Plant and Soil (2004) 254: 197–214
DOI 10.1007/s11104-004-1650-0

Acclimation to soil flooding – sensing and signal-transduction

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Received 2 April 2004. Accepted in revised form 26 July 2004

Key words: acclimation, adventitious roots, aerenchyma, ethylene, oxygen sensing, soil flooding, waterlogging

Abstract

Flooding results in major changes in the soil environment. The slow diffusion rate of gases in water limits the oxygen supply, which affects aerobic root respiration as well as many (bio)geochemical processes in the soil. Plants from habitats subject to flooding have developed several ways to acclimate to these growth-inhibiting conditions, ranging from pathways that enable anaerobic metabolism to specific morphological and anatomical structures that prevent oxygen shortage. In order to acclimate in a timely manner, it is crucial that a flooding event is accurately sensed by the plant. Sensing may largely occur in two ways: by the decrease of oxygen concentration, and by an increase in ethylene. Although ethylene sensing is now well understood, progress in unraveling the sensing of oxygen has been made only recently. With respect to the signal-transduction pathways, two types of acclimation have received most attention. Aerenchyma formation, to promote gas diffusion through the roots, seems largely under control of ethylene, whereas adventitious root development appears to be induced by an interaction between ethylene and auxin. Parts of these pathways have been described for a range of species, but a complete overview is not yet available. The use of molecular-genetic approaches may fill the gaps in our knowledge, but a lack of suitable model species may hamper further progress.

Introduction

Flooding of the soil, also called waterlogging, can have a tremendous impact on the growth and survival of plants, and thereby on agricultural as well as natural ecosystems. In the last decades considerable progress has been made in our understanding of the mechanisms that enable certain plant species and cultivars to withstand periods with excess soil water, or even complete submergence. Much of the research has been carried out with crop plant species, such as rice (*Oryza sativa*), maize (*Zea mays*) and sunflower (*Helianthus annuus*), but also wild species originating from wetland habitats have been used, mostly for comparative studies (e.g., Justin and Armstrong, 1987; Laan et al., 1989; Smirnoff and Crawford, 1983; Visser et al., 1996a).

The main effect of soil flooding is a considerable slow-down of the exchange of oxygen, as gas diffusion rates are four orders of magnitude slower in water than in air (Jackson, 1985). Consequently, flooded soils rapidly develop anoxic conditions at depths greater than a few centimetres, as the demand by aerobic respiration of soil organisms greatly exceeds the influx of oxygen from the atmosphere. Shortage of oxygen is obviously detrimental to the development of root systems, and those roots whose aerobic metabolism entirely depends on oxygen from the soil will cease growth, and may eventually die (Bradford and Yang, 1981; Drew, 1997). Additionally, anoxic soils may accumulate phytotoxic products from microbial reduction processes (such as hydrogen sulphide, Fe^{2+} and Mn^{+}; Ernst, 1990; Laanbroek, 1990; Lamers et al., 1998; Ponnamperuma, 1984), and the gaseous plant hormone ethylene (Smits and Scott-Russell, 1969; Campbell and Moreau, 1979; Visser et al., 1996b),

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which can strongly inhibit root growth (Konings and Jackson, 1979; Visser et al., 1997).

In-depth discussions of the large variety of adaptations that have evolved in plant species prone to soil flooding, have been presented in recent reviews, such as publications on root function, physiology and biochemistry (Jackson and Ricard, 2003), aerenchyma development (Jackson and Armstrong, 1999; Evans, 2003), gas diffusion in plants (Colmer, 2003), root-to-shoot signalling (Jackson, 2002), changes in gene expression (Subbaiah and Sachs, 2003) and root metabolism (Drew, 1997; Geigenberger, 2003; Gibbs and Greenway, 2003a, b). In the current review we will focus on how plants sense the changed conditions of a waterlogged soil. Much of this is still unclear and speculative, but recently progress has been made. Furthermore, we will discuss the two best described signal-transduction pathways leading to morphological and anatomical acclimation to soil flooding; namely, those resulting in aerenchyma formation and in adventitious rooting. Finally, we conclude with a brief summary of the most prominent advances and our view on the future development of the field.

Sensing of flooded conditions

Signals

Plants use external and internal signals to sense changes in the environment, such as shifts from aerial to aquatic. These signals are often the first step in transduction cascades leading to rapid down-regulation of metabolic pathways to decrease oxygen consumption, and, in the long-term, to morphological adjustments such as aerenchyma development and fast shoot elongation to increase oxygen entry (Geigenberger, 2003).

The two internal gaseous signals, oxygen and ethylene, are frequently associated with the responses of plants or plant parts surrounded by water. Submergence has a dramatic effect on the endogenous concentrations of these two components due to the very slow diffusion of gases in water compared to that in air (Jackson, 1985). In non-photosynthesising organs such as roots, oxygen levels will rapidly decline due to continuous oxygen consumption in respiration, and the very slow delivery of aerial oxygen to the root. Ethylene, on the other hand, will accumulate to physiologically active levels in submerged tissues, due to production in almost every organ and hampered diffusion to the atmosphere (Voesenek and Blom, 1999). However, a prerequisite for continued ethylene production is the presence of at least some molecular oxygen, since conversion of the ethylene precursor 1-aminocyclopropane-1-carboxylate (ACC) to ethylene, catalysed by ACC oxidase, uses molecular oxygen as a co-substrate (Kende, 1993).

Ethylene

Elevated ethylene levels are important for the induction of morphological and anatomical traits upon soil flooding, such as formation of aerenchyma and adventitious roots. During the last decade enormous progress has been made in disentangling the ethylene-response pathway from hormone perception at membrane structures to transcriptional regulation in the nucleus. This was mainly achieved with a genetic and molecular analysis of *Arabidopsis* mutants disturbed in ethylene perception and signalling.

In plants, ethylene is perceived by a family of receptor molecules located in the endoplasmic reticulum (ER) (Chen et al., 2002). These receptor molecules share strong homology with bacterial two-component regulators (Chang et al., 1993), and are by default functionally active and are switched to an off-state by ethylene binding to the N-terminal transmembrane part of the dimerized molecule. Ethylene receptors form a complex with a protein called CONSTITUTIVE TRIPLE RESPONSE (CTR). CTR proteins are activated by association with the receptors at the ER and repress downstream ethylene responses. In the presence of ethylene, the ethylene receptor proteins presumably undergo conformation changes that inactivate them. Under these conditions CTR is released from the ER and also becomes inactivated (Gao et al., 2003). Consequently, this derepression will result in ethylene responses. Further downstream ethylene signals are transduced via several positive regulators (e.g., ETHYLENE INSENSITIVE2 (EIN2), EIN5 and EIN6) ending with the transcription factors EIN3 and EIN3-like (EIL) (Guo and Ecker, 2003). An immediate target for EIN3 is the ethylene-responsive gene *Ethylene Response Factor1* (*ERF1*). The ERF1 protein belongs to the family of so called ETHYLENE RESPONSE ELEMENT BINDING PROTEINS (EREBPs) transcription factors. These EREBPs play an important role in controlling expression of ethylene target genes (Solano et al., 1998).

For all known ethylene responses the obligatory components of the signal transduction cascade range from the receptor molecules to the transcription factor family EIN3/EIL. The branch point resulting in the wide array of ethylene responses lies downstream of EIN3/EIL (Guo and Ecker, 2003).

