Chapter 2
Natural Variation as a Tool to Investigate Nutrient Use Efficiency in Plants

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Abstract A huge natural variation exists between individuals within a given plant species. Most of the responses of growth-related traits to different environmental scenarios are genotype dependent. Hence, natural variation in plants provides an interesting and valuable source of genetic diversity to study plant responses to environmental factors. The identification of genes that underlie phenotypic variation has an enormous practical implication by providing a means to improve crop yield and quality. The approach based on natural variation aims to use naturally occurring differences to improve our knowledge about complex physiological responses of plants to their environment, including nutrition efficiency. An overview of different approaches currently used in plant research aimed at dissecting complex quantitative traits is presented here, with a special focus on those related to Nutrient Use Efficiency, to explain strategies based on QTL mapping in segregating populations and association mapping in wild populations. Some case studies regarding each of the investigative strategies described are detailed.

Introduction

Plants are considered adapted to variable and sub-optimal environments when they show the ability to successfully grow and reproduce in them. In order to deal with changing and challenging environmental conditions, plants exhibit a wide range of integrated responses, which usually display complex quantitative variation. Plant adaptation interests a large community of scientists, from ecologists and molecular geneticists working on fundamental mechanisms of adaptation, to crop breeders looking for natural variants which could optimise environmental resources and provide targets for breeding programs (Trontin et al. 2011). The identification of genes that underlie phenotypic variation can have enormous practical implications by providing a means to increase crop yield and quality in an agricultural context

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(Bergelson and Roux 2010). Many elements in the soil serve as mineral nutrients for plants. Among them, nitrogen (N), potassium (K), phosphorus (P) and sulfur (S) are required in relatively large amounts for plant growth. Therefore, deficiency in any one of these four elements in the field affects plant metabolism, and shows a dramatic impact on yield, nutritional quality and taste as well as pathogen and pest resistance in crops (Laegreid et al. 1999). In developed countries, plentiful amounts of fertilisers are applied, resulting in abundant inorganic nutrients in the ecosystem that may cause disturbance in normal bio-geochemical cycles of nutrients. However, this trend is being challenged by the current emphasis on developing more efficient cultivars for sustainable, low-input agriculture, fuelled by increasing cost of fertilisers, restrictions to minimise environmental impact and the increased use of poor quality land (Rengel and Damon 2008). Some plant species and genotypes within species have a capacity to grow and yield well on soils with a low level of available nutrients; these species and genotypes are considered as tolerant to nutrient deficiency and with high nutrient use efficiency (NUE; Good et al. 2004). The NUE can be described as the proportion of potential yield that can be achieved under that mineral deficiency availability. It is the product of nutrient uptake efficiency (NUpE) and nutrient utilisation efficiency (NUtE), which is the optimal combination between nutrient assimilation efficiency (NAE) and nutrient remobilisation efficiency (NRE) (Masclaux-Daubresse et al. 2010). Improving each crop individually requires a global knowledge of the different mechanisms that control all the steps involved in nutrient management in plants, and also a good knowledge of the specificities of each plant species in terms of metabolism (Chardon 2012).

Investigation of Natural Variation in Plants Reveals Different Strategies of Response to Nutrient Limitation

A huge natural variation exists between individuals within a given plant species, affecting for example the colour or the shape of leaves, grain composition, seed dormancy, flowering date or maturity date. These variations could have an important impact on yield or quality of their products in crops, fruit trees and forestry management species (Saisho et al. 2011; Arikita et al. 2013; Eduardo et al. 2010; Łata et al. 2005; Robinson et al. 2012). Some developmental traits, such as flowering time or seed dormancy, have drawn particular attention, partly because they are of applied interest to crop breeding, and partly because they are easy to investigate (Shindo et al. 2007). Natural variation involves not only the morphology of plants but also their behaviour when facing contrasted environments. The responses or growth-related traits to different environmental scenarios are genotype dependent. Hence, natural variation in plants provides an interesting and valuable source of genetic diversity to study plant responses to environmental factors. The plant's capacity to adapt to environmental constraints is called plant plasticity. It

reflects both plant age and adaptation to environment (*e.g.* light intensity, photoperiod, temperature and nutrient availability). The approach based on natural variation aims to use naturally occurring differences to improve our knowledge about complex physiological responses of plants to their environment, including nutrition efficiency.

Plant morphology and physiology are complex quantitative traits, implying that they are genetically controlled but also influenced by the environment. The measurements of such traits show differences of averages between genotypes as well as variations due to chance and technical inaccuracies. Therefore, the investigation of such complex traits needs statistical tools for taking into account fluctuations due to chance in order to correctly understand the genetics behind them.

Investigation of natural variation is of interest from two general points of view. First, analysing this variation makes it possible to identify the function of individual genes. Despite the fact that mutant approaches have been very powerful for functional analysis, the small number of genetic backgrounds analysed limits the definition of gene functions using these procedures. Ultimately, the sort of mutant phenotypes that can be identified depends on the wild type genotype (Koornneef et al. 2004). There are specific alleles in nature that would not be easily recognised in mutant screens because they require very specific amino acid changes and therefore appear at an extremely low frequency (El-Din El-Assal et al. 2001). Second, analysis of natural variation has an increasing interest from an ecological and evolutionary perspective since diversity of physiologies and evolved responses in nature result from millions of generations of evolution (Ungerer et al. 2008). While much has been learned from bringing organisms into the laboratory to study elements of their biology in isolation, ignoring the ecological context in which these elements arose and persist runs the risk of a suboptimal understanding of particular biological responses and processes. Thus, the patterns of phenotypic and molecular variation observed are analysed to elucidate the mechanisms generating and maintaining this variation, and to identify which allelic variants are adaptive under specific environmental conditions.

