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SNP ANALYSIS FOR DROUGHT-RELATED CANDIDATE GENES IN A GERMPLASM COLLECTION AND A TILLING POPULATION OF ITALIAN RICE

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Raffa,

this work is the result of my efforts and your great supervision and support.

I will treasure all your precious advice and the scientific method you have taught me.

And… thanks for being a real friend.

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CHAPTER 1

General introduction and scope of the thesis.

INTRODUCTION

Oryza sativa

Rice is the most important food crop in the world, representing the main source of caloric intake for more than one third of the world's population. Developing countries such as China, India, Indonesia, Bangladesh, Vietnam, Thailand and Burma, listed in order of decreasing rice growth area and production, are the main world rice producer countries. Worldwide rice production was 678 million tons in 2009 (FAOSTAT 2010) representing the third largest crop behind maize and wheat.

Rice belongs to the family Gramineae, genus *Oryza,* which comprises about 24 species spread over the tropical and sub-tropical regions. Cultivated rice belongs to two species, *Oryza sativa* and *Oryza glaberrima*, of which the former is the more widely utilized, while the latter is known as African rice and is limited to West Africa.

When and where rice originated is not known with certainty. While the genus *Oryza* probably emerged at least 130 million years ago in Gondwanaland, it is believed that domestication of rice occurred 10000 years ago and that the two cultivated species *O. sativa* and *O. glaberrima* originated independently in Asia and Africa from the wild ancestors *Oryza rufipogon* and *Oryza barthii*, respectively.

O. sativa is traditionally divided into two major subspecies, *indica* and *japonica*, which differ in their adaptation to different climatic, ecogeographic and cultural conditions (Chang 2003). Lowland tropical areas of South and Southeast Asia and China represent the growth area of the *indica* varieties, whereas *japonica* varieties are cultivated in colder temperate climates, including northeastern Asia, Europe, western US, Chile and Australia but also in the tropical regions of Southeast Asia in both lowland and high-elevation upland areas (Khush *et al.* 2006). The two subspecies exhibit differences in grain shape, phenol reaction, amylose content and tillering ability which are characteristics traditionally used to easily distinguish them.

Recent phylogenetic studies, based on the genotypic screening of a large number of worldwide-distributed rice accessions with panels of high density molecular markers, indicate that domesticated rice can be classified into five subpopulations: *indica*, *aus*, *temperate japonica*, *tropical japonica* and *aromatic* (Glaszmann *et al.* 1987; Garris *et al.*

2005; Khush *et al.* 2003; McNally *et al.* 2009; Caicedo *et al.* 2007; Zhao *et al.* 2010). The *indica* and *aus* subpopulations cluster with the traditional *indica* subspecies, while *temperate japonica*, *tropical japonica* and *aromatic* varieties cluster within the *japonica* subspecies (Garris *et al.* 2005).

Rice as a model cereal

Not only rice is one of the world's most popular edible crops, but it represents also a model system and the organism of choice for the study of the cereal genomes. What makes rice an attractive biological system is certainly its small genome (430 Mb), the ease of transformation, and the large amount of molecular and genetic information available worldwide for this crop. The completed rice genome sequence, available thanks to the continuous effort in updating sequences and annotation data coming from the two Rice Genome Sequencing projects (MSU and RAP-DB), lays the foundation for comparative genomics to the other grasses based on genome structure and individual gene function (Devos, 2005; International Rice Genome Sequencing Project, 2005).

Additionally, rice serves as an excellent model system for studying plant evolutionary genomics due to the wide range of morphological, physiological and developmental diversity found in both *O. sativa* and its widely distributed wild ancestors, *O. rufipogon/O. nivara*.

Rice and drought

Although the total world rice production increases annually, the world population grows more rapidly. One of the main constraints limiting rice production is drought, which affects approximately 23 million ha of rainfed rice worldwide, determining losses of up to 40% of total production (Serraj et al 2011). This is what was reported, for example, for the Eastern Indian states of Jharkhand, Orissa and Chhattisgarh in case of severe drought (Pandey and Bhandari, 2009).

The decrease of productivity that occurs in case of drought stress is the result of several physiological processes through which the rice plant respond to water scarcity, such as:

- inhibition of leaf production and decrease in leaf area, with consequent reduced canopy photosynthesis (Lilley and Fukai 1994);
- closure of stomata to reduce water loss for transpiration, which leads to reduced photosynthesis (Wopereis et al 1996);
- leaf rolling, leading to a reduction in effective leaf area for light interception Wopereis et al 1996);
- enhanced leaf senescence, leading to reduced canopy photosynthesis (Wopereis et al 1996);
- reduced tillering and tiller death, leading to reduced number of panicles (Bauman *et al.* 2007);
- reduced number of spikelet when drought stress occurs between panicle initiation and flowering Bauman *et al.* 2007);
- enhanced spikelet sterility when drought occurs during flowering (Cruz and O'Toole 1984)
- decreased grain weight when drought occurs after flowering.

Despite the great efforts made by breeders to improve rice in terms of drought resistance, little progress has been made in characterizing the genetic determinants of this complex phenomenon. Drought resistance comprises indeed a number of morpho-physiological processes playing at both cellular and organismic levels and at different plant developmental stages (Tripathy *et al.* 2000). According to Levitt et al 1980, drought resistance can be seen as the result of the combination of three different strategies which are: escape, avoidance and tolerance.

Examples of drought escape are a shorter plant stature, which leads to a reduction of the area involved in the loss of water by transpiration and a short life cycle, which allows to have a less extended vegetative period under stress. Drought avoidance strategies are based on the maintenance of a relatively high tissue water potential despite soil-moisture shortage, through an enhanced water uptake (increased rooting depth, efficient root system, increased hydraulic conductance) or through a reduced water loss (reduced stomatal conductance, reduced evaporation from the leaf cuticle). Drought-tolerance includes the ability to withstand water-deficit with low tissue water potential,

maintenance of turgor through osmotic adjustment (a process which induces solute accumulation in the cell), increasing cell elasticity, antioxidant capacity and desiccation tolerance by protoplasmic resistance.

The result of dissecting drought resistance in sub-traits is that a large number of genes are involved, which makes drought the most recalcitrant trait for breeders. Because of the extreme complexity of this trait and the lack of effective phenotyping methodologies, the progress on drought resistance gene discovery in rice have been so far very limited. Nevertheless, it is of crucial importance to select genotypes able to optimize water use efficiency, while maximizing yield in relation to the target environment.

Rice and water: genetic diversity in *Oryza sativa*

Among the major food crops rice, although considered a semi-aquatic plant growing well in water, has the distinctive ability of growing in a wide range of hydrological situations. Depending on the specific water availability of the area where rice is grown, the rice environments can be classified in four categories:

- **irrigated lowland rice** (79 million ha), where ponded water is maintained for at least 80% of the crop's duration. Irrigated rice accounts for 55% of the global harvested rice area and contributes 75% of global rice production (Tabbal et al, 2002).
- **rainfed lowland rice** (54 milion ha), where rainfall is the only source of water to the field. In these areas, water is generally scarce and the fields are prone to floods and drought. Rainfed lowland is the second most important environment for rice production and occupies 34% of the world's total rice land (Tabbal *et al.* 2002).
- **flood-prone rice** (11 milion ha), which suffers periodically from excess water and uncontrolled deep flooding. In these environments, rice yields are low and extremely variable due to unpredicted combinations of drought and flood, with an average yield of just 1.5 t/ha (Tabbal *et al.* 2002).
- **upland rice** (14 million ha), characterized by well-drained aerobic soil conditions (Bauman *et al.* 2007). The largest area of drought-prone upland rice is the Eastern Indian plateau region, where 35 million ha are grown, but large areas of upland rice production can be found in West Africa, Indonesia, and hilly Southeast Asia (Serraj et

al 2011). Upland rice provides only 4% of the total world rice production, althoguh accounting for 13% of the total world rice area (IRRI, 2002). Upland rice is in fact generally low yielding, but highly water saving, with a water requirement of only one third/one quarter of that of paddy rice (Luo, 2010).

A large variability is therefore shown by *Oryza sativa* species in relation to water. As mentioned above, *Oryza sativa* originated from the wild rice *Oryza rufipogon.* This wild species was growing in swamp areas characterized by alternation of dry and wet, therefore adapted to both watered and less-watered conditions. Long term evolution led then to two different ecological types according to water requirements: the paddy rice, which is adapted to the aquatic environment and the upland rice, which requires dry conditions to complete its growth (Luo *et al.* 2002). According to Ding's classification(1957), upland rice (highly resistant to drought) and paddy rice (drought sensitive) are not significantly different from a botanical point of view; the differences lie in their different ecological adaptations. So, upland rice is considered a variant type of paddy rice, adapted to an environment characterized by water scarcity. As a consequence, genetic determinants of drought resistance should exist also in paddy rice germplasm and exploring its biodiversity in terms of drought resistance is crucially important.

Paddy rice fields and eco-sustainability

It can be estimated that irrigated rice receives some 34-43% of the total world's irrigation water. This makes rice the largest water using crop, receiving up to 2-3 times more water than other irrigated cereals such as wheat or maize (Tuong et al 2005). In addition, paddy rice fields negatively affect the environment due to ammonia volatilization and the production of greenhouse gases.

Ammonia (NH3) volatilization from urea fertilizer is the major pathway of nitrogen (N) loss in tropical flooded rice fields, often causing losses up to 50% or more of the applied urea-N (Buresh and De Datta 1990). Ammonia emissions from lowland rice fields are estimated to be some 5-8% of the globally emitted ammonia per year (Kirk 2004). Volatilized ammonia can be then deposited on the ground by rain, leading to soil acidification and involuntary N inputs to natural ecosystems.

Furthermore, rice paddy fields are a significant source of methan (CH4) and to a lesser extent of nitrous oxide (N2O). Current estimates of annual methane emissions from rice lands are around 5-10% of total global emissions (Kirk 2004).

The development of water-saving rice varieties, able to complete their growth cycle in aerobic conditions, represents therefore a crucial issue for a more sustainable rice production in the world .

Root System Architecture and drought resistance in rice

Among the several processes involved in plant responses to water scarcity, avoidance strategies, in which the root system architecture (RSA) seems to play a central role, are considered the major factor in drought-resistant performance (Blum, 2005; Levitt *et al.* 1980).

The importance of studying plant RSA lies in the fact that water and nutrients are heterogeneously distributed in the soil, so that the root spatial development will significantly define the capability of a plant to ensure the resources required to survive (Lynch 1995). Changes in the RSA have occurred as a consequence of breeding and have led to contrasting spatial arrangements of roots (Chloupek *et al.* 2006; Beebe *et al.* 2006; Yue *et al.* 2006; Kato *et al.* 2006; Sanguineti *et al.* 2006).

In several studies it was shown the overlap of QTLs for root morphology with those for productivity, suggesting the profound implications that breeding for RSA will have on the improvement of the water/nutrient use efficiency of crops and on the enhancement of their productivity under abiotic stresses (Tuberosa *et al.* 2002; Steele *et al.* 2007; Johnson *et al.* 2000).

Moreover, the conclusion emerging from long-term multi-location drought studies in rice was that rainfed lowland rice is mostly a drought-avoider. Indeed, the genotypes that produce higher grain yield under drought are able to maintain better plant water status by an increased water uptake, due to the development of a deep root system (Fukai et al, 2009).

The rice root system can be divided into three different classes which differ in origin, anatomy and function: seminal roots, nodal roots and lateral roots, which emerge from

the other two. Rice has only one seminal root or embryionic root, which is usually the longest root before the third-leaf period (Zhang *et al.* 2001) and has a poor conducting capacity (Harada and Yamazaki, 1993). Nodal roots are postembryonic roots that arise from nodes at the base of the main stem and tillers. They elongate deeply into the soil and, when a certain length is reached, the branching process starts and lateral roots begin to appear (Morita and Yamakazi, 1993). Lateral roots, comprising the biggest proportion of the root system in total length and number, are responsible for the greatest amount of water and nutrient uptake (Yoshida *et al.* 1982).

Significant genetic variation was shown to exist among different rice cultivars for root morphological traits, such as root diameter (Armenta-Soto *et al.* 1983), root depth (Nicou *et al.* 1970; Reyniers and Binh, 1978; Yadav *et al.* 1997; Mambani and Lal, 1983a; Nemoto *et al.* 1998; Kato *et al.* 2006), root pulling force (O'Toole and Bland, 1987; Ekanayake *et al.* 1985a), deep root to shoot ratio (Yoshida and Hasegawa, 1982), root number (Armenta-Soto *et al.* 1983), root growth plasticity (O'Toole, 1982; Ingram *et al.* 1994; Price *et al.* 2002) and root penetration ability (Babu *et al.* 2001; Clark *et al.* 2008, 2000; Ali *et al.* 2000).

A positive association between root growth and grain yield under drought-stress was documented in rice (Lilley and Fukai, 1994). In a study carried out by Venuprasad *et al.* (2002), root characters and grain yield were evaluated simultaneously under contrasting moisture regimes. The conclusion of such study was that genotypes able to produce deep roots prior to the onset of stress, showed improved productivity compared with genotypes that did not show that ability.

Bi-parental QTL mapping vs. Association mapping

In the past 20 years, many QTLs related to drought-avoidance root traits have been identified in rice using 12 different bi-parental mapping populations, which were evaluated for more than 30 root morphological parameters. In most of these studies root traits have been measured under controlled conditions and containers such as PVC pipes, rhizotrons and pots have been used to grow the rice plants. The PVC cylinder system is considered an improvement over pot culture, since root depth is less restricted and soil moisture is more representative of field conditions (Upchurch and Taylor, 1990). Roots were also grown in "baskets" to predict rooting depth indirectly according to root growth

angle (Oyanagi *et al.* 1993). Using the "basket method" Kato *et al.* (2006) and Uga *et al.* (2009) demonstrated the relationship between a wide root growth angle and root depth in rice.

The mapping populations used for rice root QTL studies included mainly recombinant inbred lines (Champoux *et al.* 1995; Ray *et al.* 1996; Price *et al.* 2000, 2002; Ali *et al.* 2000; Kamoshita *et al.* 2002b; Courtois *et al.* 2003; Zheng *et al.* 2003), F2 (Price and Tomos, 1997; Price *et al.* 1997), backcross inbred lines (Kato *et al.* 2008) and doubled haploid lines (Yadav *et al.* 1997; Zheng *et al.* 2000; Hemamalini *et al.* 2000; Venuprasad *et al.* 2002; Toorchi *et al.* 2002; Babu *et al.* 2003; Kamoshita *et al.* 2002a; Zhang *et al.* 2001). Most of these populations were derived by crossing varieties belonging to different subspecies group (japonica x indica).

A big drawback of QTL studies by bi-parental mapping is that the parental lines are mainly chosen based on the differences in the target trait, rather than on their overall agronomic value. Although this approach maximizes the probability to detect major QTLs involved in the trait of interest, it does not give any guarantee in terms of field performances when the QTL is introgressed in the cultivar of interest. The results obtained by Shen *et al.* (2001) and Steele *et al.* (2006) clearly indicate that the effect of QTL alleles can be influenced by the genetic background of the accessions used in the breeding program. Among the several Azucena QTLs for root traits introgressed in the IR64 (Shen *et al.* 2001) and Kalinga III (Steele *et al*. 2006) rice varieties, only two resulted in a significant effect in the novel genetic background. The failure of this study relies mainly on the fact that the QTLs identified were not fine-mapped with tightly linked markers, so the desired gene might have been lost in the selection process. Moreover, the QTLs identified had a small effect on the phenotype itself, so their effectiveness has been lost in the novel background (Gowda *et al.* 2011).

Association mapping represents a more promising method for complex trait dissection that, by focusing on association within collections of unrelated individuals, allows to locate QTLs with better precision and not related to the genetic background (Gowda *et al.* 2011).

Unlike traditional bi-parental QTL mapping, which maps genes using gametic phase disequilibrium created generally in a single cross, association mapping is based on Linkage Disequilibrium (LD) and relies on the segregating variation in natural

populations. As a result, association mapping samples many more informative meioses, namely all those that have occurred in the history of the population (Gaut and Long 2003; Gupta *et al.* 2005).

LD is defined as the non-independence of alleles and different methods have been proposed to calculate it. The most popular measures are: D, which incorporates information about allelic association and allele frequencies; D', in which the allelic association is normalized with the allele frequencies, with the advantage not to be dependent on marginal allele frequencies; r^2, which takes into account the recombination rate between two markers and the effective population size.

LD is affected by both biological factors, such as recombination and historical factors, which affect the characteristics of the population (Gaut and Long., 2003). For example, population bottlenecks and directional selection increase LD, since they reduce the genetic variability. Strong selection for a particular allele limits genetic diversity around a locus, resulting in an increased LD around the selected gene. The mating system also has a profound effect. Selfing in fact leads to homozigosity and consequently decreases the number of double heterozygotes that can be mixed by recombination. As a result, the rate of recombination is lower in inbred species like *Oryza Sativa* and LD is maintained over long physical distances (Gaut and Long., 2003). Mather *et al.* (2007) demonstrated that the average extent of LD in rice is around 200 kb, ranging from 75 kb in Indica subspecies until up to 500 kb in some regions of temperate Japonica.

So far, association mapping applied to drought resistance gene discovery in rice has been performed only in target regions of candidate genes (Serraj et al 2011). In this study a mini-core collection of worldwide representative rice varieties was selected, phenotyped in drought conditions and screened for Single-Nucleotide Polymorphisms (SNP) in several drought-related candidate genes. However, the big drawback of targeted genotyping is the need to identify candidate genes before screening, whereas drought stress affects thousands of genes. Genome-Wide Association (GWA) mapping, in contrast, can detect new regions associated with the trait of interest by testing the statistical associations between the variation of the trait and SNP variation at whole genome level.

GWA mapping was originally applied to human genetics projects, where it has emerged as a powerful approach for identifying genes underlying complex diseases (e.g. Altshuler et al. 2008, Hindorff et al. 2009). In recent years, it has also been successfully applied to

Arabidopsis and maize to study morpho-physiological traits and disease resistance (Tedesco et al. 2010; Buckler et al. 2009; Tian et al. 2011; Kump et al. 2011; Poland et al. 2010).

