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Title: **Macronutrient Metabolism in Crop Plants: Problems and Perspectives**

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Abbreviations:
GMO, genetically modified organism; NUE, nutrient use efficiency; QTL, quantitative trait locus

Summary

Macronutrient deficiencies are among the most limiting factors for plant production in high-yield agriculture. In complementation of integrated and precision agriculture, modern agroecosystems require sustainable resources. The development of plants with improved nutrient use efficiency represents a long neglected approach to reduce mineral input and a the same time maintain or increase yield. Such plants can be generated by advanced breeding programs and genetic modification, but progress is difficult due to lack of knowledge of physiology and genetics of NUE traits. Some of the current approaches are presented with respect to nitrogen use efficiency and the role of sulfur.

Introduction into Nutrient Use Efficiency

Agroecosystems differ in many aspects from natural ecosystems. One major difference is the input of mineral nutrients that is required to achieve adequate levels of crop yields. In moderate climates and on regular soils as present in most of Europe the macronutrients are most determining for plant production, whereas micronutrient limitations are more often observed under extreme climates and on adverse soils. However, in both cases plants are faced with the problem to enrich their mineral nutrient contents against gradients from soil solutions. Macronutrients according to general classification are N, K, P, S, Ca and Mg (Marschner, 1995). Among these nitrogen and sulfur are selected for further discussion, since their deficiency results not only in losses of yield but also of grain quality, e.g. the malting properties of barley, and because their assimilation pathways show strong regulatory interaction (Hell and Hillebrand, 2001; Kopriva et al., 2002). While other critical growth factors like water and CO_2 are difficult to control, it appears quire feasible to optimise plant production by developed fertilization regimes. However, the real challenge to plant production in Europe in the future consists not in sheer yield production, but to manage sustainable agricultural systems, i.e. to improve recovery of applied nutrients by the plant.

This development requires crop plants with improved nutrient use efficiency (NUE). Alternatively, or in addition, appropriate nutrient management and precision supply strategies could be developed. By definition NUE is the ability of a crop system to convert nutrient inputs into desired outputs. Inputs are the supply, availability and amount of minerals. Outputs are growth, physiological activity and yield. In short form: Efficiency = input/output (e.g. kg yield/ kg fertilizer). Advantages of nutrient-efficient crops are (i) lower production costs, (ii) improved crop yield (iii) better crop quality (higher price) and (iv) protection of the environment due to reduced leakage of minerals like nitrate and phosphate into ground water (Hell and Hillebrand, 2001).

Crop plants did not evolve under conditions of low nitrogen nutrition. Many of the mechanisms involved to define crop yield are not suited to growth under such conditions (Miflin and Habash, 2002). At least for high-yield cultivars this and other nutrient-related traits have been neglected in the past decades. The increased demand for nutrients by new cultivars or hybrids was simply be met by increased fertilization in the past. Crop plants with improved NUE need to be generated by advanced breeding programs. Alternatives include the generation of new crops for modern sustainable agriculture by selection and crossing of genotypes for desired traits (quantitative trait loci, QTL), the modification and introduction of new traits into existing cultivars (genetically modified organisms (GMO), or both approaches combined. The aim would be to exactly match nutrient resources, from soil or applied, with the genetic potential of the crop. If supply by resources is less, then the potential of the plant is not reached. In turn, if the supply exceeds the demand set by the genetic potential, such resources are lost for production (Lawlor, 2000). Essential targets for NUE in both strategies are the metabolism during vegetative and generative plant development and the adaptation and dynamics of plant architecture in response to nutrient supply. Instead of 'tailoring the soil to fit the plant' this approach tries 'tailoring the plant to fit the soil'. Advantages would be good acceptance by farmers because of better yield, less fertilizer and no changes in agricultural practice.

The disadvantage consists in the poorly defined connection between metabolism and yield. Genetic, physiological and biochemical bases of NUE are mostly unknown and therefore hamper breeding efforts. Consequently, genetic engineering and targeted breeding both are difficult because the targets are unclear and the interactions between the underlying processes and changes in the environment are complex. Detailed understanding of the biological mechanisms however is a prerequisite to achieve further improvement of such complex traits

in crop plants. In the following chapters the possible contributions of plant biology to improve NUE for agricultural plant production will be evaluated with respect to the macronutrients nitrogen and sulfur.