Several recent papers demonstrated that natural variation exists for the different steps of plant nutrition: nutrient uptake and roots to shoot translocation, their assimilation in leaves, as well as their recycling and remobilisation for seed filling.

The uptake efficiency is dependent on the root system architecture (Dunbabin et al. 2004), and specific software have been developed for analysis (Armengaud et al. 2009; Ristova et al. 2013; Galkovskyi et al. 2012). It has long been known that root architecture and plasticity reveal a response of plants to scarce nutrients, and natural variation exists for these traits in different species, related to potassium (Kellermeier et al. 2013; Jia et al. 2008), nitrogen (De Pessemier et al. 2013), and phosphorus availability (Wang et al. 2010). Natural variation for NUpE has been shown directly by measuring a specific mineral content in different genotypes, as Burns et al. demonstrated for nitrate in different varieties of lettuce. Otherwise, a complementary approach aimed at studying the activity of enzymes involved in mineral assimilation in roots can be used. For example, Blair et al. (2010) identified important differences between varieties of beans for their ability to reduce iron

when grown at various hydroponic iron concentrations, ranging from 0 to 20 μM Fe. Interestingly, these differences were more evident in plants grown at low Fe concentrations (iron limiting conditions) than at high iron concentrations (sufficiency conditions), revealing a genotype x environment interaction. Finally, the use of isotope labelling has been used to evaluate nutrient uptake capacity. In maize, nitrogen-15 labelling was used to study NUpE (Coque et al. 2008), showing that 28.3 % of whole-plant nitrogen was taken up after silking, and 93 % of this post-silking nitrogen uptake was allocated to kernels.

A similar approach could be used to analyse the roots to shoot translocation of nutrients. As an example, in order to understand why *Noccaea caerulescens* has good properties for phytoremediation, Xing et al. (2008) investigated the root-to-shoot translocation of Cd and Zn in different genotypes. The percentages of Cd and Zn transported to shoots within 24 h exposure varied widely among the 11 accessions analysed. Interestingly, the translocation efficiency did not correlate with the uptake for either metal, suggesting independent variation in uptake and translocation among different accessions of *Noccaea*.

Post-genomic studies integrating all "omics" sciences can depict precise pictures of nutrient assimilation in plants (Hirai et al. 2004). Sulpice et al. (2013) studied the response of 97 accessions of *Arabidopis* to different nitrogen and carbon conditions, an important number of traits showed significant natural variation between accessions. For example, biomass differed between the 97 accessions by 3.1-fold and 2.8-fold in high and low nitrogen, respectively, relative to the accession with the lowest biomass in that growth regime. The impact of low nitrogen and low carbon differed between accessions, with some accessions showing a greater than 70 % decrease in biomass and others showing no decrease. Moreover, accessions that maintained a relatively high biomass in low nitrogen tended to show only a small increase in biomass in high nitrogen, whereas accessions that showed a relatively small biomass in low nitrogen showed a large (greater than 3-fold) increase in biomass in high nitrogen. The total nitrogen concentration in the rosette was unrelated to the biomass difference between low and high nitrogen The nitrogen content (mg/N per rosette) was strongly related to the response of an accession to nitrogen; accessions that maintained biomass in low nitrogen contained more nitrogen in the rosette than accessions that showed a large gain in biomass in high nitrogen. These results imply that accessions differ in the extent to which they can acquire nitrogen from low nitrogen soil and that this is far more important for the response of biomass to nitrogen supply than changes in the nitrogen content of the rosette.

As for uptake efficiency, natural variation for remobilisation efficiency can be directly investigated by measuring nutrient content in the seeds. For instance, Khan et al. (2012) revealed differences in oil content in the seeds of various *Acacia* species, revealing some species as a novel source of edible vegetable fat. As for the studies conducted on N uptake, an isotope labelling technique is suitable to investigate the remobilisation efficiency. This was shown in the work of Coque et al. (2008), who investigated N remobilisation in maize with the aim of mapping and characterising loci involved in the variation of quantitative traits (QTL) related

to NUpE, grain N yield, N remobilisation and post-silking N uptake. They stated that QTLs for remobilisation mainly coincided in clusters with loci for leaf senescence, underlying the role of a "stay-green" phenotype in favoring N uptake capacity, and thus grain yield and N grain yield. Similarly, in *Arabidopsis* a range of variation for Harvest Index (HI) and Nitrogen Harvest Index (NHI) has been found when 20 accessions were cultivated with a limited or ample supply of nitrogen (Masclaux-Daubresse and Chardon 2011). It was observed that the range of variation among the 20 accessions was conserved between the two nitrogen levels. Globally HI is similar at high and low nitrogen, while NHI, which defines the resource allocation, is twice as high at low nitrogen compared to high nitrogen, indicating that grain NUE is higher when nitrogen fertiliser is limited.

Several studies have been conducted by investigating natural variation in crop species, which were focused mainly on yield improvement (Shewry et al. 2013) and grain composition (Li et al. 2011), but only a few have been done evaluating the interaction of those traits with fluctuation of nutrient content in the environment. This kind of study requires highly controlled conditions in order to clearly elucidate the impact of the genotype and the environment on the variance of traits. This is why such studies have been up until now conducted mainly in *Arabidopsis*, as shown in the review of Chardon et al. 2012. Variation between *Arabidopsis* accessions can be explored to discover ideotypes that can match with different crop specifications: four groups, corresponding to different agronomic indicators such as grain yield, vegetative biomass and composition of the grains at low or high nitrogen supply, can be defined. On the basis of their physiological performance for nitrogen uptake and remobilisation, some *Arabidopsis* accessions are presented as good models for the investigation of the agronomic performances required to fit with crops specifications. Some other examples come from the investigations of *Arabidopsis* accessions grown in limiting nutrition (Reymond et al. 2006; North et al. 2009) or complete nitrogen starvation (Richard-Molard et al. 2008; Ikram et al. 2012). The aim of these studies was to investigate the extent of variation of growth responses to nitrate limitation and starvation in *Arabidopsis* to identify accessions showing contrasted responses and eventually different growth adaptive strategies.

