Bioengineering nitrogen acquisition in rice: can novel initiatives in rice genomics and physiology contribute to global food security?

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Summary

Rice is the most important crop species on earth, providing staple food for 70% of the world's human population. Over the past four decades, successes in classical breeding, fertilization, pest control, irrigation and expansion of arable land have massively increased global rice production, enabling crop scientists and farmers to stave off anticipated famines. If current projections for human population growth are correct, however, present rice yields will be insufficient within a few years. Rice yields will have to increase by an estimated 60% in the next 30 years, or global food security will be in danger. The classical methods of previous green revolutions alone will probably not be able to meet this challenge, without being coupled to recombinant DNA technology. Here, we focus on the promise of these modern technologies in the area of nitrogen acquisition in rice, recognizing that nitrogen deficiency compromises the realization of rice yield potential in the field more than any other single factor. We summarize rice-specific advances in four key areas of research: (1) nitrogen fixation, (2) primary nitrogen acquisition, (3) manipulations of internal nitrogen metabolism, and (4) interactions between nitrogen and photosynthesis. We develop a model for future plant breeding possibilities, pointing out the importance of coming to terms with the complex interactions among the physiological components under manipulation, in the context of ensuring proper targeting of intellectual and financial resources in this crucial area of research. *BioEssays* **26:683–692, 2004.**

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Abbreviations: IRRI, International Rice Research Institute; NUE, Nitrogen-use efficiency; *ENOD*, early nodulin gene; PR, photorespiration; GS, glutamine synthetase; GDH, glutamate dehydrogenase; PEPcase, phosphoenol pyruvate carboxylase; PPDK, pyruvate orthophosphate dikinase; RuBisCO, ribulose bisphosphate carboxylase/oxygenase; CGIAR, Consultative Group on International Agricultural Research.

Introduction

In the 1960s, human population growth was outpacing agricultural production in many areas of the globe and a shortfall in food supply, especially that of rice (*Oryza sativa* L., Fig. 1), was predicted.[1] Fortunately, this grim Malthusian scenario was circumvented by a research effort spearheaded by the nonprofit International Rice Research Institute (IRRI). IRRI developed, and freely distributed, highly productive new rice cultivars that were instrumental in the eventual tripling of global rice yields. This achievement was hailed as the "second green revolution"; the first green revolution involved the Nobel-Prize-winning development of highly productive strains of wheat.[1,2]

IRRI's conventional breeding methods produced plants with many desirable characteristics, including dwarfism, a high number of tillers (reproductive, grain-bearing shoots), and dark green, erect leaves for optimal light reception. Dwarfism, in particular, resulted in an increased harvest index, i.e. the ratio of grain weight to above-ground biomass, and improved the physical stability of the stem architecture, and thus reduced the spoilage of well-fertilized, grain-heavy, plants. This allowed for substantial increases in fertilization, particularly that of nitrogen, the plant nutrient most frequently limiting growth and yield in rice[3–5] and most other plants.

Despite these major accomplishments, however, nitrogen remains a major factor limiting rice productivity. Moreover, record yields per hectare of 10^4 kg can only be achieved by addition of 250 kg nitrogen or more, while rice farmers in much of Asia typically apply only 86 to 138 kg.[6] Nevertheless, increased nitrogen application is not an ideal solution, partly from a cost perspective—in 2001, increased natural gas prices caused the price of nitrogen fertilizer to nearly double.[7] Increased nitrogen use is also undesirable from a eutrophication perspective—50% or more of the nitrogen applied to

Figure 1. Rice paddy in northern China. Photo courtesy of John Sheehy, IRRI.

irrigated rice fields is typically lost to the environment through volatilization, runoff and other factors,[8] which can cause widespread ecological disruption.

At the same time, increases in grain productivity over the last decade have fallen behind concomitant increases in human population. Between 1990 and 1996, for instance, global population increased by 1.4% annually, while global grain production increased by only 1%, causing predictions of famines reminiscent of the 1960s.[1] To prevent such a crisis, rice yields will need to increase by an estimated 60% over the next 30 years.[3] This must be achieved while simultaneously limiting fertilizer input, curtailing the use of herbicides and pesticides, and preventing the exploitation of marginal, often fragile, lands not yet under cultivation.

