Diagnostics in Plant Breeding - Chapter 23

Functional Marker Development Across Species In Selected Traits

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Abstract

Functional marker (FM) development across plant species requires common allelic sequences that affect phenotype variation in the same way. The identification of orthologous genes showing the same function across species could suppose conservation of functional polymorphisms. In this chapter we provide an overview on the polymorphic sites described within homologous genes across species directly related with selected traits, as well the functional markers developed and available for plant breeding. The review demonstrates that conservation of functional sequence motifs across species is a very rare phenomenon. Plasticity *per se* is highlighted as a new trait for plant breeding on robust, multi-stress tolerant and non-recalcitrant adventitious developmental behaviour for plant production. As a conclusion of the review, new perspectives for targeting functional markers across species are proposed.

1. Introduction

Breeding is a dynamic and adaptive process due to a changing environment. Domestication of wild plants to modern cultivated crops involved a suite of changes in morphological, physiological and biochemical traits (Doebley et al. 2006). Implementation of new mechanic methods of harvesting, human inclination towards valuing novelty and high quality exigency, the increase of the human population and global as well as local changes in climate and soil conditions are factors that require permanent adaptation in defining specific and across species traits. While there is an increased demand for sustainably produced agricultural products of high quality, availability of agricultural land and natural resources such as water and fertilizers are restricted. Climate changes can vary the frequency and severity of abiotic and subsequently biotic constrains. In view of the needs of human life, breeders want to

create crop plants that are able to confront unfavorable natural conditions and restricted resources, and respond to agro-technological requirements. Across species identification of FMs that might assist plant selection could make breeding efforts much more efficient.

Plants as sessile organisms are able to acquire during individual and genetic evolution an unique capacity for developmental plasticity. Its manifestation depends on environmental conditions in interaction with the genotype and its developmental stage. Environmental factors affecting adaptive plant development include all kinds of abiotic signals such as light, temperature, wind, humidity, soil structure, water and nutrient availability as well as biotic components from pathogens to competitors (Tonsor et al. 2005). The natural ability of plants to adapt growth and development to varying conditions is defined as phenotype variation. The capacity for phenotypic variation is genetically determined (Jungk 2001). Phenotypic variation is typically thought to arise spontaneously by genetic polymorphisms that can be maintained either by natural or human selection (Alonso-Blanco et al. 2005). However, repetitive events in evolution, such as speciation, were observed (Feldman et al. 2009, Rokas and Caroll 2008, Rundle et al. 2000, Wood et al. 2005, Zhang and Kumar 1997) and closer similarities between genotypes from distant regions than from within regions were recognized (e.g. Coelho et al. 2006). These observations may indicate the importance of environmental conditions and a directed rather than casual parallel genetic and epigenetic evolution of organisms. Thus, the emergence of similar functional polymorphisms during evolution might be suggested for key genes of common traits across species. The identification of polymorphisms within gene and regulative sequences which affect gene expression patterns assumed high importance due to the potential of developing FMs for plant breeding (Anderson and Lübberstedt 2003). To assist breeding the presence of FMs

should indicate the high probability of a genotype to achieve a phenotype of interest (Brenner et al. in this edition).

Functional polymorphisms can be located in both protein-coding and non-coding regions of a gene. The higher frequency in non-coding parts reflects the strict functional requirements of the protein-coding regions (Wang et al. 2005). Polymorphisms occurring in the coding sequence can affect protein sequences due to amino acid changes (non-synonymous polymorphism) which can interfere with the function of the protein. Interruption of the protein sequence by a nonsense mutation can originate a truncated protein with consequent loss-of-function. In introns, polymorphisms can be functionally critical in view of its potential to influence binding of transcription factors (Xie et al. 2005), alternative splicing (Baek et al. 2008), the coding of intronic regulatory elements, such as micro- or small nucleolar- RNAs (Li et al. 2007) as well as nonsense-mediated mRNA decay (Jaillon et al. 2008). Genetic and epigenetic regulation of the organization of DNA into condensed structures and loops in eukaryotic chromosomes plays an important role for gene expression, DNA synthesis, recombination, and repair by modulating the accessibility of DNA (Arnholdt-Schmitt 2004, Fransz and De Jong 2011, Shaposhnikov et al. 2007).

Mutations through spontaneous insertion/deletion (InDel) and single nucleotide polymorphism (SNPs) are thought to be the major driving forces in genome evolution besides retroelements (Gregory 2004, Zhang and Gerstein 2003). They are highly abundant and distributed throughout the genomes in various plant species (Batley et al. 2003, Costa et al. 2009b, Drenkard et al. 2000, Nasu et al. 2002,). SNPs and InDels can contribute directly to a phenotype (Thornsberry et al. 2001) or they can be associated with a phenotype shown by linkage disequilibrium (Daly et al. 2001). SNPs are becoming important genetic markers for major crop species for genetic research and

breeding (Fan et al. 2009, Jia et al. 2004, Lagudah et al. 2009, Lata et al. 2011, Ramkumar et al. 2010, Wang et al. 2011a, Yeam et al. 2005). Particularly, non-synonymous coding SNPs (nsSNPs) which together with SNPs in regulatory regions are believed to have the highest impact on phenotype determination (Ramensky et al. 2002). In some cases InDels have been successfully exploited as FMs (Bradbury et al. 2005b, Chen et al. 2010, Juwattanasomran et al. 2010, Lagudah et al. 2009, Shi et al. 2008).

Here we review functional polymorphisms identified in genes that are linked to selected traits across species. For some traits research efforts were more focused on cereals and sometimes exclusively on rice, which is explained not only by the importance of this species for human food but also as it is used as a model for domesticated plants due to the small genome (Alonso-Blanco et al. 2005). Traits only explored in rice will not be considered in this chapter in order to avoid repetitions in the present edition (see chapter on rice).

2. Selected traits for yield and quality parameters

2.1. Yield parameters in grain cereals

2.1.1. Grain weight

Grain weight is determined by different mechanisms that regulate grain size through its length, width, and/or thickness (Sakamoto and Matsuoka 2008, Takno-Kai et al. 2009, Xing and Zhang 2010).

2.1.1.1. Grain length

Gene sequences or QTL for grain length have been identified with common regions from rice, wheat and maize. In rice the gene *GS3* (*GRAIN SIZE 3*) overlaps a major

QTL for grain length and weight and also a minor QTL for grain width and thickness (Fan et al. 2006). Recent advances in comparative sequence analysis between wheat and rice genomes have confirmed extensive synteny between the two species (Quraishi et al. 2009). This enables to assess the positional correspondence between QTL identified in wheat and known QTL or loci that affect grain morphology in rice. The rice (*Oryza sativa* L.) *GS3* (*OsGS3*) corresponds to the strong wheat QTL for grain size (*TaGS3*), which consegregates consistently with grain width (Gegas et al. 2010, Li et al. 2010b). In contrast, *OsGS3* effects primarily grain length and less the width (Fan et al. 2006). However, up to now no more work is published about the wheat gene and its application in plant breeding.

The *OsGS3* encode a transmembrane protein consisting of four putative domains: a plant-specific organ size regulator (OSR) domain of 66 aa at the N terminus (Mao et al. 2010), which substitute the phosphatidylethanolamine-binding protein (PEBP)-like domain previously proposed by Fan et al. (2006); a transmembrane domain at 97-117 aa, a TNFR (tumor necrosis factor receptor)/NGFR (nerve growth factor receptor) family cysteine-rich domain at sites 116-155 aa, and a von Willebrand factor type C (VWFC) 60-80 aa in length in the C-terminal region (Mao et al. 2010). The VWFC domain of the OsGS3 functional protein is reported to be important for protein-protein interaction and signaling (Zhang et al. 2007).

The genotype/phenotype of grain length was well characterized by Mao et al. (2010) who identified four alleles, three associated with differences in grain length (Table 1). The allele *OsGS3*-4 with a one bp deletion is characterized by the loss-of-function mutation of the C-terminal TNFR/NGFR and VWFC domains with a consequent inhibitory effect on the OSR function and production of very short grain (Mao et al. 2010). The allele *OsGS3-3* is characterized by a nsSNP which lead to a

nonsense mutation and consequently elimination of part of the OSR domain and all the other three conserved domains. According Mao et al. (2010) the OSR domain is both necessary and sufficient for functioning as a negative regulator. The nonsense mutation is shared among all the large-grain varieties of *O. sativa* sequenced in comparison with small-to medium-grain varieties (Fan et al. 2006, Fan et al. 2009, Takano-Kai et al. 2009). These findings suggest that *OsGS3* acts as a negative regulator of grain length, in agreement with the recessive nature of the long-grain phenotype (Fan et al. 2006).