Principle mechanisms of macronutrient uptake by plant roots

Ion uptake by the plasmalemma of root surface cells has been generally described by the pioneering experiments of Epstein (1966). He first distinguished between the existence of high- and low-affinity systems that are specific for a nutrient and either constitutive or inducible by nutrient supply. At micromolar concentrations of a nutrient ion transport systems with high activity operate. Above threshold levels of about 0.2 - 0.5 mM other, less affine systems become apparent that display linear or saturation kinetics. Today's molecular and electrophysiological techniques allow further dissection of the components of the different systems. Anion uptake is mostly electrogenic. Upon exposure to nitrate, sulfate or phosphate the plasma membrane is initially depolarised, rendering the inside more positive. It is followed by re-polarization that enhances the activity of the P-type H^+-ATPase. This is required to allow co-transport of 2-3 protons per imported anion (Chrispeels et al., 1999). The cloning of genes encoding membrane transporter proteins revealed that, according to secondary structure prediction, nitrate, phosphate and sulfate transporters share a common topology of 12 membrane-spanning domains. Functional analyses of such cloned transporters are carried out in heterologous expression systems such as yeast or *Xenopus* oocytes.

Interestingly, the genes of the high-affinity transport systems react quite differently to limitations of their respective nutrient anions in the soil solution. In a simplified pattern the deficiency of phosphate, sulfate and potassium induces their corresponding uptake systems and genes, whereas limitation of nitrate and ammonium represses their systems (Fig. 1A). In fact, availability of nitrate and ammonium induces high-affinity systems and is probably feedback-regulated by internal ammonium and amino acid concentrations (Forde and Clarkson, 1999; Glass et al, 2001).
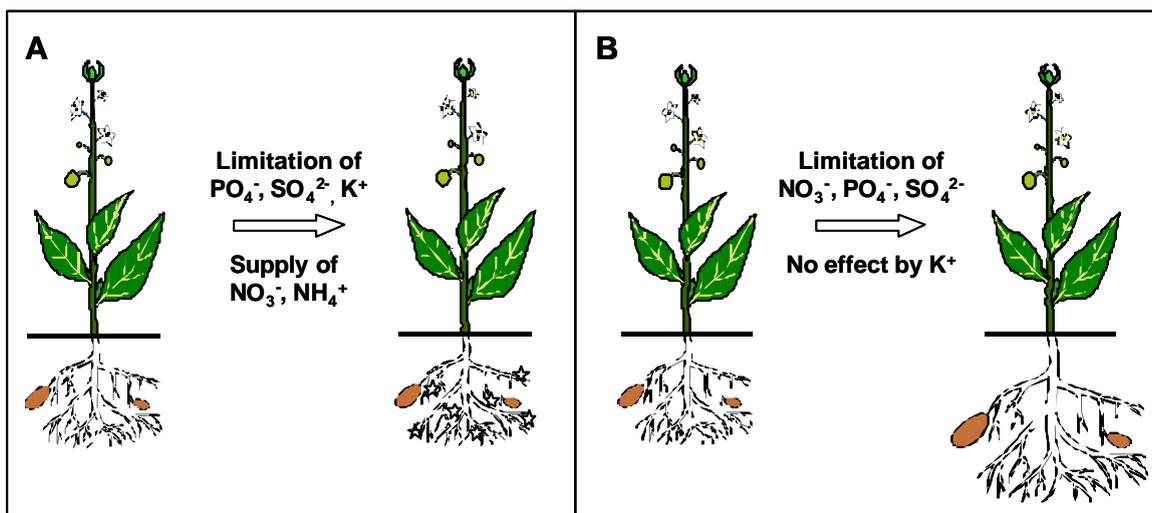

Fig. 1. Fluctuations of macronutrient supply modulate ion uptake and root-to-shoot ratios.
A. Limitation of different macronutrients induce high-affinity uptake systems (indicated by asterisks) and the underlying genes by opposite supply signals. B. Oxo-anion deficiency results in increased root-to-shoot ratio.

