Impact of Oxygen Stress and Energy Availability on Membrane Stability of Plant Cells

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This article reviews the relationship between the energy status of plant cells under O_2 stress (e.g. waterlogging) and the maintenance of membrane intactness, using information largely derived from suspension cultures of anoxia-intolerant potato cells. Energy-related parameters measured were fermentation end-products (ethanol, lactate, alanine), respiratory rate, ATP, adenylate energy charge, nitrate reductase activity and biomass. ATP synthesis rates were calculated from the first four parameters. Reactive oxygen species were estimated from H_2O_2 and superoxide levels, and the enzymatic detoxification potential from the activity levels of catalase and superoxide dismutase. Structure-related parameters were total fatty acids, free fatty acids (FFAs), lipid hydroperoxides, total phospholipids, *N*-acylphosphatidylethanolamine (NAPE) and cell viability. The following issues are addressed in this review: (1) what is the impact of anoxia on membrane lipids and how does this relate to energy status; (2) does O_2 *per se* play a role in these changes; (3) under which conditions and to what extent does lipid peroxidation occur upon re-aeration; and (4) can the effects of re-aeration be distinguished from those of anoxia? The emerging picture is a reappraisal of the relative contributions of anoxia and re-aeration. Two successive phases (pre-lytic and lytic) characterize potato cells under anoxia. They are connected by a threshold in ATP production rate, below which membrane lipids are hydrolysed to FFAs, and NAPE increases. Since lipid peroxidation occurs only when cells are reoxygenated during the lytic phase, its biological relevance in an already damaged system is questionable.   © 2002 Annals of Botany Company

Key words: *Acorus calamus* L., energy shortage, free fatty acids, lipid peroxidation, lipolytic acyl hydrolase, lipoxygenase, membrane intactness, *N*-acylphosphatidylethanolamine, O_2 stress, reactive oxygen species, *Solanum tuberosum* L.

INTRODUCTION

In plant cells, O_2 participates in more than 200 different reactions (Hendry, 1994). This broad spectrum ranges from respiration, which draws on over 95 % of the cellular O_2 consumption to cover the energetic needs of the cell (Babcock, 1999), to the introduction of a double bond in a fatty acyl chain, which uses less than 0·007 %, to confer the appropriate fluidity to a given membrane (Rébeillé *et al*., 1980). When plants are submitted to flooding, their underground organs must then face a microenvironment that remains hypoxic or even anoxic for relatively long periods of time. Under these conditions, some plants adapt and survive. Many others (including valuable crops) are more sensitive and soon show irreversible damage. Later, if the water table falls too rapidly, the sudden irruption of air imposes a new oxidative challenge to the already damaged plants. Thus, O_2 stress has two facets, deprivation always preceding re-aeration. Under these conditions, energy shortage and perturbed membrane structure can be viewed as important constraints imposed by O_2 stress on plant cells. The multifarious effects of O_2 stress on sensitive and resistant plants have been extensively reviewed during the last decade (Armstrong *et al*., 1994; Sachs, 1994; Ratcliffe, 1995; Crawford and Braendle, 1996; Drew, 1997; Vartapetian and Jackson, 1997; Braendle and Crawford, 1999).

A non-interrupted access to an energy source, such as fermentable sugars, is a prerequisite for survival in an O_2-deprived environment (Barclay and Crawford, 1983). Anoxia-tolerant plants are particularly efficient at mobilizing storage polysaccharides when challenged by the higher carbohydrate consumption (Pasteur effect) required by fermentation processes (Perata *et al*., 1992, 1996). Transcription of α-amylase, for instance, could be observed in rice seeds but not in barley or wheat (Perata *et al*., 1993). Moreover, exogenously supplied sugars could improve energy metabolism and survival (Webb and Armstrong, 1983; Saglio, 1985; Perata *et al*., 1992) as well as restore the mitochondrial ultrastructure of both sensitive and tolerant species under anoxia (Vartapetian *et al*., 1977). A bacterial *PDC* gene was overexpressed in tobacco plants with the aim of improving the anoxia tolerance of roots by enhancing the carbon flux through the ethanolic fermentation pathway (Tadege *et al*., 1998), since this enzyme is usually expressed at very low levels and is probably rate-limiting during O_2 deprivation (Morrell *et al*., 1990). The outcome was rather disappointing: the increased flux in ethanolic fermentation of the transgenics did not enhance anoxia tolerance as compared with the wild type, whereas simple carbohydrate replenishment improved survival (Tadege *et al*., 1998). We wanted to know whether the well-known failure of potato tubers to survive waterlogging could be ascribed at least partly to a restricted mobilization of starch. Our investigations focused on α-amylase because this enzyme, in contrast

