Vinod KK, Heuer S.
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AoB PLANTS, 2012; 2012:pls028-1-pls028-18

Originally published at: http://dx.doi.org/10.1093/aobpla/pls028

http://hdl.handle.net/2440/98219

Open access – Review

THIS PAPER IS PART OF A SPECIAL ISSUE ENTITLED 'IMPROVING CROP TOLERANCE TO STRESS'

Approaches towards nitrogen- and phosphorus-efficient rice

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Abstract

Background and aims Food production has to increase to meet the demand of a growing population. In light of the high energy costs and increasingly scarce resources, future agricultural systems have to be more productive and more efficient in terms of inputs such as fertilizer and water. The development of rice varieties with high yield under low-nutrient conditions has therefore become a breeding priority. The rapid progress made in sequencing and molecular-marker technology is now beginning to change the way breeding is done, providing new opportunities.

Scope Nitrogen (N) and phosphorus (P) are applied to agricultural systems in large quantities and a deficiency of either nutrient leads to yield losses and triggers complex molecular and physiological responses. The underlying genes are now being identified and studied in detail, and an increasing number of quantitative trait loci (QTLs) related to N and P uptake and utilization are being reported. Here, we provide an overview of the different aspects related to N and P in rice production systems, and apply a breeder's perspective on the potential of relevant genes and pathways for breeding applications.

Main points For the development of nutrient-efficient rice, a holistic approach should be followed combining optimized fertilizer management with enhanced nutrient uptake via a vigorous root system, leading to increased grain filling and yield. Despite an increasing number of N- and P-related genes and QTLs being reported, very few are actively used in molecular breeding programmes. The complex regulation of N- and P-related pathways challenges breeders and the research community to identify large-effect genes/QTLs. For this it will be important to focus more on the analysis of tolerant genotypes rather than model plants, since tolerance pathways may employ a different set of genes.

Introduction

Life in Asia depends on rice not only because it provides >70 % of the daily calories for the population, but also because of its important role as a source of income for millions of rice farmers and landless workers (Dawe 2000). Global food security is at stake since the demand for rice is exceeding production. Furthermore, the increase in rice production shows a diminishing trend, falling from 1.6 % per annum in the 1990s to <1.0 % by 2010 (Food and Agriculture Organization of the United Nations 2003). Having surpassed the seven billion mark, global population growth is the lone serious factor that influences increased demand for rice (United Nations Population Fund 2011). With

increasing urbanization, land suitable and available for agriculture is diminishing and an increase in rice production has to be obtained by increasing productivity, i.e. an increased yield per unit land. In addition to improved and sustainable agro-management options, higher-yielding and more nutrient efficient genotypes have to be developed in order to secure rice production.

Mineral nutrition in rice requires 16 essential elements (De Datta 1981), of which nitrogen (N), phosphorus (P) and potassium (K) are applied to rice fields as chemical fertilizers in large quantities. Nitrogen and P are fundamental to crop development because they form the basic component of many organic molecules, nucleic acids and proteins (Lea and Miflin 2011). Estimates suggest that, from 2008 to 2012, global fertilizer demand will increase by 1.7 % annually, amounting to ∼15 million tons (Mt), of which 69 % is required in Asia alone (Food and Agriculture Organization of the United Nations 2008). By 2012, global demand for N fertilizer will increase by ∼1.4 % (7.3 Mt) and by ∼2 % (4.2 Mt) for P fertilizer. Since Asia's share of global rice production is >90 % (Gulati and Narayanan 2002), a substantial proportion of the increased fertilizer demand would be utilized for rice production (Witt et al. 2009). This is of growing concern because recent estimates (Food and Agriculture Organization of the United Nations 2008) indicate a declining trend in nutrient-use efficiency as a consequence of the fertilizer consumption exceeding the grain production. In addition, fertilizer prices are increasing due to high energy costs and because natural resources are limited and increasingly difficult to assess, as will be outlined in more detail below.

The mineral fertilizers, apart from their immense benefit, when applied in excess cause eutrophication of freshwater estuaries and coastal water ecosystems (Raven and Taylor 2003), and the increased emission of greenhouse gases, such as nitrous oxide (N_2O; Matson et al. 1998). If this practice of surplus fertilization continues, nutrient leaching and atmospheric contamination will soon become widespread problems in developing countries (Ortiz-Monasterio et al. 2001). However, excess P quickly becomes adhered to soil particles owing to its insoluble nature and can lead to deficiencies of copper, iron (Fe), manganese and zinc. Moreover, erosion of P-enriched soils can contaminate water bodies and trigger eutrophication in freshwater systems (Wolf 1996). Therefore, a balanced and sustainable use of fertilizers is of utmost importance. The food crisis in 2008 (Von Braun 2008), which triggered socio-political unrest worldwide, has given us a first glimpse of what is to come if we do not succeed in providing sufficient food at affordable costs, long term and in a sustainable way.

Nitrogen: constrictively plant available

In spite of being the most abundant element in the atmosphere (78 %), N is one of the most limiting nutrients in natural and agricultural ecosystems. It enters the soil only in miniscule amounts through natural precipitation and biological N fixation. Although significant N reserves are present in the soil ($2-20$ t ha^{-1}) (Bockman et al. 1990), only a limited amount is available to plants. Since plants require large quantities of N, greater than those of any other primary nutrient, plant assimilation of soil N often exceeds the amount being replenished (Epstein and Bloom 2005). Native sources of soil N include pre-existing inorganic and organic forms, net N mineralization from organic matter, biological N fixation, and N inputs from irrigation waters and deposition from the atmosphere (Cassman et al. 1996). The majority of plant-useable N is consumed as nitrate (NO_3) from well-aerated soils and as ammonium (NH_4^+) from poorly aerated, submerged soils (Huang et al. 2000). Although NH_4^+ uptake requires less energy than that of NO_3, only a few plant species, such as rice, are capable of growing exclusively with NH_4^+ (Kronzucker et al. 1999). Since rice is capable of assimilating both forms of N (Wang et al. 1993a; Kronzucker et al. 1998) it is adapted to aerobic as well as anaerobic growth conditions.

In traditional rice ecosystems, low-N stress is a problem in marginal areas where no or sub-optimal levels of N are applied (Laffite and Edmeades 1994a, b; Agrama et al. 1999) because farmers lack resources to purchase fertilizers and agricultural practices are often subsistence farming (Ortiz-Monasterio et al. 2001). Furthermore, diminishing non-renewable global energy resources, such as petroleum and natural gas, demand more efficient fertilizer utilization for two compelling reasons: (i) conservation of energy, because the chemical synthesis of N fertilizers requires ammonia produced by the Haber–Bosch process (Travis 1993), for which natural methane is the major hydrogen source; and (ii) curtailing the ever–increasing fertilizer costs. Although N deficiency, in particular, can be managed to a certain extent by addition of organic manures and by cultivation of fallow legumes (Balasubramanian et al. 2004), a significant reduction in N-fertilizer application can be achieved by optimizing the rate and timing of fertilizer application to synchronize N supply and demand (Peng and Bouman 2007; Chen et al. 2011). To further reduce N applications, an alternative approach is the development of varieties that use N more efficiently, either physiologically, that is, increased carbon gain per unit plant N and time, or agronomically, i.e. greater dry matter production and protein yield per unit N (Laungani

and Knops 2009). However, the major obstacle in achieving this is the low N-use efficiency of <40 %, which requires improvement in N foraging and uptake.

