



Tansley review

How plants cope with complete submergence

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Summary

Key words: aerenchyma, ethylene, flooding, oxygen, shoot elongation, signal transduction, underwater photosynthesis.

Flooding is a widespread phenomenon that drastically reduces the growth and survival of terrestrial plants. The dramatic decrease of gas diffusion in water compared with in air is a major problem for terrestrial plants and limits the entry of CO₂ for photosynthesis and of O₂ for respiration. Responses to avoid the adverse effects of submergence are the central theme in this review. These include underwater photosynthesis, aerenchyma formation and enhanced shoot elongation. Aerenchyma facilitates gas diffusion inside plants so that shoot-derived O₂ can diffuse to O₂-deprived plant parts, such as the roots. The underwater gas-exchange capacity of leaves can be greatly enhanced by a thinner cuticle, reorientation of the chloroplasts towards the epidermis and increased specific leaf area (i.e. thinner leaves). At the same time, plants can outgrow the water through increased shoot elongation, which in some species is preceded by an adjustment of leaf angle to a more vertical position. The molecular regulatory networks involved in these responses, including the putative signals to sense submergence, are discussed and suggestions made on how to unravel the mechanistic basis of the induced expression of various adaptations that alleviate O₂ shortage underwater.

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I. Introduction

Flooding is one of the most common and widespread of all natural disasters. It is detrimental for nearly all terrestrial plants and results

in hampered growth and ultimately death of many plant species. Flooding can have catastrophic impacts on the productivity of arable farmland, as most crops are intolerant to excess water (http://www.plantstress.com/Articles/waterlogging_i/waterlog_i.htm).

The negative impact of flooding on terrestrial plant life is a consequence of the slow diffusion rates of gases in water compared with in air and the relatively low solubility of O₂ in water (Jackson, 1985; Armstrong & Drew, 2002). In nonphotosynthesising plant tissues, such as roots, O₂ concentrations decline strongly upon submergence (Gaynard & Armstrong, 1987; Armstrong *et al.*, 1994), whereas CO₂ produced during respiration and fermentation probably accumulates (Crawford, 1992). Submerged green tissues, on the other hand, are characterized by a significant diurnal variation in gas composition (Raskin & Kende, 1984a). During daytime, photosynthesis depletes endogenous CO₂ concentrations, whereas O₂ concentrations can be maintained close to 21%. During the night, however, endogenous O₂ concentrations are reduced as a result of respiration, but complete depletion within shoot tissues should be rare because the surrounding water generally contains O₂ (Mommer *et al.*, 2004). A third, endogenously produced, gas, which changes in concentration when plant tissues are submerged, is the plant hormone ethylene. Physical entrapment, combined with a very low rate of catabolism (Hall, 1991), leads to an increase of ethylene inside the tissues of submerged plants. A physiologically relevant increase in ethylene concentration can occur within minutes after submergence (Vreeburg *et al.*, 2005). Despite these dramatic changes in gas composition and the stress it imposes, some plant species, especially those growing in flood-prone areas, have a remarkable tolerance and can grow vigorously under flooded conditions. However, species from flood-exposed environments are not always flood tolerant. Some avoid the adverse effects of flooding by timing of life cycle events (Blom & Voesenek, 1996). Here, we review how plants regulate morphological adjustments that help to prevent severe O₂ shortages during submergence.

It is generally accepted that energy deficit, caused by a shortage of O₂, and thus inhibition of respiration, is one of the most severe problems encountered by plants when subjected to flooding. Oxygen is the terminal acceptor of electrons in the oxidative phosphorylation that indirectly provides the plant with ATP. Metabolic acclimations that aim to continue energy production in plants during anoxia were reviewed recently by Sauter (2000), Dolferus *et al.* (2001), Geigenberger (2003), Gibbs & Greenway (2003) and Greenway & Gibbs (2003). When O₂ concentrations decline (e.g. ≤ 5%), glycolysis and fermentation pathways are stimulated in *Zea mays* (Sachs *et al.*, 1996) – the classic ‘Pasteur’ effect. Consistent with this biochemical observation at low concentrations of O₂ (≤ 5%) are the results of several microarray studies with *Arabidopsis* that showed an up-regulation of genes coding for enzymes of sugar metabolism, glycolysis and fermentation upon low O₂ concentrations (Klok *et al.*, 2002; Liu *et al.*, 2005; Loreti *et al.*, 2005). The induction of these pathways allows continuation of at least some energy production when anoxia starts. Low O₂ also induces the expression of nonsymbiotic hemoglobin genes (Dordas *et al.*, 2004). It has been suggested for *Z. mays* that hemoglobin can detoxify

NO produced during hypoxia (Dordas *et al.*, 2003) and/or play a role in the regeneration of NAD⁺ necessary to maintain glycolysis (Sowa *et al.*, 1998; Igamberdiev *et al.*, 2005). Another group of genes that are up-regulated at low O₂ concentrations are associated with the detoxification of reactive oxygen species (peroxidase, ascorbate peroxidase, monohydroascorbate, glutathione reductase and superoxide dismutase) (Klok *et al.*, 2002). It is assumed that the products of these genes play roles in the protection against postanoxia injury (Monk *et al.*, 1989).

A second strategy in plants that inhabit areas prone to submergence is functionally associated with the long-term avoidance of low internal O₂ partial pressures. For example, a major adjustment induced by complete submergence is a change in leaf morphology and anatomy. Leaves of *Rumex palustris*, *R. crispus* and *Phalaris arundinacea* developed underwater have a higher specific leaf area (SLA; m² kg⁻¹) (Vervuren *et al.*, 1999; Mommer *et al.*, 2005a), and *R. palustris* leaves also have a thinner epidermal cell wall and cuticle, and the chloroplasts are oriented towards the epidermis instead of the intercellular spaces (Mommer *et al.*, 2005b). These acclimations result in higher underwater rates of photosynthesis and lower CO₂ compensation points, ultimately generating higher internal O₂ concentrations in submerged plants (Mommer *et al.*, 2004, 2005a,b). In addition, many species show stimulated elongation of shoot organs; a trait that can restore contact of the leaves with air. Aerenchyma formation is also common in wetland species. The aerenchyma creates longitudinally interconnected pathways of gas spaces that, together with the renewed contact of leaf tips with the atmosphere through enhanced shoot elongation, enable diffusion of O₂ from the air to the root tips. Ethylene is a key regulator of the formation of lysigenous aerenchyma, at least in *Z. mays*, through programmed cell death (Drew *et al.*, 2000), and stimulated shoot elongation mediated by cell-wall loosening in rice and *Rumex* (Voesenek & Blom, 1999; Sauter, 2000; Vreeburg *et al.*, 2005). In *Arabidopsis*, genes involved in the regulation of ethylene synthesis, ethylene signaling, programmed cell death and cell-wall loosening are up-regulated upon low O₂ conditions (Klok *et al.*, 2002; Liu *et al.*, 2005).