Different molecular markers which target the functional SNP at the *OsGS3-3* allele were already developed and can be used as a tool for routine and large-scale genotyping and selection of long/short grain length genotypes at the seedling stage which is vital for long grain breeding plant materials (Table 1).

Recently, Wang et al. (2011a) identified two new polymorphisms in other regions of *OsGS3* defining two new alleles (RGS1 and RGS2) which confer in combinations moderate/short grain (Table 1). However, the development of specific markers which allow the use of RGS1 and RGS2 motifs in breeding programs was not developed until now.

In maize (*Zea mays* L.) to-date only one cytosolic maize glutamine synthetase isoenzyme (GS1), product of *Gln1-4* has been shown by mutant analysis to have an impact on grain length (Martin et al. 2006) although, an orthologous of the *OsGS3*, named *ZmGS3*, was isolated in maize. *ZmGS3* encodes a protein sharing common domains with OsGS3, including one transmembrane domain and two overlapping TNFR/ NGFR family, cysteine-rich domains (Li et al. 2010b). Expression analysis revealed that *ZmGS3* is primarily expressed in immature ears and kernel and decreases rapidly after pollination (Li et al. 2010b), suggesting a role in kernel development, as it was discovered in rice (Fan et al. 2006, 2009). However, different functional

polymorphisms were identified suggesting different mechanisms from that of *OsGS3* (Table 1). A polymorphism in the promoter region of this gene was found to affect 'hundred grain weight' (HGW) (Li et al. 2010b).

GS3 is a major gene for grain length in rice, and it can explain up to 72% of the variation in grain length (Fan et al. 2006, 2009). However, *ZmGS3* was marginally significant and the phenotypic variation explained by the identified polymorphism is less than 8%, indicating that *ZmGS3* is only a minor gene for variations in maize grain traits (Li et al. 2010b). Nevertheless, it could not been excluded that it may play an important role in maize grain development, and, thus, hold potential for yield improvement in maize.

2.1.1.2. Grain width

GW2 (*GRAIN WIDTH 2*) was the first gene cloned in rice controlling grain width and weight (Song et al. 2007). *OsGW2* encodes a cytoplasm RING-type protein with intrinsic E3 ubiquitin ligase activity. Its function is related to the degradation step in the ubiquitin-proteosome pathway. Homologous genes to *OsGW2* with high aa sequence identities (86.5% and 81% respectively) were identified in *Triticum aestivum* and *Z. mays* (Song et al. 2007).

In rice, the loss of *GW2* function, due to a polymorphism in the ORF (Table 1) increased cell numbers, resulting in a larger (wider) spikelet hull. Further, the loss-of-function accelerated the grain milk filling rate, resulting in enhanced grain width, weight and yield (Song et al. 2007). These findings suggest that *OsGW2* functions as a negative regulator for grain width through the control of cell division in the spikelet hull by targeting unknown substrate(s) for the ubiquitin-dependent degradation by the 26S proteasome (Song et al. 2007). Pleiotropic effects were attributed to this gene, at least

on the panicle number per plant, days to heading and main panicle length, in addition to on the grain numbers per main panicle (Song et al. 2007). The development of a FM based on the polymorphism identified was reported (Table 1) (Yan et al. 2009).

Li et al. (2010a) describe identification of two genes as chromosomal duplicates co-orthologous of rice *GW2* in *Z. mays*, *ZmGW2-CHR4* and *ZmGW2-CHR5*. Expression and candidate gene-based association analyses suggested that both genes play a role in kernel size and weight variation, as does rice *GW2*. From all the 70 fixed polymorphic sites identified covering different regions of *ZmGW2* genes, a SNP located in the promoter region of *ZmGW2-CHR4* (C40T) was of great interest because it was significantly associated with phenotypic differences in GW and HGW (Li et al. 2010a). However, no further studies were reported confirming that hypothesis and also the possible application of this polymorphism in the development of molecular markers for plant breeding application.

In *T. aestivum* an ortologous gene of *OsGW2* was identified (*TaGW2*). Nucleotide sequence analysis of *TaGW2* led to the identification of two *TaGW2* haplotypes, Hap-6A-A and Hap-6A-G (Su et al. 2011). Expression analysis revealed a negative correlation between grain width and the expression level of *TaGW2*. Moreover, the average expression level of *TaGW2* in varieties with Hap-6A-G was higher than in varieties with Hap-6A-A, what indicates association of this haplotype with higher grain width and weight (Su et al. 2011). The effect of *TaGW2*-6A Hap-6A-A in wheat was similar to a loss-of-function mutation in *OsGW2* in rice, leading to increased grain width and weight and higher TGW, and associated with earlier heading and maturity. A CAPS marker was already developed and validated (Table 1). Association analysis revealed that Hap-6A-A was significantly associated with wider grains and higher one-thousand grain weight (TGW) in two crop seasons (Su et al. 2011).

A second gene identified in rice as controlling grain width is *GW5* (*GRAIN WIDTH 5*) (Weng et al. 2008) or *qSW5* (QTL for SEED WIDTHT in chromosome 5) (Shomura et al. 2008). *GW5* encodes an unknown nuclear protein containing a predicted NLS and an arginine-rich domain, which physically interacts with polyubiquitin, indicating that GW5 may be involved in the ubiquitin-proteosome pathway to regulate cell division during seed development (Weng et al. 2008). Recent studies have also pointed to a critical role of the ubiquitin pathway in seed development. As example, in *Arabidopsis thaliana* L. an induced mutation in the *DA1* (DA means "large" in Chinese) is related with the loss-of-function of a predicted ubiquitin receptor and consequently with an increase in the seed and organ size (Li et al. 2008). Also in rice as described before a loss of function of GW2, an enzyme of the ubiquitin-proteosome pathway is related with an increase in grain weight by a large increase in grain width (Song et al. 2007).

An identified functional polymorphism (Table 1) was associated with differences at the grain width and weight. Loss of function of GW5 resulted in a significant increase in sink size owing to an increase in cell number in the outer glume of the rice flower (Shomura et al. 2008). A negative regulation as reported in the *GW2* (Song et al. 2007) was also described for *GW5* (Weng et al. 2008). However, no FMs were reported based in this functional polymorphic difference.

2.2. Quality parameters in cereals and fruits

2.2.1. Lipids content

Plants are a vital source of renewable oils for food (representing 25% of human caloric intake in developed countries) but also nonfood applications, which represents a third of plant oil harvested. Controlling the composition and maximizing the energy-efficient

yield of oils within diverse crop species have been recognized as major goals for plant breeders and the biotechnology industry. To make breeding efforts more efficient, identification of common FMs for phenotype variation in oil contents or quality across species could be supportive. Rapeseed (*Brassica napus*), soybean (*G. max*), oil palm (*Elaeis guinensis*), and sunflower (*Helianthus annuus*) account for more than 65% of vegetable oil production worldwide (Gunstone 2001).

fad2 (*FATTI ACID DESATURATION 2*) encodes the enzyme responsible for the desaturation of oleic acid to linolenic acid in *A. thaliana* (Okuley et al. 1994). High-oleic-acid content in seeds of *Brassica* is a current breeding objective because it increases the termostability of the oil, making it more suitable as cooking oil. Cloning of homologous *Atfad2* in *B. rapa* allowed to make a comparison between *Brfad2* sequences from the wild-type and the high-oleic-acid allele. From SNPs which differentiate high-oleic-acid allele from the wild-type allele, only SNP_{484}(T/C) creates an aa change (L131P) (Tanhuanpää et al. 1998). Based on that nsSNP FMs for selection of plants with high-oleic-acid were developed (Table 1).

Another gene related with seed oil accumulation is the class IV homeodomain-ZIP transcription factor GLABRA 2 (*GLABRA 2*, *GL2*) characterized in *A. thaliana* related to the regulation of seed oil accumulation (Chai et al. 2010). Chai et al. (2010) reported the cloning of four orthologues of *AtGL2*. From oilseed rape (*B. napus*) the gene was named *BnaA.GL2.a*, from *B. napus BnaA.GL2.b*, from *B. rapa BraA.GL2.a*, and from *B. olearace BolC.GL2.a*. The existence of four orthologues *GL2* genes is explained by the origin of that species since *B. napus* (genome AACC, 2n=38) results from spontaneous hybridization between *B. rapa* (AA, 2n=20) and *B. olearacea* (CC, 2n=18) comprising two sets of homologous chromosomes from the two species. Eleven non-synonymous point mutations were identified among the four gene sequences,

responsible for aa changes (Q238H, L269V, A404V, M412V, -418T, A419S, K477R, M660L, A697-, M709L, C745S). Nevertheless, more variation was reported in intron sequences, specifically in introns 5 and 7, which were used to develop three FMs to distinguish *B. napus*, *B. rapa*, and *B. olearacea* from genomic DNA by using specific primer pairs (exon-anchored primers 10 and 11; A- and C-genome specific, BnGL2A and BnGL2C, respectively) (Table 1) (Chai et al. 2010). Also a CAPS marker was developed, based on the SNP A3486C, where A is present in A-genomes (*BnaA.GL2.a* and *BraA.GL2.a*) and C in C-genomes (*BnaC.GL2.b* and *BolC.GL2.a*) (Table 1).