Uncovering Genes Involved in NUE Quantitative Loci by QTL Mapping in Segregating Populations

Principles of QTL

The establishment of the genetic basis of quantitative traits is commonly referred to as quantitative trait locus (QTL) mapping and has been hampered by their multigenic inheritance and the often strong interaction with the environment. The principle of QTL mapping in segregating populations is based on the genotyping of

progenies derived from a cross of distinct genotypes for the trait under study. Phenotypic values for the quantitative traits are then compared with the molecular marker genotypes of the progeny to search for particular genomic regions showing statistically significant associations between polymorphism and the trait variation, which are then called QTL. QTL analysis makes use of the natural variation present within species. Once genetic variation is found among accessions, the aim is to identify how many loci account for it and where they are located in the genome (Koornneef et al. 2004).

Identifying the number and genome position of the segregating QTL in an experimental population requires the following steps: (a) the generation of an experimental mapping population; (b) its genotyping with markers throughout the genome and the phenotyping for the trait of interest; (c) the association analysis between phenotypic values of the trait and genotypic classes of the polymorphic markers. Thus, the number and genetic position of loci that control the trait variation in that population, their relative additive effect, the contribution of genetic interactions between loci (epistasis) and the mode of action of each QTL (dominance effects) are calculated depending on the population type (Koornneef et al. 2004). The number of loci identified per analysis varies from 1 to >10, depending on the complexity of the genetic variation under study, including parameters such as the true number of loci segregating, the relative additive effect of each QTL, and the effect of genetic interactions. In addition, this number depends on the heritability of the trait in the assay performed, *i.e.* the control of the environmental uniformity, the quality and density of genotypic data, the statistical method used to map QTL, and the size of the mapping population.

Mapping Populations

In plants, the use of "immortal" mapping populations consisting of homozygous individuals is preferred because it allows performance of replications and multiple analyses of the same population. Such populations known as recombinant inbred lines (RILs) or introgression lines (ILs), also referred to as near isogenic lines (NILs), are practically homozygous and therefore phenotypic values can be based on multiple replicates, reducing the environmental effects and increasing the power to detect QTL. They can be analysed in multiple environments without the need for further genotyping, and thus, the effects of each QTL in different environments can be precisely estimated and tested for QTL × environment interactions (Koornneef et al. 2004). Homozygous populations can be obtained by repeated selfing, as for RILs, but also by induced chromosomal doubling of haploids. In contrast, NILs consist of lines containing a single fragment or a small number of genomic introgression fragments from a donor parent into an otherwise homogeneous genetic background, which increases the power to detect a small-effect QTL. In plants, RILs and NILs are the most common types of experimental populations used

for the analysis of quantitative traits. In both cases the accuracy of QTL localisation, referred to as mapping resolution, depends on population size. The mapping of QTL in segregating populations has limited resolution since loci associated with the expression of a quantitative trait can be mapped with a precision of about 5–20 cM depending on its relative effect and the quality of the QTL mapping assay (Keurentjes et al. 2007). The choice of one mapping population over another depends on the plant species and the specific parents of interest. In cases where different cultivars or wild accessions are studied, preference is often given to RILs. However, when different species or wild and cultivated germplasm are combined, NILs are preferred. In *Arabidopsis* for example, the ease with which fertile RIL populations with complete genome coverage can be generated, due to its fast generation time, has led to their extensive use in mapping quantitative traits. An overview of the steps undertaken to generate a set of RILs is represented in Fig. 2.1. The population derived from a European accession and a genetically distant one from central Asia, Bay-0 and Shahdara, is an example of a novel RIL population suitable for the investigation of traits such as the response to nitrogen availability, root architecture, seed germination, drought tolerance and virus resistance (Loudet et al. 2002). The phenotypic variation resulting from such a cross is expected to reflect the adaptation to the specific habitat and the genetic distance between the parental accessions. The first extensive study of N metabolism in Arabidopsis using QTL mapping was conducted by Loudet on 415 RILs derived from Bay-0 × Shahdara population (Loudet et al. 2003) to describe whole plant N physiology and growth at a vegetative stage. The study, conducted in controlled growth conditions, aimed at comparing two different N environments (10 mM and 3 mM nitrate) and identified several loci explaining the variability of growth and total N, nitrate, and free amino acid contents.

Other approaches involve the use of multiple parents, as in the multiple advanced generation intercross (MAGIC) and *Arabidopsis* multiparent RIL (AMPRIL) populations (Kover et al. 2009; Huang et al. 2011). The MAGIC design is more elaborate and generates more recombination events per line than the AMPRIL strategy, but the founder genomes are less evenly represented in the final lines. Mapping in either population is more complex than with RILs, but with a sufficiently high density of intermediate frequency markers, one can infer the most likely local founder genotype. Some of the advantages of using RIL-type populations will continue to apply in the future.