Rice, as a selfing species with a large extent of LD, has been shown to be a good candidate for GWAS. Huang et al. (2010) performed a GWA study for 14 agronomic traits on 317 indica landraces, identifying several loci explaining on average about 36% of the phenotypic variance. Zhao et al. (2011) and Famoso et al. (2011), by genotyping 413 and 383 worldwide distributed rice landraces, identified several loci with large effect in determining yield, morphology, stress tolerance and nutritional quality traits.

The success of GWA studies relies on thorough phenotyping for the traits of interest coupled with a cost-effective high-throughput genotyping technology, enabling to rapidly scan the largest number of markers across the largest set of genotypes to yield high-density/quality haplotype maps.

SNPs detection for plant improvement

Single Nucleotide Polymorphisms (SNPs), which are genome sites where DNA sequence differs by a single base when two or more individuals are compared, currently represent the most popular genetic markers. Not only SNPs are the most abundant form of genetic variation in eukaryotic organisms, being present in both coding and non-coding regions of nuclear and plastid DNA (Kwok *et al.* 1996), but they are also stable, efficient, amenable to automation and increasingly cost-effective (Duran *et al.* 2009, Edwards and Batley 2010, Rafalski 2002). The introduction of Next Generation Sequencing has, in fact, dramatically reduced the cost for detecting and monitoring polymorphisms at whole genome level (Craig *et al.* 2008, Huang *et al.* 2009, Metzker 2005, Schuster 2008). The result is that today Single Sequence Repeat (SSRs), that in the early past were considered the molecular markers of choice for applications in plant breeding and genetics, are being largely substituted by SNPs.

SNPs are now widely used in many breeding and research programs. They are exploited for marker assisted selection programs, for QTL and association mapping studies as well as for QTL positional cloning approaches. SNPs are also used in fingerprinting examines

such as pedigree analyses, seed purity testing and variety identification (Bernardo 2008, Eathington *et al.* 2007, Jannink *et al.* 2010, Lorenz *et al.* 2010, Moose and Mumm 2008.

Due to their presence in both coding and non-coding regions, they may be responsible for a specific phenotypic trait or may represent neutral variation that is useful in the context of diversity and evolution studies. Many successful researches demonstrated the powerful of SNPs in terms of assessment of the range of alleles available for a specific gene in a germplasm collection and their combined use in plant improvement for a target environment (Collard and Mackill 2008, Heffner *et al.* 2009, Jannink *et al.* 2010, Kim *et al.* 2010, Moose and Mumm 2008, Xu *et al.* 2008). SNPs are also extensively used in phylogenetic studies, to define the population structure of a germplasm collection, to explore the ancestry of a specific allele as well as to understand the history of domestication (Ebana *et al.* 2010, Flint-Garcia *et al.* 2005, Hamblin *et al.* 2010, Hyten *et al.* 2007, Nordborg and Weigel 2008, Zhao *et al.* 2010, Kovach *et al.* 2009, Li *et al.* 2010, Shomura *et al.* 2008, Sweeney *et al.* 2007, Takano-Kai *et al.* 2009, He *et al.* 2006, Yamamoto *et al.* 2010).

To date, several SNP-detection platforms offering different kind of resolution are available for the discovery of genome-wide polymorphisms. SNP-fixed arrays allow the screening of different varieties by interrogating a subset of previously identified SNPs in a high-throughput automated method. The two most commonly used arrays include high-resolution custom-designed Affymetrix's SNP genotyping arrays and Illumina's SNP oligonucleotide pools assays (OPAs).

A high-resolution Affimetrix custom array has recently been designed for rice that consists of 44.100 SNPs, providing approximately one SNP every 10 kb, which is expected to be sufficient for genome wide association mapping in rice (Zhao *et al.* 2011). Nevertheless, this array has two big drawbacks: the high per-sample cost (~400 dollars) and the fact that it was designed on a collection of worldwide distributed wild and cultivated rice. The result is that among the ~44.000 SNPs provided, less than 8.000 are polymorphic in temperate japonica rice. Indeed, unlike the multi-allelic SSR loci, SNP markers are bi-allelic and the specific base change detected as a SNP is expected to have occurred only once in evolutionary time. Thus, SNPs are generally informative only for a particular set of genetic materials and, considering the deep subpopulation structure existing in *Oryza sativa*, each SNP assay has to be optimized for the population under study (McCouch *et al.* 2010).

Alternatively, whole genome re-sequencing through Next Generation Sequencing (NGS) approaches represents a very powerful strategy for characterizing genetic variation at DNA level, which is becoming available at a relatively low price. Since the chemistry of Illumina Genome Analyzer II has been improved leading to a substantial increase of the read length (from 56 bp to 129 bp), GAIIx platforms are well-suited for large SNP discovery by generating hundreds of millions of short but overlapping sequence-reads, which allow allele-calling with enough good confidence (Ilmefort *et al.* 2009).

Exploring induced genetic variation: the TILLING strategy

Different reverse genetics technologies have been proposed for establishing gene function in plants, such as transposon-tagging, T-DNA insertional mutagenesis or gene silencing using RNA interference (Alonso and Ecker 2006; Small 2007; Boutros and Ahringer 2008; Hirochika 2010; Bolle *et al.* 2011; Upadhyaya *et al.* 2011).

TILLING (Targeting Induced Local Lesions IN Genomes), which was developed for reverse genetics studies by McCallum *et al.* (2000) in *Arabidopsis Thaliana*, is a powerful technology that combines traditional mutagenesis, able to induce mutations at high frequency, with a sensitive screening method for discovering point mutations in target genes.

The general protocol for constructing a TILLING platform in plants includes the creation of a mutated population by treating the seeds with a mutagenic agent (chemical or physical) and by propagating them to the M3 generation. DNA is isolated from individual M2 plants and subjected to mutation detection in targeted PCR-amplified sequences using different procedures (e.g. cleavage by specific endonuclease). Finally the induced mutant phenotype is assessed in the M3 progeny plants.

Among the chemical mutagens that have been used in the creation of TILLING populations, the alkylating agent EMS is the most popular. Alkylating agents constitute a class of base-modifying compounds which directly alter the structure and properties of the DNA bases. In particular, EMS is able to alkylate guanine bases leading to mispairing: alkylated guanine pairs with thymine instead of cytosine, and this results mainly in G/C to A/T transitions (Henikoff and Comai 2003). In Arabidopsis, maize and wheat this type of transition was shown to make up more than 99% of all EMS-induced mutations,

whereas in other species such as tomato, rice and barley G/C to A/T transitions constituted no more than 70% (Minoia *et al.* 2010). It was shown that EMS-induced mutations are randomly distributed in the genome and that, in Arabidopsis, among the alterations induced in coding regions, ~5% resulted in the premature termination of the gene product (nonsense mutations), whereas ~50% led to missense mutations (Greene *et al.* 2003; Martín *et al.* 2009).

Besides EMS, other chemical agents such as N-methyl-N-nitrosourea (MNU) and sodium azide were successfully used for the development of TILLING platforms in soybean (MNU-Cooper *et al.* 2008), rice (MNU-Suzuki *et al.* 2008; SA-Till et al. 2007) and barley (SA-Talamè et al. 2008). Also physical agents, such as gamma rays, were used in rice yielding a low mutation frequency with a higher rate of knockout mutations compared to chemical mutagens (Sato *et al.* 2006).

There are several advantages in using TILLING for reverse genetics. The main advantage is that TILLING can be applied to any plant species, irrespective of genome size and ploidy level, as well as to genes of any size. Furthermore, this approach allows to obtain mutations with higher frequency compared to other techniques. Till *et al.* (2003) demonstrated that in Arabidopsis each M2 mutagenised plant carried on average ~720 mutations, while only ~1.5 T-DNA insertions per mutant line were detected in the insertion populations created in the same species (Alonso *et al.* 2003). Therefore, much smaller populations are necessary to reach saturation using TILLING rather than T-DNA mutagenesis (Alonso and Ecker 2006).

TILLING, since it does not require genetic manipulation, represents the most appropriate technology to be used for breeding purposes, allowing to avoid the numerous GMO procedures and controversies. Moreover, the chemical mutagens (e.g. EMS) used for TILLING can create a spectrum of different mutations, including missense changes and stop-codon insertions, at a target locus. Consequently, a range of different phenotypes can be rescued other than loss-of-function (predominantly obtained in case of insertional mutagenesis), that can turn out to be useful for breeding (Alonso and Ecker 2006). A valuable phenotypic mutant detected within a TILLING procedure can then be rather easily introduced in a breeding program, thanks to the stability of the alterations induced.

An example of successful application of TILLING for crop improvement was described by Dahmani-Mardas *et al.* (2010) in melon. A TILLING screening performed in 3.306 M2 plants for 11 genes related to fruit quality led to the identification of one mutant showing an improved shel-life due to delayed ripening and yellowing. The mutant phenotype was shown to be related to an alteration in the sequence of a gene involved in ethylene biosynthesis, the hormone that regulates shelf life in fruits.

In conclusion TILLING, initially developed as a discovery platform for functional genomics, has become a valuable tool in crop breeding as an alternative to transgenic approaches, especially in countries (such as in Europe) where GMO are not allowed.

SCOPE OF THE THESIS

The national context

Italy is the first European rice producing and exporting country, with a production of 1.638.400 tons providing ~50% of the total European paddy rice production (FAOSTAT 2010).

While Spain, Greece and France grow rice in their coastal areas, in Italy rice is cultivated in the North-West portion of the Po River Valley, where 94% (~250.000 ha) of the total rice growing area is located. Due to the nature of this area, characterized by large availability of water and an efficient water distribution net, and the need to preserve an excellent grain quality, rice in Italy is conventionally cultivated under submersion. Nevertheless, in the last decades, also Northern Italy has experienced a reduction in water availability, mainly due to climate changes and to the increased need of water from other sectors. As a consequence, long dry periods in winter as well as in spring time have strongly affected the management of rice paddy fields with consequences on production and quality. The development of rice varieties capable to cope with water scarcity becomes therefore of major importance for a sustainable rice cultivation in Italy.

Aim of the work

The aim of this study was to identify new alleles with added value for the improvement of drought resistance in Italian rice, intended as capability to cope with scarce water input and/or lack of water during critical phases of the plant growing cycle.

For this purpose two different genetic materials were explored: an Italian rice TILLING population, where new variability was induced by the treatment with the mutagenic agent EMS, and an Italian/European rice germplasm collection, where the variability related to the existing biodiversity was explored.

In the first part of this study (**Chapter 1**) the EMS-induced genetic variability in drought-related candidate genes was explored in a TILLING population developed in the Italian rice variety Volano. The aim of this approach was to look for new genetic variation in

genes known to be involved in conferring drought resistance, with the final goal to identify interesting phenotypic mutants that could enter national breeding programs.

The second part of this study (**Chapter 2 and 3**), on the other hand, aimed at exploring the natural genetic variability existing in the rice germplasm cultivated in Italy in terms of drought-stress resistance. To date this information is missing and the collection and characterization of germplasm carrying drought resistant traits is of utmost importance both at national and European level. Since drought avoidance strategies represent in rice the major players in plant responses to drought stress, we decided to carry out a thorough screening of root system architecture in a collection of Italian rice varieties. To assess the variability existing for this trait in the Italian genetic pool, a pilot test was performed on a subset of 13 varieties representative of the biodiversity existing in the Italian rice germplasm (**Chapter 2**). Based on the encouraging results obtained, the screening was extended to a panel of 96 selected varieties that in parallel were whole-genome genotyped using a promising NGS technology. A genome-wide association analysis was then performed on the generated genotypic and phenotypic data with the aim of identifying new genetic loci controlling drought-avoidance root traits (**Chapter 3**).

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CHAPTER 2

TILLING in Italian rice: hunting for mutations in agronomical traits.

(manuscript in preparation)

INTRODUCTION

Rice is the most important food crop in the world, representing the main source of caloric intake for more than one third of the world's population. Although a great part of the global rice production comes from the developing countries such as China, India, Indonesia and Bangladesh, northern Italy plays a relevant role in terms of rice production providing about 50% of the total European paddy rice production (FAOSTAT *2010*).

Italy grows more than one hundred different rice varieties, most of which belong to the same temperate japonica phylogenetic subgroup. Because of this, the Italian rice germplasm exhibits a very narrow genetic basis requiring the introduction of new sources of genetic variation.

TILLING, combining classical mutagenesis (by chemical or physical agents) with a high throughput screening method for the detection of the induced mutations, is a very powerful technology that can be used to induce natural genetic variation. TILLING was originally developed in *Arabidopsis Thaliana* (McCallum *et al.* (2000) and since then has been successfully employed in many other plant species (reviewed in Gilchrist and Haughn 2010; Henikoff *et al.* 2004; Kurowska *et al.* 2011; Rashid *et al.* 2011; Wang *et al.* 2012) as well as animal species (Cooper *et al.* 2008; Moens *et al.* 2008).

Originally developed for functional genomics studies as an alternative to transgenic approaches such as transposon tagging, T-DNA insertion or RNA interference technologies (Alonso and Ecker 2006; Small 2007; Boutros and Ahringer 2008; Hirochika 2010; Bolle *et al.* 2011), TILLING was shown to be also a valuable tool in crop breeding. Among the various mutagenic agents, Ethyl Methane Sulfonate (EMS) can produce random mutations in genetic materials at a very high density (Koorneef 2002). Thanks to its capability to create a spectrum of different mutations (missense, nonsense) therefore inducing a diverse array of mutant alleles, EMS, in combination with a TILLING approach, can provide a range of different phenotypes that can turn out to be useful for breeding (Alonso and Ecker 2006). Favourable mutations detected within a TILLING platform can be rather easily introgressed into different genetic backgrounds or TILLING itself can be developed into the genetic material of interest, as classical mutagenesis can be applied to any plant species.

Several examples of successful use of TILLING for crop improvement were described in different plants. In a soybean TILLING collection, two mutants with altered seed

oligosaccharide content (raffinose/stachyose and oleic/linoleic acid), a phenotype desirable for cooking and industrial oils, were identified (Dierking and Bilyeu 2009). The development of a TILLING platform in melon allowed the identification of a mutant with a significantly improved shelf-life due to an induced alteration in an ethylene biosynthetic enzyme (Dahmani-Mardas *et al.* 2010). In tomato, TILLING led to the identification of two allelic mutations in an ethylene receptor gene, causing delayed fruit ripening and prolonged shelf-life (Okabe *et al.* 2011). In addition, mutants for virus-resistance in melon (Nieto *et al.* 2007and tomato Piron *et al.* 2010), starch in potato (Muth *et al.* 2008) and wheat (Sestili *et al.* 2010; Uauy *et al.* 2009), natural products in sorghum (Blomstedt *et al.* 2012) and nornicotine content in tobacco (Julio *et al.* 2008) were described.

TILLING was also employed to produce mutant collections in rice. Wu *et al.* in 2005 developed the first rice TILLING platform in the indica variety IR64, the most widely grown cultivar in Southeast Asia. In that study a large mutagenised M2 population was obtained using different mutagenic agents but a low mutation density (1/1000 bp) was observed. Improvements of both the mutagenesis procedure and the screening method allowed to obtain a higher mutation density (1/300 kb) in a TILLING population developed in the reference rice variety Nipponbare (Till *et al.* 2007). An even higher frequency of mutation was obtained in the Taiwanese japonica rice cultivar Taichung 65, by treating pollinated flowers with *N*-methyl-*N*-nitrosourea (MNU; Suzuki *et al.* 2008).

However, so far no TILLING platform was developed using genetic material adapted to grow in the Italian pedo-climatic conditions and of use for Italian rice breeding programs.

In this study we aimed at developing a source of new genetic variation in Volano, one of the most cultivated and important Italian rice varieties, chosen as being representative for the traditional Italian high quality rice. In Italy, Volano has a large internal market as it belongs to the 'Arborio class', the most popular 'risotto' type rice. Its growing area in 2011 covered 20.230 ha, representing 18% of the national growing area of risotto type rice (long grain type A). Volano is of strategic relevance for ongoing breeding programs in Italy albeit many traits need to be improved, such as the plant stature, the duration of the growth cycle and the resistance to biotic and abiotic stresses.

Here we describe the development and the validation of a TILLING platform in Volano. Approximately 2000 M2 EMS mutagenised lines were generated and used for TILLING

screening of three agronomically relevant target genes. A mutation density of 1/374 kb was estimated, proving the effectiveness of this approach for targeted rice crop improvement of Italian germplasm.

MATERIAL AND METHODS

Mutagenesis and plant material

Seeds from *Oryza sativa* cv. Volano were obtained by CRA-RIS (Vercelli, Italy).

EMS mutagenesis was carried out essentially as described by Till *et al.* (2007). To evaluate the toxicity and/or lethality of the EMS treatment in Volano, a range of doses from 0,25% to 1,0% EMS was preliminary tested on batches of 200 seeds each. The kill curve analysis shows that seedling survival decreased markedly at doses above 0,75%. To ensure high mutation frequency and acceptable plant survival rate, the optimum dosage appeared hence to be 0.75%, which gave germination rates averaging 59% (control displayed 95%).

A total of 20.000 seeds were then treated in batches of 200 seeds. Mutagenised seeds, after being thoroughly rinsed under running tap water, were directly sown in the field to avoid pre-germination. The M1 plants were grown according to standard paddy rice agronomic practices and harvested at maturity. Twenty M2 seeds from each line were sown in the field the next growing season. After germination, the number of albino and chlorotic M2 plants were recorded. Each M2 line was then thinned to a one M2 'healthy plants' per line, leaf samples were taken for DNA isolation and the plants bagged individually and allowed to grow to maturity. The DNA extraction was carried out on a single fertile M2 plant per line. From each selected M2 plant, M3 seeds were collected and stored at 4°C with 7-10% relative humidity, to ensure their long-term viability.

DNAisolation and pooling strategy

DNA was isolated from lyophilized leaf tissue in 96-well plates with NucleoSpin Plant II (Macherey-Nagel), using a TECAN Freedom EVO150 liquid handling robot (TECAN Group Ltd, Switzerland). Prior to pooling, the concentration of each sample was determined using the PicoGreen® dsDNA quantitation assay (LIFE TECHNOLOGIES) and normalized

at a standard concentration of 2 ng/µl, to ensure that each sample was equally represented in the pool. Two-dimensional pooling was then performed by combining all samples in shared rows and all samples in shared columns, as diagrammed in **Figure 1**.