The present review summarizes the recent progress in the molecular and physiological regulation of mechanisms that contribute to long-term anoxia avoidance, namely stimulated shoot elongation, aerenchyma formation and underwater photosynthesis, which ultimately enhance the internal O₂ status of plants in flooded environments. In addition, the importance of understanding submergence sensing, and the opportunities for rapid progress in submergence research by the use of *Arabidopsis*, will be highlighted.

II. Sensing of submergence

In order to react appropriately to the flooded environment, plants have to sense the changes that take place. The changes

in endogenous gas composition make O₂, CO₂ and C₂H₄ potential molecules to signal submergence. However, the availability of light complicates the matter with respect to CO₂ and O₂ because their internal concentrations are strongly influenced by both photosynthesis and respiration. For this reason, C₂H₄ is considered to be a far more reliable indicator for complete submergence in green shoot tissues (Voeselek & Blom, 1999). In addition to these gases, reactive oxygen species (ROS), which may accumulate during flooding, have also been proposed to have a regulatory role during submergence (Bailey-Serres & Chang, 2005; Blokhina *et al.*, 2003). Furthermore, we cannot exclude a direct role for light quality and quantity as submergence signals as both can change upon submergence (Holmes & Klein, 1987).

Ethylene

In *R. palustris*, ethylene concentrations increase 20-fold within the first hour of submergence as a result of physical entrapment (Voeselek *et al.*, 1993; Banga *et al.*, 1996). The production rate of ethylene during submergence is maintained at the same level in *Rumex* (Voeselek *et al.*, 1993; Vriezen *et al.*, 1999), or increased, as in the internode tissue of deepwater rice (Kende *et al.*, 1998). Biosynthesis of ethylene is a well-characterized process (Wang *et al.*, 2002) that is dependent on the presence of O₂ (Kende, 1993). In *R. palustris*, it was shown that ethylene plays a pivotal role in initiating responses to submergence, although it is not fully clear whether it is the sensing signal. In flooding-tolerant *Rumex* species, the 1-aminocyclopropane-1-carboxylic acid concentration (ACC, a precursor of ethylene) strongly increased upon submergence, whereas the conversion to ethylene was inhibited. At the gene expression and protein levels (*in vitro* assay), increased expression of ACC synthase genes, and resulting enzyme activity, were observed (Vriezen *et al.*, 1999; Voeselek *et al.*, 2003; Rieu *et al.*, 2005). In *R. palustris*, ACC oxidases are strongly up-regulated during submergence and this correlates with an increase in enzyme activity, measured *in vitro* (Vriezen *et al.*, 1999; Voeselek *et al.*, 2003). The increased concentration of ACC oxidase enzyme during submergence possibly counterbalances the reduced enzyme activity at low O₂ concentrations, thus sustaining ethylene production during submergence (Vriezen *et al.*, 1999).

In plants, ethylene is detected by a set of receptors. These and the subsequent signal-transduction cascade have been elegantly clarified over the last decade in *A. thaliana*, using genetic techniques (Chang, 2003; Alonso & Stepanova, 2004; Guo & Ecker, 2004; Stepanova & Alonso, 2005). An *R. palustris* orthologue of the ethylene response sensor (ERS) ethylene receptor gene was isolated and shown to be strongly up-regulated upon submergence. This desensitizes the tissue towards ethylene (Vriezen *et al.*, 1997), which results from the fact that ethylene receptors actively suppress a downstream regulator of ethylene responses when the receptors have not bound ethylene. This desensitization

has probably no effect on the ethylene-induced responses, as ethylene concentrations were found to be saturated in the shoots of submerged plants (Voeselek *et al.*, 1993; Banga *et al.*, 1996).

Carbon dioxide

The role of CO₂ as a sensing molecule in submergence research has received limited attention. High concentrations of CO₂ do not affect petiole elongation in *R. palustris* (Voeselek *et al.*, 1997) but it was shown, in rice, that elevated CO₂ concentrations (6%) could induce elongation of internodes, albeit to a much lesser extent than ethylene (Raskin & Kende, 1984a). Recently, Mommer *et al.* (2005b) showed, in the leaves of submerged plants, reorientation of chloroplasts towards the leaf exterior where CO₂ diffuses in through the cuticle and cell wall. This, and other changes, led to a decreased diffusion resistance for CO₂ to reach Rubisco, resulting in higher underwater assimilation rates at low external CO₂ concentrations. These data suggest that plant cells, in addition to guard cells, are able to sense the CO₂ concentration and respond to it accordingly. Whether these alterations are caused by CO₂ sensing, possibly through a mechanism as in guard cells, or by other mechanisms (perhaps even independent of CO₂), is still to be elucidated.

Much work has been carried out on the sensing of CO₂ in relation to stomatal opening and closure. However, despite the numerous publications on this subject, the CO₂-sensing pathway(s) of guard cells remain poorly understood and may involve cytosolic pH and malate, zeaxanthin, Ca²⁺, or anion channels in the short-term response to CO₂ (Assmann, 1999; Schroeder *et al.*, 2001).

