

Allelic Variation of Salinity Tolerance Genes in Barley Ecotypes (Natural Populations) Using EcoTILLING: A Review Article

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Abstract: Crop plants are usually affected by biotic and abiotic stresses that reduce their relative yields. Soil salinity is abiotic stress factor that poses a serious threat to agricultural production, as more than 800 million hectares of land in the world have been salt affected, which account for 6% of the total land area. Impacts of climatic and anthropogenic factors such as soil salinity, soil acidity and depletion of soil organic matter leading to reduced soil productivity continue to be a constraint and will become more serious under climate change and variability. Understanding the molecular basis of salinity will be helpful in developing selection strategies for improving salinity tolerance. To determine gene function relationships, large scale genome-wide reverse genetics approaches have been developed which includes both non-transgenic technology platforms such as TILLING (targeting induced local lesions in genomes) and EcoTILLING and insertional mutagenesis systems based on transgenic technology. In this review we discuss effects of salinity on plant growth and production, different mechanisms used by plants to handle this problem, the genes related to salinity tolerance in barley (and other plants) and the potential of EcoTILLING technique for detection of salinity stress genes variation in barley.

Key words: Allelic variation • Barley ecotypes • Salinity tolerance • EcoTILLING

INTRODUCTION

The food production is the main obsession in the next two decades due to the projected increase in human population of 1.5 billion people, coupled with projected increase in urbanization in developing countries. Yet, the pressure of urbanization and industrialization of fertile agricultural fields reduced the amount of land suitable for farming and caused more shrinkage in agricultural acreage due to salt accumulation in arable lands.

Salinity not only hinders normal plants' growth and development but also crop production worldwide. Artificial salinization occurred since humans practiced irrigated agriculture. It is estimated that about 20% of the world's cultivated area and nearly half of the world's irrigated lands are affected by salinity [1]. Based on the FAO-UNESCO soil map of the world, over 800 million hectares of land throughout the world are salt-affected, either by salinity (397 million ha) or sodicity/ alkalinity (434 million ha) [2-4]. Salinization is an ever increasing problem worldwide and is progressively causing decline

in crop production in large areas of the world. Consequently, soil salinization was identified as a major cause of land degradation with the world losing at least three hectares of arable land every minute and based on other reports, nearly 10 million hectares annually, due to excessive salt accumulation and/or waterlogging [5]. It is estimated that only about 10% of the world's total land is currently non-stressed, good cropland [6]. The vast majority of arable or potentially arable lands are limited in terms of crop productivity by a complex of environmental stresses; predominately excess salts, drought and soil acidity. Excess salt in soil and/or water is at the heart of today's major agricultural constraints.

Rapid and inadequate development of large-scale irrigation systems and poor drainage has led to further salinization [2, 7]. Other activities such as land clearing and replacing of native trees with shallow-rooted crops further aggravate dryland salinity [7]. Salinity is one of the main factors limiting crop production and their further area of expansion worldwide. Excess salinity in soil has devastating effects on plant growth, reduces crop yields

or even leads to complete crop failure in severe cases. In general irrigation water with an EC < 0.75 dS/m is considered non-problematic, while irrigation water with an EC 0.75-3.0 dS/m may pose an increasing problem and irrigation water with an EC greater than 3.0 dS/m will pose a severe problem [8].

Soil salinity affects plant growth and development by causing osmotic stress, ion toxicity and nutrient imbalances [9-11]. The most common soluble salts in soils are chlorides and sodium sulfates, calcium and magnesium; with sodium and chloride being the dominant ions [12]. Since enhancing the tolerance of plants to environmental stresses especially salinity and drought, is one of the most urgent needs in plant breeding. Plant breeders have tried to develop varieties tolerant to salinity and other related abiotic stresses that also have desirable agronomic characteristics [13, 14]. Progress in breeding of salt tolerant crops, however, has been hampered by a number of factors including the complexity of the tolerance traits, insufficient genetic knowledge of individual physiological components of tolerance, lack of understanding of the molecular basis of salt tolerance, little or no correlation among tolerance mechanisms at different developmental stages and lack of valuable selection criteria [15]. Apparently, progress in developing salt tolerant crop varieties requires a thorough understanding of the physiological, biochemical and genetic basis of salt stress [15, 16]. Only few aspects of the numerous salt stress-tolerance traits found in nature had been unraveled *via* the application of molecular tools such as gene disruption and transgenic approaches.