Crop varieties can respond to nutrient supply in four different ways, namely by being efficient or inefficient under nutrient deficiency, and as a responder or non-responder under nutrient sufficiency (Ortiz-Monasterio et al. 2001). Efficient genotypes are those that possess high external (uptake) efficiency, whereas responders are characterized by high internal (utilization) efficiency. Since nutrient uptake and utilization are interdependent, it is difficult to distinguish a responder from an efficient cultivar. It is therefore important to develop efficient selection criteria for low-nutrient tolerance, to differentiate between external and internal efficiency (Agrama 2006), and to assess genetic variability (Broadbent et al. 1987; DeDatta and Broadbent 1988). This necessitates a careful integration of both physiological and agronomic evaluation of cultivars under low- and high-nutrient regimes. For high-input systems, remarkable breeding efforts have already been carried out for the selection of responder varieties with high internal efficiency. This now has to be complemented with breeding of cultivars that are tolerant of nutrient deficiency and cultivars that maintain a high yield with reduced fertilizer inputs.

Selection in environments with low nutrient status is often plagued by problems of low heritability and high environmental variability. Although nutrient-scarce environments are not normally preferred by breeders, selection under such conditions will be more effective for yield per se rather than selection for yield potential alone (Muruli and Paulsen 1981; Blum 1988). Evaluation under field conditions is preferable over screenings in nutrient solution since the latter cannot simulate the complex soil–plant interaction. Furthermore, in the case of N, screening of genotypes at low N levels would additionally avoid problems of vulnerability to pests and diseases and lodging, which creates artefacts at high N levels (Tirol-Padre et al. 1996).

To establish selection criteria for higher uptake efficiency, it is essential to study yield components, biomass production, as well as nutrient assimilation in relation to the traits determining uptake efficiency such as a large root volume, efficient nutrient absorption and nutrient transport (Bassirirad 2000; Wang et al. 2006). This is particularly important because high nutrient-uptake efficiency without repletion of nutrients might accelerate nutrient depletion, while low-nutrient-efficient genotypes may lead to increased nutrient leaching or volatilization under high-nutrient situations (Ortiz-Monasterio et al. 2001). In addition, a clear understanding of environmental parameters, such as location effect, weather and radiation, and their effect on nutrient uptake in different genotypes, can leverage an objective-based distinction between uptake vs. utilization efficiency in the target environment. In practice, however, it is difficult to formulate a strict screening strategy for N use, because of the poor understanding of the underlying routes leading to better use efficiency. More often than not, genotype evaluations for N-use efficiency are conducted under two regimens: N-fertilized and native (no N fertilizer added) conditions. This may lead to identification of tolerant genotypes to zero fertilized conditions possessing traits related to low-N tolerance. Many researchers, however, argue that such varieties may be unsuitable for fertilized conditions because, in practice, no crop is going to be grown under unfertilized conditions. Therefore, they suggest a system-level approach if the target is N-use efficiency under fertilized conditions. For instance, a simulation study carried out in wheat that used parameters related to plant development, the size of N storage pools in the plant and traits related to root uptake efficiency for water from two contrasting environments showed that weather, N management, leaf N content and the phyllochron are the major determinants of N-use efficiency in wheat (Semenov et al. 2007). However, these factors may not hold good for rice, as it is adapted to flooded conditions. In general, modelling can be a useful tool to help identify the contributions of major factors influencing N-use efficiency, namely the environment, management and genotype.

Phosphorus: declining natural reserves and immobile in the soil

In contrast to N, P is a non-renewable natural resource and there is growing concern that the natural reserves of rock phosphate, the source of P fertilizer, are limited (Cordell et al. 2009). A recent study conducted by the International Fertilizer Development Centre concluded that currently known and easily accessible world rock phosphate reserves will last for approximately another 300–400 years (van Kauwenbergh 2010). Investments are therefore being made to discover new rock phosphate reserves and to develop alternative technologies to isolate P from e.g. marine sediments and faeces (see below). Another concern is the unequal geographical distribution of P reserves, with the vast majority of phosphate rock located in Morocco, followed by the USA and China (van Kauwenbergh 2010). The dependence of the world's food production on a few countries is problematic and policies should be put in place in time to ensure equal access to P reserves in the future. In fact, China is already imposing seasonal export taxes to secure the national supply of P fertilizer

(**http://agfax.com/2011/11/18/chinese-government-imposes-seasonal-export-taxes**).

Whereas P fertilizer is applied in excess in the Western world and some Asian countries (e.g. China, Japan and Korea; MacDonald *et al.* 2011), P deficiency is a major problem in many Asian and African countries, as well as in South America. Estimates suggest that ~50 % of agricultural soils are deficient in P (Lynch 2011). The two main reasons for this are (i) insufficient application of P in the form of P fertilizer or manure and (ii) P-fixing soil properties which render P unavailable to plants even if it is present in sometimes large amounts. Phosphorus imbalances in the world, with too much P in some countries and too little or inaccessible P in others (MacDonald *et al.* 2011), will require different measures and breeding strategies.

In modern agriculture, inorganic P fertilizer has widely replaced the application of manure. A large quantity of P present in animal and human faeces is therefore removed from the nutrient cycle and ends up in waterways, where it is no longer available and difficult to recycle (Cordell *et al.* 2009). In realization of this, technologies are being developed in Europe to extract P from sewage in urban centres to produce Struvite (ammonium magnesium phosphate) that can be used as P and N fertilizer (**http://www.ceep-phosphates.org/Files/Newsletter/scope50.pdf**). In light of decreasing rock phosphate reserves, these efforts are extremely important and, once applied on a large scale, this technology will become more competitive in terms of production costs.

The high concentration of P in human and animal faeces is due to the consumption of phytic acid (PA, inositol hexaphosphate), which is the major (50–80 %) storage form of P and is present in large quantities in cereal grains, legumes, soybean and other plants (for a review see Lott *et al.* 2000). Phytic acid is not digestible by humans and animals, and additionally it binds iron, zinc and other minerals, thereby reducing their bioavailability. Therefore, research is in progress that is aimed at reducing PA in crops (for a review see Raboy 2009). Apart from its role as an 'anti-nutritional factor', PA is the main source of P drainage from fields. Estimates suggest that 50–80 % of the P in cereal grains and legume seeds is stored as PA (Lott *et al.* 2009). Studies on low PA (*lpa*) mutants in rice (Larson *et al.* 2000; Liu *et al.* 2007) and barley (Bregitzer and Raboy 2006), however, showed that total grain P was generally higher in *lpa* mutants compared with wild-type controls, and this approach is therefore not directly applicable to reduce the P drainage from fields.