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to phosphorylase (S. Arpagaus, pers. comm.), initiates the hydrolytic break-up of starch in the amyloplast (Steup, 1983; Witt and Sauter, 1995). We measured gene expression, protein synthesis and activity of α-amylase as well as sugar levels in the particularly anoxia-intolerant potato tubers (*Solanum tuberosum* L.) of the Désirée variety and compared them with those of the extremely tolerant *Acorus calamus* (L.) rhizomes (Arpagaus and Braendle, 2000). Northern blot analysis showed that in both plant organs, the level of α-amylase mRNA was constant over at least 10 d and was affected neither by incubation under air, nor by anoxia. Since messengers remained present under anoxia (as well as under other stress conditions; see Bailey-Serres, 1999), a translational regulation of the α-amylase synthesis was expected. Immunoblotting did indeed reveal that the protein level of α-amylase decreased considerably in anoxic potato tubers, whereas it increased slightly in *A. calamus* rhizomes. These variations were well mirrored in the *in vitro* hydrolytic activities measured with the artificial substrate *p*-nitrophenyl-maltoheptaoside. In line with these data, the fermentable sugars (sucrose, glucose and fructose) increased markedly in rhizomes maintained under anoxia, whereas this treatment resulted in an almost complete disappearance of fermentable sugars in potato tubers. The existence of a functional α-amylase is an absolute requirement of wetland plant rhizomes to ensure a permanent supply of fermentable sugars for survival and growth under anoxia (Crawford, 1994). Conversely, the impaired translation of α-amylase is one of the crucial causes of the intolerance of potato tubers to anoxia (Arpagaus and Braendle, 2000).

The relationship between energy status and the fate of plant membrane lipids under O_2 deprivation has received little attention to date. Lipid alterations necessarily occur whenever mitochondria damage develops in sensitive plants under anoxia, and this has been associated with the resulting unfavourable energy status (Vartapetian *et al.*, 1985; Vartapetian and Zakhmylova, 1990; Andreev *et al.*, 1991). Furthermore, lipid synthesis decreases and desaturation stops because of their respective ATP and O_2 requirements (Vartapetian *et al.*, 1978; Brown and Beevers, 1987). Consequently, plant organs in which lipid turnover (via enzymatic deacylation, reacylation and/or *de novo* synthesis) is low are at a definite advantage under prolonged anoxia, because (1) membrane intactness is better preserved and (2) the *de novo* fatty acid synthesis contributes only marginally to the renewal of membrane lipids, thus maintaining the fluidity of the membrane within a range compatible with its functions. This distinct property of highly tolerant organs has been observed in the rhizomes of *Acorus calamus* (L.) and *Schoenoplectus lacustris* (L.) for instance (Henzi and Braendle, 1993). On the other hand, we have shown that during the first 6 h of anoxia, the adenylate levels and energy charge of potato tubers decreased continuously, and the ATP production rate became too low to sustain the basal metabolic requirement of the tuber in spite of its ample starch reserves (Sieber and Braendle, 1991). Membrane damage could be induced by ATP deprivation, as suggested by the correlation between electrolyte leakage and free fatty acid (FFA) release in anoxic tubers (Crawford and Braendle, 1996). A link should thus exist between the energy status of the tuber and its ability to preserve the intactness of its membrane lipids under anoxia. It is widely recognized that phospholipase activities are involved in the response of plants to several environmental stresses (Chapman, 1998). However, anoxic stress was not mentioned in this list, although there is no *a priori* reason why it should not be.

Potato cell suspensions as a model system for studying responses to anoxia

Potato is an important crop whose tubers show a high sensitivity to O_2 deprivation. However, because of their compactness, tubers are not well suited for some types of experiments. We have recently chosen to work essentially with potato cell cultures as an alternative and practical model for anoxia studies. Cell suspensions allow an optimal diffusibility of gases and solutes, possess an inherent homogeneity and are ideally suited to work with chemical effectors in known concentrations. Cells can be easily filtered and washed even under anaerobic conditions. Finally, the duration of anoxic treatments, which is 2–10 d for tubers, can be shortened to 24 h with cell cultures.

In the following sections we review recent work on the effects of energy depletion (via anoxia or via the addition of metabolic inhibitors under normoxia) on the membrane lipids of cultivated potato cells (*S. tuberosum* 'Bintje'), and show the existence of a threshold in ATP synthesis rate under which these cells become irreversibly committed to lipid hydrolysis and FFA release (Rawyler *et al.*, 1999). We also show that lipid hydrolysis in anoxic potato cells is significantly delayed and their survival enhanced when nitrate reductase activity can stimulate the glycolytic flux by recycling additional NADH to NAD^+, thus increasing its energy output rate (Oberson *et al.*, 1999). In addition, we report that the level of the very minor phospholipid *N*-acylphosphatidylethanolamine (NAPE) is markedly increased and that new NAPE molecular species are synthesized in potato cells in response to energy shortage (Rawyler and Braendle, 2001). Finally, we assess the role of reactive oxygen species (ROS) and lipoxygenase (LOX) in post-anoxic lipid peroxidation.

UNDER ANOXIA, ATP AVAILABILITY TRIGGERS THE TRANSITION BETWEEN MEMBRANE INTACTNESS AND DISRUPTION

Changes in biomass, lipid composition, fermentation end-products, ATP levels and synthesis rates have been studied in potato cells incubated for up to 24 h under anoxia (Rawyler *et al.*, 1999). These changes were then compared with those obtained in cells treated for the same time in the presence of O_2 with various inhibitors that interfered with energy metabolism. Potato cells (*S. tuberosum* 'Bintje') were suspended in Murashige & Skoog medium supplemented with 90 mM sucrose, a concentration that was high enough to prevent sugar starvation even under anaerobiosis over the time scale of our experiments.

