



REVIEW

From promise to application: root traits for enhanced nutrient capture in rice breeding

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Abstract

Improving nutrient uptake is an objective in crop breeding, especially in tropical areas where infertile soils dominate and farmers may not have the resources to improve soil fertility through fertilizer application. Scientific endeavors to understand the genetic basis of nutrient acquisition have mostly followed reverse genetic approaches. This has undoubtedly led to improved understanding of basic principles in root development and nutrient transport. However, little evidence suggests that the genes identified are actively utilized in breeding programs, and the bottleneck has been the failure to establish links between allelic variation for identified genes and performance in the field. Screening experiments typically reveal large genotypic variation in performance under nutrient deficiency, strongly suggesting the presence of superior alleles for genes controlling root growth and/or nutrient uptake processes. Progress in sequencing technology has enabled characterizations of allelic variation across whole genomes and an international effort has recently culminated in the sequencing of 3000 rice genomes from the International Rice Research Institute genebank. Queries of the 3000 rice sequence database offer immediate possibilities to assess the extent to which allelic variation exists for candidate genes. By selecting subsets of accessions, allelic effects can be tested, diagnostic markers developed, and new donors identified. Technological and conceptual advances in phenotyping of root traits offer improved possibilities to assure that trait–allele associations are established in ways that link to field performance. Genotype-to-phenotype relationships can thus be predicted and tested with unprecedented precision, facilitating the discovery and transfer of beneficial nutrition-related alleles and associated markers into existing breeding pipelines.

Key words: Genome-wide association studies, indirect selection, nutrient acquisition, phosphorus, rice genome, root phenotyping.

Introduction

Rice (*Oryza sativa* L.) is a staple cereal crop that is cultivated on over 150 million ha of land globally. Much of that land is deficient in one or more essential nutrients (Haefele *et al.*, 2014). While deficiencies of both macronutrients such as

nitrogen (N), phosphorus (P), potassium (K), and calcium (Ca), and micronutrients including zinc (Zn), boron (B), copper (Cu), and molybdenum (Mo), can constrain rice growth in specific soils and environments (Ismail *et al.*, 2007), N, P,

and Zn are the major nutrient deficiencies that limit rice yields on a global scale (Dobermann and Fairhurst, 2000; Haefele *et al.*, 2014; Ismail *et al.*, 2007). Since the onset of the Green Revolution, such deficiencies have been addressed by fertilizer application in combination with the use of high-yielding modern varieties (HYMVs) that respond far better to fertilizer than traditional varieties (Khush, 1995). The combination of responsive varieties, increased fertilizer inputs, and improved and/or extended irrigation facilities, jointly termed the 'Green Revolution package', has caused food production to outpace population growth and was thus instrumental in avoiding large-scale food shortages (Otsuka and Kalirajan, 2006; Pingali 2012).

While the phenomenal success of the Green Revolution in providing food security is undeniable, the reliance on fertilizer inputs has caused environmental problems, including increased eutrophication of waterways due to erosion of P-rich sediments, and deposition of N, into watercourses (Carpenter *et al.*, 1998). The nutrient issues arise primarily because of the poor crop recovery of N, P, or Zn fertilizers due to soil fixation (P, Zn) or gaseous losses (lowland crops) and leaching (upland crops) in the case of N (Fageria and Barbosa Filho, 2007; Linquist *et al.*, 2013; Wissuwa *et al.*, 2006). As little as 20–40% of the N applied as fertilizer is recovered by rice crops (Cassman and Pingali, 1995; Vlek and Byrnes, 1986), with the bulk of losses occurring through NH₃ volatilization or denitrification to N₂ in flooded systems (Linquist *et al.*, 2013). While N₂ losses are not problematic in terms of environmental damage, deposition of NH₃ in later rain events can lead to higher N concentrations in watercourses, with subsequent eutrophication issues (Carpenter *et al.*, 1998).

A second issue related to fertilizers is cost and access. Poor transportation infrastructure in more remote inland regions adds a considerable cost to fertilizers; this is particularly evident in Africa, where local fertilizer prices can be twice as high as world market prices (Otsuka and Kalirajan, 2006). This erodes farming profits and effectively denies access to fertilizers to many resource-poor farmers (Ismail *et al.*, 2007). Further increases in the cost of fertilizers are likely because the source of most P fertilizers—rock phosphate—is a non-renewable resource, and the price of N fertilizers tends to increase concurrently with the price of oil (Cordell *et al.*, 2009; Vinod and Heuer, 2012). Thus, strong environmental and economic reasons exist to improve the efficiency of fertilizer use in agriculture in general, and in rice in particular, because of the number of resource-poor farmers who rely on rice cultivation for their livelihood (Cassman and Pingali, 1995).

The Green Revolution package has generally been adopted in favorable environments, particularly those characterized by a reliable water supply (Otsuka and Kalirajan, 2006), which account for just above 50% of the area on which rice is cultivated (Dobermann and Fairhurst, 2000). The remaining rice is cultivated in less favorable environments of the rainfed lowland, flood-prone, or upland rice ecologies where production is constrained by drought, excess water, low general soil fertility or some other soil-related problem, or a combination

of such factors (Haefele *et al.*, 2014). While it has been maintained that HYMVs can perform well over a range of environments (Khush, 1995), this appears to be due to their high harvest index and not because of any specific adaptation to stressful or infertile environments (Rose and Wissuwa, 2012). Screening experiments on P-deficient soils have clearly indicated that some traditional varieties outperform modern varieties of rice (Tyagi *et al.*, 2012; Wissuwa and Ae, 2001) or sorghum (Leiser *et al.*, 2012). Since HYMVs were selected on breeders' stations under high-input conditions, it is likely that specific traits conferring advantages under low-input conditions were lost, particularly if these traits come with a yield cost under high-input conditions. The *PSTOLI* gene conferring tolerance to P deficiency is a good example, as it is absent from most of the International Rice Research Institute (IRRI) breeding material developed for the irrigated lowlands, but is present in many breeding lines targeted for unfavorable environments (Chin *et al.*, 2010).