Maize is the crop species, which has traditionally been involved in QTL mapping, and numerous QTL studies for NUE are now available. Zhang et al. (2010) has published recently a study on QTL mapping for several enzyme activities. They detected 73 QTLs for the activity of 10 enzymes involved in carbon and nitrogen metabolism and eight QTLs for biomass in an intermating RIL population developed by randomly intermating plants for four generations following the F2, prior to the derivation of mapping progeny. A RIL population of rice has also been tested for tolerance to salinity, measuring the amount of Na^+ and K^+ ions in shoots and roots in three environmental conditions of 0, 100 and 120 mM NaCl

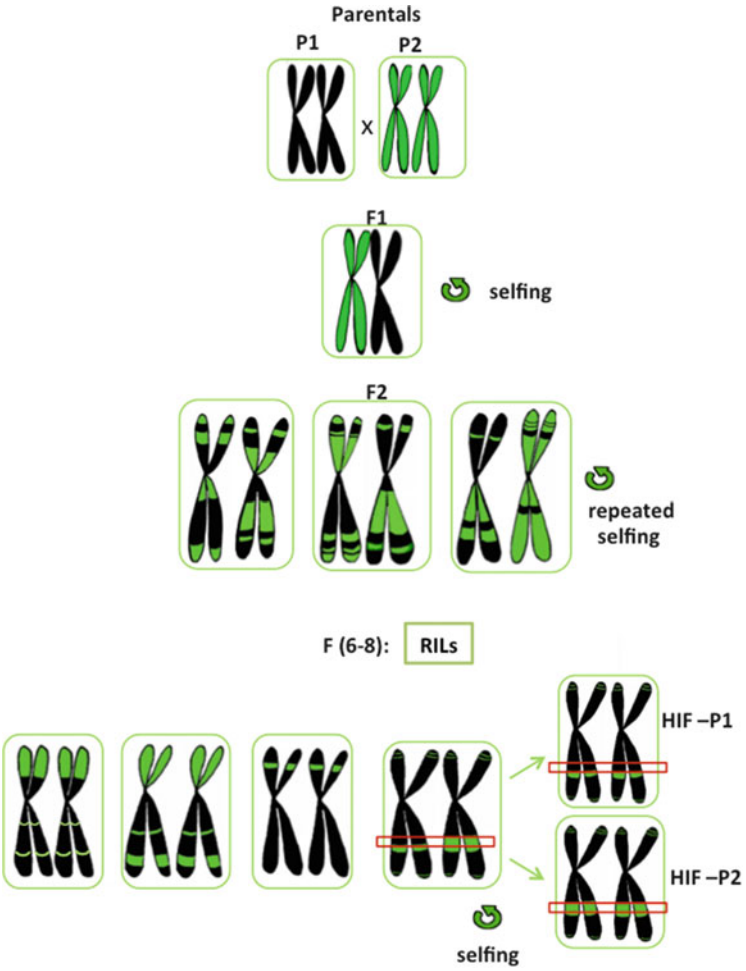

Fig. 2.1 Generation of RILs by successive selfings: two parental lines are crossed to produce an F1. The F1 is then selfed to obtain an F2. The selfing process continues until a certain level of homozygosity is reached. The end product consists of a set of RILs, each of wich is a fixed recombinant of the parental lines. HIFs individuals are derived from RILs in wich a small portion of the genome is still heterozygous (shown in red rectangle). Selfing such a RIL, it is possible to obtain individuals fixed for parental's 1 or parental's 2 allele. Only one chromosome pair is shown for each individual

(Wang et al. 2012). It is known that plants, which tolerate salinity are able to maintain a flux of Na^+ between shoots and roots in order to keep the ratio Na^+/K^+ as low as possible. The authors identified several major QTLs for salt tolerance and one of them, named qSNC11, will be suitable for use in marker-assisted selection when developing new salinity-tolerant cultivars.

QTL Fine Mapping

NILs have been used in various studies to confirm and fine map QTLs previously mapped in RIL populations (Koornneef and Smeekens 2005; Edwards et al. 2005) for which heterogeneous inbred families (HIFs) have also been used (Tuinstra et al. 1997). To construct an HIF-type NIL, a RIL is chosen that is still heterozygous around the QTL of interest but homozygous elsewhere. In fact, even after six generations of inbreeding, which is customary for RILs, a small percentage of the genome remains heterozygous. This RIL is then selfed and genotyped so that each homozygous genotype at the region of interest can be identified and studied in detail. HIFs should not to be compared with the reference parental genotype, but with one another within the descendants (family) of the chosen RIL. In contrast to "conventional" NILs, the genetic background of an HIF line is not homogeneous, but a mix of both parental genomes since these lines originate from one RIL of the population. The ease with which NILs can be extracted from a large population of HIFs may also allow a QTL mapped at low significance thresholds to be confirmed by subsequent examination of NILs. Second, NILs extracted from segregating HIFs are useful for the fine mapping of QTLs. Each segregating HIF is independent and contains unique recombination events in genomic regions flanking the QTL. An example of fine mapping using HIFs comes from the investigation of the four loci identified in the Arabidopsis population Bay-0 × Shahdara in low and high nitrogen environments by Loudet et al. (2003). The production of homogeneous plant material for a large number of lines was certainly the most challenging and limiting step of this work, but the authors also remarked that uncontrolled environmental effects can affect the evaluation of the quantitative traits and that environmental heterogeneity can occur between two different cultivation repetitions (even in the same growth chamber), as well as within one single growth chamber during a repetition. For these reasons, it is recommended (a) to always study all of the lines in the same cultivation repetition and (b) to compare different N environments in the same cultivation repetition. One of the limitations of HIF analysis for the evaluation of QTL is that the genetic background of NILs derived from HIFs are unique and cannot be easily replicated. NILs are not easily developed for evaluating the effects of more than one QTL in a single genetic background or for comparing the effects of QTL identified in different populations.

