoplasmic pH of PPase activity under anoxia has not been demonstrated yet. (4) NO_3^- (Fan et al. 1988, 1995; Gibbs and Greenway 2003; Stoimenova et al. 2003) and/or NO_2^- (Libourel et al. 2006) has been suggested as a means of acidosis reduction. In fact, μmolar NO_2^- concentrations in the incubation medium have an ameliorating effect on cytoplasmic pH under anoxia, i.e. pH 6.7 without NO_x vs. pH 6.9 in the presence of NO_x. Question remains, however, whether the difference of 0.2 units really helps the cells to better withstand anoxia. (5) Lactate (or lactic acid) extrusion has been postulated to mitigate negative anoxia effects by reduction of cytoplasmic acidosis (Xia and Saglio 1992; Rivoal and Hanson 1993, 1994; Xia and Roberts 1994, 1996). Although it is demonstrated that maize roots survived longer after acclimation to low oxygen and measured lower cytoplasmic lactate levels compared to non-acclimated plants, the causal links and especially the kind of lactic acid extrusion (transporter?) remain obscure. Since lactic acid has a very acidic pK_S of 3.08, essentially all lactic acid within the cytoplasm exists as anion, regardless of how large the anoxic pH switch becomes. Therefore, passive loss of the protonated acid to the two large ambient compartments (vacuole, apoplast) can be neglected. Export of the anion is also no option because it would always lead to cytoplasmic acidification. The only possibility, an active export of lactic acid (which has not been demonstrated) is energy consuming and thus counterproductive. The observation that substantial amounts of lactic acid actually do appear in the test medium is due to the fact that a lactic acid gradient is kept up between roots and medium. This is not the case in vivo, where lactic acid will leak largely into the apoplast, a small space which very quickly gets saturated with lactic acid and prevents its further export. Even though, a correlation of lactate extrusion and cytoplasmic pH and survival of the maize roots has been demonstrated. The question remains whether these are causal correlations, and did cells survive anoxia (within the tested time) because cytoplasmic pH was 0.3 units less acidic in the acclimated system? An alternative possibility would be an improved energy metabolism (which of course would to some extent also include pH). An unambiguous conclusion cannot be made at this point.

5.6 pH as Signal for Gene Activation

It has been known for some time that cytoplasmic acidification is one of the preconditions for gene activation. This has been demonstrated with elicitors like *N*-acetylchitoheptaose (He et al. 1998) and oligogalacturonides as well as with weak acids like propionic acid (Lapous et al. 1998). This compound rapidly (6–10 min after

addition) induced gene expression in suspension cultured rice cells. The levels of mRNAs were up-regulated for acid-responsive genes *EL2, EL3, PAL*, but not for acid-nonresponsive genes. The kinetics of the cytoplasmic acidification induced by propionic acid is quite similar to the anoxic pH drop which could indicate a causal relationship, an idea not too far fetched if one accepts the anoxic pH drop as the result of biochemical reactions that involve organic acids (lactic- and malic acids).

Although anoxia quickly inhibits protein synthesis in general, only a small group of proteins – the anaerobic proteins – continue to be made. Of these transcripts, most are enzymes of glycolysis and fermentation. Clearly, these enzymes are required under normoxic conditions as well, but the observation that these are among the few that are induced under hypoxia and expressed under anoxia, underpins their importance under these life endangering conditions. Among these enzymes, pyruvate-decarboxylase is interesting because its activity increases substantially with lower pH (Morrell et al. 1990).

5.7 pH Signaling and Oxygen Sensing

Bailey-Serres and Chang (2005) have suggested that the rapid decrease in cytosolic pH may reflect indirect oxygen sensing in plant cells. In fact, as Fig. 5.4 shows, cytosolic pH drops within 2–3 min after oxygen shortage has started by several tenths of a pH unit. The fast pH initial response is independent of O_2 once the O_2 level falls short of a level of about 10%. The difference in the response is the pH recovery. As a consequence thereof, cytoplasmic pH would not be an oxygen-sensing signal; also because it is the result of anoxic biochemical responses and not their cause. Therefore, O_2 sensing in plants would be at the level of the respiratory chain; it may be a matter of debate whether this would be real sensing or just a response to the absence or shortage of one reactant (adenylates, ATP etc.).

5.8 Conclusions

No doubt, pH plays a central role during anoxia and when cytoplasmic pH drops below a certain value, cell damage and cell death become the inevitable consequence. However, the conclusion that the *active* maintenance (or regulation) of a certain cytoplasmic pH under anoxia was the means to ameliorate or even to overcome anoxia should be reconsidered. The problem is that pH regulation critically depends on the regulation and activity of other ions; for instance, there is no pH shift because of H^+ transport without charge compensation. Therefore, any disturbance in the ionic composition of a compartment will *nota bene* have an impact on pH as well, meaning that pH changes never occur alone or isolated from but always together with other processes. In other words, a pH change always

signals other changes at the same time as well. For anoxia, this means that critical cytoplasmic acidosis signals the breakdown of transmembrane gradients that cannot be maintained because of the energy shortage. It is the maintenance of the transmembrane gradients – ions and energy-rich compounds – that is the foremost problem an anoxic cell has to accomplish. Amongst the various functions, pH is involved in a cell as a signal which informs about the energy status of a certain cellular compartment.

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