2.2.2. Fragrance/ Aroma

Rice grains with a fragrance, like Basmati and Jasmine rice varieties, are appealing to consumers due to their superior grain qualities and pleasant aroma, which increase the retail price when compared with conventional rice (Shi et al. 2008). Also aromatic vegetable soybean (also known as "Chamame" or green soybean) have higher prices and demand than those from normal varieties (Statistics Department, Ministry of Agriculture, Forestry and Fisheries 2009).

In soybean as in rice the aroma has been associated with increased levels of 2-acetyl-1-pyrroline (2AP) (Arikit et al. 2011b, Fushimi and Masuda 2001), and also in both species a single gene was suggested to be responsible for fragrance (Bradbury et al. 2005a, AVRDC 2003). In rice, that gene is known as *Os2AP* (Vanavichit et al. 2008), *OsBad2* (Bradbury et al. 2005a, 2008) or *OsBadh2* (Niu et al. 2008), and in soybean as *GmBadh2*. BADH2 was proposed to encode the Betaine Aldehyde Dehydrogenase 2, which inhibits the biosynthesis of 2AP, a potent flavor component of rice and soybean fragrance (Chen et al. 2008, Juwattanasomran et al. 2010, 2011 Shi et al. 2008).

Silencing *OsBADH2* gene in non-fragrant rice varieties by a transgene approach showed elevated 2AP biosysthesis and, thus, fragrance in those varieties (Vanavichit et al. 2008; Niu et al. 2008). Similarly, transferring functional *OsBadh2* gene into fragrant rice resulted in non-fragrant rice (Chen et al. 2008). The involvement of *Badh2* in fragrance of rice was firstly reported by Bradbury et al. (2005a, b) with the identification of the recessive allele *badh2-E7* carrying a 8 bp deletion in exon 7 which causes a premature stop codon, and consequently generates a non-functional BADH2 and fragrance. Shi et al. (2008) identified a novel recessive allele, *badh2-E2*, differing from the *badh2-E7* by having an intact exon 7 but a 7-bp deletion in exon 2, which causes a frame shift leading to a non-functional BADH2 before critical residues that form the catalytic and/or substrate binding domains. Chen et al. (2008) showed that only the intact 503 aa protein encoded from full-length transcript of *OsBadh2* could inhibit 2AP synthesis. The functional OsBADHs protein contains two peptide sequences – VSLELGGKSP and EGCRLGSVVS – and a cysteine residue (28 aa away from VSLELGGKSP) in exons 8, 9 and 10, respectively, highly conserved in aldehyde dehydrogenases. It suggests that these sequences are essential for functional activity of OsBADHs (Bradbury et al. 2005a). In *OsBadh2*, exons 8, 9 and 10 also contain coding regions for these elements, respectively (Bradbury et al. 2005a).

The development of FMs for rice fragrance using both alleles, were firstly reported by Bradbury et al. (2005b), which developed the markers (Table 1). They were used by Sakthivel et al. (2006). Shi et al. (2008) also developed FMs always based on one of the deletions at the *badh2* allele, later applied by Jin et al (2010) to select individuals carrying the *badh2-E2* allele. Amarawathi et al. (2008) also describe the development of FMs based in the 8 bp deletion *badh2-E7*.

Kovach et al. (2009) reported eight additional non-functional alleles of *OsBadh2* (beside the *badh2-E7*/*badh2.1* and *badh2-E2*/*badh2.2*) associated with fragrance: four of them are frameshift-inducing InDels (*badh2.3*, *badh2.4*, *badh2.5*, *badh2.7*), one is a nsSNP creating a premature stop codon (*badh2.6*), and the other three potential functional mutations include a 3-bp insertion (*badh2.8*). Two nsSNPs (*badh2.9* and *badh2.10*) are not related with truncation of protein but induce only an aa change (Kovach et al. 2009). Nevertheless, no FMs were developed for these new alleles.

In rice, besides the *OsBadh2* that strongly affects strength of fragrance, Lorieux et al. (1996) reported two minor QTLs each on chromosome 4 and chromosome 12, while Amarawathi et al. (2008) identified two minor QTLs each on chromosome 3 and chromosome 4 influencing the level of fragrance and suggesting *OsBadh1*, a homologous of *OsBadh2*, as a candidate gene for the QTL on chromosome 4. The biochemical function and substrate specificity of the BADH enzymes coded by the two genes is quite similar (Bradbury et al. 2008).

Recent studies on *Badh1* point to the putative involvement of two nsSNPs in the substrate binding capacity of BADH1 towards gamma-aminobutyr-aldehyde (GABald), a precursor of the major aroma compound 2-acetyl-1-pyrroline (2AP) (Chen et al. 2008, Singh et al. 2010). Based in the nsSNPs Chen et al. (2008) defined two haplotypes (*Badh1_PH1* and *Badh1-PH2*) (Table 1). By *in silico* analysis Singh et al. (2010) discriminated in *Badh1-PH2* only 8 out of the 18 binding sites as sites for GABald binding suggesting a drastic reduction in the affinity of that haplotype for GABald, the precursor of the aroma compound 2AP. Thus *Badh1-PH2* could be a loss-of-function allele of the *Badh1* gene with implications in rice aroma similar to the loss-of-function alleles of the *Badh2* (Kovach et al. 2009, Singh et al. 2010). Singh et al. (2010) related the BADH1 haplotype PH2 with aromatic rice varieties and observed a significant

association between that haplotype and aroma score. Nevertheless, this association awaits validation in segregating populations for potential utilization in rice breeding programs.

In soybean was reported the cloning of two *Badh* genes, *GmBadh1* and *GmBadh2* (Juwattanasomran et al. 2011). Silencing of *GmBadh2* resulted in 2AP biosynthesis in non-fragrant soybean varieties (Arikit et al. 2011b). Comparison of gene sequences provided from fragrance and non-fragrance varieties allowed the identification of several polymorphisms (Table 1), two related with the loss-of-function of OsBadh2 and consequently enhancement of 2AP (fragrance) (Bradbury et al. 2005a, Juwattanasomran et al. 2011, Niu et al. 2008, Shi et al. 2008, Vanavichit et al. 2008). Both mutations occur in exon 10 of *GmBadh2*, which contains the conserved motif EGCRLGPIVS, similar to the motif for the essential functional activity of *OsBadhs*. The first SNP (G/A) in *GmBadh2* causes a change of the conserved motif to, which may be associated with the loss of functional activity of protein (Juwattanasomran et al. 2011); the polymorphism is a 2 bp InDel which causes a truncation of the protein and consequently lacks the peptide sequence EGCRLGPIVS, resulting in 2AP accumulation (fragrance) (Arikit et al. 2011b, Juwattanasomran et al. 2010). Mutation at the same gene which generates fragrance in rice and soybean suggested that both plant species have similar biochemical pathways for 2AP synthesis.

Several FMs were already developed to distinguish both alleles Gmbadh2.1 and Gmbadh2.2 (Table 1). However, in some few fragrance rice accessions no mutations were identified in the coding or promoter region that may alter *OsBadh2* or its expression (Kovach et al. 2009). *OsBadh2-1* could also be identified in soybean RILs with no fragrance (Juwattanasomran et al. 2011). The impossibility to explain completely the variation observed in fragrance in rice and soybean by looking for

polymorphisms already identified in the genes, suggest a role for other genes in conditioning fragrance. In case of rice BADH2 complementation with the BADH1 protein haplotype PH2 could be important for full aroma expression (Singh et al. 2010).

2.2.3. Fruit size and shape

2.2.3.1. Fruit size

In tomato, 28 QTL were identified in two or more independently conducted studies (Grandillo et al. 1999). Seven QTL explained more than 20% of the phenotypic variance (Grandillo et al. 1999, Tanksley 2004). Nevertheless, up to now *fw2.2* (*SECOND FRUIT WEIGHT* QTL on chromosome 2), is the only locus for which the underlying gene has been identified (Frary et al. 2000). *fw2.2* encodes a protein with similarity to a human oncogene RAS protein (Frary et al. 2000), known to belong to a family which includes proteins with wide regulatory functions, including control of cell division (Sprang 1997).

