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Biotechnology of nutrient uptake and assimilation in plants

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ABSTRACT Plants require a complex balance of mineral nutrients to reproduce successfully. Because the availability of many of these nutrients in the soil is compromised by several factors, such as soil pH, cation presence, and microbial activity, crop plants depend directly on nutrients applied as fertilizers to achieve high yields. However, the excessive use of fertilizers is a major environmental concern due to nutrient leaching that causes water eutrophication and promotes toxic algae blooms. This situation generates the urgent need for crop plants with increased nutrient use efficiency and better-designed fertilization schemes. The plant biology revolution triggered by the development of efficient gene transfer systems for plant cells together with the more recent development of next-generation DNA and RNA sequencing and other omics platforms have advanced considerably our understanding on the molecular basis of plant nutrition and how plants respond to nutritional stress. To date, genes encoding sensors, transcription factors, transporters, and metabolic enzymes have been identified as potential candidates to improve nutrient use efficiency. In addition, the study of other genetic resources, such as bacteria and fungi, allows the identification of alternative mechanisms of nutrient assimilation, which are potentially applicable in plants. Although significant progress in this respect has been achieved by conventional breeding, in this review we focus on the biotechnological approaches reported to date aimed at boosting the use of the three most limiting nutrients in the majority of arable lands: nitrogen, phosphorus, and iron.

KEY WORDS: *plant nutrition, macronutrients, grain yield, gene overexpression, bacterial gene, biotechnology*

Introduction

Mineral elements, such as phosphorus (P), nitrogen (N), calcium (Ca), iron (Fe), zinc (Zn), magnesium (Mg), and manganese (Mn), among others, play essential roles in all living organisms. An appropriate balance of all these nutrients is necessary at each stage of plant development to achieve maximum yield. Plants require large amounts of P and N that are key nutrients because they are building blocks for fundamental biological molecules, such as nucleotides, amino acids, and proteins, whereas they need only small amounts of micronutrients, such as Fe, Zn, and boron, which generally act as cofactors in enzymatic reactions. However, in most soils, one or more of these nutrients is in short supply or unavailable for plant uptake as a consequence of different factors, such as low diffusion rates, rhizosphere microbial activity, and soil physicochemical properties. Among all the vital nutrients for plants, P and N are the most limiting factors for agricultural production, making necessary the application of high amounts of fertilizers each year to increase crop yield.

Since the Green Revolution, in the past 60 years, worldwide food production has increased dramatically thanks to the introduction of improved crop varieties, but also to an enhanced use of fertilizers and other agrochemicals. Currently, nearly 50 million

Abbreviations used in this paper: BBP, β-propeller phytase; CIPK, CALCINEURIN B-LIKE-INTERACTING PROTEIN KINASE; CK, cytokinin; DMA, deoxymugineic acid; fdr, ferric-chelate reductase defective mutant; FER, Fe-uptake response; FIT, FER-LIKE IRON DEFICIENCY INDUCED; FRO, FERRIC CHELATE REDUCTASE OXIDASE; Gln, glutamine; Glu, glutamate; GOGAT, glutamine-2-oxoglutarate aminotransferase; GS, glutamine synthetase; HAP, histidine acid phosphatase; HATS, high-affinity transport systems; IDEF, IRON DEFICIENCY-RESPONSIVE ELEMENT-BINDING FACTORS; IDS, IRON-DEFICIENCY SPECIFIC CLONE; IRO, IRON-RELATED TRANSCRIPTION FACTOR; IRT, IRON-REGULATED TRANSPORTER; LATS, low-affinity transport systems; LR, lateral root; MA, mugineic acid; NAS, nicotianamine synthase; NiR, nitrite reductase; NR, nitrate reductase; NUE, nutrient use efficiency; OA, organic acid; PAP, purple acid phosphatases; PEPc, phosphoenolpyruvate carboxylase (PEPc); Phi, phosphite; PHT, Pi transporter; Pi, inorganic P; PR, primary root; PUE, Pi use efficiency; TF, transcription factor.

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tons of P fertilizers, 110 million tons of N fertilizers, and 5 million tons of pesticides (of which 50% are herbicides) are applied worldwide (http://faostat.fao.org/site/339/default.aspx; http://www.fao.org/worldfoodsituation/wfs-home/csdb/en/; http://fertilizer.org/ifa/Home-Page/STATISTICS; http://www.fertilizer.org/ifa/HomePage/SUSTAINABILITY/Fertilizer-Best-Management-Practices). Although the systematic introduction of improved crop varieties allowed an increase in cereal production from 824 million tons in 1960 to almost 2,400 million tons in 2011 (www.fas.usda.gov/psdonline), traditional plant breeding programs appear insufficient to ensure future global food demands. Moreover, according to prognoses that take into account the current fertilizer consumption rate, 208 million tons of fertilizer will be needed to secure the global food demand in 2020 (Roy *et al.*, 2006). Moreover, when soil nutrient depletion derived from intensive land use is taken into consideration, fertilizer consumption in 2020 could reach up to 300 million tons, unless new plant varieties or hybrids with enhanced nutrient use efficiency and/or improved fertilization schemes are developed and implemented (Cakmak, 2002). Therefore, modern breeding programs based on genomic information together with transgenic approaches are needed to increase food production while reducing the use of fertilizers and other agrochemicals.

Among the factors that contribute to excessive N and P fertilizer consumption, the most important are (i) fixation of a large percentage of the P fertilizers into the soil, by adsorption and reaction with cations; (ii) loss of highly soluble N compounds by run-off and volatilization; and (iii) strong competition from soil microorganisms and weeds for the available N and P fertilizers. Due to these factors, cultivated plants use effectively only 20% to 30% of the applied fertilizers. Excessive application of fertilizers not only increases food production costs but also seriously pollute the atmosphere and water bodies, such as rivers and oceans, leading to eutrophication and ocean-dead zones. Hence, the rational use of these resources is imperative to enhance crop yields through improvement of the N and P uptake and utilization efficiency. In other words, the challenge consists not only in the development of genotypes that use N and P more efficiently, but also in their implementation in better-designed agricultural schemes. Achieving these goals is particularly urgent in the case of P because it is a nonrenewable resource, of which high-quality reserves have been predicted to last between 70 to 150 years if current use is maintained (Gilbert, 2009).

To produce improved plant varieties or hybrids, traditional breeding programs are based on trial-and-error strategies that are slow and require the analysis of thousands of cross-derived plants. Currently, knowledge-based breeding programs start to be designed thanks to the increasing information on plant biology that was initially triggered by the development of plant transgenesis and, more recently, by the implementation of next-generation DNA and RNA sequencing and other omics platforms. In particular, understanding the genetic basis of plant nutrition progresses rapidly and allows the identification and management of key regulatory elements involved in nutrient uptake, transport, and assimilation, as well as in root system morphology and physiology, such as ion transporters, transcription factors (TFs), and metabolic enzymes, of which overexpression or inactivation are becoming promising approaches to generate plants with improved nutrient use efficiency. In addition, because the human diet depend directly on the mineral composition of plants (such as cereal grains), increase in nutrient content and availability in agricultural products will have a positive impact. Here, we review the current knowledge on the key genes that regulate uptake and assimilation of P, N, and Fe in plants and on their application to improve nutrient use efficiency in several crops.

Engineering plant nitrogen nutrition for a rational agriculture

Not only is N an essential nutrient for plants, but also a signaling molecule that regulates important physiological and developmental processes, such as seed dormancy, flowering time, leaf expansion, root development and the expression of multiple N-responsive genes (Bouguyon *et al.*, 2012). Plants are able to use two types of N-containing compounds as N sources: inorganic compounds, namely nitrate (NO_3^-) and ammonium (NH_4^+) and organic compounds, such as amino acids, peptides (di- and tri-peptides), and proteins (Miller *et al.*, 2007). Under natural conditions, the content of organic and inorganic N sources in the soil is highly heterogeneous and dynamic, depending also on a variety of soil factors, such as temperature, pH, chemical properties, and the presence of microorganisms. The availability of NH_4^+, urea, amino acids, peptides, and proteins is limited in agricultural soils; therefore, NO_3^-, supplied as fertilizer, is the major N source for most plants under aerobic soil conditions (Krouk *et al.*, 2010). Hence, we will mainly discuss data regarding mechanisms for NO_3^- uptake, transport, and assimilation as well as the more recent progress on the regulatory elements that control responses to low NO_3^- availability and their potential use to improve N metabolism in plants.

Nitrogen uptake, transport, and assimilation in plants: a complex network of proteins

Regulation of plant N-metabolism in higher plants is highly complex and influenced by several physiological and metabolic processes such as sucrose synthesis and transport, circadian rhythms, key N metabolite levels (for instance, glutamine [Gln]), and NO_3^- itself. Physiological studies revealed that different plant species are capable of responding to N availability by reprogramming their growth through modification of root system architecture, modulation of vacuolar N storage and remobilization, and activity of NO_3^- and NH_4^+ transport systems. Extensive transcriptome analyses revealed that the expression of numerous genes (up to 10% of the *Arabidopsis thaliana* genome) is modulated by NO_3^- availability. The study of plant mutants together with genomic approaches has allowed the identification and functional characterization of several components of plant N responses, including specific NO_3^- and NH_4^+ transporters and transceptors (transporter/receptor) (Fig. 1A) as well as signaling components, including calcium-related protein kinases, TFs (Fig.1B), and some regulatory elements involved in responses to low NO_3^-, particularly modulating root development (Fig. 1C).

Nitrogen transport

Plants have evolved sophisticated mechanisms to optimize and regulate the acquisition and assimilation of different N sources. NO_3^- uptake from the soil relies on the concerted action of low-affinity and high-affinity transport systems (LATS and HATS, respectively) that ensure the intake of adequate levels of NO_3^- over a wide range of concentrations (Fig. 1A). Both LATS and HATS NO_3^- gene

families include constitutive and NO_3^--inducible members (Miller *et al.*, 2007). Until now, four NO_3^- -transporter gene families are known, of which NITRATE TRANSPORTER1/PEPTIDE TRANSPORTER (NRT1/PTR) and NITRATE TRANSPORTER2 (NRT2) gene families are responsible for NO_3^- uptake from the environment, the first also comprising transporters involved in NO_3^- efflux, such as the NITRATE EXCRETION TRANSPORTER and NRT1.5 (for a detailed review see Wang *et al.*, 2012).