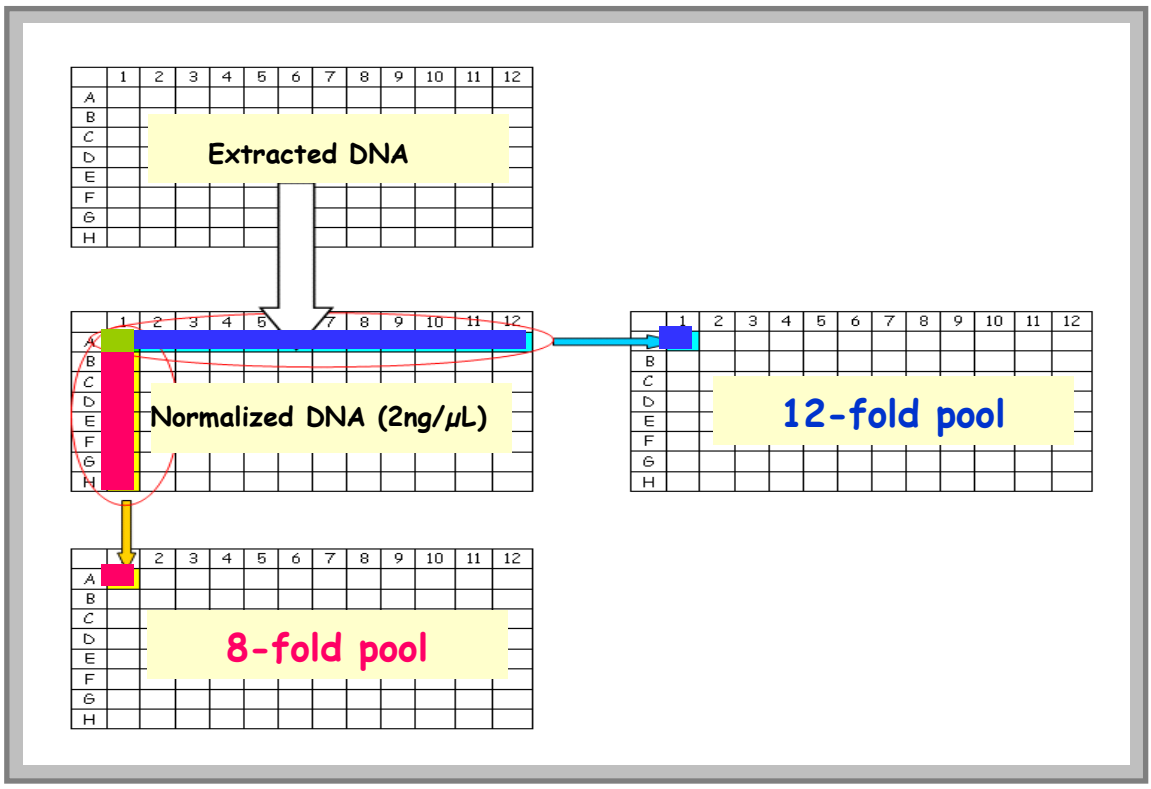

Figure 1. Two-dimensional pooling. *Samples from a common column are pooled in the first dimension and samples from a common row are pooled in the second dimension, so that each M2 line is represented both in the 8-fold pool and in the 12-fold pool.*

PRIMER DESIGN

Primers for the amplification of the target genes were designed from the Nipponbare genome sequence using CODDLE (Codons Optimized to Discover Deleterious LEsions; http://www.proweb.org/coddle) and PRIMER3 (Rozen and Skaletsky 2000). The target genes and the selected primer sequences are listed in **Table 1**. Forward and reverse primers were 5'-end labelled with IRDye 700 and IRDye 800, respectively. Labelled and unlabelled oligonucleotides were purchased from Applied Biosystems (Applied Biosystems).

Table 1. Target genes and primers used for TILLING.

Gene Name	Locus[1]	Forward primer	Reverse primer	Amplicon size (bp)
SD1	Os01g66100	acacacgctctcaactcactcc	agcagaggagaacagaggagag	1081
HD1	Os06g16370	gtccatgtggtgcaagctaaag	cgtggcatgtagtaacaactaac	972
SNAC1	Os03g60080	cagcgagaagcaagcaagaag	agcatcgatcaccacctgttc	1142

[1] Referring to MSU v.6.1 rice genome annotation.

TILLING protocol

The screening of induced mutations by TILLING was performed essentially as described by Till *et al.* (2006), with few modifications.

PCR were carried out in a final volume of 10 µl, using 2 µl of pooled genomic DNA and HotStarTaq Plus DNA polymerase (Qiagen). Labelled and unlabelled primers were mixed in a 3:2 ratio, with a final concentration of 0,4 µM. Cycling was performed on a TProfessional thermocycler (Biometra GmbH, Germany) as follows: 95°C for 15 min; 35 cycles of 94°C for 1 min, Tm–5°C for 1 min, 72°C for 1 min and 30 s; 72°C for 10 min. After amplification, PCR products were denatured and annealed to form heteroduplex between complementary strands as follows: 94°C for 10 min; 90 cycles of 1 min from 94°C to 4°C, decreasing by 1°C/cycle. Heteroduplex were then cleaved by digestion with the mismatch-specific endonuclease ENDO1 (Serial Genetics, Evry, France; Trique *et al.*, 2007), according to the manufacturer's instructions. The reactions were carried out in a final volume of 30 µl and incubated at 45°C for 20 min. The samples were then ethanol precipitated, rinsed with ethanol 70% and resuspended in 12 µl of Hi-Di Formamide and 0,05 µl of GeneScan™ 1200 LIZ® Size Standard (Applied Biosystems).

To identify the cleavage products resulting from the recognition of heteroduplex mismatches by the ENDO1 endonuclease (mutations), the samples were loaded on an ABI3730 DNA sequencer (Applied Biosystems) using a 96-capillary array with POP7 polymer. The output sequences were then analyzed using the software GeneMapper® 4.0 (Applied Biosystems).

Validation of mutations

Too confirm the detected mutations, PCR and conventional sequencing were performed on the individual M2 DNA samples identified according to the two-dimensional pooling strategy.

PCR amplifications were performed in a final volume of 10 µl as previously described, except that only unlabelled primers were used and the cycling program was stopped after the extension step of 10 min at 72°C. Prior to sequencing, the PCR products were purified from free primers and dNTPs using ExoSAP-IT (GE Healthcare), following the manufacturer's instructions.

Sequencing reactions were set up in a final volume of 10 µl with 10-40 ng of purified PCR product and a primer concentration of 0,32 µM using the BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). The following cycles were used: 96°C for 1 min; 25 cycles of 96°C for 10 sec, 55°C for 5 sec, 60°C for 4 min. Samples were then Ethanol/EDTA precipitated and loaded on the ABI3730 DNA sequencer (Applied Biosystems). The output sequences were analysed with the software MUTATION SURVEYOR (SoftGenetics, State College, PA, USA) to identify and validate the mutations.

To predict whether a point mutation would have an effect at the protein level, the target (*SD1*, *HD1*, *SNAC1*) amino acid sequences were analysed with SIFT (http://sift.jcvi.org; Kumar *et al.* 2009). Amino acid substitutions identified with TILLING were then compared with the SIFT outputs.

SD1 mutant phenotyping

Mutant and wild-type plants were grown in the field at CRA-RIS (Vercelli, Italy). Seeds were sown directly into the soil and the field was submerged at the 3rd-4th leaf stage. The field was managed according to the standard paddy rice agronomic practices. Total plant height was measured at maturity stage considering the distance from the soil to the top of the panicle (excluding the awn), according to the UPOV guidelines.

RESULTS

Development of the Volano TILLING population

To develop the Volano TILLING platform we chose to use the chemical mutagen EMS. The effectiveness of this mutagen applied to the TILLING screening procedure in rice was previously demonstrated in Nipponbare by Till *et al.* (2007), where a density of induced mutations of 1/300 kb was reported. However, EMS toxicity and efficiency can vary within the same species, as shown by the lower mutation rate registered in the indica variety IR64 (1/1000 bp; Wu *et al.* 2005). Therefore, a pilot test using different doses of EMS was carried out on small batches of Volano seeds to identify the optimal amount to be used for large-scale mutagenesis.

Based on the results obtained, the mutagenesis was then performed using 0,75% EMS on 20.000 seeds of Volano. The mutagenized seeds were directly sown in the field and the survived M1 plants were grown to maturity and self-fertilized. Approximately 2.000 fertile M1 lines were harvested and 20 seeds from each line were planted to generate the M2 generation . During the growth of the M2 population, several mutant phenotypes were observed at different developmental stages, ranging from seedling to maturity stage. The most common mutant phenotypes were related to dwarfism, plant sterility, alteration in plant architecture and seed morphology. Examples of the observed phenotypes are shown in **Figure 2**. Among the 20 M2 seeds sown for each M1 line, only one M2 fertile and healthy individual plant was selected for DNA isolation and for seed harvest. In total, 1860 M2 lines were selected to constitute the Volano TILLING collection.

Detection of mutations

To estimate the efficiency of the EMS treatment and to evaluate the mutation frequency in our population, TILLING was initially performed on DNA isolated from 1152 M2 lines arranged according to a two-dimensional pooling strategy (**Figure 1**).

A similar strategy was successfully employed in rice by Till *et al.*(2007), where 8-fold pools were used in both dimensions. The advantage of using a 2D pooling strategy relies on the possibility to directly identify the M2 line containing the mutation, without the need to sequence all the samples of the pool.

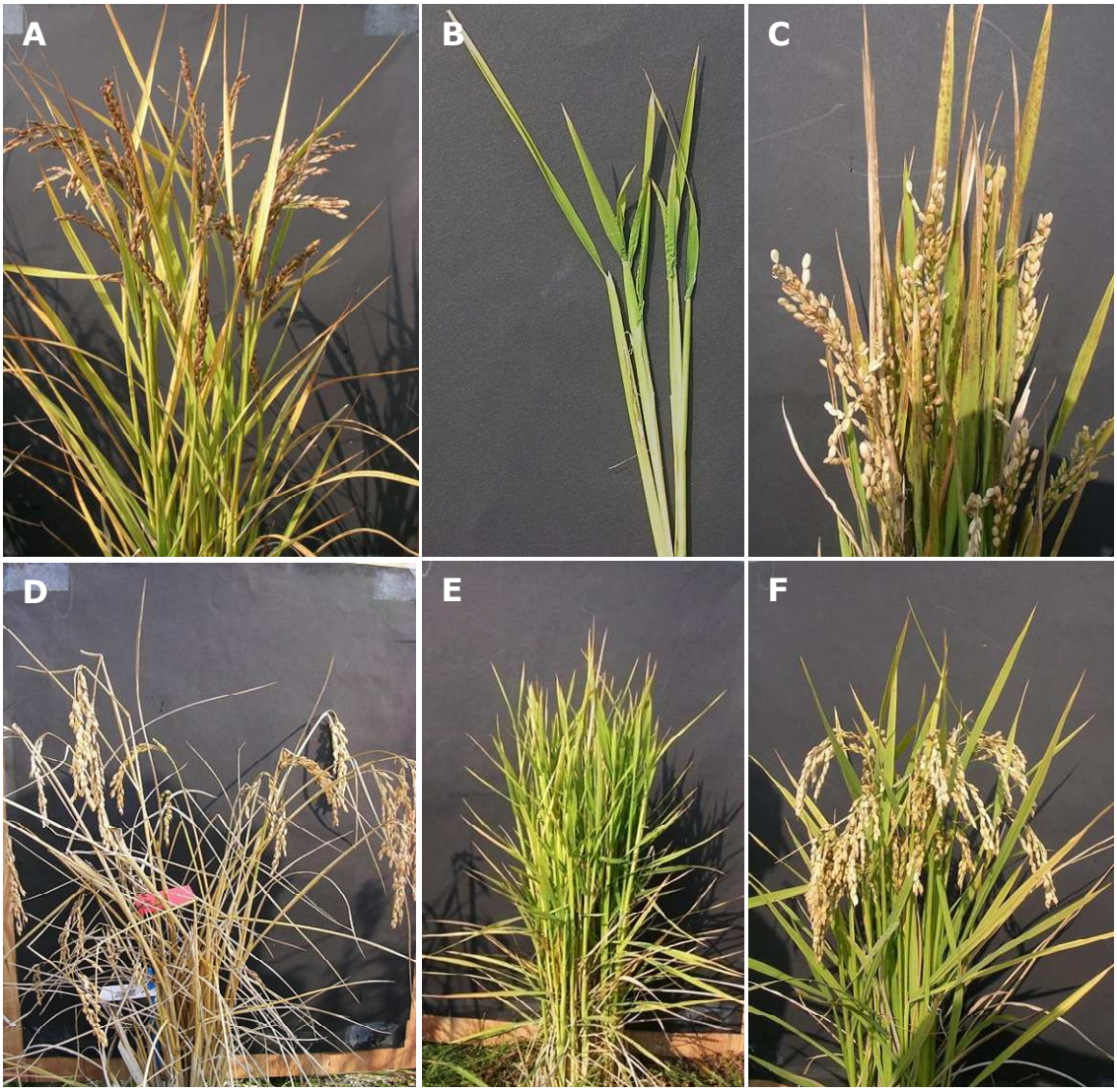

Figure 2. Examples of morphological mutant phenotypes observed in the M2 generation. *(A) Abnormal spikelet pigmentation; (B) reduced culm number; (C) altered seed shape and leaf pigmentation; (D) early flowering; (E) late flowering; (F) dwarfism.*

For this pilot screening, three genes were selected from the literature of relevance for the agronomic improvement of Volano: *SD1* (semidwarf-1), involved in plant height determination (Sasaki *et al.* 2002); *HD1 (Heading date-1)*, which plays a crucial role in determining the flowering time (Yano *et al.* 2000) and *SNAC1* (Stress-Responsive NAC

1), shown to be a central player in stomata guard cell closure under water stress conditions (Hu *et al.* 2006).

TILLING screening resulted in the identification of eight mutations in total, of which three in the *SD1* gene, one in *HD1* and four in *SNAC1* (**Table 2**). The mutations observed were predominantly G/C to A/T transitions (87,5%), except for one T/C transition. This result is in agreement with what reported in the literature. In Arabidopsis, maize and wheat G/C to A/T transitions were shown to make up more than 99% of all EMS-induced mutations (Greene *et al.* 2003), whereas in other species such as tomato, barley and rice these types of mutations represent only 70% (Minoia *et al.* 2010) of those observed. Because the detected T/C transition was heterozygous, the hypothesis that it occurred naturally should be rejected considering the low estimated rate of spontaneous mutations (10^{-7} - 10^{-8} bp/generation; Greene *et al.* 2003).

Table 2. EMS induced mutations identified in the Volano TILLING population.

Gene	Plant ID[1]	Nucleotide change	Position from ATG[2]	Position in CDS[2]	Aminoacid change	Effect[3]
SD1	860	G/A	144	exon1	W>STOP	damaging
	1427	C/T	473	exon1	S158F	tolerated
	921	T/C	802	exon2	Y234H	damaging
HD1	782	G/A	242	exon1	C81Y	damaging
SNAC1	1213	C/T	262	exon1	R88C	damaging
	951	C/T	316	exon1	P106S	damaging
	1523	C/T	467	exon1	S156F	damaging
	269	G/A	756	exon2	Glu>Glu	silent

[1] M2 line carrying the mutation.
[2] Positions refer to the genomic sequences.
[3] Prediction based on SIFT algorithm .

All the identified mutations occurred in exons (**Table 2**), as expected considering that less than 17% of the 3195 nucleotides used for the screening were spanning introns (**Table 3**). Based on the predicted effect on the protein product, we found 75% missense, 12,5% silent and 12,5% truncation (nonsense) mutations. Besides the nonsense mutation, the majority of the detected missense mutations (5 out of 6) were likely to result in non-functional protein products, according to SIFT predictions (Kumar

et al. 2009). All but one (the silent change in *SNAC1*) of the mutations identified were shown to be heterozygous (**Table 3**).

To estimate the mutation rate, 200 bp were subtracted from the length of each screened amplicon according to Greene *et al.* (2003) that reported the difficulty to detect mutations close to the TILLING primers. The resulting average mutation density in the Volano TILLING population as observed in the pilot screening was 1/374 kb.

Table 3. Distribution of mutation classes.

Gene	Size (bp)	CDS (bp)	Total	Silent	Missense	Nonsense	Hetero	Homo
SD1	1081	879	3	0	2	1	3	0
HD1	972	828	1	0	1	0	1	0
SNAC1	1142	951	4	1	3	0	3	1
Total	3195	2658	8	1	6	1	7	1
%				**12.5%**	**75.0%**	**12.5%**	**87.5%**	**12.5%**

TILLING for crop improvement: 'dwarf Volano'

Reduction in plant height represents one of the most important goals of the current breeding programs for Volano. To identify Volano lines with shorter stature, we screened for EMS-induced mutations in the *SD1* gene, representing the most important gene of the rice Green Revolution (Sasaki *et al.* 2002). *SD1* encodes for a gibberellin 20-oxidase, a key enzyme in the gibberellin (GA) biosynthetic pathway, which plays a central role in determining plant height by affecting cellular and internode expansion. Alterations in the *SD1* genomic sequence result in decreased levels of GAs due to the defective GA 20-oxidase enzyme, leading to plants with shorter and thicker culms, improved lodging resistance and a greater harvest index (Monna *et al.* 2002; Sasaki *et al.* 2002; Spielmeyer *et al.* 2002).

TILLING of *SD1* in the Volano mutagenized population resulted in the identification of three independent point mutations, of which two missense and one truncation mutation (**Table 2**). In particular, the C/T transition in line M2_1427 led to a tolerated non-

synonymous amino-acid change (S158F) presumably not affecting protein function, according to SIFT prediction (Kumar *et al.* 2009). In contrast, the T/C transition in line M2_921 caused a Tyr→His substitution at position 234, expected to be highly deleterious for protein function. Thehe G/A transition in the M2_860 line resulted in the creation of a premature STOP codon at position 48 of the protein sequence, generating a predicted non-functional product lacking the last 341 amino-acid residues. All the identified mutations were heterozygous.