Root traits will likely play a key role in overcoming the issues outlined above of (i) environmental pollution due to high fertilizer inputs and inefficient utilization, (ii) increasing cost of fertilizer inputs that erode farming profits, and (iii) poor yield under low-input conditions. Indeed, optimizing root traits using a plant-breeding approach has been suggested as an economically viable option for improving the efficiency of N, P, and Zn use in rice systems (Ahmadi *et al.*, 2014; Ismail *et al.*, 2007; Vinod and Heuer, 2012). Potential root traits of interest will not be reviewed in detail here because several recent reviews have attempted to identify traits crucial to improved nutrient capture in rice (Rose *et al.*, 2012; Vandamme *et al.*, 2015; Vinod and Heuer 2012). While root traits associated with N, P, and Zn capture in rice cultivations systems and phenotyping strategies for target environments are discussed, the major focus of this review is how trait-based selection, including that for linked markers, has been conducted in the past and how recent game-changing developments in genotyping and phenotyping technologies may facilitate rapid developments in this area in the near future.

Enhancing rice nutrient capture through breeding: target environments and modes of selection

Target environments: low-input versus high-input systems

High- and low-input rice cultivation systems present distinct problems with regard to enhancing nutrient capture by roots. In low-input systems, soil bioavailable nutrient levels of one or all of N, P, and Zn are typically below the levels required for maximum crop yields (Ismail *et al.*, 2007). From a breeding perspective, the aim is to select breeding lines for root traits that enable a nutrient-stressed plant to acquire as much of the limiting nutrient(s) as possible (Rose *et al.*, 2012; Wissuwa *et al.*, 2009), which may involve acquiring nutrients that are present in the soil in low plant-availability forms (Vandamme *et al.*, 2015). Assuming a standard fertilizer response curve for grain yield,

the resulting varieties would be characterized by an upward shift of the response curve in the nutrient-deficient range (Fig. 1A). This is in contrast to high-input systems, where maximum yields in relation to nutrient inputs are already achieved. Breeding targets in these systems would be root traits that maximize the capture of the nutrient(s) applied as fertilizer, as well as native soil nutrients, to reduce the fertilizer inputs required for maximum yields and minimize nutrient losses to the surrounding environment (Rose and Wissuwa, 2012). This effectively means the yield response plateau would shift toward optimum yields being achieved at lower fertilizer inputs (Fig. 1B).

To what extent these different scenarios require different root traits or could be accomplished by the same traits is currently not known and may depend on the specific characteristics of soil–nutrient interactions. However, evidence from selection experiments conducted in high-/low-input environments indicate that selection in the target environment is more efficient for improving the respective yield in response to P of oats (Atlin and Frey, 1989), sorghum (Leiser *et al.*, 2012), and rice (Wissuwa and Ae, 2001; Mori *et al.*, 2016). The results are less clear for selection under N-limited environments; Bänziger *et al.* (1997) concluded that selection in the respective target environment is most efficient in maize, whereas no such advantage was seen for oats (Atlin and Frey, 1989). This could suggest that root traits enhancing P uptake in low-input environments would differ from traits conferring superior performance in a high-input environment. Whether these traits are related to roots or even nutrient uptake is typically not determined in high-input environments, and thus very little is known as to the extent of trait contribution to nutrient uptake across a range of environments. However, at least for nutrients that strongly interact with soil constituents with subsequent effects on plant-availability, such as P and possibly Zn, one may conclude that selection should be done in the target environment. In the following section we will focus predominantly on nutrient-limited environments because nutrient uptake studies have typically concentrated on these environments.

Direct, indirect, and marker-aided selection

Most breeding programs rely on direct selection for yield, which typically is augmented by selections for pest and disease resistance and important grain quality traits, and it is rare that nutrient acquisition is explicitly considered or selected for. However, the presence of large genotypic variation within rice for performance under P deficiency (Wissuwa and Ae, 2001), N deficiency (Singh *et al.*, 1998; Tirol-Padre *et al.*, 1996), or Zn deficiency (Wissuwa *et al.*, 2006) suggests that selection for nutrient acquisition would be successful if practiced. Traditional varieties tend to outperform modern varieties for nutrient acquisition under deficient conditions (Leiser *et al.*, 2014; Wissuwa and Ae, 2001), which would indicate that they were selected under similarly deficient conditions in the pre-Green Revolution era, and that HYMVs may have lost adaptive traits and genes during the selection under high-input conditions that has been practiced over the past 50 years (Wissuwa *et al.*, 2009).

The conventional breeding approach would be to select one of these traditional varieties with superior nutrient uptake as a donor to be used in crosses with elite breeding lines. Subsequent selection within segregating generations evaluated under nutrient-deficient conditions would produce offspring combining improved nutrient capture with positive attributes of the elite material. This approach has produced high-yielding rice lines with superior tolerance to Zn deficiency (Gregorio, unpublished) and should, in principle, work for other nutrient deficiencies as well. However, little evidence exists that it has been successful in terms of producing rice varieties that have been officially released and are widely grown by farmers. Several factors are responsible for this apparent lack of success:

- (i) Variety release procedures require proof of superior yield performance in regional or national variety trials, and these are typically conducted under recommended management practices that include optimum doses of

