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Lysigenous Aerenchyma Development in Roots – Triggers and Cross-talks for a Cell Elimination Program

Dimitris L. Bouranis[1*] • Styliani N. Chorianopoulou[1] • Vassilis F. Siyiannis[1] • Vassilis E. Protonotarios[1] • Malcolm J. Hawkesford[2]

[1] Plant Physiology Laboratory, Plant Biology Department, Faculty of Agricultural Biotechnology, Agricultural University of Athens, 75 Iera Odos, 11855 Athens, Greece
[2] Crop Performance and Improvement Division, Rothamsted Research, Harpenden, Herfordshire AL5 2JQ, UK

Corresponding author: * **bouranis@aua.gr**

ABSTRACT

Aerenchyma, the tissue containing enlarged gas spaces and surrounded by parenchymatous cells, is formed either as part of normal development or in response to hypoxia (oxygen shortage), mechanical impedance, high temperature, nitrogen-, phosphorus- and sulphur-deficiency. Focussed in the root cortex, some cells die to create a lacuna. The diverse stimuli induce a cell elimination program (CEP) result in the same endpoint: a lysigenous aerenchyma formation in the root cortex. CEP occurs as an orchestrated series of events, suggesting a genetically controlled process of active cell death, and can be distinguished in three major processes: the activation process, the execution process and the dissemination and termination process. It seems that perturbations in the energy and the redox status of specific cortex cell stimulate the activation process, which may include the enzymes NADPH oxidase, phospholipase D, protein kinase C, mitochondrial permeability transition pore, cytochrome c, haemoglobin, ACC synthase, ACC oxidase and mitogen-activated protein kinase cascade, as well as the molecules ATP, NADH, reactive oxygen species, calcium, phosphatidic acid, nitric oxide, 1-amino-cyclopropane-1-carboxylate (ACC), and ethylene as CEP activators, organised in various signal transduction modules, while mitochondrion possess a key role in this part of CEP. The coordination of CEP activation modules results in the expression of CEP specific genes. Subsequently, the execution process, may include vacuolar processing enzyme and all the necessary hydrolytic enzymes including proteases, lipases, DNases, RNases, pectinases, cellulases, xyloglucan endo-*trans* glygosylase, expansins and γ-tonoplast intrinsic proteins as CEP executors and possibly again reactive oxygen species and peroxynitrite, with a key role of the lytic vacuole. Dissemination of CEP produces a tubular structure, the architecture of which is strongly affected by cell packing. Aerenchyma formation is a localised, site-specific CEP, the termination process of which determines the extent of the dissemination, to facilitate either an effective translocation of oxygen, carbon dioxide and ethylene or other gases, or to redirect scarce resources, or to provide a rapid diffusion path for solutes, within the root cortex.

Keywords: autophagy, ethylene, hydrogen peroxide, hypoxia, lacunae, lysigeny, nitric oxide, nutrient deficiency, programmed cell death
Abbreviations: **ACC**, 1-amino-cyclopropane-1-carboxylate; **ACO**, ACC oxidase; **ACS**, ACC synthase; **ADH**, alcohol dehydrogenase; **CEP**, cell elimination program; **ETR**, ethylene receptor; **Hb**, haemoglobin; **IS**, intercellular spaces; **MAPK**, mitogen-activated protein kinase; **MTP**, mitochondrial permeabilty transition pore; **NOX**, NADPH oxidase; **OXI1**, oxidative signal-inducible 1; **PA**, phosphatidic acid; **PCD**, programmed cell death; **PLD**, phospholipase D; **ROP**, Rho-related GTPase; **ROS**, reactive oxygen species; **SAM**, *S*-adenosyl methionine; **TIP**, tonoplast intrinsic protein; **VPE**, vacuolar processing enzyme; **XET**, xyloglucan endo-*trans* glygosylase

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Invited Review

INTRODUCTION

Some parenchymatous tissues enclose enlarged spaces which exceed those commonly found as intercellular spaces (IS) and in connection with them construct longitudinal channels that pass between the cells. This network of usually gas-filled spaces is called aerenchyma and comprises a specialised tissue found in petioles, stems and roots, within various anatomical patterns of cellular configuration. There are several recent reviews that cover aerechyma formation by hypoxia (see Jackson and Armstrong 1999; Evans 2003; Visser and Voesenek 2004), while a brief history of the research on aerenchyma, its definition and types have been provided by Seago *et al*. (2005). The two main types of aerenchyma are lysigenous and schizogenous.

Schizogeny is a cell separation process that occurs through the separation of cells from each other in an early stage of development. Schizogenous aerenchyma is more often found in the stems and petioles of wetland plants and may also be found in roots. When found in a root, schizogenous aerenchyma seems a more or less constitutive feature and occurrence does not change in those roots that are already present at the onset of soil flooding. Instead new roots may develop that contain a larger amount of schizogenous aerenchyma (Laan *et al*. 1989; Visser *et al*. 1996).

Lysigeny is cell death through lysis. Lysigenous aerenchyma formation is a programmed process under developmental control, where specific parenchymatous cells are programmed to self-destruct, while the surrounding cells survive. This type is initiated by death of cortical cells, resulting in voids between the living cells that remain. Lysigenous aerenchyma is most abundant in roots and rhizomes, and may develop in both mature and in newly-developing roots (Thomson *et al*. 1990).

Programmed cell death (PCD) comprises a functional concept that refers to cell death as part of the normal life of a multicellular organism and includes activation and execution of an orchestrated series of degradation processes, which are under genetic control and lead to controlled disassembly of the cell. There are numerous examples of PCD during plant development that conform to the general definition (Greenberg 1996; Buckner *et al*. 1998; Drew *et al*. 2000; Samuilov *et al*. 2000; Kuriyama and Fukuda 2002; Dickman and Reed 2004; Evans 2004; Mittler and Shulaev 2004; Woltering 2004; Drury and Gallois 2006). PCD in plants also occurs in response to abiotic stresses. PCD that occurs during normal differentiation and as a result of abiotic stress is called 'developmental' (van Doorn and Woltering 2005).

Aerenchyma formation by lysigeny is a developmental PCD, which may be constitutive, requiring an internal stimulus, or inducible by abiotic stress. Many wetland plant species display aerenchyma in their roots even under well-aerated conditions (Justin and Armstrong 1987). In the wetland species rice (Jackson *et al*. 1985b; Kawai *et al*. 1998) and *Sagittaria lancifolia*, as well as in the maize relatives *Tripsacum dactyloides* and *Zea luxurians* (Drew *et al*. 2000), aerenchyma forms without any requirement for an external stimulus.

The ability to form lysigenous aerenchyma is widespread even among dryland vegetation. Many plant species can be induced to form aerenchyma structures, particularly when growing under flooded soil conditions. Aerenchyma formation is inducible by flooding in maize (Drew *et al*. 1979; He *et al*. 1994), in the coastal grass *Spartina patens* (Drew *et al*. 2000) and in many monocotyledonous and dicotyledonous native species that occupy wetland habitats (Justin and Armstrong 1987). Aerenchyma formation in crop species such as sunflower (Kawase 1979), bean, tomato (Kawase 1981), wheat (Huang *et al*. 1994; Wiengweera *et al*. 1997), barley (Larsen *et al*. 1986; Garthwaite *et al*. 2003), and *Trifolium subterraneum* (Aschi-Smiti *et al*. 2003) seems to be controlled by hypoxia and ethylene.

In addition to hypoxia (oxygen shortage), other known stresses that induce aerenchyma formation are mechanical impedance, high temperature, nitrogen-, phosphorus- and sulphur-deficiency. As soil compaction can lower the oxygen concentration of the soil, aerenchyma may be potentially beneficial for growth or function of roots in a densely packed soil layer. Mechanical impedance induces root aerenchyma formation and both mechanical impedance and hypoxia produce a synergistic effect. Furthermore, dissolved oxygen concentration lowers when temperature increases, and thus high temperature introduces oxygen shortage. Treatment in sand culture with low concentrations of nutrient solution increased root porosity of *Nardus stricta* plants to equally high levels as found in waterlogged plants (Smirnoff and Crawford 1983). Studies with maize later confirmed that both low concentrations of nitrate or phosphorus nutrition increased aerenchyma in the roots. These treatments induced the formation of aerenchyma close to the apex of the adventitious roots that subsequently emerged from the base of the shoot, a response similar to that shown previously to be induced by hypoxia (Konings and Verschuren 1980; Drew *et al*. 1989; Fan *et al*. 2003). Similarly, when young maize plants were grown in a complete, well-oxygenated nutrient solution and then deprived of their external source of sulphate, the treatment induced the formation of aerenchyma in roots (Bouranis *et al*. 2003). Thus, N- or P- or S-deficiency leads to formation of aerenchyma in maize adventitious roots by lysis of cortical cells and aerenchyma seems to have an identical structure as that found in roots of waterlogged plants. Moreover, ethylene treatment produces aerenchyma artificially.

