Genomics Approaches For Improving Salinity Stress Tolerance in Crop Plants

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Abstract: Salinity is one of the major factors which reduces crop production worldwide. Plant responses to salinity are highly complex and involve a plethora of genes. Due to its multigenicity, it has been difficult to attain a complete understanding of how plants respond to salinity. Genomics has progressed tremendously over the past decade and has played a crucial role towards providing necessary knowledge for crop improvement. Through genomics, we have been able to identify and characterize the genes involved in salinity stress response, map out signaling pathways and ultimately utilize this information for improving the salinity tolerance of existing crops. The use of new tools, such as gene pyramiding, in genetic engineering and marker assisted breeding has tremendously enhanced our ability to generate stress tolerant crops. Genome editing technologies such as Zinc finger nucleases, TALENs and CRISPR/Cas9 also provide newer and faster avenues for plant biologists to generate precisely engineered crops.

1. INTRODUCTION

Crop plants in the fields are usually subjected to one or more biotic and/or abiotic stresses which reduce agricultural output significantly, leading to a huge yield-gap. Soil salinity is one such stress which reduces agricultural output drastically. The FAO/UNESCO soil map of the world (1970-1980) revealed that 20% of irrigated land is affected by salinity and approximately 2.1% of total dry land agricultural fields are salt affected. The current actual land area affected by salinity is unknown but estimated to be higher than one-third of total irrigated land. Soil salinization is also continuously increasing with increased irrigation in semi arid and arid regions. In addition, estimates show that despite global hunger reduction, approximately 805 million people are chronically undernourished [1]. Moreover, the world population is increasing at such an exponential rate that it is expected to cross 9.5 billion by the year 2050 [2]. Ironically, the amount of land devoted for agriculture has increased by only 12% over the last 50 years, that too, mostly at the expense of natural ecosystems [3].

Through improved farming practices and conventional breeding, the global increase in food production is about 32 metric tons per year, which is 12 metric tons less than what is required as per the target of the Declaration of the World Summit on Food Security which aims to acquire 70% more food by 2050 [4]. Therefore, to meet the requirements of the growing population, there has been and still is a need to abate the losses incurred through various environmental stresses including salinity. In this regard, plant scientists and breeders have been working towards obtaining crop plants which have higher tolerance to salinity than the present day cultivars. The plant research community has taken different approaches towards achieving this goal with plant breeding leading the way, as genetic engineering of crop plants (transgenic technology) is still being disputed in most countries due to ethical and political issues. On the other hand, a combination of the latest molecular biology advances and traditional plant breeding has now been widely utilized and proved to be highly successful for many crop plants. In the last 30 years or so, there has been tremendous advancements in the field of genomics in particular, which have enabled us to get a much better understanding of how plants perceive and respond to environmental stresses. Genomics is proving to be a useful tool in attaining the necessary information for raising stress tolerant transgenic plants and breed better varieties of crops. In this review, we focus our attention to the various genomics based approaches developed over a period of time which are contributing towards better understanding of salinity tolerance in crop plants and, thus, improving their stress tolerance.

2. IDENTIFICATION OF THE GENES INVOLVED IN THE SALT STRESS RESPONSE

In order to improve salinity stress tolerance of crop plants we must first have a comprehensive understanding of the mechanisms through which plants respond to salinity stress. Identifying the genes involved in salinity stress response is thus the first important step towards attaining the necessary

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knowledge for generating salinity stress tolerant crops. However, identification of the stress responsive genes is just one step which is then followed by functional characterization of the genes as well as deciphering their mechanism of action. The final step includes the application of the acquired knowledge in raising stress tolerant crops either through genetic engineering or through molecular breeding. Arabidopsis and rice were the first two plant species whose genomes were sequenced and, thus, most of the research in plant science has been carried out using these two plants as models. This is primarily due to the fact that the data obtained from the complete genome sequencing projects provide valuable information regarding the genes to be analyzed [5]. Of the various approaches available for gene discovery in both model and non-model species, some of the most utilized are described briefly.

2.1. Forward Genetics and the Candidate Gene Approach

Since basic processes such as plant development and stress response mechanisms follow similar pathways in different plant species, genes of model species can be directly expressed in non-model species for crop improvement and/or serve as candidate genes for the identification of orthologs in non-model species [6]. Using both forward genetics and reverse genetics approaches on a genome-wide scale, researchers have been able to identify many salt stress responsive genes in various model as well as non-model species. For example, using a forward genetics approach Wu and colleagues [7] showed that *salt overly sensitive 1 (sos1)* mutants of Arabidopsis were extremely hypersensitive to NaCl and two years later Zhu and colleagues [8] isolated and characterized several Arabidopsis mutants with limited shoot growth under salt stress which led to the identification of the *SOS1, SOS2, and SOS3* as the fundamental components of the SOS pathway for Na$^+$ exclusion from the cells. Using Arabidopsis SOS pathway genes as the candidate genes, similar homologues have also been identified in rice, poplar and tomato, of which only *SOS1* has been characterized in all three species [9-11]. Similarly, using the Arabidopsis genome sequence (partially sequenced at the time) the Na$^+$/H$^+$ plasma membrane antiporter of Arabidopsis, *AtNHX1* was identified through sequence homology with characterized animal Na$^+$/H$^+$ exchanger (NHE) family and the yeast *ScNHX1* [6]. Heterologous overexpression of the *AtNHX1* suppressed the salt sensitive phenotype in a yeast *nhx1* mutant and conferred salt tolerance to Arabidopsis as well as tomato and *Brassica* [12-16]. This highlights the conserved nature of the salinity response not only among different plant species but also among different genera, thus, making the 'candidate gene' approach for identification of orthologs a highly relevant genomics approach for gene identification/discovery in different species.

