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Oxygen Sensing and Plant Acclimation to Soil Flooding

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ABSTRACT

Plants in the natural environment often experience limited oxygen availability due to transient or continuous flooding. The slow diffusion rate of gases in water limits the oxygen supply, which affects aerobic root respiration as well as many biochemical processes in all the plant parts. Falling internal oxygen concentrations are sensed within plants and lead to adaptive responses to avoid internal oxygen deficiency that have a variety of different time-scales and levels of control. The examination of regulatory mechanisms and signalling events responsible for triggering responses to oxygen deficient conditions in plants is an interesting area of research. 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. In this study, the likely origin of the flooding signal, its perception and the plant responses are examined. Focus is drawn to the potential involvement of nitrate and nitrate reductase during flooding stress.

Key words: Flooding, oxygen sensing, acclimation, nitrate, nitrate reductase

INTRODUCTION

Plants grow in a dynamic environment, which frequently imposes constraints on growth and development. Among the adverse environmental factors commonly encountered by land plants, flooding is one of the most significant abiotic stresses (Blom and Voesenek, 1996).

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 (Visser *et al.*, 1996; Dubois *et al.*, 2010). The examination of regulatory mechanisms and signalling events responsible for triggering responses to oxygen deficient conditions in plants is an interesting area of research. 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 (Gibbs and Greenway, 2003; Greenway and Gibbs, 2003). Much of the research has been carried out with crop plant species, such as *Oryza sativa*, *Zea mays*, *Trifolium subterraneum* and *Medicago sativa* (Vartapetian and Jackson, 1997; Aschi-Smiti *et al.*, 2003; Irving *et al.*, 2007), but also wild species originating from wetland habitats (Justin and Armstrong, 1987; Visser *et al.*, 1996). All these plant species have their own survival strategies reflecting adaptations to their specific environment and may serve as models in detailed experimental studies.

To understand how ecosystems respond to flooding, it is crucial to understand how individuals perceive and will respond to changes resulting from alterations in the environment. Insight on how

soil waterlogging variously alters plant responses, will have to include a comprehensive description of plant perception and the adaptive mechanisms during flooding. In this study, the likely origin of the flooding signal, its perception and the plant responses are examined. Focus is drawn to the potential involvement of nitrate and nitrate reductase during flooding stress.

Plant responses to flooding is O₂ concentration-dependent: Soil water saturation has dramatic consequences on gas diffusion processes, as gases diffuse 10⁴ faster in air than in water (Mommer *et al.*, 2004). Consequently, one of the main effects of flooding is a lower pool of available O₂ in the submerged plant parts. This decline in O₂ is heightened by aerobic processes taking place in the rhizosphere (i.e., microbial respiration). Accordingly, hypoxic conditions will develop leading to a reduction in ATP production and consequent decrease in root metabolism (Ricard *et al.*, 2006). The decline in available energy can subsequently reduce other active cellular processes such as osmotic adjustment, nutrient uptake or regulation of cytoplasmic pH (Ricard *et al.*, 2006). However, the effect of O₂ limitation on cellular metabolism is concentration dependent (Drew, 1997). The precise determination of the Critique Oxygen Pressure (COP), which corresponds to the lowest value of the O₂ level that saturates the respiration, appears of particular interest for defining the sensitivity of a tissue to decreases in O₂ level in the surrounding medium (Saglio *et al.*, 1988). Therefore, three types of flooding stress conditions are distinguished based on the level of O₂ in the root environment: (1) normoxia: conditions under which aerobic respiration and metabolism proceed normally and most of the ATP is generated via oxidative phosphorylation, (2) hypoxia: conditions under which the reduction in available O₂ starts to become a limiting factor for ATP production through oxidative phosphorylation (i.e., glycolysis accounts for a greater proportion of ATP production) and (3) anoxia: conditions under which ATP is only produced through glycolysis, as no more O₂ is available. As a result, there is a reduction in protein synthesis and other adverse effects on cell division and elongation. Therefore, there exist different levels of O₂ deficiency during flooding and below a certain threshold concentration, O₂ may not only become the main limiting factor for normal plant development, but probably the prime signal triggering the response (Saglio *et al.*, 1988).