For soils naturally low in or depleted of P due to continuous cropping without repletion of P (and other nutrients), fertilizer or manure application is inevitable to maintain productivity and prevent soil degradation. However, continuous cropping of poor soil is often related to poverty, and breeding of efficient crops, therefore, has to be complemented by policy measures providing poor farmers with agricultural inputs. With regard to breeding for poor soils, crops with high P uptake and high internal P-use efficiency need to be developed to maximize yield in such low-input systems (Rose and Wissuwa 2012). In addition, a combination of both, uptake and internal-nutrient efficiency, is equally desirable for high-input systems since it would facilitate reduction of fertilizer doses without yield penalty. In rice, P fertilizer-use efficiency is only ~25 % (Dobermann and Fairhurst 2000), providing considerable scope for improvement.

For areas with P-fixing soils, high fertilizer application is currently necessary in order to provide sufficient plant-available P. Soils with P-fixing properties are widespread in the Asia Pacific and occur on 5 % of the total land area (Bot *et al.* 2000). In China, Indonesia, Japan, Thailand and Vietnam, P-fixing soils cover 9–15 % of the total land area. These numbers are even higher in Laos (24 %) and Myanmar (16 %). In Africa, P-fixing soils are especially widespread in Burundi, Congo, Liberia, Swaziland and Rwanda (16–29 %). Similar numbers are reported from South America, where P fixation occurs on 14–25 % of the total land area in e.g. Brazil, Colombia, Venezuela, Peru and Ecuador. In French Guyana, 79 % of the total land area has P-fixing properties (Bot *et al.* 2000). The development of crops that can access P reserves in these soils and that are highly efficient in P fertilizer uptake should therefore be a global breeding priority. In addition, it is critically important to develop crops with tolerance of multiple stresses because P deficiency is often a secondary effect in soils with high concentrations of iron and aluminium, and with low pH, which restrict root growth even if P is available (Ismail *et al.* 2007).

Nitrogen uptake and metabolism: genes and pathways

In rice, excessive N stimulates shoot growth, root inhibition, delayed flowering and senescence (Bernier *et al.* 1993; Stitt 1999), whereas deficiency results in stunted growth, chlorosis, poor yield and anthocyanin pigmentation due to carbohydrate accumulation (Martin *et al.* 2002). Under low-N conditions, rice plants attempt to acquire more N by increasing the root surface area, which increases the root-to-shoot ratio (Marschner *et al.* 1986) to varying degrees depending on the phenological stage (Sheehy *et al.* 1998). Rice genotypes show

significant variability for N uptake (external efficiency) and N utilization (internal efficiency) with yield being predominantly determined by the uptake process, particularly under low-N conditions (Singh *et al.* 1998; Witcombe *et al.* 2008). The most N-efficient rice genotypes are those capable of accumulating N in the first 35 days of transplanting (Peng *et al.* 1994). External efficiency declines as the crop progresses to maturity, with a reduction in the daily uptake of N towards terminal stages due to increasingly inefficient roots (Sheehy *et al.* 1998) and internal N recycling from senescing tissues to the developing panicle (Mae and Ohira 1981). Under low N supply, internal recycling accounts for 70–90 % of the total panicle N (Mae 1997; Tabuchi *et al.* 2007). On the other hand, the N concentration in the straw at crop maturity is not significantly affected by changes in N supply at terminal stages (Tirol-Padre *et al.* 1996). Hence, N uptake prior to panicle initiation is crucial in building up the internal N reservoir (Fig. 1).

The uptake process and N homeostasis are complex processes that involve recycling of N (especially amino acids) from shoots to roots via the phloem, and from roots to shoots via the xylem (Imsande and Touraine 1994; Marschner *et al.* 1997). In rice plants, particularly during the vegetative stage, roots play a significant role in N absorption with root density and distribution in the soil being the major determinants (Youngdahl

et al. 1982). Although there are many studies related to variation in N uptake in rice, there seems to be little information on differences in root morphology that may contribute to this variation. Root characteristics such as root length density and root weight density have been identified as important factors because N uptake is determined by root mass and N uptake per unit root volume (Shimono and Bunce 2009). A recent study on two rice cultivars by Fan *et al.* (2010) further indicates a significant influence of root morphological parameters and physiological characteristics on N-use efficiency at different growth stages.

The molecular responses to N are complex in rice, as shown by rapid induction/repression of many stress responsible genes and transcription factors coupled with repression of photosynthetic and energy metabolism genes in seedlings subjected to low N stress (Lian et al. 2006). However, intricate aspects of N utilization are well studied in model plants such as *Arabidopsis*. Many gene families, including NO_3 and NH_4^+ transporters and primary assimilation genes, amino acid transporters, as well as transcription factors and other regulatory genes, have been identified by different approaches. With the identification of orthologous genes from rice, opportunities are now emerging for utilizing these genes in marker-assisted breeding for N efficiency (for reviews, see Li *et al.* 2009b; Kant *et al.* 2011). Nitrogen
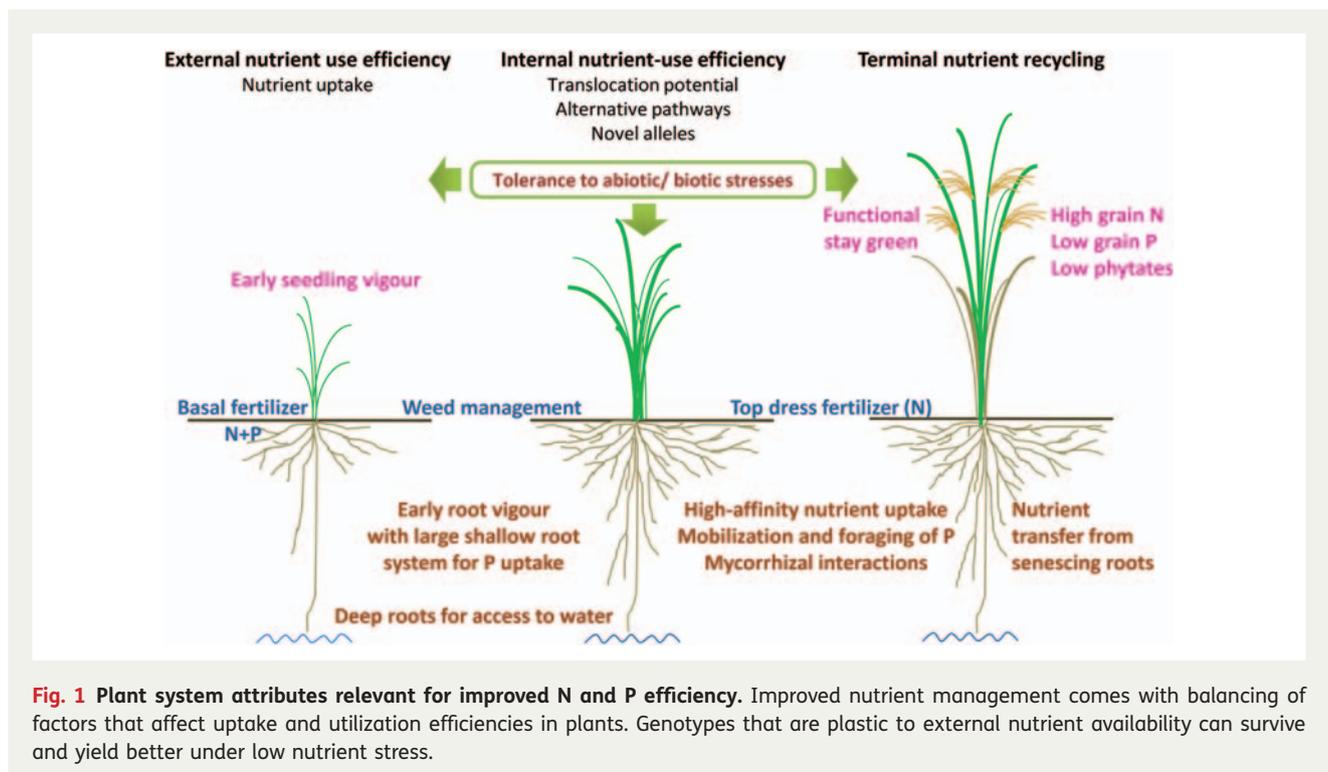
Fig. 1 **Plant system attributes relevant for improved N and P efficiency.** Improved nutrient management comes with balancing of factors that affect uptake and utilization efficiencies in plants. Genotypes that are plastic to external nutrient availability can survive and yield better under low nutrient stress.