2.2. Comparative Gene Expression Analysis

Another method for identification of genes involved in abiotic stresses, widely adopted in the recent past, is high throughput gene expression analysis using technologies such as suppression subtractive hybridization (SSH), expressed sequence tags (ESTs), microarray, serial analysis of gene expression (SAGE) and massively parallel signature sequencing (MPSS). Experimental designs included comparative gene expression analysis between; i) stressed and unstressed samples of the same species, ii) contrasting genotypes of the same species iii) phenotypically contrasting organisms of different but related species and iv) expression analysis of extremophiles [17]. With regard to salinity stress, these high throughput techniques were highly successful since a number of salinity responsive genes have been identified in many crop species [18-25]. Though SSH continues to be a highly proficient tool for species with limited genome sequence information, next-generation sequencing (NGS) (RNA-Sequencing) is emerging as a method of choice for gene expression analysis. This is because RNA sequencing offers multiple advantages over the existing techniques such as: genomic sequence is not a pre-requisite for transcript detection using RNA sequencing unlike hybridization techniques; location of transcription boundaries to a single-base resolution is possible through RNA sequencing [26]; RNA sequencing has very low background noise, costs less than Sanger based sequencing methods, and has a large dynamic range for gene expression analysis [26]; RNA sequencing can detect single base genetic variations such as SNPs which can be extremely useful in discovering superior alleles [27]; RNA sequencing can also detect small RNAs and long noncoding RNAs which play a key role in regulation of gene expression [28, 29]. There are many reports where RNA sequencing was successful in identifying key regulating factors of the salinity stress response in crops such as previously unidentified transcripts or miRNAs in rice, soybean, broccoli, sugarcane and many more [30-34]. An example of the usefulness of NGS for gene discovery is provided in the work carried out by Severin and colleagues [35], who used RNA-Seq for transcriptome profiling of soybean and identified 177 genes with a functional role in the process of seed filling.

2.3. Association Genetics

So far, almost all of the techniques mentioned provide information which can be applied to modern biotechnological methods for crop improvement. When it comes to conventional plant breeding, there has been fairly good progress on the genomic scale as well. NGS based methods have been developed which enable the discovery and genotyping of thousands to hundreds of thousands of markers in tens to hundreds of individuals [36]. Association genetics has been widely used recently to map candidate genes, QTLs and other genetic markers. Whole Genome Scan or genome wide association studies (GWAS) in crops have recently picked up and proven to be a highly useful tool for allele and marker discovery. In one of the first studies on crop plants, Huang and colleagues [37] performed NGS based genotyping and GWAS for 373 *Oryza sativa* indica lines and obtained a genetic map at fine resolution for 14 agronomic traits ranging from yield to grain quality to abiotic and biotic stress tolerance. Recently, EcoTILLING (Eco- Targeting-Induced Local Lesions IN Genomes) was used to genotype 392 accessions of rice followed by a candidate-gene association mapping approach, which managed to identify 11 SNPs in the coding regions of five known salt tolerance genes associated with salinity tolerance [38]. More recently, Kumar and colleagues [39] carried out genotyping and GWAS on 220 indica rice

accessions and were able to map three new QTLs for salinity tolerance in rice, one each on chromosomes 4, 6 and 7 [13]. Thus, association mapping is proving to be a very useful tool for discovering accessions with novel salinity tolerance genes, potentially superior alleles and QTLs.

2.4. Computational Tools and Databases

With the advent of NGS technologies, draft genome sequences have been assembled for many plant species including crops such as tomato, potato, barley, maize, wheat and many more, but the quality and coverage of the genomes are not at par with that of Arabidopsis and rice [40-44]. This increase in the number of sequenced genomes has further led to the development of a large number of web based databases for organisms with sequenced genomes. These databases have now become an invaluable resource for plant biologists worldwide [45]. In salinity stress related studies, with regard to gene discovery, databases provide a quick and calculated method for identification of putative genes involved in the stress response through sequence and structure homology as well as similar expression patterns with known salinity responsive genes of the same or other species. However, it should be noted that information acquired through databases are merely predictions and need to be validated through biological assays. Nevertheless, these databases can provide a starting point for experiments or can be used as tools to make calculated selections based on observations preceding target gene identification studies.

3. FUNCTIONAL CHARACTERIZATION OF GENES/QTLs/ALLELES

Once a gene/allele or QTL has been identified, the next logical step is to characterize it. Thus, their function first need to be verified *in vivo* before they can be considered for use in crop improvement, as generating field quality crops through genetic engineering and/or breeding is a time consuming, laborious and relatively expensive task. As a consequence, there have been multiple tools developed for characterization of gene function(s). With regard to salinity stress, these tools have been highly helpful in validating the function of genes and QTLs to be used for crop improvement. In combination with conventional biochemical and physiological assays, these tools have been useful in mapping out stress signaling pathways and elucidating the role of members of different pathways which enable researchers to acquire a holistic systems biology perspective of the stress response mechanisms of plants. A few of these tools and their application towards salinity stress associated gene(s)/QTL(s) function validation are listed below:

3.1. Mutagenesis

The most convenient method to assess the function of a gene is to analyze the plant's phenotypic variations associated with the gene's inactivation [46]. Thus, loss-of-function mutants along with knockdown mutants serve as a valuable tool for gene function characterization. Mutagens are of three types: chemical, physical and biological. While chemical and physical mutagens can generate small insertions/deletions and point mutations, disruption of gene function can be obtained through insertion of large DNA fragments such as

T-DNA and transposable elements [47]. The problem with chemical and physical mutagenesis is that they are random events and can occur anywhere in the genome and multiple events can occur as well. There is no way to direct the mutation event to our gene of interest. Further, mutation doesn't always lead to a loss of function especially in the case of point mutations. Thus, thousands of mutants are generated with the hope that at least one of them is the mutant of interest. Identification of the desired mutant and elimination of the undesired background mutation through backcrossing with the wild type is also a tedious and laborious task. The use of NGS can greatly simplify the genotyping of mutants but backcrossing remains the only way to eliminate the background noise.