***Figure 3**. **Example of a "dwarf" Volano mutant.** A M3 plants from line M2_921 carrying the homozygous mutation is shown (on the left) in comparison with wild-type (on the right).*

To confirm the inheritance of the induced mutations and to explore their phenotypic effect, thirty M3 seeds derived from each M2 mutant line were sown in the field and grown to maturity. DNA was isolated from each M3 plant and the SNP alterations

confirmed by sequencing of the *SD1* gene. For the M2 lines 921 and 860, M3 progeny plants carrying the mutations in the homozygous state showed a significant decrease in plant height when compared to homozygous wild-type plants (**Figure 3**). In particular, we observed an average height reduction of 19,1 cm in case of M3 homozygous mutant progenies derived from M2_860 and 23,8 cm in case of M2_921 (**Figure 4**). Furthermore, the M3 plants heterozygous for the mutations showed an intermediate stature between their homozygous mutant and wild-type (**Figure 4**), supporting the hypothesis that the observed phenotypes arose from the EMS-induced alterations in the *SD1* gene. In agreement with the predicted effect of the mutation on the protein function, the M3 mutant progeny plants deriving from line M2_1427 did not differ significantly in plant height from wild-type plants (**Figure 4**).

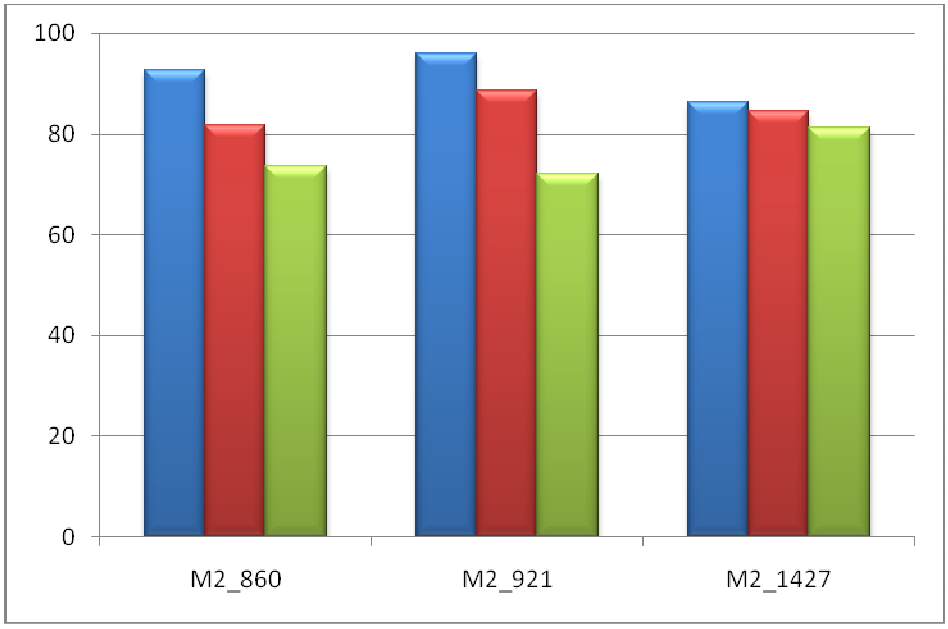

Figure 4. Segregation of plant height among M3 progenies of SD1 mutants. *For each M2 line, the blue bars represent the average height of the homozygous wild-type plants, red bars represent plants carrying the mutation as heterozygote, green bars represent the homozygous mutant plants. Error bars are indicated.*

DISCUSSION

Volano is one of the most cultivated and important Italian rice varieties. Belonging to the "Arborio class", which is the most popular rice for "risotto", Volano is indeed considered representative for the traditional Italian high quality rice.

Due to its strategic relevance for ongoing national breeding programs, we chose this traditional variety for the development of the first Italian rice TILLING platform. Despite its good grain quality, Volano has many traits that may benefit to be improved such as plant height, the duration of the growth cycle and the resistance to biotic and abiotic stresses.

To create our TILLING population in Volano we chose EMS as a mutagenic agent among other treatments previously used to create mutant collections in plant species, as the best suited to our purpose. Different mutagens can produce different spectrum of mutations, therefore different series of alleles. Gamma and fast neutron irradiation were shown to induce small (few bp) and large (hundreds to thousands bp) deletions respectively, mainly resulting in gene knockouts (Sato *et al.* 2006; Li *et al.* 2001). On the other hand, chemical mutagens such as EMS and MNU typically induce single nucleotide polymorphisms. These point mutations, in addition to loss of function alleles, lead to the generation of a series of polymorphic alleles that provides a range of different phenotypes with a potential use in crop improvement (Cooper *et al.* 2008). In rice, MNU-induced mutations were shown to occur at a rate two times higher (1/135 kb; Suzuki *et al.* 2008) than those obtained with EMS (1/300 kb; Till *et al.* 2007). However, to obtain such a high mutation frequency, the treatment with MNU requires that the flowers are exposed to the mutagen rather than the seeds (as for EMS), making the mutagenesis procedure more complicated.

Based on previous results obtained in *Medicago truncatula* (Le Signor *et al.* 2009), we chose to select only one M2 individual from each M1 mutagenised line to build our population, to maximise mutation efficiency. In their study, Le Signor *et al.* compared the mutation frequency and recovery obtained from two mutant populations created starting from a different number of M1 lines, clearly demonstrating that the number of total mutations was three-fold higher if only one M2 from each M1 line was used.

The mutation density estimated in the Volano TILLING population (1/374 kb), based on a pilot screening of three target genes in 1152 lines, was similar to what previously obtained in rice using EMS. The observed rate was higher than that reported for the indica rice variety IR64 (1/1000 kb; Wu *et al.* 2005), yet slightly lower than that obtained in Nipponbare (1/300 kb; Till *et al.* 2007). However, this difference could likely be due to the higher dose of EMS (1,5%) used by Till *et al.* compared to what was used in this study. Overall, the results obtained confirmed the efficiency of the mutagenic treatment and the potentiality of our TILLING platform as a source of new genetic variation in Volano. At present the platform consists of 1860 M2 lines and newly mutagenised plants are being produced to enlarge the mutant collection, allowing the screening of any gene involved in any trait of interest.

Besides the validation of the TILLING platform, this work also provides genetic materials that could be directly exploited for the improvement of Volano. As mentioned earlier, one of the main objectives of the breeding in Volano is represented by the reduction of its stature. Reduction in total height has been shown to increase plant responses to nitrogen inputs, resulting in a higher yield performance without culm elongation and lodging problems (Ashikari *et al.*, 2002). Moreover, a shorter stature can be beneficial for the plant also in terms of tolerance to water-limited conditions. Indeed a reduced plant height implies a reduction of the area involved in loss of water by transpiration, which represents one of the main strategies of drought-escape (Levitt 1980).

In this study we detected three independent mutations in the *SD1* (Semi-Dwarf1) gene, which in rice plays a crucial role in determining plant height (Sasaki *et al.* 2002). *SD1* encodes a GA20-oxidase, a key enzyme in the biosynthesis of gibberellins. In particular, it catalyzes the sequential oxidation and elimination of C-20 in the GA biosynthetic pathway, providing a substrate for the GA3β-hydroxylase (GA3ox) that catalyzes the last step of the synthesis of active GAs (Hedden *et al.* 2000). Two GA20ox genes (GA20ox-1 and GA20ox-2) were shown to be present in the rice genome. GA20ox-1 is predominantly expressed in unopened flowers and enables flowers to develop and fertilize normally (hence ensuring yield), while GA20ox-2 (corresponding to *SD1*) is strongly expressed in the leaf blade and stems. This redundancy can explain how loss of *SD1* function (and consequently GA deficiency) can result in reduction of plant height without seed yield being affected (Monna *et al.* 2002; Sasaki *et al.* 2002).

Two of the mutations identified in this study displayed a phenotypic effect, resulting in a significant reduction of plant height. This reduction correlated with the allelic status of the mutation, with a stronger effect associated with the homozygous mutated allele (**Figure 4**), supporting the hypothesis that the mutant phenotype was indeed caused by the EMS induced change. Both the mutations were predicted to affect protein activity. In line M2_860, the premature insertion of a STOP codon was expected to result in an inactive truncated GA20-oxidase enzyme and therefore in defective GA synthesis. On the other hand, line M2_921 carried a substitution of a highly conserved Tyrosine, having a very high probability of causing a loss of protein function (SIFT prediction). Interestingly, the phenotypic effect on plant stature associated to this mutation appeared to be slightly stronger than that observed for the other mutant.

Several *SD1* alleles that cause semidwarfism in rice were described and used in breeding programs worldwide to improve the agronomic performances of local varieties (Asano *et al.* 2007). Dee-geo-woo-gen, the Chinese semi-dwarf rice cultivar where *SD1* was firstly identified, and the derived high-yielding cultivar IR8 (IRRI 1967) which led to the Green Revolution phenomenon, carry the same *SD1* allele harbouring a 383-bp-deletion from exon 1 to exon 2 that originates a stop codon (Monna *et al.* 2002, Sasaki *et al.* 2002). An independent deletion of 280 bp was found in the coding region of *SD1* in the Indica semidwarf variety Doongara (Spielmeyer *et al.*2002). In addition, several point mutations occurring at different positions in the *SD1* coding sequence were demonstrated to cause single amino-acid substitutions resulting in semi-dwarfism, as in the japonica semi-dwarf vareties Jikkoku, Reimei and Calrose 76 (Spielmeyer *et al.*2002). The two *SD1* mutants identified in the Volano TILlING population represent an interesting genetic material for Volano breeding programs at national level.

Similarly to plant height reduction, reducing the life growth cycle represents another strategy of drought escaping. In wheat and barley, shortening the crop life-cycle duration was shown to be an effective breeding strategy for reducing water consumption, by reducing the growing season and, consequently, the total plant water request (Cattivelli *et al.* 2008). The *HD1 (Heading Date*-1) gene used for TILLING screening in this study, plays a key role in rice in determining flowering time (Yano *et al.* 2000*)* and hence represents a good candidate for targeting growth cycle duration in Volano. One missense mutation was detected in our screening in the *HD1* coding sequence, which was predicted

to affect protein function. However, assessment of M3 progeny mutant plants for phenotypic changes gave non conclusive results (data not shown), requiring further evaluations at the phenotypic level of M3 lines.

An earlier stomatal closure in a crop is considered a positive trait for the improvement of water use efficiency in drought environments (Sinclair and Muchow, 2001). A previous study in Arabidopsis reported the cloning of a QTL regulating transpiration efficiency while maintaining biomass production, hence uncoupling the usually negative association between transpiration efficiency and both stomatal conductance and biomass production (Masle *et al.*, 2005). Based on this findings the *SNAC1* (Stress-Responsive NAC 1) gene, shown to be involved in stomatal closure mechanisms in the early stages of drought stress (Hu *et al.* 2006) was selected as a target for TILLING screening of the Volano mutagenized population. The progeny of the three M2 lines carrying the mutations predicted to affect protein function are currently under investigation to assess the resulting phenotypes under water-limited conditions.

CONCLUSIONS

This work represents the initial step towards the improvement of Volano, one of the most important rice varieties cultivated in Italy. We have shown that TILLING, based on classical mutagenesis to create new genetic variation without the use of transgenic technologies, represents a powerful technique for crop improvement that can be applied to local genetic material. This initial screening allowed the identification in Volano of a number of potential mutations in agronomically relevant genes that can provide genetic materials directly of use for marker-assisted breeding.
Since the efficiency of the platform was validated and the collection is being extended to a larger number of mutagenized lines, TILLING in Volano can become a platform of novel genetic variation to assist breeding for improvement of target traits such as disease resistance, stress tolerance and nutritional properties of the rice seeds.

ACKNOWEDGEMENTS

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CHAPTER 3

Soil-filled glass rhizotrons for visualising roots.

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Methodologies for Root Drought Studies in Rice.
International Rice Research Institute, Los Baños, Philippines.

1. Preamble:

Plants are grown in thin rhizotrons with glass sides that are filled with soil and inclined at 15°. Photographs and non-destructive (visual) assessment of root traits such as rooting angle and depth can be taken whenever required. After six or seven weeks the rhizotron can be harvested to assess root thickness, root and shoot mass. Withholding water followed by weighing can provide assessment of water use. Delta-T theta probes can be inserted in the side to assess the volumetric water content at any stage. The technique has been used to assess a mapping population and collections of rice sampling genetic diversity at scales of 24 to 320 rhizotrons at a time.

2. Materials used:

A. Sheets of 4 mm thick glass cut to 1200 x 300 mm.
B. Sandy loam soil sieved using a course sieve to remove stones and large clumps.
C. Drip irrigation system (available in gardening stores).
D. Sundries including duct tape, two lengths of straight 15 mm thick and 1000 mm long wood, bottle tops, custom made metal chute to guide soil, chemicals for nutrient solution.

3. Methods adopted:

Preparation of rhizotrons:

Two glass sheets are selected at least one of which is clean on both sides. One is placed on a worksurface with two of the four edges slightly overhanging. Two lengths of 15 mm thick wood are placed on top of the first sheet, a 15 mm thick bottle top (e.g. Coca Cola) is placed at the top and the bottom of the glass and then the second sheet of glass is placed over the top. Duct tape is used to join the two sheets of glass together at the overhanging edges (figure 1a). The sheets are turned so that the remaining long edge is overhanging, and that is then sealed with duct tape. The empty rhizotron is set vertical, a single strip of duct tape is wound right around the rhizotron at a depth of about 300 mm from the bottom and the two lengths of wood are removed. The two bottle tops

prevent the glass coming together and empty rhizotrons can therefore be stacked waiting for filling. The one at the top can be removed at a later date during the soil filling process. The bottle top at the bottom must remain in the rhizotron.

The empty rhizotron is stood upon a soft support such as expanded polystyrene sheet and sieved soil is then encouraged into the rhizotron using a dustpan and the custom made guide (figure 2).When nearly full, the upper bottle top can be removed. When full, the rhizotron is lifted and then gently dropped onto the support causing the soil level to drop by 5-10 cm due to packing of the soil. The rhizotron is refilled, gently dropped once more and refilled for a final time. The force of the drop will affect the amount of soil used. The aim is to pack sufficiently well to prevent slumping of the soil when it is watered, but not too much that it splits the tape or creates impedance to the roots. The later can be roughly assessed by pushing a sharpened pencil into the soil. If it is difficult to push, the roots will probably also find it difficult. Once filled, the rhizotron should be weighed with the aim to have each the same weight. Typically they weigh 13 kg and contain about 7 kg of wet soil. A small drainage hole should be made at each side at the bottom using an implement such as a sharpened pencil.

Typically, rhizotrons are placed in stacks of eight and are leaned at an angle of 15^{o} to encourage roots to grow on the lower face (figure 1c). The exposed face of the first stack is backed with an insulation sheet to reduce heat exchange and prevent light penetration. Insulation is place over the front of the stacks and an irrigation system is installed (figure 1 d). This should supply water equally to each rhizotron and apply it slowly (to avoid soil slumping). Typically, an irrigation rate of 40 ml min^{-1} is used. This can be used to supply nutrients or water as required.

Each rhizotron is labeled on the lower sheet so that in a photograph of that side (where roots are most visible) the identity of the rhizotron can be seen. Typically, two seeds are sown in each rhizotron which are thinned to one when they have emerged. Watering is typically done three times a week with 250 mlsof Yoshida's nutrient solution for the first 3 weeks, moving up to more frequent and larger volumes of nutrient and water as the plant grow, reaching about 250 mls nutrient and 150 mls water every day when 6 weeks old, but amounts will vary with climatic conditions.

Photographs of the lower side of each rhizotron can be taken when required but must be done in the dark to reduce reflection from the glass. This can be done at night using spot lights at either side of the rhizotrons. Typically two rhizotrons are photographed together.

Withholding water followed by daily weighing using a 20 kg balance can provide data on daily water use once drainage from the rhizotrons has stopped (a few hours after irrigation). A Delta-T (Cambridge, UK) theta probe can be modified by removing one of the three exterior rods and inserted into the side of the rhizotron through the tape to give a reasonable estimate of volumetric water content. The holes created can be reused any number of times to monitor changes in soil moisture at different depths.

4. Trait to be recorded:

- On a weekly basis shoot growth is monitored as height of the plant (length from the soil to the tip of the longest leaf) while the length of the longest visible root and the number of roots passed 25, 50, 75 and 100 cm is recorded. After about 21 days the angle of spread of the root system can be measured with a protractor (the maximum angle between the least vertical main root axes on either side of the plants base).

- To measure water use, daily weighing can be conducted. This is most easily achieved if watering is stopped, otherwise added water must be recorded. For maximum accuracy at least two control rhizotrons without plants can be used to assess water loss from drainage and the exposed soil surface although the former stops within a day and the latter is relatively small.

- At anytime, but typically at the end, the rhizotrons can be photographed two at a time with a reasonably high resolution (eg. 12 mega pixel) digital camera. From the images, the angle of main (nodal) roots can be evaluated either using image analysis or manually. In the manual method a protractor placed against each image on a computer screen. With the protractor placed horizontally, the number of main axis in 9 angle classes representing each of the 10^{o} subdivisions of the protractor were counted. Roots in the division 0-10^{o} and 170-180^{o} are counted together in the 0-10^{o} class to give the number of roots in the most horizontal of the 9 angle classes. A weighted average of the angle is calculated by multiplying

the number of roots in each class by the halfway angle of each class (e.g. 5 for the 0-10^{o} class and 35 for the 30-40^{o} class), summing across all classes and dividing that by the total number of axes.

- At the end of the experiment (e.g. day 42), shoots are removed in a single day and dried to assess shoot dry weight. Over a 1-week period, each chamber is opened. Short sections of three of the thickest roots are removed from each root system near the base of the shoot, placed in water and stored in the fridge, before being used to assess root thickness under a dissecting microscope. The entire root system is divided into 3 sections, 0-40 cm (the top), 40-80 cm (middle) and 80-120 cm (bottom), washed, dried and weighed.
- If information on fine root structure is required, instead of drying, the roots can be preserved in 50% ethanol before being scanned for analysis using software such as WinRhizo.