Oxygen

Oxygen is another internal gas signal that changes dramatically upon submergence. The stress induced by low oxygen concentrations is not restricted to flooding environments, but can occur in tissues characterized by fast metabolic rates (e.g., root meristems, phloem tissue). In these tissues the rate of oxygen delivery via diffusion is too slow to keep pace with its consumption (Geigenberger, 2003).

It is important to distinguish real anoxic conditions from those where cytochrome oxidase activity is not limited by oxygen, but where the oxygen levels are below ambient. Falling oxygen levels are sensed in plants, and lead to a fast inhibition of respiration, a lowering of the adenylate status, and a down-regulation of the TCA cycle and glycolysis (Geigenberger, 2003). These changes are consistent with the down-regulation of genes that encode enzymes involved in the biosynthesis of cell walls, lipids and flavanoids, defense responses and protein degradation in *Arabidopsis* roots exposed to low oxygen (Klok et al., 2002). This inhibition of biosynthetic fluxes and metabolic rates occurs even at oxygen levels that are much higher than the K_m of cytochrome oxidase and alternative oxidase (AOX), indicating that it is very likely that oxygen sensing operates independently of the electron transport chain. However, it cannot be ruled out that diffusion limitation in bulky tissues might, in fact, mean that a proportion of the cells are exposed to oxygen levels below the K_m of cytochrome oxidase and AOX. The observed metabolic shift is assumed to be adaptive, since it decreases oxygen consumption and saves ATP, and thus delays the onset of anoxia (Geigenberger, 2003). In a micro-array study with *Arabidopsis* roots exposed to low oxygen it became evident that next to a set of down-regulated genes, several genes were up-regulated. These up-regulated genes belong to three categories: (i) genes involved in ethanolic and lactic fermentation, (ii) genes that potentially play a role in post-anoxia injury, and (iii) genes related to ethylene synthesis, ethylene signalling, programmed cell death and cell-wall loosening (Klok et al., 2002). The first two classes could be interpreted as pre-adaptive genes that are expressed to continue energy production in subsequent anoxic periods, and that enhance survival in post-flood phases in which oxygen re-enters the plant. The third category of genes is strongly associated with aerenchyma formation which leads to a more long-term acclimation to enhance entry of oxygen.

Thus, in order to timely respond to decreasing oxygen levels, plants need to sense oxygen concentrations that are between the K_m of cytochrome oxidase and AOX and normoxia. This oxygen-sensing system in higher plants operates independently of changes in energy metabolism. This contention is supported by findings that induction of the *ADH1* gene by low oxygen concentration cannot be mimicked by respiratory inhibitors (Bucher et al., 1994). One of the first detectable changes upon oxygen deprivation is an elevation of cytosolic Ca^{2+} (Subbaiah et al., 1994), probably caused by calcium mobilization from mitochondria (Subbaiah et al., 1998), suggesting that mitochondria are at the centre of oxygen sensing (Subbaiah and Sachs, 2003). Several low-oxygen-induced genes are characterized by an anaerobic response element in their promotor (Klok et al., 2002). The transcription factor AtMYB2, induced by low oxygen, binds to this promoter, and can activate *ADH1* promotor activity (Hoeren et al., 1998).

Oxygen-sensing systems have long been known for prokaryotes (reviewed by Bunn and Poyton, 1996). A well-studied example in this respect, found in various bacteria, is the oxygen-sensing protein FixL. It contains a histidine kinase domain belonging to the class of two-component regulatory systems and a heme-binding sensory domain that shares homology with the PAS domain superfamily (Taylor and Zhulin, 1999). Under well-aerated conditions FixL is oxygenated, and kinase activity is turned off. When FixL is deoxygenated during falling oxygen levels it autophosphorylates at a histidine. The subsequent transfer of this phosphoryl group to the transcription factor FixJ triggers a cascade of gene expression (Gong et al., 1998).

Hemoglobins

During recent years much progress has been made on the role of hemoglobin proteins during hypoxia stress. Genes encoding for non-symbiotic class 1 hemoglobins or stress-induced hemoglobins are expressed during low-oxygen conditions (Klok et al., 2002), and upon exposure to elevated levels of nitrate and sucrose (Dordas et al., 2003). These hemoglobin-encoding genes, in contrast to the *ADH* gene, are also induced by respiratory chain inhibitors, indicating that oxygen affects hemoglobin expression indirectly (Nie and Hill, 1997). Hemoglobin proteins reversibly bind oxygen, and are characterized by very low dissociation constants. Consequently, hemoglobins remain oxygenated at oxygen levels far below those that induce so-called anaerobic responses (Sowa et al., 1998). This chemical property and their indirect response to low oxygen make it very unlikely that hemoglobins act as an oxygen carrier, store or sensor. However, it seems that they do have an important role in hypoxia tolerance of higher plants as illustrated below. Transgenic *Arabidopsis* plants overexpressing a class 1 hemoglobin (GLB1) are more tolerant towards severe hypoxia, and plants overexpressing a mutated *glb1* gene resulting in a protein that has a strongly reduced affinity for oxygen are as susceptible to hypoxia as wild-type plants (Hunt et al., 2002). Similar results were obtained in a system with cell cultures of maize that constitutively express barley hemoglobin in either sense or antisense orientation (Sowa et al., 1998). Furthermore, *GLB1* overexpression in *Arabidopsis* could phenocopy low-oxygen pretreatments in terms of survival (Hunt et al., 2002).

Interestingly, hemoglobins also appear in rapidly growing tissues (e.g., root tips of germinating seeds), and they improve early growth in *Arabidopsis*, even under normoxic culture conditions (Hunt et al., 2002; Dordas et al., 2003). This occurrence probably reflects the presence of localized low oxygen tensions often observed in densely packed organs such as meristems.

Second messengers

Hemoglobin up-regulation is not directly controlled by low oxygen, but presumably a consequence of reduced ATP levels (Nie and Hill, 1997). This stresses the importance of second messengers in the regulation of low-oxygen responses. Very recently, the decline in cytosolic pH, probably caused by lactate fermentation, was identified as a second messenger controlling water permeability of *Arabidopsis* root cells exposed to anoxia (Tournaire-Roux et al., 2003). In a set of elegant experiments, Tournaire-Roux and colleagues (2003) demonstrated that the decline in hydraulic conductivity in roots during anoxia was related to a hampered water influx through plasma-membrane intrinsic proteins (PIPs) induced by cytosolic acidification. A particular histidine at position 197 of the aquaporin molecule was responsible for cytosolic pH sensing, and thus for gating these water-channel proteins.

Next to Ca^{2+} and pH, hydrogen peroxide (H_2O_2) was very recently identified as a second messenger in responses to low oxygen (Baxter-Burrell et al., 2002). In *Arabidopsis*, exposure to low oxygen increases the production of H_2O_2 co-ordinately with a substantial increase in ADH activity. The production of H_2O_2 is fine-tuned by two proteins, Rop and RopGAP4, in which RopGAP4 negatively regulates Rop and Rop has a positive regulatory impact on RopGAP4. It is of utmost importance that this Rop rheostat controls the levels of H_2O_2 in plants cells precisely, since levels that are too high may trigger formation of reactive oxygen species that may induce cell death, whereas levels that are too low prevent the expression of adaptive genes (e.g., *ADH*) that improve survival during low-oxygen stress (Baxter-Burrell et al., 2002).