Aerenchyma is a functional tissue fulfilling a role in plants for the transport of various materials over large distances. Principally aerenchyma provides an internal aeration system for the transfer of oxygen from the shoot to the roots, contributing in this way to the ability of plants to tolerate low-oxygen soil environments. In addition, aerenchyma facilitates the counter-flow of other gases and volatile compounds accumulated in the anaerobic soil and plant tissues, such as carbon dioxide, methane and ethanol (Vartapetian and Jackson 1997; Subbaiah and Sachs 2003). IS are generally present in extrafascicular plant aerenchyma tissues and when aerenchyma is present it comprises a continuum with them. In water-saturated roots, cortical IS are usually gas-filled and comprise the predominant pathways for oxygen diffusion through plant organs, even where their volume fraction is small. In roots lacking aerenchyma, the resistance to oxygen movement in gas-filled IS is relatively large. For oxygenation over greater distances, aerenchyma is necessary and effective as determined by higher levels of ATP and adenylate energy charge, because it cuts back on the number of oxygen-consuming cells and lowers the resistance to gas diffusion or convection. Air-filled IS are necessary and ubiquitous in higher plants and the most likely mechanism by which these spaces are kept from being flooded is that the living plant cell may maintain a hydrophobic monolayer on the surfaces of adjacent intercellular spaces (Woolley 1983).

Liquid-filled lacunae may provide another rapid diffusion path, in addition to the cell wall apoplastic pathway. There is water in some lacunae of the aerenchyma, and in some of the normal IS in undamaged roots that are not waterlogged. This water may provide a radial path for rapid diffusion of ions across the cortex. Therefore, aerenchyma in these roots may function directly in plant water/ion relations, apart from their role in aeration of the root tissues. The formation of large spaces in the cortex eliminates a large part of the cellular pathway into the root but it does not seem to affect nutrient uptake. Therefore, aerenchyma can provide an additional mechanism which maintains ion movement across the cortex, and in the presence of aerenchyma the apoplast of the root cortexes comprises at least three distinct spaces: the cell-wall apoplast, the gas-filled aerenchyma and IS continuum and the liquid-filled one (for details see van der Weele *et al*. 1996 and Michael *et al*. 1999).

Moreover, stress adaptation includes redirection of

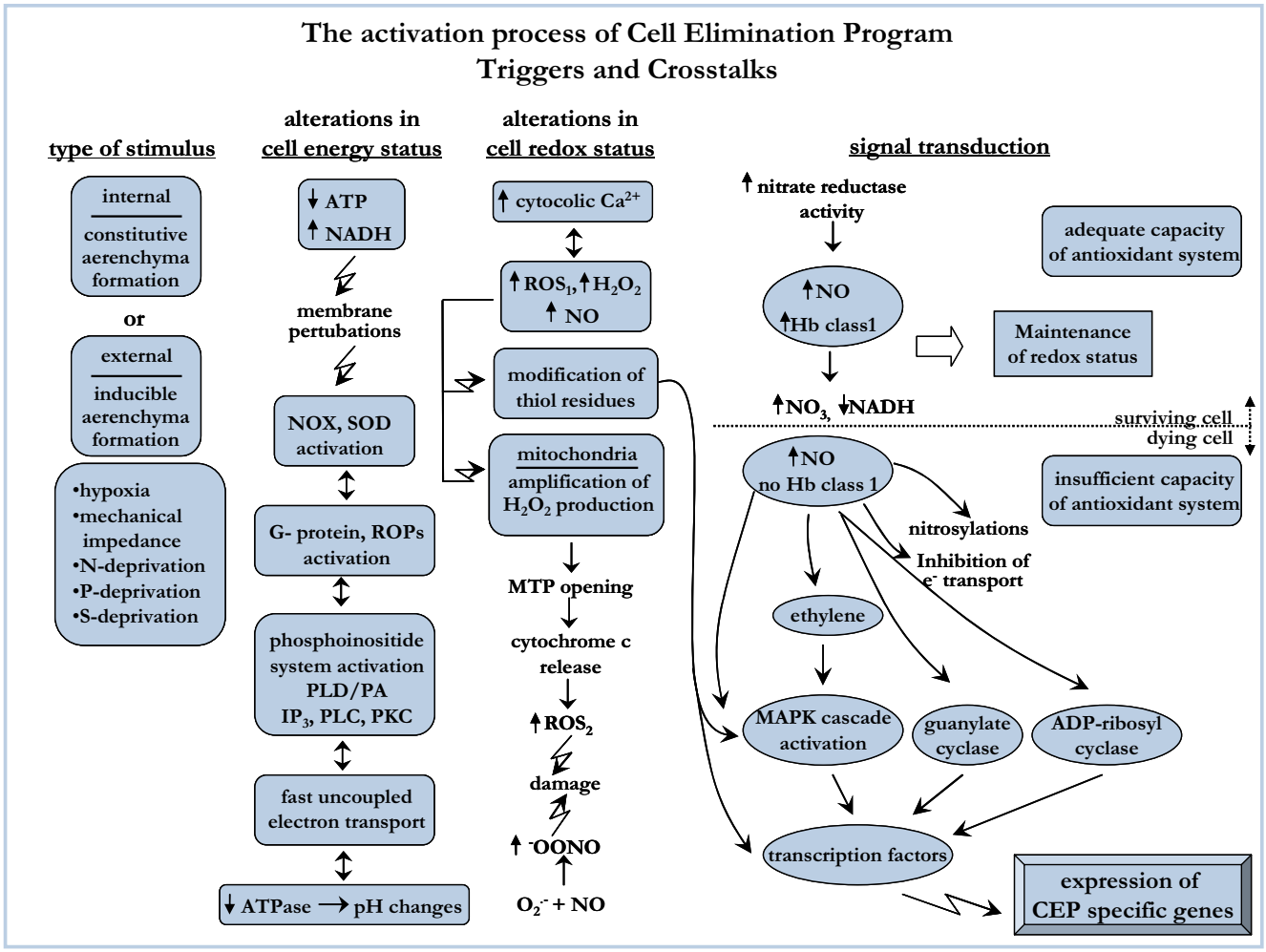

Fig. 1 The program of aerenchyma formation includes several processes and molecules. The first process in the pathway from the stimulus to lacunae is the activation of the Cell Elimination Program (CEP) and this cartoon presents a suggested hierarchy of the various players and the facts which lead to the expression of specific CEP specific genes. Several different stimuli (hypoxia, mechanical impedance, concrete nutrient deprivations) may result to a common condition (i.e. perturbations in ATP and NADH levels), thus triggering the same CEP. Then, the cell may survive or not and such a tentative turn point is presented. It seems that afterwards ethylene-dependend or –independent pathways may exist. ROS_1 and ROS_2 indicate two obviously different roles of ROS. It is most likely that in this process ROS_1 serve as signal while ROS_2 seem to contribute to the amplification of the lesion.

scarce resources to the maintenance of essential sinks, activation of adaptive pathways and disinvestment in non-essential sinks and pathways. Selective cell death reduces the demand for oxygen (Fan *et al*. 2003). Aerenchyma formation in phosphorus-deficient roots was disproportionately correlated with reduced root respiration; roots with 30% aerenchyma in the cortex presented 70% less respiration than roots without aerenchyma, while aerenchyma formation was proportionally correlated with reduced root phosphorus concentration. Variation in aerenchyma formation was correlated with root respiration and phosphorus concentration, regardless of whether variation was caused genetically or by ethylene or phosphorus treatments. These results support the hypothesis that aerenchyma formation reduces the respiratory and phosphorus requirements of soil exploration by roots, thus representing a useful adaptation to low phosphorus availability. Combined with the assumption that cell components are being resorbed during cell lysis, this would imply a lower investment of construction and maintenance costs per unit root length, which would in turn add to the capacity of the plant to explore the soil for sources of phosphorus (Fan *et al*. 2003).

Diverse stimuli induce PCD terminating to the same end result: lysigenous aerenchyma formation in the root cortex. Where do they converge and what is in common? Various signaling modules are implicated in stress signal transduction (see Xiong and Zhu 2001). Signaling modules as tranducers of the hypoxic signal for lysigenous aerenchyma formation include ethylene, G-protein, inositol 1,4,5-trisphosphate, increase in cytosolic calcium concentration, calmodulin, protein kinase C and protein phosphatases (He *et al*. 1994, 1996a), however the full cascade of events as well as the exact place of each of these transducers in the cascade are not known. Drew *et al*. (2000) proposed an ethylene signal transduction pathway initiating cell death in cortical cells in maize roots. In this scenario, ethylene is the trigger of the cascade, however one might argue that the proposed cascade may act upstream of ethylene, thus activating the corresponding genes for ethylene production, and downstream of ethylene a MAPK cascade might be in charge to activate the genes for the production of hydrolytic enzymes for cell death. The role of ethylene as a regulator of aerenchyma formation in inducible systems has been studied extensively (Jackson *et al*. 1985a; Drew *et al*. 1989; He *et al*. 1992, 1996a, 1996b; Gunawardena *et al*. 2001). An important question in ethylene controlled aerenchyma formation is how the signal of lower oxygen availability is transduced into ethylene biosynthesis and Evans (2003) remarks that the signal transduction pathway between hypoxia and ethylene biosynthesis remains to be fully investigated. On the other hand, there are cases of aerenchyma formation which are independent of ethylene (Jackson *et al*. 1985b). It is obvious that the same end is produced by different cascades of signalling modules.