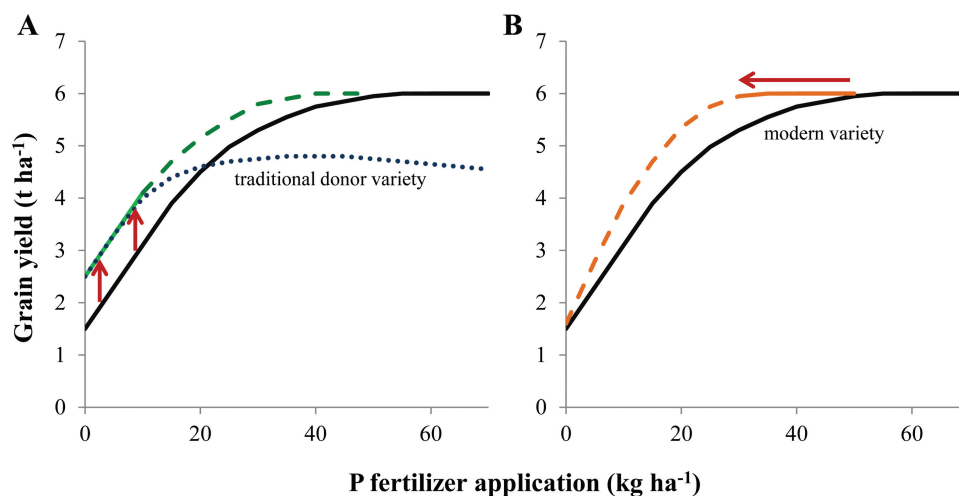


Fig. 1. Yield response curve of a hypothetical modern variety versus a potential traditional donor variety with improved capacity for nutrient acquisition under low-input conditions. (A) Identifying traits and underlying loci/genes in the donor, and subsequent transfer of these to the modern variety, will ideally lead to an upward shift of the response curve in the low-input range. (B) In high-input scenarios, root traits for efficient nutrient acquisition should lead to a backward shift of the yield plateau, allowing reduced fertilizer inputs and improved long-term sustainability. (This figure is available in colour at JXB online.)

- fertilizers. As such, superior performance under low-input regimes may not be evaluated, and any yield penalty under optimum conditions would prevent varietal release.
- (ii) In addition to grain yield, breeders are required to optimize a host of disease resistance and quality traits, and concerns that an exotic donor will upset the balance of these additional traits makes breeders reluctant to utilize such donors.
 - (iii) Breeding programs are typically conducted with optimum input. Adding a parallel selection scheme under low-input conditions increases cost considerably, which may not seem justified given the varietal release criteria described under (i).
 - (iv) The provision of optimum amounts of fertilizer reduces environmental variation and increases the heritability realized in a breeding program. Each cycle of selection may therefore provide less genetic gain under the more variable low-input conditions.

The success of selection in plant breeding has been expressed as the response to selection (R), which is a function of the heritability of a trait (h^2) and the selection differential (S) (Fig. 2), which in turn depends on the selection intensity (i) and the amount of variation (σ) present. Where low heritability and/or the considerable additional cost of direct selection for grain yield under low-input conditions disfavors this approach, indirect selection for a trait that is closely associated with performance in the target environment can be an alternative approach. Such indirect selection can lead to bigger gains from selection if variation for the trait can be evaluated with a high degree of precision that increases the heritability of the indirect trait relative to the heritability of direct selection. Furthermore, indirect selection may allow higher selection intensities if a larger number of lines can be evaluated for an easy-to-assess or cost-efficient indirect trait than for direct yield evaluation in the field. However, a key requirement for successful indirect trait selection is that this indirect trait (Y) is closely correlated (r) to the target trait (X). Following Falconer (1960), indirect selection would produce a better response to selection if the indirect term $i_Y \cdot h_Y \cdot r$ is bigger than the direct term $i_X \cdot h_X$ (Fig. 2). Since we

can assume that the correlation between indirect and target trait is $r < 1$, the term $i_Y \cdot h_Y$ should be considerably larger than $i_X \cdot h_X$ if indirect selection is to be superior to direct selection. A low correlation should generally result in a poor response to indirect selection; thus, care needs to be given to establish the predictive power of the indirect trait, and this should be increasingly important the more artificial trait evaluation protocols are (i.e. nutrient solution). Finally, it should be pointed out that indirect selection can and has been practiced for root traits following conventional as well as modern molecular breeding approaches.

We have pointed out above under (ii) that breeders may be reluctant to utilize ‘exotic’ donors in breeding programs for fear of upsetting the balance between several required traits in their elite breeding material. Backcross breeding, particularly when coupled with marker-aided selection (MAS), is the method of choice to overcome this constraint. Markers are typically identified through quantitative trait locus (QTL) mapping. Analogous to the discussion above, two basic approaches have been employed in screening studies designed to identify QTLs for enhanced nutrient uptake. The first approach—direct phenotyping—is simply to screen mapping populations in the target environments and to measure either grain yield or nutrient uptake in shoots. The main advantage of this approach, if successful, is that the best-performing lines from QTL mapping can easily enter breeding programs. Furthermore, breeders are more likely to be convinced that identified QTLs and linked markers have utility in applied breeding if their positive effect was detected or at least confirmed in the target environment. Successful examples of this direct approach are the *Pup1* locus enhancing P uptake (Chin et al., 2010; Wissuwa et al., 2002), and *DTY* for yield under drought (Kumar et al., 2014). Mapping QTLs for indirect traits has typically followed the concept of ideotype selection (Ahmadi et al., 2014), focusing mostly on root anatomical and architectural traits such as maximum root length, which is assumed to enhance water and nitrate uptake from deeper soil layers. Given the difficulty of evaluating roots in the field, phenotypic characterization is typically done in specific off-field

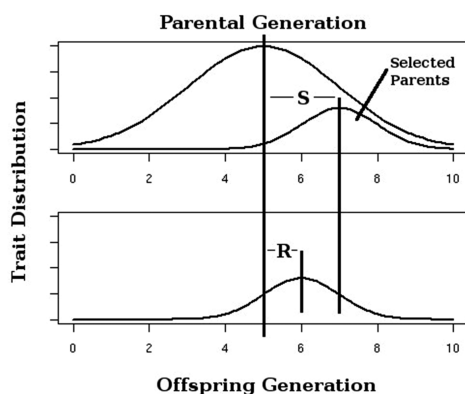


Fig. 2. Response to selection (Source: Pete Hurd, Wikipedia commons) for a trait X such as grain yield, and comparing direct selection for X versus indirect selection for a correlated (root) trait Y . The selection differential (S) can be expressed as the product of selection intensity (i) and phenotypic standard deviation (σ_P ; square root of phenotypic variance).