Signal transduction – hormones and further down-stream components

Aerenchyma formation

Aerenchyma is the specialised tissue in petioles, stems and roots consisting of longitudinal gas-filled channels, which may result from various anatomical patterns of cellular configuration (Smirnoff and Crawford, 1983; Justin and Armstrong, 1987; Visser et al., 2000). A common feature of aerenchyma is that it forms a network of gas-filled spaces between the cells that interconnects most parts of the (partly) submerged plant with the atmosphere, and thereby is able to deliver oxygen almost throughout the plant (Armstrong, 1979). Conversely, gases produced by the soil or plant may be vented through these channels to the atmosphere (Colmer, 2003).

Schizogenous versus lysigenous aerenchyma

In general, two types of aerenchyma can be distinguished, although intermediate forms do occur. The first type, which is most abundant in roots and rhizomes, is initiated by the death of cells in the cortex, resulting in gas-filled voids between the living cells that remain. This type is named lysigenous aerenchyma, after the lysis of cells that precedes gas space development (Figure 1A). The second type is schizogenous aerenchyma, which forms through the separation of cells from each other in an early stage of development (Figure 1B, C). Schizogenous aerenchyma may also be found in roots, but more often in the stems and petioles of wetland plants. In both aerenchyma types, there is a large variation

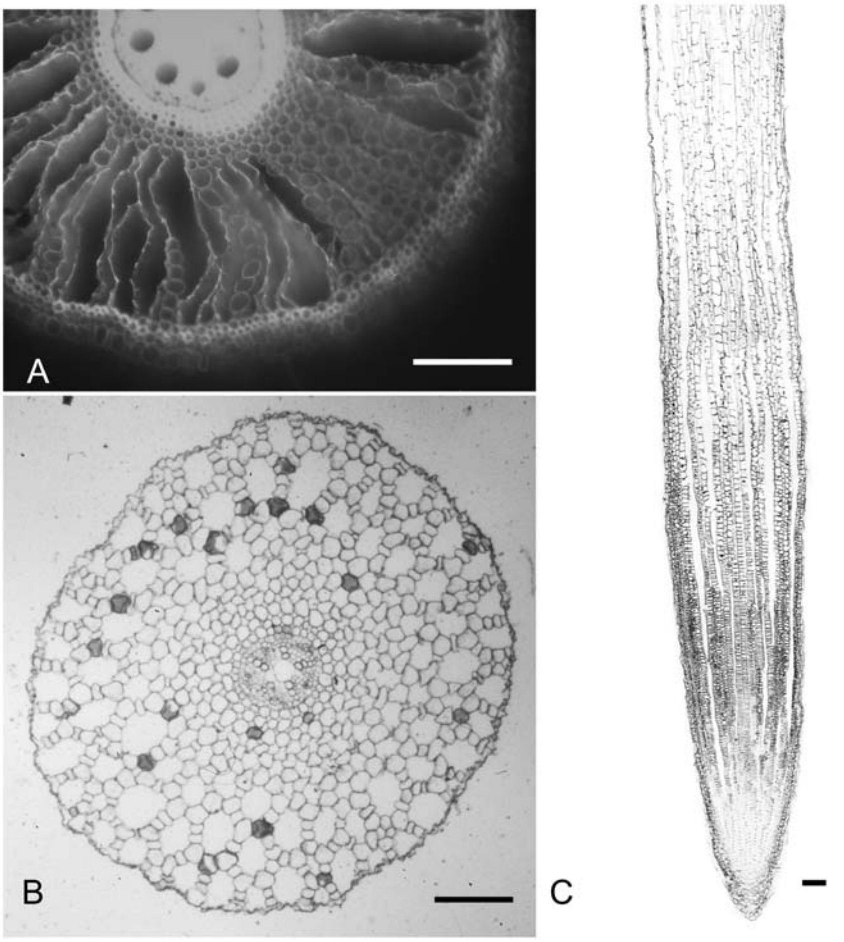

Figure 1. A. Cross-section of the aerenchymatous root of a hydroponically grown *Juncus effusus* plant. Large lysigenous aerenchyma has developed in the cortical parenchyma. B. Cross-section of an adventitious root of a hydroponically grown *Rumex palustris* plant. C. Longitudinal section of a similar root. The aerenchyma forms in a schizogenous way by directional division and separation of the cortical cells (B) and develops close to the root apex (C). Scale bars indicate 0.2 mm.

in the exact configuration of the cells and cell remnants, as shown by Justin and Armstrong (1987) in their extensive screening of root anatomy of wetland, intermediate and non-wetland species.

Schizogenous aerenchyma seems a more or less constitutive feature within a given root, and 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., 1996a). In contrast, lysigenous aerenchyma can develop in both mature and in newly-developing roots (although older wheat roots were not capable to form aerenchyma (Thomson et al., 1990)), and its presence often depends on environmental stimuli. Many crop species, such as wheat (*Triticum aestivum* – Huang et al., 1994; Boru et al., 2003; Malik et al. 2003), barley (*Hordeum vulgare* - Bryant, 1934; Garthwaite et al., 2003), maize (McPherson, 1939; Drew et al., 1979; Konings and Verschuren, 1980), sunflower (Kawase, 1979), and rice (Jackson et al., 1985b), form lysigenous aerenchyma. This is probably the reason why so little research has been done on the regulation of schizogenous aerenchyma formation (Jackson and Armstrong, 1999), whereas the inducible signal-transduction pathway of lysigenous aerenchyma has gained far more attention, particularly by using maize root aerenchyma as a model.

Hypoxia and ethylene

The first evidence for a role of ethylene in the induction of aerenchyma in tissues of waterlogged plants was derived from studies on sunflower. However, soon after the discovery that treatment of sunflower with ethylene resulted in similar aerenchyma formation in the hypocotyl as did soil flooding (Kawase, 1974), Drew et al. (1979) reported that root aerenchyma could be evoked by treating maize with ethylene. Also, inhibition of ethylene action by silver ions inhibited aerenchyma formation in waterlogged maize plants (Drew et al., 1981). The latter species proved to be a suitable model to investigate lysigenous aerenchyma formation, and subsequent research was done to find the triggers that set off the lysis of cells. One would expect that accumulation of ethylene due to the physical entrapment by the surrounding water-saturated soil suffices to induce aerenchyma. Nevertheless, the prevalent low oxygen concentrations further stimulate ethylene production (Jackson et al., 1985a), thereby adding to the build-up of ethylene concentrations. Ethylene levels exceeding 0.5 μL L^{-1} are usually sufficient to evoke the maximum response in treated roots.

Much of the role that ethylene plays has been elucidated since these early observations (Figure 2; Drew et al., 2000). He et al. (1994) described an increase in the root tips of maize of the enzyme that is largely responsible for the rate of ethylene biosynthesis, i.e. ACC-synthase, as a response to low oxygen concentrations. This response was not present when oxygen was completely absent (accomplished by flushing with nitrogen gas), possibly because strict anoxia largely prevents protein synthesis. Application of either low oxygen or increased ethylene concentrations resulted in an increase of the cellulase activity in the root apex (He et al., 1994), which therefore likely contributes to cell-wall break-down, being the last step in lysigenous aerenchyma formation. Blocking the activity of ACC-synthase with a specific inhibitor (aminoethoxyvinylglycine, AVG) suppressed cellulase activity to the constitutive control levels, providing further proof that ethylene is the plant hormone responsible for controlling this part of aerenchyma development (He et al., 1994).