Cells exhibited a two-phase behaviour with respect to anoxia. The first phase (from 0 to 10–12 h) reflected the survival of cells that could temporarily cope with the stress condition by switching to the fermentation mode. There was no net loss in cell biomass, and cell viability was maintained, although both ATP level and synthesis rate were depressed to lower values owing to fermentative metabolism. Cell membranes were still intact, as attested by the complete absence of lipid degradation. Accordingly, this first phase was called the pre-lytic phase. The second phase was an autolytic process starting approx. 10–12 h after the onset of anoxia. It was characterized by an irreversible drop in cell biomass, a dramatic decrease in cell viability, a further reduction in ATP levels and production rates and a massive accumulation of FFAs at the expense of phospholipids. The extent of hydrolysis reached 55–60 % after 24 h of anoxic stress, indicating extensive membrane disruption and loss of cell compartmentalization. Accordingly, this second phase was called the lytic phase. Clearly, the fate of these cells can be sealed already under anoxia.

Analysis of total lipid extracts of damaged cells showed that on a molar basis, each phospholipid molecule hydrolysed gave rise to two fatty acyl chains, whereas lysophospholipids were not detectable. In addition, after significant lipid hydrolysis, the acyl composition of the FFAs released closely matched that of total lipids. Moreover, when the hydrolytic activity of a potato cell extract on the artificial substrate *p*-nitrophenylpalmitate was assayed in a Ca^{2+}-free buffer (Galliard, 1971), a substantial amount of palmitic acid was produced. Finally, when fresh normoxic cells were mechanically disrupted (by ultrasonication or with a Yeda press) and further incubated under either normoxic or anoxic conditions, FFA release started immediately, and was similar in extent and rate to that observed during the lytic phase (C. Reusser, pers. comm.). Collectively, these facts indicate that lipid hydrolysis is due to the constitutive presence of an unspecific lipolytic acyl hydrolase (LAH)—an enzymatic activity expressed by the major tuber protein patatin (Andrews *et al*., 1988)—that becomes activated after a threshold time under anoxia, cleaving both fatty acyl chains from membrane lipids and releasing the water-soluble polar headgroups in the surrounding medium. LAH was claimed to be sequestered in lysosomes (Wardale and Galliard, 1977) or to be a part of the latent vacuolar lytic potential (Travnicek *et al*., 1999), although a cytoplasmic location cannot be excluded (Senda *et al*., 1996). This enzyme, known to be active as soon as the cell is ruptured, has already been implicated in the response of plant cells to mechanical and pathogenic wounding (Racusen, 1984; Slusarenko *et al*., 1991; Farmer and Ryan, 1992) and in the autophagic process induced by sucrose starvation (Aubert *et al*., 1996). The reasons behind the latency of LAH in normoxic cells and how the enzyme becomes activated in anoxic potato cells have not yet been elucidated.

Under anoxia, the pyruvate formed by glycolysis was further metabolized to alanine, lactate and ethanol (Rawyler *et al*., 1999). The latter was not only the major end-product of fermentation, but its production increased steadily up to 24 h, whereas lactate and alanine syntheses were essentially arrested after 12 h. Simultaneously, the ATP level decreased

to 10 and 5 % of its initial value after 12 and 24 h of anoxia. According to Roberts *et al*. (1984), the ATP content of a cell cannot sustain its energy demand for more than 1–2 min. On a time scale of hours, the main determinant of energy balance should be the rate of ATP synthesis rather than ATP level (Tadege *et al*., 1998). Assuming a maximum theoretical yield of 38 ATP for six O_2 consumed and an equimolar correspondence between each fermentation end-product and ATP, we calculated the ATP synthesis rates of potato cells from their respiration rate (normoxic cells) and from the summed contributions of each fermentation end-product (anoxic cells), taking into account the energy saved if the cell were to shift from the invertase to the sucrose-synthase feeding mode of glycolysis (Stitt and Steup, 1985; Plaxton, 1996). Although the feeding mode has a very limited bearing on the energy production rate in respiring cells (608 *vs*. 640 µmol ATP g^{-1} f. wt h^{-1}), it does have a strong impact under anaerobiosis (e.g. 10 *vs*. 20 µmol ATP g^{-1} f. wt h^{-1} after 12 h of anoxia). A critical role of sucrose-synthase in improving anoxia tolerance was also shown in maize roots (Ricard *et al*., 1998).

By incubating cells for various time periods under anoxia, we were able to modulate the ATP synthesis rate and to show that the relationship between this rate (as a measure of the energetic competence of the cell under anoxia) and the extent of lipid hydrolysis was of all-or-none nature, as previously postulated (Xia *et al*., 1995). Indeed, a narrow range of rates (10–20 µmol ATP g^{-1} f. wt h^{-1}) was identified as the metabolic threshold above which membrane intactness was fully preserved, and below which lipid hydrolysis inevitably occurred (Rawyler *et al*., 1999).