While enormous, these difficulties are not insurmountable, and their solutions are expected to constitute a third green revolution. To improve yield gain relative to nitrogen application (a parameter known as agronomic nitrogen-use efficiency, or agronomic NUE), both conventional means and novel ones involving molecular biotechnology, will most likely be required. Amongst the former are improvements in field management, such as the techniques of precision agriculture, in which the timing, dose, and depth of nitrogen application are closely coordinated with the requirements of the developing crop.[9–11] Precision agriculture could significantly diminish nitrogen loss through volatilization of ammonia or NO_x gases, but, while economically promising in the longer term, is currently expensive relative to the incomes of most farmers in Asia, where the vast majority of rice is grown.[10,11] An alternative solution to the nitrogen-loss problem could be the enhancement of the oxidative formation and stability of non-volatile nitrate (NO_3^-) in paddy soils, by breeding for increased O_2 exudation from rice roots,[12] or by alternating periods of flooding with drying regimes that facilitate nitrification.[13]

The main aim of this paper is to present a different approach to the issues of nitrogen and rice yield that involves the physiology of rice–nitrogen relations, at whole-plant, cellular, biochemical and genetic levels of organization. This knowledge is vital to the implementation of both conventional and recombinant-DNA breeding methods to increase yield and agronomic nitrogen-use efficiency in rice. We will discuss four important aspects of this topic: (1) biological nitrogen fixation, (2) primary nitrogen uptake, (3) metabolic assimilation of nitrogen into organic compounds (the carbon–nitrogen interface), and (4) points of intersection between photosynthesis and plant nitrogen use.

Biological nitrogen fixation in rice

Paradoxically, despite the fact that nitrogen comprises 78% of earth's atmosphere (3.8×10^{18} kg), this rich store can be fixed only by a small number of bacteria,[14] known as diazotrophs ("N_2-eaters"), which contain the nitrogenase enzyme system. However, about 12,000 plant species have evolved symbioses with diazotrophic bacteria, exchanging sugars for plant-available nitrogen. Because this association relieves the host of the nitrogen limitation experienced by other plants, there has been a great amount of interest in fostering associations between diazotrophs and rice, and in the complete transfer of N_2-fixing systems to the plants themselves.[15]

While this is clearly a long-term project, expectations of its success in rice are not unreasonable, given that rice already takes some advantage of bacterial nitrogen fixation under natural conditions. Diazotrophs can be found in many rice-growing areas, either free-living or associated with the legume *Sesbania* or the aquatic water fern *Azolla*.[3] The nitrogen fixed by these bacteria is directly or indirectly available to the crop, with free-living bacteria alone contributing an average of 30 kg nitrogen per hectare annually.[16] Under greenhouse conditions, the amount of biologically fixed nitrogen taken up by rice can be as much as 80% of its nitrogen uptake, and is strongly cultivar-dependent.[17]

In another naturally occurring situation, nitrogen-fixing bacteria of the genus *Rhizobium* (known as "rhizobia") are known to endophytically associate with rice plants, colonizing their root tissues without the formation of true root nodules.[18] One such bacterium, *Rhizobium leguminosarum* bv. trifolii, has been in use in Egypt for centuries, as part of a cropping system consisting of a rotation of rice with berseem clover (*Trifolium alexandrinum*, which acts as a biological reservoir for the bacterium). Recent field trials with rice and *R.*

Table 1. N-related grain-yield enhancements in rice and wheat

Treatment	Yield enhancement (control = 1)	Reference
Rhizobium inoculation (rice; no applied N)	1.48	18
Rhizobium inoculation (rice; 144 kg N ha^{-1})	1.41	18
Transgenic PEPcase expression (rice)	1.22	86
GS1 overexpression (wheat)	1.14	56

Controls refer to non-inoculated, or wild-type plants.

leguminosarum isolates have resulted in substantial yield and agronomic NUE increases (of 30% or even higher), particularly when only one third of the recommended fertilizer-nitrogen is used (Table 1).[18] Interestingly, however, these increases were not attributed to nitrogen fixation, but to changes in root architecture, stimulated by rhizobial production of the plant growth hormones IAA and GA7.[18] Altered root morphology appears to improve extraction of nitrogen and other nutrients from the soil, leading to enhanced growth, yield, and NUE.