The dual-affinity member of the *NRT1/PTR* gene family, *NRT1.1* (also called *CHLORATE RESISTANT1* [*CHL1*]), and the *NRT2.1*, *NRT2.2*, and *NRT2.4* members of the *NRT2* gene family are HATS particularly important under low NO_3^- availability. In addition to their transport function, *NRT1.1* and *NRT2.1* are also involved in NO_3^- sensing (Fig. 1A). *NRT1.1/CHL1* is an interesting case of a finely regulated gene: its expression is transcriptionally regulated by NO_3^-, nitrite (NO_2^-), NH_4^+, Gln, N starvation, light, sucrose, diurnal rhythm and its transceptor activity depends on the phosphorylation of threonine 101 by CALCINEURIN B-LIKE-INTERACTING PROTEIN KINASE23 (CIPK23) (Fig. 1B). Phosphorylated and dephosphorylated NRT1.1 acts as a HATS and LATS, respectively (Bouguyon *et al.*, 2012). Interestingly, these properties allow NRT1.1 to sense a wide range of NO_3^- concentrations in the soil and switch between its transporting and signaling activities. In contrast to CIPK23, CIPK8 positively regulates the low-affinity phase of NRT1.1. Furthermore, *NRT1.1* transceptor-dependent gene regulation is quite complex (Fig. 1B): it can up-regulate *NRT2.1* in response to short-term NO_3^- induction and down-regulate it under prolonged high NO_3^- levels (Gojon *et al.*, 2011).

NRT2.1 is the main component of inducible HATS in *Arabidopsis* roots, as demonstrated in *nrt2.1* that lack up to 75% of the high-affinity NO_3^- uptake activity. To be active, NRT2.1 forms a functional unit with NITRATE ACCESSORY PROTEIN2.1 (also called AtNRT3.1) that plays an important role in both constitutive and inducible HATS (Laugier *et al.*, 2012). NRT2.1 is up-regulated by NO_3^- and sugars and down-regulated by N assimilation products (such as Gln) and cytokinin (CK) (Kiba *et al.*, 2011).

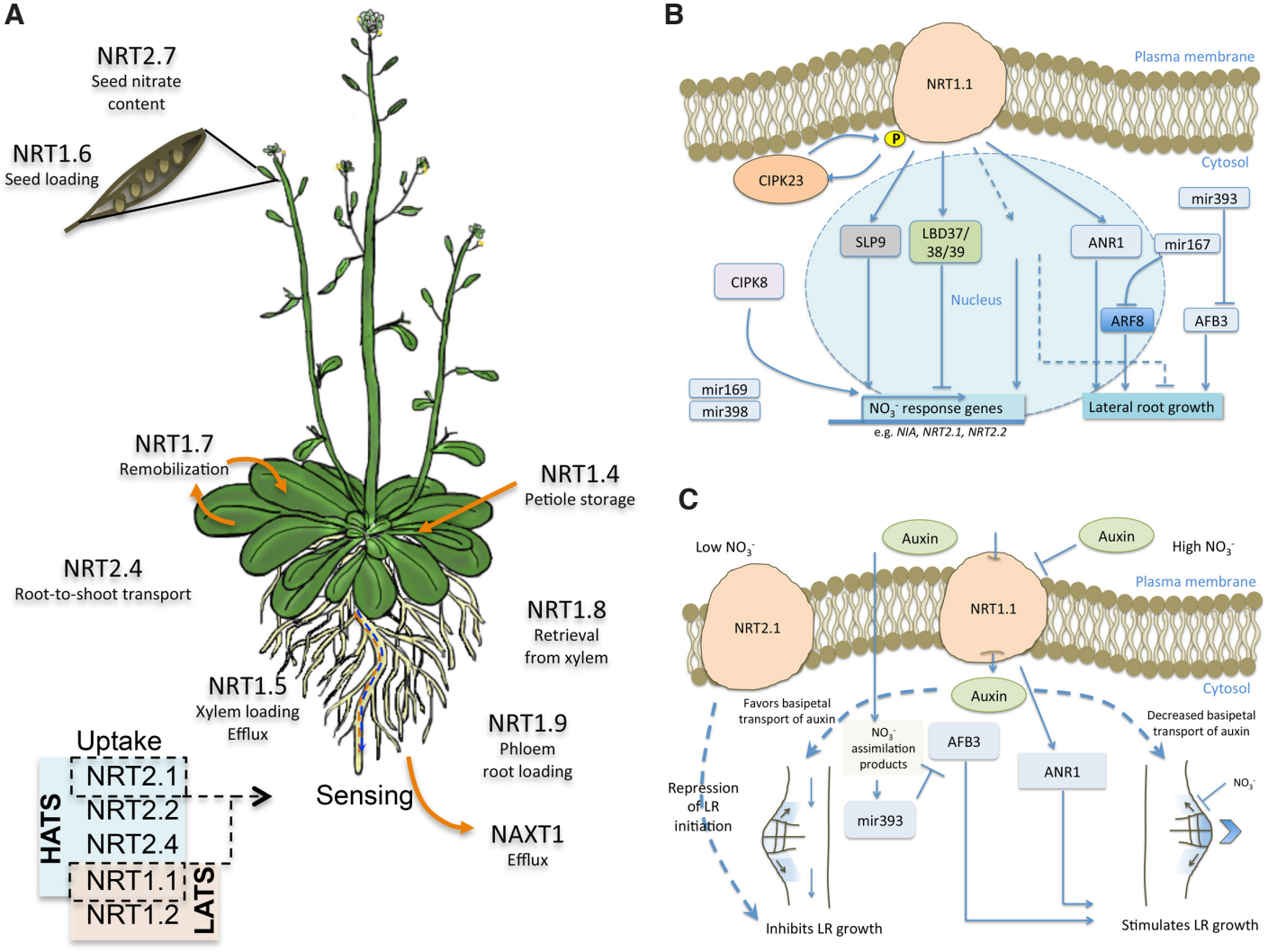

Fig. 1. Transporters and regulatory elements involved in nitrate metabolism. (A) *Nitrate is taken up through the root system and allocated to different tissues by the concerted action of numerous transporters. NRT1.1 and NRT2.1 also sense the nitrate status in the environment and inside the plant.* **(B)** *Schematic representation of the signaling pathways that regulate the expression of nitrate-responsive genes.* **(C)** *Schematic representation of the signaling pathways specifically involved in changes in root architecture in response to nitrate.*

Assimilation of nitrogen through the NR-NiR-GS-GOGAT pathway

Once NO_3^- has entered the root, it can be directly assimilated in root cells or transported across the plasma membrane to different tissues. To be incorporated into organic molecules, such as amino acids, NO_3^- is first converted in the cytoplasm into NO_2^- by a nitrate reductase (NR), then into NH_4^+ by nitrite reductase (NiR) in both the cytoplasm and plastids, and, finally, the resulting NH_4^+ into amino acids (e.g. Gln and glutamate [Glu]) through the glutamine synthetase (GS)/glutamate syntase (glutamine-2-oxoglutarate aminotransferase (GOGAT) pathway.

Sugars play a central role in the coordination of carbon (C) and N metabolism because amino acid biosynthesis requires C skeletons. For instance, light and carbohydrates transcriptionally induce NR-encoding genes, thus increasing N assimilation and amino acid biosynthesis when photosynthates are available. Recently, the mitochondrial folylpolyglutamate synthetase, encoded by *DFC*, has been involved in N assimilation through folate biosynthesis. *Arabidopsis DFC*-deficient mutants showed altered N utilization and a considerable reduction in lateral root (LR) initiation during early seedling development, a phenotype enhanced under low-N conditions (Jiang *et al.*, 2013).

N is predominantly transported inside plants as Gln, Glu, asparagine (Asn), aspartate (Asp), and, to a lesser extent, as NO_3^- and NH_4^+. This task is accomplished by the concerted action of LATS (NRT1.4-9) belonging to the NRT1/PTR family that play specific roles in plant development (Fig. 1A) (Wang *et al.*, 2012). Two additional HATS, NRT2.4 in *Arabidopsis* and NRT2.3a in rice (*Oryza sativa*) have been recently involved in root-to-shoot NO_3^- transport under N-limited conditions (Kiba *et al.*, 2012; Tang *et al.*, 2012) (Fig. 1A).

NRT1.1, CIPK23, and CIPK8 are well known to participate in the regulation of plant responses to low-NO_3^- availability. However, because mutants affecting this signaling pathway still present specific responses to NO_3^- deprivation, additional sensing systems (different to the NRT2.1-dependent one) might exist in plants that, together with some TFs, could combine at least two different low-NO_3^- response-controlling signaling pathways (Fig. 1 B,C). Recently, NODULE INCEPTION-LIKE PROTEIN7 (NLP7) has been reported as a novel TF involved in NO_3^- signaling. *Arabidopsis nlp7* mutants show a phenotype similar to that of N-starved wild-type plants and a considerably reduced expression of NO_2^- transporters (NRT2.1 and NRT2.2) and NR-encoding genes (*NIA1* and *NIA2*) after a short N stress followed by NO_3^- resupply. The increased LR growth phenotype of *nlp7* plants hints at a possible role of NLP7 in root branching under NO_3^- deficiency (Castaings *et al.*, 2009). Additionally, three members of the TF LATERAL ORGAN BOUNDARY DOMAIN gene family (LBD37, LBD38, and LBD39) have been found to act as negative regulators of NO_3^--responsive genes (e.g. *NRT2* and *NIA* genes) (Rubin *et al.*, 2009) (Fig. 1B).

Root architecture responses to nitrate availability

Root system architecture responses to NO_3^- availability are particularly interesting because NO_3^- has the capacity to induce two contrasting effects on root growth in several plant species: the inhibition of the root growth when NO_3^- is high and uniformly distributed in the media, and the stimulation of root growth by the direct contact with NO_3^--rich patches. This was initially observed in *Arabidopsis* split-root experiments in which the application of different N sources to one side of the split-root system caused a 2- to 4-fold increase in LR length (McAllister *et al.*, 2012). Although some elements regulating these responses have been characterized at the molecular level, many remain to be uncovered. *ARABIDOPSIS* NITRATE-REGULATED1 (ANR1), a MADS-box TF normally expressed in root tips of primary root (PR) and LR, and NRT1.1, have been identified as key components in the signal transduction pathway regulating NO_3^--inducible root growth (Remans *et al.*, 2006a) (Fig. 1C). *Arabidopsis nrt1.1* mutant displays alteration in PR and LR development, independently of N uptake and availability. The *nrt1.1* phenotype has been associated with a decrease in *ANR1.1* transcript abundance, placing *NRT1.1* upstream of *ANR1.1*. Enhancer trap and dexamethasone-inducible overexpressing lines confirmed that *ANR1* positively regulates LR initiation and growth, being more responsive in the presence of NO_3^- (Gan *et al.*, 2012). In *Arabidopsis*, *nrt2.1* mutants have decreased LR initiation, independently of its transport activity, suggesting a role for NRT2.1 in the control of root development (Remans *et al.*, 2006b). In fact, increasing evidence demonstrates that NRT2.1 acts as a sensor or signal transducer in an N-dependent LR growth-regulating pathway (Fig. 1C) (Gojon *et al.*, 2011).