The different internal or external signals direct the cell to prepare for death and processing of remains. To this end, plant cells must first acquire the competency to trigger death; thereafter these specific cells address the death pro-

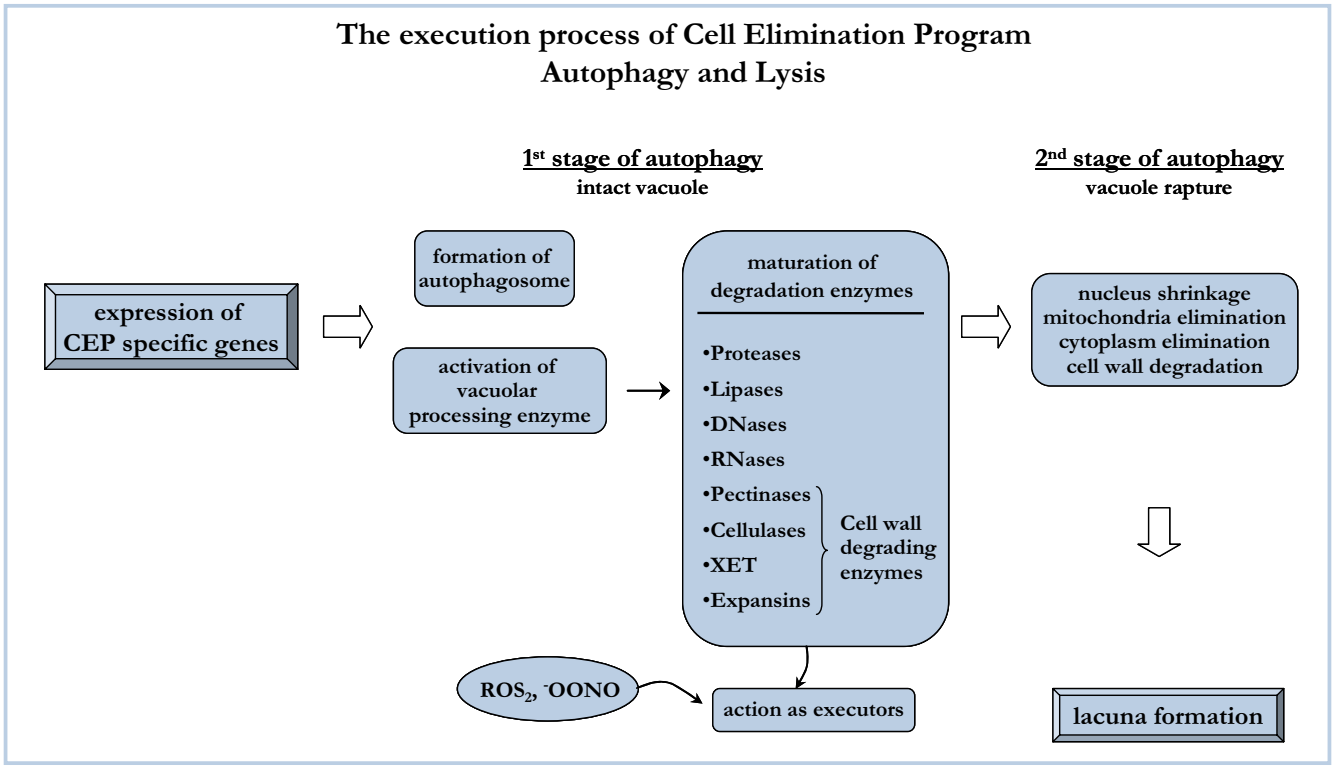

Fig. 2 The expression of CEP specific genes provides a whole arsenal of degradation enzymes and the second process in the pathway from the stimulus to lacunae is the execution of the CEP through autophagy and lysis. It is apparent that two stages of autophagy take place and the rupture of the tonoplast is of key importance. This second process culminates in the formation of the first lacuna.

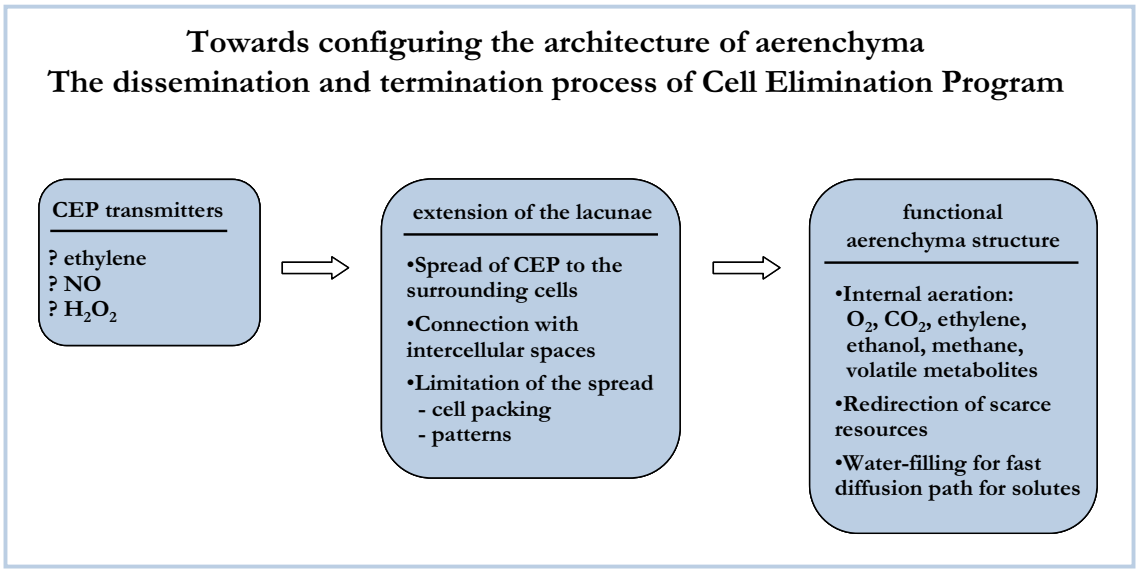

Fig. 3 Aerenchyma is a tubular structure. After the formation of the first lacuna sequential or parallel execution of a group of cortex cells takes place and dissemination and termination of CEP is needed, resulting in aerenchyma development. The lesion spreads to the surrounding cells and is subject to limitation. The extent of aerenchyma formation caused by the various stimuli is not of the same intensity or architecture. Cell packing is an important parameter for aerenchyma development and various CEP patterns have been distinguished. Except its role as an internal aeration system, aerenchyma may serve also other roles.

cess autonomously. Such a cell elimination program (CEP) can be distinguished into three major processes: the activation process (**Fig. 1**), the execution process (**Fig. 2**) and the dissemination and termination process (**Fig. 3**). For each of these processes, we review the possible involvement of other modules that may be involved in the cascade of events, in addition to those known to lead to aerenchyma formation.

THE ACTIVATION PROCESS OF THE CELL ELIMINATION PROGRAM

The sequence of events which activate the CEP includes varrious enzymes and effectors, organised in interacting modules. The energy status and the redox status of the cell seem to be the key factors for the activation of the CEP.

The energy status of the cell and its impact on the integrity of the plasma membrane

There is a relationship between the energy status of plant cells under oxygen stress and the maintenance of membrane intactness, studied mostly in suspension cultures of anoxia-intolerant potato cells. There appears to be a threshold in ATP production rate, below which membrane lipids are hydrolysed. During energy shortage, *de novo* lipid synthesis

is still possible however desaturation is impossible, leading to loss of membrane fluidity (Rawyler *et al*. 2002). The preservation of cell structure and function by restoring a 'normal' respiratory activity with pyruvate (Aubert *et al*. 1996) points to a peculiar role of the mitochondria in situations of energy shortage. ATP is required to preserve cytoplasmic ion homeostasis, especially with respect to calcium ions (Bush 1995). When cells are deprived of oxygen, the mitochondrial ATP-synthase begins to hydrolyse part of the glycolytically produced ATP in an attempt to maintain the mitochondrial proton motive force (St-Pierre *et al*. 2000). Under anoxia, ATP availability triggers the transition between membrane intactness and disruption (Rawyler *et al*. 2002). Thus, it is likely that under hypoxic conditions energy shortage and perturbed membrane structure may activate membrane proteins which integrate stress.

The plasma membrane H^+-ATPase is involved in the regulation of plant growth and development controlling the chemical potential of plant cells and nutrient transport. Plasma membrane ATPase activity is lipid dependent: delipidation of the plasma membrane reduces the ATPase activity, and addition of lipids recovers the inhibition and increases the ATPase specific activity. While lipids are needed to maintain membrane bilayer structure, changes in membrane lipid composition can also affect enzyme conformation and aggregation state in the lipid bilayer (Chen and Boss 1991).