Elevated concentrations of CO₂ also affect metabolic rates of plant species, as reviewed by (González-Meler *et al.*, 1996). Mangrove seedlings demonstrated a marked reduction in the rate of respiration when exposed to low O₂ concentrations in the presence of external CO₂ (Brown *et al.*, 1969). By contrast, exposure of dryland species, such as *Cicer arietinum*, *Tussilago farfara* and *Elymus repens*, to elevated concentrations of CO₂ up-regulated the glycolytic activity and reduced the viability (Crawford, 1992).

Oxygen

The O₂ concentration in plant tissues decreases upon submergence (Rijnders *et al.*, 2000). Furthermore, it has been shown that low O₂ could sensitize petiole tissues to ethylene, thus suggesting an interaction between ethylene and O₂ (Voeselek *et al.*, 1997). Another indication of the interaction between ethylene and O₂ was found by Peng *et al.* (2001), who showed that ethylene signaling is required for hypoxia-induced gene expression. Furthermore, Kamaluddin & Zwiazek (2002) showed that enhanced ethylene concentrations can overcome the hampered hydraulic conductivity in roots caused by low O₂ concentrations (Tournaire-Roux *et al.*, 2003).

Decreases of O₂, ranging from low concentrations (hypoxia) to complete absence (anoxia), in plants have been associated with changes in the expression of genes related to carbohydrate and lipid metabolism, glycolysis, fermentation pathways, ethylene synthesis, auxin-mediated processes, and calcium- and ROS-mediated signal transduction pathways (Klok *et al.*, 2002; Paul *et al.*, 2003; Branco-Price *et al.*, 2005; Liu *et al.*, 2005; Loreti *et al.*, 2005). Moreover, morphological responses, such as aerenchyma and adventitious root formation, and cell and organ elongation of shoots, are induced in some species. To date, no direct O₂ sensor in plants has been identified (Drew, 1997; Geigenberger, 2003). This is in contrast to animal cells where the hypoxia-inducible heterodimeric transcription factor (HIF, reviewed by Bruick, 2003) subunit, HIF1 α , is regulated by an O₂-dependent HIF1 α prolyl hydroxylase. It was shown that the activity of this enzyme in *Drosophila melanogaster* and *Caenorhabditis elegans* is proportional to the O₂ availability and, as a consequence, this system might be considered as a direct O₂-sensing system (Bruick, 2003). Another (albeit indirect) mechanism by which O₂ deprivation might be sensed is the rapid acidification of the cytosol upon O₂ deprivation (Roberts *et al.*, 1984). This is a quick response to the energy crisis experienced by cells under anoxia. The lower pH is relatively stable in anoxia-tolerant tissues, but can decline further in less tolerant tissues (Felle, 2005). There has been much debate about the precise role of cytosolic acidification, but it can affect cellular metabolism (Geigenberger, 2003) and water conductivity across membranes (Tournaire-Roux *et al.*, 2003), and could thus play a role in indirect O₂ sensing (Bailey-Serres & Chang, 2005; reviewed in Felle, 2005).

Hydrogen peroxide

It is generally accepted that biotic and abiotic stresses, among these hypoxia and anoxia, lead to a change in H₂O₂ production (Apel & Hirt, 2004; Mittler *et al.*, 2004). In particular, H₂O₂ produced by cytosolic membrane-bound NADPH oxidases has been associated with stress responses (Laloi *et al.*, 2004). As H₂O₂ is potentially damaging, there are cellular antioxidant mechanisms that remove it very efficiently (reviewed in Noctor & Foyer, 1998; Corpas *et al.*, 2001).

Recent reviews by Neill *et al.* (2002), Laloi *et al.* (2004), Apel & Hirt (2004) and Mittler *et al.* (2004) elaborate on the progress made on various aspects in ROS research. More specifically, Bailey-Serres & Chang (2005) discuss the current state-of-the-art about the role of ROS in sensing O₂ deprivation. There is evidence that the change in ROS in the particular cases of hypoxia and anoxia in plants are mediated by a group of compounds called the RHO-like GTPases (ROP). Activation of ROP after treatment with a low concentration of O₂ induces H₂O₂ production and this seems necessary for low O₂ signaling (Baxter-Burrell *et al.*, 2002, 2003; Bailey-Serres & Chang, 2005). Components of the signal-transduction

pathway of ROS itself have been revealed by recent studies (reviewed in Apel & Hirt, 2004; Mittler *et al.*, 2004). ROS might be sensed by two-component sensors possessing a histidine kinase, subsequently inducing a mitogen-activated protein kinase (MAPK) signaling pathway, and it has been speculated that ROS may inhibit protein phosphatases, and with some evidence also that ROS can activate transcription factors directly. All of these actions lead to changed gene expression.

Interestingly, it has been shown that ETR1, one of the receptors in the ethylene signaling pathway, mediates stomatal closure in response to H₂O₂ (Desikan *et al.*, 2005). A cysteine at position 65 was essential for this possible interaction between ethylene and H₂O₂ (Desikan *et al.*, 2005). Moreover, it was shown by D'Haeze *et al.* (2003) that ROS production may precede ethylene production during Nodulation factor-stimulated root hair growth, suggesting another interaction between ROS and ethylene.

Light

Light quantity attenuates strongly in water as a result of its absorptive properties and of the absorptive, reflective and scattering properties of suspended material within the water (Holmes & Klein, 1987). Vervuren *et al.* (2003) analysed the light transmission of river Rhine floods and showed that inundations with a depth of 50 cm already have a transmission reduction of 90%, whereas deep floods (> 1.5 m) have light transmissions of < 1%.

Reduced intensities of light and submergence with normal light conditions are both able to induce a similar set of traits in *Rumex* species, such as an increase in petiole length and SLA, and a decline in the underwater compensation point for light (Mommer *et al.*, 2005a). Moreover, both low light and submergence can induce hyponastic growth in *A. thaliana* Col (Millenaar *et al.*, 2005; Pierik *et al.*, 2005). The resemblance in the morphological and biochemical responses to low light and submergence might simply be explained by separate signal-transduction cascades that both affect a common downstream component. Alternatively, it is conceivable that these two pathways operate synergistically or additively when both signals are present at the same time (flooding with a strong attenuation of light). Recently, an additive effect was observed for petiole elongation in *R. palustris*: submerged plants in low light had significantly longer petioles than plants submerged in high light (Mommer *et al.*, 2005a).

