

Submergence tolerance in rice requires *Sub1A*, an ethylene-response-factor-like gene

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Submergence of rice (*Oryza sativa*) by flash flooding is a major constraint to rice production in Asia. Rice cultivars vary in their capacity to tolerate complete submergence; quantitative trait loci analyses have revealed that a large portion of this variation in submergence tolerance can be explained by one locus (*Sub1*) on chromosome 9. Two recently published papers (Takeshi Fukao *et al.* and Kenong Xu *et al.*) present evidence that a transcription factor belonging to the B-2 subgroup of the ethylene response factors (ERFs)/ethylene-responsive element binding proteins (EREBPs)/apetala 2-like proteins (AP2) within the *Sub1* locus determines submergence tolerance in rice. These genes control highly conserved hormonal, physiological and developmental processes that determine the rate of elongation when submerged.

Flooding tolerance

Flooding is one of the most important environmental stresses worldwide. It has a dramatic effect on the growth and yield of crop plants because most of these economically important species are intolerant to flooding. Energy deficit, caused by inhibition of respiration, is one of the biggest problems encountered by plants when subjected to submergence because the oxygen supply is hampered [1,2].

Not all plant species are intolerant to flooding. In particular, species originating from semi-aquatic environments have the capacity to cope with flooding stress. They can survive complete submergence for weeks and some even have the capacity to grow vigorously and produce flowers and seeds. A broad range of metabolic and/or morphological adaptations characterize these tolerant species. Many develop a strong capacity to generate ATP without oxygen (continuation of glycolysis followed by fermentation) and/or to induce specific anatomical or morphological features (e.g. aerenchyma and enhanced shoot elongation) that improve the entrance and diffusion of oxygen [3–9]. Gene expression studies of plants exposed to a low oxygen level revealed the upregulation of genes coding for transcription factors, signal-transduction components, nonsymbiotic hemoglobin, ethylene biosynthesis, nitrogen metabolism and cell wall loosening [9]. At the protein level, a low oxygen level selectively induces the synthesis of proteins known as the anaerobic polypeptides, most of which are enzymes involved in sugar metabolism, glycolysis and fermentation pathways [10–12].

Despite this knowledge of adaptive mechanisms and regulation at the level of genes and proteins, our understanding of the mechanism underlying between- and within-species variation in submergence tolerance is limited. Even though we know a lot about the various individual mechanisms of tolerance it is still difficult to pinpoint the exact cause of tissue death. It has been suggested that survival is a balancing act that results from, for example, the management of carbohydrate consumption and the avoidance of oxidative stress [15].

Escape versus quiescence

Some semi-aquatic plant species have the capacity to survive submergence through a highly coordinated enhancement in upward growth of shoot organs such as stems and petioles. This 'escape' strategy returns the shoot to the atmosphere above the water surface, allowing the plant to resume aerobic metabolic activity [9,13]. However, rapid shoot elongation is only a favorable trait if the costs associated with it are outweighed by benefits such as improved aeration and restored aerial photosynthesis. An imbalance between costs and benefits is likely to happen if floods are either too deep to allow renewed contact with the air or when floods are too ephemeral to outgrow the flood water [14]. These specific flooding regimes favor a so-called 'quiescence' strategy characterized by slow growth and, thus, conservation of energy and carbohydrates [15]. Species exploiting this strategy have higher survival rates during long-term submergence than those that use the elongation strategy, provided that they are restrained from reaching the air. The advantage of slow growth for survival has been elegantly demonstrated for young rice seedlings using substances that either stimulate growth (gibberellins) or inhibit growth (paclobutrazol) [16].

The molecular mechanism underlying this variation in under water growth and, hence, submergence tolerance has been recently described by Takeshi Fukao *et al.* [17] and Kenong Xu *et al.* [18] – they conclude that a putative ethylene response factor (ERF) explains the variation in submergence tolerance between rice cultivars.

Rice tolerance to submergence is due to several adaptive traits

Rice is an essential food crop for billions of people. The success of rice as an agricultural crop is also related to its ability to cope with a wide range of environmental conditions. Rice is one of the few crop species that can