The development of Targeting Induced Local Lesions IN Genome (TILLING) technology has however brought a resurgence in the use of chemically induced mutagenesis for gene function analysis. TILLING is a high-throughput and economical method for identification of SNPs and mutations in the gene of interest in chemically induced mutants [48]. Genome sequence availability is a requirement for any TILLING experiment and the strategy can be applied to any model species regardless of ploidy levels or genome size [49]. TILLING has been combined with NGS for an even faster, high resolution mapping of SNPs and mutations [50].

Insertional mutagenesis using T-DNA and/or transposable elements provides a faster way of identifying the desired mutant than chemical or physical mutagenesis. The inserted sequences are known and thus the region flanking the inserts can be identified through PCR and cloning based strategies. Wang (2008) [51] evaluated the effectiveness in knocking out a gene through insertional mutagenesis and his findings show that there is a 90% knock-out if the insertion is in the protein coding region and 25% if it is before the start codon. In order to saturate the genome, hundreds of thousands of insertion mutations have been generated in Arabidopsis. Thus insertion mutants of most of the known Arabidopsis genes are available at the Arabidopsis Biological Resource Centre (ABRC) [52]. Large scale insertional mutagenesis has been carried out in rice using the Tos17 retrotransposon as well and the mutant lines can be ordered online through the Rice Tos17 Insertion Mutant Database [53]. Like in chemical and physical mutagenesis, in transposable element and T-DNA mediated mutagenesis, the mutation events are still randomly localized and cannot be targeted. One disadvantage of all mutation based gene silencing/knockout is that it is difficult to silence genes which are members of a large family and have redundant functions. Multiple rounds of crossing among single mutants are necessary to obtain the desired multiple-gene mutant with complete knockdown of the function of the redundant genes.

3.2. RNA-mediated Gene Silencing

RNA mediated gene silencing is one of the most widely utilized approaches for functional characterization of genes. Gene silencing, in this case, implies a knockdown of gene expression usually through the targeted downregulation of transcript accumulation mediated by small RNAs (sRNAs) which are classified into two classes: small interfering RNAs (siRNAs) and microRNAs (miRNAs) [54]. Silencing

through transgenic expression of sRNAs has thus been widely used for functional characterization of genes. For example, through RNA-interference (RNAi) knockdown of OsNAC5, Song and colleagues [55] showed that OsNAC5 positively regulates the abiotic stress response in rice. Similarly, by developing RNAi transgenic rice Ouyang and colleagues [56] were able to show the positive regulatory role of the rice tocopherol cyclase in salt stress tolerance. In another interesting study, Oh and colleagues [57] showed that RNAi knockdown of the *Thellungiella salsuginea SOS1* gene resulted in the increase in salt sensitivity and loss of halophytism in the salt cress transgenics, thereby, verifying the role of *ThSOS1* as an integral regulator of salt tolerance of the halophyte. Various methods for sRNA-mediated gene silencing have been developed and the mechanisms and differences among them along with the applications are beyond the scope of this article but have been reviewed extensively elsewhere [58-60]. Majority of the strategies involve the post transcriptional silencing but siRNA can be utilized for transcriptional silencing of gene expression through DNA methylation (RNA-directed DNA Methylation) or chromatin modifications [58]. Thus in crop plants, RNAi serves as a valuable tool for assessing gene function. Unlike mutagenesis, it is a targeted approach with little noise in terms of background effects. Therefore, back crossing to wild type plants is not necessary. Moreover, sRNA-mediated gene silencing is sequence specific, thus the simultaneous knockdown of multiple redundant genes of a family is possible simply by targeting the conserved regions of the gene family. With information on endogenous sRNAs increasing exponentially due to contribution of NGS technologies, RNA-mediated gene silencing has developed tremendously as well. Further, the development of artificial microRNAs (amiRNAs) has added more impetus to the field. Computational tools such as the Web MicroRNA Designer (WMD) and the PsRNAtarget have been developed which have immensely simplified the design of amiRNAs especially for model plant species such as rice, Arabidopsis, poplar etc [61, 62].

3.3. Gain-of-function Lines

Over-expression of genes to acquire a gain-of-function phenotype is perhaps one of the most used tools for gene function analysis in plants. Two strategies have been employed for obtaining 'gain-of-function' lines. The first employs the activation of endogenous genes through randomly inserted transcription enhancers (activation tagging) while the second involves the constitutive expression of transgenes regulated through transformation [63]. The advantage of overexpression is evident when assessing the role of individual genes which belong to a gene family comprising members with redundant functions [64]. Heterologous expression of plant genes in yeast is also possible and has been widely used for functional characterization of genes. For example, heterologous expression of the rice cyclophylin gene, *OsCYP2*, conferred tolerance to multiple abiotic stresses in yeast thus indicating similar function for the gene *in planta* [65]. Since both activation tagging and transgene overexpression through transformation strategies are dependent on T-DNA of *Agrobacterium tumefaciens* for delivery into the plant, the insertion sites are random and this may result in

unwanted background noise. For instance; it is theoretically possible for the insertion, of T-DNA carrying an overexpression cassette of a particular gene, to occur in the middle of an endogenous gene. In such a case, the phenotype observed could be due to the disruption of the latter rather than the overexpression of our target gene. Insertion sites therefore need to be assessed prior to proceeding with experiments to rule out such incidences. The roles of many genes of crop plants, especially those involved in the abiotic stress response such as Dehydration Responsive Element Binding Protein (*DREB*), MYB transcription factors, absicic acid responsive element binding factor (*ABREF*), etc., have been characterized through the ectopic overexpression of cDNAs under the control of the CaMV35S promoter [66-68]. The function of genes in plants where transformation protocols have not been established can still be assessed through transformation and transgene expression in model organisms. This makes 'gain-of-function' lines an extremely valuable tool for characterization of genes from species where knowledge and protocols are limited.