Candidate Genes

Co-localisation with candidate genes sometimes allows rapid molecular identification of QTL. This was the case in a study by Loudet et al. (2007) where QTL mapping for sulfate content in Arabidopsis leaves resulted in the identification of several minor QTLs and one major QTL on chromosome 1 in two contrasted N levels (3 mM and 10 mM). The major QTL co-localised with the gene APR2 coding

for the adenosine-5′-phosphosulfate reductase, a key enzyme for assimilatory sulfate reduction pathway. The authors showed that the difference in sulfate contents between the two parental lines was not due to a different expression of the gene in the two genotypes, but to a change of alanine into glutamic acid in the APR2 protein. However, it is possible for there to be no overlap between the QTL and the candidate genes, as happened in the study on common bean conducted by Blair et al. (2010). A QTL for iron reductase activity in roots was identified under iron sufficiency (15 μg Fe), but it was mapped on a different chromosome from the one found under iron-limited growth (1 μM). Therefore, it was postulated that iron reductase activity was influenced by more than one locus, with a first iron reductase-related locus on chromosome b02, which contributed to the trait in iron-limited plants, and a second iron reductase-related locus present on chromosome b11 contributing in iron-sufficient plants. The authors also mapped loci for the FRO genes (iron-reductase homologues) but since there is no co-localisation with the QTL they concluded that some other gene may control iron reductase activity. Another resource for finding candidate genes is analysing gene expression in the vicinity of the QTL. This can be done using standard assays for a limited number of candidate genes, or using high-throughput genome-wide techniques, such as microarrays (Borevitz and Nordborg 2003). When the functional allelic variation results in gene expression differences, this may clearly indicate the candidate gene. Identifying an artificially induced mutant showing phenotypic effect in the trait of interest provides a unique functional argument to select a candidate gene. The availability of T-DNA insertion mutants for almost any Arabidopsis gene and the efficiency of TILLING (Targeting Induced Local Lesions in Genomes) procedures to identify mutations in numerous candidate genes provide efficient strategies to analyse knock-out phenotypes of (nearly) all genes in a QTL region. Nevertheless, most collections of mutants are in the laboratory backgrounds L*er* (Landsberg *erecta*) and Col (Columbia), which do not necessarily carry functional alleles at the gene of interest and, consequently, will not always show a distinct phenotype when mutated. Therefore, loss-of-function mutants of particular lines, such as NILs, carrying alleles different from the common laboratory accessions, can also be induced by mutagenesis with standard chemical or physical agents. This approach is especially useful when identifying novel alleles that are dominant over laboratory backgrounds (Koornneef et al. 2004). Ultimately, the proof for the identification of a QTL gene should come from complementation experiments by plant transformation.

QTL Validation

The presence of a QTL is validated when allelic variation of that QTL area has an effect on the studied trait. The search for polymorphic region-specific markers is crucial to fine map a QTL. NILs and HIFs are screened with molecular markers that are contrasted at the target region in parents. Finally, combining the analysis of a

large number of segregating recombinants and the use of new polymorphic markers in the QTL area makes it possible to define a candidate region of less than 50 kb which will contain about 10 ORFs (open reading frames). If the region size is not sufficiently small for further analysis, new screening of rHIFs (possible recombinants within a heterozygous region of a QTL) is performed. When the desired region size contains only few genes, the QTL is considered as fine mapped and genes within it are examined for potential clues (Ikram and Chardon 2010). In addition to fine mapping, several functional strategies are available for plants whose complete genome sequence is available in order to select relevant candidate genes for the QTL. The knowledge of the complete genome sequence allows the search of such candidates on the bases of the predicted gene functions. Nevertheless, the function of many ORFs remains unknown at the cellular and/or phenotypic level and, therefore, it is not always possible to find obvious candidates from the genome sequence (Koornneef et al. 2004).

QTL cloning is a very efficient way to verify a new gene without *a priori*. For instance, Calenge et al. (2006) were interested in two genotypes, which accumulated soluble sugars at different rates in 10 and 3 mM nitrate nutrition. QTL analysis resulted in a major QTL for fructose content in leaves. The fine mapping restricted the QTL area to a 3 kb interval enclosing the single gene *SWEET17* (Chardon et al. 2013). The authors showed by functional analysis that variation in fructose content is uncoupled from further metabolic pathways, which result from sequestration of fructose into the vacuole, the main compartment for soluble sugars. They demonstrated that SWEET17 is a new vacuolar transporter of fructose in plant.

In contrast, simply resequencing a region with dozens or more genes is, on its own, not generally informative because of the high number of polymorphisms that distinguish an arbitrary pair of accessions, about 1 in every 200 bp (Weigel 2012). Fortunately, compared to other multicellular organisms in which natural variation is studied, *Arabidopsis* has the enormous advantage that almost all accessions are quite easily transformed by dipping flowering plants into a suspension of *Agrobacterium tumefaciens* containing a T-DNA vector with the transgene of interest.