A role for auxin on root branching in response to NO_3^- availability has also been uncovered. For instance, NRT1.1 not only senses NO_3^- responses, but also possibly facilitates auxin transport to promote LR growth under low-NO_3^- conditions (Fig. 1C) (Remans *et al.*, 2006a; Krouk *et al.*, 2010). Additionally, cell-specific transcript profiling in response to NO_3^- allowed the identification of the miR167/AUXIN RESPONSE FACTOR8 regulatory module as an important component in regulating the ratio between initiating and emerging LRs in *Arabidopsis* (Gifford *et al.*, 2008). Recently, the AUXIN SIGNALING F-BOX 3, an auxin receptor induced by NO_3^- in roots, was reported to be down-regulated by N- metabolites due the induction of miR393, modifiying auxin perception and, in turn, affecting PR and LR growth (Fig. 1C) (Vidal *et al.*, 2010). Because CK levels strongly correlate with N- status, a direct role of this hormone in regulating root architecture has also been proposed (Kiba *et al.,* 2011).

Crosstalk between auxin, abscisic acid, and CK signaling pathways has also been involved in coordinating the requirement and acquisition of N and their effects on root branching (Fig. 1C). For instance, in *Arabidopsis,* NRT1.1 regulates the expression in roots of *AtIPT3 (ADENOSINE PHOSPHATASE-ISOPENTENYLTRANSFERASE),* a gene involved in CK biosynthesis. However, it has been shown that when exogenously applied, CKs repress the expression of some NO_3^-, NH_4^+, and amino acid transporters, especially those expressed in roots (for a detailed review of hormonal control of N metabolism, see Kiba *et al.*, 2011).

Engineering nitrogen use efficiency

As mentioned previously, the use of N- fertilizers has resulted in significant increases in crop yield. However, this has been accompanied by an inevitable negative environmental impact, because only 30% to 50% of the applied N is used by crop plants, whereas with the remainder is lost by leaching, run-off, volatilization, or microbial activity. This situation has received great attention for many years and numerous research groups are attempting to identify genes that improve Nitrogen Use Efficiency (NUE) in plants. Although many definitions and evaluation methods have been proposed, NUE has been defined as the yield of grain per unit of N available in the soil (natural or applied).

The *efficiency* issue is being tackled by trying to identify genes

involved in N uptake and assimilation that have the potential to improve NUE. Extensive experiments with several plant species, including *Arabidopsis*, *Vicia narbonensis* (purple broad vetch), *Pisum sativum* (pea), *Brassica napus* (canola), *Nicotiana tabacum* (tobacco), rice, *Zea mays* (maize), and *Glycine max* (soybean), have allowed the identification of specific genes involved in amino acid biosynthesis, C/N homeostasis, and the regulation of N uptake, translocation, and assimilation, as candidate genes to improve NUE (for a detailed review, ision see Xu *et al*., 2012). However, in many cases, overexpression of these genes in many instances did not result in a direct effect on this trait. In the following paragraphs, some attempts to improve NUE are discussed.

Candidate genes involved in transport, translocation, and remobilization of nitrogen

N uptake is one of the most critical NUE components under N-limiting conditions. Although NO_3^- transporters, such as NRT1.1 and NRT2.1, are the first components of the NO_3^- assimilation pathway in the root, only few studies have been carried out to characterize the effect of their overexpression on plant growth and development. Although overexpression of the high-affinity NRT2.1 (*NpNRT2.1*) transporter in tobacco (*Nicotiana. plumbaginifolia*) led to an increase in NO_3^- uptake under low-NO_3^- conditions, no net improvement in NUE was observed (Quilleré *et al*., 1994).

Overexpression of other transporters/translocators involved in N homeostasis has been reported to have effect on NUE. Accumulation of storage protein (e.g. globulins and, albumins) in seeds has been strongly associated with N availability and partitioning of N- assimilates in several plant species. Therefore, improving amino acid uptake into the embryo could improve NUE. This was demonstrated by overexpression of the *Vicia faba (*fava bean) *AMINO ACID PERMEASE1* (*VfAAP1*) gene in *V. narbonensis* and pea that increased accumulation of storage proteins, particularly globulins, and of some amino acids such as Asn, Asp, Glu, and Gln in the seeds. Interestingly, seed from *VfAAP1*-overexpressing pea lines presented 20, 43 and 5% higher N, globulin, and weight, respectively, than those of control plants (Rolletscheck *et al*., 2005). In rice, overexpression of the endogenous *EARLY NODULIN93-1* (*ENOD93-1*) gene, which is potentially involved in amino acids transport, resulted in increased shoot dry biomass and the concentrations of total amino acids and total N in roots, especially under N stress. Moreover, although the number of tiller produced by *ENOD93-1*-overexpressing and untransformed plants was similar, transgenic plants exhibited a 10%-20% higher number of spikes and spikelets, which was reflected in an enhanced seed yield under both limiting and high-N conditions (Bi *et al*., 2009). Additionally, data reported in some patents suggest that overexpression of an improved yeast nitrate transporter in maize results in an increased NO_3^- uptake under field conditions (McAllister *et al*., 2012).

Modulation of NR and NiR enzymes to improve nitrogen use

As stated above, NO_3^- assimilation is controlled by the concerted action of several enzymes, such as NR and NiR. These enzymes are important regulatory checkpoints to be considered for improving NUE. However, overexpression of the tobacco NR-encoding genes *NIA1* and *NIA2* in different plants (e.g. *Solanum tuberosum* [potato], *Lactuca sativa* [lettuce], and *N. plumbaginifolia*) showed no NUE-phenotype associated under N-limiting conditions. Accordingly, overproduction of NR in *N. plumbaginifolia* resulted in reduced NO_3^- levels in leaves, corresponding to an increase in foliar Gln and malate accumulation (Good *et al*., 2004). When the tobacco *NIA2* gene was overexpressed in potato, NO_3^- levels in the tubers were lower than those in control plants and without effect on yield or tuber number (Djennane *et al*., 2002). The lack of positive effects of overexpression of genes encoding NR and NiR has been associated with a tight regulation at the translational and posttranslational levels. However, several patents protect the use of NR-encoding genes from the red algae *Porphyra perforata* and *P. yezoensis*, which overexpression increased yield in maize under N-limiting conditions (Good *et al*., 2004). These results suggest that more detailed studies are still necessary to unravel the mechanisms that regulate the expression of *NR* and *NiR* genes at the transcriptional and posttranscriptional levels and, more importantly, that sources of NR- and NiR-encoding genes from organisms other than plants must be considered.

Potential target genes from the GS-GOGAT pathway

In higher plants, GS and GOGAT play key roles in NH_4^+ assimilation. GS is the rate-limiting enzyme in controlling N assimilation to support plant growth and productivity because it catalyzes the major step that converts N into organic compounds. The two GS isoforms, located in the cytosol of phloem companion cells (GS1) and in the stroma of chloroplast (GS2), have essential roles in the assimilation and recycling of NH_4^+. GOGAT also has a central role in N assimilation and, as GS, is present in two isoforms in leaves: a ferredoxin-dependent GOGAT (Fd-GOGAT), exclusively present in the chloroplasts, and a NAD-dependent GOGAT (NADH-GOGAT), preferentially located in vascular bundles of unexpanded leaves. Numerous attempts have been made to determine the specific roles of GS/GOGAT genes on plant development. The study of *gs*-mutants in maize, for example, has revealed that GS has a pivotal role in grain filling, determining kernel size, and yield (references in Good *et al*., 2004). Therefore, changing the expression of *GS* genes as well as GS activity could potentially affect NUE.

Many experiments have been carried out to overexpress both cytosolic and plastidic GS isoforms in different plant species. The results obtained are controversial: some authors report positive effects on plant growth and protein content, whereas others have been unable to show a specific phenotype or increase in GS activity and none showed a direct effect on NUE. Overexpressionng a *Medicago sativa* (alfalfa) *GS1* gene in tobacco resulted in 40% more protein per area and a higher total leaf GS activity than those of wild-type plants, but only changes in free NH_3 and amino acid levels were observed in transgenic maize plants overexpressing a cytosolic *GS1* (McAllister *et al*., 2012). Although plants with enhanced GS activity in roots were obtained by the root-specific expression of the *GS15* gene of soybean in pea, biomass and N accumulation in the studied transgenic lines varied widely among N treatments and GS activity (Fei *et al*., 2006). Leaf-specific overexpression of *GS2* in tobacco stimulated growth rate, amino acid accumulation (2.5-fold Glu and 2.3-fold Gln), and seed biomass, but the amount of protein per unit of fresh weight was unaltered (Migge *et al*., 2000). The GS activity in transgenic lines with 15- and 18-fold higher transcript levels was only 2- to 2.3-fold higher than in control plants, evidencing a posttranscriptional control of *GS2*.

In the case of GOGAT, several reports have demonstrated its importance in grain production because, when suppressed, grain yield in rice, *Triticum* sp. (wheat), *Sorghum bicolor* (sorghum) and

maize is drastically reduced. For instance, a *nadg-gogat1* knockout mutant in rice showed a reduced number of panicles and spikelets per plant, causing a decrease in total yield and biomass (Tamura *et al.*, 2010). Recently, cosuppression of both Fd-GOGAT and NADH-GOGAT isoforms in rice drastically decreased tiller number, total shoot dry weight, and yield (Lu *et al.*, 2011). In rice, overexpression of *NADH-GOGAT* has been associated with an enhanced grain filling (Yamaya *et al.*, 2002) and, when overexpressed under its own promoter, grain weight was 80% increased, whereas overexpression of an alfalfa *NADH-GOGAT* in tobacco enhanced the C and N contents in shoots and roots (Good *et al.*, 2004; McAllister *et al.*, 2012). The study of the combined effects of *GS* and *GOGAT* overexpression with other genes involved in N metabolism could provide more consistent improvements in NUE.

In two rice cultivars with different GS2 activities, the ability to recycle and reassimilate NH_3 within the plant was better in the cultivar with high GS activity (Akenohoshi) than that of the cultivar with low GS activity (Kasalath), because less NH_3 is lost to the environment (Kumagai *et al.*, 2011). These data suggest that a diversity of tools must be considered to analyze the effects of assimilative enzymes on NUE and that the reduced NH_3 emission in crops is an additional target trait to improve NUE.

Genetic modifications involving transcription factors and other regulatory elements

TF candidates. The use of signaling and regulatory proteins, such as TFs, is a promising approach to modify plant metabolism for NUE improvement. For instance, *ANR1* overexpression induces LR initiation and elongation in *Arabidopsis* (Gan *et al.*, 2012). The manipulation of other genes, such as the overexpression of a maize *FERREDOXIN-NADP⁺ REDUCTASE* gene, enhanced root growth, ear size, and seed weight in transgenic maize, soybean, and rice (McAllister *et al.*, 2012). The increase in productivity of *ANR1*-overexpressing plants could be due to an increase in N uptake resulting from an enhanced exploratory capacity of the root system, which is one of the critical steps that limit the efficient use of applied N fertilizers. However, increased N uptake rates do not necessarily imply a higher assimilatory capacity, which would depend on the competitive nature of the plant itself. Therefore, an increased N uptake capacity combined with genes affecting seed production will probably be needed. For instance, the reduced expression of *CYTOKININ OXIDASE2* gene in rice, identified as quantitative trait locus *Gn1a*, increases the number of reproductive organs, improving grain yield (Ashikari *et al.*, 2005).