In addition to decreases in light quantity, the spectral composition of light changes when it passes through water. Water specifically absorbs the longer wavelength, like far-red (FR), which results in an increase of the red (R) : FR ratio (reviewed in Holmes & Klein, 1987). In turbid water, caused by, for example, silt or clay, the light is scattered and also the shorter wavelengths are absorbed (ultraviolet and blue light). Therefore, in turbid water, the green wavelengths penetrate the furthest (reviewed in Holmes & Klein, 1987).

Elongation of plant organs, such as petioles, is stimulated by low intensities of blue light and a low R : FR ratio (Pierik *et al.*, 2004; Pierik *et al.*, 2005). Therefore, because the R : FR ratio increases in submerged environments, we conclude that submergence-induced elongation is not induced by this change in the R : FR ratio. However, a stimulating contribution of low blue light intensities can occur as this light signal is attenuated in water under turbid field conditions.

Summary of possible submergence signals

Based on the information presented above there is no clear-cut conclusion to be drawn about how submergence is sensed. There is ample evidence that in *R. palustris*, ethylene is responsible for very rapid responses, although the exact role of ethylene needs to be further elucidated. There are indications for possible interactions between ethylene and low O₂, and ethylene signaling and H₂O₂, but these have to be studied further. At this point we do not know whether submergence-induced responses are influenced by direct or indirect sensing of changes in O₂ concentration, or changes in ROS and/or other second messengers, such as NO and Ca²⁺. The effect of light is probably restricted to conditions in which the floodwater has a high turbidity.

III. Internal aeration and underwater photosynthesis

Internal aeration provides O₂ to submerged organs of plants in flooded environments; enabling aerobic respiration even when the adjacent external environment limits O₂ entrance (Jackson & Armstrong, 1999). In addition, a portion of the O₂ transported to roots is lost radially [radial O₂ loss (ROL)], particularly from laterals and tips of the main axes in the case of many wetland species (Armstrong, 1979; Colmer, 2003). ROL re-oxygenates the rhizosphere (Pedersen *et al.*, 1995; Revsbech *et al.*, 1999), thereby protecting roots from the reduced substances (e.g. Fe²⁺, Mn²⁺, S²⁻, organic acids) commonly present in flooded soils (Ponnamperuma, 1984).

In emergent wetland plants, atmospheric O₂ enters the shoot and moves, via aerenchyma, to the tips of the roots. Gas transport in shoots is mostly diffusive, but can (in some species) occur through pressure-driven flows that force gases along the lacunae in stems or petioles and rhizomes, at least when environmental conditions are suitable (Dacey, 1980; Armstrong & Armstrong, 1991, reviewed in Colmer, 2003). In contrast to throughflow convection, demonstrated elegantly in water lilies (Dacey, 1980) and reed (Brix, 1988; Armstrong & Armstrong, 1991), no significant aeration contribution can be expected from a second type of convective gas flow, the so-called nonthroughflow convection (Beckett *et al.*, 1988).

When plants are completely submerged, however, direct access to atmospheric O₂ is abolished. The sources of O₂ to completely submerged plants are those produced during

photosynthesis and present in the floodwaters (Sand-Jensen *et al.*, 2005). Thus, submerged plants can experience marked diurnal changes in tissue O₂ concentrations, the O₂ concentration being highest during the day and lowest during the night (e.g. for rice; Waters *et al.*, 1989). The consequences of distance within the plant body along the diffusion path from an O₂ source, and the combined resistances (physical and O₂ consumption) to diffusion along the pathway, have been reviewed in detail (Armstrong & Drew, 2002; Colmer & Greenway, 2005). The remainder of this section will focus on environmental factors and plant traits, determining: (i) aerenchyma formation; (ii) rates of photosynthesis by submerged plants; and (iii) O₂ exchange between the shoot and floodwater.

Aerenchyma formation

Aerenchyma is tissue containing large gas-filled spaces (lacunae) that interconnect longitudinally to provide a low-resistance pathway for long-distance gas transport along plant organs. The presence of lacunae provides increased porosity (gas volume per unit tissue volume) of tissues to levels much higher than the inherent values resulting from the usual intercellular gas-filled spaces formed as a constitutive part of development. In roots, aerenchyma usually forms in the cortex, whereas in stems (including rhizomes) aerenchyma can occur in the cortex and in the pith cavity (Armstrong, 1979). The volume of aerenchyma formed depends on genotype (species, as well as cultivar/accession) and environmental conditions (Colmer, 2003).

Two basic forms of aerenchyma have been recognized (Sachs, 1882), namely that which develops by the separation of cells ('schizogenous' aerenchyma) or that which develops by the collapse of cells ('lysigenous' aerenchyma) (Fig. 1), although within both of these main 'types', several different processes can form the resulting aerenchyma (Justin & Armstrong, 1987; Longstreth & Borkhsenius, 2000; Seago *et al.*, 2005).

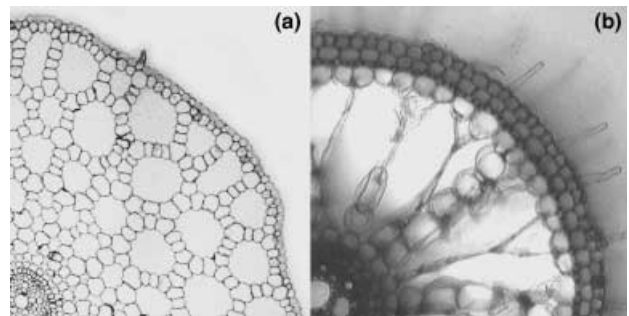


Fig. 1 Two main types of aerenchyma: schizogenous aerenchyma in a *Rumex palustris* adventitious root (a), and lysigenous aerenchyma in a rice adventitious root (b). The 'honeycomb-type' schizogenous aerenchyma in *R. palustris* forms as a result of cells being forced apart because of oblique divisions by some of the cortical cells in radial rows. The lysigenous aerenchyma in rice forms owing to the collapse of radial files of cortical cells.