Natural genetic variation at *fw2.2* locus (3 SNPs in 5'-UTR, 35 SNPs within the two predicted introns and 4 SNPs representing silent mutations) alone can change the size of fruit by up to 30% (Frary et al. 2000). However, the control of tomato fruit size seems to be mediated by a cis-regulatory mechanism due to 5'-regulatory regions and gene expression patterns rather than variation in protein sequences of different alleles (Frary et al. 2000, Nesbitt et al. 2002). The described changes in the 5'UTR are associated with lower total transcript levels during the cell division phase of fruit development as well as a shift in the timing of expression (Cong et al. 2002). Changes in gene regulation, rather than protein function, have long been hypothesized as a major mode of evolutionary change, especially concerning morphological differentiation. In this regard, *fw2.2* is one of a growing number of examples in which natural variation

associated with morphological changes can be traced to regulatory mutations (Cong et al. 2002). The most striking evidence in support of this notion came from the fact that the coding sequence of a small-fruit wild tomato species *Lycopersicon cheesmanii* is identical to that of the large-fruit domestication species *Lycopersicon esculentum*, indicating that the *fw2.2* coding sequences cannot be the reason for fruit size variation (Nesbitt et al., 2002). Thus, the hypothesis emerged that the 3 SNPs identified in the 5'-UTR of *fw2.2* may be a cause of the observed phenotype difference (Frary et al. 2000). Nevertheless, no reference was made to any of the polymorphisms at the intron level and/or the potential role in regulating gene expression. Frary et al. (2000) also proposed that the differences in fruit size imparted by the different *fw2.2* alleles may be modulated by a combination with the 35 SNPs identified within the two predicted introns. The development of molecular markers using the mutations identified in *fw2.2* was not reported.

Homologous sequences to *fw2.2* of tomato were identified in several monocot and dicot plant species, like *O. sativa*, *G. max* and *Z. mays* (Frary et al. 2000). Nevertheless none of these sequences has a known function. Orthologous genes were also identified in other *Solanaceae* species, like *fw2.1* in eggplant (*Solanum melongena*, Doganlar et al. 2002) and pepper (Ben Chaim et al. 2001), however, no research was found about *fw2.2* sequence variation and it relation with fruit size.

2.2.3.2. Fruit shape

Like fruit size, shape is also a quantitative genetic trait. In tomato, the major loci affecting shape are *OVATE*, *SUN*, *FRUIT SHAPE CHR 8.1* (*fs8.1*), *FASCINATED* (*f*) and *LOCULE NUMBER* (*lc*) (Tanksley 2004). The three major loci *OVATE*, *SUN* and *fs8.1* modulate fruit shape with a minimal effect on fruit size (Tanksley 2004). *OVATE*

from tomato is the only locus that had been characterized at molecular level (Liu et al. 2002).

OVATE controls transition from round to pear-shape fruits in tomato (Liu et al. 2002) and also in eggplant (*fl2.1*) (Doganlar et al. 2002). The *OVATE* gene from tomato encodes a hydrophilic protein with a putative bipartite nuclear localization signal (NLS; Robbins et al. 1991), two putative VWFC protein–protein interaction domains (Hunt and Barker 1987), and a 70 aa carboxyl-terminal domain conserved in tomato, *Arabidopsis* and rice (Liu et al. 2002).

The similarity in morphological change and DNA sequence deletion between the rice grain and tomato fruit strongly suggests that the putative VWFC domain may have a role in regulating the fruit/grain shape by negatively affecting the growth. It is known that the VWFC domain, also referred to as Chordin-like cysteine-rich (CR) repeats, is present in the growing number of extracellular matrix proteins, and binds to members of the transforming growth factor-ß (TGF-ß) superfamily (Abreu et al. 2002). It has been proposed that the general function of VWFC is to regulate growth factor signaling by disrupting the receptor binding sites in the TGF-ß superfamily of the extracellular matrix, thus preventing activation of the TGF-ß receptor (O'Leary et al. 2004). Such inhibitory activity of VWFC on growth factor signaling is clearly consistent with the mechanism of negative regulation in the development of grain size and fruit shape hypothesized for the *GS3* and *OVATE* genes.

OVATE presents a nonsense mutation (Table 1) in the C terminus of the predicted protein and could eliminate most of the conserved domain of the protein. It may account for the loss-of-function (recessive) phenotypes. Sequence comparison revealed that all the varieties of tomato with the pear-shaped fruit had this mutation in

the *OVATE* gene (Liu et al. 2002). This allows to propose the polymorphism responsible for the nonsense mutation for further FM development.

3. Specific traits for stress tolerance

3.1 Abiotic stress tolerance

3.1.1. Drought tolerance

DREB (DEHYDRATION-RESPONSIVE ELEMENT-BINDING) proteins are important transcription factors that belong to the APETALA 2/ETHYLENE RESPONSIVE FACTOR (AP2/ERF) family. The AP2/ERF domain specifically binds to the CRT/DRE (C-REPEAT/DEHYDRATION-RESPONSIVE ELEMENT) of downstream genes, regulating their expression and consequently enhancing plant tolerance to abiotic stresses like low temperature, drought, and high-salinity (Agarwal et al. 2006, Liu et al. 1998, Sakuma et al. 2002, Yamaguchi-Shinozaki and Shinozaki 2005). DREBs were identified in a high range of herbaceous and woody plant species (see revision on Agarwal et al. 2006, Benedict et al. 2006, Kitashiba et al. 2004, Yang et al. 2011).

Differences between *DREB* alleles related with droughts were reported by Chen et al. (2005) in *Triticum aestivum* with the identification of nine haplotypes when analyzed 20 accessions of wheat. From those two of them (haplotype 1 and 3) were identified as drought tolerant. Combining the gene expression data, which report an induction under drought treatment, and the existence of gene sequence polymorphisms, which is related with drought tolerance in the two haplotypes, *TaDREB* was considered as a useful gene for improving drought-tolerance in wheat. FMs were developed by Wei et al. (2009) based on genome-specific primers for each of the orthologous DREB loci on chromosome 3A, 3B and 3D based on InDels and SNPs previously identified as locus-specific (Chen et al. 2005).

3.1.2. Salinity tolerance

Salinity tolerance is a polygenic trait, controlled by interaction between many different genes involved in different pathways, such as ion compartmentation, ion extrusion, ion selectivity, compatible solute synthesis and Reactive-Oxygen-Species (ROS) scavenging (Munns and Tester 2008, Zhu 2001). Although many of these mechanisms are probably universal in most plants, their relative importance in salt tolerance may vary from species to species, depending on the metabolic background (Sun et al. 2010).

The impacts of salinity on plant growth arise through the effects of dehydration (osmotic toxicity) and interference with cellular metabolism caused by high levels of Na^+ in the cytoplasm (ion-specific toxicity) (Munns and Tester 2008). Na^+ can inhibit K^+ uptake (Rains and Epstein 1965), and in cytoplasm, Na^+ readily displaces K^+ in many enzymes that require K^+ as a co-factor for their activity (Tester and Davenport 2003). Using genetic approaches, many genes have been identified and associated with enhanced salinity tolerance in diverse plant species. These genes are generally divided into three groups, according to their function:

(1) genes that enhance osmotic protection and ROS scavenging such as *OSMOREGULATORY TREHALOSE SYNTHESIS* (*OTS*) (Garg et al. 2002), *MANNITOL-1-PHOSPHATE DEHYDROGENASE* (*M1PD*) (Abebe et al. 2003) and the *Δ^1-PYRROLINE-5-CARBOXYLATE SYNTHETASE* (*P5CS*) (Hong et al. 2000). The *P5CS* cloned from several higher plants (Armengaud et al. 2004, Chen et al. 2009, Hu et al. 1992) encodes a rate-limiting enzyme (P5CS) involved in the biosynthesis of proline from glutamate (Yoshiba et al. 1995). Proline in turn is an important osmo-protectant present in higher plants that is thought to be critical for adaptation to several abiotic stresses such as drought and salt (Verslues et al. 2006). Expression studies demonstrated that the transcript level of *P5CS* increases significantly by salt and drought treatments (Chen et

al. 2009, Dombrowski et al. 2008, Hu et al. 1992, Igarashi et al. 1997, Strizhov et al. 1997). Natural variation of *P5CS* was recently reported among 27 common bean accessions of *Phaseolus vulgaris* (Table 2), which was used for FM development (Chen et al. 2010).