Response to selection R

$$R = h^2 \cdot S \quad (\text{direct selection})$$

$$= i \cdot h \cdot \sigma_A$$

$$R_{YX} = i_Y \cdot h_Y \cdot r \cdot \sigma_{AX} \quad (\text{indirect selection})$$

Indirect selection for trait Y to improve trait X
superior if $i_Y \cdot h_Y \cdot r > i \cdot h$

setups that should increase the repeatability of measurements and thereby the heritability of the trait.

We have seen that breeding for enhanced nutrient capture involves a series of decisions on the part of the breeder as to whether a specific environment or nutrient availability level should be targeted and whether selection should be done directly for performance in that environment or for an indirect trait predictive of performance in the target environment. Indirect trait selection can have several advantages, such as more reliable or cost-effective phenotyping; however, in order to be implemented by breeders, the indirect trait needs to be highly predictive of yield performance in the field. In the following section we provide a brief overview of approaches taken to identify traits and genes for improved nutrient capture and review the evidence (or lack thereof) that such traits and genes have been employed in crop breeding.

Root traits and associated genes and their current level of utilization in rice breeding

A wealth of reverse genetic studies have been published for rice because it is a monocot model with a relatively small, well-annotated genome (Goff *et al.*, 2002; Yu *et al.*, 2002) and is receptive to transformation (Slamet-Loedin *et al.*, 2014). Q-TARO (<http://qtaro.abr.affrc.go.jp>), an online database for QTL annotation, contains the OGRO (Overview of Functionally Characterized Genes in Rice Online) database, which lists 1158 entries for functionally characterized rice genes (Yamamoto *et al.*, 2012). Using 'root' as a filter, Q-TARO retrieves 98 gene entries, while filtering for 'other soil stress tolerance' retrieves 64 gene entries. Of these 162 functionally characterized root-related genes, most are annotated as being related to transport and transcriptional or hormonal regulation (Fig. 3). The vast majority (98%) of these genes have been identified through reverse-genetic approaches, and for only three genes (*PSTOLI*, *DRO1*, and

Bet1) was the gene identification based on natural allelic variation affecting phenotypes.

While, to the best of our knowledge, none of the 159 genes characterized using reverse genetics has been utilized in rice variety development, both *PSTOLI* for P starvation tolerance (Gamuyao *et al.*, 2012) and *DRO1* for deep rooting (Uga *et al.*, 2013) are utilized in MAS. Both genes were identified through QTL mapping based on biparental populations, which has been the method of choice in forward genetic studies over the past two decades. However, the overall track record of QTL mapping in providing selection tools for rice breeding has not been much better than the mutant-centered reverse genetic approach. The EURoot Database (<http://gohelle.cirad.fr:8080/euroot>) curating data on root-related genes and QTLs lists an astonishing 749 rice root QTLs, the majority of which are related to root morphology. Of the 1051 QTLs for rice found in Q-TARO, 60 are listed under 'root' and 72 under 'other soil stress tolerance'. Despite this large number of available QTLs, the only QTLs related to nutrient uptake used to date are the *PSTOLI* gene at the *Pup1* locus (Chin *et al.*, 2010) and the root length QTL *qRL6.1* enhancing nitrate uptake from deeper soil layers (Obara *et al.*, 2010). First yield trials with *Pup1* breeding lines conducted in Asia and Africa indicate that *Pup1* is particularly effective in enhancing early crop development under P deficiency (Fig. 4) and in reducing the delay in maturity typically observed under P deficiency. The extent to which grain yields are improved seems to partly depend on the genetic background of the breeding lines and to increase with a concomitant occurrence of mild drought (Wissuwa *et al.*, unpublished data).

If there is no shortage of genes and QTLs controlling root traits, the question must be asked why so few have been adopted in breeding programs. What are the main limitations or bottlenecks that need to be overcome in order for breeders to invest resources in trait-specific, indirect selection or MAS for underlying genes and/or loci? Several factors are likely to play a role:

(i) Lack of positive allelic variation for key genes.

We have seen that most genes identified via mutant studies are loss-of-function mutations with detrimental effects. Very few gain-of-function mutations exist that would increase nutrient acquisition or other agronomically relevant traits. While having established the role of a certain gene in root development through its loss-of-function phenotype provides insight into key biological processes, its utilization in breeding would require the presence of alleles that improve the phenotype relative to the wild type. Any impact on varietal improvement will be nil as long as such positive alleles are missing. Even in cases where knockout, knock-down, or ectopic expression have led to phenotypic improvements, the fact that these allelic changes have been obtained through transgenic approaches renders the product a genetically modified organism and thus makes it difficult to be utilized in breeding.

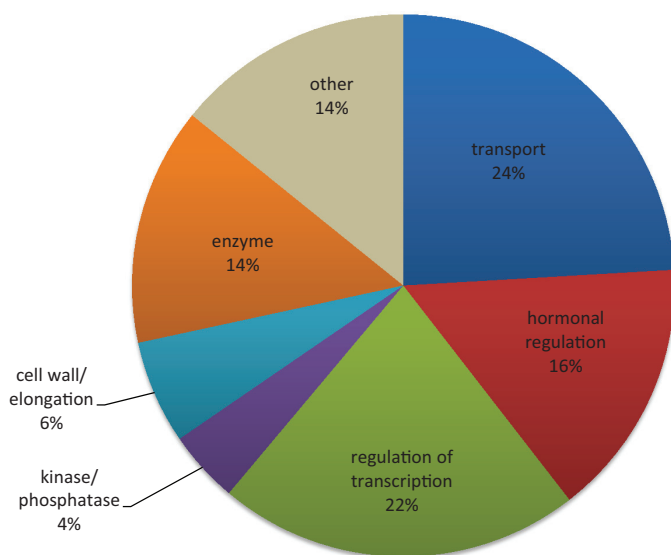


Fig. 3. Ontology of root and soil stress-related genes identified in the Q-TARO database (<http://qtaro.abr.affrc.go.jp>). (This figure is available in colour at JXB online.)