In many wetland plants, aerenchyma forms constitutively, but is further enhanced in response to flooding. In most nonwetland species, aerenchyma is largely absent until it is induced by environmental conditions, such as flooding (Armstrong, 1979; Colmer, 2003), and also, in some species, by soil compaction or nutrient deficiencies (Drew *et al.*, 1989; He *et al.*, 1996). Information on the regulation of formation of schizogenous types of aerenchyma is scant, whereas the induction of lysigenous aerenchyma has been studied in some detail, at least in roots of *Z. mays*.

The formation of lysigenous aerenchyma involves the removal of files of cortical cells, leaving enlarged gas-filled spaces, although the remnants of cell walls persist (Fig. 1b). Ethylene is the primary signal that initiates programmed cell death in the cortical cells that are removed (Drew *et al.*, 2000). Numerous files of cells die, but some files remain intact and these maintain symplastic connections from the stele to the rhizodermis. The mechanisms by which some radial files respond to the ethylene signal (and die), whilst tangential neighbours do not, is unknown (Evans, 2004). Nevertheless, some aspects of the signaling pathway, details of the ultrastructural events during the programmed cell death, and several biochemical aspects (particularly related to cell-wall degradation), have been described. Drew *et al.* (2000) proposed a hypothetical working model for ethylene signaling, ultimately resulting in the phosphorylation of (unidentified) downstream proteins, which then presumably regulate the required changes in gene expression to control the programmed cell death in these target cells. Stages of the ultrastructural changes in cells destined to die to form lysigenous aerenchyma are described in detail for the roots of *Z. mays* (Gunawardena *et al.* 2001), as are changes in expression of enzymes (cellulases and XET) proposed to have roles in cell-wall degradation during aerenchyma formation (He *et al.*, 1994; Saab & Sachs, 1996). As also indicated in other reviews (Jackson & Armstrong, 1999; Evans, 2004), further studies are needed to elucidate the molecular basis of aerenchyma formation in plants in greater detail.

Underwater photosynthesis

Photosynthesis in submerged plants can commonly be restricted by low availability of light and/or CO₂ (Sand-Jensen, 1989; Maberly & Spence, 1989). Light reaching the leaves of submerged plants is attenuated by water, dissolved organic matter, particles (e.g. silt) and/or phytoplankton suspended in the water column (Bach *et al.*, 1998; Vervuren *et al.*, 2003). It was recently demonstrated for the river Rhine system in the Netherlands that floods with a water depth of 1 m can have a light transmission below 1% (Vervuren *et al.*, 2003). On the other hand, light intensities are not necessarily always low underwater, as demonstrated in a study where a photosynthetically active radiation (PAR) value of > 600 μmol m⁻² s⁻¹ was measured at a flooding depth of

30 cm in an abandoned clay pit in the Netherlands, the typical environment of *R. palustris* (Mommer *et al.*, 2006).

CO₂ concentrations in most water bodies are much lower than the concentrations required to saturate photosynthesis by leaves of vascular plants (under saturating light and well-mixed conditions), and even that by aquatic species (Sand-Jensen, 1989). Diurnal fluctuations in dissolved CO₂ commonly occur in floodwaters, with a build-up of CO₂ during darkness and depletion to concentrations well below those in air-saturated water when light is available for photosynthesis (e.g. to 0.003 mol m⁻³ in flooded rice fields, Setter *et al.*, 1987). Rates of CO₂ uptake by leaves can limit photosynthesis, owing to the already mentioned 10⁴-times slower diffusion of gases in water than in air, resulting in the movement of CO₂ across boundary layers adjacent to leaves becoming rate-limiting, even in submergence-acclimated leaves of aquatic species (Smith & Walker, 1980; Maberly & Spence, 1983; Raven *et al.*, 1985). Furthermore, for leaves of terrestrial species that become submerged, the cuticle is a major component of the resistance to CO₂ entry, further impeding underwater photosynthesis (Mommer *et al.*, 2005b). In addition, low concentrations of CO₂, relative to O₂, increase photorespiration in submerged leaves (Mommer & Visser, 2005; Mommer *et al.*, 2005b).

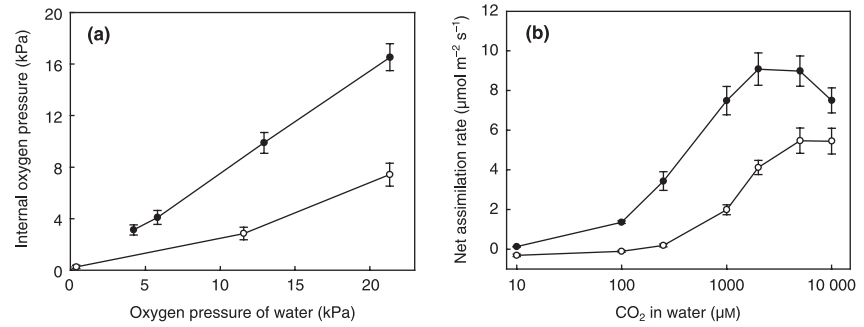
Physiological and morphological adaptations in leaves of aquatic and amphibious macrophytes have been reviewed in detail (Maberly & Spence, 1983, 1989; Raven *et al.*, 1985; Bowes & Salvucci, 1989; Madsen & Sand-Jensen, 1991). CO₂-concentrating mechanisms, the capacity to utilize HCO₃⁻ and a leaf morphology to reduce boundary layer thickness (e.g. dissected leaves), were identified as important adaptations to access CO₂ in aquatic environments. Thin cuticular layers that are relatively permeable to CO₂ are also important (Frost-Christensen *et al.*, 2003; Mommer *et al.*, 2005b). Other strategies to access CO₂ were noted, such as, in many species, the development of floating leaves or aerial leaves to access atmospheric CO₂ (and O₂), or the more specialized strategy by certain plants that exploit CO₂ from the sediment (Raven *et al.*, 1988).