Although the rice–clover–*Rhizobium* rotation system includes a season in which rice is not grown, and hence has a lower yield potential than a double- or triple-cropped rice system, its natural occurrence demonstrates that rice is genetically predisposed toward becoming the host partner in a tighter endophytic relationship with a diazotroph, especially since the associations appear to be heritable, with high genotypic specificity.[18]

In 1992, IRRI initiated a multi-center "Human Frontier" project for the bioengineering of nitrogen fixation in rice, using nodulating, leguminous plants as a model. While the steps involved in nodule development and function are complex, and still not completely understood, much progress has been made towards their characterization,[19–21] which will be briefly described here. In legumes, the sequence typically begins with the release of flavonoids and other signal compounds, by which the host plant induces the expression of *nod* genes in rhizobia. This triggers the expression of "early nodulin" genes (*ENOD*s) in the root vascular tissue, and is followed by the synthesis of plant hormones (by both plant and bacterium). The formation of root nodules then results from extensive cell division and the production of cellulose microfibrils by the plant.[22] Nitrogen fixation ultimately proceeds via an enzyme system encoded by bacterial *nif* genes.

Since no true nodule formation has been demonstrated in rice, then, a proposed rice–diazotroph symbiosis will require the engineering of a wide array of plant–bacterial interactions, not to mention the establishment of a pathway for the export of fixed nitrogen to the plant host.[23] Still, some morphological responses to rhizobial inoculation have been documented in rice. *Rhizobium*-produced plant hormones can favour the growth of short and thick roots,[24] and root-hair deformation, a process associated with early bacterial infection, has also been demonstrated.[25] Interestingly, rice appears capable of perceiving Nod factors coded for by bacterial *nod* genes, and several homologues to legume *ENOD*s are present in rice.[26] Moreover, the promoter activity of rice *ENOD40* in soybean revealed that its tissue-specific expression was identical to that of the endogenous soybean promoter, indicating that key regulatory features of these genes may be conserved in rice.[25] A partial explanation for this may lie in the fact that rice possesses the capacity to form symbiotic (mycorrhizal) associations with underground fungi,[27] and that there appear to be substantial similarities at the genetic level between the formation of such mycorrhizal associations and the formation of symbioses with diazotrophs.[28]

The possibly larger challenge in directly engineering nitrogen fixation in rice lies in the successful incorporation of the 16 *nif* genes essential to nitrogenase activity into the rice genome. Root plastids or chloroplasts, rather than the cell nucleus, have been suggested to be the most suitable intracellular locations for these genes,[29] because plastidic genetics most closely resembles that of N-fixing prokaryotes.[30] Additionally, localization within chloroplasts may allow the substantial energetic cost of nitrogen fixation to be met directly through photosynthesis[29] (36 molecules of ATP are required for every N_2 molecule reduced[15]). A potential problem with this approach is that the activity of the nitrogenase enzyme complex is typically suppressed by oxygen, which is present in virtually all plant cells[31] while, in legume nodules, oxygen is sequestered by the leghemoglobin protein. Possible solutions to this problem include limiting *nif* expression to root plastids in rice, where photosynthetically produced oxygen is not present, or diurnally regulating expression in chloroplasts such that nitrogen fixation only occurs at night. Another solution might be the expression, in rice, of the oxygen-tolerant nitrogenase found in the bacterium *Streptomyces thermoautotrophicus*.[32]

While these problems are, in principle, surmountable, it will likely require many years of intensive research and development before a useful product makes it to the field trial stage, let alone into the hands of farmers. In the nearer future, enhancing rice nitrogen status by optimizing associations between rice and naturally colonizing endophytic bacteria (see above) may be more promising.

Feedback limitations on primary nitrogen acquisition

Plant roots access inorganic nitrogen from the soil in the form of the ions ammonium (NH_4^+) and nitrate (NO_3^-), via substrate-specific, membrane-bound transport molecules. Recent decades have seen remarkable progress in the functional and genetic characterization of these transporters, and homologues to transporters originally identified in the genetic model plant species *Arabidopsis thaliana* are now known in the major cereals, including rice.[33–35] Like other plants, rice has a multiplicity of transport proteins for each ion, corresponding to various physiological conditions, and to cells of varying type and developmental stage. It appears plausible, and indeed has formed the rationale for many research initiatives, that plant nitrogen acquisition can be enhanced by the increased expression of these transport systems. However, as we shall discuss here, such an approach may be intractably hindered by strong feedback inhibitors of nitrogen uptake.