5. Precautions to be taken:

- The glass presents a risk of cutting. Use carborundum paper to remove the sharp edges from new glass and where gloves when constructing them.
- Ensure one sheet of glass (the one that will be photographed) is clean.
- During watering, there is a tendency for the soil to move down under gravity. This is minimal if the rhizotron is well packed with soil (not loose) and if water is applied slowly.
- Ensure the labeling is sufficiently large to be read in the photographs.
- Light should be excludes as much as possible.
- Within a greenhouse in temperate regions, these experiments can only be done in the Summer months since the light intensity is limited in the winter even with supplementary lights.

6. Photographs:

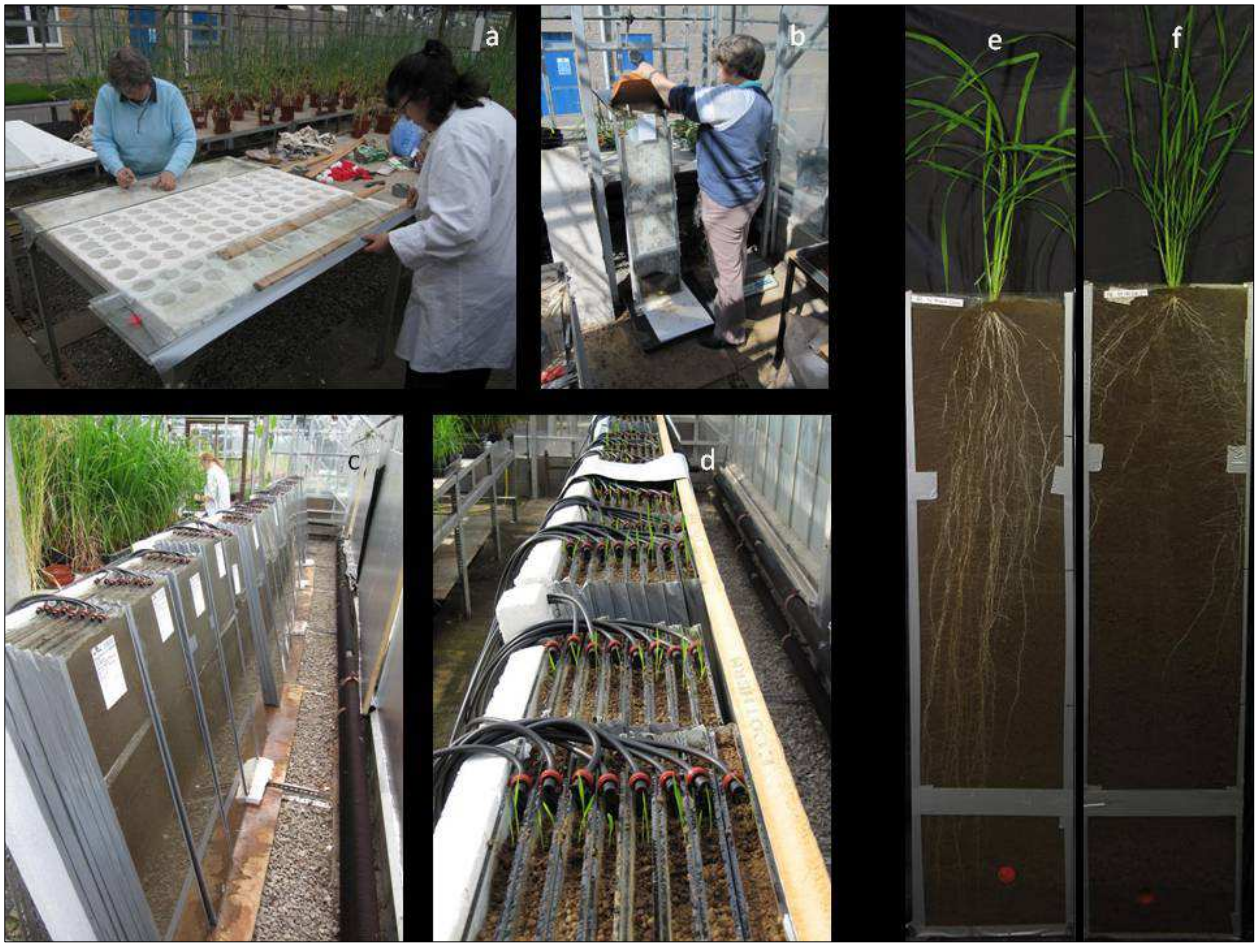

Figure 1 (a) *construction of empty rhizotron,* ***(b)*** *filling with soil,* ***(c)*** *stacking at 15^{o},* ***(d)*** *close up of drip irrigation,* ***(e)*** *photograph of Indian cultivar Black Gora,* ***(f)*** *photograph of IR64.*

7. Case study:

Objective

To assess the degree of variation for root traits in Italian cultivars as part of an Italian-funded project DryRice.

Materials and Methods

- Plant materials

Thirteen Italian cultivars (namely Augusto, Baldo, Balilla, Carnaroli, Euro Sis, Gladio, Koral, Loto, Perla, Salvo, Sis R215, Thaibonnet and Vialone Nano) plus check varieties IR64 and Black Gora.

- Rhizotron methodology

The experiment was sown on the 13th May 2010. There were 4 replicates organized as a randomized complete block design. Plants were grown for 42 days, water was withheld from day 39 so that daily water use in the last 3 days could be assessed.

Results and discussion

As typically observed many root traits are significantly correlated with overall plant growth and it is important to assess traits in that context. Clear differences in traits were observed indicating major differences in root morphology of Italian rice cultivars. Variety Loto was notable for having long roots in absolute terms and relative to total plant growth while most were relatively short (figure 2). Salvo and Carnaroli had notably thick main axis while Balilla and Perla were thin (figure 3). Perla also had a low mean root angle indicative of a shallow root system while Vialone Nano was notably vertical (figure 3). Water use between day 40 and 42 was very strongly correlated to shoot and root mass (r 0.89 and 0.91 respectively) suggesting no major differences in transpiration of well watered plants. These results demonstrate that even within a geographically and genetically narrow set of rice accessions clear differences in root morphology can be revealed.

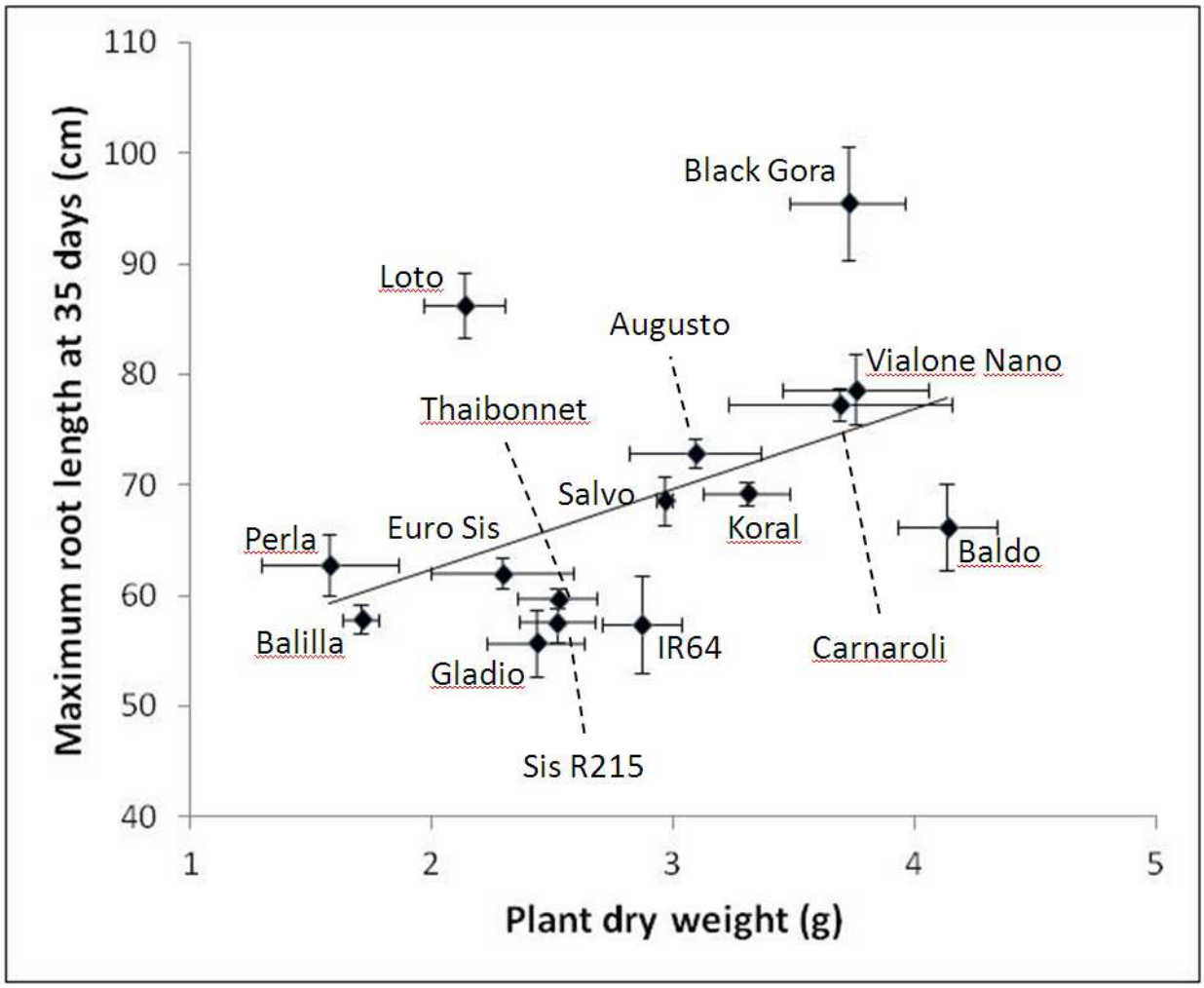

Figure 2 *Maximum root length at 35 days plotted against total plant dry weight at 42 days for 13 Italian varieties plus check varieties Black Gora (deep, vertical roots) and IR64 (shallow, more horizontal roots). Bar is standard error. A regression line is drawn as there is typically a relationship between root traits and plant growth.*

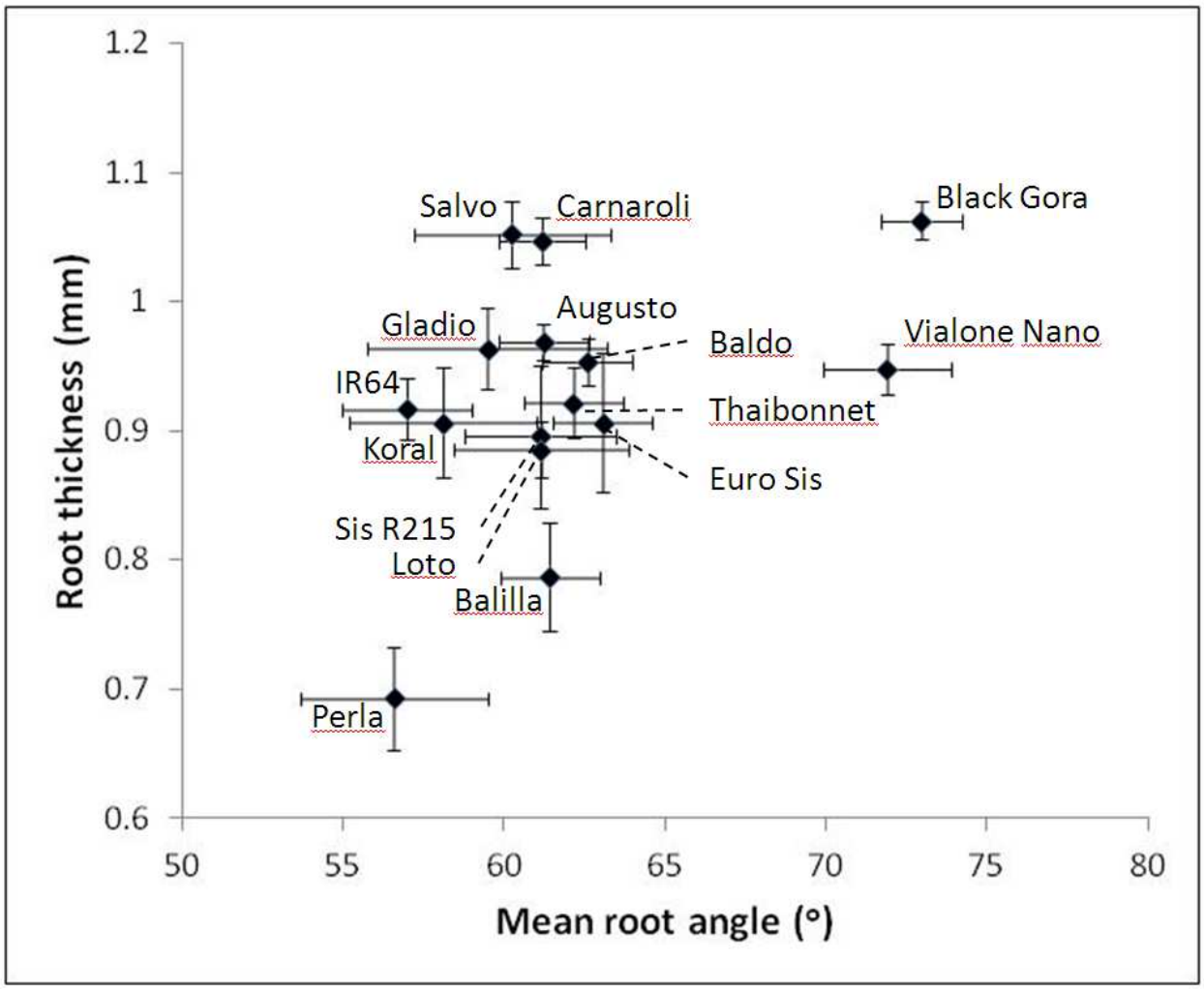

Figure 3 *Root thickness plotted against mean angle of main roots (90° is vertical) for 13 Italian varieties plus check varieties Black Gora (deep, vertical roots) and IR64 (shallow, more horizontal roots). Bar is standard error.*

9. Publications using this method:

Price AH, Steele KA, Gorham J, Bridges JM, Moore BJ, Evans JL, Richardson P and Jones RGW (2002). Upland rice grown in soil-filled chambers and exposed to contrasting water-deficit regimes: I. Root distribution, water use and plant water status. Field Crops Research 76 11-24

Price AH, Steele KA, Moore BJ and Jones RGW (2002) Upland rice grown in soil-filled chambers and exposed to contrasting water-deficit regimes: II. Mapping QTL for root morphology and distribution. Field Crops Research 76 25-43

10. Common mistakes:

- Not packing soil sufficiently well to prevent slumping when water is applied is the major problem encountered.

CHAPTER 4

Genome-Wide Association studies for root traits in a temperate rice collection.

(manuscript in preparation)

INTRODUCTION

Among all the abiotic stresses, drought is the major factor limiting plant production. It is estimated that drought affects approximately 23 million ha of rice growing areas worldwide (Serraj et al 2011), determining losses of up to 40% of total production.

Traditionally, drought has not been considered a problem for Italian paddy rice fields, as almost the entire Italian rice growing area (94%) is located in the North-West portion of the Po River Valley, characterized by large water availability and a very efficient water distribution net. Thus, rice in Italy developed in the last two centuries essentially as a water demanding crop, completing its growth cycle under submersion. However in the last decades also Northern Italy has experienced a reduction in water resources, mainly due to climate changes and to an increased demand of water from other sectors. Long dry periods in winter, as well as in spring time, have strongly affected the management of Italian rice paddy fields, with consequences on production and quality. Understanding the genetic determinants of drought resistance in Italian rice germplasm becomes therefore of crucial importance, in order to identify genotypes able to optimize water use efficiency while maintaining yield stability.

Drought resistance is a complex phenomenon, involving a number of morpho-physiological processes playing at different organismic levels and developmental stages (Levitt 1980; Tripathy *et al.* 2000). Among them, drought avoidance strategies are considered one of the main player influencing drought-resistant performance (Blum 2005). Drought avoidance is based on the maintenance of a relatively high tissue water potential despite soil-moisture shortage, through an enhanced water uptake or a reduced water loss. Among the drought avoidance strategies played out by rice plants, the development of a root system able to absorb water at depth is considered the most relevant trait contributing to drought resistance in upland conditions (Yoshida and Hasegawa, 1982). The relationship between deep rooting habit and drought resistance was explored by Venuprasad *et al.* (2002) in a study involving a simultaneous evaluation of root characters and grain yield under different moisture regimes. The results of this study, later confirmed by others (Kanbar *et al.* 2009; Steele at el. 2007; Toorchi *et al.* 2006), revealed an increase in grain yield in genotypes characterized by the ability to develop a deep root system before the beginning of drought stress.

In the past 20 years many bi-parental mapping populations have been developed in rice and used to evaluate a number of root morphological parameters, with the aim of identifying QTLs related to drought-avoidance root traits. Different types of populations were used, including F2 (Price and Tomos, 1997; Price *et al.*, 1997), backcross inbred lines (Kato *et al.*, 2008), doubled haploid lines (Yadav *et al.*, 1997; Zheng *et al.*, 2000; Hemamalini *et al.*, 2000; Venuprasad *et al.*, 2002; Toorchi *et al.*, 2002; Babu *et al.*, 2003; Kamoshita *et al.*, 2002a; Zhang *et al.*, 2001), and recombinant inbred lines (Champoux *et al.*, 1995; Ray *et al.*, 1996; Price *et al.*, 2000, 2002; Ali *et al.*, 2000; Kamoshita *et al.*, 2002b; Courtois *et al.*, 2003; Zheng *et al.*, 2003).

Even though a large number of major and minor root QTLs were identified in these studies, so far only two were shown to improve root architecture when introgressed into new varieties (Shen *et al.* 2001, Steele *et al.* 2006). This failure is mainly due to the low precision of QTL localization obtained in bi-parental QTL mapping studies, deriving from inadequate population sizes, and to the small phenotypic effect that a QTL can have when introgressed into a different genetic background (Gowda *et al.* 2011)**.**

Genome-wide association (GWA) mapping, which is based on linkage disequilibrium (LD) in natural populations to identify associations between markers and quantitative traits (Gaut and Long 2003), has recently emerged as a promising approach for revealing the genetic basis of phenotypic variation. GWA employs large unrelated germplasm collections, allowing to simultaneously explore a broader range of natural genetic variation with higher resolution and to consider many more informative meioses (namely all those that have occurred in the history of the population) if compared to traditional QTL bi-parental mapping. As a consequence, GWA mapping allows to locate simultaneously more QTLs and with better precision (Gowda *et al.* 2011).

