Meetings

Flooding stress signaling through perturbations in oxygen, ethylene, nitric oxide and light

The 15th international New Phytologist workshop on Flooding Biology, Ravenstein, The Netherlands, June 2015

A grand challenge in the coming decades is to feed the rapidly growing world population. A second 'Green Revolution' is needed to further sustain and increase agricultural productivity in the face of global climate change. Climate projection models predict an increasingly wetter world with frequent and severe flooding events in major farming regions, especially in the tropics and Western Europe (Hirabayashi *et al.*, 2013). Prolonged wet conditions hamper seedling establishment, growth, and reproduction of plants thereby negatively impacting yield (Bailey-Serres & Voesenek, 2008). Annual global losses in crop production due to flooding are often comparable to that caused by droughts (Bailey-Serres *et al.*, 2012). The development of high yielding crop cultivars that are more flood-tolerant – beyond cultivated rice (*Oryza sativa*) – is therefore urgent.

In the last decade, research on molecular mechanisms that control flooding tolerance has progressed rapidly. Adaptations to waterlogging (root system inundation) and submergence (root and aerial system flooding) have been dissected at the molecular level in rice (Fukao & Xiong, 2013; Voesenek & Bailey-Serres, 2015a) and knowledge of low-O_2 sensing, acquired through Arabidopsis research, has been successfully translated to barley to improve waterlogging tolerance (Mendiondo *et al.*, 2015). Molecular regulators of the flood-adaptive quiescence (*SUB1A*) and escape (*SNORKEL1/2*) survival strategies were discovered in rice (Xu *et al.*, 2006; Hattori *et al.*, 2009). The elusive mechanism of O_2 sensing involving Ethylene Response Transcription Factors of sub-clade group VII (ERF-VIIs) was unraveled (Gibbs *et al.*, 2011; Licausi *et al.*, 2011; Sasidharan & Mustroph, 2011) and studies on naturally tolerant wild species revealed previously unknown tolerance mechanisms and genes (Sasidharan *et al.*, 2013; van Veen *et al.*, 2013). However, many breaches still exist in our knowledge of low-O_2 sensing, signaling and downstream response networks. There are major challenges in understanding and improving aeration of roots, regulation of low-O_2 metabolism and post-stress recovery.

Considering the accelerated momentum in flooding research and the urgency of enhanced global food security, researchers with interests in flooding and low-O_2 stress biology meet, discuss and update once every three years at a conference organized by the International Society of Plant Anaerobiosis (ISPA), and the most

recent meeting was held in 2013. To enhance and synergize flooding research between ISPA meetings, and introduce new researchers to the community, an international New Phytologist Workshop on flooding biology was held in Ravenstein, the Netherlands in 2015. More than 20 leading scientists from 12 different countries met in the rustic former monastery Soeterbeeck in the rural south of the Netherlands.

Mechanisms that increase flooding survival

Several plant traits, either constitutively present or inducible, improve flooding tolerance. These traits are categorized as 'Escape' traits or 'Quiescence' traits. In the first category, anatomical and morphological features and modifications improves the access of flooded plant cells to O_2 and CO_2, thus impinging the energy and carbohydrate status of flooded plants. The latter class of traits involves a reorganization of a plant's metabolism and ultimately growth through which survival during flooding stress is perpetuated (Voesenek & Bailey-Serres, 2015a).

Rashmi Sasidharan (Utrecht University, the Netherlands) provided evidence that even within a non-wetland species such as *Arabidopsis thaliana* considerable variation in flooding tolerance exists (Vashisht *et al.*, 2011). Through RNA-seq analysis of eight accessions with varying capability to survive prolonged (> 7 d) dark submergence, copious steady-state transcript adjustments were conserved, but interestingly several potential tolerance-related genes were identified. These variations hint at a small cadre of molecular mechanisms that underlie survival advantages that function in the root or shoot system during or immediately following submergence.