QTL Meta-analysis

With data on multiple populations, it useful to know whether QTL identified for a given trait in one population correspond to those detected in other populations, or whether QTL locations identified in one species correspond to QTL or other types of loci detected in corresponding regions in other plant species. With this aim, a method has been developed by Goffinet and Gerber (2000) to estimate the minimum number of loci giving the observed QTL in individual studies and to combine the available information to precisely give the position of each individual QTL. Such an approach is called ‘meta-analysis’ and its usefulness is to pool information when raw data are not available. Comparative analysis of QTL between species

reveals the existence of homologous QTL for traits involved in domestication, such as plant height and maturity, as well as tolerance to abiotic stress, within the cereals (Chardon et al. 2005; Hanocq et al. 2007; Li et al. 2013; Swamy et al. 2011). A recent example of meta-QTL analysis of a nutrition use efficiency-related trait comes from the dissection of an ortho-metaQTL in bread wheat by Quraishi et al. (2011). The authors identified a major NUE ortho-metaQTL conserved at orthologous positions in wheat, rice, sorghum and maize. Starting from three independent studies reporting QTL detection for traits related to NUE components in wheat, the authors proposed that a glutamate synthase (GoGAT) gene is conserved structurally and functionally at orthologous positions in rice, sorghum and maize genomes, and it that may contribute to NUE in wheat and other cereals.

Exploiting Genetic Variation in Wild Populations to Reveal NUE Genes by Association Mapping

First Results Obtained on Arabidopsis to Reveal NUE Genes

Over the past 10 years, traditional QTL mapping has led to the identification of sequence variants that modulate a range of physiological and developmental traits. Prior knowledge of the biological function of the affected genes was often helpful in identifying them, but increasingly the responsible locus is found to encode a protein without known biochemical function (Lempe et al. 2005). Apart from alleles that alter expression levels or protein function, a surprising number of drastic mutations such as deletions and stop codons underlie phenotypic variation. Some of these changes are found in many accessions (Weigel and Mott 2009) suggesting that they are adaptive. Nevertheless, despite some success stories, the number of known alleles responsible for phenotypic variation among accessions remains limited, mostly because fine mapping and dissection of QTLs are time consuming. *Arabidopsis thaliana* was the first plant species for which a genome sequence became available (Arabidopsis Initiative 2000). This initial sequence was from a single, high quality, inbred strain (accession) with each chromosome represented by only two contigs, one for each arm. In addition to functional analyses, the 120 Mb reference sequence of the Columbia (Col-0) accession proved to be a boon for evolutionary and ecological researches. A particular advantage in this respect is that the species is mostly self-fertilising, and most strains collected from the wild are homozygous throughout the genome (Weigel and Mott 2009). This distinguishes *Arabidopsis* from other model organisms such as the mouse or the fruit fly. In these systems, inbred strains have been derived, but they do not represent any individual actually found in nature. Numerous plants genomes have been completely sequenced and released recently.

Natural *Arabidopsis* accessions show tremendous genetic and phenotypic diversity. Thus far, significant natural variation has been reported for every phenotypic

trait investigated (Koornneef et al. 2004). Moreover, assays of metabolite profiles by large-scale unbiased metabolomics methods have uncovered natural variation at the level of small molecules, suggesting that they reflect physiological phenotypes that could be under selection in nature (Keurentjes et al. 2006). Efforts to accelerate the discovery of functionally important variants began with a large-scale study in which some 1,000 fragments across the genomes of 96 accessions of *Arabidopsis thaliana* gathered from all over the world were compared by dideoxy sequencing (Rosenberg et al. 2005). A major conclusion from this work was that there has been considerable global gene flow, so that most sequence variants are found worldwide, although genotypes are not entirely random. There is isolation by distance, and even though population structure (which is a division of the population into distinct subgroups related by kinship) is relatively moderate, it can easily be a confounding factor in association studies. From this first set of 96 strains, 20 maximally diverse strains were chosen for much denser polymorphism discovery using array-based resequencing (Clark et al. 2007). This led to the identification of approximately one single nucleotide polymorphism (SNP) for every 200 base pairs of the genome, constituting one quarter or so of all SNPs estimated to be present. In addition, regions that are missing or highly divergent in at least one accession encompass about a quarter of the reference genome. For this reason it is becoming increasingly clear that it is inappropriate to think about 'the' genome of a species, even though this is what the initial sequencing papers stated in their titles just a few years ago (Weigel and Mott 2009). The previous emphasis on relatively minor changes between individuals, such as SNPs, was largely due to the fact that sequence variation had overwhelmingly been studied by PCR-based methods or hybridisation to known sequences. It is now known that Arabidopsis accessions can vary in hundreds of genes. Of particular importance is the observation that some genes with fundamental effects on life history traits such as flowering are not even functional in their reference accession, and thus could not have been discovered on the basis of the first genome sequence alone. Whilst knowledge about the origin and phenotypic effects of sequence polymorphisms is central to understanding how species adapt to their natural environment, most studies of genetic variation in Arabidopsis have probably been motivated by the desire to identify regulatory and other genes that are not present in the common laboratory accessions (Weigel 2012). A project begun in 2009 aimed to sequence the genome of 1001 accessions of *A. thaliana* (Weigel and Mott 2009), and the task is almost complete now. The main motivation for the 1001 Genomes project is, however, to enable genome-wide association studies (GWA) in this species. The seeds from the 1001 accessions are freely available from the *Arabidopsis* stock centres and each accession can be grown and phenotyped by scientists from all over the world (Weigel and Mott 2009). Importantly, because an unlimited supply of genetically identical individuals will be available for each accession, even subtle phenotypes and ones that are highly sensitive to the microenvironment, which is often difficult to control, can be measured with a high degree of confidence. The phenotypes can include morphological analyses, such as plant stature, growth and flowering; investigations of plant content, such as metabolites and ions; responses to the abiotic environment, such as

resistance to drought or salt stress and to N deficiency; or resistance to disease caused by a host of prokaryotic and eukaryotic pathogens, from microbes to insects and nematodes.