GWA mapping was originally applied to human genetics projects, where it has emerged as a powerful approach for identifying genes underlying complex diseases (e.g. Altshuler *et al.* 2008, Hindorff *et al.* 2009). In recent years, it has also been successfully applied to model plants and crops. In Arabidopsis thaliana the potential of GWA was demonstrated by the successful functional validation of *ACD6* (Tedesco *et al.* 2010), a gene previously shown to be involved in vegetative growth and resistance to microbial infection (Lu *et al.* 2003). Very encouraging results were also obtained in GWA for flowering time (Buckler *et al.* 2009), leaf architecture (Tian *et al.* 2011) and resistance to leaf blight (Kump *et al.*

2011, Poland *et al.* 2010) in maize, using nested association mapping (NAM) populations (McMullen *et al.* 2009).

Rice, as a selfing species with a large extent of LD, has been shown to be a good candidate for GWAS. Huang *et al.* (2010) performed a GWA study for 14 agronomic traits, using a direct low-depth resequencing approach coupled with a novel data-imputation method of 317 indica landraces, and identified several loci explaining on average about 36% of the phenotypic variance. Zhao *et al.* (2011) and Famoso *et al.* (2011), by genotyping 413 and 383 worldwide distributed rice landraces using an Affimetrix 44K SNP custom array, identified several genes with large effect in determining yield, morphology, stress tolerance and nutritional quality traits.

Despite the encouraging results obtained in these first applications, GWAS in plants has been hindered by limited resources for developing high-density haplotype maps, namely cost-effective methods for rapidly scanning the largest number of markers across the largest set of genotypes ensuring throughput and quality.

Here we present a GWA study for root morphological traits in a collection of 96 Italian/European rice varieties, including traditional and modern local accessions and a set of foreign varieties from temperate areas adapted to Italian agro-climatic conditions. High-throughput whole-genome genotyping was performed using Genotyping-By-Sequencing (GBS), a simple highly specific and low cost technology developed by Elshire *et al.* (2011). GBS relies on next-generation sequencing of multiplex libraries based on reducing genome complexity by methylation-sensitive restriction enzymes and has been successfully applied to maize (Elshire *et al.* 2011) and barley (Poland *et al.* 2012). In parallel, a thorough phenotypic screening for root morphological features was performed using an improved "basket method" (Uga *et al.* (Oyanagi *et al.* 1993; Kato *et al.* 2006; Uga *et al.* 2009), enabling to address deep root development, coupled with WinRHIZO analyses, an image analysis software specifically designed for root characterization. GWA performed on a first set of phenotypic traits allowed the identification of SNP markers located within QTLs previously shown to be associated with drought avoidance root traits.

METHODS

Plant materials

The rice diversity panel used in this study included 96 *Oryza sativa* varieties all belonging to the *japonica* subspecies. The 96 accessions were selected from the rice germplasm collection of the CRA-Rice Research Unit (Vercelli, Italy) based on diversity analysis using 24 unlinked SSRs (Faivre-Rampant *et al.* 2010), with the aim to include the broadest range of genotypic and phenotypic diversity for temperate rice. The sampled collection consisted of local accessions, representing the genetic diversity of the rice germplasm cultivated in Italy, and a set of foreign varieties from temperate areas adapted to our agro-climatic conditions. The foreign accessions were selected based on their behaviour in water-limited conditions assessed in previous experiments (data not shown), as a source of favourable alleles for drought resistance. The complete list of the accessions used in this study is reported in **Table 1**. All the selected accessions were purified by single seed descent before genotyping and phenotypic evaluations.

DNA isolation and genotyping

Total genomic DNA was isolated from 3-week old leaves using the DNeasy Plant Mini Kit (QIAGEN) with a TECAN Freedom EVO150 liquid handling robot (TECAN Group Ltd, Switzerland). For each of the 96 accessions, a single individual plant was used. Whole genome genotyping was then carried out using the GBS (Genotyping-By-Sequencing) technology, following the protocol described by Elshire *et al.* (2011). Digestions were performed on 100 ng of genomic DNA in a 96-well plate using *Ape*KI, which was shown to cut every 1 kb on average in an *in silico* digestion of the Nipponbare genome (data not shown). The digested DNAs were then ligated to 12 µl of 0.6 ng/µl adapter pairs (optimised to guarantee good quality libraries in rice) and the 96-plex library constructed according to the above-mentioned protocol. The library was then loaded on a single flow cell channel of a Genome Analyzer II (ILLUMINA, Inc., San Diego, CA) for the sequencing. Raw sequence data filtering, sequence alignment to the rice reference genome (Nipponbare, MSU release 6.1) and SNP calling were carried out using the GBS pipeline provided by the Buckler's lab (http://www.maizegenetics.net). The pipeline was run up to the command MergeDuplicateSNPsPlugin following the default settings, except for the command MergeMultipleTagCountPlugin where 5 was used for the –c option

setting (minimum number of times a tag must be present to be output). To impute the large number of missing data resulting from the low-coverage GBS genotyping, a Bayesian algorithm was developed at Cornell University, that utilizes the extensive linkage disequilibrium (LD) structure in the rice genome to estimate underlying genotype ancestry (unpublished data).

SNPs with a call rate < 90% and a minor allele frequency < 0.05% across all samples were removed from the dataset obtained after imputation using TASSEL v3 (Bradbury *et al.* 2007). After this step, 52.655 SNPs met our criteria (call rate > 90% and MAF > 0.05%) and were used for all subsequent analyses.

Phylogenetic and population genetic analyses

To calculate genetic distances, a dissimilarity matrix was computed using a shared allele index with DARwin software (Perrier and Jacquemoud-Collet 2006; http://darwin.cirad.fr/) and an unweighted neighbor-joining (NJ) tree was constructed based on this dissimilarity matrix.

To infer the population structure, principal component analysis was performed using the software EIGENSOFT (Price *et al.* 2006).

Estimation of LD decay

The pairwise SNP linkage disequilibrium (LD) among the 52.655 SNPs was measured as the correlation among pairs of alleles across a pair of markers (r^2). For all pair of SNPs, r^2 was calculated using the **--r2 --ld-window 99999 --ld-window-r2 0** command in PLINK (Purcell *et al.* 2007). The LD decay plot was generated averaging the pairwise r^2 across the whole genome every 10 kb.

Genome-Wide Association Analyses

GWA analyses were performed with TASSEL under three statistical models: 1) a General Linear Model (GLM), based on a simple phenotype/genotype linear regression (considering as a fixed effect only the allelic status at the target loci); 2) a GLM+P model,

using as cofactor the top five principal components (P matrix) to correct for population structure; 3) a compressed Mixed Linear Model (MLM), where the random effects were estimated through the relative Kinship matrix (K) calculated with TASSEL.

For all the association analyses, the average phenotypic values across replicates were used. QTLs and candidate genes underlying significant peaks/hits were extracted from the Gramene database (www.gramene.org).

Root Phenotyping

For the phenotypic screening of root morphological features, rice plants were grown under controlled conditions in plastic cylindrical mesh baskets (ANELLI) placed at the top of PVC pipes. The 'basket method' was chosen as it allows to determine the root growth angle, defined by the position where the root penetrates the mesh of the basket (Oyanagi *et al.* 1993, Kato *et al.* 2006, Uga *et al.* 2009). The PVC pipe was 60 cm high with a diameter of 14 cm; the basket was 5,4 cm high with a diameter of 7,2 cm and a mesh size of 2 mm. This size was large enough to allow root emergence from the basket without interference. Each PVC pipe was filled with field soil (silty clay loam, sieved to 5 mm), thoroughly mixed with 33% of sand and inorganic fertilizer. The bottom of the pipe was covered with a non-woven fabric to allow free draining.

The PVC pipes were placed in a greenhouse according to a randomized block design with three replications, for a total of 288 pipes. About 10 seeds for each of the 96 accessions were pre-germinated in petri-dishes for 48 h at 30°C. Then, two seedlings for each replication were sown exactly in the centre of each basket at a depth of 1 cm. One week after emergence, the seedlings were thinned to the healthiest one. Plants were grown for six weeks at 28°C day/24°C night at 75% relative humidity, under daylight conditions (16h light / 8h dark). Using a drip irrigation system, the pipes were supplied with 250 ml of water every day for the first 3 weeks and with 500 ml for the last three weeks. The amount of water, defined in a previous test carried out on a small subset of varieties (data not shown), allowed to maintain aerobic conditions without stressing the plants. From the third week, corresponding to the beginning of the tillering stage, 200 ml of inorganic fertilizer were supplied to each pipe once a week. Plant height (length from the soil to the tip of the longest leaf) was recorded on a weekly basis, as an indicator of shoot growth and plant health.

At the end of the experiment (day 42), all the shoots were cut at soil level, dried for three days at 80°C and weighted. Baskets were then gently extracted from the pipes and washed thoroughly to completely remove the soil from the roots. Roots were then cut and separated according to their growth angle, defined by the position from where they emerged from the basket mesh. Four layers were considered according to horizontal ground level angles: 0°-27°, 27°-45°, 45°-57°, 57°-90°. Roots were then placed on different glass trays according to the layer, taking care of spreading the smallest secondary roots, and scanned. The images were then analysed using WinRHIZO (Regent Instruments Inc.), a software specifically designed for root characterization. A list of the traits collected for each plant is reported in **Table 2**.

Data on grain length and width for each accession were collected from a parallel field experiment conducted at CRA-RIS (Vercelli, Italy), under standard conditions. Values were obtained using the software WinSEEDLE (Regent Instruments Inc.) averaging measurements on 100 seeds/accession (randomly harvested in the plot) for three replicates.

RESULTS

Selection of a representative temperate rice diversity panel

The GWA study for root morphological traits was carried out on a rice diversity panel including 96 genotypes. This pilot subset was selected with the aim to explore the broadest range of genotypic/phenotypic diversity of rice cultivated in Italy. Moreover, a set of drought-tolerant foreign varieties well adapted to our agro-climatic conditions was included to enrich allelic variation associated with drought avoidance.

For this purpose, a neighbour-joining (NJ) analysis using 24 unlinked SSRs (Faivre-Rampant *et al.* 2010) was performed on a subset of the European Rice Germplasm Collection (Courtois *et al.* 2012) consisting of 192 rice accessions, mainly belonging to the japonica subgroup. A set of 50 accessions belonging to known enzymatic groups and representative of the diversity of *Oryza sativa* worldwide (mini-Germplasm Bank; Glaszmann *et al.*, 1995) were used as references to characterize the subpopulation structure.

The resulting NJ tree (**Figure 1**) showed the typical bipolar structure of *O.sativa*, with indica and aus/boro accessions clearly separated from sadri/basmati and japonica accessions (Courtois *et al.* 2012). Within this last group the two clusters, corresponding to tropical and temperate japonica varieties, were distinguishable and comprised most of all the European accessions used. Based on this analysis, 96 varieties were then selected among the temperate-tropical japonica group, aiming at maximizing the genetic distances existing in this cluster.

Whole genome genotyping of the diversity panel

Whole-genome genotyping of our diversity panel was performed using the GBS approach, as described by Elshire *et al.* (2011). All the generated reads, after being filtered to eliminate sequencing errors, were then aligned to the rice reference genome for SNP identification. Consensus genome sequences for each rice accession were then built aligning all the reads of each accession and SNP were called based on discrepancies between the consensus sequence and the reference genome. On average, the resulting consensus sequence of each accession covered 4,2% of the Nipponbare reference genome, for a total of ~9630 called SNPs. Missing data were then imputed using a very efficient algorithm that takes advantage of the extensive LD structure in rice, developed within the group of Dr. McCouch at Cornell University (unpublished data). As a result, a total of 52.655 non-redundant SNPs were identified across all samples (call rate >90%, MAF>0,05%), which greatly compensate for the low-coverage resulting from the short GBS reads. The average distance between SNPs was 8,7 Kb, with 77,9% of the SNPs located within 10 kb from the nearest SNP.

The distribution of the SNPs along the rice chromosomes was consistent with the predicted gene density (**Figure 2**), as expected in case of GBS genotyping. Indeed, the employment of the methylation-sensitive restriction enzyme *Ape*KI to construct the GBS libraries, should allow to enrich for genic regions, avoiding highly methylated non-coding repetitive regions. Of the SNPs identified, almost 40% (16.856) was found in annotated genes with transcript support. Of these, ~61% (10293) were located in exons, ~23% (3.841) in introns and the remaining (2.872) in the untranslated regions.

All the SNPs identified were then used to analyse the average extension of LD across the diversity panel. The LD decay rate, measured as the average chromosomal distance at

which the pairwise correlation coefficient (r^2) dropped to half its maximum value (Huang *et al.* 2010), was estimated at 400 kb (**Figure 3**). This values is in agreement with what previously reported for temperate/tropical japonica subgroups (Mather *et al.* 2007).

Evaluation of drought avoidance root traits

In order to deeply characterize the root system architecture, the 96 accessions were grown in PVC pipes under controlled conditions using the 'basket method' (Oyanagi *et al.* 1993, Kato *et al.* 2006, Uga *et al.* 2009). This system was chosen since it allows to dissect root system architecture through root separation according to their growth angle. In this way, superficial roots can therefore be easily separated from the more vertical roots (having a wider angle with respect to horizontal ground level), able to explore the deepest soil layers.

Three replicates of each variety were then grown in cylindrical baskets placed at the top of deep PVC pipes, whose height (60 cm) allowed the development of the plant root system without interference. According to Price *et al.* (2012), roots were harvested at 6 weeks after sowing, which represented a good compromise to ensure enough variability (root growth is related to tillering, starting usually at 4 weeks after sowing) without an excessive development of the root apparatus that would be critical for the successive analyses. After being separated based on their growth angle (see Methods), roots were accurately placed on glass trays and scanned. The images were then analyzed using WinRHIZO, an image analysis software specifically designed for root characterization, which allows to gain information also on fine root structure.

A large number of traits were measured in this study. Plant height (PH), monitored on a weekly basis, and shoot dry weight (SDW) were measured as indicators of shoot growth and plant health. Concerning the root apparatus, several traits were assessed for roots belonging to each of the four layers identified. The complete list of the traits collected is reported in **Table 2**.

Root traits for GWA studies

Preliminary GWA analyses aiming at the identification of drought-avoidance related loci were performed on a subset of the measured root characters, selected as descriptors of root thickness, deep mass and penetration ability, which most express the capacity of the plant to cope with water deficits.

Indeed, roots thickness was shown to affect the amount of water that a plant is able to absorb, as thick roots have larger xylem vessels and lower axial resistance to water flux (Yambao *et al.*, 1992). The thickness of deep roots was also shown to be related to root penetration ability through hard layers, which facilitates water uptake from the deepest soil layers who are the last to lose water in case of scarcity (Clark *et al.*, 2008). Moreover, root mass at depth is considered the most relevant trait contributing to drought resistance in upland conditions (Yoshida and Hasegawa, 1982),

As indicators of root thickness, the total length (RL_TK) and volume (RV_TK) of the roots with an average diameter >0,6 mm were considered. To evaluate root mass at depth, we selected the total root length (RL), root volume (RV), root surface area (RSA) and number of tips (RT) of the roots attributed to the 3rd and 4th layer (growth angle >45°). Lastly, as an index for the root penetration ability, the volume of vertical thick roots (average diameter >0.6mm; growth angle >57°) was considered (RV_TK). For each of the selected traits, statistical analyses were performed in order to assess the frequency distributions across all samples and their variability (**Figure 4**).

Genome-wide association analyses

To assess the effectiveness of GWA on our SNP dataset and diversity panel, we firstly conducted an association analysis for grain width, a phenotypic trait that is controlled by a known gene (*qsw5*; Shomura *et al.* 2008). Phenotypic data from this trait were obtained from a parallel field experiment, in which the 96 accessions used in this study were evaluated for agronomic performances (data not shown). Three statistical models were used to perform GWA analyses: GLM, GLM-P, MLM (see Methods). As shown in **Figure 5**, strong association signals located close to the *qsw5* gene were detected, using both GLM_P and MLM_K. These results confirmed that our germplasm set, in spite of its

small size, could be suitable to perform GWA studies for the identification of genetic loci associated with traits of interest.

GWA was then carried out on the selected drought avoidance root traits. For each trait, we decided to perform the analysis using all the tree statistical models previously mentioned and to select the most appropriate based on the Quantile-Quantile (Q-Q) plots generated. As shown in **Figure 6**, the simple GLM model (which does not take into account population structure) performed worse than the other models for all the traits considered. When this model was used, the distribution of observed $-\log_{10}$(P-values) in the Q-Q plot analysis was strongly deviated from the expected distribution in case of no association (P-values lying on the diagonal line), which lead to a high level of false positive signals. On the contrary GLM_P and MLM, allowing to correct for population structure and relatedness respectively, should eliminate false positive signals increasing the power in detecting true associations. Accordingly, Q-Q plot results when these models were employed displayed a more consistent trend with the expected null distribution, except for the lowest P-values that should represent the true associations (**Figure 6**). Based on these considerations, the GLM-P model was chosen for GWA analysis in case of root thickness-related traits, both the GLM-P and MLM models in case of deep root mass-related traits and the MLM model for penetration ability-related traits.

The list of the significant associations detected in this preliminary study is reported in **Table 3**. GWA for root thickness-related traits identified three significantly associated regions on chromosome 3, 4 and 12. In case of deep root mass-related traits, three highly significant SNP clusters on chromosome 2, 6 and 10 were revealed. The association signal on chromosome 2 was detected using both the GLM-P and MLM models. Finally, five chromosomal regions resulted to be significantly associated with root penetration ability, on chromosome 2, 8, 9 and 10. The two regions on chromosome 10 partially overlap with that identified for deep root mass. All the detected associations co-localized with regions previously reported to be involved in drought avoidance and/or root development traits based on bi-parental mapping studies (summarized in Courtois *et al.* 2009; Khowaja *et al.* 2009).