3.4. Genome Engineering/Genome Editing

Genome editing, as the name implies, is the targeted mutagenesis of genomes. Current technology allows us to introduce specific changes at specific sites in the genome. The technology utilizes customized DNA cleavage reagents and cellular DNA repair pathway [68]. The reagents are usually engineered nucleases which cleave target DNA at user-specified sequences and these double strand breaks are repaired either by non homologous end joining (NHEJ) or homologous recombination (HR) [69]. Custom modifications to the DNA, such as deletions, insertions, donor cassette insertions, are obtained through the manipulation of these DNA repair mechanisms or through erroneous repair. Here, we provide only a brief overview of genome engineering. For a more comprehensive understanding of the technology a few of the many extensive reviews on the topic are referred [69-73]. There are four types of engineered nucleases which have been used for genome engineering in plants: the Zinc finger nucleases (ZFNs), the Transcription activator-like effector nucleases (TALENs), the Meganucleases and the CRISPR/Cas RNA-guided nucleases (Fig. **1**) [69, 72].

3.4.1. The Zinc Finger Nucleases (ZFNs)

ZFNs are chimeric proteins which are engineered by fusing the cleavage domain of the *Fok*I restriction enzyme with a customized Cys2-His2 Zinc finger (DNA binding) domain. Since dimerization of the *Fok*I is necessary for DNA cleavage, ZFN monomers also need to dimerize for endonuclease activity [73, 74]. Therefore, individual ZFN monomers are engineered as such that they flank a 5-6 bp spacer sequence and site specific cleavage at this spacer sequence is obtained only through heterodimerization of ZFNs (Fig. **1**) [75]. Each zinc finger domain is composed of 3 -6 individual fingers, and each finger is capable of recognizing an approximately 3 bp long DNA sequence [75]. Thus, different fingers can be aligned in arrays of 3 -6 fingers to recognize any sequence of interest. Thus, a ZFN heterodimer comprising of 2 DNA-binding domains composed of 4 fingers each, and each finger recognizing a 3 bp sequence will recognize a sequence of approximately 24 bp in length which, statistically, can occur
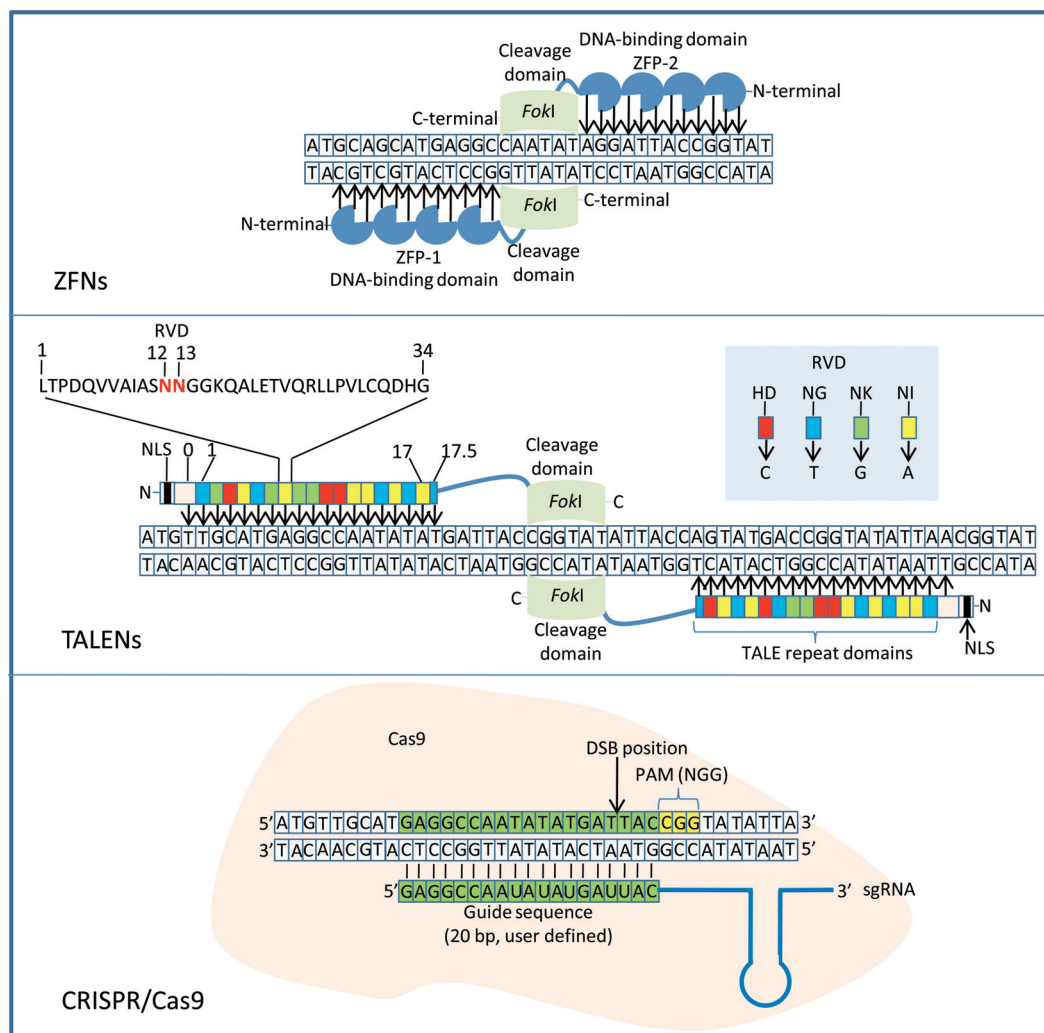
Fig. (1). Three types of engineered nucleases used for genome engineering in plants. Zinc finger nucleases (ZFNs) recognise and bind to target sites on DNA. Binding of both ZFN-1 and ZFN-2 leads to dimerization and activation of the *Fok*I endonuclease catalytic domains and subsequent cleavage of target site. Transcription activator-like effector nucleases (TALENs) are made up of repeat domains each with its specific nucleotide binding specificity depending on the Repeat di-variable residues (RVD) present on position 12 and 13 on each repeat. The '0' domain specifically binds thymine only. Binding of left and right TALENs result in *Fok*I catalytic domain dimerization, which leads to cleavage resulting in a double-strand break on the DNA target site. The CRISPR/Cas9 system is guided by an engineered sgRNA, which is composed of the crRNA and the tracrRNA. The crRNA contains a customizable 20-nucleotide guide RNA at its 5'-end which binds to target sites on the DNA and cleavage by Cas9 occurs within the target site adjacent to the protospacer adjacent motive (PAM).