Sensing of flooded conditions: 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 (Drew, 1997). 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-photosynthesizing organs, such as roots, oxygen levels rapidly decline due to continuous oxygen consumption in respiration and the very slow delivery of aerial oxygen to the root (Visser *et al.*, 1996). Ethylene, on the other hand, accumulates to physiologically active levels in submerged tissues, due to production in almost every organ and hampered diffusion to the atmosphere (Voisenek 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). Elevated ethylene levels are important for the induction of morphological and anatomical traits upon soil flooding, such as formation of aerenchyma and adventitious roots (Ricard *et al.*, 2006).

In plants, ethylene is perceived by a family of receptor molecules located in the endoplasmic reticulum (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 (Chen *et al.*, 2002). Ethylene receptors form a complex with a protein called constitutive triple response. These proteins are activated by association with the receptors at the endoplasmic reticulum and repress downstream ethylene responses (Visser and Voesenek, 2004). In the presence of ethylene, the ethylene receptor proteins presumably undergo conformation changes that inactivate them. Under these conditions, the constitutive triple response is released from the endoplasmic reticulum and also becomes inactivated (Gao *et al.*, 2003). Consequently, this de-repression will result in ethylene responses. Further downstream ethylene signals are transduced via several positive regulators (e.g., ethylene insensitive 2 (EIN2), EIN5 and EIN6) ending with the transcription factors EIN3 and EIN3-like (EIL) (Guo and Ecker, 2004). 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, 2004).

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 (COX) 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 COX 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 COX and AOX (Ricard *et al.*, 2006). 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). Thus, in order to timely respond to decreasing oxygen levels, plants need to sense oxygen concentrations that are between the K_m of COX and AOX and normoxia (Ricard *et al.*, 2006). This oxygen-sensing system in higher plants operates independently of changes in energy metabolism. This contention is supported by findings that induction of the *ADH* 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 suggesting that mitochondria are at the centre of oxygen sensing (Subbaiah and Sachs, 2003).

Morphological, physiological, metabolic and molecular plant responses to flooding: Due to hypoxia in the rhizosphere, waterlogging can severely impair the performance of terrestrial eukaryotes and, thus, has a great impact on the species composition at sites which are prone to waterlogging (Mommer *et al.*, 2004). It is well known that, during flooding, many flood-tolerant species undergo a variety of morphological and physiological changes to maintain more normal conditions (Blom and Voesenek, 1996). These changes, which include adventitious root formation and aerenchyma development, are regarded as facilitating oxygen transport to anaerobic tissues (Aschi-Smiti *et al.*, 2003). Under flooded conditions, changes in shoot water relations can occur (Pezeshki, 2001). In some investigations, a decrease in leaf water potential resulting from waterlogging was detected (Dreyer, 1994), whereas in other studies, the leaf water potential remained unchanged (Zhang and Davies, 1986; Dreyer *et al.*, 1991). In contrast, a decrease in stomatal conductance, due to stomatal closure, is considered a general reaction induced by root hypoxia (Armstrong *et al.*, 1994; Vartapetian and Jackson, 1997). The decrease in stomatal conductance, caused by reductions in the soil-plant hydraulic conductance or by other signals from the oxygen-deficient roots (Vartapetian and Jackson, 1997), can cause severe restriction of CO₂ diffusion in the leaves of the flooded plants (Jackson, 1985). In addition to stomatal closure, oxygen deficiency in the roots causes physiological disruption, including inhibition of photosynthetic activity under conditions of prolonged flooding (Pezeshki, 2001; Horchani *et al.*, 2008, 2010a, b).