Overproduction of the DNA-binding with One Finger1 (Dof1) TF appears to enhance N uptake and assimilation under low-N conditions. Dof1 is a key activator for multiple genes associated with organic acid metabolism. Maize *Dof1*-overexpression in *Arabidopsis* revealed improved growth and increased amino acid (Gln and Glu) and total N contents under low-N conditions (Yanagisawa *et al.*, 2004). Recently, *ZmDof1*-overexpressing experiments in rice showed enhanced N and C accumulation and photosynthesis rates in transgenic rice plants under N-limiting conditions. N accumulation occurred particularly in roots that had also a higher biomass than the control plants (Kurai *et al.*, 2011).

Candidate genes involved in amino acid metabolism. Due to the importance of Gln and Glu as starting materials for the synthesis of other amino acids and nucleotides, the overexpression of enzymes involved in N assimilation have been assayed. One of these cases is the expression of the alanine aminotransferase-encoding gene *(AlaAT)* from *Hordeum vulgare* (barley) in canola and rice. AlaAT is an enzyme involved in stress and hypoxia recovery in plants. Transgenic lines overexpressing *AlaAT* under the canola root-specific *btg26* promoter displayed an increase in biomass and seed yield under N-limiting field conditions. In field trials with suboptimal N fertilization (56 $kg.ha^{-1}$), the seed yield of transgenic plants was 42.3% higher than that of the wild-type control plants, whereas at a rate of 168 $kg.ha^{-1}$, the seed yield increased by 32.7% (Good *et al.*, 2007). However, overexpression of *AlaAT* in *Arabiodopsis* did not result in a consistent phenotype and its positive effect in improving NUE was tissue specific.

Overexpression of the glutamate dehydrogenase A (*gdhA)* gene from *Escherichia coli* in tobacco resulted in a 10% increase in dry weight under field conditions with a regimen of 125 $kg.ha^{-1}$ of applied N (Ameziane *et al.*, 2000). Interestingly, when overexpressed in maize, the same enzyme induced an increased germination and grain production when plants were grown under water stress.

In higher plants, N is assimilated in Asn and Glu from Gln and Asp via asparagine synthetase (AS) that is encoded in *Arabidopsis* by a small gene family (*ASN1, ASN2,* and *ASN3*). Because Asn plays a key role in allocating N between source and sink organs as N storage compound, analysis of the effects of *ASN* overexpression is important. *ASN1*-overexpressing *Arabidopsis* plants show an enhanced tolerance to N-limiting conditions and an increased content of soluble and total proteins in seeds, as reflected in an increased seed weight. In addition, an increase in allocation of free amino acids (mainly Asn) to flowers and siliques has been observed (Lam *et al.*, 2003). Interestingly, pathogen resistance is also conferred by overexpression of *ASN* in *Arabidopsis* (Hwang *et al.*, 2011).

In addition, overexpression of the *E. coli* AS-encoding gene (*AS-A*) in lettuce resulted in early seed germination, early development of leaves and early bolting and flowering time when compared with control plants. Additional determinations showed that in *AS-A*-overexpressing lines, leaf protein content and dry weight were 1.2- to 1.4-fold and 1.3-fold higher, respectively, than those in wild-type plants (Giannino *et al.*, 2008). Overexpression of this enzyme in other leafy crops needs to be assessed to study a possible positive effect on NUE.

Aspartate amino transferase (AspAT), an enzyme involved in Asp and 2-oxogluatarate synthesis from Glu and oxaloacetate, and AlaAT have been proposed as main players in grain filling. However, overexpression of *AspAT* resulted in increased amino acid and protein contents in seeds, but not in improved seed yield or biomass under low-N or high-N conditions (McAllister *et al.*, 2012). Further studies of these enzymes in crop plants as well as field trials are needed to determine whether they can be used to enhance NUE in cereals.

Candidate genes involved in photosynthesis and carbon metabolism. It has been well documented that ribulose-1,5-biphosphate carboxylase oxidase *(*RuBisCO) and phospho*enol*pyruvate carboxylase (PEPc) are key players in C fixation and N storage as crosstalk points between C and N metabolism. Overexpressing *RuBisCO* theoretically represents a potential increase in photosynthesis, which is an ideal trait for crop improvement. In rice, *RuBisCO*

overexpression experiments resulted in an increase in RuBisCO to leaf N ratio, but the photosynthesis rate did not change. Similar results were obtained by overexpressing *PEPc* in tobacco and rice. However, gene shuffling of the *RuBisCO* large subunit apparently resulted in enhanced RuBisCO activity in maize, thus influencing NUE (McAllister *et al*., 2012).

The manipulation of other proteins involved in N homeostasis has positively affected NUE. The *Arabidopsis* SUGAR TRANSPORT PROTEIN13 (STP-13) is active in hexose transport in sink tissues and is regulated by a NO_3^--inducible GATA TF. In *Arabidopsis STP-13*-overexpressing plants, glucose uptake rate and N accumulation were higher than in control plants and more biomass was produced under N-limiting conditions (Schofield *et al*., 2009). The study of this type of transporters will help to understand the interactions between C and N metabolism and demonstrate that NUE may be improved by increasing C availability.

Super crops for low-phosphate soils: a dream or short-term reality?

Although the content of total organic P and inorganic (Pi) in the earth crust is high, the availability of orthophosphates ($H_2PO_4^-$ and HPO_4^{2-}), the only chemical form that can be acquired and assimilated by plants, is low and heterogeneous in almost all natural and agricultural ecosystems. Pi availability strongly depends on several factors, such as soil pH, cation presence, and its rapid conversion by soil microorganisms into organic forms that are not directly available for plant uptake (Alatorre-Cobos *et al*., 2009). The negative effect of these factors is clearly noticeable in the high amounts of Pi fertilizers used each year. Of particular importance is that only approximately 20% to 30% of the P fertilizers applied is effectively exploited by cultivated plants. Therefore, P represents one of the major constraints to increase crop productivity in arable lands worldwide.

During land plant evolution, primitive aquatic plants, which came from a mineral-rich aquatic world, had to cope with low-Pi availability in the newly colonized terrestrial habitats. This adaptation involved the acquisition of complex and orchestrated strategies to survive and reproduce under Pi-limiting conditions. Currently, forward and reverse genetics approaches combined with analyses of whole transcriptomes, obtained by high-throughput sequencing technologies, allow the identification of master controllers of the different signaling pathways that modulate plant responses to Pi deficiency. Genes involved in Pi uptake, translocation, and recycling have been characterized and their possible roles in enhancing Phosphate Use Efficiency (PUE) have been evaluated. In the following sections, we will describe some of the genetic modifications that are aimed toward increasing PUE and are potentially useful for agronomically important crops.

Pi signaling pathway components

Pi uptake from the rhizosphere is a function carried out by proton (H^+)/Pi symporters, simply called Pi transporters. To date, four gene families of Pi transporters (PHT1, PHT2, PHT3, and PHT4) have been identified in *Arabidopsis* and their respective orthologs have been found in other plant species. Special attention has been given to the *Arabidopsis PHT1* gene family that includes nine members encoding high-affinity Pi carriers that are mainly expressed in root epidermal cells and are highly responsive to Pi deficiency. The contribution of these carriers to Pi acquisition has been determined with null mutants and gene overexpression approaches in *Arabidopsis* and rice. The first plant Pi transporter was identified by overexpression of the *Arabidopsis AtPT1*/*AtPHT1;1*. Tobacco cells overexpressing *AtPHT1;1*, produced 1.5-fold higher biomass than control cells when grown under Pi-limiting conditions (Mitsukawa *et al*., 1997). Recently, a role in tolerance to low-Pi availability of *AtPHT1;9* was observed in *Arabidopsis AtPHT1;9*-overexpressing lines, in which 20% to 30% more shoot fresh weight was produced than in wild-type plants under Pi-limiting conditions (Remy *et al*., 2012). In rice, overexpression of *OsPT1* not only results in a two-fold higher Pi content than in wild-type plants, but also in a higher number of tillers per plant, independent of the Pi-fertilization regimen (Seo *et al*., 2008). In contrast, barley plants with an enhanced expression of *HORvu;Pht1;1* (*HvPT1*) showed no differences in dry weight and total P content when compared to control plants (Rae *et al*., 2004). Thus, although a positive correlation between enhanced expression of PHTs and increased Pi content has been reported, it is not always observed, implying that a tight control operates at different levels (transcriptional, translational, and metabolic rearrangements) to maintain plant Pi homeostasis.

In general, overexpression of *PHT* genes moderately increases Pi content and biomass accumulation, whereas the constitutive expression of regulatory elements, such as TFs, microRNAs, signaling intermediates, or some TF activity modifiers, apparently increases Pi accumulation in plant tissues (Fig. 2). In *Arabidopsis*, overexpression of *PHOSPHATE STARVATION RESPONSE1 (PHR1)*, a master TF controlling a large subset of Pi stress-responsive genes, including *PHT* genes, resulted in a 2.5- and 4-fold increase in shoot Pi accumulation, under Pi-sufficient and Pi-stress conditions, respectively (Nilsson *et al*., 2007). Such increases have been associated directly with increased transcript levels of *AtPHT1;7, AtPHT1;8*, and *AtPHT1;9,* confirming that Pi acquisition is a concerted action between several transporters and that their individual overexpression might not be sufficient to have a significant impact on Pi content and plant growth. In rice and canola, overexpression of *PHR1* orthologs had similar effects on Pi accumulation and *PHT* expression levels (Zhou *et al*., 2008; Ren *et al*., 2012). In *PHR2*-overexpressing rice plants, increased Pi content was associated with changes in root system architecture (longer PR and LRs) (Zhou *et al*., 2008). Interestingly, in null mutants of the *AtSIZ1* (a positive regulator of PHR1 activity by sumoylation) gene, a root phenotype similar to that described for *PHR2*-overexpressing plants was associated with changes in auxin accumulation (Miura *et al*., 2011). Although SIZ1 and PHR1 are components of the same signaling pathway that regulate many responses to Pi deficiency, these molecular controllers might play opposite roles on root development (Miura *et al*., 2011).