Understanding the molecular basis of salt-stress signaling and tolerance mechanisms is essential for breeding for salt tolerance in crop plants through genetic engineering [2, 17]. Salinity tolerance is expressed as the ability to survive and grow in a saline medium and conferred by genes that control salt uptake through roots and its distribution within different plant organs; genes that affect ionic and osmotic balance of cells in roots and shoots; and genes that regulate leaf development and the onset of senescence [11]. Understanding the mechanisms of plant salt tolerance will lead to an effective means to breed or genetically engineer salt tolerant crops. Salt tolerance research also represents an important part of basic plant biology, contributing to our understanding of subjects ranging from gene regulation and signal transduction to ion transport, osmoregulation and mineral nutrition. Additionally, some aspects of salt stress responses are intimately related to drought and cold

stress responses [16, 17]. Plant salt tolerance studies thus contribute to the understanding of the mechanisms of cross-tolerance of abiotic stresses. Although appreciable progress has been made in the general understanding of the physiology of the relationship between plants and salt, yet less information is available for a comprehensive understanding of the principal physiological mechanisms of salt damage and tolerance.

A better understanding of the mechanisms involved in the inhibition of plant growth by salinity may accelerate genetic manipulations aimed at increasing crop salinity tolerance. Extending the knowledge on physiological mechanisms of salt tolerance is of utmost importance in developing plants better adapted to saline and sodic soils [17, 18]. Many scientists have reported that one of the most important physiological mechanisms of salt tolerance in plants is the selective absorption of K⁺ by the plant from the saline media [13, 18-20]. Considerable progress was also made in understanding the molecular bases of salt tolerance in plants and this has been extensively reviewed elsewhere [17, 21-26].

Transgenic approaches showed that significant improvement in tolerance could be achieved through improvement of fewer target traits [2, 27-31].

Salinity Stress in Barley: Barley (*Hordeum vulgare* L.) is an important food and fodder crop which is widely cultivated in saline areas as one of the most salt tolerant field crops [32, 33]. Identification of responsive genes for biotic and abiotic stresses is a fundamental step in developing new tolerant varieties through conventional and modern plant breeding. So far, not many studies have been applied to target the responses of genes to a salinity stress, or to detect the responsible genes for salinity tolerance in barley. A prominent feature of the response to salinity is the induction of genes involved in jasmonic acid biosynthesis and genes known to respond to jasmonic acid treatment. A large number of abiotic stresses (heat, drought and low temperature) related genes were previously found to be responsive to salinity stress [34].

Salinity stress in *Arabidopsis*: The research on high-salinity responses in *Arabidopsis* implied that a large proportion of the genome is involved in high-salinity stress responses [35-37]. In several cases, it has been shown that alteration of individual gene expression level can significantly impact responses to high-salinity stresses in plants [38-41].

Genes Involved in Salinity Tolerance in Barley and Arabidopsis:

HVA1 gene has been reported as a late embryogenesis abundant (LEA) protein gene, from barley (*Hordeum vulgare* L.). The HVA1 transgenic rice plants had showed significantly increased tolerance to water deficit and salinity [40]. Expression of the barley HVA22 gene found also to be induced by environmental stresses, such as dehydration, salinity and extreme temperatures and by a plant stress hormone; abscisic acid [42]. AtHVA22D (*Arabidopsis thaliana* HVA22 barley homologue D) and AtHVA22E genes in *Arabidopsis* encodes two out of five HVA22 homologs in *Arabidopsis*.