Candidate Gene Association and Genome-Wide Association Studies

An explanation of how association mapping refers to the analysis of statistical association between genotypes (usually individual SNPs or SNP haplotypes, determined in a collection of individuals), and the phenotypes (traits) of the same individuals is undertaken here. Until recently, genetic mapping was usually done in purpose-created populations, such as progeny of parents chosen on the basis of the difference between them for the trait(s) of interest, or in defined pedigrees (families) (Rafalski 2010). By contrast, genetic association mapping involves using a collection of individuals, such as those derived from wild populations, germplasm collections or subsets of breeding germplasm. Consequently, at each locus several alleles may be simultaneously evaluated for association in a diverse population, while only two alleles segregate in any biparental population. Two association mapping methodologies are in use: Candidate Gene Association and Whole Genome Scan, also called Genome-Wide Association Study. In the candidate gene approach, the hypothesis that there is a correlation between DNA polymorphisms in gene A and the trait of interest is tested. For example, it is possible to test if in a diverse germplasm collection there is a correlation between DNA sequence alleles of phytoene synthase (or any other gene involved in carotenoid biosynthesis) and carotenoid content of seeds (Palaisa et al. 2003; Pozniak et al. 2007). This approach assumes good understanding of the biochemistry and genetics of the trait, but many genes may escape attention. Therefore, in the absence of detailed knowledge of the biochemical pathway of interest, including regulatory genes, whole genome scan (described below) is a better choice (Rafalski 2010). Genome scan involves testing most of the segments of the genome for association by genotyping densely distributed genetic marker loci over all chromosomes. The simple hypothesis that one of the genetic loci being considered is either causal for the trait or in linkage disequilibrium (LD, defined as association between genetic loci) with the causal locus is under consideration. The choice of population for association mapping, and of the appropriate marker density, are crucial decisions. One of the sources of false positives in association mapping is population structure. Complex population structure could be expected in crop species that were subject to a severe domestication bottlenecks followed by breeders' selection. Pronounced differences in the germplasm used in different regions of the world and maturity-related sets of allele frequencies for many genes may also be expected. Examples include the division of maize germplasm into heterotic groups (Reif et al. 2005) and a severe post-domestication bottleneck associated with adoption of soybean in North

America (Hyten et al. 2006). Before choosing the appropriate number of genetic markers (usually SNPs) for a genome scan, it is necessary to have some understanding of the LD in the population selected for the study. In general, LD decreases with distance between marker loci, more slowly in inbreds (soybean), faster in outbred species (maize), although breeding practices have a large impact (Flint-Garcia et al. 2003). LD is, however, very non-uniform across the genome, with both general trends (more LD in centromeric regions) and pronounced local fluctuation (Rafalski 2010). For instance, LD in Arabidopsis extends for roughly 10 kb, which is a nearly ideal distance for mapping since it extends up to the gene level (Bergelson and Roux 2010). Genetic resolution of any mapping methodology ultimately depends on the amount of recombination available in the experimental population, as measured by the rate of decay of LD (Rafalski 2010). In collections of distantly related individuals many generations have passed and much recombination occurred since the last common ancestor, therefore resolution of association mapping will, in general, be considerably higher than in simple biparental populations. The power of association mapping is strongly dependent upon the quality of phenotypic data. It is important to stress that in most cases it is necessary to use well-controlled environmental conditions, including, when possible, use of growth chambers, especially for the collection of samples for metabolomic or biochemical phenotypes. Relevance of such phenotypes for field performance will have to be separately established (Rafalski 2010). High throughput methods to precision phenotyping, frequently referred to as phenomics, are developing rapidly and automated facilities for high precision phenotyping are being established (E. Finkel 2009). The use of such a facility has been recently presented as a powerful tool to investigate plant response to drought stress by Tisné et al. (2013), allowing the precise control of watering condition of more than 700 plants. In this way, the environmental variance was strongly reduced allowing the identification of QTLs for complex traits related to drought response.

Validation and Applications

Validation of the hypotheses generated by association mapping constitutes an integral part of the experiment (Rafalski 2010). In one approach, NILs differing in the alleles at the candidate locus are constructed by repeated backcrossing into a reference genetic background (Vlad et al. 2010). The resulting NILs are then phenotyped side by side, and the amount of phenotypic variation ascribed to the presence of introgressed segment is estimated. Biparental populations segregating for the relevant alleles at the associated locus may also be used (Beló et al. 2008). Alternatively, the association experiment could be expanded by the inclusion of additional individuals in the expectation that the strength of the association should improve if the association hypothesis is correct. Association mapping is usually performed with the objective of applying the results for genotype-based selection of superior individuals in plant breeding, or as a step toward positional cloning

(Rafalski 2010). In marker assisted recurrent selection, breeders identify desirable alleles at one or more loci, basing on the outcome of a mapping experiment, and then use closely linked genetic markers for selecting individuals in breeding populations (Collard and Mackill 2008; Ribaut et al. 2010). This approach results in fixing the desirable allele(s) in the population(s) of interest.

Limitations

The detection power of association mapping greatly depends not only on the magnitude of the effect that can be ascribed to a locus, relative to other loci present in the population, but also on the allele frequency distribution. Rare alleles cannot be detected with good confidence, unless their effect is very large. Therefore, segregating biparental populations are more appropriate for the mapping of alleles rare in the germplasm pool of interest (Rafalski 2010). Genetic association mapping enriches the repertoire of tools available for the dissection of trait architecture in crop plants and model species. As high-density genotyping becomes increasingly accessible, this approach will gain power to identify with high-resolution genetic loci and in some cases causal polymorphism affecting agronomic and end-use traits in crop plants, as long as relevant alleles are present at high frequency. Mapping in defined biparental populations will remain the method of choice for rare alleles, especially those with moderate effects, and for the study of epistatic interactions. Independent validation of the associations found by both approach and evaluation of their effects in different genetic backgrounds remains an essential, even though sometimes neglected, aspect of a genetic experiment. Improvement in phenotyping remains a major challenge for mapping many agronomical important traits such as NUE or drought tolerance.