As O_2 deficiency, and therefore limitation of mitochondrial electron transport, is one of the most important nuisances of flooding, tolerant plants have or initiate the development of structures that encourage the inward and internal diffusion of O_2, as well as other gases such as CO_2. One trait associated with this is the formation of shoot-borne porous roots, often referred to as adventitious roots. Eric Visser (Radboud University Nijmegen, the Netherlands) presented evidence for the significance of the development of adventitious roots on the stem of the wetland plant *Solanum dulcamara* during flooding. He also showed that ethylene and abscisic acid (ABA) are important regulators of adventitious root development in this species. Margret Sauter (University of Kiel, Germany) showed previously that adventitious root formation on stem nodes of rice is triggered by ethylene and is related to reactive O_2 species (ROS)-mediated epidermal cell death and mechanical signaling (Steffens *et al.*, 2012). Sauter now presented evidence that rice ULTRAPETALA1, a protein that limits the repressive activity of Polycomb complexes on chromatin and thereby influences meristem size in *Arabidopsis*, is involved in this root formation process.

Another important way in which flooding tolerant plants conserve and ease diffusion of O_2 is the development of a barrier impeding outward diffusion of O_2 from roots and the development of longitudinally interconnected gas filled spaces (aerenchyma). Mikio Nakazono (Nagoya University, Japan) introduced a chromosomal segment by recombination, containing the genes responsible for barrier formation in the wild teosinte *Zea nicaraguensis*, into the agriculturally important *Zea mays*, that normally lacks a barrier and therefore is sensitive to soil flooding. This is an excellent example of a wide-breeding approach to enhance performance under abiotic stress (Mickelbart *et al.*, 2015), in this case applied to develop flood tolerant crops.

Aeration of underwater tissues can also be associated with a thin layer of air surrounding hydrophobic submerged leaves (gas films), which augments influx of O_2 and CO_2 and thus respiration and photosynthesis. Brian Sorell (Aarhus University, Denmark) showed that these gas films contribute to the maintenance of convective gas flows during partial submergence and therefore to elevate O_2 levels in rhizomes. Sea grasses (e.g. south Asian *Thalassia hemprichii* and *Enhalus acoroides*) typically grow submerged and can be exposed to relatively high temperatures at low tides. Based on photosynthesis, respiration and internal tissue aeration data, Ole Pedersen (University of Copenhagen, Denmark) concluded that slightly higher temperatures as foreseen by future climate models would extinguish these sea grasses. Many plants not only have to deal with O_2 deficiency during flooding but also with high salt levels. Data presented by Tim Colmer (University of Western Australia, Crawley, Australia) and very recently published work by Kotula *et al.* (2015) demonstrated that low O_2 has a serious impact on ion transport and the cellular distribution of these ions across roots of barley (*Hordeum vulgare*). This also affected the necessary exclusion of potentially toxic Na^+ from shoots in flooded saline soils. The importance of the ion balance during low-O_2 stress was further illustrated by Joost van Dongen (Aachen University, Germany), who presented data about the role of potassium gradients that can support the proton motive force of the phloem to drive sucrose import (Gajdanowicz *et al.*, 2011).

Soybean (*Glycine max*) is another example of an economically important crop that is very sensitive to flooding. Takeshi Fukao (Virginia Tech, Blacksburg, VA, USA) studied the developmental, transcriptional and metabolic responses of this crop to complete submergence and discovered tissue specific (cotyledons, leaves, and roots) responses.

Under normoxic conditions, ATP is produced via oxidative phosphorylation, potentially resulting in 36 ATP units per hexose unit. When O_2 levels decline this phosphorylation route is severely restricted and ultimately halted. Plants compensate for this by increased ATP production via substrate-level phosphorylation via an activation of glycolysis (Pasteur effect). In order to maintain a high glycolytic rate, recycling of NAD^+ from NADH is essential. This is realized by (1) a stimulation of lactate and ethanol fermentation, both producing NAD^+ and/or by (2) enhanced conversion of pyruvate via pathways that lead to alanine, γ-amino butyric acid (GABA) and succinate, and (3) increased conversion of nitrate into nitrite, which subsequently is converted into nitric oxide (NO). Nitrate reduction requires NADH as co-substrate and

therefore this so-called NO-cycle is hypothesized to be an alternative source of NAD^+ (Van Dongen & Licausi, 2015). Joost van Dongen showed evidence in soybean roots, garnered by monitoring flux of several isotopically labeled metabolites, that the activity of the NO-cycle stimulates the Pasteur effect while reducing ethanolic fermentation.