It seems likely that the stresses leading to aerenchyma formation result in a depletion of ATP, perhaps due to fast uncoupled electron transport. A decline in mitochondrial respiration triggers an increase in NADH and a drop in ATP level. Oxidative stress can cause uncoupling between respiration and generation of ATP, and coupled with calcium influx such depletion of ATP can be sufficient to induce CEP.

Induced alterations in the redox status of the cell and the role of the mitochondrion

It has been recognised that ROS, especially hydrogen peroxide, have a major role in cellular signalling pathways, across a wide range of organisms, including plants (reviewed by Neill *et al*. 2002; Foyer and Noctor 2005a). ROS can be generated in plants via the leakage of electrons from mitochondria, or can be synthesized by a variety of dedicated enzymes, such as peroxidases and NADPH oxidases (NOX) (Neill *et al*. 2002). Once produced, ROS can partake in signalling, although these events will be modulated by the complement of antioxidants in or around the cell. PCD may be controlled, in part, by ROS (Desikan *et al*. 1998). Sulphate deprivation resulted in alterations of ROS, calcium levels and pH in aerenchymatous sectors of maize roots compared with the basal non-aerenchymatous region. ROS appeared in groups of intact mid-cortex cells and formation of superoxide anion and hydrogen peroxide were found in degenerating cells of the mid-cortex (Bouranis *et al*. 2003, 2006).

NOX is a multicomponent enzyme composed of several membrane-bound and cytosolic subunits, which catalyses the NADPH-dependent production of the superoxide anion. Although superoxide anions can be a by-product of metabolic processes such as respiration, NOX is believed to be a key enzyme for the stimulus-induced release of ROS and becomes active when its cytosolic subunits translocate to the membrane (Sang *et al*. 2001). One of the cytosolic subunits is of the Rac type, a G protein. Rac may play a role in regulating ROS formation and cell death. Moreover, one of the two plasma membrane proteins contains calcium binding motifs, which suggests that the NADP oxidase complex may be regulated by calcium, a common feature of PCD (Keller *et al*. 1998). H_2O_2 production by the combination of a plasma membrane NOX system and a plasma membrane superoxide dismutase may be integrated to trigger CEP.

ROPs (Rho-related GTPases from plants) trigger H_2O_2 production and hence the oxidative burst, most likely by activating NOX (Agrawal *et al*. 2003). ROPs are a class of plant proteins that are closely related to the mammalian Rac family, among which Rac2 is a key regulator of NOX. The ROP monomeric GTPase modulates the induction of ADH1 in *Arabidopsis* seedlings (Baxter-Burrell *et al*. 2002, 2003). Based on findings with *Arabidopsis*, it was proposed that a ROP GTPase rheostat promotes ethanolic fermentation when active, and conserves carbohydrates when inactive (Fukao and Bailey-Serres 2004). The ROP family proteins activate and repress signalling cascades that control diverse mechanisms in plant cells (Gu *et al*. 2004). ROP activation is necessary to promote ADH1 induction, but moderation and reversibility of ROP signalling is an additional prerequisite for survival of transient hypoxia. A consequence of ROP activation is the induction of H_2O_2 accumulation. ROS production is a necessary component of a low oxygen signalling pathway. However, negative regulation of the signalling is necessary to avoid oxidative stress (Baxter-Burrell *et al*. 2002; Fukao and Bailey-Serres 2004). These findings support the hypothesis that rheostat-like regulation of ROP activity mediates temporal activation of an H_2O_2-dependent signalling pathway that leads to ADH1 expression. The importance of the production and amelioration of ROS in the response of plant cells to oxygen deprivation was recognized prior to the elucidation of the ROP rheostat (Blokhina *et al*. 2003). For a detailed analysis see Bailey-Serres and Chang (2005).

For proteins to perceive the presence of ROS, such as H_2O_2, and to act as signal intermediaries, there needs to be a specific recognition of H_2O_2 by the protein, or a direct chemical interaction which leads to signal propagation. The former is unlikely, owing to the small size of H_2O_2, but the latter is likely as the oxidising nature of H_2O_2 will allow it to modify thiol residues in proteins directly, as has been suggested (Cooper *et al*. 2002; Vranová *et al*. 2002; Foyer and Noctor 2005b), although other amino acids may be able to be oxidised too, such as Tyr, Trp, and His (Dröge 2002). As numerous cellular and extracellular proteins will be potential targets for this type of oxidative modification, the ROS sensors must have some specific characteristics that enable them to propagate this signal. Therefore, Hancock *et al*. (2006) propose that thiol modification is a key to H_2O_2 perception and describe how the oxidised nature of H_2O_2 allows it to modify thiol residues in proteins directly.

A brief pulse of a high concentration of H_2O_2, or a continuous generation of low concentrations of H_2O_2, cause amplification of H_2O_2 production by the mitochondria through fast uncoupled electron transport (Tiwari *et al*. 2002). Mitochondria constitute a source of ROS and they can amplify the superoxide anion and hydrogen peroxide levels inside the cell (Tiwari *et al*. 2002). It has been suggested that they may act as an integrator of cellular conditions from different stimuli (Lam *et al*. 1999; Jones 2000). Generation of ROS by mitochondrial respiratory chain is a physiological and continuous process. Under normal conditions, toxic effects of ROS are removed by antioxidant systems. Under abiotic stress the concentration of ROS in the cells can rise significantly, reaching a threshold that can trigger CEP (Lamb and Dixon 1997). Thus, in terms of CEP initiation, attention has shifted towards the mitochondrion and the release of cytochrome c as a potential trigger of CEP (Yu *et al*. 2002). Beyond a certain threshold, mitochondria activate CEP in a number of ways: (a) at the level of substrate import into the mitochondria (Hautecler *et al*. 1994), (b) at the level of substrate oxidation and electron transport (Moore *et al*. 1991), (c) by the control over oxidative phosphorylation (Kesseler *et al*. 1992), (d) by regulation of mitochondrial permeability transition pore (MTP) (Kroemer 1997; Green and Reed 1998), (e) by the upregulation of the alternative oxidase, thus diverting the electron flow (Day and Wiskich 1995).

Alterations in a MTP will lead to profound changes in mitochondrial functioning. According to Jones (2000), MTP forms as a complex with the voltage-dependent anion channel and the adenine nucleotide translocator, permits

water to move into the matrix and protects the mitochondria from the loss of electrochemical potential for protons by preventing nonspecific transfer of solutes of less than 1.5 kDa (Tiwari *et al*. 2002). This complex has been proposed as a sensor of cellular stress. Jones (2000) has suggested that opening of MTPs leads to translocation of cytochrome c from the mitochondria to the cytosol. Outer mitochondrial membrane rupturing occurs when the inner membrane swells. Situations that lead to the release of cytochrome c include oxidative stresses that induce MTP formation, stresses on electron transport and a rise in calcium levels. Release of cytochrome c to the cytoplasm activates CEP. Thus, when cells are unable to maintain metabolic homeostasis and the stresses overwhelm the cell, the mitochondria release cytochrome c triggering cell elimination. Furthermore, the release of cytochrome c leads to a second ROS generation. Cytochrome c is a candidate for ROS attack (Hancock *et al*. 2001, 2003). It may be oxidised by H_2O_2, but it may be reduced by superoxide anions, and therefore cytochrome c may exist in a reduced state, or an oxidised state, depending on which ROS is most prevalent in the cell. Additionally it has been shown that the structure of the protein, and therefore its potential ability to signal is determined by its oxidation state (Calver *et al*. 1997).

The involvement of calcium ions

The H_2O_2 signal can also be transmitted through alterations in calcium ion fluxes and cellular redox state. Both calcium and redox alterations are very early events that follow the rises in H_2O_2 levels (Rentel and Knight 2004). There may be a connection between ROP-promoted H_2O_2 production and the hypoxia-induced increase in cytosolic calcium. An interaction between ROP signalling and calcium is supported (Bailey-Serres and Chang 2005). An increase in cytosolic calcium is a likely prerequisite for the activation of apoplastic H_2O_2 production by the calcium-dependent NOX (Keller *et al*. 1998; Sagi and Fluhr 2001). In *Arabidopsis* roots, ROS produced by this oxidase activates calcium channels and facilitates calcium flux(es) necessary for root growth (Foreman *et al*. 2003; Mori and Schroeder 2004). Moreover, *Arabidopsis* ROP1 is responsible for an intracellular calcium gradient at the tip of root hairs (Jones *et al*. 2002). These observations lead to the speculation that ROP may further promote an increase in cytosolic calcium under hypoxic conditions, which could be mediated by NOX, possibly located at the plasma membrane. Thus, calcium dynamics may provide a balance between production of H_2O_2 as a signalling molecule and the damage it can cause as a ROS.