In many plant species, including rice, influx of nitrogen from the environment is inversely related to plant nitrogen content (Fig. 2).[36,37] The biochemical feedback agents involved in downregulating nitrogen influx may include the originally transported nitrogen sources themselves,[38,39] which can accumulate to substantial concentrations within plant cells.[38–41] Alternatively, or additionally, they may include the products of the metabolic assimilation of these ions, particularly amino acids such as glutamine.[41–45] This presents a serious dilemma for plant breeders seeking to maximize the crop's capacity to abstract nitrogen from the soil, and also its nitrogen content, which is closely correlated with photosynthesis and productivity.[4,5]

Moreover, it is important to consider the often ignored fact that, even when transport is feedback-inhibited, plant roots still display excess nitrogen-influx capacity. This surprising phenomenon is visible in the often sizable efflux of nitrogen from root cells back into the external environment, even under nitrogen limitation. Typically, the ratio of efflux to influx increases as the external nitrogen concentration increases,[40,46,47] rising to values as high as 0.9 in the case of NH_4^+.[48] This excess indicates that plants take up more nitrogen from the environment than they assimilate or store, a condition also seen with other nutrient ions. Thus, the overexpression of nitrogen-influx transporters could result in increased nitrogen efflux, with no net advantage to the crop, unless such modifications are coordinated with the simultaneous overexpression of nitrogen sinks within the plant. Appropriate sink fluxes include metabolic fluxes into amino acid pools and storage proteins, subcellular sequestration fluxes (e.g. into the vacuole), and longer-distance fluxes from the root to the shoot. Removal of inorganic and assimilated nitrogen from cytosolic locations may be particularly important, as this compartment is likely to be the one where downregulation is exerted.

Another problem in this regard is the poor correlation sometimes observed between mRNA levels for a transporter, and transport activity itself.[41,49] Unfortunately, these post-transcriptional and/or post-translational regulatory mechanisms are largely unknown. As well, the multiplicity of transporters for a given ion appears to give the plant considerable ability to compensate for imposed changes in the expression patterns of individual transporters.[49,50] This plasticity, and the set-points at which the concerted activities of the transport systems appear to be co-regulated, implies that there are high-level integrative controls in the plant that a breeding program based on molecular biology will have to consider (see next section). One example of such integrative control is the apparently universal characteristic of plants to maintain a

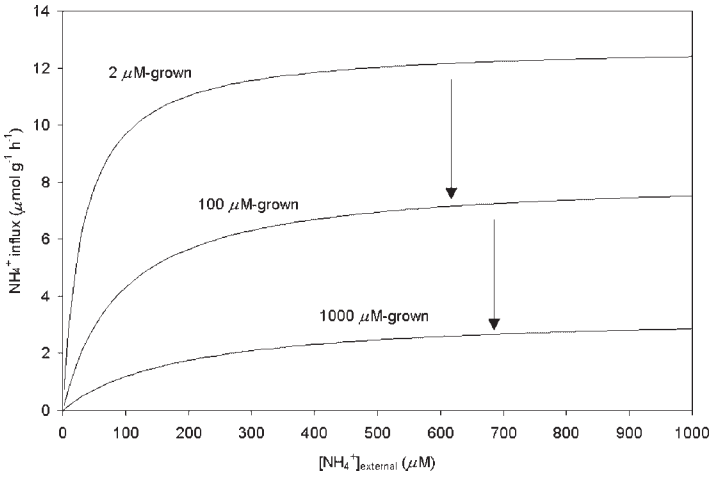

Figure 2. Regulation pattern of NH_4^+ influx in rice, as influenced by nitrogen supply. Arrows indicate down-regulation of fluxes with increasing steady-state nitrogen nutrition (based on Michaelis–Menten parameters from $^{13}NH_4^+$ tracer data published in ref. 36).

constant turnover of inorganic nitrogen pools in the cytosolic compartment of root cells, regardless of the intensity of nitrogen fertilization, or of the magnitude of the fluxes or pool sizes involved.[51]

Re-engineering the nitrogen–carbon interface

In the attempt to reduce nitrogen efflux and to improve yield through more extensive and efficient nitrogen capture, it is crucial to consider which factors limit nitrogen metabolism. Nitrate reductase (NR, which reduces NO_3^- to NO_2^-) was long considered the rate-limiting enzyme in the NO_3^- assimilation pathway, and was hence thought to be pivotal to the growth response of plants to nitrate fertilization.[52] More recently, however, experiments varying NR expression have questioned this idea, as they resulted in little or no change in plant growth.[53] In one particularly illuminating instance, increased NR lowered nitrate uptake by tobacco plants,[54] which the authors attributed to feedback regulation resulting from increased amino acid synthesis. More generally, it is possible that this surprising outcome is related to the strict maintenance of cytosolic nitrogen turnover (also see above), indicating that a regulatory matrix, which may resist attempts at artificial manipulation, controls the transport and reduction of inorganic nitrogen multiple points.[51]