Down-stream parts of the signal transduction pathway

Earlier processes in the chain of events leading to death of cells predestined to become aerenchymatous spaces are still largely unknown. A first attempt to elucidate these was the application of inhibitors and elicitors of programmed cell death (PCD) that were previously used successfully in animal systems or with other PCD processes in plants (He et al., 1996b). K-252a, a substance that inhibits protein kinases and protein kinase C in particular, inhibited both cellulase activity and aerenchyma formation under low oxygen concentrations, suggesting a role for protein kinase C in the induction of aerenchyma. On the other hand, specific inhibition of protein phosphatases 1 and 2A by applying okadaic acid (Cohen et al., 1990) is supposed to enhance protein phosphorylation and proved to promote cellulase activity and aerenchyma formation (He et al., 1996b). Similarly, GTPγS, which is capable of locking G-proteins in the active state, increased the volume of aerenchyma under normoxic conditions, whereas its analogue GDPβS, which inactivates the proteins, did not have effect on the aerenchyma content, neither under normoxic nor under low-oxygen conditions. Apart from protein phosphorylation processes and G-proteins, also inositol phospholipids appear to be involved, since neomycin, which interferes with the binding of inositol phospholipids with the plasma membrane, almost fully prevented low-oxygen-induced aerenchyma formation (He et al., 1996b).

Manipulation of cytoplasmic calcium concentrations and fluxes also proved successful in changing the capacity to form aerenchyma (He et al., 1996b). Both thapsigargin and caffeine increase intracellular calcium concentrations, by blocking Ca^{2+}-ATPase activity in the ER, and opening Ca^{2+}-channels, respectively. Either substance resulted in an increase in aerenchyma content in normoxic roots, and in a faster progress of PCD in oxygen-deficient roots. Lowering Ca^{2+} levels with EGTA (a Ca^{2+}-chelator) or ruthenium red (which blocks Ca^{2+}-channels) inhibited cell lysis. Ruthenium red also binds to calmodulin (CM), thereby preventing Ca-CM dependent protein activity, and a similar effect could be expected from $W-7$ ($N-$[6-aminohexyl]-5-chloro-1-naphthalenesulfonamide), which inhibits CaCM-mediated ion-channel function. Again, the latter substance prevented aerenchyma formation. These results make it highly likely that an influx of Ca^{2+} into the cytoplasm is a necessary step in the process leading to cell death (see also Figure 2).

Programmed cell death

The chemical compounds mentioned above have originally been successfully applied in studies of apoptosis in animal cells, and the similarity in responses between apoptosis in these systems and aerenchyma formation suggested that the regulation of these processes

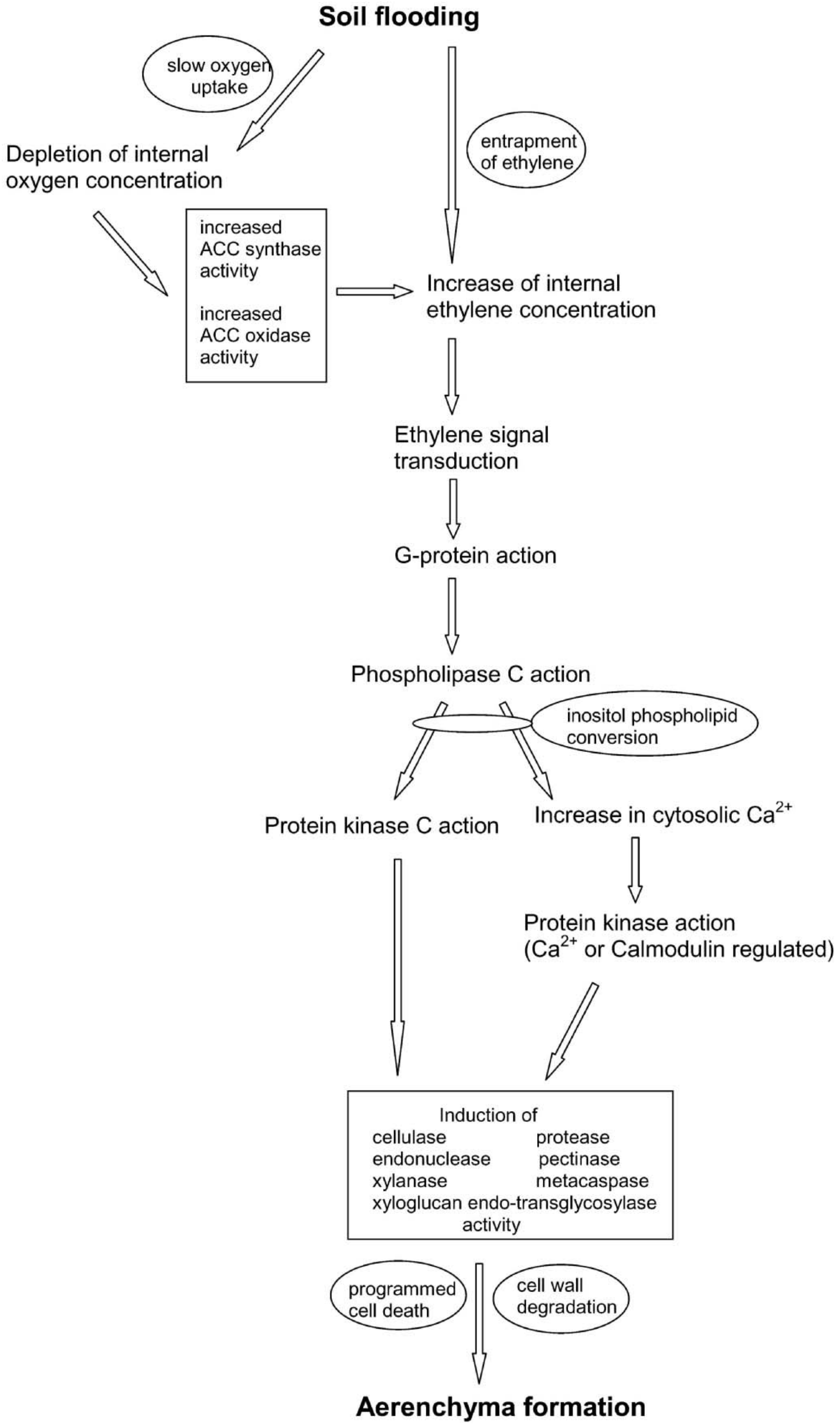

Figure 2. Schematic view of the processes likely involved in lysigenous aerenchyma formation. Drawn according the data and schemes shown and reviewed in Drew et al. (2000), Gunawardena et al. (2001), Woltering et al. (2002), Aschi-Smiti et al. (2003) and Bragina et al. (2003). Most of the data originated from studies on maize (*Zea mays*).

may be similar at the cellular level (Pennell and Lamb, 1997; Buckner et al., 1998). However, more detailed studies changed this view somewhat. Campbell and Drew (1983) already observed some early events at the cytoplasmic level in maize root cells that underwent programmed cell death, but only more advanced techniques made it possible to search for the specific changes involved in apoptosis.