It is noteworthy in this context that the well-documented shift of the shoot-to-root ratio in response to mineral deficiencies is also specific for some of the limiting macronutrients (Fig. 1B). Nitrate, phosphate and sulfate limitation induce this shift, while potassium deficiency does not (Robinson, 1994). It is generally assumed that the allocation of C and N compounds from shoot to root functions in improved nutrient acquisition ability of a mineral-starved plant via increased access to soil patches and uptake surface area. Taken together, the plant responds specifically and differently to limitations of macronutrients in its environment. This differential regulation probably reflects the individual functions of the assimilation products of these nutrients in the plant.

Regulation of nitrogen metabolism with respect to NUE

Despite massive global fertilizer inputs (10^{11} kg/a^{-1}), roots of crop plants experience enormous soil heterogeneity with respect to N and other soil nutrient (Glass et al., 2001). Thus, uptake is often executed against concentration gradients. In the context of such heterogeneity, plant roots must respond to local, regional and seasonal changes by means of adaptations that can optimise nutrient capture. To improve NUE these adaptation mechanisms have to be identified and tested for their suitability.

Already at the cellular level is becomes clear that nitrogen metabolism consists of a complex network of processes. Specific nitrate and ammonium transporters provide import across the plasmalemma. In the cytoplasm NH_4^+ can be directly fixed by cytosolic glutamine synthetase, while nitrate is reduced to nitrite by nitrate reductase. Nitrite is then imported into the chloroplast where nitrite reductase reduces it to NH_4^+. Plastid glutamine synthetase and the GOGAT cycle then provide fixation into glutamine and from there into other amino acids. Nitrate can be transiently stored in the vacuole and NH_4^+ can additionally released by photorespiration in the mitochondria. Thus, the subcellular organization of the pathway already requires numerous fluxes and communication between different metabolite pools (Crawford, 1995).

The biochemical pathway of nitrogen assimilation reveals three major regulatory steps that integrate environmental and developmental signals (Fig. 2): nitrate (NH_4^+) uptake, nitrate reduction and NH_4^+ fixation. Nitrate and ammonium transport follow the high- and low-affinity/constitutive versus inducible uptake concept outlined by Epstein (1966). Molecular biology is now challenged with the correlation of these uptake systems and gene activities. The model plant *Arabidopsis thaliana* with its completely sequenced genome indicates 11 genes encoding nitrate transporters, four NRT1 genes for high-affinity type and seven NRT2 genes for low-affinity type as well as several ammonium transporter genes. This information is useful also for crop plants such as rape and beet due to the genomic synteny between Brassica species. Expression analysis of the NRT gene family in roots three hours after addition of nitrate revealed complex up- and down-regulation as well as constitutive patterns of the members (Glass et al. 2001). Why do such multiple nutrient uptake systems exist? For one, redudancy in essential nutrient uptake could be important for evolutionary survival of a species. For two, the large variation in soil nitrogen concentration requires transporters with different properties to ensure condition-dependent uptake. Furthermore, differential expression patterns in plant organs and cell types are required for allocation within the plant (Chrispeels et al., 1999). This complex organisation strongly suggests that higher uptake rates for nitrogen by either breeding or GMOs is not easy to achieve and probably not even equivalent efficient use of the extra nitrogen.
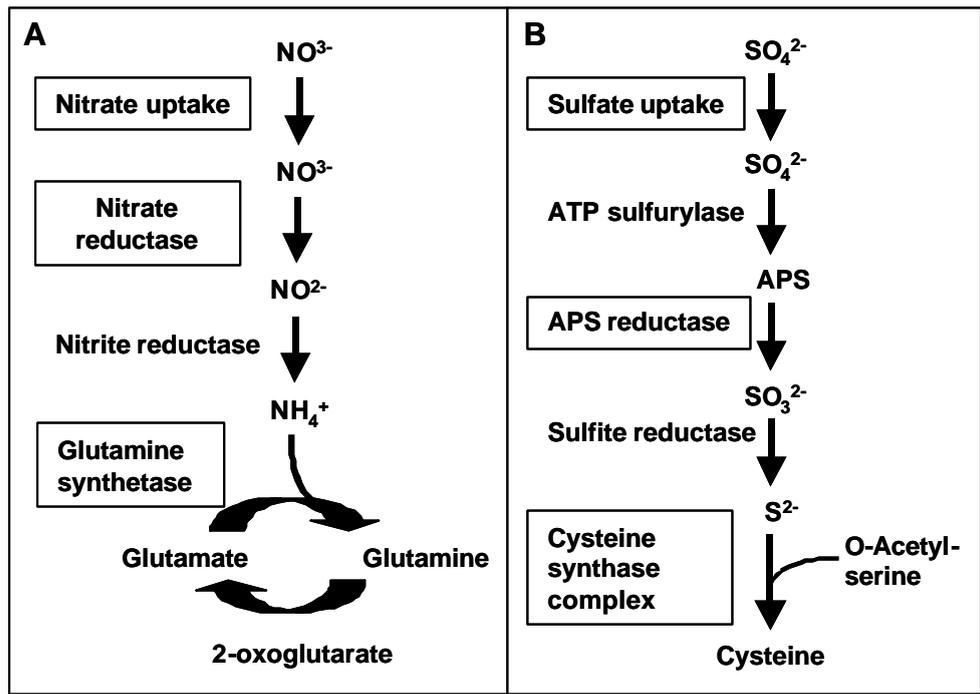
Fig. 2. Comparison of the biochemical reaction sequences of (A) nitrate and (B) sulfate assimilation from inorganic minerals into reduced and organic bound forms. Major regulatory steps are indicated by boxes.