only once in any organisms' genome [75]. This makes ZFNs extremely specific and robust for targeted genome engineering.

3.4.2. The Transcription Activator-like Effector Nucleases (TALENs)

Similar to the ZFNs, TALENs are also chimeric proteins consisting of customizable DNA-binding elements fused to the *Fok*I endonuclease. In this case, the DNA-binding elements consist of highly conserved amino acid repeats which are derivatives of the transcription activator-like effectors (TALEs) produced by the bacterial plant pathogen, *Xanthomonas* spp. [76]. Upon infection, TALEs are secreted into the host cells via a type-III secretion system and bind to host DNA, thereby, regulating host gene expression [76]. The principle of TALENs technology is the same as that of

ZFNs, with the only difference being the target DNA sequence is recognized by the customized TALEs. TALENs usually contain a DNA binding domain made up of 16-20 tandem repeats of about 34 amino acids [77]. The sequence specificity is determined by two adjacent repeat variable di-residues (RVDs) normally present at residue 12 and 13 in each repeat which mediate binding to a single nucleotide (Fig. **1**) [77]. Interestingly, it has been shown that designer TALEs with customized specificity can be generated simply through the modulation of TALE RVDs [78]. Platforms such as the Golden Gate Platform have emerged which enable the construction of ready-to-use TALENs within a period of five days [79]. In addition, majority of the engineered TALENs have been found to be functional making them a popular tool for genome engineering [72]. TALENs have only recently been adopted in plant science. Nevertheless, a few reports show the successful use of TALENs for regulation of gene
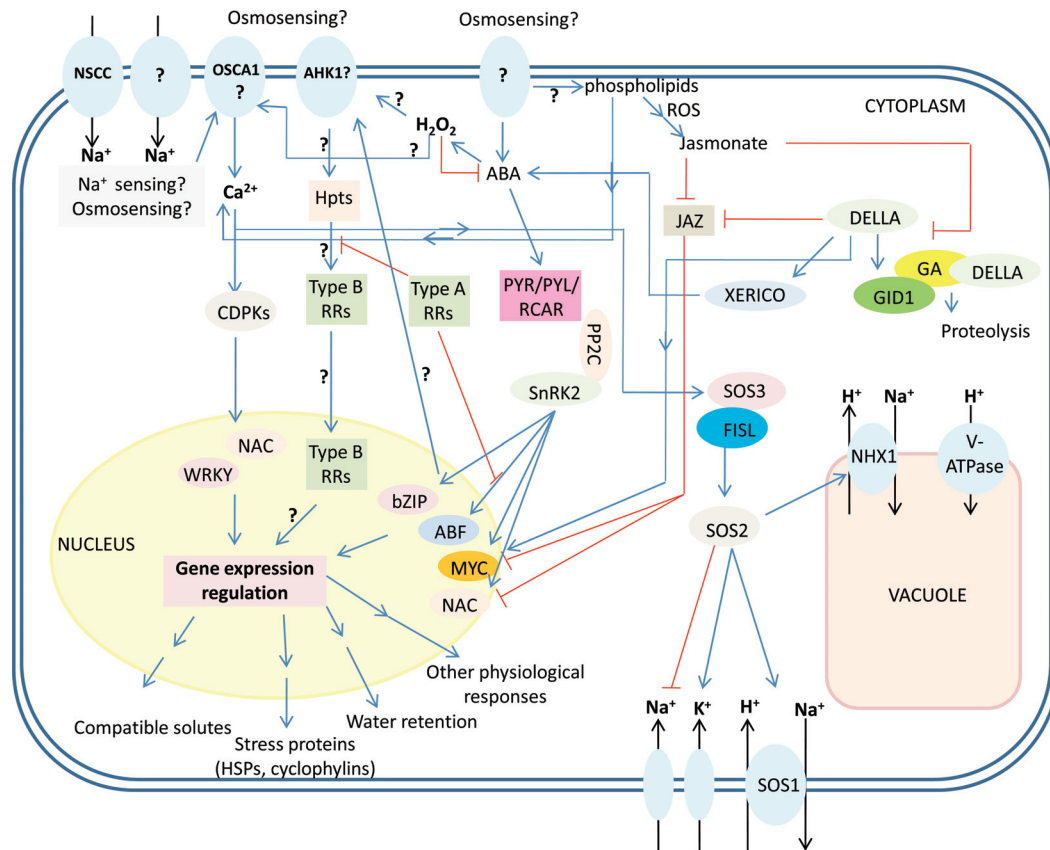
Fig. (2). Simplified view of the complex mechanisms of salinity tolerance in plants. Salinity stress is composed of two components, an osmotic component which imposes osmotic stress similar to drought and an ionic component which involves accumulation of Na$^+$ leading to cellular damage. The response to the osmotic component is still unclear but appears to involve the maintenance of the osmotic potential through the production of osmolytes. The response to the ionic component of salinity stress is much clearer and involves osmotic adjustment and protection through the synthesis of compatible solutes and osmoprotectant proteins through both ABA-dependent as well as ABA-independent signaling pathways. Ionic stress is also abated through either the exclusion of Na$^+$ from the cells or through its sequestration in the vacuole. Ca^{2+} plays an integral role in both the osmotic and ionic stress responses. A probable cross-talk between the cytokinin, jasmonic acid, gibberellic acid and ABA signaling in the salinity stress response has been shown. The crosstalk shows the positive roles of jasmonate and ABA in the salinity stress response while cytokinins and gibberellic acid (GA) have a negative regulatory role. The involvement of a two component histidine-aspartate phosphorelay is also hypothesized based on the positive regulatory role of the Arabidopsis histidine kinase, AHK1, in the salinity and osmotic stress response. AHK1 activates Histidine phosphotransfer proteins (Hpts) which in turn activate type-B response regulators (RRs) which ultimately regulate gene expression.