Future Perspectives

In addition to visually obvious phenotypes, natural variation has also been observed in genetic mechanisms such as cytosine methylation (Riddle and Richards 2002). A recent interest concerns the exploitation of natural variation in gene expression, leading to the first studies using expression QTL mapping (eQTL) proposed as a valuable approach to dissect the genetic basis of transcript variation, one of the prime causes of natural phenotypic variation. A recent study using eQTL conducted on 191-individual pseudo-F1 progeny of grape to dissect the genetic basis of berry colour formation (Huang et al. 2013), led to the identification of two major QTL explaining 20 % of genotypic variance and co-locating with a key enzyme for anthocyanin synthesis. With available genomic tools such as the whole genome

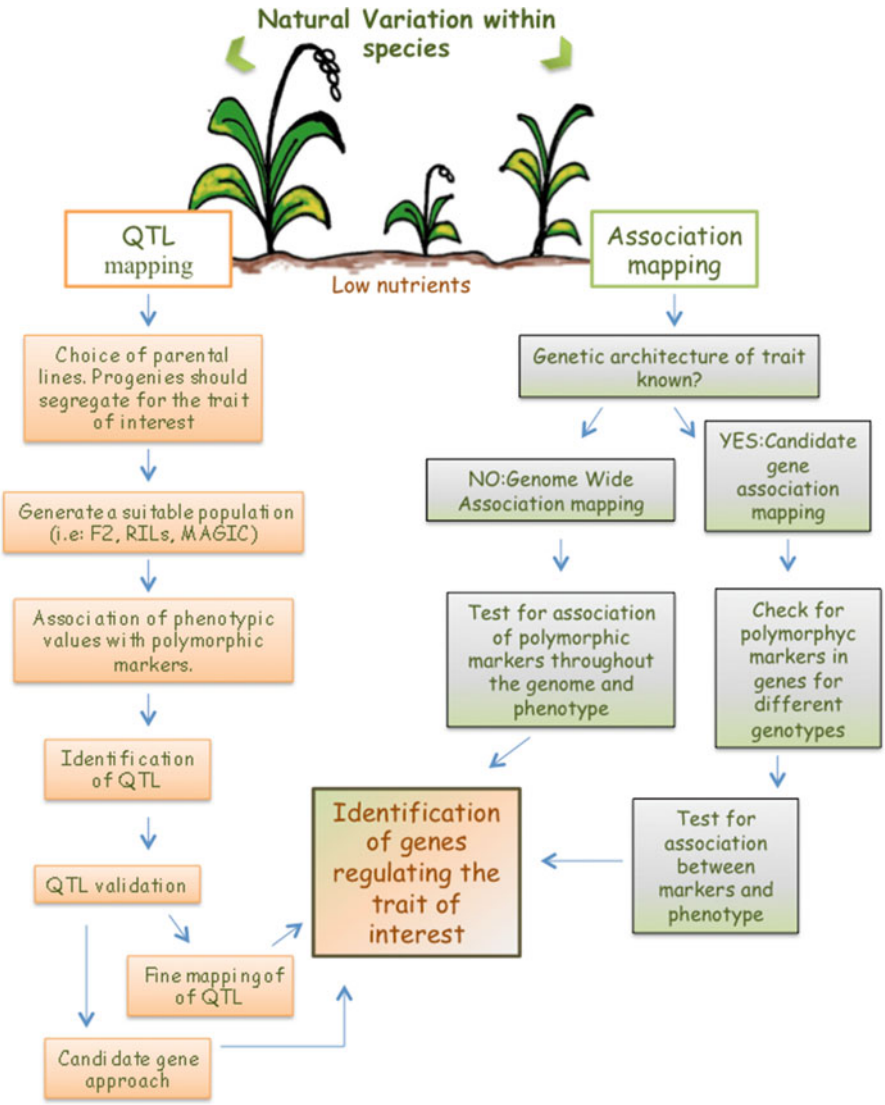

Fig. 2.2 A scheme of two possible strategies to be followed in the investigation of nutrient use efficiency related traits using natural variation in plants

sequence of many species, further investigation through genome-wide eQTL studies should bring a valuable contribution to understanding the molecular basis of traits of interest and should offer the opportunity for knowledge transfer to horticultural plants. In Fig. 2.2, a graphical overview of the possible strategies to investigate plant mineral use efficiency is displayed.

Conclusions
In light of scarce resources, increasing fertiliser production costs and the demand for greater crop production, the development of nutrient-efficient varieties is increasingly important. Both nutrient uptake and metabolic pathways are under the control of a complex regulatory network involving many genes. The identification of large-effect QTL/genes is therefore a challenge (Vinod and Heuer 2012). With the experiences gained in QTL mapping and the rapid development of genome-sequencing and molecular-marker technologies, more high-impact, large-effect QTL will surely be identified in the future. These efforts require expertise in different disciplines and, therefore, modern breeding is being implemented more and more in multidisciplinary teams involving breeders, physiologists and molecular biologists/geneticists. With the advances in molecular breeding technologies, breeders now have access to genes from wild species and unadapted genotypes that are difficult to use in breeding programmes due to crossing barriers and their poor agronomic performance. Molecular breeding therefore provides an exciting opportunity to use these gene pools effectively for the development of well-adapted and nutrient-efficient plants.

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