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germinate and grow in permanently waterlogged soils [1,19]. Furthermore, rice develops an interconnected gas space network (aerenchyma) that facilitates O₂ diffusion from aerial to submerged parts of the plant. Some of the O₂ is lost as a result of radial diffusion to the rhizosphere. However, some rice cultivars prevent this 'loss' of O₂ with an inducible barrier to radial oxygen loss (ROL) [20]. Whereas rice germination under anoxia is the consequence of a poorly understood metabolic adaptation, aerenchyma formation and the inducible barrier to ROL are 'escape' strategies that functionally depend on the rapid elongation of the shoot to cope with the rising water level. However, when completely submerged, most rice cultivars are flood sensitive and die within a week [18]. Only a few cultivars, such as FR13A, can survive up to two weeks of complete submergence. The submergence tolerance of FR13A is linked to a major quantitative trait locus (QTL), known as *Submergence1* (*Sub1*), on chromosome 9 [21]. This locus (200 kb) explains a large proportion of the variation (35–69%) in flooding tolerance between *indica* (tolerant) and *japonica* (intolerant) rice cultivars [22]. In their recent paper, Xu *et al.* [18] reveal that the FR13A *Sub1* region encodes three transcription factors (*Sub1A*, *Sub1B* and *Sub1C*) belonging to the B-2 subgroup of the ethylene response factors (ERFs)/ethylene-responsive element binding proteins (EREBPs)/apetala 2-like proteins (AP2). The transcription of both *Sub1A* and *Sub1C* is strongly up regulated by submergence and down regulated by de-submergence. However, *Sub1C* is less upregulated in the presence of *Sub1A* than in its absence, suggesting that *Sub1A* might downregulate *Sub1C*. The third ERF, *Sub1B* is only slightly regulated upon submergence. An allelic survey revealed that *Sub1A* is absent from five out of 17 *indica* rice cultivars and from the four *japonica* cultivars investigated. Furthermore, submergence tolerance is strongly correlated with the presence of the *Sub1A-1* allele, whereas intolerance to submergence is associated with the *Sub1A-2* allele or with the complete absence of the *Sub1A* gene [17]. Interestingly, transformation of a submergence-intolerant *japonica* variety with *Sub1A-1* conferred submergence tolerance to the transgenic plants [18].

Fukao and colleagues [17] propose a model in which increased ethylene levels inside submerged plant tissues result in an accumulation of *Sub1A* transcript in submergence-tolerant rice. The SUB1A protein increases transcription of genes associated with ethanolic fermentation and represses transcription of genes associated with cell elongation and carbohydrate catabolism. Furthermore, SUB1A is responsible for a feedback restriction in ethylene production (Figure 1).

To examine the functionality of the *Sub1* locus further, *Sub1* (haplotype *Sub1A-1*, *Sub1B-1*, *Sub1C-1*) was introgressed into a submergence intolerant background (*japonica* cultivar M202). Compared with the intolerant M202 cultivar (haplotype *Sub1B-2*, *Sub1C-2*), M202(*Sub1*) was more submergence tolerant (Figure 2), starch and soluble carbohydrate levels declined more slowly, mRNA levels coding for α -amylases and sucrose synthases were lower, pyruvate decarboxylase (PDC) and alcohol dehydrogenase (ADH) activity was increased, ethylene production