Metabolism can also be affected. Under flooding, crop plants switch from oxidative phosphorylation to fermentation for ATP production (Perata and Alpi, 1993; Armstrong *et al.*, 1994; Ricard *et al.*, 1994, 2006). Of the twenty-one proteins synthesized under anaerobic conditions in maize primary roots and known as the anaerobic proteins (ANPs), twelve have effectively been identified as enzymes involved in the mobilization of carbohydrates for fermentation (Sachs *et al.*, 1996). The decrease in oxygen levels generally results in an increase in catalytic potential of the ANPs, due to a complex interplay between transcriptional activation and post-transcriptional regulation (Ricard *et al.*, 1994; Sachs *et al.*, 1996). Two enzymes, present in aerobic tissues in very low amounts and often postulated to be limiting, are pyruvate decarboxylase (PDC) and lactate dehydrogenase (LDH) (Ricard *et al.*, 2006). Over-expression of bacterial PDC in transgenic tobacco led to increased accumulation of acetaldehyde and ethanol under short-term anoxia in contrast to control plants where ethanolic fermentation was very low, indicating that PDC may indeed be a limiting factor (Bucher *et al.*, 1994). Lactate fermentation generally precedes ethanol production and has been proposed to be necessary to assure the pH drop, which initiates ethanol fermentation (Ricard *et al.*, 2006).

Expression analyses reveal the increased transcription of genes that are involved in the alcoholic (*pdh*, *adh*) and lactic (*ldh*) fermentation pathways when external oxygen concentration is decreased to 5% (Klok *et al.*, 2002). In addition to the changes in gene expression that represent pre-adaptations to survive anoxia, there are also changes in transcription that can be interpreted as adaptations to avoid anoxia (Ricard *et al.*, 2006). It is well known that low oxygen concentrations lead to a coordinated inhibition of respiration and biosynthesis, allowing oxygen consumption to be decreased (Klok *et al.*, 2002). In *Arabidopsis* roots, several genes that encode enzymes that are involved in cell-wall, lipid and flavanoid biosynthesis and in defence responses are repressed, in low oxygen concentrations (Klok *et al.*, 2002). This repression is consistent with the rapid decreases in biosynthetic fluxes and metabolic rates that occur when internal oxygen tensions are falling. The transcript levels of *SuSy* genes are increased in response to low oxygen concentrations in many

plant species (Zeng *et al.*, 1998; Klok *et al.*, 2002). The induction of *SuSy* has frequently been proposed to be involved in the tolerance to oxygen deficiency by increasing sucrose degradation rates to allow high rates of glycolysis and fermentation in roots (Sachs *et al.*, 1996; Ricard *et al.*, 2006). However, other studies using a *Sus1* antisense potato line (Biemelt *et al.*, 1998) do not support this conclusion, as decreased *SuSy* expression did not limit glycolytic flux under anoxic conditions. Rather, the results of recent studies on invertase-over-expressing lines imply that the induction of the less-energy-consuming *SuSy* sucrose degradation pathway is linked to the need to decrease oxygen consumption and to avoid anoxia when oxygen concentration is low (Geigenberger, 2003).

Hormonal regulation of adaptive responses: Phytohormones act as intermediates between environmental signals and the plant's responses to these stimuli. Ethylene is among the best-characterized regulators of plant growth and development. It plays a prominent role in processes such as germination, senescence, abscission, fruit ripening and in the acclimation of plants to environmental stress (Grichko and Glick, 2001). In submerged plants, ethylene levels rapidly build up, because the diffusion rate of this gas in water is approximately 10^4 times slower than in air and because ethylene is hardly metabolized in most tissues (Voesenek *et al.*, 1993). Furthermore, the production rate of ethylene is stimulated by sub-ambient partial pressures of O_2 (Jackson 1993; Blom *et al.*, 1994). During over-wet conditions, ranging from waterlogging to complete submergence, ethylene participates in the stimulation of shoot extension, the programmed cell death during aerenchyma formation and the initiation of adventitious root formation (Grichko and Glick, 2001). In flooded tomato plants, the molecular basis for the increase in ethylene production is the induction of 1-aminocyclopropane-1-carboxylate (ACC) synthase, a key enzyme in ethylene biosynthesis. ACC, which is synthesized in roots, is transported to shoots, where it is converted to ethylene (Grichko and Glick, 2001).

In addition to the convincing example of root-to-shoot ethylene signalling events during flooding, there is increasing interest in the role played by abscisic acid (ABA), gibberellic acid (GA), auxin (IAA) and cytokinin (CK).