Nitrate reductase has been recognised as the intersection of input and output signals in nitrogen metabolism. It has a complex quaternary structure with several electron transport domains and is regarded as one of the most highly regulated enzymes in plant biology (Kaiser et al., 1999). It can be regulated at the enzymatic, transcriptional, post-transcriptional and post-translational level. The most important signals affecting regulation are nitrate and sulfate supply, internal glutamine, sucrose, circadian rhythm, cytokinin, photosynthetic activity, CO_2 and Anoxia. Transgenic over-expression and antisense technology demonstrated that nitrate reductase activity is generally in excess and needs not to be increased to improve NUE. However, the complex convergence of signals makes a targeted modification of nitrogen metabolism at this step very difficult. In addition, the potential toxicity of the intermediates nitrite and ammonia require concerted modifications.

Glutamine has been identified as a very likely candidate for regulating nitrate and ammonium uptake as well as the activity of nitrate reductase. Transformation of glutamine synthetase improved grain yield and protein composition in wheat and resulted in more biomass production in potato and tobacco (Harrison et al., 2000; Miflin and Habash, 2002). Whether direct modulation of glutamine synthetase under nitrogen-limiting conditions improves efficiency remains to be seen. Constitutive up-regulation of this step alone might not be a suitable target for NUE, since either nitrate uptake or glutamine for protein biosynthesis would be impaired. However, selection of superior genotypes and marker assisted breeding suggests that an integration of signals that converge at glutamine synthetase might improve NUE for nitrogen (Miflin and Habash, 2002).

Initial experiments with different genotypes of barley demonstrated that sufficient genotypic variation is available even in established breeding cultivars to harbour improved nitrogen use efficiency. Seven cultivars of the barley core collection of the German Germplasm Collection at Gatersleben (http://fox-serv.ipk-gatersleben.de/) were selected for recent or early 19th century official market introduction (Fig. 3; Hell et al., unpublished). At vegetative stages these lines showed no significant differences in total dry weight and root/shoot ratios, but under severe nitrogen starvation two lines from Sweden (introduced 1899) and from the UK (1902) exhibited superior biomass production. Apparently the integration of nitrogen efficiency traits in these lines worked positively together to form the basis of a NUE effect that had never been analysed in the past. This finding suggests that such traits exist and that this positive effect also holds for generative stages to increase yield at reduced input.

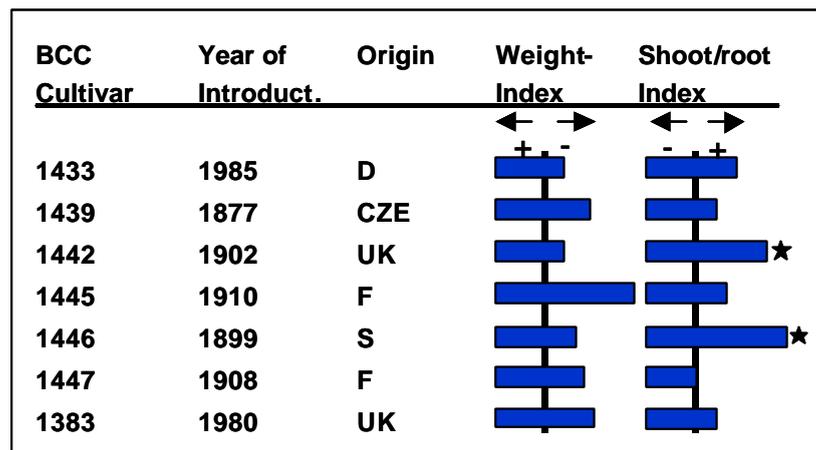| BCC Cultivar	Year of Introduct.	Origin	Weight-Index	Shoot/root Index
			← → + -	← → - +
1433	1985	D		
1439	1877	CZE		
1442	1902	UK		★
1445	1910	F		
1446	1899	S		★
1447	1908	F		
1383	1980	UK		