Nevertheless, alterations in the expression of another critical nitrogen-assimilating enzyme, glutamine synthetase (GS, which catalyzes the incorporation of NH_4^+ into amino acids), have resulted in substantial growth changes (Table 1). In a particularly dramatic instance, a 42% increase in GS activity in poplar trees resulted in a height gain of 76% over controls after two months of growth, although this increase declined to 21% after six months.[55] In rice, the potential for GS overexpression is suggested by the observation that such experiments with another cereal grain, wheat, resulted in about 20% higher grain yield.[56] Overexpression of GS1, the cytosolic isoform, also resulted in biomass increases in *Lotus japonicus*[57] and in tobacco,[58] and, similarly, the overexpression of GS2, the plastidic isoform, was shown to increase seedling biomass in tobacco.[59] Although rice plants containing constructs for the overexpression of both GS1[60] and GS2[61] have been produced, information on their growth and yield is lacking. These measurements need to be made because of the success of this approach in other plants, and, moreover, need to be made in the highest-yielding "elite" strains of rice, which appear to have reached the ceiling of yield potential afforded by conventional breeding.[1] Interestingly, the transgenic overexpression of glutamate dehydrogenase (GDH), another enzyme that brings ammonium nitrogen into the amino acid pool, has also conferred increased growth and yield on tobacco plants.[62] Both GDH and GS are found at the intersection of carbon and nitrogen metabolism, and this special property may provide a clue as to how variations in their activities may overcome the restrictions on nitrogen acquisition that matrix regulation appears to impose on the flux through NR and on nitrogen transport.[51]

More concretely, GS and GDH overexpression may improve nitrogen-use efficiency to the plant, by increasing its capacity to refix the NH_4^+ released by a wide range of cellular dissimilatory processes, chiefly the reaction cycle known as photorespiration. Although it derives its name from a respiration-like production of CO_2, photorespiration simultaneously involves an equimolar production of NH_4^+. Indeed, photorespiration generates an nitrogen flux that can be as much as 10 times greater than the primary acquisition of soil nitrogen.[63] Losses of carbon and nitrogen from the plant, as gaseous CO_2 and NH_3 (derived from the deprotonation of NH_4^+), can thus be substantial.[63,64]

Recent GS expression studies in tobacco[65,66] and rice[61] have shown a positive correlation between GS activity and photorespiration, suggesting that GS is a limiting factor in the photorespiratory pathway (however, see below). Because these genotypes also typically show increased growth rates, these findings are striking in that they appear to contradict the conventional wisdom that photorespiration is a wasteful process.[67] While photorespiration is undoubtedly an energy-dissipating process, this very feature may help protect leaves from damage resulting from exposure to high solar radiation, that may offset any physiological liability. This is because increased photorespiration (as in GS-overexpressing plants) can allow the greater withdrawal of electrons from the highly reduced photosystems that exist under light stress, and thereby decrease the associated, and detrimental, production of reactive oxygen species under these conditions.[68,69] This advantage might be optimized in GS overexpressors, if they can minimize associated nitrogen losses, by being better able to refix the nitrogen released in photorespiration. Loss of carbon, in addition, could be counterbalanced by greater carbon refixation via the enzyme phosphoenolpyruvate carboxylase (PEPcase, see next section), which is upregulated by elevated glutamine levels;[70–72] fortuitously, these pools are increased by higher GS activity (Fig. 3).[73] Thus, a protective, and potentially growth-enhancing role for photorespiration, like that for the xanthophyll cycle, might be particularly advantageous in rice, given the high irradiances typically seen in rice paddies.