If energy supply (as ATP) is the key factor for the maintenance of cell intactness under anoxia, it should also control lipid hydrolysis in normoxic cells treated with metabolic inhibitors. In the presence of the uncoupler FCCP (carbonyl-cyanide-4-trifluoromethoxyphenylhydrazone), the respiration-dependent, membrane-linked phosphorylations are suppressed, whereas the substrate-level phosphorylations of the glycolytic pathway and of the tricarboxylic cycle still occur and are even enhanced by the uncoupler-accelerated O_2 uptake rate. Such uncoupling conditions allowed sufficiently high rates of substrate-level phosphorylation (60–144 µmol ATP g^{-1} f. wt h^{-1}) at the glycolysis and tricarboxylic acid cycle levels, and no lipid hydrolysis occurred during 24 h or more. Azide inhibits at the end of the cytochrome pathway, whereas SHAM (salicylhydroxamic acid) blocks the alternative oxidase pathway (Vanlerberghe and McIntosh, 1997). Lipid hydrolysis was observed exclusively when cells were simultaneously treated with one inhibitor of each pathway. This shows that when all membrane-linked redox reactions are inhibited in mitochondria, the glycolytic and fermentative pathways cannot prevent lipid hydrolysis, as previously observed under anoxia. We also blocked the glycolytic pathway by incubating cells in the presence of both DeOGlc (2-deoxy-D-glucose) and IAc (sodium iodoacetate). This treatment was more efficient in triggering lipid hydrolysis than the inhibition of respiration, because the upstream localization of the blockage caused a more complete inhibition of the ATP production than did respiratory

inhibitors. Moreover, lipid hydrolysis was also an all-or-none process and the acyl composition of the FFAs released in chemically inhibited normoxic cells was identical to the FFA pattern observed in anoxic cells, indicating that LAH was also responsible for these degradations (Rawyler *et al.*, 1999).

Interestingly, the autophagic process triggered in sycamore (*Acer pseudoplatanus* L.) cells submitted to sugar starvation under normoxia also led to membrane damage, including lipid deacylation (Dorne *et al.*, 1987). The preservation of cell structure and function by restoring a 'normal' respiratory activity with pyruvate (Aubert *et al.*, 1996) thus points to a peculiar role of the mitochondrion in situations of energy shortage. For instance, ATP is required to preserve cytoplasmic ion homeostasis, especially with respect to Ca^{2+} ions (Bush, 1995). However, when cells are deprived of O_2, the mitochondrial ATP-synthase begins to hydrolyse part of the glycolytically produced ATP in an attempt to maintain mitochondrial proton motive force (St-Pierre *et al.*, 2000). This 'cellular treason' can speed up the bioenergetic failure of anoxic cells.

The behaviour of anoxic potato cells thus depends entirely on the efficiency of their metabolic survival strategy, that is, on their capacity to resolve the dilemma of the reallocation of energy between essential and non-essential ATP-demanding processes. This challenge includes several coexisting aspects. First, ATP-consuming processes of lower priority can be suppressed, so as to preserve enough ATP to maintain vital functions, e.g. ionic homeostasis (Bush, 1995; Barkla and Pantoja, 1996; Sze *et al.*, 1999). The metabolism of sucrose via sucrose-synthase rather than via invertase, for example, can improve the residual ATP production (Sachs, 1994; Plaxton, 1996), resulting in an increase in the ATP net yield of 1·5- to 2-fold, according to pyrophosphate availability (Mertens, 1991; Stitt, 1998). Entering into anaerobic retreat can slow down the ongoing ATP-consuming processes (Pradet and Raymond, 1983). Finally, the efficiency of some of these ATP-consuming processes can be enhanced, e.g. by raising the H^+ to ATP ratio of plasmalemma and tonoplast H^+-ATPase pumps (Slayman, 1980). Which elements of this survival strategy were used by anoxic potato cells, and to what extent, is not known yet. In any case, this strategy could not prevent the cell—except during the first hours of anoxia—from reaching the metabolic threshold at the end of the pre-lytic phase that activates the formerly silent LAH and causes lipid hydrolysis during the lytic phase (Rawyler *et al.*, 1999).

NITRATE INCREASES MEMBRANE STABILITY IN POTATO CELLS UNDER ANOXIA

Since transition from the pre-lytic to the lytic phase appears to be controlled by energy availability, increasing the metabolic competence of potato cells under anoxia is expected to improve their resistance by postponing this lethal transition. Overexpression of a key enzyme of the ethanolic fermentation pathway (e.g. PDC), though seductive in theory, did not enhance the tolerance of tobacco roots to anoxia (Tadege *et al.*, 1998). Randomized deletions in the *patatin* gene would selectively suppress the LAH activity of patatin without affecting the cell protein content, but would also impair the response potential of cells (especially tubers) to pathogenic stresses (Slusarenko *et al.*, 1991; Farmer and Ryan, 1992). Other avenues are thus desirable.