(2) genes involved in Na^+ and K^+ transport, including the *HIGH-AFFINITY* K^+ *TRANSPORTER* family of genes (*HKT*) that are involved in K^+ transport (Horie et al. 2009) and the Na^+/H^+ *EXCHANGERS* (*NHX*) genes family (e.g., *NHX1*) or *SALT-OVERLY-SENSITIVE* genes (e.g., *SOS1*) involved in Na^+/H^+ antiport systems (Shi et al. 2003). From this group natural variation in *HKT* genes is known to be related with salinity tolerance (Qiu et al. 2011). Two alleles were identified (*HvHKT1* and *HvHKT2*) by sequence comparison in 40 different Tibetan wild barley accessions (see Table 2).

(3) regulatory genes such as transcription factors (i.e. *DREB*/*CBF* family) that function in signaling pathways, regulating the expression of downstream genes (Morran et al. 2011) involved in salinity tolerance in plants (Liu et al. 1998, Cong et al. 2008). At least 20 *CBF* genes were identified in barley (*Hordeum vulgare L.*), classified as subgroup *HvCBF1*, *HvCBF3* and *HvCBF4* (Skinner et al. 2005). *HvCBF4* encodes a protein closely homologous to *DREB1/CBF* in *A. thaliana* and *Vitis* sp. (Haake et al. 2002, Xiao et al. 2008). Transgenic overexpression of *HvCBF4* in rice has been demonstrated to enhance tolerance to drought, high-salinity and low-temperature (Oh et al. 2007). Natural variation within that gene sequence was reported by Wu et al. (2011) and Rivandi et al (2011), which developed a FM to apply in breeding programs of barley based in polymorphic sites.

In the species *Setaria italic* (foxtail millet) a *DREB* gene was characterized that belong to the A2 subgroup (*SiDREB2*) related with dehydration and salinity (NaCl)

tolerance (Lata et al. 2011). Sequence variation in this gene related with differences in salinity tolerance and its use for FM development was reported (described in Table 2).

3.1.3. Low temperature tolerance

Low temperature is one of the primary stresses limiting growth and productivity in winter. To cope with low-temperature stress, plants have evolved adaptive mechanisms that are temperature regulated. Low-temperature acclimation and vernalization response are the most important (Fowler et al. 1995).

Cold acclimation is coordinated by a complex process of up- or down-regulation of hundred of *COLD-REGULATED* (*COR*) genes which, in turn, are controlled by a complex regulatory network (Fowler and Thomashow 2002). In many plant species the *C-REPEAT BINDING FACTOR* (*CBF*) genes are key regulators of a signal cascade that leads to the expression of *COR* genes. There are several examples showing positive correlation between freezing tolerance and *DREB/CBF* transcript accumulation (Chen et al. 2009, Fricano et al. 2009, Yang et al. 2011). Heterologous over-expression of *CBF* sequences in transgenic plants has demonstrated increased levels to frost tolerance (Takumi et al. 2008, Oh et al. 2007). Data are available showing the existence of natural variability in three *CBF* genes of *H. vulgare* and the association of *HvCbf14* with frost resistance (Table 2) (Fricano et al. 2009). However, it was not established that those polymorphic sites are functional motifs responsible for variation in frost tolerance.

Beside *DREB* genes, other cold-responsive genes have been identified. Fowler and Thomashow (2002) revealed the existence of 306 cold-reponsive genes in *A. thaliana*. A gene family already reported as involved in response to low temperature is the *ALTERNATIVE OXIDASE* (AOX) gene family. AOX is an inner mitochondrial membrane protein that functions as terminal oxidase in the alternative (cyanide-

resistant) pathway of respiration where it generates water from ubiquinol (Umbach et al. 2002). AOX serves to relieve oxidative stress originating from environmental stresses by limiting mitochondrial reactive oxygen species (ROS) formation and preventing specific components of the respiration chain from over-reduction (Popov et al. 1997) and canalizing ROS signals (Amirsadeghi et al. 2007). AOX activity can support the homeostasis of plant growth to changing environmental conditions (Hansen et al. 2002 (see in Arnholdt-Schmitt et al. 2006),Vanlerberghe et al. 2009). In fact, there are several studies in many plant species showing a sharp increase in *AOX* transcript and/or protein content after a transfer to low temperature or during growing at low temperature (Umbach et al. 2009, Wang et al. 2011b, Watanabe et al. 2008). Abe et al. (2002) showed by site-direct mutagenesis that $nsSNP_{297}$(G/T) of *AOX1a* in rice (*OsAOX1a*) responsible by the substitution of K71N affects a quantitative trait locus (QTL) for thermo tolerance. *AOX* genes have been proposed for FM development related to multi-stress-tolerance and plant plasticity (Arnholdt-Schmitt 2009, Arnholdt-Schmitt et al. 2006, Polidoros et al. 2009, see also under 4 in this chapter).

3.2. Biotic stress tolerance

Typically, race/cultivar-specific resistance is proposed to involve the recognition of the pathogen avirulence (*avr*) gene product by the complementary host resistance (*R*) gene protein. This recognition initiates a signal transduction cascade and the defense response (Martin et al. 2003). The largest family of *R* genes is the *NBS-LRR*, which encodes predictive cytoplasmic proteins with nucleotide binding site (NBS) and leucine-rich repeat (LRR) domains. The *NBS-LRR* gene family can be divided into two subfamilies, the TIR and the non-TIR, depending on the presence of a domain at the N-terminal with similarity to the *TOLL/INTERLEUKIN-1 RECEPTOR* (*TIR*) (Meyers et al. 1999). R proteins

of the non-TIR class are often predicted to have a coiled-coil (CC) structure near their N-terminus and are referred to as the CC-NBS-LRR class.

R genes that confer resistance to different types of pathogens encode very similar proteins. However, resistance genes that control closely related or identical pathogens are only rarely located in corresponding positions in different genera. This is particularly true for dominant resistance factors that are involved in gene-for-gene interactions and are characterized by a NBS and/or a LRR domain (Ruffel et al. 2005). The LRR region plays a major role in pathogen recognition specificity (Yahiaoui et al. 2006). Interestingly, this feature appears not to be shared by recessive resistance genes that control viruses belonging to the genus *Potyvirus*. In comparison with resistance genes controlling other pathogens, recessive resistance to potyviruses is relatively common, comprising about half of all known resistances against this viral genus (Diaz-Pendon et al. 2004).

Additionally, identification of extensive intra- and inter-specific genetic variations within *NBS-LRR* genes makes it difficult to identify functional polymorphisms of *NBS-LRR* alleles based on sequence homology (Ingvardsen et al. 2008). In the following paragraphs *R* genes across different plant species and the nucleotide polymorphisms related with resistance to different pathogens will be described.

3.2.1. Fungi resistance

3.2.1.1. Resistance against *Blumeria graminis* f.sp. *tritici*, *Puccinia triticina* and *P. striiformis* sp. *tritici*

Blumeria graminis f.sp. tritici, *Puccinia triticina* and *P. striiformis* are three of the most devastating pathogens in wheat production causing powdery mildew, leaf or brown rust

and yellow or stripe rust diseases, respectively. Strong investments were made in order to develop FMs to assist plant breeding, and nowadays many *Pm* (*POWDERY MILDEW*) genes have been identified (see review in Maxwell 2008). *Pm3* encodes a protein belonging to the CC-NBS-LRR family that confers race-specific resistance to *B. graminis* f.sp. *tritici* (Yahiaoui et al. 2004). *Pm3* carries a higher number of alleles than other *Pm* genes (Tommasini et al. 2006). In hexaploid wheat seven resistance alleles (*Pm3a*, *Pm3b*, *Pm3c*, *Pm3d*, *Pm3e*, *Pm3f* and *Pm3g*) have been described, all deriving from one susceptible allele *Pm3CS*, which is widespread among hexaploid bread-wheat lines (Tommasini et al. 2006, Yahiaoui et al. 2006) (see details in Table 3). Only three nsSNPs differentiate the resistant alleles *Pm3d* and *Pm3e* from *Pm3CS*, suggesting that the specific resistance they confer might be based on these few polymorphic residues. Direct mutagenesis studies, indicated that aa W659 is required for *Pm3d*-dependent resistance, and the replacement of E1334V in *Pm3CS* was sufficient to convert the susceptible to resistant phenotype (Yahiaoui et al. 2006).