ROS are generated by mitochondria during their normal activities and at the same time mitochondria are sensitive to ROS. Oxidative stress triggers MTP-opening directly and possible also indirectly via elevated calcium levels. Under conditions where ATP pools are low, calcium is the fundamental MTP-activator and there appears to be a dependency between ROS and calcium activation of MTP (Petronilli *et al*. 1994; Kowaltowski *et al*. 1996; Scorrano *et al*. 1997; Crompton 1999). Inhibition of electron transport (including that by nitric oxide) reduces ATP pools, elevates ROS pools and under conditions of elevated calcium opens the pore, because at high calcium levels cytochrome c is released (Jones 2000). In this way, the mitochodrial responses can be regulated by calcium influx from the cytosol. Calcium influx into mitochondria is regulated by different stimuli via changes in membrane potential (Silva *et al*. 1992). For example H_2O_2 causes calcium influx (Fortes *et al*. 2001). The regulation of calcium levels in the different subcellular compartments is closely linked (Wendehenne *et al*. 2004). There is a tight regulatory network that links ROS generation and calcium fluxes, both of which are involved in control of PCD and it is suggested that both stimuli are involved in the same cell death pathway. It seems that an increase in intracellular calcium concentration is necessary for cell death and the resulting aerenchyma development in roots of maize subjected to hypoxia. ROS can also induce MPT in a calcium-independent manner (Fortes *et al*. 2001).

A specific calcium signature can lead to various downstream effects, including cell death, through the numerous calcium-interacting proteins, including calmodulins and the family of calcium-dependent protein kinases (Harper *et al*. 2004). Plants possess a unique set of calcium/calmodulin-regulated proteins with different biological functions. Although some of these proteins, such as NAD kinase, aid in the production of H_2O_2 and enhance cell death (Harding *et al*. 1997), others like catalase have the opposite effect (Yang and Poovaiah 2002). Catalase is of paramount importance for regulating H_2O_2 homeostasis, as it can function as a cellular sink for H_2O_2. Catalase deficiency leads to an elevation of H_2O_2 levels and a triggering of PCD (Gechev *et al*. 2004; Vandenabeele *et al*. 2004). Calcium is therefore not only essential for PCD, but also for maintaining H_2O_2 levels that ensure cell survival (Yang and Poovaiah 2002). In addition to calcium/calmodulin, catalase activity may also be modulated by nucleoside diphosphate kinase (NDK). This notion is suggested by the observations that transgenic plants overexpressing AtNDK1 exhibited enhanced ability to detoxify H_2O_2 (Fukamatsu *et al*. 2003). For details on the role of calcium in signal transduction, see Sanders *et al*. (2002) and Lecourieux *et al*. (2006).

The phosphoinositide signal-transducing system

NOX is a likely target of phospholipase D (PLD) activation, and phosphatidic acid (PA) plays a role in stimulating the NADPH-dependent production of superoxide (Sang *et al*. 2001). A PA-specific protein kinase mediates the activation of NOX (Waite *et al*. 1997; McPhail *et al*. 1999). In addition to NOX, PLD has been identified as an important early signalling enzyme, because activation of PLD generates signalling messengers and is involved in a wide range of cellular processes, including hormone action (Fan *et al*. 1997; Lee *et al*. 1998; Jacob *et al*. 1999). PLD hydrolyses phospholipids to produce PA, which serves as a second messenger that may activate phosphatidylinositol 5-kinase, phospholipase C and protein kinase C. The cellular activity of plant PLD is regulated by calcium (Zheng *et al*. 2000), polyphosphoinositides (Qin *et al*. 1997), G-proteins (Munnik *et al*. 1995; Ritchie and Gilroy 2000), pH-changes (Pappan and Wang 1999) and membrane perturbation (Pappan *et al*. 1998). PLD is a major family of phospholipases and all characterised plant PLDs contain the C2 domain, which is a calcium-phospholipid-binding site, making calcium an important regulator (Wang 2005). PLD mRNA and enzyme activity are stimulated by hypoxia.

The nitric oxide and haemoglobin system

Nitric oxide (NO) is often produced at the same time and in the same locations in plants as ROS and, like ROS, NO is involved in a plethora of responses and functions (reviewed by Neill *et al*. 2003). Of particular note here is that NO may also react with thiol groups on proteins in a process known as S-nitrosylation, to yield a -S-NO group. It has been found that many of the proteins found are also those which are potentially modified by H_2O_2 (Lindermayr *et al*. 2005). It is likely that NO and H_2O_2 may be covalently altering and potentially controlling the same complement of proteins in cells: there may be a competition between H_2O_2 and NO at the level of thiol modification which may determine the exact signalling processes that ensue (Hancock *et al*. 2006).

NO levels may cause the following: (i) NO forms peroxynitrite after reaction with superoxide anions (ii) NO can release calcium by nitrosylation of the calcium channel and therefore the nitrosylation of calcium channel can potentially induce MTP formation (iii) NO reduces ATP by the inhibition of electron transport, another requisite for MTP formation. Modulation of NO levels can lead to significant

changes in hormonal responses (Wendehenne *et al*. 2004). Both promotive and inhibitory effects on NO upon ethylene generation in plants have been demonstrated (Leshem *et al*. 1998; Leshem and Pinchasov 2000; Sozzi *et al*. 2003). NO may advance CEP, individually or in combination with ethylene, MAP kinases (Capone *et al*. 2004; Pagnussat *et al*. 2004), the guanylate cyclase (Wendehenne *et al*. 2004) or the ADP-ribosyl cyclase (Igamberdiev *et al*. 2005).

A decline in mitochondrial respiration triggers an increase in NADH and a drop in ATP levels. According to Igamberdiev *et al*. (2005), the drop in ATP levels results in activation of nitrate reductase leading to production of NO and in haemoglobin (Hb) gene expression. NO reacts with the oxygenated form of newly synthesized Hb (class 1) and is oxidised back to nitrate. With this reaction the accumulated NADH is oxidised and the Hb/NO cycle comprises a NO-scavenging system, linked to ATP levels, the modulation of which results in maintenance of the energy and redox status of the cell and the prevention of cell death.

Hbs encompass functions that include the reversible binding of gaseous ligands and the ability to bind other cellular molecules (Arredondo-Peter *et al*. 1998). With respect to aerenchyma, alfalfa roots overexpressing class 1 barley Hb exposed hypoxic conditions for several hours show no sign of aerenchyma development, whereas control roots and roots underexpressing Hb showed evidence of cell disruption (Dordas *et al*. 2003a). Suppressing Hb expression in maize cell suspensions resulted in elevated ethylene formation (Manac'h-Little *et al*. 2005). This indicates a potential role for Hb in regulating ethylene levels in the cell. In maize cell suspensions, both ethylene and NO levels increase when Hb expression is impaired. In this case, NO appears to have a promoting effect on ethylene biosynthesis. Decreasing the levels of NO in the cell by overexpressing Hb (Dordas *et al*. 2003b, 2004; Igamberdiev *et al*. 2004; Igamberdiev and Hill 2004) may directly or indirectly turn off the signal, triggering the activation of ethylene biosynthesis. This may indicate participation of Hb in an acclimation response to low oxygen stress, regulating the levels of NO and ethylene, delaying aerenchyma formation. There is also the possibility that a selective expression of Hb in root cells may regulate the process of aerenchyma formation via direct effects of NO on programmed cell death.

The role of ethylene

Ethylene is a growth regulator that has received significant attention in the studies of low oxygen responses, the biosynthesis of which is mediated by two key enzymes in higher plants. 1-amino-cyclopropane-1-carboxylate (ACC) synthase (ACS) catalyses the formation of ACC from SAM. The second step in the hormone biosynthesis is the oxidation of ACC to ethylene by ACC oxidase (ACO). Ethylene biosynthesis increases within 4 h of transfer to hypoxic conditions in several species (Drew *et al*. 1979; Lorbiecke and Sauter 1999). In arabidopsis, phosphorylation of ACS by the stress-responsive MAP kinase (MAPK) 6 leads to the accumulation of ACS protein (Liu and Zhang 2004). Consequently, levels of cellular ACS activity and ethylene production are elevated. It is not known if a MAPK signalling pathway is activated by oxygen deprivation (Bailey-Serres and Chang 2005). The biosynthesis of ethylene is not likely to occur under strict cellular anoxia because the conversion of ACC to ethylene by ACO requires consumption of oxygen (Yang and Hoffman 1984; Kende 1993). Ethylene enhanced the hypoxic induction of ADH1 in *Arabidopsis* (Peng *et al*. 2001).

Cell death in maize root cortex during hypoxia is not caused directly by oxygen deficiency. The involvement of ethylene in aerenchyma formation is well documented for maize roots, in the cortical cells of which aerenchyma forms by CEP, and this process appears to be regulated by ethylene. ACS and ACO are both encoded by a multigene family which can be induced under various conditions (Grichko and Glick 2001). Hypoxia stimulates the activities of ACS and ACO in the apical zone of intact roots following a lag of some hours and ethylene production is accelerated under these conditions. As a result, hypoxic root tips contain higher concentrations of ACC and higher concentrations of ethylene than normoxic ones. It is still not known whether the relatively rapid increase in the activity of enzymes involved in ethylene biosynthesis is a result of the activation of pre-existing enzymes, or if there are changes in the transcript level of the corresponding genes. Mechanical impedance also increases the activities of ACS and ACO (He *et al*. 1996b). On the other hand, activity of ACS is not stimulated in the apical zone of intact roots by anoxia, while ACO requires molecular oxygen to convert ACC to ethylene. Thus, ethylene synthesis is depressed and aerenchyma formation cannot take place under anoxia (Drew *et al*. 1979; Jackson *et al*. 1985a; He *et al*. 1994). Treatment of anoxic roots with exogenous ethylene fails to elicit aerenchyma formation (Jackson *et al*. 1985a).