TUNEL-staining of fragmented nuclear DNA showed that already in 0.5-day-old ethylene-treated cortical root cells endonuclease activity increased, which was confirmed by increased laddering of genomic DNA on an agarose gel (Gunawardena et al., 2001). Apoptotic ultrastructural changes of the cells, such as chromatin condensation and its relocation to the nuclear periphery, could be detected after one day by electron microscopy. Changes in the plasma membrane, differences in the staining of vacuoles and cytoplasmic content (of which the meaning was not known), and the occurrence of large numbers of vesicles preceded the visual changes in the nucleus. Plasmodesmata then became very distinct in 1.5-day-old tissues, and apoptotic body-like structures containing entire cell organelles appeared, which were possibly involved in the hydrolysis of cell components. Although there are quite a few similarities, it is evident that there are also clear differences between apoptosis in animal cells and the programmed cell death during aerenchyma formation. For example, macrophage digestion does not take place at the final stages in the plant tissue, as it does in animal cells, and also the sequence of nuclear and other cytosolic events is different, as the latter do not precede nuclear changes in animal apoptosis (Gunawardena et al., 2001). Apparently, aerenchyma formation forms a class of programmed cell death of its own.

Although in animals a group of proteins named caspases form an intrinsic element of the cascade of events in programmed cell death, until recently no direct evidence was found for the presence of functional homologues of these enzymes in plants (Woltering et al., 2002). However, indirect evidence suggested that caspase-like activity is needed for programmed cell death in plants (Woltering et al., 2002), and metacaspases have now been identified that may serve as such in plants (Uren et al., 2000; Hoeberichts et al., 2003).

Cell-wall-degrading enzymes

The final step in aerenchyma formation is the breakdown of cell walls, which ultimately creates the voids needed for gas diffusion. Conditions of low oxygen and high ethylene concentrations may induce a steep increase in cellulase activity within three days (He et al., 1994). This was confirmed by Bragina et al. (2003), who also found increased levels of pectinase and xylanase activity. Additionally, a structural homologue of xyloglucan endo-transglycosylase (XET) was found to be induced by hypoxia (Saab and Sachs, 1996). This combination of enzymes enables a stepwise degradation of the cells and resorption of their structural components.

It is interesting that quite often radial files of cells remain unaffected by the lytical process (Justin and Armstrong, 1987), and also the presence of a developing lateral root may inhibit cell break-down. Apart from this, programmed cell death usually does not affect the inner- and outermost parenchyma layers of the cortex. The reason of this ‘immunity’ of cells to the action of ethylene or to components more downstream of ethylene is unknown. Also it is not clear why in many species mainly the tangential cell walls collapse, whereas the majority of radial walls remain intact (*e.g.*, in Poaceae); conversely, in Juncaceae and Cyperaceae most tangential walls remain, while a large part of the radial walls disappear, resulting in so called ‘spider’s web’ aerenchyma (Smirnoff and Crawford, 1983). This directional targeting of cells and cell-wall material requires a very specific distribution of signals, or of the sensitivities to these signals, and much remains to be elucidated about how these patterns develop.

Other growth regulators

Some attention has been paid to a possible role of polyamines in aerenchyma formation in maize roots, because spermine and spermidine are biosynthesised from the same precursor as ethylene (*S*-adenosylmethionine), and a decline of these polyamines may be involved in the rise of ethylene production during hypoxia (Jackson and Hall, 1993). However, no proof could be found for such an involvement, since these polyamine levels did not decrease in hypoxic maize roots, and addition of putrescine, a precursor of spermine and spermidine, inhibited rather than increased aerenchyma formation (Jackson and Hall, 1993).

Auxin has been studied as a potential regulator of aerenchyma content as well (Konings and De Wolf, 1984; Justin and Armstrong, 1991a). The first study indicated an inhibition of aerenchyma formation in maize roots by the synthetic auxin 1-naphthalene acetic acid (1-NAA), but, after adjustment of the data for the profound effects of auxin on the root growth rate, Justin and Armstrong (1991a) found a small stimulation, which they attributed to auxin-induced ethylene production. Gibberellin (GA_3) and kinetin (a cytokinin) also promoted the formation of aerenchyma in maize (Konings and De Wolf, 1984), which may again, in the case of GA_3, be caused by ethylene action, since the effect was counteracted by the simultaneous application of ethylene inhibitors. In the same study, abscisic acid (ABA) had a negative effect on aerenchyma, but none of these effects have been followed up in further research.

Other plant species

The hormonal regulation of aerenchyma formation in maize has at least partially been validated for several other plant species. Aerenchyma formation in crop species such as sunflower, bean (*Phaseolus vulgaris*), tomato (*Lycopersicon esculentum* – Kawase, 1981) and wheat (Wiengweera et al., 1997) and in tree species like *Pinus serotina* (Topa and McLeod, 1988) seems under the control of low oxygen concentrations and ethylene, although silver did not affect aerenchyma formation in barley (Larsen et al., 1986). In *Trifolium subterraneum* a change in protease composition was shown following hypoxia, which correlated with cell lysis during root aerenchyma development and may point to the expression of a specific set of lysis-involved protease genes (Aschi-Smiti et al., 2003). However, the lack of model species that develop inducible aerenchyma and are also suitable for molecular-genetic studies seems to have hindered further progress in the signal transduction chain down-stream of ethylene.

Constitutive versus inducible aerenchyma

Many wetland plant species display a considerably high porosity in their roots even under well-aerated conditions (Justin and Armstrong, 1987). This is due to the presence of aerenchyma whose development is apparently not dependent on low oxygen concentrations or ethylene accumulation. It is as yet unclear how the onset of programmed cell death is initiated in these examples of constitutive aerenchyma, as until now only two studies dealt with this question, and their conclusions are contradictory. Jackson et al. (1985b) used inhibitors of ethylene in two cultivars of rice, of which one formed partly constitutive and partly inducible aerenchyma, whereas the other only developed constitutive aerenchyma. In the latter cultivar aerenchyma formation was not affected by ethylene inhibition, which led the authors to conclude that ethylene does not play a role in constitutive aerenchyma development. In response to this paper, Justin and Armstrong (1991b) partly repeated the experiments, but corrected the data for the negative effect of the treatments on the elongation rate of the aerenchymatous roots. With this correction, they concluded that ethylene did play a role. Most likely the root cortical cells had a much higher sensitivity to ethylene than in plant species with inducible aerenchyma, making them responsive to the low internal ethylene concentrations that prevail in a non-flooded root system. It would be interesting to conduct similar experiments with plant species whose root elongation is less sensitive to ethylene. This would prevent confounding of the results by side effects of ethylene. Until then, it remains to be seen whether these conclusions are broadly valid for other wetland plants with constitutive aerenchyma.

Factors other than flooding that induce aerenchyma

Next to oxygen shortage, also nutrient deficiency may lead to programmed cell death in the root cortex. Konings and Verschuren (1980) observed the development of aerenchyma in maize roots when these were grown in aerated but nitrogen-deficient nutrient solution, and Smirnoff and Crawford (1983) found that treatment of *Nardus stricta* plants in sand culture with low concentrations of nutrient solution increased root porosity to equally high levels as in waterlogged plants. Studies with maize later confirmed that both low concentrations of nitrate and low levels of phosphorous nutrition induced aerenchyma in the roots, and that this was accompanied by lower activities of ACC-synthase and ACC-oxidase, lower ACC levels and, therefore, lower ethylene-production rates (Drew et al., 1989). This apparent contrast to the response of the roots to hypoxia, where ethylene production increases and can be causally linked to programmed cell death (Jackson et al., 1985a; Atwell et al., 1988), could be explained by subsequent work by Drew and co-workers. Although ethylene biosynthesis slowed down upon nutrient deficiency, this was counteracted by a strong increase in the sensitivity of the cortical tissues to ethylene, so that the threshold leading to aerenchyma development was exceeded (He et al., 1992).