At each stage of plant development, salt tolerance appears to be controlled by more than one gene and to be highly influenced by environmental factors [43]. Barely has been found to be tolerant to salinity at germination, sensitive at the seedling and early vegetative growth stages and then tolerant again at maturity [44]. The ABA- and stress-inducible gene first isolated from barley, where members of this gene family have only been found in eukaryotes. AtHVA22E mRNA is upregulated to varying degrees in response to cold stress, salt stress, ABA treatment or dehydration-Plasma membrane Na⁺/H⁺ antiporter gene has showed improving in salt tolerance in *Arabidopsis* [39].

Functional Genomics: Two main approaches of functional genomics are forward and reverse genetics. In forward genomics (from phenotype to genotype), phenotypic characterization of big populations or gene bank collections is laborious and expensive task. Therefore, the

alternative approach for saving time and cost is needed for this purpose. In reverse genetics (from genotype to phenotype), the gene sequence is known and mutants are screened to identify individuals with structural alterations in the gene of interest [45, 46].

Some of the reverse genetic strategies employed in plants include homologous recombination, *Agrobacterium* mediated insertional mutagenesis, transposing tagging, RNAi (RNA interference) or PTGS (post transcriptional gene silencing) and using mutagenesis [47].

TILLING/ EcoTILLING, are reverse genetic methods utilizing mutation detection strategy to elucidate gene function and finding desired genotypes. They generally characterize Single Nucleotide Polymorphism (SNP) in induced mutation and natural mutation populations, respectively. In addition, they have capability to detect insertions/ deletions (INDEL) and small repetition numbers and they are time-saving and cost-effective methods compared to other reverse genetic techniques.

Information Resource for Functional Genomics Related to Stress Genes:

Figure 1 shows the step-by-step functional genomics strategy to study stress tolerance in plants. There are some database and websites, which are resources for functional genomics research. One major database dedicated to stress genomics is the Stress Functional Genomics Consortium (<http://stress-genomics.org>). It contains a collection of stress responsive ESTs from *Arabidopsis thaliana*, *Oryza sativa*, *Mesembryanthemum crystallinum*, *Hordeum*

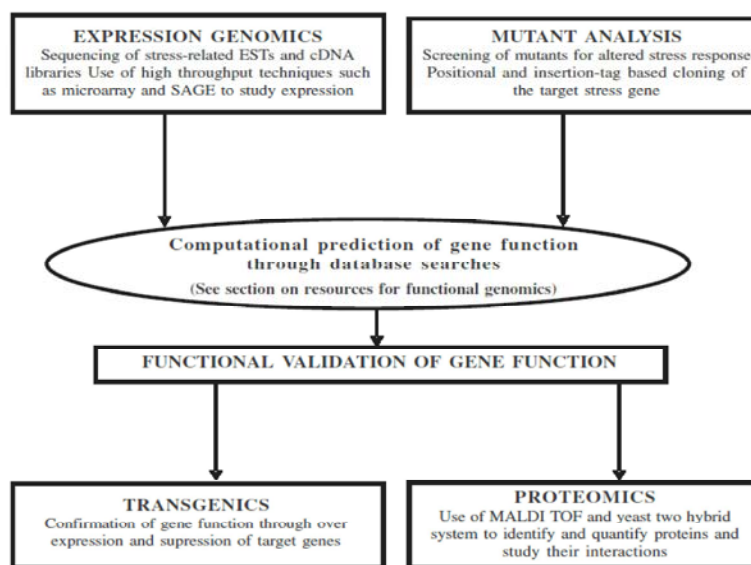


Fig. 1: A step-by-step functional genomics strategy to study stress tolerance in plants.

vulgare, *Selaginella* and *Dunaliella*. A collection of Arabidopsis mutants altered in stress signaling and global gene expression profiles of Arabidopsis, rice and ice plant under stress conditions can also be accessed through the site [48].