Leaf morphology and anatomy

Diffusion across the cuticle might become the major pathway for CO₂ entry into submerged leaves (Frost-Christensen *et al.*, 2003), even for leaves with stomata (i.e. aerial leaves of amphibious species and leaves of emergent wetland plants); although information on whether the stomata remain open, or close, during submergence appears to be scarce. Most stomata of *R. palustris* are closed when this plant is completely submerged (Mommer *et al.*, 2005b). Resistance to gas movement across the cuticle can be less in water-grown leaves than in air-grown leaves of several species (Frost-Christensen *et al.*, 2003; Mommer *et al.*, 2005b).

In response to long-term (e.g. more than several days) submergence, some plants can produce new acclimated leaves

Fig. 2 Acclimation responses to submergence in *Rumex palustris* leaves formed underwater: the new leaves show reduced gas diffusion resistance (a; measured in darkness, data from Mommer *et al.*, 2004. © Blackwell Publishing, reprinted with permission) resulting in enhanced rates of underwater photosynthesis (b; data from Mommer *et al.*, 2005b. © American Society of Plant Biologists, reprinted with permission). black circles, acclimated leaf; white circles, nonacclimated leaf.



underwater that have an enhanced gas exchange with the water column (Mommer & Visser, 2005). For example, young leaves of *Rumex* species elongate upon submergence, and although most of the elongation growth occurs in the petiole, the lamina also elongates and becomes narrower (e.g. for *R. palustris* see Fig. 2 in Mommer & Visser, 2005). This elongated leaf morphology would be expected to result in smaller boundary layers than present for the broader lamina of nonelongated leaves. Next to becoming narrower, the leaves of submergence-tolerant *R. palustris* and *R. crispus* also become thinner (i.e. increased SLA), which can enhance gas exchange between the leaf and the surrounding water (Vervuren *et al.*, 1999; Mommer *et al.*, 2005a). Furthermore, submergence-acclimated leaves of *R. palustris* had thinner epidermal cell walls, a thinner cuticle and the chloroplasts were orientated towards the epidermis, all of which would decrease diffusion resistance between the leaf surface and chloroplasts (Mommer *et al.*, 2005b). Thus, changes in morphology and anatomy in submergence-acclimated leaves enhance CO_2 entry.

The enhanced capacity for underwater gas exchange by new leaves of *R. palustris* produced following submergence was evidenced by higher internal O_2 concentrations in petioles of these leaves during periods in light, when compared with O_2 concentrations in plants grown in air prior to submergence (Mommer *et al.*, 2004). Higher internal O_2 presumably resulted from improved rates of CO_2 entry from the water column and thus enhanced rates of photosynthesis, and this was evident across a range of dissolved CO_2 concentrations (up to $\approx 1 \text{ mol m}^{-3}$) in a turbulent water column (Fig. 2b; Mommer *et al.*, 2004; 2005a). Improved uptake of dissolved gases by the acclimated leaves was also demonstrated by manipulations of water column O_2 concentrations in the dark, whilst monitoring O_2 concentrations in the petiole. In darkness, acclimated leaves displayed higher internal O_2 concentrations at any given external O_2 concentration, indicating a greater entry of O_2 (Fig. 2a; Mommer *et al.*, 2004).

In summary, leaves of submergence-tolerant plant species (e.g. *R. palustris*) can acclimate to an underwater environment, resulting in enhanced CO_2 uptake for photosynthesis and therefore providing a supply of sugars to the plant. In addition, O_2 status is improved not only during the daytime

(O_2 derived from photosynthesis), but also during the night (enhanced entry of O_2 from the water column). An improved O_2 status in the shoots would benefit the roots, as internal O_2 diffusion via the aerenchyma to the roots would increase, not only enabling respiration within the roots, but also oxygenation of the rhizosphere. Despite these recent insights, much remains to be elucidated regarding the physiological and molecular regulation of the acclimations. As suggested by Mommer & Visser (2005), the two hormones ethylene and abscisic acid (ABA) would, however, be good starting points as these are known to be involved in the switch between aquatic and terrestrial leaf type formation (heterophylly) in aquatic plants (Lin & Yang, 1999; Kuwabara *et al.*, 2003).

IV. Stimulated shoot elongation

Several plant species from wetland environments demonstrate dramatic changes in shoot anatomy, morphology and growth pattern when submerged partially or completely (Ridge, 1987). Spectacular submergence responses are the altered orientation of petioles from prostrate to vertical (hyponastic growth) and the strongly stimulated petiole and internode elongation (Fig. 3). Not all petioles and internodes within one plant are equally responsive, as demonstrated in a study on *R. palustris* (Groeneveld & Voeselek, 2003). In this study, both the very young and the oldest petioles possess only a very minimal competence to elongate, whereas intermediately aged petioles are characterized by an elongation response of up to 300%, measured over 10 d of submergence. Differentiation in responsiveness was also described for internode elongation in deepwater rice where only the youngest internodes respond to submergence (Sauter, 2000).

Shoot elongation underwater requires energy and carbohydrates for cell divisions and the synthesis of new cell-wall material. Evidence for these costs of elongation growth came from work on rice in which elongation underwater without reaching the water surface occurs at the expense of survival (Setter & Laureles, 1996). Lowland rice cultivars demonstrated a negative correlation between survival and elongation growth. Moreover, enhancing shoot-elongation capacity by the addition of the growth-promoting hormone, gibberellin



Fig. 3 Upon submergence, *Rumex palustris* quickly re-orientates its leaves into a more vertical position (hyponastic growth) followed by strong petiole elongation towards the water surface. Note that petiole elongation without the preceding hyponastic growth would not bring the leaf tips much closer to the water surface.