Improved performance under Pi limiting conditions has also been reported in transgenic plants in which other TFs, that participate in the control of the plant responses to Pi deprivation, are expressed constitutively (Dai *et al*., 2012). In rice, *OsMYB2P-1* encodes a novel R2R3 MYB TF, of which the expression is induced in roots, stems, and leaves by Pi starvation. Under Pi-limiting conditions, *OsMYB2P-1*-overexpressing rice plants show an enhanced tolerance to low Pi, as reflected in a 24% and 30% higher shoot and root dry weight, respectively, than those of control plants. Such increase in biomass production in *OsMYB2P-1*-overexpressing plants was positively correlated with enhanced Pi content, espe-

cially in roots. However, under Pi-sufficient conditions, *OsMYB2P-1*-overexpressing rice plants showed a 50% decrease in shoot and root growth associated with an increased Pi content. As observed for *PHR1* overexpression, constitutive expression of *OsMYB2P-1* also up-regulates the expression of *PHT* genes (Dai *et al.*, 2012).

Modulator functions on Pi homeostasis are also known for other signaling components located downstream of TFs. SPX proteins have been described in yeast and in humans as proteins harboring domains involved in Pi perception, signaling, and transduction. In *Arabidopsis* and rice, analyses of SPX-RNAi and *SPX*-overexpressing lines indicate that SPX proteins negatively modulate the expression of genes involved in the uptake, allocation, and remobilization of Pi (Wang *et al.*, 2009a). As *SPX* genes play an important role in Pi homeostasis in plants, they represent a potential target to produce plants with improved PUE.

Although large sets of Pi-responsive TFs have been identified in massive transcriptomic analyses, new P signaling networks, different from those controlled by SIZ1/PHR1, have been poorly described. Characterization of new genetic controllers for Pi homeostasis and a better understanding of Pi signaling networks will provide new opportunities to generate plants with an enhanced Pi use, especially under low-Pi availability conditions.

Biotechnological approaches to improve phosphate uptake and PUE

Pi acquisition capacity in conjunction with the internal PUE are the two major parameters influencing PUE index. For the last 10 to 15 years, crops with a high PUE have been obtained by exploring the natural variation among crop genotypes or by modifying the expression of genes directly involved in Pi uptake or those considered as master regulators of Pi homeostasis in plants (e.g. Pi transporters, TFs, signaling intermediates, and traffic facilitators). Moreover, the use of bacterial genes involved in the metabolism of nonconventional P sources has provided interesting results. Among the efforts to improve the PUE, three attempts are generally considered promising if implemented in important crops and the field trials are successful: the use of phythases to metabolize phytate, the use of citrate synthases to overproduce citrate, and the use of phosphite dehydrogenase to use phosphite (Phi) instead of Pi as fertilizers (Fig. 2,3). Below, these approaches and their implications will be discussed.

Releasing phosphate for surviving: a role for phytases and organic acids

The rapid fixation of Pi into the soil by cation interaction and its quick conversion into organic forms (not readily available for plant uptake) by microbial activity are probably the main causes of low Pi availability in natural soils. Therefore, for plant uptake, Pi must first be released from insoluble compounds or organic forms present in the soil. In response to Pi deficiency, the root system of several plant species, such as canola, *Lupinus albus* (white lupin) and the Proteaceae plant family, shows an enhanced exudation of organic acids (OAs; such as citrate), favoring Pi solubilization and enhancing P uptake. This response is positively controlled at transcriptional and posttranscriptional level in monocotyledonous and dicotyledonus plants (Ryan *et al.*, 2001). OAs are low-molecular weight carbon compounds harboring one or more carboxyl groups and are intermediates (e.g. citrate, malate, and fumarate) in the tricarboxilic acid cycle of living cells. In soils, depending on their dissociation properties and number of carboxyl groups, OAs can bind different cations, such as aluminum (Al^{3+}), iron (Fe^{3+}), and calcium (Ca^{2+}), ameliorating the negative effects of these cations on plant nutrition.

The initial attempt to improve Pi acquisition in plants was the enhancement of the OA production and exudation with the aim to overcome Al^{3+} toxicity in acidic soils and to remobilize Pi from calcium phosphates in alkaline soils. In 1997, tobacco and *Carica papaya* (papaya) transgenic plants expressing a bacterial citrate synthase gene were produced (De la Fuente *et al.*, 1997). These transgenic plants had an enhanced capacity to acquire Pi from insoluble-P sources. Under different Pi regimens, the transgenic tobacco plants accumulated more dry weight in shoots and fruits (15% and 23 to 35%, respectively) than control plants, corresponding to an increased total P content in shoots (30% to 40%) (López-Bucio *et al.*, 2000). Although these initial results were controversial, several independent studies have confirmed a positive correlation between overexpression of genes encoding enzymes involved in OA biosynthesis and an improved plant growth in soils with low Pi availability and/or in the presence of toxic Al^{3+} concentrations (Ryan *et al.*, 2011).

In the last decade, research on Al^{3+} tolerance mechanisms in plants has expanded our knowledge on new molecular players implicated in the release of OAs by roots into the rhizosphere. Recently, *MULTRIDUG AND TOXIN COMPOUND EXTRUSION (MATE)* genes that encode OA transporters have been identified

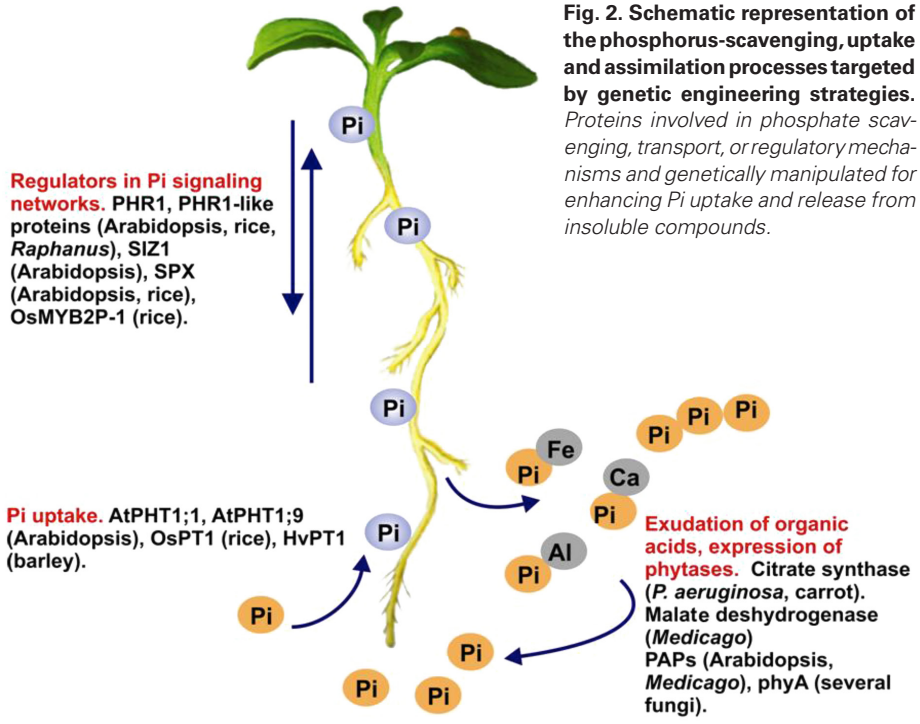

Fig. 2. Schematic representation of the phosphorus-scavenging, uptake and assimilation processes targeted by genetic engineering strategies. *Proteins involved in phosphate scavenging, transport, or regulatory mechanisms and genetically manipulated for enhancing Pi uptake and release from insoluble compounds.*

(Ryan *et al*., 2011), of which some are root specific and induced by Pi deficiency or toxic Al^{3+} concentration. The role of MATE proteins in Al^{3+} tolerance was demonstrated by heterologous expression and knockout mutant analyses, but their possible function in Pi acquisition still remains to be uncovered (Ryan *et al*., 2011). The effectiveness of enhanced OA biosynthesis and OA efflux transporters to confer an enhanced Pi uptake or Al^{3+} tolerance remains to be demonstrated under field conditions and to be agronomically relevant.

Enhancement of the Pi-scavenging capacity of plants, especially during Pi starvation, has been tested via the expression of genes encoding enzymes that directly hydrolyze organic Pi forms that cannot be taken up directly by plant roots. Special attention has been paid to hydrolytic enzymes that can liberate Pi from phytate (myoinositol 1,2,3,4,5,6-hexakisphosphate), the predominant organic form of P accumulated by animals and plants. These enzymes are collectively called phytases and are classified into four groups according to their catalytic properties: (i) histidine acid phosphatases (HAPs), (ii) purple acid phosphatases (PAPs), (iii) Cys phosphatases, and (iv) β-propeller phytases (BPPs) (Brinch-Pedersen *et al*., 2002; Ma *et al*., 2009).

The biotechnological impact of phythase secretion into the soil on Pi plant nutrition has been evaluated by constitutive or root-specific expression of chimeric versions of phytase genes harboring extracellular targeting sequences. This strategy has been found to be effective for several plant species (e.g. *Arabidopsis, Trifolium subterraneum* [subterranean clover], potato, soybean, canola, rice, wheat, and tobacco) that were capable of growing in media supplied with phytate as the sole P source (Brinch-Pedersen *et al*., 2002; George *et al*., 2005). For instance, under *in vitro* conditions, the Pi content of transgenic *phytase A*-overexpressing tobacco plants grown with phytate as sole P source was 3-fold higher than that of control plants. However, the capacity of these transgenic plants to use phytate as a P source was reduced in natural low-Pi soils (George *et al*., 2005).

In addition, canola and soybean plants expressing phytases (of HAP and BPP type) from several sources (e.g. fungi, bacilli, and yeast) have also been generated to evaluated their phytate-hydrolysing capacity in seeds, in which phytate accounts for 60%-80% of the total P, to improve nutrition of monogastric animals. When broilers and piglets were fed with these phytase-expressing seeds, significant increases in body-weight gain and gain-feed ratios were observed without adverse effects in liver, kidney, or bone tissues, when compared to control diets. These data suggest that expression of phytases increases the availability of the Pi contained in seeds for animal nutrition (Brinch-Pedersen *et al*., 2002).

The presence of PAPs has been reported in several plants species, usually as Pi-starvation responsive genes. In *Arabidopsis*, a large family for PAPs with at least 29 genes has been identified, of which only a few members encode enzymes with phytase activity. Heterologous expression of the PAP-encoding genes *PAP15* (from *Arabidopsis*) and *PAP1* (from *Medicago truncatula* [barrel medic]) in soybean and *Trifolium* sp. (clover), respectively, showed a positive correlation between an enhanced phytase activity and a increase in dry weight and total P content when phytate was supplied as sole P source (Xiao *et al*., 2006; Wang *et al*., 2009b). Further characterization of the *AtPAP15*-overexpressing soybean plants showed that the numbers of pods and seeds per plant were higher than those of nontransformed controls (Wang *et al*., 2009b). This increased productivity was observed even when plants were grown in natural acidic soils, but has not been observed for other HAP-overexpressing plants.