The identified polymorphisms (including single and multiple nucleotide polymorphisms and a small InDel) were mainly located in the terminal part of the *Pm3* coding region (encoding the LRR region of protein) and in the 3'-UTR (Tommasini et al. 2006). They were used for FMs development in order to distinguish the allelic series of powdery mildew resistance (Table 3). The FMs were used to screen the seven alleles in 1005 accessions (Bhullar et al. 2010).

The *Pm3* locus is conserved in tetraploid wheat, in which Yahiaoui et al. (2009) identified 61 allelic sequences that corresponded to 21 different haplotypes (H1-H21) and one additional resistant allele (*Pm3k*, H22) with only a single polymorphism, not found in susceptible allele ($SNP_{1332}C$), a predicted solvent-exposed residue of LRR27. As in hexaploid wheat highest sequence diversity was located in the LRR-encoding

region, and a change in a solvent-exposed residue of LRR27 was sufficient to convert the susceltible *Pm3CS* into a functional allele (Yahiaoui et al. 2006).

A second gene related with fungi resistance in wheat is *Pm38* (*POWDERY MILDEW 38*), which confers a high level of broad-spectrum resistance (Spielmeyer et al. 2005). This gene has been identified in several genetic backgrounds, and is inherited as a gene complex which also confers resistance to leaf or brown rust (*P. triticina*), stem or black rust and leaf tip necrosis (*P. graminis*), stripe or yellow rust disease (*P. striiformis* sp. *tritici*) and moderate resistance to powdery mildew (*B. graminis*). This is also the reason why the same gene *Pm38* acquired different synonyms: *Lr34* (*LEAF RUST 34*), *Ltn* (*LEAF TIP NECROSIS*) or *Yr18* (*YELLOW RUST 18*) (Liang et al. 2006, Schnurbusch et al. 2004, Singh 1992, Spielmeyer et al. 2005, 2008;). The gene is known to encode a pleiotropic drug resistance (PDR)-like ATP-binding cassette (ABC) transporter (Krattinger et al. 2009).

Lr34 sequence comparison of wheat resistant and susceptible cultivars revealed the existence of three haplotypes, two susceptible differing in only one nucleotide– Lr34) and one resistant (+Lr34), due to the existence of three polymorphic sites within the genomic gene sequence (Krattinger et al. 2009, Lagudah et al. 2009) (see details in Table 3). Lagudah et al. (2009) also reported a SNP (G/T) identified in exon 22 of the wheat cv. Jagger (susceptible) which result in a premature stop codon laking 185 aa of the C-terminus originating a nonfunctional protein. The same authors developed a co-dominant functional marker to detect that nsSNP.

3.2.1.2. Resistance against *Magnaporthe oryzae*

Rice blast disease caused by the pathogenic fungus *Magnaporthe oryzae* B. Couch (anamorph *Pyricularia grisea* Cav.) is one of the most devastating diseases in rice

production (Zeigler et al. 1994). To date, more than 80 blast *R* genes have been identified (Ballini et al. 2008) but less than twenty were molecularly characterized. We will describe in the following those for which FMs have been developed.

Pi-ta

Pi-ta was the first gene studied in order to develop FMs. *Pi-ta* encodes a protein with unique features when compared with other proteins of the NBS-containing class *R* genes (see Bryan et al. 2000). It includes a C-terminal LRD (LEUCINE-RICH DOMAIN) instead the characteristic LRR motif found in other genes of this class (Bryan et al. 2000).

Bryan et al. (2000) characterize two *Pi-ta* alleles with a nsSNP located at the C-terminal region (T2752G) in the ORF which is responsible for the aa change A918S and the subsequent change from the susceptible phenotype (allele *pi-ta* with A918) to a resistance phenotype (allele *Pi-ta* with S918). Functional analysis by transforming the susceptible rice variety Nipponbare with genomic and cDNA of the *Pi-ta* allele confirmed the identity of the gene as resistant to *M. grisea* (Bryan et al. 2000). The importance of A918 in determining *in vivo* specificity in the *Pi-ta* gene-for-gene system was also demonstrated by transient expression assays (Bryan et al. 2000).

Four additional nsSNPs were outlined when the variety Yashiro-mochi (resistant) was compared with the susceptible variety Tsuyuake: G17T: S6I, G444C: S148R, G474C: Q158H, T527A:V176D) (Bryan et al. 2000). The five polymorphic sites were confirmed by Jia et al. (2003) which included 8 new rice cultivars. The same authors reported additional SNPs at 5'-UTR (G2040A) and 3'-UTR (T6808A) and in intron sequences (A3536CC, G4234A, G4270A, C4391T, T4394A and GCC4426-4428CTAT). The identified polymorphisms were used to develop dominant and

codominat functional markers for identification and incorporation of the *Pi-ta* gene in MAS (Table 3).

Pit

The gene *Pit* which belongs to the CC-NBS-LRR family of resistance genes (Hayashi and Yoshida 2009) was also studied in order to develop FMs. Sequence comparison of *Pit* alleles between a susceptible (Nipponbare) and a resistant cultivar (K59) revealed that the resistance-conferring allele contains four aa substitutions (G143R, I176M, T720A and V780M), a DNA transposon *dDart*, and a long terminal repeat (LTR)-retrotransposon (*Renovator*), both inserted at the promoter. The effect of *Renovator* was verified by gene expression analysis and in a transgenic approach. The level of *Pit* mRNA was up-regulated through its insertion, and the effect of *Renovator* on the *Pit* promoter activity was greater than that of the aa substitutions (Hayashi and Yoshida 2009).

Based in that knowledge Hayashi et al. (2010) studied the variability of the *Pit* coding sequence in ten rice cultivars. They identified the same $nsSNP_{2338}$(G/A) located at the LRR region . The same authors developed PCR-based markers to detect that nsSNP, the *Renovator* and the *dDart*, suggesting the marker detecting *Renovator* as optimal for the identification of functional *Pit* gene since the up-regulation of *Pit* mRNA occurs only through the insertion of *Renovator*.

Pi54 (Pik^h)

Pik^h, recently renamed *Pi54* (Sharma et al. 2010) is one of the major blast resistance genes identified as encoding a NBS-LRR protein. A recent study including 27 landraces collected in the north-eastern part of India, report several polymorphisms in

Pi54 in which an InDel of 144 bp in the coding sequence is related with a resistant phenotype (Ramkumar et al. 2011). A functional co-dominant marker was developed to identify the resistant allele (Table 3).

3.2.1.3. Resistance against *Fusarium oxysporum f. sp. melonis* Snyder and Hansen

Fusarium wilt caused by the fungus *Fusarium oxysporum f. sp. melonis* Snyder and Hansen has become one of the most destructive diseases of *Cucumis melo* L. (melon) crops throughout the world (Leach 1983). To date four races (0, 1, 2 and 1-2) of this fungus have been defined, and two resistance genes were genetically identified to control the resistance of races: *Fom-1* (for races 0 and 2) and *Fom-2* (for races 0 and 1) (Risser et al. 1976). *Fom-2*, already isolated and characterized is predicted to encode a protein belonging to the NBS-LRR type of *R* genes. Wang et al. (2011c) analyzed specifically the LRR region in order to identify functional polymorphisms useful for FM development. Sequence comparison between resistant and one sensible genotype revealed three polymorphic sites useful for functional dominant and co-dominant markers (Table 3).

3.2.2. Nematodes resistance

FM development for nematode resistance is most advanced in soybean (*Glycine max* L.). The cys nematode (SCN; *Heterodera glycines* Ichinohe) is an important pathogen of soybean worldwide. The resistance is conditioned by different genes (Rao Arelli et al. 1992), being assigned three recessive genes (*rhg1*, *rhg2*, *rhg3*) (Caldwell et al. 1960) and a dominant resistant gene (*Rhg4*) (Matson and Williams 1965). Nevertheless, the allele for partial resistance at the *rhg1* resistance locus has been demonstrated to control more than 50% of the variation for resistance and appears to effectively control a

number of SCN races (Concibido et al. 1997). The *rhg1* gene family encodes a PROTEIN-RECEPTOR-LIKE KINASE (RLK) (Hauge et al. 2001), with an N-terminal signal peptide (1-61), an extracellular domain with ten extracellular LEUCINE-RICH REPEATS (LRR, 141-471), two trans-membrane domains (TM, 40-60; 485-507), and a cytoplasmic SERINE/THREONINE/TYROSINE KINASE domain (STYKc, 569-840) (Ruben et al. 2006). LRR-containing RLKs, which form the largest group of RLKs in plants, were predicted to play a central role in signaling during pathogen recognition in plant defense mechanisms and in developmental regulation.