expression as well as gene replacement in plants such as the engineering of disease resistant rice by disrupting the *Os11N3* promoter, or the targeted, in frame insertion of YFP gene into *SurA* and *SurB* genes in tobacco protoplasts [78-83]. Thus, TALENs could provide a highly efficient tool for gene function analysis in plants.

3.4.3. Meganucleases

Meganucleases or homing endonucleases are the third kind of DNA-cleaving restriction enzymes which have been used for genome engineering. Meganucleases were, in fact, the first double-strand break generating nucleases to be used for genome engineering [84]. Usually encoded within mobile introns and inteins, meganucleases are categorized into five classes based on their sequence and structural motifs: LA-GLIDADG, GIY-YIG, HNH, His-Cys Box and PD- (D/E) XK [85-87]. The most widely studied class of meganucleases are the LAGLIDADG homing endonucleases (LHEs). LHEs can function in either homodimeric forms or as a sin-

gle peptide comprising two monomeric repeats joined by a linker [72]. LHEs bind to 20-30 bp DNA-sequences and are highly sequence specific but their use has been limited as modifications to their binding domain to alter specificity often affect their nuclease activity [72]. This makes engineering customized meganucleases an arduous and highly time consuming task unlike ZFNs and TALENs. Thus, the use of meganucleases for genome editing in plants has been limited. Nevertheless in 2012, Cellectis and Bayer Crop-Science have started a collaborative project for the manufacture of plant genome specific nucleases for targeted genome editing in crops. The results of this collaboration are still awaited.

3.4.4. The Clustered Regularly Short Interspaced Palindromic Repeats/CRISPR-associated Sequence (CRISPR/Cas) RNA-guided Nucleases

The most recent addition to the repertoire of nucleases for targeted genome engineering is the CRISPR/Cas RNA-

guided nucleases which are derivatives of the of the adaptive immune response proteins prevalent in bacteria and archae [88]. CRISPR/Cas have been classified into 3 types (I-III) and each system is composed of three main components: The Cas nuclease cluster, non-coding RNAs and an array of direct repeats [89, 90]. The complexes function to cleave invading nucleic acids such as viral DNA through RNA guided sequence specific cleavage [89]. DNA CRISPR-mediated adaptive immunity proceeds in three distinct stages: acquisition of invading foreign DNA called spacers between two adjacent repeats at the proximal end of the CRISPR, CRISPR RNA (crRNA) generation from the spacer-containing CRISPR template, and target interference through crRNA-guided recognition and cleavage of protospacer sequence in foreign DNA [88]. The presence of a protospacer adjacent motif (PAM) in the target DNA is also necessary for cleavage [91].

Among the three types of CRISPR/Cas systems, the type-II, CRISPR/Cas9 system from *Streptococcus pyogenes* is currently being manipulated for genome engineering. In this system, Jinek and colleagues [91] showed that the Cas9 nuclease is guided to its target sequence complementary to the crRNA by two small RNAs; the crRNA and the transactivating crRNA (tracrRNA). They further went on to show that a single chimeric RNA (sgRNA) composed of the tracrRNA and the crRNA was efficiently capable of programming the Cas9 DNA cleavage and target specificity could be customized simply by changing 20 nucleotides in crRNA [91]. This discovery laid the foundation for utilizing the CRISPR/Cas9 complex for genome engineering and less than a year later reports were published showcasing the potential of this tool for engineering eukaryotic genomes from various genera including plants [92-96]. Although these initial studies were performed on well characterized genes mostly belonging to model organisms, the potential of the technology to characterize gene function in plants is almost limitless. To design highly specific sgRNA, Xie and colleagues [97] carried out an analysis for identifying the density of protospacer adjacent motifs (PAM) sequences in the genomes of 8 plants, comprising both model and major crops. These authors observed that on an average, 5-12 'NGG' PAM sites were available per 100 bp genomic DNA and it was possible to design highly specific sgRNA for 85-99 % of the currently annotated transcripts in those genomes except for maize in which only 30% annotated transcripts could be specifically targeted [97].

The CRISPR/Cas9 system has similar mutation frequency as compared with ZFNs and TALENs but it does have some advantages over them. A few are worth mentioning: It is simpler as it requires no protein engineering steps; unlike the ZFN platform, the technology has been kept open access by the community and, therefore, is easily accessible to any laboratory, which has promoted its use; the ability of the Cas9 to cleave methylated DNA is a big advantage over other platforms especially when it comes to editing plant genomes, where 70% of the CpG/CpNpG sites are methylated; multiplexing, which is the ability to simultaneously edit several CRISPR/Cas9 sites in a genome, also provides another advantage [72].