Fig. 3. Nitrogen use efficiency of different cultivars from the barley core collection (BCC) of IPK Gatersleben. Market introduction years of official breeding cultivars from different geographical regions of Europe were tested at seedling stage on conditioned hydroponic growth solutions with or without nitrogen. Dry weight and size were determined to calculate weight index (total fresh weight of control (+)/starved(-)) and shoot/root-index (fresh weight ratio of control (+)/starved (-)). Significantly better performing accessions are marked by asterisks.

An advanced application to identify complex traits is QTL mapping using recombinant inbred lines. Hirel et al. (2001) used an inbred population of maize to demonstrate sufficient physiological and genetic variation for improvement of NUE. They found positive correlations of glutamine synthetase activity and yield. Specifically, cytosolic glutamine synthetase 1 is a key component of NUE, but not plastid glutamine synthase 2 or nitrate reductase activity. It is important to note that nitrogen efficiency of the inbred lines first analysed by yield parameters such as grain yield, grain nitrogen and thousand kernels weight, i.e. by results of generative development of the plants. In the second step Hirel et al. (2001) correlated these data with physiological parameters like nitrate content and glutamine synthetase activity in juvenile, i.e. vegetative stages and identified genetic loci that corresponded to glutamine synthetase 1. This suggests that at least in maize superior traits in these early stages already determine yield and not only physiological parameters of the later stages like grain filling and leaf storage mobilisation. These approaches are very promising, but rigorous breeding into high-yield backgrounds and subsequent fields trials are required to establish a robust NUE trait.

Sulfur metabolism and crops

Sulfur has long been a neglected macronutrient in plant biology and agriculture. Reasons are its usually sufficient abundance in regular soils combined with a 20 to 30 times lower molar requirement by plants as compared to nitrogen. Furthermore, the assimilation pathway has only been finally elucidated a few years ago (Leustek et al., 2000). Anthropogenic changes of atmospheric sulfur input recently spurred research in sulfur metabolism. Beginning with the industrialisation of Europe the burning of fossil resources increased SO_2 gas release, especially from power plants working with coal. The pollution peaked in the 1980ies, leading to increases of atmospheric sulfur input e.g. in areas of northern Germany from about 5 to 50 kg/ha. Due to this contribution the steady increase in sulfur requirement by crop plants with improved yield went largely unnoticed. When the SO_2 emissions were recognized as a major source of acid rain and forest decline, filters were installed at power plants and emissions dropped to less than 20 kg/ha in the past 20 years (Dämmgen et al., 1998). This successful reduction finally led to sulfur deficiency syndromes, first in high-sulfur requiring crops like oil seed rape but later on also in less demanding wheat and potato. Concomitantly an increase in sensitivity to fungal pathogens was observed. This could be cured by sulfur fertilization and led to the concept of sulfur induced resistance (Schnug et al., 1995). Apparently sulfur is not only required to sustain regular growth but also to strengthen pathogen defense mechanisms. This effect has so far only been defined by yield in response to fertilization regime and pathogen infection. The physiological basis effect is currently under investigation (Jost et al., 2003).