In tomato, foliar ABA concentrations are increased transiently (Else *et al.*, 1995) and, a doubling in ABA concentration was measured in the xylem sap of de-topped *Phaseolus vulgaris* during flooding (Neuman and Smit, 1991). In addition, exogenous ABA applications increased anoxia tolerance in maize and *Arabidopsis* (Ellis *et al.*, 1999) and transcript levels of AtbZIP50 were transiently increased in anoxia-treated *Arabidopsis* root cultures (Klok *et al.*, 2002). Plant bZIPs play an important role in the ABA response. Finally, a Myb transcription factor (AtMYB2) was induced by hypoxia preceding the induction of ADH. Interestingly, this transcription factor is also induced by exogenous ABA (Dennis *et al.*, 2000). Although, there are several reports of an accumulation of ABA in the hypoxia response, there are other studies that suggest the opposite. For example, endogenous levels of ABA in *Arabidopsis* remained unaltered during hypoxia stress (De Bruxelles *et al.*, 1996). ABA levels decreased in elongating internodes of rice during submergence (Azuma *et al.*, 1995) and high levels of exogenously applied ABA reduced shoot growth in *Ranunculus sceleratus* (Smulders and Horton, 1991) and *Potamogeton pectinatus* (Summers and Jackson, 1996). Because of the contradictory data concerning the ABA effect, it is now believed that ABA action may in fact be linked to its effect on GA (Dubois *et al.*, 2010). In rice, ABA is a potent inhibitor of GA action and ethylene application reduces endogenous ABA levels (Hoffmann-Benninig and Kende, 1992).

It is now well established that ABA and GA can act as antagonists in various growth responses and the role of GA in stimulating shoot growth is well established (Hoffmann-Benning and Kende, 1992; Rademacher, 2000). There is also evidence for an increase in GA concentration and sensitivity to ethylene during flooding (Rademacher, 2000). This is supported by the requirement of GA for ethylene action in rice leaves during submergence. In fact, the synergism between ethylene and GA is believed to increase the responsiveness of rice internodes to GA (Hoffmann-Benning and Kende, 1992). When GA inhibitors are applied to rice seedlings, ethylene and submergence-induced growth are inhibited (Raskin and Kende, 1984). Finally, enhanced expression of GA-inducible genes encoding for cell wall loosening enzymes (i.e., expansins) has been described in the literature. However, as illustrated with *Rumex acetosa*, the response may vary between species, as GA levels remain unchanged although ethylene levels increased during submergence (He *et al.*, 1996).

Other hormonal interplays have also been described during flooding stress. Synergism between IAA and ethylene has been proposed during adventitious root formation (Grichko and Glick, 2001). Adventitious root development at the base of the shoot is an important adaptation to flooding and is initiated soon after submergence (Aschi-Smiti *et al.*, 2003). Although the endogenous IAA concentration remained unchanged during flooding-induced rooting, a basipetal transport from the shoot to the root zone was essential to keep auxin concentrations stable (Visser *et al.*, 1996). Other commonly observed flooding induced morphological changes, such as lateral root formation, can also be induced by IAA application (Visser *et al.*, 1996). An involvement of IAA in the flooding response is further supported by the transient increase in transcript levels of two auxin-responsive genes during hypoxia treatment of root cultures (IAA2, IAA3) (Klok *et al.*, 2002).

Finally, CK may also participate in the cross-talk during responses to flooding. In sunflower xylem sap, CK levels are remarkably reduced following 24 h of flooding (Burrows and Carr, 1969). Soil inundation is believed to reduce CK production by lowering O₂ concentrations at the site of CK production, the root apical meristem (Visser *et al.*, 1996). However, whether the reduction is due to lower biosynthesis or decreased transport from roots to shoot is still not demonstrated. Recently, transgenic *Arabidopsis* plants with auto-regulated CK production were shown to be more tolerant to flooding. This enhanced tolerance was attributed to the regulation of flooding induced senescence by endogenously produced CK (Zhang *et al.*, 2000).