(GA), decreased survival, whereas an inhibitor (paclobutrazol) of GA biosynthesis, and therefore shoot elongation, enhanced survival. It is suggested that the nonelongating strategy is more suitable for short-lasting and/or deep-flooding events that cannot be outgrown, whereas fast underwater elongation increases fitness mainly under prolonged, but relatively shallow, floods (Setter & Laureles, 1996; Sauter, 2000). This hypothesis was recently confirmed in a study that combined field surveys in floodplains of the river Rhine (the Netherlands) with experiments on the shoot-elongation capacity of 22 selected plant species. Fast-elongating species were exclusively found in flood-prone environments characterized by slow drainage and shallow temporary pools. Under these environmental conditions, the costs of elongation are probably outweighed by benefits such as improved aeration and restored rates of photosynthesis when the leaves emerge. Nonelongating species were only found on rarely flooded sites and in environments with frequent, but short-lasting, floods (Voeselek *et al.*, 2004).

Hyponastic growth interacts with shoot elongation

One of the first phenotypic modifications that takes place upon submergence in some plant species is an increase in the angle, compared with the horizontal, of mainly the younger petioles, thus decreasing the distance between the tips of the leaf blade and the water surface (Banga *et al.*, 1997). This flooding-induced response has been described, to date, for *Ranunculus repens*, *Caltha palustris*, *Leontodon taraxacoides*, *Paspalum dilatatum*, *R. palustris* and *A. thaliana* (Ridge, 1987;

Voeselek & Blom, 1989; Grimoldi *et al.*, 1999; Insausti *et al.*, 2001; Millenaar *et al.*, 2005). Interestingly, hyponastic growth responses are also described in response to other environmental cues, such as shading and high temperature (Ballaré, 1999; Pierik *et al.*, 2004; Millenaar *et al.*, 2005). In *R. palustris*, hyponastic growth is caused by differential cell elongation across the petiole base, with epidermal cells on the abaxial surface elongating faster than cells on the adaxial surface (Cox *et al.*, 2004). Furthermore, it was discovered that, in this species, hyponastic growth interacts with nondifferential petiole elongation. A prerequisite for flooding-induced petiole elongation is that a minimal petiole angle of 40–50° (from horizontal) has to be reached. When the initial petiole angle is lower than this threshold, no petiole elongation will take place until hyponastic growth has increased the petiole angle to 40–50° (Cox *et al.*, 2003).

Regulation of submergence-induced shoot elongation: hormone interactions and cell-wall events

Accumulated ethylene is probably the primary signal that triggers the plant to start a cascade of reactions ultimately leading to enhanced cell elongation in submerged shoot organs. A direct effect of this increased ethylene concentration is a fast decrease in the ratio between two other plant hormones: ABA and GA. This is related to a strong and fast reduction of the endogenous ABA concentration (Hoffmann-Benning & Kende, 1992; Benschop *et al.*, 2005) and an increase of the endogenous GA concentration (Rijnders *et al.*, 1997; Raskin & Kende, 1984b). The decrease of ABA

in petioles of *R. palustris* during submergence and treatment with ethylene is regulated via a fast down-regulation of several members of the 9-*cis*-epoxycarotenoid dioxygenase (NCED) gene family, a group of genes that limit ABA biosynthesis in higher plants (Benschop *et al.*, 2005). Furthermore, an increase (of 10–25%) of a product of ABA catabolism, phaseic acid, was observed in the time frame in which ABA decreased by 70% (Benschop *et al.*, 2005), suggesting increased ABA breakdown. Interestingly, application of the ABA biosynthesis inhibitor, fluridone, reduced the lag phase of submergence-induced petiole elongation in *R. palustris* by 50%, resulting in a very rapid elongation response. However, when this fluridone treatment was combined with a 1-MCP pretreatment (inhibitor of ethylene action), elongation growth underwater was completely abolished. This result indicates that a reduction of ABA by itself is insufficient to induce fast petiole elongation; the presence of elevated concentrations of ethylene is required (Benschop *et al.*, 2005).

It is clear that in both *Rumex* and rice, ABA acts as a negative regulator of underwater elongation. This raises the question as to how this is regulated at a physiological level. Recent data indicate that ABA directly interferes with GA biosynthesis. When submerged *R. palustris* plants were additionally treated with 10 μ M ABA, no increase was observed in the endogenous GA1 concentration; this result was in contrast to submerged controls. Therefore, ABA probably inhibits the up-regulation of the growth-promoting hormone GA (Benschop *et al.*, 2005). In this respect, it is interesting to note that GA-induced growth stimulation in roots and elongation of shoots in *Arabidopsis* seedlings (Fu & Harberd, 2003; Vriezen *et al.*, 2004) is associated with the destruction of DELLA proteins in the 26S proteasome. These DELLA proteins, localized in the nucleus of plant cells, function as repressors of growth (Alvey & Harberd, 2004). In rice, GA binds to a soluble GA receptor (GID1) present in the nucleus. Upon GA binding, this receptor molecule interacts with DELLA proteins, resulting in the degradation of these growth-repressing DELLA proteins (Ueguchi-Tanaka *et al.*, 2005). It is highly possible that DELLA degradation is a prerequisite for ethylene-induced shoot elongation in submerged plants.

Ultimately, the hormonal changes described above result in cell elongation within the elongating organ (e.g. petioles, internodes). This requires loosening of cell walls, uptake of water and synthesis of cell-wall polysaccharides (Cosgrove, 1999). Elongation of internodal cells in deepwater rice is primarily related to increased cell-wall extensibility, and not to decreases in osmotic potential or increases in hydraulic conductance (Kutschera & Kende, 1988). Cell-wall extensibility is thought to be associated with cell-wall loosening proteins, such as those belonging to the family of expansins (McQueen-Mason *et al.*, 1992). Expansin activity has a pH optimum between 3.5 and 4.5 (McQueen-Mason *et al.*, 1992) and is hypothesized to break H-bonds between hemicelluloses and cellulose microfibrils (McQueen-Mason & Cosgrove, 1994).