The assimilation pathways of nitrate and sulfate show remarkable similarities and differences at the same time (Fig. 2). In both cases three major regulatory steps can be identified that act at comparable biochemical levels. Sulfate uptake at the plasmalemma follows the same basic patterns as nitrate uptake. *Arabidopsis thaliana* carries 14 genes encoding sulfate transporting membrane proteins with 12 helix spanning domains each. These proteins are of high- or low affinity for sulfate and are differentially regulated at the transcriptional level (Hawkesford, 2000). It is important to note that sulfate deficiency triggers gene induction, while nitrate deficiency represses nitrate transporter genes (Fig. 1A). A further analogy between both pathways is that the intermediates sulfite and sulfide are potentially toxic like nitrite and ammonium. Sulfite and nitrite reductases are in fact structurally related and react via the same electron transfer mechanism. Similar to nitrate reductase, APS reductase is the most regulated enzyme of the sulfate pathway, reacting to sulfate and nitrate deficiency, circadian rhythm and various stress condition. However, its structure and reaction mechanism are totally different from nitrate reductase (Leustek et al., 2000; Kaiser et al., 1999). Finally, the equivalent of glutamine synthetase and the GOGAT cycle in terms of biochemical function is the cysteine synthase complex. Besides forming a true protein complex with two enzyme subunits, serine acetyltransferase and O-Acetylserine (Thiol) Lyase, the cysteine synthase complex serves regulatory function and seems to integrate sulfur with nitrogen and carbon metabolism via the reaction intermediate O-Acetylserine (Kopriva et al., 2002; Hell and Hillebrand, 2001; Hell et al., 2002). Interactions of nitrogen and sulfur assimilation pathways have long been documented (Kopriva et al., 2002). Combined fertilizers achieve synergistic effects on yield and crop quality. Sulfate deficiency represses nitrate reduction genes, since protein biosynthesis is stalled due the limitation of cysteine and methionine. Under such conditions abundant photosynthetic proteins such as Rubisco can be degraded as a source for amino acids.

Attempts to improve the sulfur composition of crops so far mostly concentrated on seed protein composition. The nutritional value of the sulfur-containing amino acids methionine and cysteine is based on the absolute requirement of most animals for methionine and the ability of cysteine to partially compensate for methionine. Consequently, when feed is

produced at optimized high-yield conditions, blended plant diets are applied that need to be supplemented with both sulfur amino acids. Conventional breeding yielded only limited success in overcoming this nutritional limitation. Most transgenic approaches have focused on increased sink strength, using over-expression of seed storage proteins rich in methionine and cysteine (Tabe and Higgins, 1998). A close inspection of such plants showed a limitation set by supply of sulfur, not demand. The total sulfur content of seeds was unchanged but the composition was shifted from oxidized (i.e. sulfate) to reduced sulfur (i.e. sulfur amino acids). Eventually, free methionine and cysteine and finally import of sulfate become limiting for further increase of reduced sulfur content (Tabe and Droux, 2002). Little is known about the seed tissue that are responsible for sulfur assimilation and transport of sulfur compounds to the storage proteins during seed development. The situation is strikingly different from nitrogen metabolism, where most reduced nitrogen is simply imported from source tissue as glutamine and arginine but not assimilated from nitrate by the seed itself.

Genetic engineering is only at the beginning to identify the bottlenecks. Nevertheless, the over-expression of serine acetyltransferase resulted in enhanced levels of cysteine and glutathione, indicating that the cysteine synthase complex plays a central role in the control of flux through the sulfate assimilation pathway (Hell et al., 2002). Sulfate uptake and allocation within a crop plant are candidates to enhance sulfate efficiency (Hawkesford, 2000). Selection and breeding efforts for improved sulfate use efficiency are not reported so far. They might become interesting considering the quality of crop seed composition and of new traits like sulfur induced resistance, but in particular to create synergistic effects with nitrogen metabolism.

In addition to these physiological and biochemical properties, the architecture of the plant is an important component of adaptive measures towards mineral deficiency. It is clear that enlarged roots systems represent larger uptake areas for nutrients at the root-soil interface (Fig. 1B). This developmental adaptation can be enhanced by asymmetric lateral root growth into nutrient-rich soil patches as documented for nitrate and phosphate (Lynch, 1995). Not all crop plants are able to modulate the architecture of their roots systems in such a way and the genetic basis of this phenomenon just begins to be elucidated (Zhang and Forde, 2000). Even less is known about the contribution of mycorhiza under nutrient-limiting conditions and whether there are differences between plant genotypes in their interaction with symbiosis partners for NUE. In conclusion, the generation of crop plants with improved NUE is a promising goal, but requires close interactions between plant biology and agricultural sciences.

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