Nitrate is considered as an alternative electron acceptor able to sustain glycolysis and increase the ATP level in rice seeds (Reggiani *et al.*, 1985*a*, *b*, 1993*a*, *b*; Fan *et al.*, 1997) and in embryonic axes of *Erythrina caffra* (Kemp and Small, 1993). In addition, gene expression of the nitrate reduction pathway has been observed in germinating rice under anaerobiosis (Mattana *et al.*, 1994). In pea roots, nitrate reductase increased during anoxia but decreased upon re-aeration (Glaab and Kaiser, 1993). We investigated whether the energy gained from coupling nitrate reduction to NADH reoxidation was beneficial to the stability of potato cell structures under anoxia (Oberson *et al.*, 1999). To this end, cells were suspended in a sucrose-enriched Murashige & Skoog-based medium in the presence of nitrate or ammonium as the sole N source, and well-buffered (at pH 5·6) so as to counteract pH variations associated with ion absorption. The NADH-dependent nitrate reductase activity was preserved and nitrite formed in large amounts up to 18 h of anoxia in the presence of nitrate, in total contrast with the ammonium medium. Although both ATP level and adenylate energy charge (AEC) diminished during anoxia, their values were significantly higher and more stable in the nitrate than in the ammonium medium. The cell biomass was maintained 6 h longer in the nitrate than in the ammonium medium before starting to decrease. The transition from pre-lytic to lytic phase occurred in both cases, but the time course of FFA release was also delayed by about 6 h in nitrate-treated cells. When asparagine was substituted for ammonium, membrane breakdown was comparable with that observed with ammonium. Therefore, delayed membrane degradation could be associated with the presence of nitrate and not the absence of ammonium (Oberson *et al.*, 1999). The most obvious interpretation is that under anoxia, the energy status is improved by the extra energy retrieved from NADH reoxidation coupled to nitrate reduction. In addition, the acyl composition of the FFAs released was the same in both nitrate- and ammonium-treated cells, suggesting that lipid hydrolysis stems in both cases from a single enzyme, very likely the LAH identified by Rawyler *et al.* (1999).

NAPE—A NEWLY FORMED, STRESS-RELATED LIPID?

Mammalian tissues have been known for 30 years to contain low levels of the unusual phospholipid NAPE. This compound is characterized by the presence of a third fatty acyl group linked to the *N*-atom of the phosphatidylethanol-amine headgroup by an amide bond and shows a propensity to accumulate under various pathological conditions involving degenerative membrane changes (Schmid *et al.*, 1990). Several roles have been attributed to NAPE, including membrane protection and stabilization, participation in cell signalling processes and response to stresses. Properties

similar to those of the recently 'rediscovered' plant NAPE were also recognized (Chapman, 2000). However, the involvement of NAPE in the response of plant tissues to O$_2$ stress has not yet been addressed.

A minor phospholipid class has been isolated (Rawyler and Braendle, 2001) from potato cells, chromatographically purified and identified by electrospray ionization mass spectrometry (ESI-MS) as *N*-acyl-*O*-(1,2-diacyl-*sn*-glycero-3-phosphoryl)-ethanolamine (Fig. 1). The basal NAPE level was low in unstressed cells (13 ± 4 nmol g^{-1} f. wt). According to acyl chain length, only 16/18/18 species (group II) and 18/18/18 species (group III) were present. These two groups of NAPE molecular species were rich in di- and triunsaturated fatty acyl chains. The NAPE level increased up to 13-fold in anoxia-stressed cells, but this occurred only when FFAs started being produced after the pre-lytic to lytic transition. The NAPE level was linearly correlated with the extent of FFA release until the latter reached about 30 %. At higher extents, this correlation was lost because NAPE itself started being hydrolysed by LAH. The level of the pre-existing groups II and III was progressively increased by unspecific *N*-acylation of phosphatidylethanolamine molecules with various acyl residues extracted from the growing FFA pool. But in addition, new 16/16/18 species (group I) appeared via a specific *N*-palmitoylation. A similar accumulation of NAPE also occurred in aerated cells treated with NaN$_3$ + SHAM. The *N*-acyl patterns of NAPE were dominated by 18 : 1, 18 : 2 and 16 : 0 (fatty acids are abbreviated as C : n, where C is the number of carbons atoms in the chain and n the number of double bonds in that chain), but in no case did they reflect the FFA composition. Moreover, they did not change greatly either during the anoxic treatment or after the normoxic incubation in the presence of metabolic inhibitors. This was in marked contrast with the *O*-acyl patterns of NAPE, which became particularly enriched in 18 : 2 after these treatments (Rawyler and Braendle, 2001).