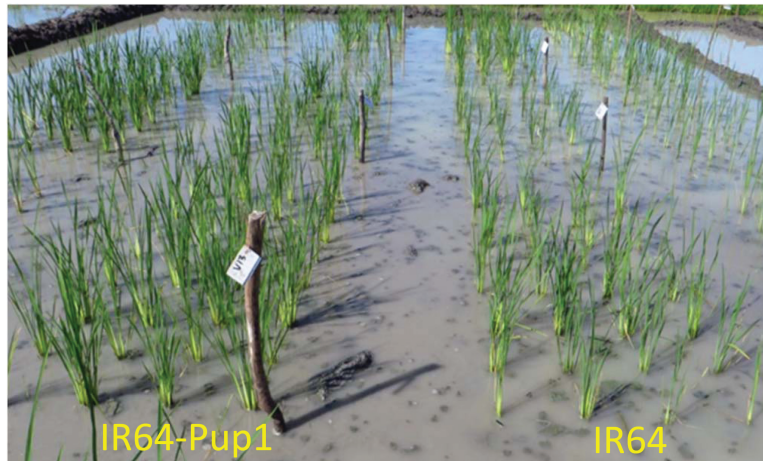


Fig. 4. *Pup1* breeding lines in an IR64 background in a P-deficient field in Africa showing superior biomass accumulation at the tillering stage.

(ii) *Insufficient precision of mapping of most QTLs.*

The average interval size of the 132 root-related entries found in Q-TARO is around 3.3 Mb. Since most of the QTLs identified stem from exotic landrace donors and have large interval sizes of several Mb, this will invariably be associated with linkage drag, with possible detrimental effects on other traits of relevance in breeding programs. Without further fine mapping and/or candidate gene evaluation studies, production of robust marker sets for marker assisted introgressions is difficult.

(iii) *Failure to demonstrate that discovered loci are stable across a range of genetic backgrounds.*

Rice QTL studies typically use donor and recipient lines of phenotypic extremes in order to maximize detection potential, which commonly results in overestimation of trait contributing effects. In extreme cases, ‘negative’ QTLs, similar to loss-of-function mutants, are identified. Ideally, QTL effects should be detected or at least confirmed in the genetic background of target cultivars that require improvements.

(iv) *Insufficient association or predictive power between trait or gene/QTL and yield in the field.*

For a ‘yield under stress’ QTL where the association between the trait and yield was established by direct phenotyping for the target trait, confirmation of QTL effects across a variety of environments is needed. However, for indirect traits and associated QTLs, demonstrated yield advantage under stress and no yield penalty under non-stress needs to be demonstrated to attract the interest of breeders. As indicated in Fig. 2, a strong correlation between indirect trait/QTL and target trait is a condition that needs to be met if indirect selection is to be superior to direct selection.

While the above constraints have limited the large-scale deployment of advantageous root traits in breeding programs to date, a series of technological advances are changing the way associations between genotype and phenotype are established. We now discuss the potential for these new technologies to facilitate the incorporation of root traits conferring enhanced nutrient acquisition into rice breeding programs.

Game-changing genotyping, genetic manipulation, and phenotyping technologies

Genomic resources and tools for rice genotyping enable the interrogation of chromosomal regions associated with traits of interest at maximum resolution across a huge range of germplasm. Novel alleles can be identified for any gene of interest, and allelic activities can be tested and, if promising, can be included in breeding programs. Consequently, genotyping and the detection of useful genetic variation no longer represents a significant bottleneck, making the identification of causal variants or variants tightly linked to causal mutations no longer comparable to finding the proverbial needle in a haystack. A high-quality *O. sativa* ssp. *japonica* reference genome was published in 2002 (Goff *et al.*, 2002; Yu *et al.*, 2002) and has been refined through continuous annotation and curation (Kawahara *et al.*, 2013). Today, additional *de novo* assemblies are available for *O. sativa* ssp. *indica* and *O. sativa* ssp. *aus* (Pan *et al.*, 2014; Schatz *et al.*, 2014; Wang *et al.*, 2014), as well as for several wild rice species (Chen *et al.*, 2013; Wang *et al.*, 2014), allowing for intra- and inter-specific comparative genomics and subspecies-specific mapping of re-sequencing data, which are available for hundreds of rice cultivars (Huang *et al.*, 2010). Recently, the 3000 rice genomes project published the sequences of a global diversity panel (The 3000 rice genomes project, 2014). The accessions are mostly traditional landraces collected from 85 countries, representing adaptations to most environments suitable for rice cultivation. The data have been organized in a database with a versatile web interface, SNP-Seek (<http://www.oryzasnp.org/iric-portal/>), that facilitates the mining of over 20 million single nucleotide polymorphisms (SNPs) across 3000 accessions. SNP-Seek can be queried for genetic regions, specific varieties, predefined SNP lists (e.g. haplotypes or known causal variations), SNP types (e.g. non-synonymous SNPs), and annotated genes (Alexandrov *et al.*, 2015). Most importantly, accessions of interest from the 3000 genome set can be obtained unrestricted from the IRRI genebank and used for downstream applications. These include genome-wide association studies (GWAS), phenotypic confirmation studies on

haplotypes underlying QTLs or GWAS peaks, and the subsequent development of pre-breeding materials. For example, to confirm the predictability of a GWAS-derived peak for early root vigor from an Indica-specific diversity panel, we assessed the phenotypic performance of a subset of 3000 genome accessions with contrasting haplotypes in the peak region. Accessions carrying the haplotype that was associated with enhanced root vigor in the GWAS did indeed display enhanced performance in an identical screen, thus confirming the utility of this haplotype in predicting early root vigor beyond the variety range of the GWAS panel (Wang *et al.*, unpublished). Moreover, analysis of non-synonymous SNPs and small intragenic insertions/deletions that were linked with the respective haplotypes enabled identification of a high-priority candidate.