Recently, the promoting effect of low mineral nitrogen on ethylene sensitivity has been confirmed in a study where ethylene interacted with the release of herbivore-induced volatiles, such as indole and sesquiterpenes (Schmelz et al., 2003). Analogously, low phosphorus increased the sensitivity of root elongation in *Arabidopsis thaliana* to ethylene (Ma et al., 2003). Aerenchyma formation does not appear to be exclusively induced by low phosphorus and nitrogen, since Bouranis et al. (2003) found similar responses in sulphate-starved maize plants.

The aerenchyma in nutrient-starved plants seems to have an identical structure as that in roots of waterlogged plants, and the acting signal-transduction pathway is probably the same, except for the part upstream of ethylene perception. However, it is conceivable that the increased ethylene sensitivity of phosphorus- and nitrogen-depleted plants serves a different goal than aerenchyma formation per se, e.g., a change in root topology (Borch et al., 1999) or the formation of root hairs (although the latter is unlikely given the evidence provided by Schmidt and Schikora (2001) that low iron but not low phosphorus induces root hairs via the ethylene-perception pathway). On the other hand, Fan et al. (2003) measured respiration rates of phosphorus-starved roots, which were lower per volume root due to the lower number of cells after aerenchyma developed. 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 phosphorous.

Finally, soil compaction can also lead to formation of aerenchymatous tissues, which develop independently from low oxygen concentrations (He et al., 1996a). Again, ethylene plays a key role in the process, possibly by increased biosynthesis rates due to the pressure exerted on the root tip and by ethylene accumulation resulting from the increased gas diffusion resistance in the rhizosphere. It is, however, unlikely that aerenchyma is a favourable structure under conditions of high soil strength. Studies on *Rumex* and *Plantago* species indicated that aerenchymatous roots grew in a highly distorted way when encountering compacted soil (Engelaar et al., 1993b).

Adventitious root development

Flooding often causes malfunctioning of roots formed prior to flooding, even in wetland species (Justin and Armstrong, 1987; Visser et al., 1996a). This may eventually lead to the death of a considerable part of the root system, and a fast replacement by well-adapted adventitious roots that contain more aerenchyma than the original roots (Table 1, Figure 3).

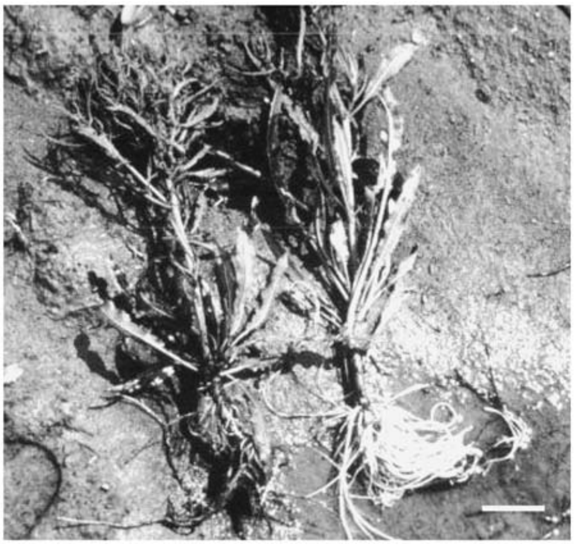

Figure 3. Root systems of intact *Rumex palustris* plants excavated from a floodplain near Nijmegen, the Netherlands. Left: a plant from a site with the groundwater table at 0.15 m below the soil surface. Right: a plant from a soil-flooded site. Notice the abundant development of thick, white adventitious roots in the soil-flooded plant, as compared to the thinner, brownish primary root system of the drained plant. Scale bar indicates 0.1 m.

There is some controversy about the exact definition of the term adventitious root, as many authors use it specifically for shoot-derived roots (i.e. roots that originate from shoot tissue), whereas others argue that roots that develop from older parts of the root system, where normally new root development would not take place, should also be considered adventitious roots (Barlow, 1994). In the current context, we will adopt the second definition, since there seems to be no functional difference between the roots that develop from the base of the stem and those that develop just centimetres lower on the upper part of the taproot (Visser et al., 1996a).

Kramer (1951) recognised that the recovery of tomato plants from the initial growth reduction imposed by soil flooding coincided with the formation of new adventitious roots. Since then, it has been well established that adventitious root development contributes greatly to the tolerance of a plant to poorly aerated soils, and the biomass of roots formed by a flooded plant usually correlates well with its resistance to waterlogging, as has been shown for closely related species of several genera (*Rumex* –Laan et al.,

Table 1. Fresh mass of primary and adventitious roots from hydroponic *Rumex palustris* plants of different age, before and after 1-week growth on stagnant, oxygen-deficient agar solution (for methods see Visser et al., 1996a). SE in brackets; $n = 6$

Age of the plant at start of treatment	Root fresh mass (g)			
	At the start of treatment		After 1 week on stagnant agar	
	Primary roots	Adventitious roots	Primary roots	Adventitious roots
2.5 weeks	0.01 (0.00)	0.00 (0.00)	0.13 (0.02)	0.08 (0.02)
3.5 weeks	0.19 (0.01)	0.00 (0.00)	0.39 (0.05)	0.77 (0.09)
4.5 weeks	2.14 (0.11)	0.04 (0.01)	1.54 (0.18)	2.26 (0.23)
5.5 weeks	5.63 (0.54)	0.07 (0.02)	4.35 (0.82)	2.82 (0.46)

1989; Visser et al., 1996a; *Pinus* – Topa and McLeod, 1986b; *Hordeum* – Garthwaite et al., 2003; various other genera – Justin and Armstrong, 1987). Flooding-induced adventitious roots are usually thick and have a relatively low degree of branching, whereas much of the cortical cell layers are occupied by schizogenous or lysigenous aerenchyma. Certain groups of plant species form adventitious roots in the natural course of root development (i.e. also without soil flooding), but even in these cases adventitious root formation is favoured above primary root development when the root system is flooded (McDonald et al., 2001a).