A preliminary exploration of a region spanning 100 kb surrounding the identified peaks revealed a number of candidate genes shown to be related to root growth and morphology (Norton *et al.* 2008; Din and Xiong 2011; Wang *et al.* 2011). Among them a

cellulose synthase, two expansins, a cell division control protein, a cell wall structure protein, two auxin-responsive factors (data not shown).

DISCUSSION

Roots play a crucial role in determining resistance to drought stress in rice. Many studies have been conducted in the last decades using bi-parental mapping populations and many root QTLs have been identified (reviewed in Courtois *et al.* 2009; Khowaja *et al.* 2009). However so far, none of them has been narrowed down to the responsible gene, as the regions detected were too large and spanned too many potential candidate genes. One way to detect QTLs with better precision is to perform genome-wide association (GWA) mapping on natural germplasm collections (Gowda *et al.* 2011).

Here, we report the first GWA study for root traits in rice. A collection of 96 temperate rice varieties of commercial relevance for Italy was thoroughly characterized for root system architecture and genotyped using a novel NGS-based technology to run GWA analyses for the identification of regions involved in root drought avoidance traits.

The GWA studies conducted in rice to date were performed on large highly-structured diversity panels (Huang *et al.* 2010; Famoso *et al.* 2011; Zhao *et al.* 2011). However, for the detection of sub-populations specific alleles, the analysis had to be restricted to the specific sub-population (Famoso *et al.* 2011; Zhao et al 2011). Since our final goal was to identify drought-resistance loci useful for marker-assisted breeding of local genetic material, we directly restricted our analysis on accessions that were cultivated in our climatic area. All the selected varieties clustered within the temperate/tropical japonica subgroup, exhibiting therefore a very narrow genetic basis.

Nevertheless, a large number of polymorphic loci (52.655 SNPs) were obtained from the GBS genotyping. This promising NGS technology was previously applied to maize and barley (Elshire *et al.* 2011), but not yet in rice. Here we demonstrated that using this rather inexpensive multiplex sequencing strategy, we could detect polymorphisms at a higher rate than using the more expensive high-density 44K SNP genotyping array (Zhao

et al. 2011). In that case, approximately 13.000 and 8.000 SNP sites (partially overlapping) were polymorphic in tropical and temperate japonica subgroups, respectively. Moreover the GBS approach, based on sequencing libraries enriched for low-methylated transcribed regions, allowed us to get a rate of SNPs in genic regions approximately 8-fold higher than that obtained by Huang *et al.* (2010) using a standard low-coverage sequencing genotyping approach.

To dissect root system architecture with the aim of identifying its drought-avoidance components, a novel root phenotyping method was developed combining an optimized plant growing system (plastic baskets in combination with PVC pipes) with an efficient imaging analysis. Cylindrical baskets allowed us to explore the root system architecture in terms of deep rooting (Kato *et al.* 2006; Uga *et al.* 2009), expected to mostly contribute to drought avoidance. Moreover using the PVC cylinder system, which is considered an improvement over pot culture for root studies, root depth was less restricted and soil moisture more representative of field conditions (Upchurch and Taylor, 1990). Using these tools in combination with a powerful image analysis software specifically designed for root characterization, enabled us to conduct a fine analysis of root structure detecting traits otherwise impossible to assess (e.g. number of root tips and forks, surface area, etc.).

The phenotyping was conducted in aerobic conditions as a sole treatment. This choice was supported by previous findings showing that QTLs detected under drought-stress conditions were generally co-localizing with QTLs detected under favorable conditions, clearly indicating that the same QTL works differently depending on the growth conditions (MacMillan *et al.* 2006). This was later confirmed by a drought QTL meta-analysis in which 24 published experiments were considered (Courtois *et al.* 2009). Therefore, the bigger effort required to run an experiment under contrasting water regimes (due to the difficulty to control the exact degree of stress applied to each individual) was not justified and might have been affecting the standardization of the conditions.

GWA analyses were first performed on a subset of root traits assigned to three main classes: root thickness, deep root mass and root penetration ability. The rationale for focusing on these classes emerged from long-term multi-location drought studies, proving that genotypes that produce higher grain yield under drought are able to maintain better plant water status by an increased water uptake, due to the development

of a deep root system (Fukai et al, 2009; Price *et al.* 1999). Furthermore, thick roots were shown to have a direct role in drought resistance, conferring a better penetration ability and an increased branching and water absorption through larger xylem vessels, with less risk of cavitation (Clark *et al.* 2008; Ingram *et al.* 1994; Yambao *et al.* 1992).

The results of the association analysis revealed 11 SNP clusters significantly associated with the selected traits. The signals were consistent among single traits within a given class, strengthening the hypothesis of true association. All the SNP clusters were co-localizing with QTLs related to root mass at depth, root thickness, root penetration ability and root length previously identified by bi-parental mapping, in most cases by more than one study (summarized in Courtois *et al.* 2009; Khowaja *et al.* 2009). Four of the SNP clusters co-localized with drought avoidance QTLs, confirming that thickness and deep rooting are key strategies to cope with water scarcity. The identified QTLs explained up to 28% of the phenotypic variation observed, which is consistent with the fact that root morphology is regulated in most cases by a large number of small effect loci interacting with the environment (de Dorlodot *et al.*, 2007). Further analysis is undergoing to narrow down the significant associated regions to candidate genes.

In conclusion, this preliminary GWA study on drought-avoidance root traits clearly indicated that a substantial phenotypic and genotypic diversity exists in the Italian/European rice germplasm, despite the predicted narrow genetic basis. The GBS technology, coupled to an improved system for fine root phenotyping, was shown to effectively capture the existing variation for a successful identification through GWA studies of the genetic determinants underlying drought-avoidance in rice. Starting from a homogeneous genetic material, as in this study, will reduce the risk of losing the effectiveness of a favorable allele when introgressed into a novel background of interest for local breeding programs.

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Table 1. List of the 96 rice accessions used.

A201	DELLROSE	LAMONE	ROBBIO SEL1
AKITAKOMACHI	DRAGO	LIDO	RODEO
ALICE	DREW	LORD	SAKHA 102
ALLORIO	ERCOLE	LOTO	SALVO
ALPE	EUROPA	LUCERO	SANT'ANDREA
APOLLO	EUROSIS	LUXOR	SANTERNO
ARBORIO	FORTUNA	M204	SCUDO
ARGO	GANGE	MARATELLI	SELENIO
ARIETE	GIADA	MARTE	SILLA
ARTEMIDE	GIANO	MELAS	SIS R215
AUGUSTO	GIGANTE VERCELLI	MONTICELLI	SMERALDO
BALDO	GLADIO	NEMBO	SUPER
BALILLA	GRALDO	ORIONE	TEJO
BARAGGIA	GREPPI	OTA	THAIBONNET
BEIRAO	GZ8367	PADANO	TITANIO
BERTONE	HANDAO 11	PANDA	ULISSE
BIANCA	HANDAO 297	PECOS	UPLA 32
CALMOCHI 101	ITALMOCHI	PERLA	UPLA 75
CARNAROLI	ITALPATNA 48	PIEMONTE	UPLA 77
CASTELMOCHI	JEFFERSON	PLOVDIV 22	VENERE
CENTAURO	KORAL	PROMETEO	VIALONE 190
CINIA 40	L201	RANGHINO	VIALONE NANO
CRIPTO	LADY WRIGHT	REDI	VOLANO
DELFINO	LAGRUE	RINALDO BERSANI	ZENA

Table 2. List of the phenotypic traits evaluated.

	Abbreviation	TRAIT
SHOOT	PH	plant height
	SDW	total shoot dry weight
ROOT[1]	RN	number of roots
	RDW	root dry weight
	AD	average diameter
	FN	number of forks
	RL	total root length
	RSA	root surface area
	RV	root volume
	RT	number of tips
	RL_TH	total length of thin roots (diameter < 0.6 mm)
	SA_TH	surface area of thin roots (diameter < 0.6 mm)
	RV_TH	volume of thin roots (diameter < 0.6 mm)
	RT_TH	number of tips of thin roots (diameter < 0.6 mm)
	RL_TK	total length of thick roots (diameter > 0.6 mm)
	SA_TK	surface area of thick roots (diameter > 0.6 mm)
	RV_TK	volume of thick roots (diameter > 0.6 mm)
	RT_TK	number of tips of thick roots (diameter > 0.6 mm)

[1] All the listed root traits were measured for each of the four layers (angle sectors) identified. From these values, total number of roots (TRN), total root dry weight (TRDW) and root to shoot ratio (TRDW/SDW) were calculated.

Table 3. Genome-wide significant associations signals of root traits.

Class/Traits	Significant SNP[1]	Model	p-value	r^2	Chr	QTL trait	Mapping population
Root thickness (RL_TK; RV_TK)	S3_10027580	GLM_P	9.99E-06	0.14	3	RN RDW Root penetration index	IRRI Mor/CO39 QTL 1994 CNHZAU Zh97/Ming63 RI QTL 2002 CTIR CT9993/IR6226 QTL 2000
	S4_31165851	GLM_P	2.92E-07	0.21	4	RL RL Root penetration index, RN, drought avoidance Root thickness, deep RDW	Cornell IR64/Azu DH QTL 2001 IGCN ZYQ18/JX17 DH QTL 1998 IRRI Mor/CO39 QTL 1994 CTIR CT9993/IR6226 QTL 2000
	S12_16588898	GLM_P	5.84E-07	0.18	12	Penetrated RN RDW	IRRI Mor/CO39 QTL 1994 CNHZAU Zh97/Ming63 RI QTL 2002
Deep root mass (RL_3+4; RV_3+4; RSA_3+4; RT_3+4)	S2_29253236	GLM_P MLM_K	3.22E-07 3.81E-05	0.22 0.20	2	Penetrated RN, RL, root thickness RL Root pulling force, root thickness Drought avoidance	Aberdeen Bala/Azu QTL 2002 TTU IR64/Orufi RI QTL 2003 CTIR CT9993/IR6226 QTL 2000 IRRI Mor/CO39 QTL 1994
	S6_24405130	GLM_P	4.04E-06	0.21	6	Penetrated RN, penetration index, RN RN Penetrated root thickness, RL	IRRI Mor/CO39 QTL 1994 CNZU IR1552/Azu RI QTL 2003 CTIR CT9993/IR6226 QTL 2000
	S10_15750216	MLM_K	6.31E-05	0.19	10	Root activity Penetrated root thickness, root penetration index RL Drought avoidance	CNRRI Zh97B/Mil46 RI QTL 2002 TTU IR58821/IR52561 QTL 2002 CNHZAU Zh97/Ming63 RI QTL 2002 IRRI Mor/CO39 QTL 1994
Penetration ability (RV_TK_4)	S2_23218049	MLM_K	5.96E-06	0.25	2	Root thickness	Cornell IR64/Azu DH QTL 2001 IRRI IR64/Azu DH QTL 2003 Aberdeen Bala/Azu QTL 2002
	S8_17535809	MLM_K	1.10E-05	0.28	8	Root thickness RN RN	TTU IR58821/IR52561 QTL 2002 Zhejian IR1552/Azu RI QTL 2001 IRRI Mor/CO39 QTL 1994
	S9_7742208	MLM_K	2.97E-05	0.26	9	RL RN Seminal root length	IGCN ZYQ18/JX17 DH QTL 1998 IRRI Mor/CO39 QTL 1994 Zhejian IR1552/Azu RI QTL 2001
	S10_12999615	MLM_K	2.15E-06	0.28	10	Penetrated to total root ratio Root activity Penetrated root thickness, root penetration index Drought avoidance	Aberdeen Bala/Azu QTL 2002 CNRRI Zh97B/Mil46 RI QTL 2002 TTU IR58821/IR52561 QTL 2002 IRRI Mor/CO39 QTL 1994
	S10_16315099	MLM_K	2.83E-05	0.21	10	RL	CNHZAU Zh97/Ming63 RI QTL 2002

[1] For each region, the SNP with the highest P-value is reported.

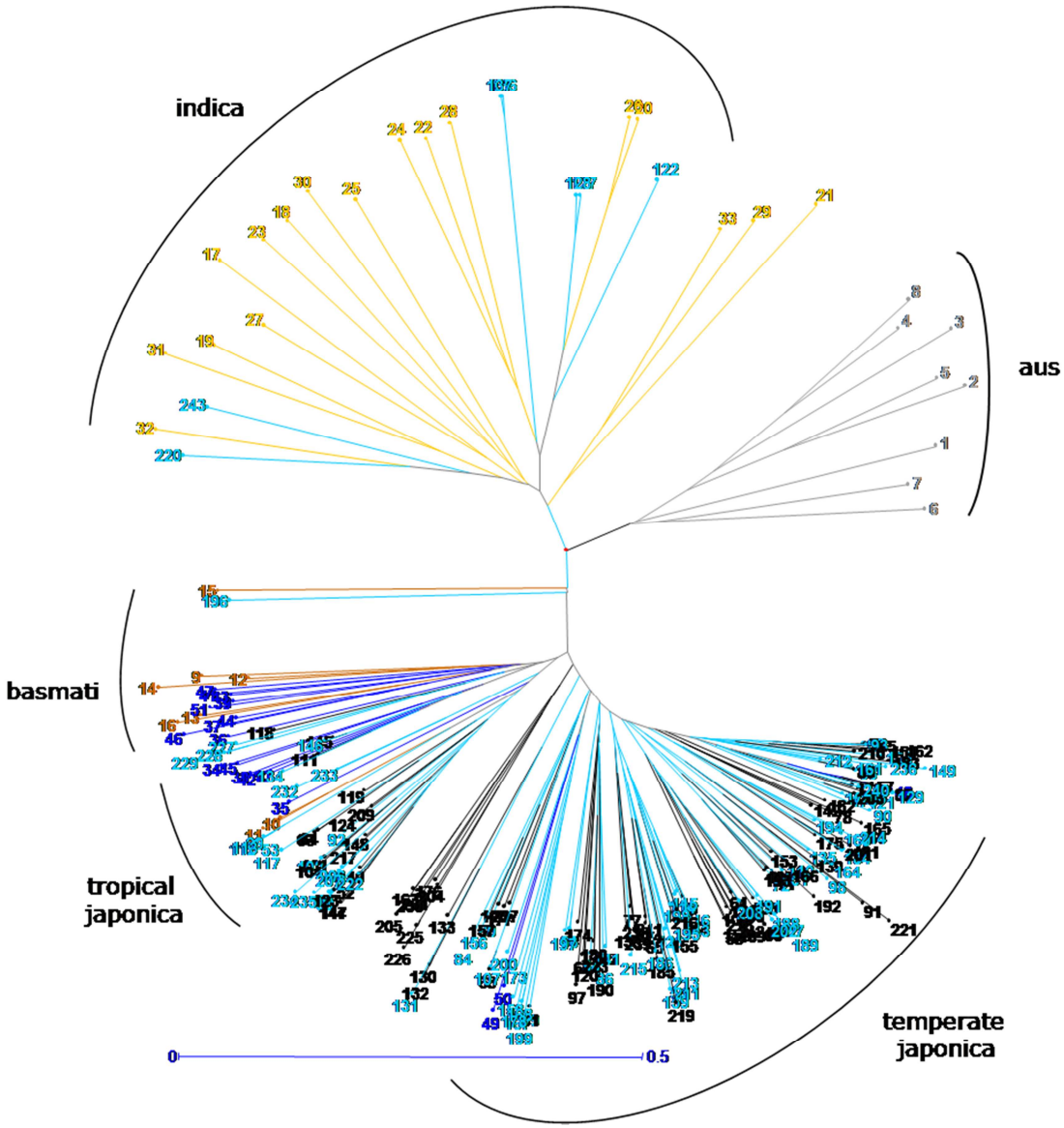

Figure 1. Neighbour-Joining tree representing the phylogenetic relationship among the 96 accessions selected for this study. *The 50 varieties of the mini-core collection are indicated as follows: indica accessions are designated in yellow; aus–boro in grey; sadri–basmati in orange and japonica in blue. The selected 96 varieties are indicated in black, while the unselected varieties are in light blue.*

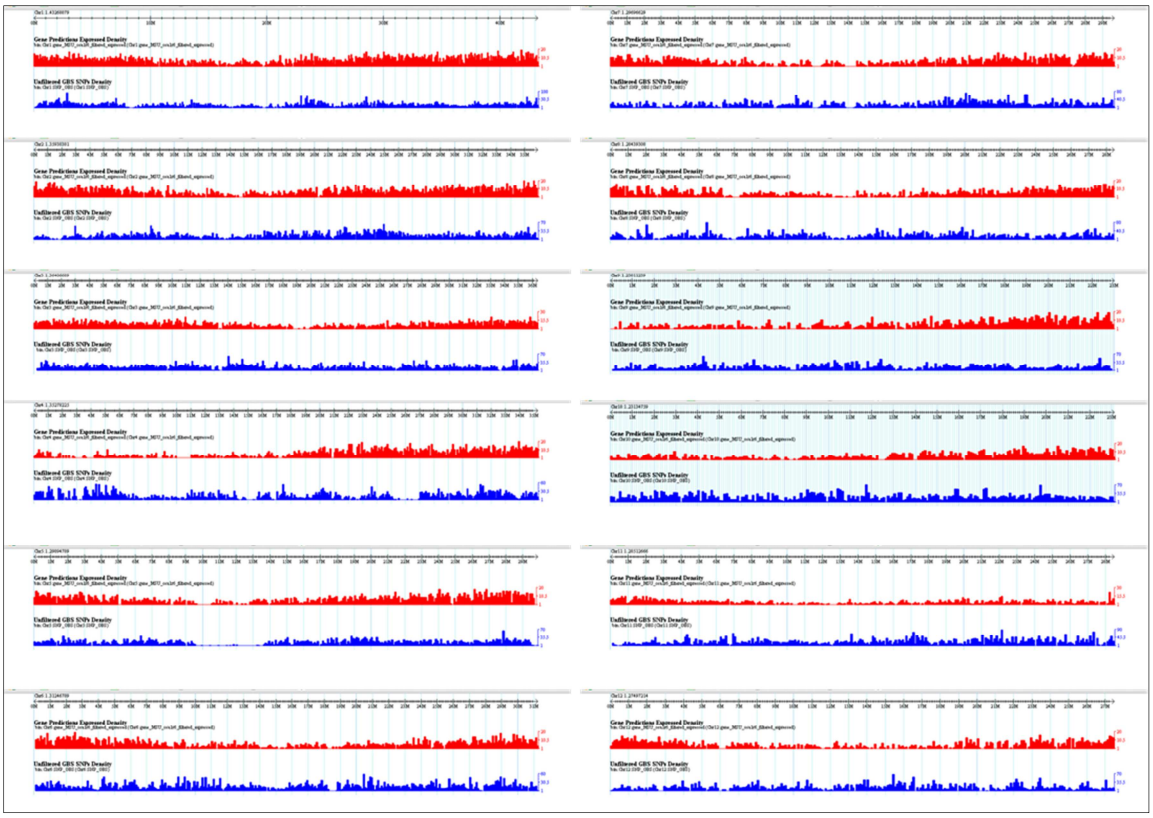

Figure 2. Distribution of the 52.655 SNPs along the rice chromosomes. *For each chromosome, the distribution of genes with transcript support (MSU v6.1 annotation) and the SNP density are indicated in red and blue, respectively. The 12 chromosomes are indicated from top-left to bottom-right.*