The induction of aerenchyma in adventitious roots of *Zea mays* by N- or P-deprivation is not due to an enhanced biosynthesis and/or accumulation of ethylene in the root tips (Drew *et al*. 1989). The conversion of SAM to ACC is inhibited or modified somehow by nutrient deprivation and activities of ACS and ACO are depressed by N- or P-deprivation, while in both deficiencies the sensitivity of cortical cells to ethylene is enhanced, causing lysis of cells in the presence of very low concentrations of the gas (Drew *et al*. 1989; He *et al*. 1992). Ethylene sensitivity of P- and N-deprived plants may serve a different goal, e.g. a change in root topology or the formation of root hairs instead of aerenchyma formation *per se* (Visser and Voesenek 2004).

Under normoxic conditions, i.e. in well-oxygenated complete nutrient solution, low concentrations of ethylene (0.1-1.0 $\mu L\ L^{-1}$ air bubbled through the nutrient solution) readily induce aerenchyma formation in the cortex of maize adventitious roots, precisely among those cells that would succumb to hypoxia. The structure of these roots was indistinguishable from the structure induced by hypoxia (Drew *et al*. 1979; Konings 1982). Inhibitors of ethylene biosynthesis or ethylene action block aerenchyma formation in hypoxic roots (Drew *et al*. 1980; Konings 1982; Jackson *et al*. 1985a), whereas in ethylene-treated normoxic roots only inhibitors of ethylene action are effective (Jackson *et al*. 1985a).

SAM is precursor for the ethylene biosynthetic pathway, and participates in the formation of spermidine and spermine. The possibility that the additional ethylene production by hypoxic maize adventitious roots resulted from decreases in spermidine and spermine has been tested and no decreases in spermidine and spermine have been found in root tissue up to 4 d of treatment. Removing oxygen completely had little effect on spermidine and spermine concentrations (Jackson and Hall 1993). Hypoxia increased the concentration of putrescine, the precursor of spermidine and spermine. This increase was not a response to the extra ethylene formed by such roots, since ethylene treatment did not reproduce the effect. Application of inhibitors of putrescine biosynthesis led to increased aerenchyma formation. Exogenous putrescine inhibited the development of aerenchyma while stimulating rather than inhibiting ethylene production, when tested in either air or hypoxia. Thus, putrescine appears to limit aerenchyma formation by suppressing ethylene action rather than its production (Jackson and Hall 1993).

The ethylene receptor, other downstream players and possible cross-talks

Data from *Arabidopsis* show that proteins involved in ethylene signalling, including histidine kinases, are induced by the addition of H_2O_2 (Desikan *et al*. 2001). A study on the histidine kinase receptor ETR1 from *Arabidopsis* showed that it was essential for H_2O_2 perception leading to stomatal closure (Desikan *et al*. 2005). Using mutants that lacked

either histidine kinase activity, or the complete histidine kinase domain of ETR1, indicated that the kinase domain was not required for H_2O_2 signalling. In its other role as an ethylene receptor, it appears that the presence of the histidine kinase domain of ETR1 is required (Gamble *et al*. 2002), suggesting that the signalling through ETR1 invoked by H_2O_2 is different from that invoked by the presence of ethylene.

According to Raz and Flur (1993), the ethylene signal is transduced via protein phosphorylation events. The H_2O_2 signal can also be transmitted through the mitogen-activated protein kinases (MAPK) cascade network (Gechev and Hille 2005). The MAPK pathways are intracellular signal modules that mediate signal transduction from the cell surface to the nucleus (Robinson and Cobb 1997; Xiong and Zhu 2001). The core MAPK cascades consist of 3 kinases that are adenylated sequentially by an upstream kinase. The activated MAPK can then either migrate to the nucleus to activate transcription factor directly, or activate additional signal components to regulate gene expression, cytoskeleton-associated proteins or enzyme activities, or target certain signal proteins for degradation. The specificity is realised by scaffold proteins that hold these kinases or by a specific component in the signalling cascade (Xiong and Zhu 2001). Plants possess an unusually high number of MAPKs, and the kinase network can be a convergence as well as a divergence point for different stress factors (Ichimura 2002). Several types of protein kinases have been shown to be activated in the presence of hydrogen peroxide. To establish that ROS do in fact have a role in signal transduction in plants, the impact of the addition of ROS to cells on the activity of signalling proteins has been investigated. Exogenously added H_2O_2 has been shown to lead to the activation of MAPKs. Biochemical evidence indicated that a MAPK cascade is responsible for relaying the H_2O_2 signal, much like in other eukaryotes (Kovtun *et al*. 2000). The recent identification of the serine/threonine kinase, oxidative signal-inducible 1 (OXI1), as an essential component in H_2O_2 signalling in *Arabidopsis* provided new insights into the complexity and specificity of the H_2O_2-relaying kinase network (Rentel *et al*. 2004). OXI1 has been shown to be up-stream of the MAPKs and is needed for full activation of two stress related MAPKs (Rentel *et al*. 2004). In addition to protein kinases, tyrosine phosphatases have been shown to be inactivated by hydrogen peroxide in mammals. Phosphatases may be involved in H_2O_2 signalling in plants also: the *Arabidopsis* protein phosphatase 2C enzymes ABI1 and ABI2 and the protein tyrosine phosphatase AtPTP1 have been suggested to play such a role. AtPTP1 regulates the activity of MAPKs, suggesting a tight link between H_2O_2, kinases and phosphatases (Hancock *et al*. 2006).

Gene expression and the transition to the execution process

The CEP activation phase terminates in the induction of gene expression of the CEP executing enzymes and effectors. This delimits the start of the next phase, that of CEP execution. One of the most well-recognised cellular effects of ROS is the alteration of gene expression. H_2O_2-derived signals initiate global changes in gene expression through regulation of a specific subset of transcription factors and, as a result of those changes, different genetic programs including PCD are executed (Gechev and Hille 2005). In *Arabidopsis*, a transcriptomic study found approximately 170 genes for which the expression was increased more than 2-fold by application of exogenous H_2O_2, and approximately 65 genes having their expression reduced (Desikan *et al*. 2001). Furthermore in tobacco, several transcription factors have been identified whose expression is regulated by H_2O_2 (Vandenabeele *et al*. 2003). It is possible that these transcription factors are also direct targets for redox modification by H_2O_2 and there is clearly more work to be done in this area (Hancock *et al*. 2006).

ROS produced in different subcellular compartments influence the expression of a large number of genes (Neill *et al*. 2002). This suggests that cells have evolved strategies to utilize ROS as biological signals that control various genetic stress programs. This interpretation is based on the unstated assumption that a given ROS can interact selectively with a target molecule that perceives the increase of ROS concentration, and then translates this information into a change of gene expression. Such a change in transcriptional activity may be achieved through the oxidation of components of signalling pathways that subsequently activate transcription factors or by modifying a redox-sensitive transcription factor directly. The effects of ROS on components of the MAPK cascade result in the indirect activation of transcription factors (Laloi *et al*. 2004). The activation of the MAPK cascade may be initiated by redox-controlled protein tyrosine phosphatases.

THE EXECUTION PROCESS OF THE CELL ELIMINATION PROGRAM

The executors

The execution of CEP is a complex degradation process accomplished by a suite of enzymes, which are distinguished from those that are involved in the signaling of cell death. Vacuolar processing enzyme, proteases, lipases and other esterases, DNases, RNases, and cell-wall degrading enzymes are involved in the degradation process. Several genes encoding hydrolases that are up-regulated are common to all plant PCDs, whereas some genes are unique to each type of preparation for death. Differences in the profiles of the nascent hydrolases might account for the underlying differences in corpse removal (Jones 2000).

The various hydrolytic enzymes which are synthesized for active degeneration of cellular contents are thought to accumulate in the vacuole to sequester them from the cytoplasm. Maturation or activation of vacuolar proteins requires a vacuolar processing enzyme (VPE), a plant caspase (i.e. a cysteine protease that exhibits caspases-1 activity) (Hatsugai *et al*. 2004). VPE is not a cytosolic enzyme (Kinoshita *et al*. 1999) and in addition to the initial activation of some of the hydrolytic vacuolar enzymes, VPE may also mediate the disruption of the vacuolar membrane. VPE deficiency suppresses vacuolar collapse leading to cell death (Hatsugai *et al*. 2004); thus, VPE triggers the vacuolar system in plants, functioning as a key molecule in vacuolar-collapse-triggered cell death: a regulator of CEP (Hara-Nishimura *et al*. 2005).