It is thus evident that through genome engineering we are now capable to decipher single or multiple gene functions

with minimal background noise in the span of 1 or 2 generations of the test organism(s). This will tremendously improve the efficiency of plant biologists towards understanding multiple facets of plant growth and development, stress response mechanisms, etc. Applications to crop improvement are endless as well. Reports in which genome engineering has been carried out in crops so far has been provided in a few reviews [69, 72, 75].

4. CROP IMPROVEMENT: THE GENERATION OF HIGH YIELDING, FIELD WORTHY, SALINITY TOLERANT CROPS

With the increasing salinization of agricultural soils, especially in semi-arid and arid farmlands, it is imperative that a new breed of crops resistant to the deleterious effects of salinity be produced which is the ultimate objective for any salinity-tolerance based study in crops. Broadly speaking, there are two ways to incorporate improved traits, including salinity tolerance, in crops: i) genetic engineering and ii) breeding, although genome engineering may have recently been added to the list. Integration of any two or all three methods can also be used to generate improved crops. These are discussed in brief as follows:

4.1. Genetic Engineering

Genetic engineering has been at the limelight of crop improvement for decades. This is mostly due to the numerous debates sparked up by the first generation of genetically engineered/genetically modified (GM) crops, like the BT-crops and the glyphosinate resistant crops. Genetic engineering has been particularly successful in obtaining abiotic stress tolerant crops but only the drought tolerant maize (Genuity® DroughtGuard™) developed by Monsanto in 2010 and drought tolerant sugarcane developed by PT Perkebunan Nusantara XI (Persara) have been approved for cultivation as food and feed. However, the classical approaches of genetic engineering such as transgenic overexpression of genes which regulate abiotic stresses including salinity usually come at a cost [98]. Abiotic stress responses in plants are highly co-ordinated and involve multiple genes that are part of different signaling, developmental and metabolic pathways and this is further complicated by the high level of crosstalk among these processes [98]. Overexpression of stress responsive genes could therefore lead to unwanted pleiotropic effects. Growth and developmental defects can result from the consumption of cell resources required for normal cell growth and expansion by the overexpressed transgene or its downstream target molecules [99]. Moreover, these early transgenic plants contained a single overexpressed transgene, which would function to positively regulate tolerance to a single stress such as salinity. In field conditions, two or more abiotic stresses can occur either simultaneously or one after the other, thus tolerance to just one is irrelevant [100]. Thus, the new age transgenic plant would have to be high yielding, tolerant to multiple stresses and devoid of deleterious pleiotropic characters.

To acquire transgenics suitable for field conditions researchers are developing new strategies. Tightly regulated transgene expression through the use of stress inducible promoters provides a viable strategy to reduce the pleiotropic

effects [101-103]. Another strategy involves engineering protein post translational modifications such as ubiquitination [104]. Guo and colleagues [105] showed that modulation of the protein post-translation modification through the overexpression of a monoubiquitin resulted not only in tolerance to multiple abiotic stresses in tobacco but also improved growth [104]. Another strategy is to express orthologous genes of effectors from wild relatives or halophytes in crops [106]. Regulation of gene expression by regulating miRNA activity through transgene expression of miRNA target sequences provides another alternative for modulating the stress response of crops [107]. Other methods such as gene pyramiding, engineering of transcription factors, osmoprotectants, chaperones, Late embryogenesis abundant proteins, metabolic pathways, even epigenetics, have been exploited to generate the new generation of transgenics [108]. While one single approach may or may not be sufficient to attain crops worthy for distribution to farmers, transgenics obtained from two or more approaches can be crossed. With such a large number of combinations available, we can certainly be hopeful for highly improved lines to be generated.

4.2. Marker Assisted Breeding

Conventional breeding for higher yield has led to genetic bottlenecks in our current batch of crops and one way out is to identify genetic markers in crops and breed to regain genetic variability but not at the expense of yield. Recent developments in genomics have led to the development of new and improved breeding methodologies which have vastly accelerated the breeding process [109]. Breeding for tolerance to abiotic stresses such as salinity could provide the solution to increasing production in stress affected areas. Since plants' responses to abiotic stresses such as salinity are complex and multigenic in nature, usually comprising hundreds of genes, breeding approach towards achieving stress tolerance as well as stability in crops have proven to be challenging. The use of marker assisted selection/marker assisted breeding in this regard has helped to simplify things to a certain extent. Marker assisted selection (MAS) uses a marker such as a specific phenotype, chromosomal banding, a particular DNA or RNA motif, or a chemical tag that associates with the desired trait [4]. With the continually increasing number of genetic markers being identified through NGS, association genetics and other technologies, marker assisted breeding has become the norm. Furthermore, new breeding practices such as gene and QTL pyramiding, marker-assisted recurrent selection (MARS), and genome-wide selection are usually accomplished using MAS [4]. Marker assisted breeding has been successful in generating crops with tolerance to biotic stresses and also better grain quality in cereals [110-113]. MAS has not been as successful with complex traits such as salinity tolerance. However, with high density maps available, genome-wide selection seems a promising approach for breeding towards salinity tolerance. Breeding with exotic wild relatives seems another approach for attaining salinity tolerant lines. Exotic wild species have evolved to withstand multiple stresses simultaneously be it at the cost of yield. Thus, breeding of elite varieties with exotic wild varieties is an interesting option to explore. The potential success of this strategy is demonstrated by the development of a highly salt-tolerant rice variety by crossing the wild relative *Oryza coarctata* with the elite variety *Oryza sativa*-IR56 [114]. Though this process may be laborious and time-consuming with a very low probability of success, the extremely high level of salinity tolerance obtained in this case seems to make it worth the effort.

5. THE SALINITY STRESS RESPONSE IN PLANTS?

Integration of genomics based knowledge with other omics' based platforms is expected to provide useful insights into the molecular response of plants to salinity. Salinity imposes two types of stresses on plants. Firstly, osmotic stress resulting from decreased soil water potential leads a decline in the water uptake by the plant. Secondly, salinity causes ionic stress which results from the accumulation of Na^+ ions and Cl^- ions over a period of time. Plants also respond to these two components of salinity stress, accordingly.