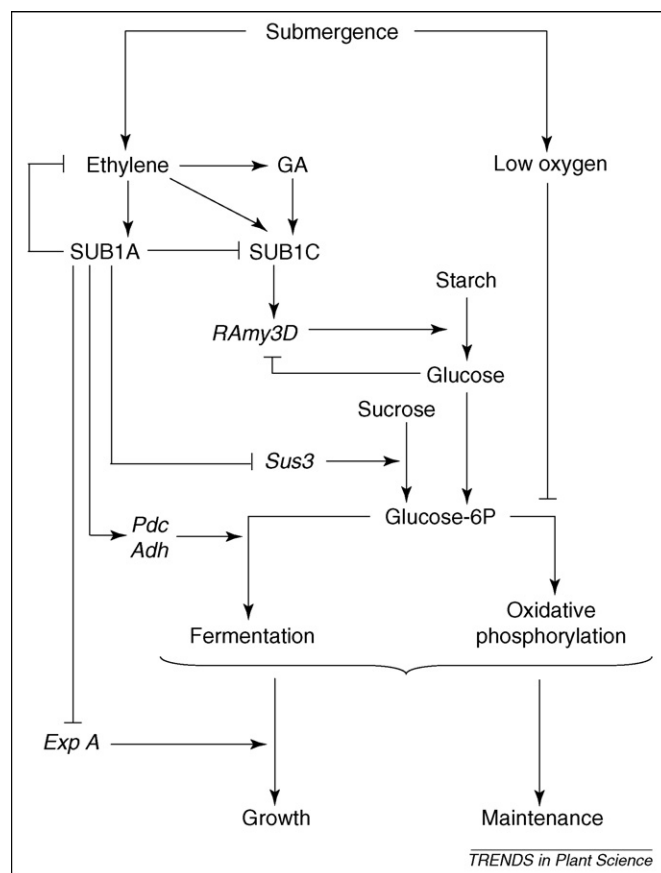


Figure 1. Schematic model linking *Sub1A* to submergence tolerance. Submergence results in ethylene accumulation that activates transcription of the *Sub1A* gene. SUB1A represses *Expansin A* and sucrose synthase and, thus, represses growth. SUB1A also enhances mRNA accumulation and enzymatic activities of pyruvate decarboxylase (Pdc) and alcohol dehydroxygenase (Adh). Fermentation allows glycolysis to continue and, thus, provides the ATP needed for survival. However, the rate of ethanol production is similar in a genotype lacking *Sub1A*, indicating that differences in the induction of *Pdc* and *Adh* genes might not be crucial. Starch degradation provides glucose needed for both glycolysis and growth. In rice varieties that express *Sub1A*, the elongation rate is lower, enabling a pool of starch and carbohydrates, which are needed for prolonged ATP production through fermentation to be preserved. *Sub1C*, which appears to control the α -amylase gene *Ramy3D*, is repressed by *Sub1A*. Furthermore, gibberellins (GA) appear to be involved in the regulation of expression of *Sub1C*. However, it is unlikely that regulation by GA directly affects *Ramy3D* expression given that the promoter of this gene lacks the GARE element required for GA regulation. Instead, up-regulation of *Ramy3D* by the lower sugar content found in *Sub1A*-deficient lines is likely. This process is described in more detail in Refs [17,23,24].

was lower and the transcription of expansin genes was suppressed. All these physiological traits are consistent with a 'quiescence' strategy to survive submergence via the conservation of carbohydrates, repression of cell elongation and the enhancement of fermentation capacity [17].

Perspectives

Tolerance to flooding is an important plant trait that also determines plant species distribution and abundance in natural ecosystems. A vegetation analysis demonstrated that plant species that stimulate shoot elongation when submerged occur predominantly in environments characterized by prolonged, but relatively shallow flooding events. Species that lack submergence-induced shoot elongation were found in areas with brief or deep floods [14]. This is consistent with the idea that different survival strategies

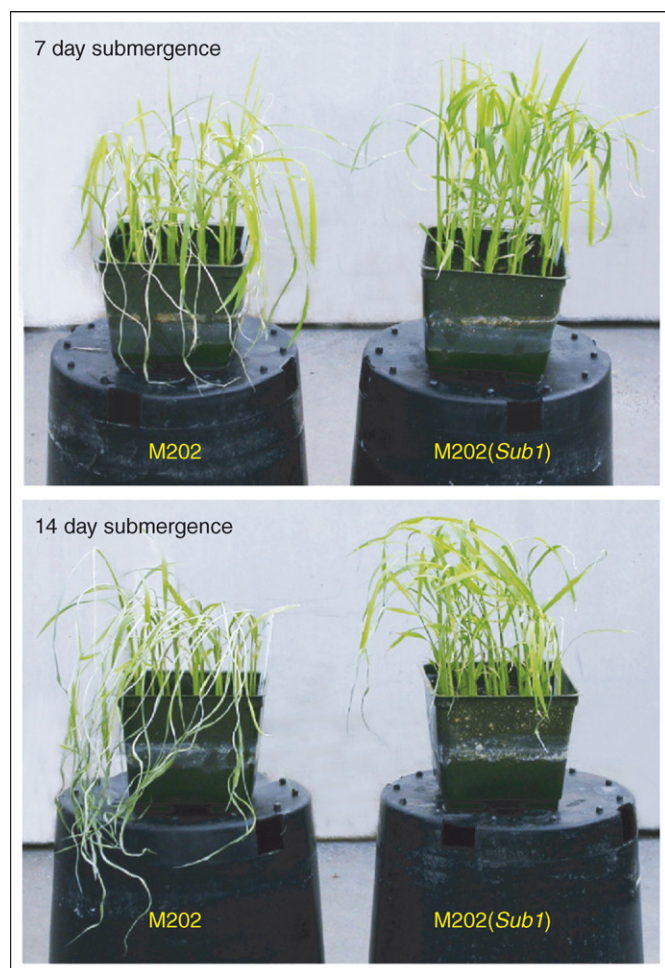


Figure 2. *Sub1* confers tolerance to M202, a submergence-intolerant *japonica* rice variety. Fukao *et al.* [17] compared the M202 variety with a M202 near-isogenic line containing the *Sub1* locus from FR13A in terms of survival after a submergence treatment. The M202 plants containing the *Sub1* locus are more tolerant to 14 days of submergence than those without the *Sub1* locus. See Ref. [17] for details about submergence treatment. Photographs provided by Julia Bailey-Serres.

(escape versus quiescence) are selected by different flooding regimes and with *SubA1* orthologs having a putative role as the genetic basis for patterns in field distribution of species in flood-prone ecosystems.