The relatively modest increase in Pi acquisition exhibited by phytase-overexpressing plants grown under natural soil conditions has been attributed to several factors, such as low phytase activity, nonspecific substrates, and low stability in the soil of the secreted protein. However, the importance of phytate availability has been largely ignored. In nature, phytate accumulates in the soil as a mixture with cations (usually K^+, Ca^{2+}, Mg^{2+}, or Zn^{2+}) known as phytin, which is transferred to the soil via plant and animal wastes. Engineered phytases to be secreted into the rhizosphere or those naturally associated to plant roots are only able to hydrolyze soluble phytate that represents only a small fraction of the total phytate present in the soil. Therefore, a biotechnological approach combining the expression of phytases with increased OA exudation has been suggested as a more effective strategy to increase phytate solubility from phytins (Brinch-Pedersen *et al*., 2002).

The phosphite oxidoreductase/phosphite system: a simple solution for two complex problems

Because Pi cannot be substituted in plant nutrition, relatively little attention has been given to the use of other chemical forms of P to formulate effective and environmentally friendly fertilizers. After World War II, Phosphite (Phi), a reduced form of P, was proposed as a promising alternative P fertilizer because of its distinct chemical and biochemical properties compared to Pi, namely, increased solubility, reduced reactivity with soil components, and the inability of most microorganisms to use it as a P source. However, several reports have demonstrated that plants cannot metabolize Phi and that this reduced P form decreases plant growth and development. Although Phi formulations are currently sold as P fertilizers, these reports challenge the use of Phi as a direct source of P to support plant growth, and suggests that its known beneficial effects on plant growth are only due to its well-documented properties to control oomycete diseases and its capacity to activate plant defense mechanisms (references in López-Arredondo and Herrera-Estrella, 2012).

Some bacterial species have been described that can oxidize Phi into Pi. The *ptxD* gene from *Pseudomonas stutzeri* WM88 encodes a highly Phi-specific oxidoreductase that oxidizes Phi using NAD^+ as a cofactor, yielding Pi and NADH as products (Metcalf and Wolfe, 1998). Recently, by means of the *ptxD* gene from *P. stutzeri,* transgenic *Arabidopsis* and tobacco plants that metabolize Phi have been generated (López-Arredondo and Herrera-Estrella, 2012). In contrast to wild-type plants, which growth was reduced when fertilized with Phi, PTXD-expressing lines grew equally well when fertilized with Phi than the same lines or the wild-type fertilized with Pi. More importantly, when grown under greenhouse conditions in agricultural soils containing their native microflora, PTXD-expressing lines required 30% to 50% less P for an optimal productivity when fertilized with Phi than with Pi (for details, see López-Arredondo and Herrera-Estrella, 2012). These results illustrate that most soil microorganisms are unable to use Phi as a P source and, thus, do not compete with the transgenic plants for its use as nutrient source. Therefore, this genetic modification represents an improvement in the competitiveness of the transgenic plants over the soil microflora, allowing a more effective use of P resources present in the soil when Phi is applied as fertilizer (Fig. 3).

Weedy plants also represent a major challenge to agriculture, particularly those that have become resistant to one or more traditional herbicides. Because, in principle, weeds are also unable to metabolize Phi, this reduced P form could be used as a fertilizer that, in low-Pi containing soils, would prevent or reduce weed growth. Growth competition experiments, in which seeds of PTXD-expressing tobacco had been mixed with seeds from weedy species were sown in low-Pi agricultural soils, revealed that the tobacco plants rapidly outgrew different weedy species when fertilized with Phi (López-Arredondo and Herrera-Estrella, 2012).

Fertilization and weed control based on transgenic plants expressing a phosphite oxidoreductase gene have clear advantages over current Pi fertilization systems because they exploit the chemical and biological properties of Phi (Fig. 3). In principle, this system is applicable to any cultivated plant species amenable for genetic transformation. Based on its effectiveness in weed control and fertilization under greenhouse conditions, this system could reduce production costs and energy consumption by replacing the independent application of fertilizers and herbicides by a single treatment, thereby also decreasing the cost for additional herbicides.

Engineering iron metabolism to improve plant nutrition and grain fortification

Iron is one of the most important micronutrients for all living organisms, including humans and plants. This micronutrient is an essential component for a myriad of proteins (e.g. iron-sulfur and heme proteins) involved in a diversity of processes, such as photosynthesis, reactive oxygen species scavenging, transcriptional regulatory networks, mitochondrial electron transport chains, DNA synthesis and repair, sensing, and signaling. Therefore, as a widespread utilized element in plants, a tight control of Fe endogenous levels is necessary to satisfy the demand for this nutrient, but also to avoid its toxicity. Excessive levels of Fe have detrimental effects on plant development, mainly due to the reaction of Fe with hydrogen peroxide, via the well-known Fenton reaction, that produces harmful reactive oxygen species. Disruption of genes encoding iron-reservoir proteins (ferritins), provokes an increase of the oxidant sensitivity in *Arabidopsis* (Ravet *et al.*, 2009).

Regulation of iron metabolism in plants

Although Fe is not required in high amounts by plants, its low solubility in agricultural soils greatly affects crop yield, especially in alkaline soils (Guerinot and Yi, 1994). In nature, free Fe concentration at neutral pH is in the range of 10^{-17} M, which would cause Fe-deficiency symptoms because the required amount for optimal plant growth is between 10^{-9} to 10^{-4} M (Guerinot and Yi, 1994). Detailed studies have demonstrated that plants utilize two different pathways that are activated by Fe deficiency to cope with this disadvantageous scenario (Fig. 4A). One of them, known as the "*reduction strategy*" (Strategy I) is used by most dicotyledonous and monocotyledonous plants, with the exception of graminaceous plants. The *reduction strategy* depends on the activities of a proton pumping ATPase (H^+-ATPase) that lowers the soil pH to release Fe^{3+} from chelating agents and of a root membrane reductase to produce Fe^{2+} from Fe^{3+}. In several plant species, H^+-ATPase-encoding genes have been identified. In *Cucumis sativus* (cucumber) roots, the plasma membrane H^+-ATPase-encoding gene *CsHA1*, is up-regulated in response to Fe-limiting conditions, whereas *CsHA2* remains unaltered (Santi *et al.*, 2005). In *Arabidopsis* roots, two H^+-ATPase-encoding genes, *AHA2* and *AHA7* are also up-regulated under Fe deficiency (Santi and Schmidt, 2009). Analysis of the Fe

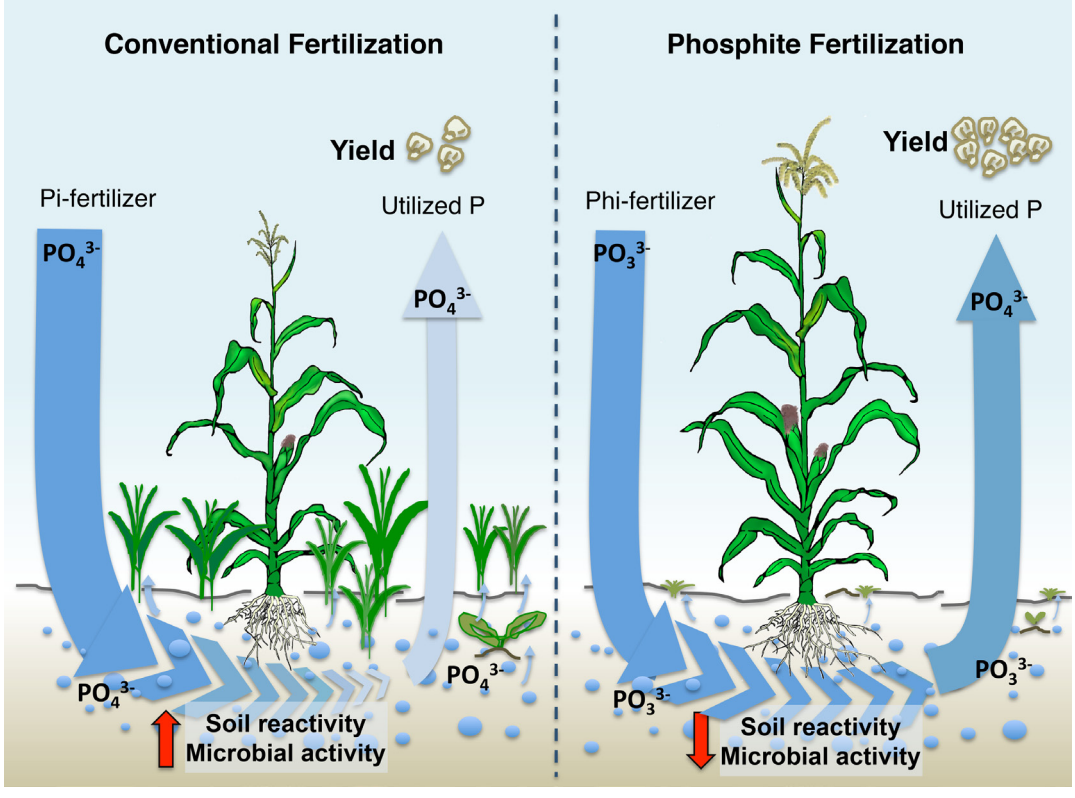

Fig. 3. Phosphite oxydoreductase/phosphite-based fertilization and weed control system. *Schematic representation of the performance of a phosphite (Phi) fertilization scheme in comparison to a conventional (phosphate) (Pi) fertilization without use of herbicides. In the conventional fertilization, approximately 70% to 80% of the applied Pi gets fixed by adsorption or is converted by soil microorganisms into organic compounds not readily available for plant uptake. Additionally, weed growth is promoted by Pi fertilization. As weeds and most soil microorganisms are unable to use Phi as a phosphorus (P) source, in the Phi fertilization scheme genetically modified plants expressing a phosphite oxidoreductase are more competitive than weeds and soil microorganisms because they can use Phi as sole P source, as illustrated in an increased grain yield. The Phi fertilization system reduces the amount of P fertilizers needed for optimal productivity and also limits the need of herbicides for weed control.*

deprivation responses of *aha2* and *aha7* mutants revealed that the lack of these H^+-ATPases diminishes the capacity of the root to acidify the rhizosphere and to develop root hairs, respectively. These observations suggest that H^+-ATPases are important constituents of the *Arabidopsis* response to Fe deficiency (Santi and Schmidt, 2009). The next step in Fe uptake is the reduction of Fe^{3+} into the more soluble Fe^{2+}, which is carried out by FERRIC CHELATE REDUCTASE OXIDASE (FRO) (Robinson *et al*., 1999). *Arabidopsis* possesses eight FRO-encoding genes, among which *FRO2* maps and complements the *ferric-chelate reductase defective 1* (*fdr1*) mutant that displays severe chlorosis under Fe-deficient conditions (Robinson *et al*., 1999). *FRO2* is predominantly expressed in root epidermal cells, whereas *FRO3*, another member of the family, is mainly expressed in the vascular cylinder, hinting at important roles in Fe reabsorption from external Fe uptake and from the apoplast, respectively. In contrast, both *FRO5* and *FRO6* are expressed in shoots and flowers; whereas *FRO7* and *FRO8* are shoot specific, suggesting roles in Fe homeostasis in different tissues (Wu *et al*., 2005).