Anoxia-induced accumulation of NAPE is thus rooted in the failure of metabolic homeostasis due to energy deprivation, but not in the absence of O$_2$. As such, it shows a remarkable parallelism with the LAH-catalysed hydrolysis of membrane lipids described above (Rawyler *et al.*, 1999). The acyl composition of basal and stress-induced NAPE suggests the existence of spatially distinct FFA and phosphatidylethanolamine pools. It reflects the specificity of NAPE synthase, the acyl composition, localization and availability of substrates, all of which are intrinsic properties of the cell, but has no predictive value as to the type of stress imposed. Whether NAPE has a physiological role depends on the cell being still alive and its compartmentalization maintained during the stress period (Rawyler and Braendle, 2001). If re-aeration takes place prior to the onset of the lytic phase, at which point cell recovery is still possible (Pavelic *et al.*, 2000), NAPE is expected to play a key role as FFA scavenger, but only when its synthesis rate exceeds or equals the hydrolysis rate of membrane lipids. Its contribution would be even more crucial for membrane repair and maintenance as post-anoxic lipid peroxidation has been shown not to be an important issue for cultivated potato cells (Pavelic *et al.*, 2000; see below).
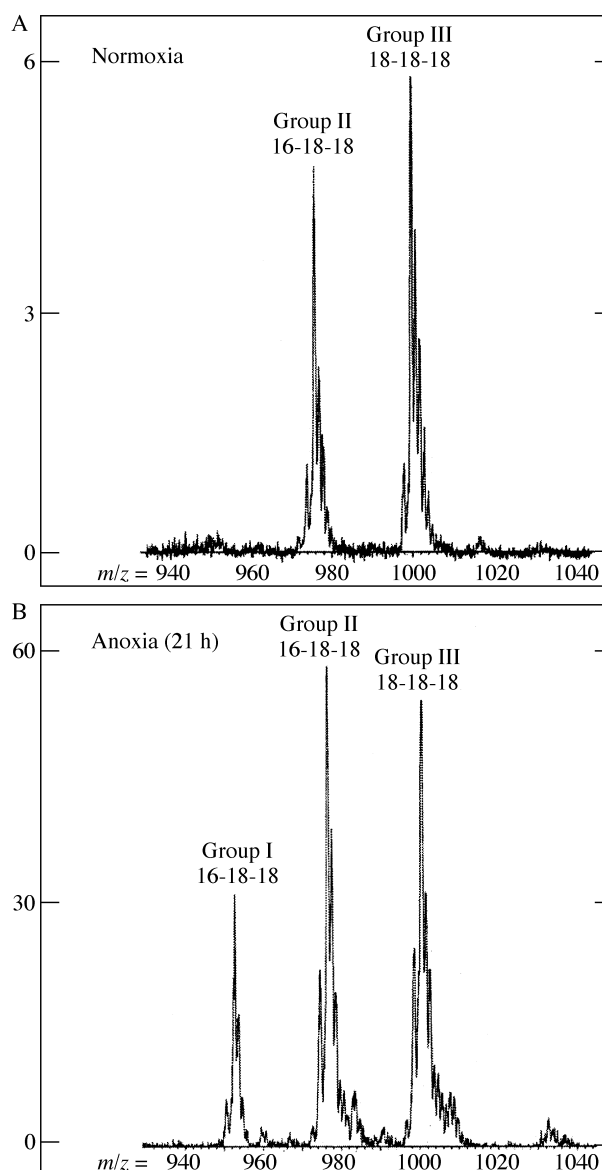

FIG. 1. Electrospray ionization mass spectra (negative mode) of the NAPE classes purified from potato cells incubated under normoxia (A) or under anoxia for 21 h (B). The abcissa displays *m/z* values and the ordinate represents the cellular levels of NAPE (nmol g^{-1} f. wt). Note the ten-fold higher ordinate scale. Each group of peaks represents a family of NAPE molecular species having acyl chains of a well-defined length (irrespective of their position as *O*- and *N*-acyl esters) and varying only in their degree of unsaturation. Thus, group I contains all those species having two 16-carbon chains (palmitate only) and one 18-carbon chain, group II all those species having one 16-carbon chain (palmitate) and two 18-carbon chains, and group III all those species having three 18-carbon chains.

POST-ANOXIC LIPID PEROXIDATION: THE ROLE OF ROS AND OF LOX

In several cases, the most apparent and severe injuries often occur after re-aeration of O$_2$-deprived organs, a phenomenon known as the O$_2$ paradox (Hendry and Crawford, 1994). These deleterious effects are usually ascribed to

reactive oxygen species such as the hydroxyl radical (OH·), the superoxide anion ($O_2 \cdot^-$) and hydrogen peroxide (H_2O_2), which are formed at many sites including the cell wall/plasmalemma, plastids, endoplasmic reticulum and mitochondria (Elstner, 1987; Scandalios, 1993; Elstner and Osswald, 1994). ROS are essentially considered as per-oxidizing agents acting, among others, on esterified and free polyunsaturated fatty acyl residues (Halliwell and Gutteridge, 1990; Halliwell, 1991) and less commonly as hydrolytic agents able to cleave the acylester bonds of membrane phospho- and glycolipids (McKersie *et al.*, 1990; Barclay and McKersie, 1994). Several authors have thus shown that re-aeration of anoxia-treated plant organs results in an increased production of end-products of lipid peroxidation, such as malondialdehyde and ethane, and have associated the observed damage with this oxidative process (Hunter *et al.*, 1983; Albrecht and Wiedenroth, 1994; Pfister-Sieber and Braendle, 1994, 1995). However, what is often overlooked is that in spite of their high chemical reactivity, ROS (and more generally any lipid peroxidation-promoting agent) are hazardous for living structures only. The possibility that the anoxic treatment itself would have already damaged the membrane structure in a ROS-independent way was neither clearly recognized nor rigorously assessed in these articles, whereas it was simply alluded to in a more recent report (Blokhina *et al.*, 1999).