uptake in roots is mainly regulated by a high-affinity transport system that regulates uptake at N levels <1 mM, and a low-affinity transport system that functions at higher N concentrations (Forde and Clarkson 1999; Glass et al. 2001; Williams and Miller 2001). It has been shown that the low-affinity NO_3 transporter OsNRT1 contributes predominantly to N uptake in the root epidermis and root hairs (Lin et al. 2000), acting in conjunction with the high-affinity and NO_3-inducible transporters NRT2 and NAR2 (Cai et al. 2008). Recent data provided further insight into the complexity of N uptake, showing that the mRNA of OsNRT2.3 is alternatively spliced (OsNRT2.3a/b) during the uptake process, and that OsNAR2.1 interacts with two other NRT proteins (Yan et al. 2011). Furthermore, it was shown that most transporter genes are up-regulated by NO_3 and suppressed by NH_4^+, with the exception of OsNRT2.3b, which is insensitive to NH_4^+ (Feng et al. 2011). Similarly to Arabidopsis, high-affinity rice NH_4^+ transporters are encoded by members of the AMT1 and AMT2 gene families (Gazzarini et al. 1999; Howitt and Udvardi 2000; Loqué and von Wirén 2004). Although functionally not well characterized, 12 putative rice AMT proteins have been identified located on different chromosomes and grouped into five sub-families (AMT1–AMT5) with one to three gene members (Suenaga et al. 2003; Deng et al. 2007; Li et al. 2009b). Gene expression analyses showed that OsAMT1;1 is constitutively expressed in shoots and roots (Ding et al. 2011). In contrast, OsAMT1;2 shows root-specific expression and is NH_4^+ inducible, whereas OsAMT1;3 is root specific and N suppressible (Sonoda et al. 2003). In addition, a high-affinity urea transporter (OsDUR3) encoding an integral membrane protein that is up-regulated under N deficiency has recently been identified in rice roots (Wang et al. 2012). OsDUR3 over-expression improved growth on low urea and this gene might therefore play a significant role in N uptake in rice. Urea is converted to NH_4^+ and carbon dioxide in the presence of urease, which requires nickel (Ni) as a co-factor. Interestingly, the application of Ni had a marked effect on plant growth in rice when urea was provided as the sole N source (Gerendas et al. 1998). Furthermore, an early nodulin gene (OsENOD93-1) with potential function in amino acid accumulation and transport has been identified in rice (Bi et al. 2009).

To enable plants to accumulate sufficient internal N, it is critically important to apply N fertilizer at appropriate developmental stages. In irrigated rice fields, N fertilizer is usually applied in three splits, i.e. at transplanting (basal), at maximum tillering and at panicle initiation. This practice is recommended to rice farmers (Williams et al. 2010) and is now widely adopted. Urea is the major source of N in rice ecosystems since it is rapidly converted to NH_4^+ or NO_3 by soil micro-organisms. In cereals, depending on the genotype and environmental conditions, the root or shoot can be the main site for NO_3 assimilation, with root assimilation dominating at soil nitrate concentrations <1 mM and shoot assimilation at concentrations >1 mM (Andrews 1986; Andrews et al. 1995). Upon absorption, NO_3 is reduced to nitrite by nitrate reductase (NR) and subsequently to NH_4^+ by nitrite reductase (NiR). NH_4^+ from both the nitrate reduction pathway and direct absorption are subsequently incorporated into amino acids through the synthesis of glutamine and glutamate (Crawford and Glass 1998; Meyer and Stitt 2001; Campbell 2002) primarily in the chloroplasts and plastids via the glutamine synthetase (GS)/glutamate synthase (GOGAT) cycle (Tobin and Yamaya 2001; Andrews et al. 2004) and alternatively via pathways involving glutamate dehydrogenase and asparagine synthetase (Hirel and Lea 2002; Dubois et al. 2003). Although roots have high constitutive levels of GS and GOGAT, both enzymes are inducible by NH_4^+ (Ishiyama et al. 2003). For the conversion of glutamine to glutamate by GOGAT, either ferredoxin (Fd-GOGAT) or NADH (NADH-GOGAT) is used. For GS, two major forms are known, namely cytosolic GS (GS1), expressed in roots and shoots, and plastidic GS (GS2), expressed in chloroplasts and plastids. GS1 is a complex gene family of three to six genes (Hirel and Lea 2002).