A major role for auxin

Adventitious roots are not only formed by water-logged plants, but are also essential in the regeneration process of shoot cuttings, a common propagation technique in horticulture. From these studies we know since long that auxins are essential regulators in the re-differentiation of shoot cells into root-forming meristems, and Phillips (1964) was the first to experimentally link auxin action to the onset of flooding-induced adventitious root formation. The results of this study indicated that auxin accumulated at the base of the shoot of soil-flooded sunflower plants as a result of the oxygen deficiency of the roots, like suggested before by Kramer (1951). Since IAA is transported in a polar fashion by carrier proteins that are ATPase-dependent (reviewed by Palme and Gälweiler, 1999), hypoxia-induced low levels of ATP may disturb active auxin movement in the root, thereby causing auxin that is transported downward from the shoot to accumulate at the boundary of oxygen-deficient and normoxic plant tissues. Similar to auxin application or removal of the root system, such flooding-induced accumulation could then lead to or enhance adventitious root formation at the base of the shoot and upper part of the tap root. More recent work on sunflower has shown that auxin is indeed crucial to adventitious root formation in both shoot cuttings of this species (e.g., Fabijan et al., 1981; Liu and Reid, 1992; Oliver et al., 1994) and in flooded plants (Wample and Reid, 1979). Auxin is also important for adventitious rooting in other species that were given a soil flooding treatment, such as *Rumex palustris* and *R. thyrsiflorus* (Visser et al., 1995), *Acer negundo* (Yamamoto and Kozlowski, 1987) and tobacco (*Nicotiana tabacum* – McDonald and Visser , 2003). Flooding-induced root formation could experimentally be inhibited by specifically blocking auxin transport in the shoot with N-1-naphtylphtalamic acid (NPA) (Visser et al., 1995; McDonald and Visser 2003) or by applying competitors for auxin-binding sites (Visser et al., 1995). This clearly indicates that auxin is an important component in this acclimation process, at least in dicotyledonous species. Whether this also applies to monocotyledonous species is not clear, since experimental evidence is scarce. Lorbiecke and Sauter (1999) did not find an effect of applied auxin on adventitious root formation in rice (except via ethylene, see below), whereas Zhou et al. (2003) claimed that endogenous auxin is critical for adventitious root development in the same species.

Involvement of ethylene

Not only auxin but also ethylene can induce adventitious roots, although in cuttings the response seems to depend on the plant species. For instance, ethylene inhibited adventitious root formation in pea (*Pisum sativum*) cuttings (Nordström and Eliasson, 1984) and *Prunus avium* explants (Biondi et al., 1990), whereas it stimulated root initiation in cuttings of *Picea abies* (Bollmark and Eliasson, 1990). Also, experimental conditions seem to matter, as contrasting results were found in various studies on mung bean (*Vigna radiata*

- e.g., Robbins et al., 1983 vs. Geneve and Heuser, 1983). More recently, a study of the rooting capacity of two ethylene-insensitive genotypes, i.e. the tomato mutant *never ripe* and the transgenic petunia (*Petunia x hybrida*) line 44568, showed that both intact ethylene-insensitive plants and their vegetative cuttings produced far less adventitious roots than did their respective wild-types (Clark et al., 1999). Since these plants have a defective ethylene receptor, they were not very responsive to the application of the precursor of ethylene, ACC. However, more surprising was the lack of response of ethylene-insensitive plants to indole-butyric acid (IBA), whereas in wild-type plants this auxin increased adventitious root formation if applied in moderately high concentrations. Apparently, ethylene is in these species indispensable for normal adventitious root development, even when rooting is evoked by auxin application.

During soil-flooded conditions, ethylene concentrations in the root system may increase up to several μL L^{-1}, as shown for *Rumex* plants (Visser et al., 1996b). These concentrations proved to be sufficient to induce adventitious root formation, even when plants were not flooded, whereas flooded plants with impaired ethylene production (by the use of inhibitors) produced fewer roots than normally under flooded conditions (Visser et al., 1996b). A similarly important role for ethylene has been found for rice (Bleecker et al., 1987), maize (Drew et al., 1979), and tobacco (McDonald and Visser, 2003). Studies with sunflower (Wample and Reid, 1979) and *Acer negundo* (Yamamoto and Kozlowski, 1987), however, attributed a less essential role to ethylene with respect to flooding-induced adventitious root formation.

One way ethylene may promote adventitious root development was elucidated by Mergemann and Sauter (2000). Similar to its role in aerenchyma formation, ethylene seemed involved in programmed cell death, in this case of the epidermal layer of rice nodal initiation sites of adventitious roots, thereby providing unhampered outgrowth of the root primordia.

A positive effect of ethylene on adventitious root development under soil-flooded conditions seems, however, contradictory to its usual effect on root growth, as many studies report a strong inhibition of root elongation by increased ethylene (e.g., Konings and Jackson, 1979; Etherington 1983; Visser et al. 1997). Possibly, internal ethylene concentrations can be kept sufficiently low in extending adventitious roots by the ventilating aerenchyma that is often present in such flooding-induced roots.

Interactions between auxin and ethylene

Auxin and ethylene action are not entirely independent of each other; increases in the action of one of these hormones may cause a change in the action of the other. The best known effect is that auxin generally increases the production of ethylene (e.g., Imaseki et al., 1977), but the opposite is also possible. Increased ethylene concentrations may affect the polar transport of auxin in a positive (Beyer and Morgan, 1969; Suttle, 1988) or negative way (Musgrave and Walters, 1973), thereby changing the delivery rate and concentrations at the shoot base. Perhaps more importantly, ethylene may increase the sensitivity of plant tissues to auxin, such as demonstrated in pea coleoptiles (Bertell et al., 1990) and sunflower hypocotyls (Liu and Reid, 1992). In the latter study, applied ethylene stimulated auxin-dependent root formation at the base of hypocotyl cuttings without increasing auxin concentrations. A very similar mechanism appeared to induce adventitious roots in flooded *Rumex palustris* (Figure 4; Visser et al., 1996c). Although auxin was clearly needed for an effective rooting response, shown by increased adventitious root formation upon auxin supply and decreased rooting in the presence of competitive or transport inhibitors (Visser et al., 1995; Visser et al., 1996c), there was no change in endogenous free IAA concentration when plants were waterlogged. Ethylene-induced root formation in the same species appeared auxin-dependent, whereas auxin-induced adventitious root formation was not affected if ethylene production was blocked, indicating that in these plants ethylene accumulation probably led to an increase in sensitivity of the root-forming tissue to auxin, which then triggered adventitious root development (Visser et al., 1996c). This contrasts with the increased levels of auxin found by Phillips (1964) and Wample and Reid (1979) in flooded sunflower plants, which may have been the result of oxygen deficiency or increased ethylene concentrations in the root system, both capable of impairing the auxin-transport system. In ethylene-insensitive transgenic tobacco, the number of adventitious roots induced by flooding was less than half that of wild-type plants, and this effect could not be fully restored by auxin application (McDonald and Visser, 2003). On the other hand, treatment of wild-type plants with NPA, an inhibitor of the auxin-efflux carrier that is part of the polar transport mechanism, decreased adventitious root formation to the level of ethylene-insensitive plants (McDonald and Visser, 2003). The results of this study suggest again interaction of auxin and ethylene in

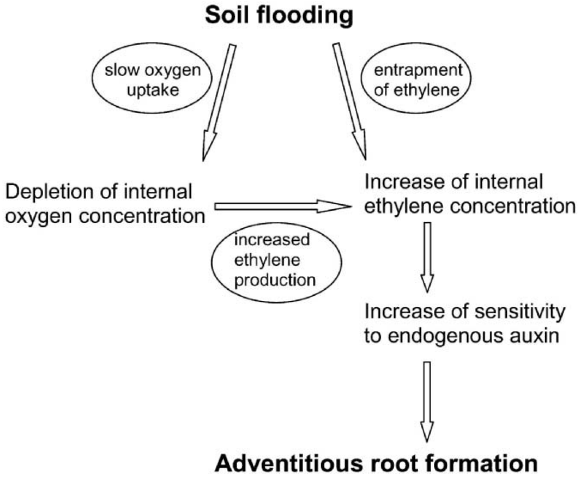

Figure 4. Schematic action scheme of the induction of adventitious root development in *Rumex palustris*. Drawn according the data shown in Visser et al. (1995, 1996b, c).

controlling flooding-induced adventitious root formation. Apparently, multiple signal-transduction pathways are possible, with varying roles for ethylene and auxin, depending on the species, and probably also on the exact environmental conditions, which makes it difficult to arrive at a unifying model on auxin and ethylene action in flooding-induced adventitious root formation.