Salinity Tolerance Genes Identification Approaches:

Different approaches are currently in place to identify candidate genes contributing to salinity tolerance in plants. Through genetic analysis, major Quantitative Trait Loci (QTLs) associated with salinity tolerance have been identified and this facilitated targeting specific chromosomal regions for gene discovery through map-based cloning [9, 49-52]. Combining microarray, TILLING/EcoTILLING technology with QTL analysis could speed up the process of gene discovery by limiting the focus to fewer genes in the QTL regions.

Moreover, in depth analysis of the important traits that cause tolerance and deciphering the individual biochemical pathways underlying each mechanism could lead to gene discovery. This could be achieved by targeting genes or trans-acting factors regulating key steps in their biochemical pathways. However, this approach is limited by the extent of comprehension of such biochemical pathways [9, 50]. Analysis of gene expression profiles using cDNA microarrays proved to be an effective means of identifying large number of responsive genes. Combining genetic and phenotypic data with microarray analysis could further enhance the efficiency of candidate gene discovery for complex traits associated with tolerance to abiotic stresses. Fewer genes could be targeted if the focus is limited to specific locations on the chromosomal regions associated with major QTLs, or to genes involved in biochemical pathways of important component traits [50, 53, 54].

One of the promising approaches for gene discovery for different biotic and abiotic stresses is the use of induced or spontaneous mutations. This approach also helps in studying the effect of specific mutations on the phenotype of the mutant plants and traces them back to the gene level. With sequence information becoming increasingly abundant and accessible through whole genome sequencing projects, mutational analysis takes on an even more important role in the assignment of putative functions to specific genes in a genome. In rice and barley, the systematic production of mutants began in several laboratories largely in response to the rapid progress in structural delineation of their genome [55, 56]. However, good genetic variation for salinity tolerance among plants, including barley genotypes was reported [57].

SNP Genotyping Methods: There are many mature SNP genotyping technologies that have been integrated into large-scale genotyping operations. The choice of a method depends on the scale of the envisioned genotyping project and the resources available. SNP genotyping methods are very diverse [58, 59]. The SNP genotyping methods include restriction endonucleases digestion, primer extension, hybridization, oligonucleotide ligation (OLA), gel electrophoresis, mass spectrometry, fluorescent analysis commercial platforms for SNP genotyping TaqMan assay, iPLEX GOLD assay, Golden Gate assay, Infinium assay, GeneChip assay, using SNP databases, methylation analysis and second generation sequencing technologies (Barley SNP database is, http://bioinf.scri.ac.uk/barley_snpdb).

Consensus Maps in Barley Using SNP Markers: For making high-density genetic maps it needs to use accurate marker datasets. For example, Close [60] used complete and error-free datasets and genetic markers to make a high-density consensus genetic map that is supported by a readily available SNP genotyping resource. They identified 22,000 SNPs from barley ESTs and sequenced amplicons; 4,596 of them were tested for performance in three pilot phases Illumina Golden Gate assays. Data from three barley doubled haploid mapping populations supported the production of an initial consensus map.

Maps that include SNPs in protein-coding genes facilitate genome content comparisons by virtue of the high conservation of protein sequences across genera, thus enabling sequence similarity searches to find orthologs [60].

Consensus maps for barley has increased dramatically in recent years. A consensus map was developed containing 1230 markers (RFLP, AFLP, SSR, SNP) from three doubled haploid populations [61]. Moreover, Wenzl [62] combined DArT with RFLP, SSR and STS from nine mapping populations to create a consensus map containing 2935 markers. Marcel [63] compiled RFLP, AFLP and SSR data from six mapping populations to produce a consensus map containing 3458 markers. Stein [64] used three doubled haploid mapping populations and combined new data from 1,055 markers (RFLP, SSR, SNP) with prior data from 200 anchor markers to produce a 1255 marker consensus map. Varshney [65] produced a 775 SSR consensus map by joining six independent maps. Potokina [66] combined SNP and other transcript derived markers to position 1596 loci on the Steptoe × Morex [67] linkage map. Hearnden [68] combined 1000 SSR and DarT markers on a map from a

wide cross. Several additional consensus maps have used portions of the SNP data, as a major marker to make these maps.