Proteolytic activity is a centerpiece of CEP which incorporates a specific proteolytic program. Plants are equipped with a large proteolytic machinery that irreversibly regulates the fate of proteins. Such a machinery serves to remove non-functional proteins and to release their amino acids for recycling (van der Hoorn and Jones 2004). This protein breakdown is mediated by different proteolytic systems associated with vacuolar, nuclear, cytosolic and organellar compartments. Serine-, cysteine-, aspartic- and metallo-proteases can be distinguished among the endopeptidases by the use of inhibitors. In *Trifolium subterraneum*, root aerenchyma development following hypoxia correlated with a change in protease composition and may point to the expression of a specific set of lysis-involved protease genes (Aschi-Smiti *et al*. 2003).

CEP incorporates a combination of cell wall degrading enzymes, including expansins, cellulases, xyloglucan endotransglycosylases (XET), and pectinases (Jackson and Armstrong 1999), for a step-wise degradation of the cell-wall components, to facilitate the resorption of their structural units. Hypoxia (or nitrogen deficiency or high ethylene concentrations) enhances cellulase activity in the apical zone, but not in the older zones of the same roots. Increased cellulase activity is always tightly coupled to CEP leading to aerenchyma formation. Cellulase activity in hypoxic maize root tips increases immediately before aerenchyma is detec-

table. Cellulase is induced by ethylene and its activity increases following an increase in ACC synthase activity (He *et al*. 1994). Treatment of normoxic roots with promoters of CEP results in increase of cellulase activity, while after treatment of hypoxic roots with inhibitors of CEP, cellulase activity remains at normoxic levels (He *et al*. 1996a). XET is a putative cell wall loosening enzyme associated with CEP (Saab and Sachs 1996). In maize, a flooding-induced and ethylene responsive XET homologue has been shown to be associated with aerenchyma formation (He *et al*. 1994; Sachs *et al*. 1996). The induction of the flooding-induced gene *xet1* appears to be specific to hypoxia, since it is not induced by other stresses. Hypoxia induces xet1 in the primary root, mesocotyl, and coleoptile of maize seedlings and this induction of *xet1* by hypoxia is associated with aerenchyma development (Peschke and Sachs 1994; Saab and Sachs 1995, 1996). XET1 transcription is induced by ethylene and by exogenous ethylene under aerobic conditions (Saab and Sachs 1996).

Events during execution

CEP is characterised by a distinct set of morphological features, including compaction and shrinkage of the cytoplasm and nucleus, and DNA and nuclear fragmentation. Campbell and Drew (1983) have examined the ultrastructure of cortical cells in maize root tips during the early stages of lysigenous aerenchyma formation, promoted by oxygen-deficient nutrient solution, while Gunawardena *et al*. (2001) have described the series of events during the execution of CEP in hypoxic and ethylene treated maize roots. According to the results of Gunawardena *et al*. (2001), cleavage of DNA occurred at a very early stage of aerenchyma formation, preceding ultrastructurally detectable changes. The first detectable events in the cells of the cortex were in the cytoplasm: in the earlier stages of CEP plasma membrane invagination, a more electron-opaque cytoplasm and shrinkage of the plasma membrane from the cell wall were observed in cells not showing DNA condensation. This was rapidly followed by the appearance of granular staining of the vacuolar contents, together with organelles surrounded by a membrane in the vacuole. Numerous vesicles beneath the plasma membrane were apparent. Chromatin condensation was first observed at the end of this stage, in cells in which cytoplasmic changes were already evident. It appeared that nuclear condensation follows at least some of the cytoplasmic changes noted. Further changes in the cytoplasm followed detection of condensed chromatin. Cytoplasmic changes at this stage included retention of organelles. Mitochondria in hypoxic roots appeared structurally compact with some membrane damage. Plasmodesmata between cortical cells showing cell death became very prominent. Membrane bound bodies containing intact organelles were evident; these bodies enclosed intact mitochondria and Golgi bodies and other inclusions. Different sizes of membrane-bound bodies occurred, containing a variety of material, the formation of which was induced by hypoxia or ethylene treatment, suggesting that this is an integral part of PCD in aerenchyma formation.

The role of the vacuole

Vacuoles have been proposed to be responsible for various types of plant PCD. As plants do not have macrophages, plant cells must degrade their materials by themselves. To do this, plants have evolved a death strategy that is mediated by a vacuolar system. Within the vacuole the accumulation of hydrolytic enzymes (including proteases, DNases and RNases) occurs and rapture of the tonoplast releases the store of hydrolytic enzymes into the cytoplasm. CEP incorporates the upregulation of a variety of vacuolar hydrolytic enzymes (Fukuda 2004).

Plant cells may contain different vacuoles, some of which have storage or digestive functions whereas others may have other functions, for example pigment-containing vacuoles (Jauh *et al*. 1998). The vacuolar system in plant cells has been shown to consist of different compartments characterised by the presence of specific tonoplast intrinsic proteins (TIPs): α-TIP has been associated with protein storage vacuoles (Johnson *et al*. 1989; di Sansebastiano *et al*. 2001), while γ-TIPs have been found in lytic or degradative vacuoles (Paris *et al*. 1996; di Sansebastiano *et al*. 2001). The formation and evolution of single vacuoles in plant cells is a complex phenomenon, because different vacuoles can fuse and change the characteristics of their contents as well as their tonoplasts (Jauh *et al*. 1998, 1999; Flückiger *et al*. 2003). In the PCD process occurring during aerenchyma formation, tonoplast disruption is suggested to play a critical role. The collapse of the vacuole is an irreversible step to execute the degradation of various organelles (Fukuda 1996; Groover and Jones 1999; Kuriyama 1999). Once the vacuole ruptures, nucleic acids in the nucleus are degraded rapidly. Tonoplast rupture is a key feature of CEP execution, dividing the process into two stages.

The 1st stage of the execution process – intact vacuole (Fig. 4A)

Lysigenous aerenchyma development is a process of the autophagic type. Autophagy is a major degradation and recycling system in eukaryotic cells. This system contributes to the turnover of cellular components by delivering portions of the cytoplasm to lysosomal vacuoles where they are digested (Klionsky and Emr 2000). Proteases, lipases, and various hydrolases are synthesized or activated during the process. Autophagy entails the formation of autophagosome, a double-membrane structure which engulfs part of the cytoplasm, with or without large organelles, and then merges with a lysosomal vacuole (Yoshimori 2004). Autophagy saves the tissue from death and is a mechanism whereby the cell degrades or mobilises its constituents before death. At this stage of CEP the vacuole increases in size until only a small layer of cytoplasm remains. The process usually starts with the disappearance of the endoplasmic reticulum (ER) and the attached ribosomes, followed by the other organelles. Concomitantly, the various organelles disappear until the cytoplasm is virtually devoid of structure. A few mitochondria and the nucleus tend to be the last organelles to be degraded (van Doorn and Woltering 2005).

The 2nd stage of the execution process – vacuole rupture (Fig. 4B)

At this stage, autophagy continues by permeabilization and rupture of the tonoplast. Organelle digestion follows and finally the plasma membrane collapses. The loss of tonoplast integrity has been identified as an early event of aerenchyma development (Campbell and Drew 1983; Kozela and Regan 2003), the subsequent rapid disappearance of the cellular contents has been observed in PCD during aerenchyma formation and tonoplast rupture has been considered as a prerequisite for the final stages of degradation in the nucleus (van Doorn and Woltering 2005). The disruption of the tonoplast and the subsequent release of the sequestered hydrolases appears to be a common feature of plant PCD (Jones 2000). In addition to the release of the vacuolar hydrolytic enzymes, the liberation of the acidic vacuolar sap causes sudden cytoplasmic acidosis which might be sufficient to kill the selected cell. The vacuolar hydrolytic enzymes attack the remaining organelles and nuclear DNA, degrading whatever is left in the cell and leading to cell death (Hara-Nishimura *et al*. 2005; van Doorn and Woltering 2005). The cell elimination process also involves complete disappearance of the cell walls in the aerenchyma formation process. The result of this stage is the complete removal of the corpse (Jones 2000). Obviously, this leaves the cellular content as a collection of low molecular mass solutes to be absorbed by surrounding cells. In rice and maize roots, cells in the mid-cortex that are the first to die, are also the first to show signs of acidosis as detected by

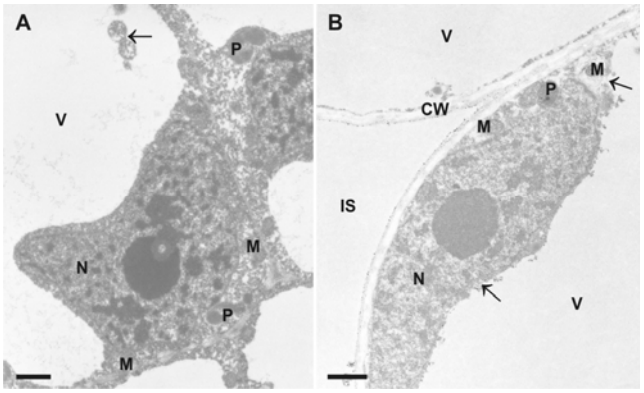

Fig. 4 Images show different early stages of autophagy processes in nitrate-deprived maize root cells in the transmission electron microscope. Samples were fixed with 2.5% glutaraldehyde, post-fixed with 2% osmium tetroxide and embedded in Agar 100 epoxy resin. Bars = 1 μm. (**A**) Cells of the root tip showing a nucleus (N), mitochondria (M) and plastids (P) in the cytosol. Within the large vacuole (V) remnants of organelles can be found (arrow). (**B**) Cells 1 cm from root tip, showing large vacuoles (V) with a ruptured tonoplast (arrows) and the cytoplasm condensed at the cell walls (CW). Within the loosened cytosol mitochondria (M), a plastid (P) and a large nucleus (N), which appears flattened at the cell periphery, can be found. IS=intercellular space. Images were obtained in co-operation with Bernd Zechmann and Maria Müller (Institute of Plant Sciences, University of Graz).

neutral-red staining (Kawai *et al*. 1998; Drew *et al*. 2000).