We have addressed this issue by studying the impact of re-aeration on the membrane lipids of anoxia-pretreated potato cells in such a way that it was always possible to distinguish clearly between the effects of anoxia and those due to subsequent re-aeration (Pavelic *et al.*, 2000). First, we investigated whether the peroxidation of diacyl lipids and free polyunsaturated fatty acids (PUFAs) is achieved by the chemical pathway (via ROS) which exhibits a broad attack spectrum (Halliwell, 1991), and/or by the enzymatic pathway (via LOX) that attacks only lipids, and more specifically free PUFAs (Gardner, 1991; Grechkin, 1998). Secondly, we evaluated the relative importance of these two pathways, particularly with respect to the biphasic behaviour of membrane lipids exhibited by potato cells under anoxia (Rawyler *et al.*, 1999).

When anoxic cells in the pre-lytic phase were reoxygenated for 2 h, the superoxide anion was not detectable, the H_2O_2 level remained as low as that of controls, and cell viability was preserved. Lipids were intact and no lipid hydroperoxides could be detected. However, small amounts of lipid hydroperoxides did accumulate if anoxic cells were supplemented with a non-lethal amount of H_2O_2 and further incubated under anoxia for 2 h. When cells having entered the lytic phase of anoxia were reoxygenated for 2 h, levels of ROS were as low as before and there was no significant difference between control and anoxia pre-treatments. However, cell respiration decreased, reflecting the extensive lipid hydrolysis that had already started under anoxia and continued during re-aeration. Simultaneously to the massive release of free PUFAs, small amounts of lipid hydroperoxides were formed, reaching at most 1–2 % of total fatty acids. Blokhina *et al.* (1999) suggested that the anoxic treatment of rhizomes of sensitive and tolerant *Iris* spp. may

induce qualitative changes in the membrane lipids of the sensitive species that can make them susceptible to peroxidation, in line with these results.

It is worth mentioning that the level of free ROS actually measured always reflects the balance between ROS-generating and ROS-consuming processes. In plant cells, low intrinsic production rates can be achieved by several sensing and regulating mechanisms, such as the mitochondrial aconitase (Verniquet, 1991), the alternative oxidase (Maxwell *et al.*, 1999) or the plant uncoupling protein (Pastore, 2000). Efficient scavenging by antioxidants (e.g. reduced levels of gluthathione and ascorbate) and detoxifying enzymes [e.g. catalase (CAT) and superoxide dismutase (SOD)] or a high reactivity toward potential targets (e.g. lipids, nucleic acids, proteins) reduces the level of free ROS. We observed (Pavelic *et al.*, 2000) that CAT and SOD activities were not greatly affected, thereby suggesting that an efficient disposal of ROS was still ensured. On the other hand, the amount and activity of LOX tended to increase during anoxia. We concluded that the level of lipid peroxidation is low during reoxygenation of anoxia-pretreated potato cells and that it is mainly due to LOX, whereas the contribution of ROS is negligible. But, above all, lipid peroxidation is a late event that occurs only when irreversible damage has already been caused by the anoxia-triggered lipid hydrolysis catalysed by LAH. This casts some doubt on the pertinence of improving resistance against lipid peroxidation in plant tissues rich in patatin-like proteins by overexpressing genes involved in antioxidative reactions. Although adequate in the case of various oxidative stresses of abiotic and pathogenic origin (Hérouart *et al.*, 1993; Sen Gupta *et al.*, 1993; Foyer *et al.*, 1994; Mehdy, 1994; Yu and Rengel, 1999), such strategies may be unsuitable to rescue a waterlogged plant upon re-aeration once its roots have entered the lytic phase of anoxia. The possibility remains, however, that at low levels, ROS—and perhaps more particularly H_2O_2—could act as signalling rather than damaging molecules in plant cells. This would occur, for instance, when the mitochondrial electron transport chain becomes inhibited by the limited oxygen supply (hypoxia) during the transition from normoxia to anoxia, as recently suggested by Blokhina *et al.* (2001).