EcoTILLING: EcoTILLING is a reverse genetic approach, a molecular technique similar to TILLING except that it targets natural genetic variations instead of induced mutations in TILLING. EcoTILLING could ease the discovery of natural variants and their putative gene function [69]. This approach allows faster and lower cost identification of naturally occurring SNPs and/or small INDELS in gene of interest. The method has proven to be successful to detect DNA polymorphisms including variations in satellite repeat number [70]. Furthermore, in highly heterozygous outcrossing species, EcoTILLING could be used to determine heterozygosity levels within a gene fragment [69]. EcoTILLING reduces the time and effort for SNP discovery generally required by weeding out identical haplotypes. In the future, new cultivars could be explored by EcoTILLING that contain the alleles originated of their wild relatives.

Many EcoTilled genes have been screened in natural populations that include, FAE1 (fatty acid elongase 1), which is involved in the control of erucic acid synthesis in *Brassica* species [71]; Ara d 2.01 (conglutin gene), the orthologue of Ara h 2.01, which codes the seed storage protein in *Arachis duranensis*, which is a very potent allergen for humans [72]; and Pina-D1 (puroindoline a) and Pinb-D1 (puroindoline b), which mainly condition kernel hardness through allelic variations in these genes in *T. aestivum* [73]. Also, EcoTILLING was used for the detection of single nucleotide mutations in the ALS genes of sulfonylurea (SU) resistant (R) *Monochoria vaginalis* (Pontederiaceae), a paddy weed in Japan.

Genomic DNA of SU-R plant was mixed with the DNA of susceptible plant and EcoTILLING showed two nucleotide mutation in the ALS gene of SU-R *M. vaginalis* [74]. Searching new virus- resistance alleles in natural population has done for 4E and 4G protein families mediate resistance to several RNA plant viruses in natural crop diversity [75]. Nucleotide changes in the genes eIF4E and eIF(iso) 4E of translation initiation factors have been detected in *Capsicum annuum* [76].

Nieto [75] used EcoTILLING technique for identification of allelic variation of melon eIF4E, a factor that controls virus susceptibility. They characterized 113 accessions of *Cucumis* spp. for susceptibility to Melon Necrotic Spot Virus (MNSV) and Cucumber Vein Yellowing Virus (CVYV). They found six polymorphic

Table 1: List of the most important genes related to salinity stress tolerance in barley and their function

Gene names	Function of gene
HvHAK1	K ⁺ transporter
HvHAKT1	K ⁺ transporter
HvHVA/68	H ⁺ ATPase
HvHVP1	H ⁺ ATPase
HvHNX1	Na ⁺ /H ⁺ antiporter
HvHNX3	Na ⁺ /H ⁺ antiporter
HvHNX4	Na ⁺ /H ⁺ antiporter
HvHKT2	Na ⁺ and K ⁺ transporter
HvCBL4	Na ⁺ /H ⁺ antiporter
HvHKT1	Na ⁺ transporter
HVA22	Na ⁺ transporter

Source: [85]

sites with high conservation of eIF4E exonic regions that just one of them was correlating with MSNV resistance and others were silent mutations. They have also characterized a new allele of eIF4E from *Cucumis zeyheri*, a wild relative of melon. Functional analyses suggested that this new eIF4E allele might be responsible for resistance to MNSV. EcoTILLING has also been used in *Solanum tuberosum* [77] and *Musa* species diploid and polyploid accessions [78].