DNA sequencing from 112 SCN-resistant Plant Introductions (PIs) and 34 derived cultivars inferred nine *rhg1* haplotypes, four of which were SCN resistant (Hauge et al. 2001, Ruben et al. 2006). Relatively few nucleotide substitutions resulted in aa changes so that only five protein allotypes were predicted with two of them potential for FM development: one alters A47V, and the second alters H297N. Both substitutions may alter pre-protein transport or protein function or both since A47 was only associated with resistance in the presence of H297 (Ruben et al. 2006).

Very recently, Li et al. (2009) demonstrated that the gene *rhg1* is essential for the development of resistant soybean cultivars. Polymorphisms at that gene were responsible for sensitive phenotypes. Four SNPs discriminated a haplotype present in five resistant soybean genotypes and another haplotype in the susceptible genotypes Suinong 14 and Guxin. From the four SNPs three are located in the coding regions, two at exon 1 located between the N-terminal signal peptide domain and the LRR domain, and one at exon 2 located in the LRR domain; the fourth is located at the 3'UTR (Table 3). The two SNPs in exon 1 form one haplotype (689C-757C) which the authors discovered as perfectly associated with SCN resistance that allowed successfully

developing functional co-dominant markers to separate resistant from susceptible genotypes (Table 3).

3.2.3.Tolerance/resistance to virus

Plant viruses are obligate parasites that multiply within their hosts by establishing specific interactions between viral factors and macromolecules, structures and processes of the plant, which determine the plant susceptibility to viral infection (Maule et al. 2002). A deleted or defective host protein that is essential for viral infection but is dispensable for the host may result in resistance to the virus. In this case, resistance is based in the 'negative model' on which resistance is expected to be genetically recessive (Fraser 1992). Despite the vast majority of the defined recessive resistance operates against viruses belonging to the *Potyviridae* (Ruffel et al. 2005), recent work reported also the same mechanism of resistance against virus belonging to *Tombusviridae* family (Nieto et al. 2006).

The characterization of natural recessive resistance genes in several dicots and monocots plant species [e.g. pepper (*pvr1*, Ruffel et al. 2002), lettuce (*mo1*, Nicaise et al. 2003), pea (*sbm1,* Gao et al. 2004), tomato (Ruffel et al. 2005), barley (*rym4/5*, Stein et al. 2005) and rice (*Rymv1*, Albar et al. 2006)] and the mutagenesis assays performed in *A. thaliana* (Duprat et al. 2002) have implicated a component of the eukaryotic translational initiation complex [i.e., eIF4E, eIF(iso)4E, eIF4G and eIF(iso)4G] as responsible for conferring resistance in plant systems to RNA viruses (for reviews see, Kang et al. 2005b, Maule et al. 2007). eIF4E is a component of the eIF4F complex and provides the 5' cap-binding function during formation of translation initiation complexes on most eukaryotic mRNAs and possibly also has a role in other processes of the cell cycle (Strudwick and Borden 2002). In plant cells, this complex is composed

of only two proteins, eIF4E and eIF4G (Browning 1996) and an additional cap-binding complex, eIF(iso)4F, in which a second cap-binding protein [eIF(iso)4E] binds with eIF(iso)4G (Bailey-Serres 1999).

It is known that sequence variations are related in some plant species with resistance to a single potyvirus species but in other cases could also result in a plurispecific effect (Ruffel et al. 2002).

The occurrence of polymorphisms in different parts of the *eIF4E* gene results in allelic series of potyvirus resistance across different plant species, which allowed the development of FMs.

3.2.3.1. Tolerance/resistance to virus in Dicots

3.2.3.1.1. Pepper

In pepper (*Capsicum annum*), the homolog of *eIF4E* located at the locus *pvr1* in chromosome 3 (Murphy et al. 1998), was demonstrated to confer resistance against several *Potyvirus* species including *Tabacco etch virus* (TEV), *Potato Y virus* (PVY), and *Pepper mottle virus* (Pepmov) (Kang et al. 2005a, Ruffel et al. 2002). Kang et al. (2005a) reported the existence of four alleles which encode the eIF4E protein: $Pvr1^{+}$ defined as the allele for susceptibility, and the three resistant alleles, *pvr1*, $pvr1^{1}$, and $pvr1^{2}$. The resistant alleles, due to aa changes (see details of aa changes sites in Table 4), encode a protein that failed to interact with the viral protein VPg (Kang et al. 2005a, Ruffel et al. 2002). In order to understand the biochemical effect of each aa substitution Yeam et al. (2007) generated alleles containing each aa substitution separately. The results indicate that the loss of VPg binding ability of eIF4E encoded by the $pvr1^{1}$ and $pvr1^{2}$ alleles is the result of an additive effect of the V67E and L79R changes; in the case of *pvr1* it is caused by the single change G107R. Amino acid 107 is adjacent to

R171, an aa that interacts directly with the negative charge of the cap phosphate group and is known to be important for cap binding (Marcotrigiano et al. 1997). The addition of another positive charge in this region, like the change of G107R could cause a strong electrostatic repulsion, not present in the wild-type protein, with adjacent positively charged residues and/or steric hindrance that interrupts the ability of the protein to bind both cap and VPg (Yeam et al. 2007). It is striking to note that the critical aa substitution in *pvr1*, G107R, also exists at the homologous sites in several other recessive resistance genes, including *sbm1* (G107R) from pea (Smýkal et al. 2010), *mo1*^{1} (QGA108-110H) from lettuce (Nicaise et al. 2003), *sub-1* (G107R) from pea (Smýkal et al. 2010) and *pot*^{1} (M109I) from tomato (Ruffel et al. 2005), which will be described in the following.

This capacity of G107R aa change in eIF4E- *pvr1* alone be sufficient to abolish the capacity of eIF4E to bind VPg was also proved by yeast two-hydrid assay (Yeam et al. 2007), and using recombinant Capsicum-eIF4E proteins produced in *Escherichia coli* (Kang et al. 2005a). A transgenic approach overexpressing *pvr1* in *Solanum lycopersicum* also resulted in gain of viral resistance (Kang et al. 2007).

Yeam et al. (2005) developed allele-specific CAPS markers for the three recessive viral resistant alleles from 13 *Capsicum* genotypes known to be homozygous for each of the four *pvr1* alleles (Table 4). Three exceptions were observed in genotypes showing resistance to PepMoV with the absence of the *pvr1* allele.

3.2.3.1.2. Tomato

Comparison of resistant *Lycopersicum hirsutum* and susceptible genotypes of *L. hirsutum* and *L. esculentum* revealed the existence of two alleles, *pot-1*$^{+}$(characterizing the susceptible phenotype) and *pot-1* which confers resistance to the *Potato virus Y*

(PVY) and *Tobacco etch virus* (TEV). Both alleles were distinguished by the existence of four nsSNPs which were used for FM development for application in plant breeding (see Table 4) (Ruffel et al. 2005). Additional confirmation of the involvement of *pot-1* allele in resistance and *pot-1*$^{+}$ in the susceptibility to PVY and TEV was achieved by a transgenic approach. Transient expression of the dominant susceptible allele restored susceptibility to both PVY and TEV, whereas expression of *pot-1* did not support potyvirus infection (Ruffel et al. 2005).

3.2.3.1.3. Lettuce

In lettuce (*Lactuca sativa*), *mo1*^{1} and *mo1*^{2} are known as recessive alleles of a single gene (Nicaise et al. 2003) associated with reduced accumulation and lack of symptoms (tolerance) or absence of accumulation (resistance) of common isolates of potyvirus *Lettuce mosaic virus* (LMV; Dinant and Lot 1992). The issue of the interaction, resistance or tolerance, depends on the virus isolate and genetic background (Revers et al. 1997). However, *mo1*^{1} is generally associated with resistance and *mo1*^{2} with tolerance (Revers et al. 1997).

Nicaise et al. (2003) characterized the cDNA gene sequence in eight lettuce genotypes and identified sequence variations that allowed classifying three lettuce *eIF4E* alleles, one for the susceptible phenotype (*Ls-eIF4E*^{o}), one for resistance *Ls-eIF4E*^{1} and another for tolerance *Ls-eIF4E*^{2} (see polymorphisms in Table 4). The aa that differ between the three Ls-eIF4E types were all mapped near the cap recognition pocket, on the face of eIF4E opposite to the eIF4G-binding site (Nicaise et al. 2003). A strict correlation between *Ls-eIF4E*^{1} and the presence of *mo1*^{1} and between *Ls-eIF4E*^{2} and the presence of *mo1*^{2} were reported by Nicaise et al. (2003); the same authors also

referred that the susceptible genotypes all had *Ls-eIF4E^{o}*. A functional codominat marker was developed to identify the allele resistant *Ls-eIF4E^{l}* (Table 4).