The expression of expansin genes and the protein activity correlate strongly with submergence-induced elongation in several plant species (Cho & Kende, 1997; Huang *et al.*, 2000; Kim *et al.*, 2000; Vreeburg *et al.*, 2005). Increased expression of expansins in deepwater rice was not only induced by submergence treatments, but also by GA application, indicating that expansin genes are a downstream target of GA action (Cho & Kende, 1997). However, in *R. palustris*, GA application cannot mimic the induction of *RpEXPA1* by submergence. This gene was the only expansin, out of a group of 13 *R. palustris* expansin genes, that was up-regulated in petioles upon submergence (Vreeburg *et al.*, 2005). The increased expression of *RpEXPA1* could be mimicked by ethylene treatment and repressed during submergence when plants were pretreated with the specific ethylene action inhibitor, 1-MCP (Vreeburg *et al.*, 2005). These results suggest that in *Rumex*, ethylene is at the base of at least two parallel operating signal transduction pathways: one leading to a decrease of the ABA:GA ratio and the other resulting in increased expression of the expansin gene and subsequent concentration of expansin protein (Vreeburg *et al.*, 2005). Ethylene also seems to be the triggering agent in expansin gene expression in the semiaquatic fern *Regnellidium diphyllum*, a species characterized by fast underwater elongation of the rachis (Kim *et al.*, 2000).

Moreover, ethylene plays a role in a process leading to cell-wall acidification in submerged shoots. The importance of cell-wall pH for petioles of *R. palustris* was recently illustrated in experiments in which buffers with a low (4.0) and more neutral (6.0) pH were injected into petioles (Vreeburg *et al.*, 2005). The low pH strongly stimulated elongation growth, whereas a temporary inhibition of underwater growth was observed when the neutral buffer was injected. An increased net efflux of H⁺ was measured within minutes after starting a submergence treatment; a response that could be abolished completely when the petiole tissue was pretreated with 1-MCP (Vreeburg *et al.*, 2005).

V. Perspectives

The suite of submergence-induced avoidance responses described in this review all are functionally associated with preventing the adverse consequences of the very slow gas diffusion in water. The regulatory mechanisms leading to these responses upon detection of submergence are, however, with the exception of shoot elongation, not well understood. There is, for example, much to be learned about the regulatory mechanisms behind aerenchyma formation and the reduced gas diffusion resistance in leaves formed underwater. Ethylene is not only the key factor in enhanced shoot elongation, but is also a major player in the formation of aquatic leaves in aquatic plants and in lysigenous aerenchyma formation, at least in *Z. mays*. Many physiological and molecular processes interacting with ethylene during submergence have become known from work on stimulated shoot elongation. This

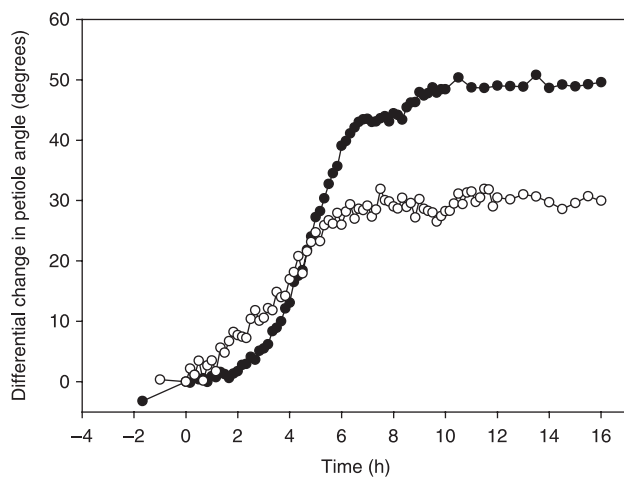


Fig. 4 Kinetics of ethylene-induced hyponastic growth in petioles of *Rumex palustris* and *Arabidopsis thaliana* are highly comparable. Data represent leaf angles in ethylene minus leaf angles of control plants, with values set to zero at $t = 0$. black circles, *Rumex*; white circles, *Arabidopsis*.

information could serve as a starting point for unravelling signaling cascades involved in the submergence-induced regulation of aerenchyma formation and reduced gas-diffusion resistance in leaves.

Although there is extensive information on signal transduction in submergence-induced hyponasty and shoot elongation, considerable progress is to be expected from the recent introduction of the model plant *Arabidopsis* in submergence research (Pierik *et al.*, 2005). The genetic variation in *Arabidopsis* for hyponasty (Millenaar *et al.*, 2005), ethylene sensitivity (Peeters *et al.*, 2002), and tolerance to full submergence (D. Tholen & L.A.C.J. Voeselek, unpublished) and soil flooding (Kolodnynska & Pigliucci, 2003), is currently being exploited in quantitative trait loci (QTL) analyses. These analyses should enable the identification of genomic loci, and ultimately the underlying genes (Maloof *et al.*, 2001; El Din El Assal *et al.*, 2001) involved in hyponastic growth (Pierik *et al.*, 2005; B. Snoek & A. J. M. Peeters, unpublished). The kinetics of hyponastic growth in *Arabidopsis*, upon submergence and ethylene exposure, are remarkably similar to those of *R. palustris* (compare Cox *et al.*, 2003 with Millenaar *et al.*, 2005; Fig. 4), making it feasible to translate insights obtained from *Arabidopsis* to flood-tolerant species, such as *R. palustris*. Recent microarray studies on *Arabidopsis* have identified a number of candidate genes involved in hyponastic growth (Pierik *et al.*, 2005; F. F. Millenaar & A. J. M. Peeters, unpublished), and an ongoing mutant screen in our laboratory has already yielded several *Arabidopsis* mutants that fail to show hyponastic growth (M. van Zanten & F. F. Millenaar, unpublished).

Such molecular and genetic research strategies could also identify the regulatory networks that enhance internal aeration during submergence through changes in aerenchyma

formation and underwater gas-exchange properties of leaves. These traits may not be well developed in *Arabidopsis* but can probably be investigated in rice, for which extensive genomic information is available and which shows a high degree of genetic variation for submergence tolerance (Jackson & Ram, 2003). Such studies would provide detailed knowledge on the physiological and molecular regulation of the plant responses to submergence, as highlighted in this review, adding to present knowledge on submergence tolerance in this important crop species (Jackson & Ram, 2003; Toojinda *et al.*, 2003). Such mechanistic knowledge is required for direct experimental tests of the costs and benefits of these responses (e.g. Voeselek *et al.*, 2004). This will ultimately elucidate how the different traits are inter-related and how the interactions between these traits determine submergence tolerance.

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