EcoTILLING in Barley: EcoTILLING technique was used in barley for different purposes including discovery and detection of DNA polymorphism in the mildew resistance genes “mlo” and Mla of barley. Eleven mlo mutant lines of barley, *Hordeum vulgare* L., the cultivar “Ingrid” (mlo WT) and 25 barley lines including cultivars from Europe, near isogenic lines in a “Pallas” background and derivatives of wild barley containing known mildew resistance locus (Mla alleles) were tested. It was concluded it is possible to combine different mlo alleles with different Mla alleles from wild barley to obtain cultivars with more durable resistance [79].

Characterization of VRNH1 and VRN-H2 haplotypes in 429 varieties of barley, analysis of genotype intron I sequencing data and growth habit tests identified three novel VRN-H1 alleles and determined the most frequent VRN-H1 intron I rearrangements. Combined analysis of VRN-H1 and VRN-H2 alleles resulted in the classification of seventeen VRN-H1/VRN-H2 multi-locus haplotypes, three of which account for 79% of varieties [80]. Hofinger [81] used a rapid and cost-effective method (HRM) for identification of novel allele in barley. They studied 96 barley accessions by this technique and reported sensitivity and accuracy of HRM for predicting genotypes carrying a wide range of nucleotide polymorphisms in eIF4E approached 100%. Results of

Table 2: List of genes used for EcoTILLING

No.	Candidate gene, abbreviation	Available molecule type	Locus	Reference
1	<i>H.vulgare</i> AR-h gene for aldose reductase, HVARH1	genomic DNA	Z48360	[86, 87]
2	<i>Hordeum vulgare</i> srg6 gene for stress responsive gene protein 6, SRG6	genomic DNA	AJ300144	[88]
3	<i>Hordeum vulgare</i> AP2 transcriptional activator (DRF1) gene, HvDRF1	genomic DNA	AY223807	[89]
4	<i>H.vulgare</i> HVA1 gene, HVA1	genomic DNA	X78205	[90]
5	<i>Hordeum vulgare</i> subsp. vulgare dehydration responsive element binding protein 1 (DREB1) mRNA, HvDREB1	mRNA	DQ012941	[91]
6	<i>Hordeum vulgare</i> HvNHX1 mRNA for sodium/proton antiporter, HvNHX1	mRNA	AB089197	[92]
7	<i>Hordeum vulgare</i> HVP1 mRNA for vacuolar proton-inorganic pyrophosphatase, HVP1	mRNA	AB032839	[92]
8	Barley fungal pathogen induced mRNA for pathogen-related protein, HvPPRPX	mRNA	X16648	[93]
9	NUD putative ethylene-responsive transcription factor", HvNUD	genomic DNA	AP009567	[49]

their study were promising and suggested that this method could also potentially be applied to the discovery of superior alleles controlling other important traits in barley as well in other model and crop plant species. Guo [82] established an EcoTILLING protocol with M13 primers in barley for SNP discovery. They used gene specific primers and M13 primers labeled with IRD fluorescence dyes to study polymorphism in chlorophyll a/b binding protein gene (Cab) of 66 accessions originated from 19 countries. They found six nucleotide polymorphism and reported by this protocol EcoTILLING can be done with lower cost and it facilitates reverse genetic and characterization of natural nucleotide variation in barley in large scale experiments.

Allele mining and haplotype discovery in nine candidate genes and 96 barley genotypes for drought tolerance, 185 single nucleotide polymorphisms (SNPs) were identified and 46 insertions/ deletions (INDELS) by EcoTILLING technique were detected with a mean of 1 SNP/ 92 bp and 11 INDEL/ 372 bp genomic sequence [83]. The barley haplotype database for drought- related candidate genes are listed in Table 2.

EcoTILLING to Identify Allelic Variation for Salinity:

EcoTILLING was used to identify natural allelic variants of rice candidate genes involved in salinity tolerance. Negrão [84] studied 375 germ plasm accessions and found that a total of 15 and 23 representative SNPs or INDELS in OsCPK17 and Sa genes, respectively. They reported that these natural allelic variants are mostly located in 3'- untranslated region, thus opening a new path for studying their potential contribution to the regulation of gene expression and possible role in salt tolerance.

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