Once the Fe^{2+} ion is produced, members of the metal transporter family ZINC-REGULATED TRANSPORTER (ZRT) and IRON-REGULATED TRANSPORTER (IRT)-like PROTEIN (acronym ZIP) carry Fe across the plasma membrane (Eide *et al*., 1996). In *Arabidopsis*, the *IRON-REGULATED TRANSPORTER1* (*IRT1*) gene acts as the major Fe transporter and is expressed in epidermal cells of Fe-starved roots (Vert *et al*., 2002). *irt1* mutants characteristically display chlorosis and severe growth impairment that can be rescued by exogenous Fe (Vert *et al*., 2002). Interestingly, the homologous *IRT2* gene does not complement the *irt1* mutant phenotype when overexpressed, suggesting that *IRT2* is not involved in Fe uptake (Varotto *et al*., 2002). Besides Fe, IRT1 also transports zinc (Zn), manganese (Mn), cobalt (Co), and cadmium (Cd) due to its low cation selectivity and its misregulation in the *fdr1* and *fdr3* mutants affects the Fe-uptake system (Eide *et al.,* 1996).

Several TFs control the expression of genes involved in the Fe uptake Strategy I. The first TF described was the tomato IRON UPTAKE RESPONSE (FER), a protein with a basic helix-loop-helix (bHLH) domain that, when mutated, affects Fe responses (Ling *et al*., 2002). In *Arabidopsis*, a bHLH29 known as FER-LIKE IRON DEFICIENCY INDUCED (FIT), plays a similar role in regulating several low-Fe inducible genes, such as *FRO2* and *IRT1* (Colangelo and Guerinot, 2004). In addition, bHLH38, bHLH39, bHLH100, and bHLH101 were identified as FIT interactors that are induced by Fe deficiency. The transcriptional activity of the heterodimer FIT/bHLH101 is higher than that of FIT/bHLH100 (Wang *et al*., 2013a). The double mutants, *bhlh39-bhlh100* and *bhlh39-bhlh101* and the triple mutant *bhlh39-bhlh100-bhlh101* behave as the wild-type in Fe-optimum medium, but under Fe-limiting conditions, they have a significantly reduced plant growth and lower Fe content (Wang *et al*., 2013a).

Changes in the root system architecture in response to Fe deficiency have been mainly associated with strategy-I plants, because they depend directly on the root surface area to acidify the rhizosphere and to reduce Fe^{3+} into Fe^{2+}. In *Arabidopsis*, the root hair density increases by 42% and 61% under Fe- and Pi-limited conditions, respectively (Müller and Schmidt, 2004). In addition, under Fe deficiency, plants increase the absorptive surface by forming branched root hairs, whereas under low-Pi conditions, the increase in hair density is mainly achieved by the formation of extra hairs (Müller and Schmidt, 2004). Impaired root hair patterning in response to Fe deficiency in mutants affected in genes involved in the first steps of root hair differentiation suggests that the nutritional signals to Fe deficiency are perceived at early stages of epidermal cell development. For instance, root hair branching in Fe-deficient roots is lost in *transparent testa glabra1* (*ttg1*), *glabra2* (*gl2*), *caprice* (*cpc*), *ectopic root hair1* (*erh1*), and *erh3* mutants (Müller and Schmidt, 2004). Recently, two ubiquitin-conjugating enzymes, UBC13A and UBC13B, have been identified that specifically regulate root hair branching in response to Fe availability in *Arabidopsis* (Li and Schmidt, 2010). It would be very interesting to

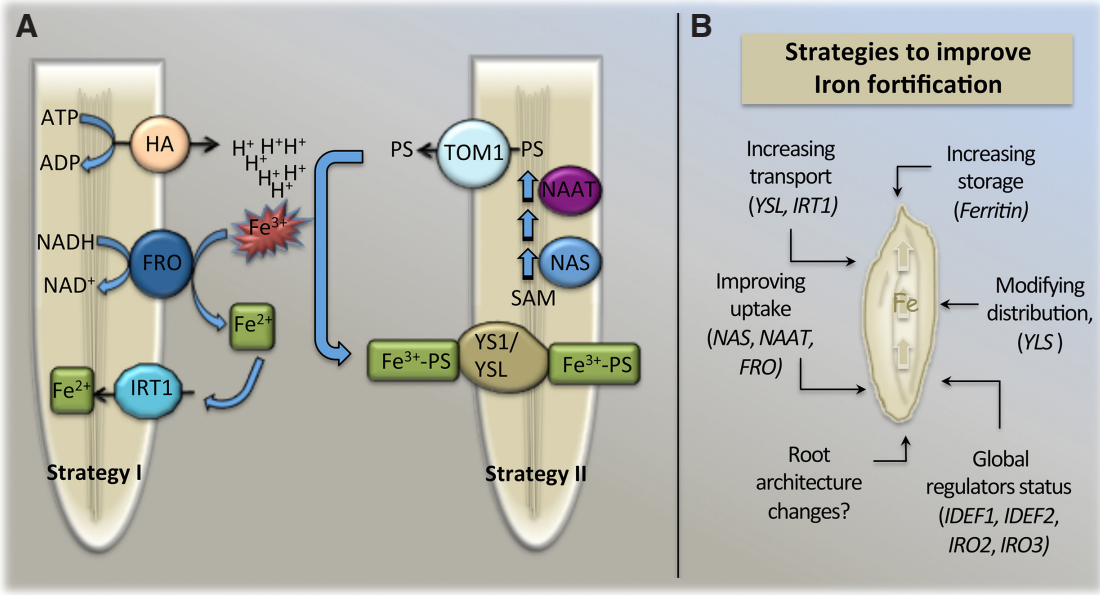

Fig. 4. Plant strategies of iron uptake and biotechnological efforts to improve iron content in seeds. (A) *Schematic representation of the two main iron uptake systems in plants.* **(B)** *Biotechnological efforts that have resulted in increased iron content in rice.*

test whether controlling root hair length and density could positively affect plant growth in crop plants as reported for *Arabidopsis* plants grown under low-Pi conditions.

The second strategy for Fe uptake, known as "*chelation strategy*" (Strategy II), is used by major cereal crops, such as maize, wheat, barley, *Avena sativa* (oat), and rice (Fig. 4). This strategy involves the secretion of the mugineic acid (MA) family phytosiderophores from the root system into the rhizosphere. These phytosiderophores chelate Fe^{3+}-containing compounds, and the complexes are taken up by a specific transport system in the root plasma membrane (Guerinot and Yi, 1994). MA biosynthesis involves the condensation of three *S*-adenosyl-L-methionine (SAM) molecules, by the nicotianamine synthase (NAS), to form nicotianamine (NA) (Bashir *et al.*, 2006). Thereafter, a nicotianamine aminotransferase (NAAT) and a 2-deoxymugineic acid (DMA) synthase (DMAS) catalyze the following reactions to produce DMA, the precursor of all MAs (Bashir *et al.*, 2006). MAs are secreted into the rhizosphere via the TRANSPORTER OF MUGINEIC ACID FAMILY PHYTOSIDEROPHORES1 (TOM1), which is highly expressed in the roots of rice and barley plants in response to Fe- deficiency (Nozoye *et al.*, 2011).

The Fe^{3+}-phytosiderophorePS complex is taken up into the root by the YELLOW STRIPE1 (YS1) and YS1-like (YSL) transporters (Curie *et al.*, 2001; Inoue *et al.*, 2009). In rice, there are18 YSL members, of which *OsYSL15* is predominantly active in the transport of Fe^{3+}-DMA complexes from the rhizosphere (Inoue *et al.*, 2009). In addition, some YS1 transporters have been identified in barley and maize (*HvYS1* and *ZmYS1*, respectively) (Murata *et al.*, 2006).

A set of TFs has been found to control low-Fe responses in plant species that use the *chelation strategy*. The IRON DEFICIENCY-RESPONSIVE ELEMENT-BINDING FACTORS1 (IDEF1) and IDEF2 were identified in rice because of its capacity to bind two *cis*-acting elements (IDE1 and IDE2) required for the transcriptional activation of Fe-responsive genes (Kobayashi *et al.*, 2007; Ogo *et al.*, 2007). These motifs are present in the promoter of the barley gene *IRON-DEFICIENCY SPECIFIC CLONE2* (*IDS2*), which is implicated in MA biosynthesis, and are widely distributed in the promoters of other low-Fe-regulated genes. Recently, IDEF1 has been found to bind Fe and Zn directly through histidine-asparagine and proline-rich regions, suggesting that this TF could be involved in Fe sensing (Kobayashi *et al.*, 2012). In rice, IDEF1 targets IRON-RELATED TRANSCRIPTION FACTOR2 (OsIRO2), a bHLH TF that acts as positive regulator of 59 Fe deficiency-inducible root-specific genes (Ogo *et al.*, 2007). Although no phenotypic differences between wild-type and *OsIRO2*-overexpressing plants had been observed under Fe-sufficient conditions, these transgenic plants perform better than the wild-type under Fe-limited conditions, without alterations in total Fe content, but with increased secretion of MAs (Ogo *et al.*, 2007).

OsIRO3 is a bHLH-negative regulator of Fe responses in rice that is strongly and specifically induced by Fe deficiency (Zheng *et al.*, 2010). *OsIRO3* overexpression results in hypersensitivity to Fe deficiency, decreased shoot Fe content, and reduced transcriptional responses to Fe limitation. These observations hint at an important role for *OsIRO3* in Fe homeostasis (Zheng *et al.*, 2010). Therefore, *OsIRO3* silencing could be a potential strategy to enhance Fe deprivation tolerance.

Interestingly, orthologs of some components of Strategy I have been identified in rice, one of the most important Strategy II crop plants. In rice, two *IRT* genes, *OsRT1* and *OsRT2,* are mainly expressed in roots and induced by Fe deficiency (Bughio *et al.*, 2002; Ishimaru *et al.*, 2006). The presence of this kind of Fe^{2+} transporters in rice is considered an adaptation to the submerged and anaerobic growing conditions. Rice also has two *FRO1-like* genes, *OsFRO1* and *OsFRO2*, but the gene products have no reductase activity (Ishimaru *et al.*, 2006). Expression of *IRT* genes in other important crops with active Strategy II could be an interesting approach to study their contribution in Fe uptake.

Genetic engineering approaches for improving iron content in plants

The issue of improving Fe uptake and assimilation in plants has received great attention not only to produce healthy plants in the field, but also to increase Fe content and bioavailability for human nutrition. According to some estimations, approximately 30% of the world´s population suffers from some degree of Fe deficiency, with a higher prevalence in developing countries. Therefore, improving Fe content in cereals that support human nutrition in low-income countries, such as rice, maize, and wheat, is urgent.