Remobilization of internal N during grain filling is another key process in N metabolism. Among the primary N assimilation genes (for a review see Lea and Miflin 2011), physiological and biochemical evidence indicates that GS1 plays a major role in the synthesis of glutamine in older leaves, which is then transported to panicles (Kamachi et al. 1991; Habash et al. 2001; Masclaux et al. 2001), a process positively related to yield and N-use efficiency. The GS1 genes from rice, OsGS1;1 OsGS1;2, are expressed in all organs, but with higher expression in leaf blades and roots, respectively. OsGS1;3 is found specifically in the spikelet (Tabuchi et al. 2005). Prior to senescence, GS1 activity is reduced, which leads to rapid accumulation of NH_4^+ and senescence since accumulated NH_4^+ is highly toxic to plant cells (Chen and Kao 1996). In addition, it was shown that a knock-out mutation in OsGS1.1 resulted in reduced plant growth and poor yield (Tabuchi et al. 2005), whereas over-expression of the GS2 gene increased yield in wheat and Arabidopsis (Habash et al. 2001; Martin et al. 2006), and enhanced photorespiratory capacity and salt tolerance in rice (Hoshida et al. 2000). Re-assimilation and conversion of glutamine to glutamate by GOGAT is tissue specific with Fd-GOGAT

predominantly active in photosynthetic tissues (Lea 1997) and NADH-GOGAT in the developing sink organs where it plays a significant role in re-utilization of glutamine (Hayakawa et al. 1993; Tabuchi et al. 2007). Over-expression of NADH-GOGAT increased the panicle weight in rice, in agreement with its important role in transporting glutamate to major sink tissues during grain filling (Yamaya et al. 1992). This enzyme may therefore play a key role in N utilization and grain filling in rice (Andrews et al. 2004). The rice OsNADH-GOGAT1 gene is mainly expressed in growing tissues such as root tips, young spikelets and developing leaf blades, and is important for N remobilization, whereas the second rice gene, OsNADH-GOGAT2, is mainly expressed in mature leaves and leaf sheaths (Tabuchi et al. 2007). A recent study on non-functional OsNADH-GOGAT2 mutants has shown a significant decrease in spikelet number per panicle associated with a reduction in yield and plant biomass, as well as total N content in senescing leaves. This implies that NADH-GOGAT2 is also important for remobilization of N and glutamine generation in senescing leaves (Tamura et al. 2011).

Grain development in rice depends on the establishment and maintenance of a photosynthetically active canopy, which acts as a major N store before internal N is translocated to the panicle. Since this process occurs at the expense of the photosynthetic machinery, canopy longevity and maintenance of the photosynthetic capacity are vital for continuous remobilization of N and starch accumulation (Hawkesford and Howarth 2011). In this context, the functional stay-green (FSG) trait is of interest since it delays leaf senescence and sustains photosynthesis during grain filling, which may lead to increased biomass production and grain yield (Fu and Lee 2008). A detailed review on the mechanisms and potential productivity benefits of FSG has recently been published (Hörtensteiner 2009). Several quantitative trait loci (QTLs) for FSG have been mapped and analysed in rice introgression lines (Yoo et al. 2007; Fu et al. 2011). Although a beneficial effect of FSG on grain yield has been observed only in one of the two analysed genetic backgrounds (Fu et al. 2011), FSG holds promise for rice yield improvement and possibly for terminal drought tolerance, and should therefore be studied in detail.

Phosphorus uptake and metabolism: genes and pathways

In irrigated rice systems, P fertilizer is generally applied only at the beginning of the season (basal), whereas N is applied in three splits (basal/after transplanting, maximum tillering and panicle initiation/booting). This is common practice in Asia and Africa (Haefele and Wopereis 2005; Hossain et al. 2005), indicating that P acquisition and requirement are highest during the early growth stages. Under P-deficient conditions, plant development is delayed and P deficiency symptoms (reduced tillering and darker leaves due to anthocyanin accumulation) are easily recognized and cause significant yield losses (Dobermann and Fairhurst 2000). Another important factor is that P, in contrast to N and K, is not transported with the soil solution (mass flow) but mainly by diffusion (Schachtman et al. 1998). A large root surface area is therefore particularly important for P uptake since plants gain access to a larger soil area and thereby to P (Lynch 2007). In agreement with that, induction of root growth under P deficiency has been described in many species (Hernández et al. 2007), although it should be noted that root growth under −P conditions is generally reduced compared with that of plants grown under +P conditions (Li et al. 2009a; Chin et al. 2010). For yield stability under P deficient conditions, long root hairs and highly branched root systems, especially in the top soil where P is mainly located, are considered beneficial (Ramaekers et al. 2010). In agreement with this, the Phosphorus uptake 1 (Pup1) major QTL for tolerance of P deficiency is an enhancer of root growth (Gamuyao et al. 2012). The same was observed in the P-deficiency-tolerant maize mutant line 99038 (Li et al. 2008), which likewise developed a larger root system.

Phosphorus is transported into the plant by P transporters located in the root plasma membrane. In the rice reference genome, 13 P transporter genes are present (Paszkowski et al. 2002). Two of these transporters have been functionally characterized, revealing that OsPT2 encodes a low-affinity and OsPT6 a high-affinity transporter (Ai et al. 2009). High-affinity transporters are generally induced under low-P conditions (Paszkowski et al. 2002) and are therefore considered more important for P uptake under field conditions since the soil P concentration is ∼1000 times lower compared with intracellular concentrations (Schachtman et al. 1998). In agreement with this, OsPT6, but not OsPT2, was shown to be expressed in the root epidermis (Ai et al. 2009). In another study (Jia et al. 2011), the effect of the rice P transporter OsPht1;8 was analysed by over-expression and RNAi. The authors showed that P uptake in the transgenics was altered according to expectation; however, both approaches led to a significant reduction in the number and size of panicles, as well as to >80 % spikelet sterility. In another study on rice, transgenic plants over-expressing the tobacco transporter NtPT1 were generated, but although some lines outperformed the controls, on average transgenic lines

yielded less (Park *et al.* 2010). Likewise, it was shown in barley that over-expression of the transporter gene *HOR-vu;Pht1;1* did not increase P uptake (Rae *et al.* 2004). Taken together, the data suggest that over-expression of high-affinity transporters alone is not sufficient to improve P efficiency. This might be due to the fact that the high-affinity P-transport system is naturally induced to a high level, sufficient to transport the P available at the root–soil interphase (Rae *et al.* 2004; Pariasca-Tanaka *et al.* 2009). Rather than P uptake, the limiting factor might therefore be the depletion of P in the vicinity of the roots. This can be addressed by developing plants with a larger root system/surface area that provides access to a larger soil area. In fact, this has been shown to be the underlying mechanism of the *Pup1* major QTL (Gamuyao *et al.* 2012). Alternatively, or in addition, enhanced mobilization of P from complexes and organic compounds via exudation of organic acids, acid phosphatases and phytases (for a review see Gahoonia and Nielsen 2004) would increase the P supply to roots. In winter barley (*Hordeum vulgare* L.), it has already been shown that a cultivar with high exudation of citric and acetic acid mobilized more P from the strongly absorbed soil P fraction, and plants developed a higher shoot dry matter than the cultivar with low exudation (Gahoonia *et al.* 2000). Also, in barley, it was furthermore shown that expression of the wheat aluminium resistance gene *TaALMT1* enhanced P uptake on acidic soils due to an increased efflux of malate (Delhaize *et al.* 2009). In wheat (*Triticum aestivum* L.) oxalate, but not citrate, significantly enhanced P uptake under the experimental conditions applied in a study by Khademi *et al.* (2010). However, over-expression of the mitochondrial citrate synthase enhanced P uptake and growth in *Arabidopsis* (Koyama *et al.* 2000). Furthermore, it was shown that over-expression of the *Arabidopsis* purple acid phosphatase gene *AtPAP15* improved P efficiency and yield in soybean grown on acidic soil (Wang *et al.* 2009). These examples are very encouraging and in particular the finding that genetic diversity exists, at least in barley, warrants a systematic assessment of this trait in rice.