In addition to SNP-Seek, a web interface for allele mining is available for a database comprising low-coverage whole genome sequencing (WGS) information of around 1500 lines, RiceVarMap (<http://ricevarmap.ncpgr.cn/>) (Zhao *et al.*, 2015). Following a GWAS, we have queried this database for novel haplotypes in peak regions of interest and thereby were able to prioritize candidate genes for P use efficiency (Wissuwa *et al.*, 2015) and root efficiency (Mori *et al.*, 2016).

Genotypes that are not represented in public databases can be assessed using novel high-density SNP genotyping platforms based on oligonucleotide arrays ('chips') or next-generation sequencing (NGS). Three *Oryza*-specific medium-density chips have been developed for the public domain, one 6000 SNP chip (Yu *et al.*, 2014) and two 50000 SNP chips (Chen *et al.*, 2014; Singh *et al.*, 2015). They reliably produce robust data that require minimal bioinformatics support for downstream analyses such as diversity studies, fingerprinting, saturated QTL mapping, and GWAS. For higher-density requirements, genotyping by sequencing (GBS) typically yields >50000 markers and was shown to be suitable for high-density GWAS (Begum *et al.*, 2015) and genomic prediction studies (Spindel *et al.*, 2015) in an active irrigated rice breeding program. Although these studies did not assess root traits, they demonstrate the predictive power of high-density genotype-to-phenotype associations in a direct breeding context. A GBS-based GWAS approach was furthermore utilized to identify a range of novel loci for a variety of root traits expressed under controlled environments (Courtois *et al.*, 2013). Although peaks and derived haplotypes and markers were not confirmed in an independent study, several of them colocalized with known meta-QTLs for root traits, adding confidence to the presented approach.

Concomitantly, novel biotechnology tools are emerging that enable precise manipulation of the genome in an unprecedented fashion (Lee *et al.*, 2015). Genome editing via site-directed nucleases (SDNs), such as TALEN and CRISPR-Cas9, allows targeted genome editing in rice that ranges from the introduction of small mutations in loci of interest to replacement of alleles and insertion of genetic material (Schaeffer and Nakata, 2015; Shan *et al.*, 2014). Intriguingly, although SDNs still have to be introduced via classic transgenic approaches, they act remotely in the genome and introduce changes that are not necessarily transgenic in

nature. Once the desired genome modifications have been obtained, the SDN transgene can be removed by segregation, leaving a product that (provided no transgene was introduced via SDN-mediated modes of action) is free of transgenic traces and indistinguishable from a classic breeding product.

Given the inherent complications in measuring or visualizing roots growing in soil, phenotyping of root traits has been and will remain a bottleneck in root research. Yet technological and conceptual advances have also provided breakthroughs in phenotyping. These include non-destructive *in situ* root phenotyping platforms based on X-ray and MRI technologies (Ahmed *et al.*, 2015; Bao *et al.*, 2014; Schulz *et al.*, 2013) that resolve structures on different scales, ranging from entire root systems in large soil volumes to root hairs around single roots (Keyes *et al.*, 2013). In the field, the 'shovelomics' concept has been developed to allow rapid high-throughput phenotyping of root angles and lateral branching patterns in QTL mapping populations or association panels (Trachsel *et al.*, 2011). Other high-throughput technologies involve the core break technique (Wasson *et al.*, 2014) and three-dimensional root system imaging of young plants in transparent gellan gum (Clark *et al.*, 2011; Topp *et al.*, 2013). Furthermore, medium- to high-throughput platforms for automated phenotyping of plant growth over time in greenhouses are available (Hairmansis *et al.*, 2014) and, while direct phenotyping of root traits is not yet established, indirect growth responses as a result of different root architectures can be assessed. Similar indirect ways of assessing the effects of root traits are available in the field through remote sensing technologies based on sensors mounted on tractors, semi-stationary above the canopy, or fully mobile on unmanned aerial vehicles (Walter *et al.*, 2015; Zaman-Allah *et al.*, 2015). Such indirect phenotyping strategies are ideally suited to confirming hypotheses regarding the positive effects of certain root ideotypes on field performance (Ahmadi *et al.*, 2014).

Harnessing new technologies to overcome old problems

Earlier in this review we identified four key limitations or bottlenecks that need to be addressed if root traits and underlying genes or markers are to be utilized in crop improvement. We believe that some of the game-changing technologies discussed offer opportunities to overcome such limitations.

(i) *Lack of positive allelic variation for key genes.*

WGS databanks such as the 3000 rice genomes constitute effective tools for allele mining of candidate genes that have been prioritized as trait-contributing through classical QTL studies, GWAS, or reverse genetic approaches. Using SNP-Seek, RiceVarMap, and other public rice WGS databases, genes of interest can be assessed for allelic diversity across thousands of varieties. Subsets of lines with highly contrasting alleles can then be subjected to phenotypic screening to assess the utility of different alleles. At loci of importance where exploitable natural variation is lacking, genome editing can introduce novel allelic variation. A prominent example of a gain-of-function phenotype obtained via genome editing

is the targeted mutagenesis of the fragrance gene *BADH2*, which confers aroma in a non-aromatic background when its function is abolished (Shan *et al.*, 2015).