Involvement of nitrate reduction in plant tolerance to O₂ deficiency: To survive energy crisis caused by oxygen shortage, plant cells need to reduce their energy requirements for maintenance and also direct the limited amount of energy produced during anaerobic catabolism to the energy-consuming processes that are critical to survival (Greenway and Gibbs, 2003; Gibbs and Greenway, 2003). The use of alternative electron and reducing power-consuming processes may be of physiological importance (Menegus *et al.*, 1991). The physiological role of exogenous nitrate under the conditions of plant hypoxia and anoxia attracted the attention of researchers since nitrate is widely applied as a nitrogen fertilizer, in particular on wetland soils abundant in many countries (Heeb *et al.*, 2005; Vartapetian, 2006). The role of nitrate reduction in oxygen deficiency tolerance is the subject of many controversies. Some investigations could not verify any effect of nitrate reduction on the energy metabolism in oxygen-deficient roots (Lee, 1979; Saglio *et al.*, 1988). However, other investigations have shown that nitrate supply to anaerobic root tissues led to an improved redox state (NADH/NAD⁺ ratio) and a higher adenylate energy charge

(Reggiani *et al.*, 1985; Stoimenova *et al.*, 2003) through the consumption of the reducing power (NADH) generated by the glycolysis and by the nitrate reduction to nitrite. In these tissues, nitrate reductase activity often increases, either through increased gene expression (Mattana *et al.*, 1996). These observations raised the hypothesis that nitrate reductase could be a key enzyme to understand the beneficial impact of nitrate on plant survival under anoxia. Experiments on hydroponically grown tomato (*Solanum lycopersicum* L.) plants during the development of root anoxia (Allegre *et al.*, 2004; Morard *et al.*, 2004) showed that nitrate reduction was beneficial in preventing the onset of wilting and necrosis in the leaves and that anoxia caused increases in NR activity and nitrite release to the growth medium (Allegre *et al.*, 2004). The effects of anoxia on NR activity and nitrite production were confirmed in parallel experiments on excised root systems with similar results (Morard *et al.*, 2004). More recently, we have investigated the putative role of nitrate and nitrate reductase in the tolerance to prolonged hypoxia (2% O₂) in tomato plants (Horchani *et al.*, 2010a). Nitrogen nutrition has been modified either by deprivation of nitrate, or by addition of tungstate -an inhibitor of NR- in the culture medium. In the absence of nitrate as well as in the presence of tungstate, plant growth was significantly disturbed. In the presence of nitrate, the growth of hypoxic plants maintained, nitrate absorption and NR activity increased and a significant release of nitrite into the medium was observed (Horchani *et al.*, 2010a). This mechanism of nitrate reduction, called nitrate respiration (Morard *et al.*, 2004; Horchani *et al.*, 2010a), could be an alternative pathway to oxygen-dependent respiration during root hypoxia and a transient adaptation of tomato roots to hypoxic conditions.

CONCLUSIONS AND FUTURE PERSPECTIVES

Increased insight into processes acting in plant communities under changing hydrological conditions will have valuable applications. Many areas all over the world suffer flooding disasters and the restoration of damaged flood-plains and the stocking of the river landscape with flood-resistant plants are topics of active research. Knowledge of the adaptive responses of individual plants is necessary to understand the processes at the vegetation level. Many physiological and morphological reactions in plants are already known, but much has to be done on adaptive mechanisms at the cell level. In addition, hormonal regulation plays an important role in the adaptation reaction. Screening of the hormonal regulation of adaptive responses in plant species that occur in the various zones of the floodplain is an interesting step in flooding research. Little is known about the signal-transduction chain upon flooding and about the genetic variation between plant populations that are exposed to long-lasting floods. Molecular biological techniques will be increasingly used to increase insight into these processes.

In general, survival to flooding includes one or more of the following responses, control of energy metabolism, availability of abundant energy sources, provision of essential gene products and synthesis of macromolecules and protection against post-anoxic injury. These strategies have been studied for more than a decade and the cellular and molecular adaptations leading to hypoxia tolerance are becoming more evident. Unfortunately, the sensors involved and the signal transduction pathways involved are still rather elusive. It is, however, becoming increasingly apparent that the induction of adaptive responses depends more on the amplitude and timing of such signals rather than their nature. This understanding is, therefore, crucial to identify and select plants capable of withstanding increased environmental cues as natural selective pressures are increased in frequency and duration.

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