Drought stress, which can also lead to an over-reduction of photosystems under conditions of intense solar radiation, might also be relieved by higher photorespiration. Water deficit generally causes plants to close the microscopic pores (stomata) on their leaf surfaces, which results in the lowering of CO_2 inside the leaf. The limitation of CO_2 as an electron acceptor for photosynthetic electron transport, and the resulting potential for reactive oxygen production, could under these conditions also be relieved by a photorespiratory siphoning-off of excited electrons. In rice leaves, a narrowing,

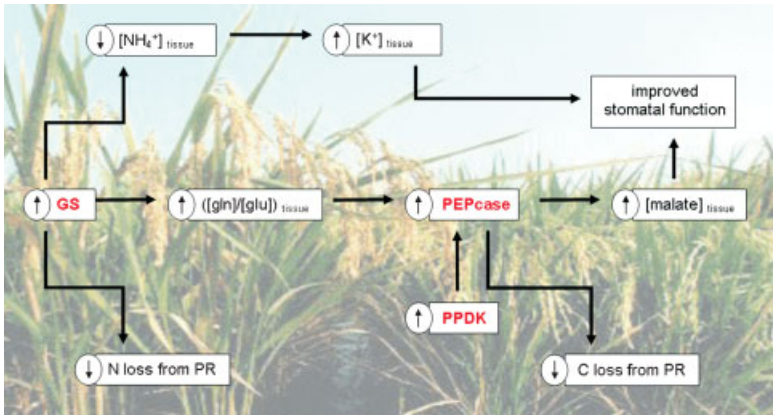

Figure 3. Model predicting physiological consequences of GS (glutamine synthetase), PEPcase (phosphoenol pyruvate carboxylase), and PPDK (pyruvate orthophosphate dikinase) overexpression. Simultaneous overexpression of the three enzymes may work synergistically towards minimizing carbon and nitrogen losses due to photorespiration (PR), and towards optimizing stomatal function. CO_2 and NH_3 losses can be reduced by refixation via PEPcase and GS, respectively, and stomatal function can be improved by increasing tissue potassium and malate levels. For further details, see text. Background photo courtesy of John Sheehy, IRRI.

or closing, of stomatal aperture appears to occur during much of the day[72,73] due to the exceptionally low hydraulic conductivity of rice roots and the resulting water deficit, even under flooded conditions.[74–76] If additional drought stresses, as in the form of highly saline paddy soils are superimposed, photorespiratory relief through enhanced GS activity could provide even further benefit. Indeed, a dramatic photosynthetic performance enhancement was seen in GS2-overexpressing rice grown under saline conditions.[61]

Nitrogen and photosynthesis

We have seen how enhanced GS activity may lead to a greater ability of transformed plants to benefit from high photorespiratory activity. However, the claim that GS catalyzes a rate-limiting step in photorespiration, as an explanation of the positive correlation between GS activity and photorespiration,[61,65,66] requires re-examination. While seemingly straightforward, it contradicts the theoretical foundation of photorespiration research, which regards the activity of this pathway to be determined solely by the catalytic properties of RuBisCO, the main CO_2-fixing enzyme in plants, and by the partial pressures of oxygen and carbon dioxide within the leaves.[77] RuBisCO stands for ribulose bisphosphate carboxylase/oxygenase, the name implying that it can use both CO_2 and O_2 as a substrate; when CO_2 is used, photosynthesis occurs, while, when O_2 is used, photorespiration occurs. In the light, as the ratio of CO_2 to O_2 within the leaf drops, as when stomata are narrow or closed, then, the ratio of photosynthesis to photorespiration similarly falls.[78]

We hypothesize (Fig. 3) that plants overexpressing GS photorespire more than wild-type plants not because GS catalyzes a rate-limiting step in the reaction cycle, but because high GS activity enhances the ability to keep stomata closed when necessary, as under conditions of drought and salinity stress. Interestingly, these are the very conditions under which the most substantial gains in stress tolerance and/or biomass resulting from GS enhancement have been observed (Table 2);[61] Given that 40% of the world's arable land is currently affected by salinity[79] and additional lands are subjected to intermittent or prolonged periods of drought, this is of particular importance.

Furthermore, we hypothesize that stomatal control in general (in terms of both opening, and closing) is enhanced by high GS activity, because of its strong, if indirect, influence on plant potassium status. Potassium is the main ion involved in the changes in cellular osmotic potential that cause stomata

Table 2. Effects of salt stress and GS2 expression on chlorophyll fluorescence in rice plants

	F_v/F_m (days after transfer to 150 mM NaCl)				
Plant genotype	**0**	**3**	**7**	**12**	**14**
Control	0.72	0.73	0.63	0.28	0
GS2 overexpressors	0.72	0.72	0.70	0.75	0.65
GS2 underexpressors	0.72	0.54	0	n.d	n.d.