(ii) *Insufficient precision of QTL mapping.*

Recently, GWAS have become an established method for QTL identification in rice (Huang *et al.*, 2010; Zhao *et al.*, 2011), including for root traits (Courtois *et al.*, 2013) and nutrient efficiency traits (Wissuwa *et al.*, 2015). In terms of precision and broad-range utility, GWAS have several advantages over classical QTL studies. The high-density genotyped panels used in GWAS are usually fixed accessions, which removes limitations in seed availability and allows for repeated phenotyping for any trait across locations and years. Depending on the genotyping density and local linkage disequilibrium, GWAS peaks in rice tend to be an order of magnitude smaller than classical QTLs. Furthermore, instead of flanking and peak marker information, GWAS indicate specific haplotypes, which tend to be clusters of SNP markers across linkage blocks significantly associated with the phenotype. Haplotypes can serve as valuable trait fingerprints for the identification of novel donors and for the design of specific markers for precise introgressions. Negative aspects of GWAS include the failure to detect rare alleles and the common occurrence of false-positive associations, often due to hidden population structure.

Thus, while the detection of a set of selectable (haplotype) markers with sub-cM precision is rapid, subsequent validation of GWAS peaks across an independent panel is a requirement in order to avoid false-positive associations. The 3000 rice genomes and other fully sequenced panels make valuable haplotype validation sets. SNP haplotypes that are positively associated with superior expression of a phenotype of interest and the respective haplotypes of the same region that are associated with low performance can be used in SNP-Seek or other queryable databases to filter for accessions with corresponding SNP haplotypes. Since they are likely different from the accessions used in the original studies, they comprise independent validation lines. If the identified haplotypes were indeed predictive for a portion of the trait of interest, then validation lines with 'beneficial' haplotypes should be outperforming validation lines with 'non-beneficial' haplotypes, when subjected to the same phenotypic screen as in the original GWAS. This approach allows an independent validation of GWAS peaks, facilitates the identification of further accessions with beneficial haplotypes, and would allow for evaluations of peak robustness if confirmation experiments are conducted across environments.

(iii) *Failure to demonstrate that discovered loci are stable across a range of genetic backgrounds.*

Compared to biparental QTL studies, GWAS query a much larger portion of the gene pool for variation. Consequently, identified loci will have shown their efficacy across the whole panel, avoiding very specific background-related effects. In addition, GWAS immediately allows the distinction between negative-effect loci, where minor haplotypes cause below-average performance, and positive-effect loci, which enhance

performance above the common haplotype/ allele. The latter case is likely of greater relevance in breeding, allowing introgressions of novel haplotypes/alleles from a donor with extremely positive trait expression. While we suggested usage of the WGS information on panels such as the 3000 rice genomes for GWAS peak confirmation, these panels, or likely subsets thereof, represent a reservoir of accessions to be selected for purpose-made association panels. Subpopulation-specific panels or global panels can be assembled according to known or expected phenotypic performance: Based on geographical origin and geographic information system data, such panels can be enriched with accessions originating from demonstrated nutrient-deficient areas, thus enriching rare specific alleles that would otherwise fall below the detection threshold.

(iv) *Insufficient association or predictive power between trait or gene/QTL and yield in the field.*

We have pointed out above that novel technologies will improve the efficiency of locus identification and confirmation, and that a much broader range of allelic variation can be queried and possibly utilized in rice improvement. However, genomic and genetic tools are less likely to have a positive impact on the fourth major limitation identified earlier, the need for a root trait or an underlying locus/gene to be closely associated with, or predictive of, performance in the field. Lack of association between trait or locus/gene and performance may have two distinct causes: (a) trait evaluation and trait expression in the field can be poorly correlated, indicating that the methodology applied in phenotyping was inappropriate; or (b) little effect of a certain phenotype in a field environment.

The first of these causes is a technical problem that is inherent to root research due to the difficult soil environment that typically necessitates simplified screening protocols to visualize root traits. Oversimplification is one particular danger, as recently shown by Nestler *et al.* (2016), who indicated that root hair length and density varied dramatically between nutrient solution-grown and field-grown plants. Large error is another potential problem. New phenotyping technologies based on X-ray or MRI technologies can remove some constraints, as certain aspects of root architectures are visualized *in situ* without the potential disrupting effect introduced by excavating roots or the randomness and lack of three-dimensional context of coring.

The second cause is a conceptual rather than technical problem. Hypotheses regarding the effects of certain root traits on performance in the field may prove to be incorrect. But more likely it is the interplay between a host of environmental factors in the field and from season to season, that cause inconsistencies of effects of certain root traits. A very variable and well characterized root trait in rice is root growth angle (RGA). Upland rice cultivars typically exhibit steep RGAs that presumably increase water uptake from deeper soil layers, while on the opposite extreme surface rooting can be observed in some genotypes (Fig. 5). QTL for surface rooting (*qSOR1*; Uga *et al.*, 2012) and deep rooting (*DROI*; Uga *et al.*, 2013) have been identified. Apart from these major

QTL, minor effect QTL changing RGA in a *DRO1* background have been identified (Kitomi *et al.*, 2015) and additional variants for surface rooting are currently being mapped (Fukuta, unpublished). Thus, it should be possible to modify rice root systems to match any of the RGA types shown in Fig. 5.