Other hormones

There is very little evidence for a possible role for hormones other than auxin and ethylene in flooding-induced adventitious root formation. Application of cytokinin and gibberellin to deepwater rice did induce some roots, but not nearly as many as did ethylene (Lorbiecke and Sauter, 1999). It is conceivable that these treatments may have evoked some extra ethylene production. Earlier, Suge (1985) found in the same species a synergistic effect of gibberellin, when applied together with ethylene, but this could not be reproduced in a later study (Lorbiecke and Sauter 1999). It cannot be ruled out that other hormones do play a role in other species, particularly when these do not constitutively produce adventitious root primordia, such as rice does, but there is as yet no information available.

Genes involved in flooding-induced adventitious root formation

Surprisingly little is known about the genes that regulate the initiation and development of adventitious root primordia during soil flooding. In deepwater rice, Lorbiecke and Sauter (1999) distinguish four stages of adventitious root development: (i) an initiation phase, followed by (ii) development into a root primordium, and then subsequent (iii) arrest of growth until an appropriate stimulus causes (iv) further development and emergence of the root from the stem epidermis. The first three stages seem part of the constitutive development course of the rice stem nodes. The final phase is under control of flooding and ethylene, and emergence is apparent within 10 h after reception of a stimulus. This emergence of adventitious roots is preceded by the expression of a number of genes involved in regulating the cell cycle, such as the mitotic cyclin *cycB2;2*, which is a marker for dividing cells. Most of these genes are also expressed during flooding-induced internode growth, and are most likely involved in the transition of cells from one phase of the cell cycle to the following. One gene appeared to be expressed specifically in developing roots, and not in the stem intercalary meristem, i.e., *cdc2Os-1*, and may therefore be part of a root-specific signal-transduction pathway that is triggered by ethylene (Lorbiecke and Sauter, 1999).

Summary and perspectives

The responses of plants to soil flooding are diverse, but mostly focused on surviving the deleterious effects of oxygen deficiency imposed by the flood water. In the short term, anaerobic metabolism may partly overcome the low energy production under anaerobiosis. However, if plants are subjected to long-term flooding (i.e., weeks or months), a more structural solution is provided by restoring the oxygen supply to the submerged plant parts. Formation of aerenchyma and new roots containing this specialised tissue are two means of morphological acclimation that fulfil this task.

The timing of acclimation strongly depends on what signals are involved in sensing the environmental stress. Waterlogging and submergence in higher plants is sensed through changed concentrations of at least two signal molecules: ethylene and oxygen. Ethylene perception and transduction has been the subject of intense study during the last decade, and the molecule forms a very reliable detection system for submerged plant organs that contain some oxygen. It triggers important anatomical and morphological modifications (e.g., aerenchyma formation, adventitious roots, stimulated shoot elongation) that improve the oxygen status of submerged organs.

Virtually nothing is known about the sensing mechanism for oxygen in higher plants, although systems homologous to those described for various prokaryotes are likely. Cleverly designed screens, preferably for *Arabidopsis thaliana*, should be developed to identify mutants disturbed in oxygen sensing, assuming that these mutants are not lethal. Lethal mutations are not completely unexpected, since low-oxygen conditions seem to be an integral part of plant growth and development, even when this takes place under normoxic conditions. More information about second messengers in low-oxygen sensing has become available during the past few years. Important signals in this respect are Ca^{2+}, pH and H_2O_2.

Interestingly, it is now recognised that responses to low oxygen can take place even before cytochrome oxidase activity becomes limited (reviewed by Geigenberger, 2003). Respiration may be inhibited already at oxygen concentrations of 8–12%, when fermentation and the ‘Pasteur effect’ are not yet apparent. This down-regulation of respiratory activity, followed by decreased ATP levels, can therefore not be controlled by the status of the electron transport chain, as this is not affected by these relatively high oxygen concentrations.

Following the perception of the flooding signal, several signal-transduction mechanisms will be activated that control the acclimation mechanisms. A better understanding of the signal-transduction chains that lead to root acclimation during soil flooding would increase our possibilities to manipulate the extent of acclimation, and thereby obtain tools to study the benefits and costs of acclimations. Some comparable examples are already known from flooding physiology, for instance with respect to the capacity of shoot elongation in rice in response to total submergence (Setter and Laureles, 1996). Blocking the elongation response with paclobutrazol, an inhibitor of gibberellin biosynthesis, increased survival under water considerably, probably since allocation of carbohydrates to the elongating shoot occurs at the expense of the carbohydrate reserves needed for (partially anaerobic) respiration. In another study, inhibition of ethylene perception in transgenic tobacco led to a decrease in adventitious root formation upon simulated soil flooding, which in turn decreased the biomass gain of the plants under these conditions (McDonald and Visser, 2003).

It is unfortunate that the most widely used genetic and molecular-biological plant model, *Arabidopsis thaliana*, has such a low resistance to flooding stress. Oxygen-sensing mechanisms may be present in this species, possibly allowing for screens of sensing mutants; however, the plant is largely unsuitable for studying the responses of plants to soil flooding, except for some acclimations that are surprisingly well preserved even in this flood-intolerant species (e.g., submergence-induced hyponasty of the leaves (Cox, 2004) and hypoxia-induced fermentative enzymes (Dolferus et al., 1997)). Rice may turn out to be the best alternative model species, as most acclimation responses (i.e. fermentation, aerenchyma formation, adventitious root development) are displayed by this species. A great advantage is the known sequence of the rice genome (Yu et al., 2002), which increases the speed and possibilities of molecular genetics considerably. Further development of heterologous microarray analyses, such as recently accomplished for adventitious root formation in *Pinus* (Brinker et al., 2004), may also help to circumvent the lack of model species. Another approach to pinpoint the genes or chromosomal regions that control flood tolerance is the use of crosses between waterlogging-tolerant and less tolerant species, cultivars or ecotypes. In this way a group of hybrids may be developed that encompass a wide variety in root traits such as aerenchyma content, radial oxygen loss or adventitious root formation (McDonald et al., 2001b; Colmer 2003). If genetic markers are made available for these species, QTL analysis may reveal the area on the genome where genes crucial for the expression of these traits are located. In conclusion, many advances are to be expected from the genetic and genomics field of research, which will increase our in-depth knowledge on regulatory and developmental processes in plants, including acclimation to flooding.

Acknowledgements

The work was supported by a PIONIER grant (800.84.470) of the Dutch Science Foundation to L.A.C.J. Voesenek. We thank Danny Tholen for commenting on the draft of this manuscript, Willem Engelaar and Gerard Bögemann for the cross-sections of the roots shown in Figures 1A and B, and Ankie Ammerlaan and Tim Colmer for the longitudinal section of the *Rumex palustris* adventitious root shown in Figure 1C.

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