The ratio F_v/F_m was used as an indicator of photosynthetic quantum yield; a declining ratio indicates increased stress on the photosynthetic apparatus (data from Ref. 61).

to open and close, and its abundance in plant tissues is, typically, inversely related to tissue NH_4^+ concentrations.[80] Because GS plays a major role in the maintenance of low tissue [NH_4^+],[61,81] its higher activity could raise tissue [K^+] and hence optimize stomatal function.[84] Higher tissue levels of malate, another important chemical component of stomatal function,[82] may also be maintained in GS overexpressors, because the high glutamine:glutamate ratio expected in these plants[73] should stimulate the activity of PEPcase, a key enzyme involved in malate formation (see previous section).[70–72]

PEPcase is crucial to a diverse group of plants known as C-4 plants, which tend to fix carbon at higher rates and efficiencies than most other plants, particularly at higher temperatures and at lower CO_2 availability.[83] Recently, attempts have been made to engineer C-4 photosynthesis in rice plants. One such attempt has involved the overexpression of PEPcase from maize, a C-4 plant, and has resulted in an impressive 10–30% increase in rice grain yield (Table 1).[84] Moreover, an additional 5–20% increase in yield was observed when the PEPcase-activating enzyme, pyruvate orthophosphate dikinase (PPDK), also from maize, was simultaneously overexpressed. The authors of this study ascribed much of this yield enhancement to increased stomatal conductance, which in turn led to higher CO_2 concentrations in the leaf and thus to a greater (up to 35%) photosynthetic capacity relative to untransformed plants.[84] This genotype also showed more efficient use of CO_2, and improved tolerance to photoinhibition.[84–86] This work suggests that even a partial realization of the C-4 pathway in rice can be of tremendous benefit to yield.

Given the yield increases attained through the overexpression of GS, PEPcase and PPDK, it is reasonable to expect that the simultaneous overexpression of all three genes may lead to still further gains in yield (Fig. 3). Indeed, high expression of these enzymes may work in a synergistic manner, given that they all appear to be related to stomatal function. Returning to the theme of nitrogen, a triply transgenic plant would also be expected to have particularly high agronomic NUE, due to a more efficient recapture of the NH_4^+ generated by processes such as photorespiration and proteolysis (especially during senescence),[65,87] and possibly due to a lower requirement for RuBisCO, a feature typical of C4 plants.[64] This is consistent with the goal of achieving higher rice yields with less input of fertilizer N, and reduced loss associated with nitrogen volatilization and runoff.

The potential of functional genomics

From the discussions provided here, it should be apparent that the components of the nitrogen acquisition pathway interact in multiple and complex ways. Indeed, the regulatory mechanisms involved are still insufficiently resolved, and this could present major obstacles to plant improvement via the accretion of single-character traits. Thus, we emphasize that an appreciation of the complex interactions among genetic, biochemical and ecological processes is a requirement for future plant breeding programs. In the case of nitrogen acquisition, the intersecting elements of ion transport, nitrogen assimilation and storage, photorespiration, photosynthesis and gene expression must all be considered, in order to maximize the benefits of research efforts.

The identification of new components and regulatory elements of the nitrogen acquisition pathway will improve our understanding of its complexity, and is expected to be greatly facilitated by recent strides made in the field of functional genomics. In particular, the recent completion of draft sequences of the rice genome, in both *indica*[88] and *japonica*[89] subspecies, have opened up novel possibilities. In addition, substantial collections of rice mutants are currently being produced via conventional (radiation- or chemical-based) mutagenic means and, especially, via the new forms of mutagenesis effected by random T-DNA and transposon insertion into the rice genome.[90–92] Such powerful reverse-genetics approaches are further augmented by intensive rice bioinformatics initiatives[92,93] and by the use of artificial chromosomes.[92] It is a fortunate coincidence that the compactness and transformability of the rice genome has made rice a model organism in this regard.[88,89,91] Given the high degree of genetic conservation among cereal grasses, discoveries made in rice will lead the way for parallel discoveries to be made in other important species such as wheat and maize.[91]

The application of this type of information to innovative ways of producing new breeding lines is also being pursued in rice. One compelling instance is the transfer of as many as ten foreign DNA fragments into a single vector that can be stably incorporated into the rice genome.[94] Another development is the extensive identification of quantitative trait loci (QTL) in rice. The open-source, web-based database for comparative grass genomics, www.gramene.org, lists 4,344 QTLs for rice (as of January 2004), including several loci governing traits related to nitrogen acquisition. These traits include total leaf nitrogen, protein content and content of key enzymes in the nitrogen assimilation pathway (glutamine synthetase and glutamate synthase).