For an extended root system and thereby better access to P, mycorrhizae are generally considered important. The positive effect of arbuscular mycorrhizal (AM) symbiosis on nutrient uptake, especially P and N, has been demonstrated in several crops (for a review see Karandashov and Bucher 2005; Sawers *et al.* 2008). In rice, surprisingly very few studies on the effect of AM are available, possibly because research focuses on irrigated rice grown under flooded, anaerobic conditions, which are generally considered unfavourable for fungi. However, using the irrigated rice variety Nipponbare, it

was shown that the P transporter *OsPT11* is specifically induced in roots colonized by AM (Paszkowski *et al.* 2002). A study from Japan additionally showed that AM inoculation of Nipponbare seedlings significantly increased yield in flooded fields and that the fungi survived under these conditions (Solaiman and Hirata 1997). The latter was also found in a study by Hajiboland *et al.* (2009) which additionally reported a significant growth advantage of plants colonized by mycorrhiza. Surprisingly, this was observed only under flooded conditions, whereas growth was severely inhibited under aerobic conditions. Growth depression due to mycorrhiza colonization has also been reported from wheat, especially under P-deficient conditions (Li *et al.* 2005). However, another study on rice that analysed the effect of mycorrhiza in four different upland crop-rotation systems showed a significant increase in P uptake and grain yield in the system with the highest concentration of mycorrhiza (Maiti *et al.* 2012). More systematic and detailed studies are needed to conclusively assess the potential of mycorrhiza for enhancement of P uptake in irrigated and rain-fed rice systems, and to address genetic diversity for mycorrhizal interaction (Fig. 1).

In contrast to enhanced P uptake, improvement of internal P-use efficiency would target genes/pathways that enable plants to maintain cellular processes and productivity under low-P conditions. Since P is an indispensable component of virtually all cellular functions and is required in large amounts, e.g. ATP, NADPH, nucleic acids and phosphoproteins, it is reasonable to expect that modification of P-related pathways will affect the whole plant. Alternative pathways have been described that are up-regulated under P starvation utilizing PPi rather than ATP (for a review see Hammond *et al.* 2004). This includes UDP-glucose pyrophosphorylase (PPi-dependent conversion of glucose to hexose-P) and phosphofructokinase (PPi-dependent phosphorylation of fructose-6-P). Other adaptive processes include the substitution of phospholipids with galacto- and sulfolipids, and up-regulation of ribonucleases to mobilize P from nucleic acids. Many other genes and pathways are altered under low-P conditions, which is not surprising in light of the central role of P in living cells. The challenge will be to identify genes that enhance internal P-use efficiency without causing an imbalance in P homeostasis and negatively affecting plant development.

In microarray gene expression studies in rice (Wasaki *et al.* 2003; Pariasca-Tanaka *et al.* 2009) and *Arabidopsis* (Wu *et al.* 2003; Morcuende *et al.* 2007), between 220 and 5800 genes, depending on the study, were shown to respond to P. For breeding applications, it will be critically important to identify the early sensors of P deficiency and genes that act as upstream regulators of

this systemic response in order to reduce complexity and the number of genes/QTLs needed to modify P-use efficiency. The regulatory network of P homeostasis has been well studied in *Arabidopsis* and, for some genes, rice orthologues have been identified. The MYB-type transcription factor *AtPHR1* (Rubio *et al.* 2001) and its rice orthologue *OsPHR2* (Zhou *et al.* 2008) act as positive regulators of P transporters and other P-responsive genes, whereas other genes act as suppressors of P starvation genes, e.g. the ubiquitin conjugating enzyme *AtPHO2* and its rice orthologue *OsLTN1* (Aung *et al.* 2006; Bari *et al.* 2006; Hu *et al.* 2011). Expression of *PHO2* is negatively regulated by a micro RNA (miR399), which itself is sequestered by 'target mimicry' of the *IPS1* gene (Aung *et al.* 2006; Bari *et al.* 2006; Franco-Zorrilla *et al.* 2007). Two recent reviews provide an excellent and comprehensive overview of the genes involved and their interaction (Nilsson *et al.* 2010; Hammond and White 2011).

Interestingly, it has recently been shown in *Arabidopsis* that down-regulation of the *PHO1* gene (an SPX protein) conferred tolerance via suppression of the P starvation response (Rouached *et al.* 2011). A similar observation has been made in tolerant *Pup1* rice plants, which did not differentially express P starvation genes in comparison with non-*Pup1* controls (Pariasca-Tanaka *et al.* 2009; Gamuyao *et al.* 2012). In this context, it is important to note that most studies on P-starvation responses in rice were conducted in the intolerant variety Nipponbare. The identified P-starvation genes and pathways, therefore, represent the intolerant response. In agreement with this, genes not formerly related to P-starvation tolerance have been identified in the *Pup1* donor variety Kasalath (Gamuyao *et al.* 2012) as well as in a QTL-mapping study using a tolerant *Arabidopsis* accession (Reymond *et al.* 2006). It therefore seems important to further explore genetic diversity, in *Arabidopsis* as well as in rice, and to identify additional tolerant genotypes in order to gain access to large-effect genes and QTLs.

Quantitative trait loci for molecular breeding

Molecular breeding now provides a real opportunity to develop varieties with multiple tolerance traits—provided that large-effect QTLs/genes are available. The number of reported QTLs is steadily increasing, but still very few are applied in breeding programmes. This is largely due to a lack of data that validate QTLs/tolerance genes in different genetic backgrounds and environments (i.e. in field trials), which is a prerequisite for a large-scale application of QTLs.