Critical from a breeder's viewpoint is the question regarding the optimal RGA to target in breeding. A higher proportion of the deeper roots should improve water uptake from deeper soil layers during drought episodes that typically occur in rainfed rice. Modeling further suggests that nitrate uptake should also increase with deeper rooting (Lynch, 2013), and both factors have been attributed to improved yields of IR64-dro1 breeding lines (Uga *et al.*, 2015). However, shallow roots would be of advantage for nutrients that are possibly distributed more in top soil layers (P) or that are more plant-available in the oxidized surface layers (Zn) (Dunbabin *et al.*, 2013). The situation is straightforward if nutrient deficiencies are coupled with certain environments, but rainfed lowland rice in particular may experience drastic changes in water supply over the course of one season and certainly over different years. Thus, breeders need to carefully consider whether nutrient and water limitations are predictable enough in their target environment to breed for a specific root ideotype or whether root system plasticity is a more relevant concept (Kano *et al.*, 2011). The issue of adaptive versus constitutive roots traits should also be considered from a cost perspective: the ideal root trait would not incur a cost or yield penalty when crops are grown under non-stress conditions, and this may require the identification of adaptive or plastic root traits that are expressed only when environmental conditions make it likely that any incurred carbon cost is more than justified.

Improving nutrient acquisition for a sustainable future

Agriculture in the 21st century faces the challenge of how to feed an estimated 9 billion people. As scope to increase the arable land area is minimal, this necessitates increases in production from the area currently farmed (Cassman and

Pingali, 1995). Improving yields in low-input and low soil fertility regions will have to be realized to feed the human population and to alleviate the extreme poverty that is endemic among resource-poor farmers in such regions (Bishopp and Lynch, 2015). Toward this end, a 'Second Green Revolution' has been called for that would rely on breeding for root traits enhancing nutrient and water capture (Den Herder *et al.*, 2010; Lynch, 2007). In highly productive agricultural systems, this Second Green Revolution should shift emphasis from high input to high sustainability, envisioning a shrinking resource base rather than the widening resource base that underpinned the first Green Revolution.

Rice translational research is currently entering a new era, where novel genomic resources coupled with readily applicable bioinformatics and database tools are starting to bridge the gap between basic plant science and agricultural application (Alexandrov *et al.*, 2015; Wissuwa *et al.*, 2015; Zhao *et al.*, 2011). Genotype-to-phenotype relationships can thus be predicted and tested with unprecedented precision, facilitating the discovery and transfer of beneficial alleles and associated markers into existing breeding pipelines (Begum *et al.*, 2015; Spindel *et al.*, 2015). With regard to traits conveying efficient nutrient acquisition, it is very likely that modern high-yielding varieties have lost beneficial alleles or, as in the case of the *Pup1* locus, entire genes (Gamuyao *et al.*, 2012; Pariasca-Tanaka *et al.*, 2014). Such alleles and genes are awaiting discovery from among the large stock of genetic resources stored in genebanks. Progress in genotyping technologies has assured that genotypic characterization of such genetic resources is much less of a limitation than in the past.

As a result, phenotyping is commonly considered to be the bottleneck in breeding efforts, especially for improved nutrient and water capture, which would rely on largely 'invisible' root traits. However, the true bottleneck in terms of assuring traits and underlying genes are utilized in crop improvement is typically insufficient evidence that such traits improve performance in the field. Having identified in excess of 100 root-related genes and an equally high number of root-related QTLs in rice—of which possibly fewer than five are utilized

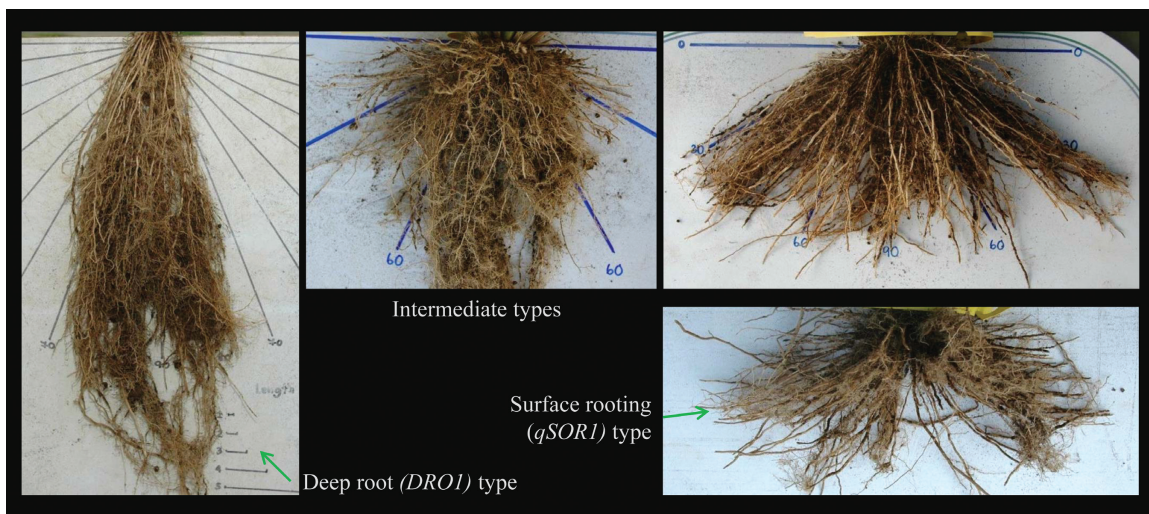


Fig. 5. Rice roots excavated from a P-deficient upland field following a 'shovelomics' protocol show a range of root growth angles from steep to shallow (Mori *et al.*, 2016).

in practical rice breeding—highlights the extent to which insufficient trait–performance associations have prevented successful breeding outcomes. For many genes that are considered important under stress, the distinction between genes involved in stress response and stress tolerance is crucially important (Pariasca-Tanaka *et al.*, 2009). Advances in conceptual, technological, and modeling approaches (Dunbabin *et al.* 2013; Lynch, 2007; Trachsel *et al.*, 2011; Walter *et al.*, 2015) are encouraging signs that trait–performance associations are increasingly being focused on, particularly with regard to root anatomical and architectural traits. However, the danger remains that technological capabilities dictate which trait is studied, rather than evidence that the trait actually improved field performance. This danger highlights the need for better integration between scientific disciplines focusing on trait development on one hand and applied breeding on the other.

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