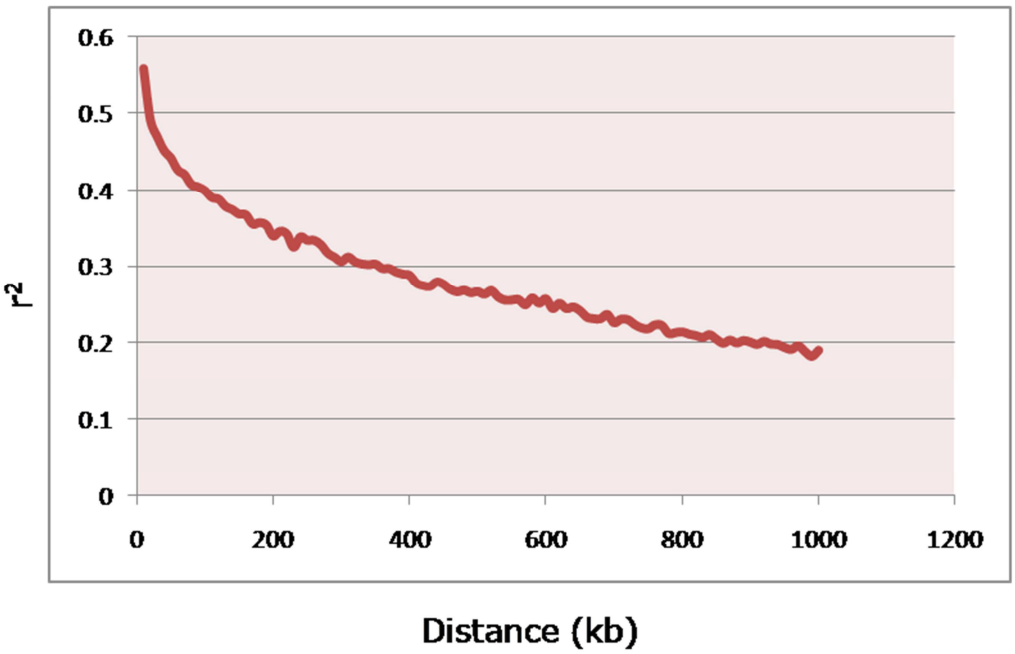

Figure 3. Average LD decay estimated for the 96 rice accessions.

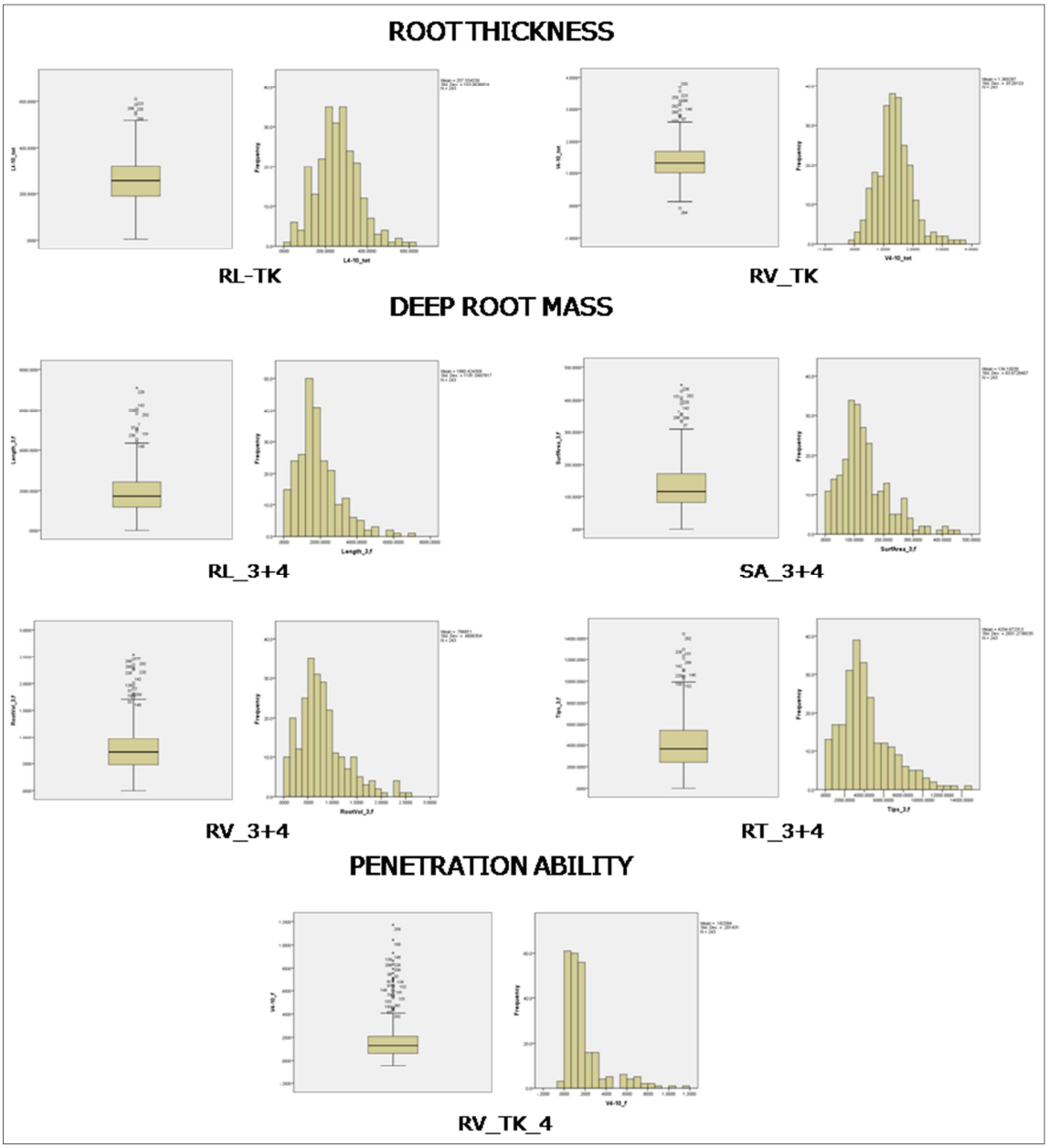

Figure 4. Distribution analysis of root traits across the diversity panel. *Boxplots and histograms for the root traits used for GWA analyses across the 96 rice accessions.*

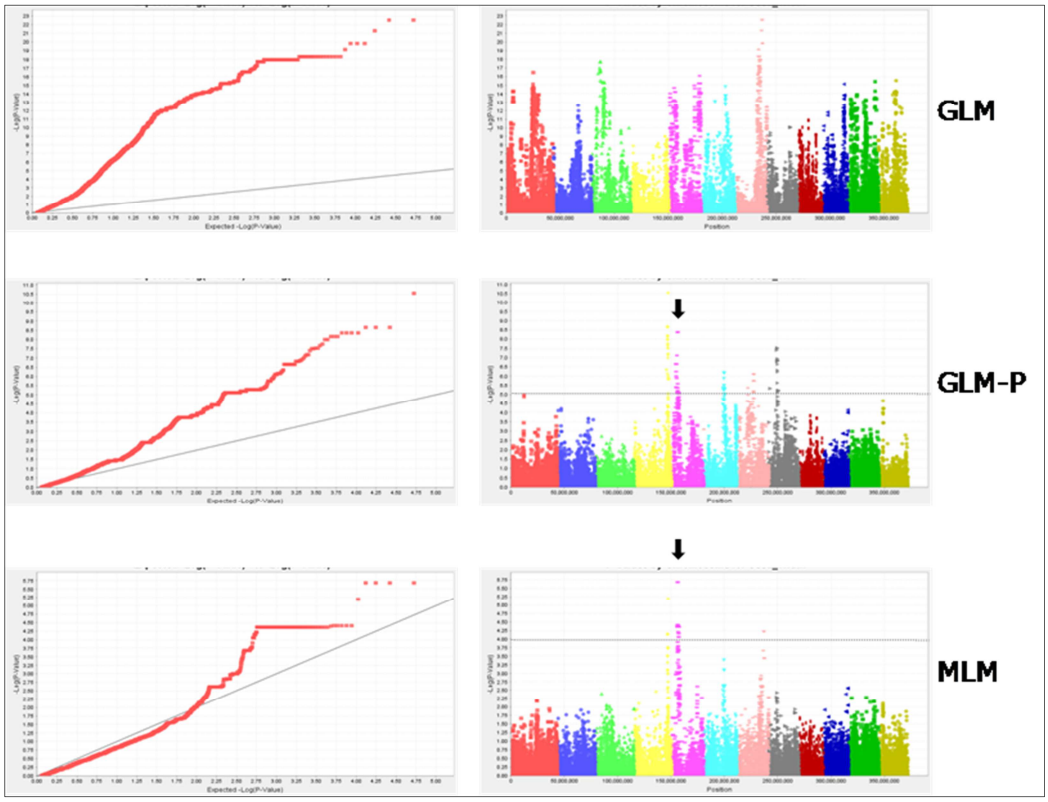

Figure 5. GWA analysis for grain width. *For each of the three statistical model employed, the Quantile-Quantile plot (left) and the Manhattan plot (right) is shown. The black arrows indicate the SNP significantly associated with qsw5. A threshold of p<1.0E-04 for MLM and of p<1.0E-05 for GLM-P was applied.*

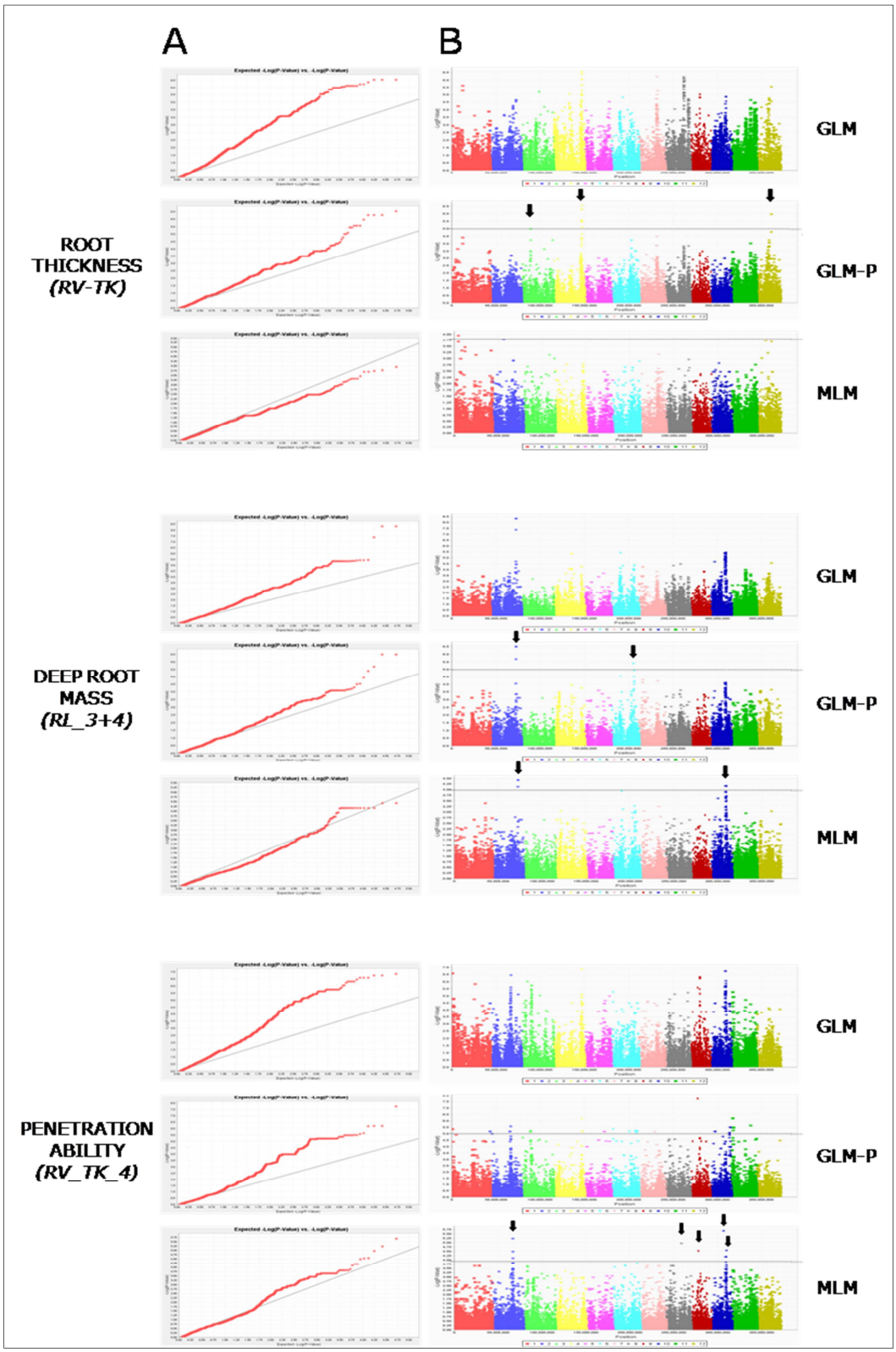

Figure 6. Genome-wide association study of root traits. *GWA analyses using the three statistical models indicated on the right are shown for one representative trait for each class. A) Quantile-quantile plots showing the estimated and observed distribution of -log$_{10}$(P-value) for each analysis. B) Manhattan plots showing the P-values distribution (plotted as -log$_{10}$) along each chromosome. The black arrows mark the significant association signals identified with the GLM-P and the MLM model. A threshold of p<1.0E-04 for MLM and of p<1.0E-05 for GLM-P was applied.*

CHAPTER 5

Summary and concluding remarks.

Due to the nature of the area devoted to rice growth, characterized by large availability of water and an efficient water distribution net, rice in Italy developed in the last two centuries as a water demanding crop, completing its growth cycle under submersion. However in the last decades also these regions experienced a reduction in water availability, with consequences on production and quality. The development of new varieties capable to cope with water scarcity is therefore becoming of utmost importance for a sustainable rice cultivation in Italy.

This work provides a initial study paving the way towards improvement of Italian rice varieties in terms of drought resistance. Within this study, two different approaches were undertaken with the aim to provide starting genetic materials for marker-assisted breeding at national level.

The first part of this study aimed at exploring a source of new genetic variation created in one of the most important Italian rice variety, namely Volano. Approximately 20.000 seeds of the Volano variety were previously mutagenized with EMS to create the first Italian rice TILLING population. Within this study, the Volano TILLING platform was validated through the screening of three agronomically relevant target genes. A mutation density of ~1/374 kb was estimated, proving the effectiveness of this approach for targeted rice crop improvement of Italian germplasm. A total of 11 mutant lines, of which three with a clear phenotypic effect, were identified in all the three genes selected for the validation. The collection, currently consisting of 1860 mutant lines that are being enlarged with new mutagenized lines, represents an interesting source of variation exploitable in terms of response to drought stress and directly of use for targeted breeding programs. The mutant lines identified, affecting genes shown to be involved in plant drought escape and avoidance strategies, not only are relevant for Volano breeding programs, but represent a powerful genetic material in view of breeding for drought improvement in Italian rice.

The second part of the work aimed at understanding the genetic determinants of root system architecture in the Italian rice germplasm, considering the profound implications of root development on the ability of the plant to cope with water deficits. A big effort was therefore undertaken to run the first Genome-Wide Association study on root traits in rice.

To assess the variability existing for root system architecture in the Italian rice genetic pool and to evaluate the best method to characterize this complex trait, a subset of 13 varieties representative of the biodiversity existing in the Italian rice germplasm was tested in a pilot experiment in which plants were grown in soil-filled glass rhizotrons. The results obtained indicated the presence of significant variability among Italian rice accessions for root traits. However the bi-dimensional rhizotron phenotyping system turned out to be inadequate for a thorough screening of root system architecture, as it didn't allow to define the root growth angle.

An optimized phenotyping system was therefore developed and employed to characterize a collection of 96 temperate rice varieties of commercial relevance for Italy. This novel root phenotyping system combined an optimized plant growing system (plastic cylindrical baskets coupled with PVC pipes) with an efficient imaging analysis. In parallel, the rice diversity panel was genotyped using a novel NGS-based technology (GBS) to run preliminary GWA analyses on a subset of root traits selected for their predicted involvement in drought avoidance. The results of this initial study were very encouraging. All the detected significant associations were in fact co-localizing with root QTLs previously identified in bi-parental mapping populations. Moreover, four of the detected regions co-localized with drought-avoidance QTLs, strongly supporting the hypothesis of their possible involvement in plant ability to cope with water scarcity.

This preliminary GWA study on drought avoidance root traits clearly indicated that substantial phenotypic and genotypic diversity exists in the Italian/European rice germplasm, despite its narrow genetic basis, and that GBS technology coupled with an improved system for fine root phenotyping can be successfully employed for GWA studies in rice.

Although this preliminary effort was mainly focused on the set up of an efficient system to detect new regions elucidating the genetic determinants of drought-avoidance root QTLs, the regions already identified are worth being validated and further analyzed. In fact, even without knowing the genes involved, the detection of new regions involved in root development as well as the reduction of the size of the confidence interval of QTLs previously detected should greatly help breeders in marker-assisted selection programs for drought resistance.

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