In rice, molecular marker-assisted breeding is at an advanced stage for a few large-effect QTLs that confer tolerance to submergence (*Sub1*; Septiningsih *et al.* 2009; Singh *et al.* 2010), drought (e.g. *qtl12.1*; Bernier *et al.* 2009*a*, *b*; Swamy *et al.* 2011), salinity (*SalTol*; Thomson *et al.* 2010) and P-deficiency tolerance (Chin *et al.* 2011). The latter QTL, *Pup1*, was identified more than 10 years ago (Wissuwa *et al.* 2002) and is, to our knowledge, currently the only P-related QTL for which molecular markers are available and which has been evaluated in different genetic backgrounds under field

Table 1 Quantitative trait loci for P-related traits in rice. The number of mapped QTLs for P-related traits in rice remains small, although one QTL, *Pup1*, is widely acclaimed for imparting P-deficiency tolerance and is currently the only P-related QTL for which molecular markers are available and which has been evaluated in different genetic backgrounds under field conditions

Traits	Population	Cross	No. of QTLs		Reference
			MQTL	EQTL	
PUP, PDW, TN, PUE	NIL	Nipponbare/Kasalath	8	–	Wissuwa *et al.* (1998)
RTA, RSDW, RRDW	RIL	IR20/IR55178	4	–	Ni *et al.* (1998)
PUP, TN	NIL	Nipponbare/Kasalath	1 (*Pup1*)	–	Wissuwa *et al.* (2002)
RE, SDW, RPC, RIC	F8	Gimbozu/Kasalath	6	–	Shimizu *et al.* (2004)
RRL, RSL, RSDW, RRDW	BIL	OM2395/AS996	1	–	Lang and Buu (2006)
REP	CSSL	Nipponbare/Kasalath CSSL29	1	–	Shimizu *et al.* (2008)
PH, MRL, RN, RV, RFW, RDW,SDW, TDW, RS	ILs	Yuefa/IRAT109	24	29	Li *et al.* (2009*a*)

DW, plant dry weight; MRL, maximum root length; PH, plant height; PUE, phosphorus-use efficiency; PUP, phosphorus uptake; RDW, root dry weight; RE, root elongation; REP, root elongation under phosphorus deficiency; RFW, root fresh weight; RIC, relative Fe content; RN, root number; RPC, relative phosphorus content; RRDW, relative root dry weight; RRL, relative root length; RS, root/shoot dry weight ratio; RSDW, relative shoot dry weight: RTA, relative tillering ability; RV, root volume; SDW, shoot dry weight; TDW, total dry weight; TN, tiller number.

conditions (Chin et al. 2010, 2011; Gamuyao et al. 2012). Additional P-related QTLs with smaller effect have been identified and are summarized in Table 1. A QTL on chromosome 6 was mapped in two independent studies (Ni et al. 1998; Wissuwa et al. 1998) but has, compared with *Pup1*, a smaller effect. However, this QTL gained importance after it was shown that a cluster of P-responsive genes is located in this region (Heuer et al. 2009), including the transcription factor gene *OsPTF1* which confers tolerance to P deficiency (Yi et al. 2005). Within the larger-effect QTL *Pup1*, no P-responsive gene has been identified and, in agreement with that, *Pup1*-based tolerance does not seem to employ currently known P-starvation response pathways, as indicated by two independent gene array analyses (Pariasca-Tanaka et al. 2009; Gamuyao et al. 2012). Interestingly, the region on chromosome 12 where *Pup1* is located has been associated with tolerance to several biotic stresses (Ramalingam et al. 2003; Li et al. 2006), as well as to drought (Babu et al. 2003; Bernier et al. 2007),

aluminium toxicity (Wu et al. 2000) and cold (Andaya and Mackill 2003).

With respect to N, several genomic regions associated with N use and response have been mapped in rice (Fang and Wu 2001; Ishimaru et al. 2001; Obara et al. 2001), following the pioneering work on mapping QTLs for N-use efficiency in corn (Agrama et al. 1999). An overview of the published rice QTLs is provided in Table 2. Indications so far suggest possible links between very few of these QTLs and primary N assimilation genes and transporters, especially of GS structural genes and yield components (Obara et al. 2001, 2004; Senthilvel et al. 2008; Feng et al. 2010; Vinod et al. 2011). In a recent study, stably expressed QTLs for yield and associated traits at different N levels, especially those expressed at low N, were reported in recombinant inbred line populations (Tong et al. 2011). One of these QTLs, for number of grains per panicle under low N level, is located in the same region as the *Pup1* locus on chromosome 12, suggesting the use of *Pup1* materials for testing low-N tolerance. In this

Table 2 Quantitative trait loci for N-related traits in rice. Almost all the N-related QTLs mapped in rice so far have smaller effects and many of them exhibit significant epistatic and QE interactions, making them less amenable to breeding programmes.

Traits	Population	Cross	No. of QTLs		Reference
			MQTL	EQTL	
PH	DHL	IR64/Azucena	10	–	Fang and Wu (2001)
Rubisco, TLN, SPC	BIL	Nipponbare/Kasalath	15	–	Ishimaru et al. (2001)
GS, GOGAT	BIL	Nipponbare/Kasalath	13	–	Obara et al. (2001)
GS, PN, PW	NIL	Koshihikari/Kasalath	1	–	Obara et al. (2004)
TGN, TSN, NUP, NUE, NTE	F3	Basmati370/ASD16	43	–	Senthilvel et al. (2004)
RDW, SDW, BM	RIL	Zhenshan97/Minghui 63	52	103	Lian et al. (2005)
PH, PN, CC, SDW	CSSL	Teqing/Lemont	31	–	Tong et al. (2006)
TGN, TLN, TSN, NUP, SLN	RIL	IR69093-4-3-2/IR72	32	–	Laza et al. (2006)
RL, RT, RM, BM, etc.	RIL	Bala/Azucena	17	–	MacMillan et al. (2006)
TGN, TLN, TSN, PNUE, BM	RIL	Dasanbyeo/TR22183	20	58	Cho et al. (2007)
TPN, NUE	DHL	IR64/Azucena	16	–	Senthilvel et al. (2008)
TPN, NDMPE, NGPE, TGN	RIL	Dasanbyeo/TR22183	28	23	Piao et al. (2009)
PH, NR,GS, GOGAT, BM, etc	RIL	Basmati 370/ASD16	15	44	Vinod et al. (2011)
GYP, BM, HI, etc.	RIL	IR64/ INRC10192	46	–	Srividya et al. (2010)
PH, RDW, SDW, CC, RL, BM	RIL	R9308/Xieqingzao B	7	–	Feng et al. (2010)
GYP, GNP	RIL	Zhenshan 97/HR5	19	11	Tong et al. (2011

BIL, backcross inbred lines; BM, biomass; CC, chlorophyll content; CSSL, chromosomal segment substitution lines; DHL, doubled haploid lines; EQTL, epistatic QTL; GNP, grain number per panicle; GOGAT, glutamate synthase; GYP, grain yield per plant; GS, glutamine synthetase; HI, harvest index; MQTL, main-effect QTL; NDMPE, nitrogen dry matter production efficiency; NGPE, nitrogen grain production efficiency; NHI, nitrogen harvest index; NIL, near isogenic lines; NR, nitrate reductase; NTE, nitrogen translocation efficiency; NUE, nitrogen-use efficiency; NUP, nitrogen uptake; PH, plant height; PN, panicle number per plant; PW, panicle weight; PNUE, physiological nitrogen-use efficiency; RDW, root dry weight; RIL, recombinant inbred lines; RL, root length; RT, root thickness; RM, root biomass; SDW, shoot dry weight; SLN, specific leaf nitrogen; SPC, soluble protein content; TGN, total grain nitrogen; TLN, total leaf nitrogen; TSN, total shoot nitrogen; TPN, total plant nitrogen.

context, it is noteworthy that a peptide transporter gene was identified as one of the putative *Pup1*-downstream genes (Gamuyao et al. 2012) constituting a possible link between P and N uptake. Attempts to map loci for associative rhizosphere N fixation were also reported in rice, and independent QTLs linked to the activity of different N-fixing bacterial strains were identified on chromosome 2 (Ji et al. 2005). In independent studies (Lian et al. 2005; Cho et al. 2007; K. K. Vinod, unpub. data), many small-effect epistatic QTLs have been mapped, accounting for a large cumulative proportion of variation for traits under low- and normal-N conditions, many of which also show significant QTL × environment interaction, emphasizing the importance of validating QTLs in multiple environments and genetic backgrounds before using them in selection.