Introgression of *Sub1* into flood-intolerant rice cultivars improves submergence tolerance significantly and has no negative side effect in terms of yield, harvest index and grain quality when grown under control (non-submerged) conditions [18]. This should stimulate the economies in many regions of the world and increase the amount of food available to feed the poor in less developed regions.

Flooding also negatively affects many other crop species, therefore, improving flood tolerance is a high priority. However, it is questionable whether flood tolerance in crops other than rice is regulated by orthologs of *Sub1A*. In many crop species, flooding intolerance is not associated with rapid under water growth as in rice. Therefore, downregulation of growth is not an option to conserve energy and carbohydrates. This raises the question: why are these species that seemingly apply a ‘quiescence’ strategy not flood tolerant? It is reported that flood intolerant species up-regulate many genes generally associated with flooding tolerance [25,26]. Cereals, such as barley and

wheat, which are anoxia-intolerant, apparently have a ‘quiescence strategy’ at the germination stage because they are unable to elongate the coleoptile, a trait shown by rice [1,19]. Despite their quiescence, barley and wheat seeds rapidly lose viability under anoxia, and this correlates with the inability to degrade starch under anoxia, a pathway that is instead activated under anoxia and allows a vigorous fermentative pathway to take place [27,28]. Therefore, it appears that the ‘quiescence strategy’ does not necessarily lead to tolerance to low oxygen levels when cereals are at the seedling stage. The characterization of the *Sub1* locus has resulted in a big step forwards in our understanding of the regulation of flooding tolerance but the difference in submergence tolerance between- and within-species is only partly resolved by the identification of the *Sub1A* gene. Rice seed germination under complete anoxia is not likely to be explained in terms of *Sub1* genes. Ethylene, the trigger for *Sub1A* expression is not produced under anoxia and, indeed, the M202 and Nipponbare cultivars, which both lack the *Sub1A* gene [17,18], germinate equally well under anoxia (P. Perata, unpublished). This emphasizes the importance of flooding research that includes both tolerant and intolerant species, accessions and cultivars and that adopts a combined developmental, physiological and ‘omics’ approach.

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Understanding sample size: what determines the required number of microarrays for an experiment?

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DNA microarray experiments have become a widely used tool for studying gene expression. An important, but difficult, part of these experiments is deciding on the appropriate number of biological replicates to use. Often, researchers will want a number of replicates that give sufficient power to recognize regulated genes while controlling the false discovery rate (FDR) at an acceptable level. Recent advances in statistical methodology can now help to resolve this issue. Before using such methods it is helpful to understand the reasoning behind them. In this Research Focus article we explain, in an intuitive way, the effect sample size has on the FDR and power, and then briefly survey some recently proposed methods in this field of research and provide an example of use.

Replication in microarray experiments

The results of a DNA microarray experiment are influenced by biological and technical sources of variation. To handle this variation, researchers often replicate the measurements, using different biological cases, and then use statistical tests to identify genes of interest. An essential step in the design of an experiment is, therefore, choosing the number of biological replicates^a to be used – the sample size. In general, a larger sample size should produce more reliable results. However, the cost of a microarray experiment calls for moderation. Consequently, one should aim to find the smallest sample size

that still provides results that are of a ‘good enough’ quality.

Recently, several statistical approaches have been proposed that could be used to help estimate the optimal sample size. To make the best use of this new methodology it is helpful to first understand its theoretical basis. How does sample size affect the outcome of an experiment? How are quality measures, such as the false discovery rate (FDR) [1,2] and power, used to determine if the results are ‘good enough’? Below we examine a much-used setup that compares samples from two conditions. From this example we will try to answer the above questions in an intuitive way. We then discuss some new developments in the field of sample-size estimation.

Comparing two conditions

Assume that we want to compare gene expression in an *Arabidopsis thaliana* wild type with that in a mutant. We make $n = 3$ biological replicates for both groups and run a microarray experiment. After collecting the data we face a challenging task. For each gene we must now decide whether we think it is differentially regulated.

When trying to find regulated genes, statisticians often calculate a t -statistic^b. Based on microarray measurements, one t -statistic can be calculated for each gene. The t -statistic, in essence, quantifies the evidence of a gene being regulated. The further away from zero a t -statistic is, the greater the

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^a In the literature, ‘biological replicates’ denote replicated measurements using different biological cases, whereas ‘technical replicates’ use the same biological cases. In this article, we only consider biological replication.

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^b A standard t -statistic can roughly be written as d/s , where d is a sample statistic that quantifies group differences in gene expression, and s is the estimated standard deviation of d . We use this statistic here because of its analytically tractable properties. In microarray data analysis, other statistics are now often preferred. However, many of these are closely related to the standard t -statistic. This is the case for popular analysis tools such as the regularized t test [3], the limma-package [4] and the SAM-package [5].