Changes in the dying nucleus

Slightly before tonoplast rupture, the nucleus appears flattened at the cell periphery, and this is presumed to be caused by the pressure exerted on it by the intact vacuole. Just after vacuole collapse, the nucleus assumes a more-spherical shape, indicating a release of vacuolar pressure. A gradual decrease in nuclear staining is noticed after vacuole rupture (Obara *et al*. 2001). DNA degradation is a hallmark of PCD and apart from protoplast shrinkage, DNA fragmentation is also documented in dying cells (Mittler and Lam 1995). Use of the TUNEL technique suggested internucleosomal cleavage of DNA. Nucleic acid degradation is presumed to occur via the release of endonucleases previously contained within the vacuole (Obara *et al*. 2001). Chromatin condensation to the nuclear periphery is typical of PCD (Gunawardena *et al*. 2001).

PCD that has been detected in roots of many species in the Poaceae family (including wheat, barley and maize) is characterised by a loss of nuclear staining with acridine orange. The symptom coincides with the loss of both membrane permeability and esterase activity, with fluorescein diacetate as a substrate (Drew *et al*. 2000). Very few nuclei in the cortex of the root tip of S-starved root fluoresced, after treatment with acridine orange, being shrunken and near to the cell wall (Bouranis *et al*. 2003). Chromatin condensation detectable by electron microscopy follows cytoplasmic changes including plasma membrane invagination and the formation of vesicles. Later, cellular condensation of chromatin and the presence of intact organelles surrounded by membrane resembling apoptotic bodies have been observed. All these events were completed before cell wall degradation was apparent (Gunawardena *et al*. 2001).

TOWARDS CONFIGURING THE ARCHITECTURE OF AERENCHYMA – THE DISSEMINATION AND TERMINATION PROCESS OF THE CELL ELIMINATION PROGRAM

Location of CEP

Cell death begins at a specific cell position near the root tip. The cells for elimination are located at the centre of the cortical tissues (mid cortex) and cell elimination never takes place at peripheral cortical cells. In maize, the epidermis, hypodermis, endodermis and stele are unaffected. The location of cells undergoing lysis appears to be precise, indicating the existence of a targeting mechanism for initiating the first cell death (Campbell and Drew 1983; Seago *et al*. 2005; Bouranis *et al*. 2006), and CEP constitutes a mechanism activated at a specific time and in a specific tissue that includes coordination between adjacent cells, with a highly localized pattern in the root cortex (Drew *et al*. 2000; Jones 2001; Evans 2003).

Aerenchyma develops more frequently and more extensively where cells are packed cubically and preferentially where the pre-aerenchyma packing of cells in the cortex is radial in transverse section, which is the pattern found in many wetland species, rendering cell packing as an important parameter for aerenchyma development (Justin and Armstrong 1987). Cell packing has a marked influence on whether schizogenous or lysigenous aerenchyma forms or not. By contrast, aerenchyma rarely occurs where there is non-radial, hexagonal packing, which is chiefly found in non-wetland species, or in the outer cortical layers that do not participate in aerenchyma formation in typical wetland species (Drew *et al*. 2000).

Spread of CEP to the surrounding cells and its limitation

Aerenchyma is a tubular structure, which does not develop secondary cell wall (Kozela and Regan 2003). The execution of the CEP culminates in the complete removal of the cell content and the creation of the first lacuna. After that, coordinated CEP of adjacent cells takes place, resulting in the creation of large intercellular aerenchyma tissue. Such a progressive development of lacunae creates the rather irregularly shaped functional tissue of cortical aerenchyma. The central point of the mechanism is that there are specific cells which are subject to lysis, while the surrounding cells do not die. The cells which remain unaffected by the lytical process in the cortex are still capable of metabolic activity, forming radial bridges immediately adjacent to lysing cells. The reason of this immunity of cells is still unknown. Therefore, an important aspect of aerenchyma development is the limitation of the spread of the CEP execution. It seems likely that there are differences in the sensitivity of the cells to the stimuli initiating CEP or in the response pathways present for subsequent events (Evans 2003) or the death signal which diffuses symplastically from the initiating cells to the remainder of the cortex in the direction that most plasmodesmatal connections occur in roots (Drew *et al*. 2000). Candidates CEP transmitters may be diffusible molecules such as ethylene, hydrogen peroxide and NO (Corpas *et al*. 2001). Moreover, the underexpression of Hb (class 1) genes and the presence or the induction of the formation of lytic vacuoles characterised by γ-TIPs may be candidate characteristics of the target cells.

Furthermore in many species, primarily the tangential cell walls are affected, whereas the majority of radial walls remain intact (e.g. in the Poaceae); conversely, in the Juncaceae and Cyperaceae, most tangential walls remain, while a large portion of the radial walls disappear (Smirnoff and Crawford 1983). This directional targeting of cells and cell-wall material requires a very specific distribution of signals, and/or of the sensitivities to these signals.

In the root cortex of maize, rice and *Saggitaria* under hypoxia, CEP is first detected at a distance of one centimeter or less from the root apical meristem, in the zone where cell elongation has just been completed (Campbell and Drew 1983; Kawai *et al*. 1998). The created space becomes increasingly prominent in older zones behind the tip. The system of lacunae therefore develops simultaneously with the extension of the root; however the presence of a developing lateral root seems to inhibit cell break-down (Campbell and Drew 1983; Bouranis *et al*. 2006). However aerenchyma in maize adventitious roots induced in sulphate-dep-

rived and well-aerated nutrient solution, were found in the cortex of 77% of the root length, particularly in the region of emerging or developing lateral roots (Bouranis *et al*. 2006). The basal and apical root sectors had no aerenchyma and no aerenchyma connection was found with the shoot (Bouranis *et al*. 2006), while cell walls of endodermis of S-deprived adventitious maize roots increased 68% in thickness (Bouranis *et al*. 2003).

Variations in CEP execution

Within aerenchyma types, there is a large variation in the exact configuration of the cells and cell remnants. The processes of aerenchyma formation differ in rice and maize with regard to the order in which events occur. In maize roots, cellular collapse was preceded by the loss of tonoplast integrity (Campbell and Drew 1983), while the pattern of cell death and lysis in maize aerenchyma formation begins in the mid-cortex but unlike rice, spreads tangentially as well as radially (Setter *et al*. 1997; Kawai *et al*. 1998). However, in rice, the middle lamella degenerated, and this was followed by cell wall disintegration and the loss of tonoplast integrity (Webb and Jackson 1986). The aerenchyma of rice roots is rapidly formed as an integral part of ordinary root development (Webb and Armstrong 1983; Jackson *et al*. 1985b). A detailed review on the differences among the general patterns of aerenchyma origin and their systematic distributions among the flowering plants is available (see Seago *et al*. 2005), providing the basis for further examination of CEP variations.

FUTURE PROSPECTS

Despite the accumulating literature on all aspects of aerenchyma formation and the progress that has been made on the functionality of the known signal transduction modules, there remains a series of open questions that require explanation at the molecular level. What characterises the constitutive aerenchyma formation? How are ACC synthase and ACC oxidase activated during the progress of CEP, and what are the alternative pathways for ethylene-independent CEP? What characterises the target cells, and why is aerenchyma formation restricted to cortical cells only? What is the complete catalogue of CEP execution enzymes and their mode of action?

How is CEP transmitted to the neighbouring cells? Are there symplastic or apoplastic CEP transmitters? What limits the extent of CEP dissemination and how do neighbouring cells remain unaffected? Why is cell packing of a certain type needed? Why does the presence of a developing lateral root inhibit cell breakdown? Why is there a specific targeting of collapse to tangential cell walls? Is there a specific spatial distribution of transporters and channels for the materials that are to be pumped out of the lacuna and how is this mechanism activated? As CEP for aerenchyma formation is not the same in all species, the exact signalling modules, transmitters and executors are needed to be clarified for each case.

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