CONCLUSIONS

The main conclusions from the reviewed data are presented below and summarized schematically in Fig. 2. (1) The behaviour of potato cells under anoxia is characterized by two phases connected by a metabolic threshold. The pre-lytic phase defines the maximum time at which the anoxic cell is still intact and able to recover upon re-aeration. It also defines the time range over which the biochemical responses occurring upon re-aeration still have a physiological meaning. The lytic phase corresponds to the loss of membrane compartmentalization due to an LAH-catalysed hydrolysis of membrane lipids. The transition from the pre-lytic to the lytic phase is triggered when the ATP production rate of the cell falls below a threshold value (10–20 μmol ATP g^{-1} f. wt h^{-1}), regardless of the presence or absence of O_2. (2) The
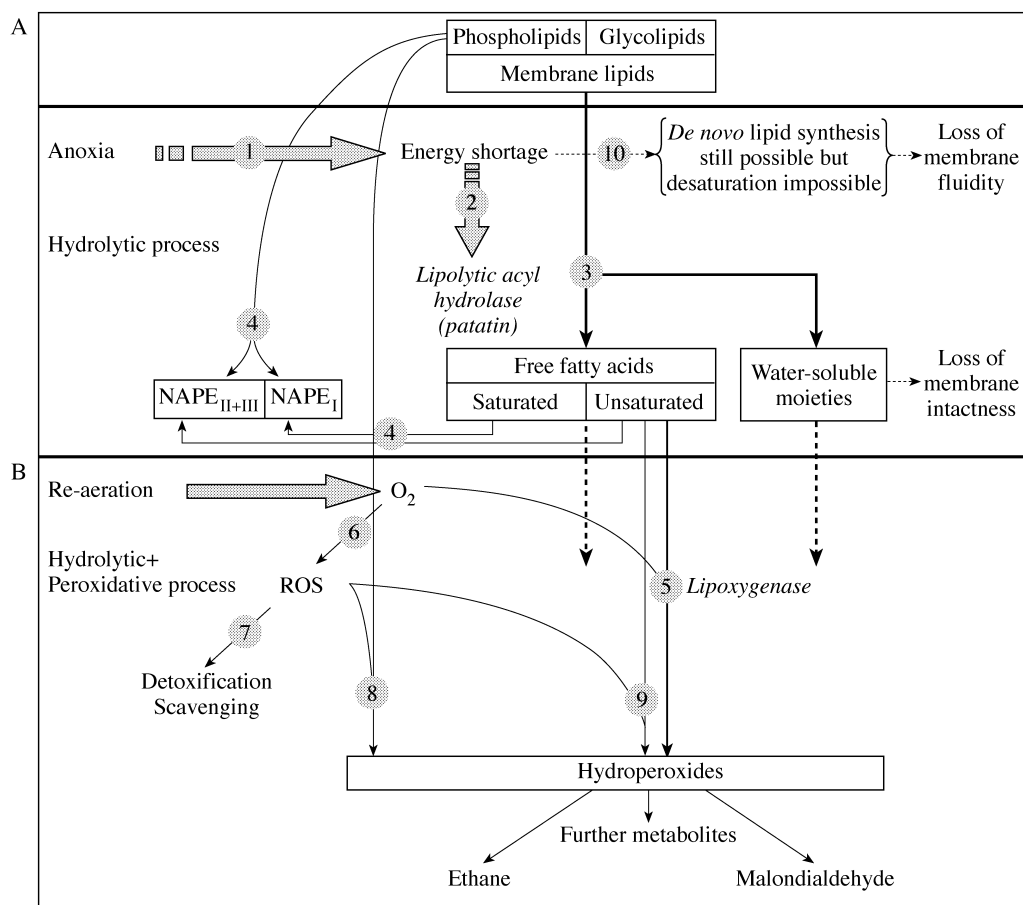
FIG. 2. Fate of membrane lipids (steps 1–9) in intolerant potato cells that are undergoing the two successive phases—anoxia and re-aeration—of oxygen stress. For comparison purposes, the behaviour of lipids in tolerant tissues is outlined in step 10. The relative importance of the processes in steps 3–9 is indicated by the size of the corresponding arrows. Incubation under anoxia (A) progressively leads to energy shortage (step 1). Passing below a threshold value of the energy production rate (10 μmol ATP g^{-1} f. wt h^{-1}) triggers activation of an LAH that belongs to the patatin family (step 2). This enzyme splits (step 3) both fatty acyl chains from diacyl-lipids and releases the water-soluble moieties of these molecules. Simultaneously, NAPE molecules are synthesized by coupling some of the free fatty acids produced to phosphatidylethanolamine molecules (step 4). Re-aeration (B) does not stop the hydrolytic process launched under anoxia (dotted arrows), but now allows peroxidative processes to occur. In re-aerated potato cells, the main peroxidative process is enzymatic, and attributable to LOX (step 5). The presence of O$_2$ may also contribute to the formation of ROS (step 6), which appears to be essentially eliminated by enzymatic detoxification and antioxidant scavenging (step 7), so that the ROS-dependent peroxidation of diacyl-lipids (step 8) or of FFAs (step 9) seems negligibly small in potato cells. The lipid and fatty acid hydroperoxides formed can be further metabolized to various physiologically active compounds and up to end-products such as ethane and malondialdehyde.

key role of the energy status in controlling this transition in anoxic cells is confirmed by the increased duration of the pre-lytic phase when the energy status is improved by the presence of nitrate as an alternative electron acceptor. (3) The capacity to increase its NAPE level under anoxia may confer some additional protection to the cell by scavenging FFAs at the beginning of lipid hydrolysis. (4) The lipid peroxidation process is of minor importance upon re-aeration of anoxia-pretreated potato cells. Ascribed to LOX and not to ROS, it occurs only when FFAs have been released. Its contribution to the overall damage to cell membranes is therefore marginal in comparison with the extensive lipid hydrolysis catalysed by LAH whilst under anoxia.

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