Conclusions and forward look

In light of scarce resources, increasing fertilizer production costs and the need for rice production to keep pace with growing demand, the development of nutrient-efficient crops is increasingly important. As outlined above, both nutrient uptake and metabolic pathways are under the control of a complex regulatory network involving many genes. The identification of large-effect QTLs/genes is therefore a challenge. However, examples such as the submergence-tolerance *OsSUB1A* gene (Xu et al. 2006; Fukao and Bailey-Serres 2008) and *Pup1* (Gamuyao et al. 2012) demonstrate that a single gene can modify many downstream responses without affecting plant performance under non-stressed conditions (Mackill et al. 2012). Large-effect QTLs have also been identified for drought tolerance (Swamy et al. 2011; Vikram et al. 2011) and cloning of the genes is in progress. *Sub1* and *Pup1*, as well as the drought QTLs, have been identified by forward genetic approaches using tolerant rice genotypes, and screenings were mainly conducted under field conditions. Given the success of this approach for a complex trait such as P-deficiency tolerance and drought, it seems advisable to apply a similar strategy for the identification of additional QTLs/genes, especially for internal N- and P-use efficiency (Fig. 2). However, it will be important to keep in mind that internal nutrient-use efficiency is directly dependent on nutrient uptake and the challenge will be to develop a screening protocol under which all genotypes are supplied with the same quantity of the respective nutrient (Rose et al. 2011; Rose and Wissuwa 2012).

Fig. 2 Future approaches in breeding nutrient-efficient varieties essentially require integration of classical and modern tools. The low heritability of nutrient-use traits necessitates precise approaches such as trait-targeted selection augmented by modern molecular tools.
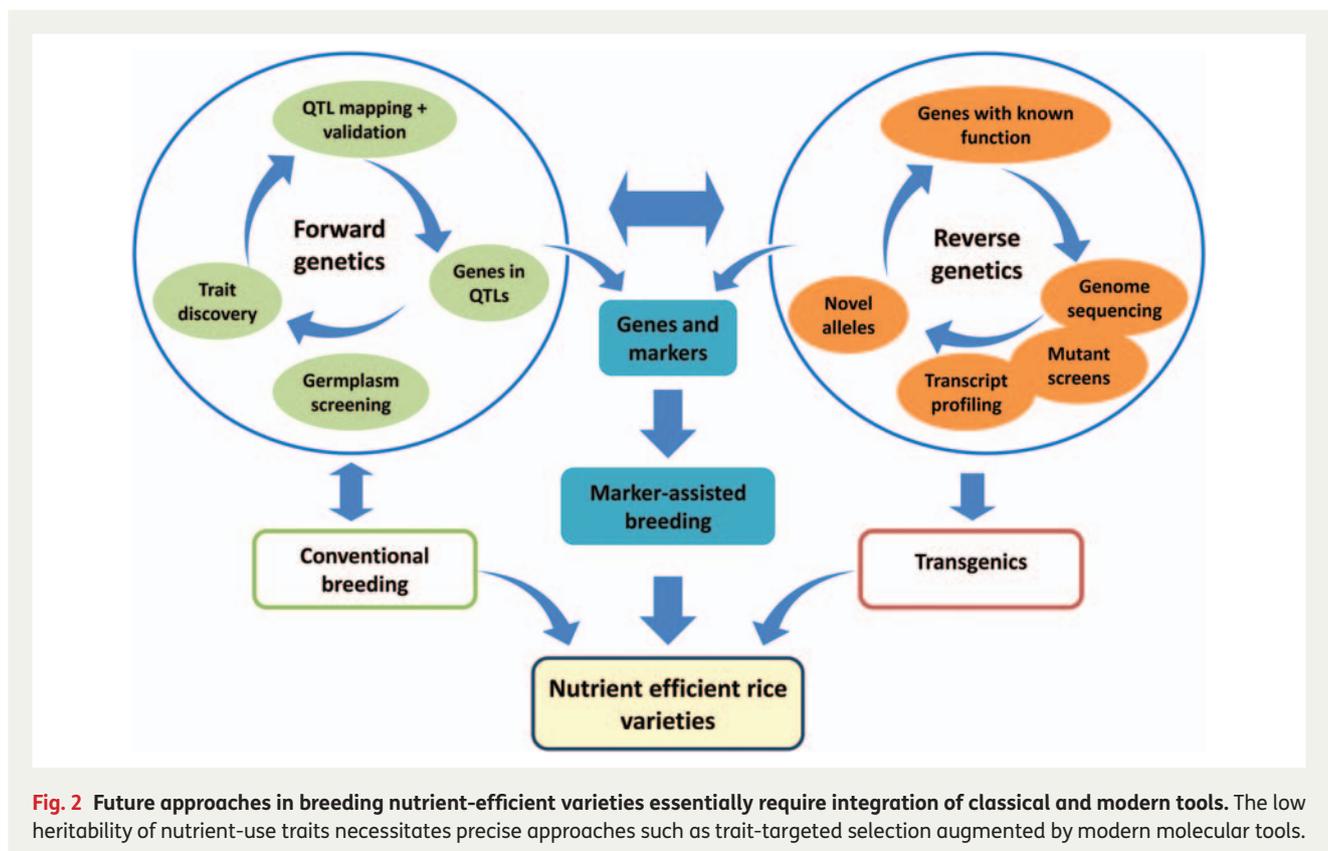
With the experiences gained in QTL mapping and the rapid development of genome-sequencing and molecular-marker technologies, more high-impact, large-effect QTLs will surely be identified in the future. These efforts require expertise in different disciplines and, therefore, modern breeding is being implemented more and more in multi-disciplinary teams involving breeders, physiologists and molecular biologists/geneticists. With the advances in molecular breeding technologies, breeders now gain access to genes from wild rice and unadapted genotypes (landraces) that are difficult to use in breeding programmes due to crossing barriers and their poor agronomic performance. Molecular breeding therefore provides an exciting opportunity to use this gene pool effectively for the development of well-adapted and nutrient-efficient rice varieties.

Contributions by the authors

Both authors have contributed equally to the preparation of the manuscript with K.K.V. mainly responsible for the parts on nitrogen and S.H. for the parts on phosphorus.

Conflicts of interest statement

None declared.

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