Sensing and signaling

A consequence of enhanced rates of glycolysis is that carbohydrate consumption is exaggerated, ultimately leading to carbohydrate and energy starvation. It is therefore not surprising that root systems – such as those of water lilies – that grow in anaerobic, permanently flooded soils have thick, carbon-rich rhizomes. Plants can sense changes in energy levels via Snf1-related kinases (SnRK1s) (reviewed by Lawlor & Paul, 2014; Tomé *et al.*, 2014). These kinases are activated via phosphorylation under starvation conditions and subsequently activate S1 bZIP transcription factors involved in the activation of genes that stimulate catabolism to provide alternative carbon sources. Ming-Che Shih (Academia Sinica, Taipei, Taiwan) provided data demonstrating that an *Arabidopsis* SnRK1.1 dominant-negative mutant had reduced tolerance of submergence. A comparative phosphoproteomics survey of wildtype and mutant tissue of submerged plants identified a number of differentially phosphorylated proteins that range in function from sugar synthesis, glycolysis, osmotic regulation, ABA signaling, protein synthesis and ROS signaling.

Linked to the regulation of energy homeostasis is the production of the metabolite trehalose-6-P from glucose-6-P and UDP-glucose (reviewed by Lawlor & Paul, 2014). In the presentation by Julia Bailey-Serres (University of California, Riverside, CA, USA), we learned of the discovery by researchers at the International Rice Research Institute in the Philippines that the *ANAEROBIC GERMINATION1* (*AG1*) locus of rice, which enables direct seeding of rice into shallow paddies, encodes a Trehalose-6-P Phosphatase (TPP7). The presence of the *TPP7* in rice accessions was associated with increased sink strength of elongating coleoptiles, enabling more rapid escape from submergence (Kretzschmar *et al.*, 2015). The presentation of James Whelan (La Trobe University, Melbourne, Australia) also focused on the remarkable ability of rice seed to germinate and efficiently elongate their coleoptile under extremely low O_2 levels (i.e. Narsai *et al.*, 2015). This escape response includes the ability to rapidly switch to aerobic growth upon emergence from floodwaters. A first look into the role of the epigenome in this process found that cytosine methylation was altered after 4 h of anaerobic germination in a reversible manner, but these regional changes were not well correlated with alterations in transcript abundance.

Typical for the hypoxia response of *Arabidopsis* seedling cell types is the transcriptional upregulation of a group of *c.* 50 hypoxia-responsive genes (HRGs). A substantial amount of the HRG transcripts code for proteins involved in the reorganization of metabolism to facilitate substrate level ATP production and fermentation (Mustroph *et al.*, 2009). Many HRGs encode proteins of poorly characterized or unknown function. In recent years several hypoxia-responsive unknown proteins have been

shown to modulate the abundance and function of the ERF-VII transcription factors (i.e. Giuntoli *et al.*, 2014; Weits *et al.*, 2014). Two additional hypoxia-induced genes, *LOB-DOMAIN-CONTAINING PROTEIN 41* (*LBD41*) and *ABA REPRESSOR 1* (*ABR1*), are studied by Angelika Mustroph (University of Bayreuth, Germany). LBD41 is most likely a repressing transcription factor, whereas ABR1, induced primarily in shoot tissue, has a conserved Methionine-Cysteine conserved nitrogen (N)-terminal motif, which makes it a putative target for the N-end-rule pathway of protein degradation. This pathway is responsible for O_2-dependent degradation of the ERF-VIIs (Gibbs *et al.*, 2011; Licausi *et al.*, 2011; Kosmacz *et al.*, 2015). Another HRG is *UNIVERSAL STRESS PROTEIN 1* (*HRU1*). Pierdomenico Perata (Scuola Superiore Sant'Anna, Pisa, Italy) presented evidence that HRU1 is induced by the ERF-VII transcription factor RELATED TO AP2.12 (RAP2.12) and that it modulates ROS production. It is proposed that HRU1 integrates the anaerobic response with the production of ROS under extreme O_2 deprivation.