Several strategies have been attempted by different research groups, including better crop management, traditional breeding, and genetic engineering. Efforts to increase Fe uptake by genetic modifications has focused on key regulatory elements of the Fe uptake pathways described above (Fig. 4B). Nevertheless, it is important to note that although some promising results have been obtained, those that expressed Strategy I genes in plants that naturally use Strategy II, have failed to improve Fe assimilation, demonstrating the high complexity of Fe uptake and homeostasis in plants.

Candidate genes involved in transport and reduction of iron

In Strategy I plants, overexpression of *IRT* genes resulted in plants that accumulate more divalent metals in both sufficient and limited Fe regimens. For instance, *Arabidopsis IRT1*-overexpressing plants accumulate up to 3-fold higher Fe leaf content in Fe-sufficient media than control plants (Barberon *et al.*, 2011). However, overexpression of *IRT1* has also detrimental effects on plant growth due to the oxidative stress caused by the increased uptake of other divalent metals such as Zn, Co, and Mn (Barberon *et al.*, 2011). One possibility to solve this problem is to make this transporter more specific for Fe uptake. Two amino acid substitutions have proven to be effective in this regard: the replacement of the Glu residue at position 103 by an alanine (Ala) eliminates Zn transport activity, and the replacement of Asp residues residues at positions 100 and 136 by Ala eliminate Zn and Mn transport activities, respectively (Rogers *et al.*, 2000). However, substitution of the Asp residue by Ala also eliminates Fe transport.

The rate-limiting step in Fe uptake through Strategy I is Fe^{3+} reduction (Connolly *et al.*, 2003). Therefore, overexpression of *FRO2* enhances the capacity of *Arabidopsis* to grow under Fe-limited conditions thanks to an increased FRO activity (Connolly *et al.*, 2003). However in *FRO2*-overexpressing lines grown under Fe-sufficient conditions, FRO activity is not enhanced, hinting at a posttranscriptional component in *FRO2* regulation (Connolly *et al.*, 2003). Interestingly, a yeast FRO mutant has been generated with improved activity at alkaline pH (Ishimaru *et al.*, 2007). Transgenic rice plants expressing this yeast mutant *FRO* gene under the promoter of the Fe^{2+} transporter *OsIRT1* displayed an almost 8-fold increase in grain yield in calcareous soil, due to a 2.2-fold and 1.8-fold increment in ferric chelate reductase activity at pH 5.5 and 8.0, respectively (Ishimaru *et al.*, 2007). Despite this

important step, the Fe content in rice grains remained unaltered indicating that, in addition to enhancing Fe uptake, improving Fe translocation and distribution is also necessary.

Genetic modifications involving transcription factors

Controlling the expression of master regulators that orchestrate many responses to low-Fe could be an effective way to produce plants with enhanced Fe-uptake capacity. Yuan and co-workers (2008) overexpressed either the bHLH38 or bHLH39 FIT interactors, in transgenic plants that overexpress *FIT* (Yuan *et al*., 2008). These plants constitutively expressed several low-Fe-inducible genes and had a 2- to 5-fold higher Fe-content in shoots, independent of the Fe supply (Yuan *et al*., 2008). Additionally, co-expression of *FIT* and its bHLH101 interactor in *Arabidopsis* conferred a Fe deficiency-tolerant phenotype and a significantly enhanced Fe accumulation in shoots both under Fe-deficient and Fe-sufficient conditions (Wang *et al*., 2013a).

Overexpression of *IDEF1,* under the control of the constitutive 35S promoter or the *IDS2* promoter, produced plants that were able to grow better under Fe-limited conditions as revealed by a slower decline in leaf chlorophyll in transgenic lines than in wild-type plants. Analysis of the Fe content in transgenic and wild-type plants grown after Fe-deficient treatments showed that both lines had similar Fe concentrations, suggesting that the improved performance of *IDEF1*-overexpressing lines was due to a better Fe utilization within the plant instead of an enhanced Fe-uptake from the rhizosphere (Kobayashi *et al*., 2007). Likewise, IDEF2 is a TF involved in the regulation of several Fe-responsive genes, including the Fe^{3+}-nicotianamine transporter gene *OsYSL2,* by direct binding to its promoter region (Ogo *et al*., 2007). Down-regulation of *IDEF2* by RNAi caused Fe overaccumulation in shoots and roots of plants growing under Fe-sufficient conditions and in roots under low-Fe conditions, indicating that IDEF2 participates in Fe uptake or translocation (Ogo *et al*., 2007). Overexpression of these TFs in economically important plants is the next step to demonstrate the feasibility of these types of strategies to improve tolerance to Fe deficiency under field conditions.

Genetic modifications involving mugineic acid biosynthesis and transport

Increasing the transport of Fe-MA complexes in developing seeds also produces fortified grains in Strategy II plants. For instance, overexpression of the *OsYLS2* gene under control of the *SUCROSE TRANSPORTER 1* (*SUT1*) promoter causes a 4.4-fold increase in Fe content in rice grains (Ishimaru *et al*., 2010). Moreover, because plants that naturally produce more MAs, such as barley, are more tolerant to low-Fe environments, a promising strategy to improve Fe assimilation in the most important cereals (e.g. maize, rice, wheat) might be to increase phytosidorophore biosynthesis and/or secretion (Takahashi *et al*., 2001), as confirmed through the expression of two barley MA-biosynthetic genes (*NAAT-A* and *NAAT-B*) in rice. In alkaline soils with low-Fe availability, transgenic rice lines expressing *NAAT-A* and *NAAT-B* had higher DMA production and perform better under Fe-deficient conditions, producing 4.1-fold higher grain yields than untransformed controls plants (Takahashi *et al.,* 2001). Overexpression of nicotianamine-encoding genes, such as *OsNAS3* in rice, also resulted in rice plants with increased Fe content. These plants accumulated 1.7- and 1.6-fold more Fe in shoots and roots, respectively, when grown in Fe-sufficient media, whereas under Fe deficiency, they accumulated 2.2- and 2.0-fold more Fe in shoots and roots, respectively (Lee *et al*., 2009). Moreover, grains of *OsNAS3*-overexpressing plants contained 2.9-fold more Fe than control plants and, when tested in anemic mice, were able to revert to the low hematocrit and hemoglobin levels, also suggesting an increase in Fe bioavailability (Lee *et al*., 2009).

Overexpression of two other nicotianamine-encoding genes, *OsNA1* and *OsNA2* also produces an increase in Fe content in rice grains. Fe content in lines overexpressing *OsNA1* and *OsNA2*-overexpressing lines was up to 2.4-fold and 3.5-fold higher, respectively, than that of wild-type plants (Johnson *et al*., 2011). Interestingly, the endosperm of one *OsNA2*-overexpressing line contained 19 μg/g Fe, which could supply the amount of Fe (14.5 μg/g) that a rice-based diet should contain (Johnson *et al*., 2011).

Genetic modifications to improve the content of ferritins as iron reservoir proteins

The most promising approach to increase Fe content in seeds may be the overproduction of ferritins. These proteins play essential roles in controlling oxidative stress and Fe homeostasis and are the main Fe-storage proteins in animals and plants. However, ferritins are not the main Fe reservoirs in seeds or leaves (Ravet *et al.,* 2009). Rice grains of plants overexpressing a soybean ferritin under control of the rice glutelin promoter *GluB-1* accumulate up to 3-fold more Fe than wild-type seed (Goto *et al*., 1999). Similarly, a 13-fold increase in ferritin content in rice grains could be achieved with a stronger promoter (Qu *et al*., 2005), but only a 30% higher Fe concentration than control plants. These results suggest that Fe content in seeds could be limited by Fe uptake and transport in ferritin-overexpressing lines. More than 6-fold increased Fe content in rice endosperm was obtained by concomitantly expressing *NAS* and ferritin genes, confirming a synergistic effect on Fe uptake and storage (Wirth *et al*., 2009).

An interesting strategy to increase Fe bioavailability in grains is the overexpression of phytase-encoding genes, because phytate in seeds contains from 60% to 90% of the total Pi (Loewus, 2002). Moreover,, due to its high affinity to several minerals, including Fe, Zn, and Ca, phytate also affects their bioavailability. Lucca and co-workers expressed a thermostable phytase from *Aspergillus fumigatus* in the rice endosperm and, despite that the transgenic rice grains showed enhanced phytase activity, it was destroyed after cooking, maintaining only 8% of the activity prior treatment (Lucca *et al*., 2001). More recently, the expression of two different phytase genes (*phyA* and *appA,* from *Aspergillus niger* and *E. coli*, respectively*)* in canola produced transgenic seeds with lower amounts of phytic acid than the wild-type. Although the authors did not perform Fe analysis, it could be expected that Fe bioavailability could have also been improved (Wang *et al*., 2013b), representing a better solution for the nutrient availability of P and Fe, at least for monogastric animals.

Conclusions

The requirement of crop varieties with high nutrient use efficiency has been discussed historically. Increasing food production and improving food quality have always been needed worldwide. However, the increasing fertilizer consumption rates focused on human and animal food production and the growing population has alerted research groups to develop urgently more efficient

agricultural schemes to use natural resources more rationally. In the past, the limited knowledge on plant metabolism affected the development of these improved varieties, but the tools are now available to genetically modify many plant species. Systems biology has accelerated the discovery of regulatory elements in several pathways with the potential to improve plant performance in the field.

As discussed above, there are numerous attempts to improve nutrient use in plants through the manipulation of enzymes and proteins directly involved in uptake and assimilation of a specific nutrient, but only a few have produced results sufficiently promising for their commercial application. As the accumulation or activity of these proteins can be modulated by various processes (translational and posttranslational), it is necessary to understand these mechanisms to ensure successful trait modification. To achieve this aim, available information on the different nutrients must be integrated with the growing experimental data under various nutritional regimens for several plant species. Such an integration would help to identify critical components that could allow the effective alteration of nutrient use efficiency in important crops.

It is important to note that to improve assimilation efficiencies for all nutrients, external nutrient acquisition must be taken into account. The root system is central for water and nutrient uptake; therefore, understanding the mechanism that control root system architecture as well as its interaction with the rhizosphere components that influence nutrient availability, will help to improve nutrient use efficiency of any crop plant. Hence, phenotype analysis of plants must be carried out under field conditions. In addition, the reincorporation of traits conferring tolerance to nutrient deficiency, naturally present in wild or traditional cultivars, could be used to enhance nutrient uptake and applied in modern crop varieties. It is also important to consider a more extensive study of positive side effects of overexpressing specific regulatory elements on pathogen and drought resistance. Indeed, if current advances in plant nutrient research and technologies developed with the use of bacterial genes are implemented in crops and if the application of fertilizers is effectively reduced, a more sustainable and ecologically benign second Green Revolution will emerge.

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