The high-throughput, multiparallel information and plant transformation techniques emerging from the field of rice functional genomics will have direct impacts on the initiatives described in the present article. In the case of engineering nitrogen fixation in rice, new possibilities are opening up due to the complete sequencing of the genome of two rhizobial microsymbionts,[95] as well as to ongoing progress in the sequencing of the model legume species *Medicago truncatula*.[95,96] These developments, among others, have led to extensive new information regarding root nodule organogenesis and nitrogen fixation, which will be useful in

the initiative to transfer such traits to rice. In the areas of nitrogen uptake and assimilation, the set of currently described genes will be increased as new homologues of transporters, enzymes and control factors, and their expression patterns, are characterized using approaches based on comparative genomics, proteomics and metabolomics.[90] Perhaps the most challenging aspects of improving plant nitrogen-use efficiency through functional genomics analysis are the identification and engineering of genes that co-ordinate interactions between carbon and nitrogen metabolism (C–N interactions). However, instances such as the identification in rice of seven QTLs for glutamine synthetase content, and six for NADH-dependent glutamate synthase content, are indications that the realization of such possibilities is not far away.

Concluding remarks

In recent years, the global human population has increased by approximately 80 million people annually, and today over a billion people are estimated to be undernourished.[97] While at present there is, nevertheless, sufficient food production for all, and improvements in food storage and distribution may extend this period of safety for some time, it is unlikely that our burgeoning numbers can be adequately fed in the near future, without substantial increases in crop productivity. These gains will most likely be met through a combination of conventional and recombinant-DNA methods. The improvement of nitrogen acquisition in rice, in order to both increase crop yield, and to stem the loss of nitrogen from paddies, can be partially achieved by classical breeding methods and by improvements in field management practices, such as more precise fertilizer application and increased use of existing bacterial nitrogen-fixing systems. However, this type of improvement, by itself, will be unlikely to increase crop yields to the necessary extent.

Here, we have outlined several research areas that involve the exploration of novel alternatives. The engineering of biological nitrogen fixation in rice remains a distant possibility, but because the number of required enzymatic and developmental steps involved in this process are so extensive, it will not likely be achieved in the near future; advances in rice yield through enhancing existing rice–rhizobial interactions appears more likely at present. In our opinion, the potential of increasing rice nitrogen acquisition through the overexpression of nitrogen transport systems is not likely to succeed, unless the potent feedback regulation systems acting upon nitrogen transport are overcome. By contrast, increasing rice yields via the simultaneous expression of a group of related enzymes that influence both nitrogen and carbon metabolism appears particularly promising. This is partly because of recent successes that have already been achieved in rice and other plant species through the overexpression of GS and GDH. Furthermore, the enhancement of specific links between nitrogen metabolism and photosynthetic functions, provides an intriguing and potentially very beneficial opportunity. To summarize this concept (Fig. 3), a plant with high expression levels of the three enzymes GS, PEPcase, and PPDK might provide optimal stomatal control and relief from light stress through increased photorespiration, as a result of several factors: (1) Increased tissue K^+, due to the lower tissue NH_4^+ that results from increased GS activity, (2) Increased tissue malate, due to higher rates of C-4-acid production via PEPcase/PPDK; and (3) Increased glutamine:glutamate ratios, due to high GS activity, which in turn reduce the malate inhibition of PEPcase/PPDK.

Given the physiological complexity underlying such efforts, and especially given the urgency of this kind of research endeavour, it is of paramount importance that all information in this area be shared freely among the scientists involved. In addition, the plant lines produced must be made accessible, particularly when discoveries with such plants are published in journals that mandate such resource-sharing. Presently, however, there is a dangerous trend in which experimental results and materials are closely guarded by commercial or scientific oligarchies. This trend must be opposed. Information- and resource-sharing should be overseen by major non-profit international institutes, such as IRRI, that are already in place. IRRI, and its associated members of the non-profit Consultative Group on International Agricultural Research (CGIAR), focus on the development and free distribution of new lines of seeds to areas of the world that require assistance the most. These organizations are models of philanthropic achievement, and their action independent of the profit motive can help ensure that issues of hunger and poverty, rather than financial gain, be maintained as the driving forces behind this research.[1,98]

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