A common observation of many researchers studying molecular mechanisms of sub-ambient O_2 response in diverse plants are the strongly upregulated transcripts encoding non-symbiotic hemoglobins (Van Dongen & Licausi, 2015). During the workshop, Robert Hill (University of Manitoba, Winnipeg, Canada) proposed to call these proteins 'phytoglobins' as a general term that more accurately describes heme-containing plant globin proteins. Two additional presentations (Kim Hebelstrup, Aarhus University, Denmark and Kurt Fagerstedt, University of Helsinki, Finland) discussed the importance of phytoglobins in flooding tolerance and their capacity to scavenge NO. A pretreatment with the plant hormone ethylene, a reliable and early signal for flooding (Voesenek & Sasidharan, 2013), boosts the tolerance to subsequent hypoxia. Rens Voesenek (Utrecht University, the Netherlands) discussed the possibility that this might be linked to phytoglobin action.

Low O_2 and development

Higher animals have a circulatory system that actively transports O_2-bound red blood cells, driven by a pump (the heart), to remote regions where diffusion constants promote release and oxygenation of cells. Plants lack such a sophisticated O_2 transport system and completely rely, for their aeration, on gas diffusion and in some species gas convection (Voesenek & Bailey-Serres, 2015a). Consequently, O_2 diffusion can be limiting in plant tissues that are densely packed, in cells or organs that have a low surface to volume ratio and in tissues with high O_2 consumption rates. Examples are centers of seeds and stems, meristems, tubers, fruits and bulky roots (Van Dongen & Licausi, 2015). Thus, tissue hypoxia is not only related to environmental perturbations such as flooding, but is also an integral part of plant development in certain cell types, tissues and organs. A striking demonstration of this was made for pre-meiotic development of anthers in maize (Kelliher & Walbot, 2012).

Submergence tolerance of *Arabidopsis* also depends on the plant's developmental stage as was demonstrated by Francesco Licausi (Scuola Superiore Sant'Anna, Pisa, Italy) and reiterated by the independent work of Takeshi Fukao with soybean. Older *Arabidopsis* plants tend to be less tolerant than younger plants. In these younger plants, genes associated with ABA and ROS were more highly activated, possibly mediated by membrane-bound NAC transcription factors. Licausi hinted at a role for mitochondrial retrograde signaling in the release of ANAC017 from an ER-bound reservoir that enables nuclear localization (De Clercq *et al.*, 2013; Ng *et al.*, 2013). An interesting question is whether the sensitivity of juvenile leaves to O_2-deprivation or re-oxygenation relates to morphological distinctions, such as cuticular lipid composition or developmentally determined distinctions in cell growth and metabolism.

An elegant demonstration of the role of O_2 in developmental plasticity in *Arabidopsis* was made by Michael Holdsworth (University of Nottingham, UK). Work from his group demonstrated the ecological role of hypoxia in protecting the shoot stem cell niche through enhancement of the apical hook during early seedling growth. This hypocotyl curvature is regulated by the stabilization of the ERF-VIIs when O_2 levels are below 12 kPa and NO levels are limited (Gibbs *et al.*, 2014; Abbas *et al.*, 2015; Voesenek & Bailey-Serres, 2015b).

Future

Progress in the field of flooding biology research is gaining rapid momentum. Breakthrough discoveries were made over the past years, such as the transcriptional regulation mediated by direct sensing of O_2, necessary metabolic and growth reorganization via group ERF-VII transcription factors, their post translational regulation by means of the N-end rule of protein degradation, the importance of NO in regulating this protein degradation pathway and the function of hypoxia as an environmental cue in plant development.

The small-scale workshop in Ravenstein stimulated interactions between flooding researchers and resulted in new collaborations and exchange of ideas and materials. In the final keynote, Julia Bailey-Serres summarized and linked together the main results of this event. We learned that short-term responses to hypoxia in plants are conserved and that small rather than big changes in metabolism distinguish tolerant plants from those that are intolerant. Moving beyond the mining of genetic variation in flooding tolerance in rice, evidence was provided that waterlogging tolerant maize can be established by introgression of gene(s) from a teosinte that enhance the barrier that prevents radial O_2 loss. New discoveries were presented on the interactions between flooding and other stresses (heat, salt) and the functions of several conserved unknown HRGs were explored. Finally, the talks positioned phytoglobins as an important group of proteins that influence flooding tolerance most likely through their interaction with NO and the N-end rule pathway.

In September 2016, ISPA will hold its next conference in Copenhagen, Denmark (http://www.is-pa.org). It will be a challenge to incorporate our fundamental knowledge into the development of flood tolerant crops to feed the expected nine billion people on our planet.

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