3.2.3.1.4. Pea

Two homologous *eIF4E* and *eIF(iso)4E* genes were identified in the *Pisum sativum* (pea) genome to be responsible for *Pea seed-borne mosaic virus* (PSbMV) and white lupin strain of *Bean yellow mosaic virus* BYMV-W resistance respectively at the *sbm1* and *sbm2* locus (Bruun-Rasmussen et al. 2007, Gao et al. 2004).

Resistance to the common strains of PSbMV is conferred by a single recessive allele (*sbm1*) encoding a mutant that fails to interact functionally with the PSbMV avirulence protein (VPg) giving genetic resistance to infection. This difference at the protein level is due by five polymorphisms (see Table 4) which were identified in sites implicated to be involved in resistance to potyviruses, also described as highly conserved between different plant species (Smýkal et al. 2010). However, Ashby et al. (2011) reported that only W62 and N169 display full resistant-like phenotypes when analyzed by direct mutagenesis.

Despite the difference within the coding sequence, resistant and susceptible genotypes show also a difference at the non-coding sequences. Insertions of 50 and 56 bp of a minisatellite-like repeat sequence at intron 3 was responsible for differences of the size of intron 3: all resistant (*sbm1*) accessions display shorter (1.151bp) intron 3 sequence, while susceptible have a larger (1.201 bp) intron 3 sequence (Table 4), which consequently is reflected at total gene size (susceptible genotypes 2.152 bp and resistant 2.102 bp) (Smýkal et al. 2010). Functional dominant and co-dominant markers were developed based on the nsSNPs and also on intron length polymorphism (Table 4).

3.2.3.1.5. Melon and Watermelon

In melon (*C. melo*) a gene coding for eIF4E was identified in which Nieto et al. (2006) found SNP mutations conferring potyviruses resistance, mostly located in the N-terminal region (Table 4). Neverteless, a nsSNP in the C-terminal region of eIF4E (H228L) was responsible for resistance in melon to *Melon necrotic spot virus* (MNSV), a virus belonging to the *Tombusviridae* (Nieto et al. 2006). The susceptible genotypes carry H228 *allele* (*NSV*) and the resistant genotypes carry a L228 (*nsv*). Genetic transformation studies also demonstrated that the expression of the *nsv* allele caring H228 in resistant melon is sufficient to restore susceptibility to the NRB strain of MNSV. Beside that knowledge no references about FMs development were found.

In watermelon, (*Citrullus lanatus* [Thunb.] Matsum. & Nakai var. *lanatus*) the *Zucchini yellow mosaic virus* (ZYMV) is one of the most economically important potyviruses (Ma et al. 2005). According Ling et al. (2009) ZYMV resistance are controlled by different allelic SNP mutations in the same *Citrullus eIF4E* gene resulting in allelic series. Sequence alignment between gene sequences of susceptible/resistant genotypes revealed the existence of three polymorphic sites (Table 4), two SNPs located in intron 1 and one nsSNP at exon 1, which is responsible by T81P substitution, unique for the ZYMV-resistant PI 595203 genotype (Ling et al. 2009). T81P is predicted to be located in the critical area for cap recognition and binding. SNPs are in the neighborhood that are related with virus resistance in other plant species, like L79R in *pvr1*^{1} and *pvr1*^{2} in pepper and A77D in *pot-1* in tomato (see Table 4). An additional nsSNP (A171G) responsible for aa substitution D71G was identified in four ZYMV-resistant *C. lanatus* var. *citroides* PIs. Functional co-dominant markers were developed to differentiate between ZYMV-resistant and susceptible plants (Table 4).

3.2.3.1.6. Common bean

In common bean (*P. vulgaris*) four recessive genes have been proposed to control resistance to the potyviruses *Bean common mosaic virus* (BCMV): *bc-1*, *bc-2* and *bc-3* (Naderpour et al. 2010). *PveIF4E g*ene cloning and sequence analysis revealed the existence of four nsSNPs responsible by aa changes at positions N53K, F65Y, A76E and D111G, defining a susceptible alleles $PveIF4E^{1}$ and a resistant $PveIF4E^{2}$. Bean genotypes reported to carry *bc-3* resistance were found to have that set of mutations, which in known to determine potyvirus resistance in other species, which place the $PveIF4^{2}$ as a strong candidate gene for *bc-3* (see table 4). The existence of polymorphisms directly related with BCMV resistance allowed to the development of a codominat FM. It application in a segregating F_2 population revealed that only plants homozygous for the $PveIF4E^{2}$ allele resisted virus infection, which allow to consider this FM as a useful tool to investigate further potyviral resistance in this species (Naderpour et al. 2010).

4. Plant plasticity – a new trait across species and plant systems

Plants as sessile organisms learned during evolution to respond to diverse environmental constraints and opportunities in terms of adaptive growth and development with species-specific and across species characteristics. The potential for adaptive plasticity can influence the stability of plant biomass and yield production but also the capacity for efficient adventitious morphological responses upon stressful treatments, such as adventitious rooting (Macedo et al. 2009) or formation of somatic embryos (Zavattieri et al. 2010), which can be important traits for cost- and time-efficient plant production. Differences in the robustness of plant genotypes to grow under diverse environmental conditions and in recalcitrant behavior related to inducing

conditions for adventitious organogenesis or somatic embryogenesis are well described across species by a vast number of authors. However, so far the capacity for plasticity, although known as a main driver in evolution for organisms to occupy ecological niches, has not been explored as a trait *per se* for molecular plant breeding. Recently, alternative oxidase (AOX) was proposed in a hypothesis-driven approach as a functional marker for efficient cell reprogramming that could be developed for common stress-confronting traits across species and stresses, such as adventitious root hair development under nutrient stress (Arnholdt-Schmitt et al. 2006; Arnholdt-Schmitt 2009; Polidoros et al. 2009; see also www.aox2008.uevora.pt and Physiologia Plantarum 2009, special issue: alternative oxidase Vol. 137 (issue 4)). Functional polymorphisms in AOX genes are under study in various plant species and systems related to stress behavior of importance for plant breeding (Abe et al. 2002; Holtzapffel et al. 2003; Cardoso et al. 2009 and 2011; Frederico et al. 2009a and 2009b; Campos et al. 2009; Ferreira et al. 2009; Macedo et al. 2009; Costa et al. 2009a and 2009b).

Searching for FMs directly linked with the capacity to react with efficient phenotypic plasticity across species is a novel strategy in molecular plant breeding and functional domains in target genes need to be identified. Physiological and morphological plasticity can be reflected by plasticity at genome level due to the flexibility in linear sequence modulation, DNA and histone modifications and the structural organization of genomic DNA in the chromatin (Arnholdt-Schmitt 2004, Arnholdt-Schmitt 2005 , Fransz and De Jong 2011).

5. Conclusions

In the last 20 years routine protocols have been developed to identify and characterize genetic loci that contribute to quantitative traits. However, the capacity to

zoom into natural segregating loci with quantitative or qualitative effects to find the molecular base of phenotypic variability has been accelerated only recently thanks to new DNA sequencing technologies. This explains the low number of FMs available comparing to QTLs known for the same traits. Nevertheless, the number of genes available for FM development is increasing. Candidate gene approaches for marker-assisted selection are rated as the most promising strategies in molecular plant breeding. Genes of interest for FM development can be identified by high-throughput sequencing or differential gene analysis or by hypothesis-driven research approaches.

However, the reviewed results do not indicate that searching for conserved FMs in traits across species could be a successful strategy for predicting common phenotype variation. Polymorphic sites seem to have low degrees of conservation. Loss-of-function polymorphisms seem to play an important role for trait variability. Phenotypic variation in the same trait across species may be linked to a diversity of sequence polymorphisms in the according orthologous genes. The review seem to confirm Risch (2000) who concluded that SNPs located in coding regions causing non-synonymous non-conservative amino acid changes are more likely to be functional than non-synonymous conservative and synonymous amino acid substitution.

As a summary and consequence, we would like to stimulate discussion on the perspective that FM development for adaptive phenotype variation for selected traits across species should better focus on polymorphic patterns in functional domains of genes involved in superimposed metabolic pathways and in the capacity for reorganizing genome structures through epigenetic mechanisms rather than on conserved polymorphisms in individual areas of downstream genes.

Acknowledgements

The authors would like to thank to Luz Muñoz for the critical revision and comments.

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