Osmosensing and the subsequent signaling pathways which lead to osmoregulation are still largely unknown and remains the topic of intensive research. There are some interesting candidates which have been proposed as putative osmosensors such as the Arabidopsis histidine kinase, *AHK1*, as well as a hyperosmolality-gated calcium-permeable channel, *reduced hyperosmolality- induced $[Ca^{2+}]_I$ increase 1 (OSCA1)*. AHK1 was proposed as a putative osmosensor as it was able to function as the sole osmosensor in yeast and the arabidopsis *ahk1* mutants were hypersensitive to drought stress [115, 116]. Moreover, in lower organisms such as yeast and bacteria, histidine kinases have been shown to function as bonafide osmosensors [117, 118]. In the case of *OSCA1*, Yuan and colleagues [119] reported that in Arabidopsis *osca1* mutants hyperosmolaity induced Ca^{2+} signaling was impaired in guard cells and root cells, while the root growth and water transpiration regulation were also hampered. Moreover, there are reports showing ions channels, which mediate increase in Ca^{2+}, function as osmosensors in animals as well as bacteria [120, 121]. All these factors put together hint at *AHK1* and *OSCA1* to potentially function as osmosensors in Arabidopsis. Downstresam of osmosensing, the signaling pathway is much more defined and involves Ca^{2+}- mediated as well as ABA-mediated gene expression regulation which ultimately leads to osmoregulation through synthesis of osmolytes and water retention. (Fig. **2**) [122].

The response of plants to ionic stress is quite well defined. Plants mitigate the deleterious effects of increased Na^+ in the cells by two mechanisms: i) Na^+ exclusion and ii) Na^+ sequestration. Increased cytosolic Na^+ concentration is detected by a yet unknown mechanism. This is followed by Ca^{2+} influx into the cytoplasm. Calcium acts as a secondary messenger, activating calcium dependent proteins such as calcium dependent kinases which subsequently mediate transcription factors to regulate gene expression resulting in synthesis of compatible solutes to abate the damaging effects of increased Na^+ ions in the cytoplasm [123]. Ca^{2+} also activates the SOS-pathway which mediates Na^+ exclusion through the SOS1 antiporter as well as Na^+ sequestration into the vacuole through the NHX1 antiporter [123].

An interesting aspect of the salinity stress response in plants is the role played by different plant hormones. In

Table 1.   Selected genes/gene families involved in salinity stress response.

Stress Perception / Sensory Proteins		
Gene/gene Family	**Function**	**Reference**
AtAHK1	Positive regulator of osmotic stress response. Capable of function as an osmosensor in yeast. Mechanism of action still unknown.	[115, 116]
AtOSCA1	Plasma membrane localized calcium channel. Stimulates intracellular Ca^{2+} increase upon osmotic stress imposition. Putative osmosensor.	[119]
SOS3	Ca^{2+} sensing and downstream activation of the SOS-pathway.	[128]
Kinases		
Gene/gene Family	**Function**	**Reference**
Calcium dependent protein kinases (CDPKs)	Mediate salinity induced calcium signaling through protein phosphorylation.	[129]
Mitogen-activated protein kinases (MAPKs)	Stress signaling through activation of transcription factors.	[130]
Histidine Kinases (HKs)	Function as receptors for ethylene and cytokinin. AtHK1 also function as putative osmosensors	[115, 116]
Sucrose non-fermenting related kinases (SnRK1)	Key component of the ABA- dedendent stress response pathway. SOS2 is an SnRK.	[131]
Transcription Factors		
Gene/gen Family	**Function**	**Reference**
Dehydration responsive element binding proteins/C-element binding factors (DREB/CBF)	Except for CBF-4, DREB/CBFs Mediate ABA-independent regulation of gene expression under osmotic stress.	[132]
WRKY transcription factors	WRKY proteins belong to Zinc finger sub family of transcription factors. They have differential roles in the regulatation of ABA-dependent abiotic stress responses.	[133]
NAC transcription factors	Stress responsive transcription factors involved in both ABA-dependent and ABA-independent signaling pathways	[134]
Leucine basic zipper (bZIP) transcription factors	bZIP transcription factors are part of the ABA-dependent signaling in responses to drought and high salinity.	[135]
MYB/MYC transcription factors	MYB/MYC transcripton factors are key elements of the ABA-dependent signal transduction pathway under abiotic stress response.	[136]
Ion Channels		
Gene/gene Family	**Function**	**Reference**
Non selective cation channels (*NSCC*)	Na^+ entry into the roots during high salinity	[137, 138]
High affinity potassium transporters subfamily-1 (*HKT*1)	Na^+ entry into root cells under salinity as well as intake of Na^+ from the xylem into root cells.	[138, 138]
SOS1	Plasma membrane localized Na^+/H^+ antiporter for NA^+ efflux from cells.	[137, 138]
The NHX-type cation/H^+ antiporters	The NHX antiporters are responsible for generating pH gradients and also facilitate Na^+ sequestration into the vacuole under ionic stress.	[137-139]

recent years, evidence of crosstalk among different hormone signaling pathways is starting to emerge. We provide here a brief discussion on hormone signaling crosstalk in the salinity stress response (Fig. **2**). For a detailed discussion on the topic, some recent reviews are referred [124-126]. Plant hormones play a vital role in development and responses to environmental stimuli. With regards to salinity stress ABA

plays a major role in regulating the plant response. ABA is, therefore, known as the stress hormone. Under salinity stress, plants induce ABA synthesis and ABA mediates the plants response through the ABA-dependent signal transduction pathway. ABA induced changes under salinity include osmolyte synthesis and stomata closure in the guard cells, etc., leading to the maintenance of cellular ionic and osmotic
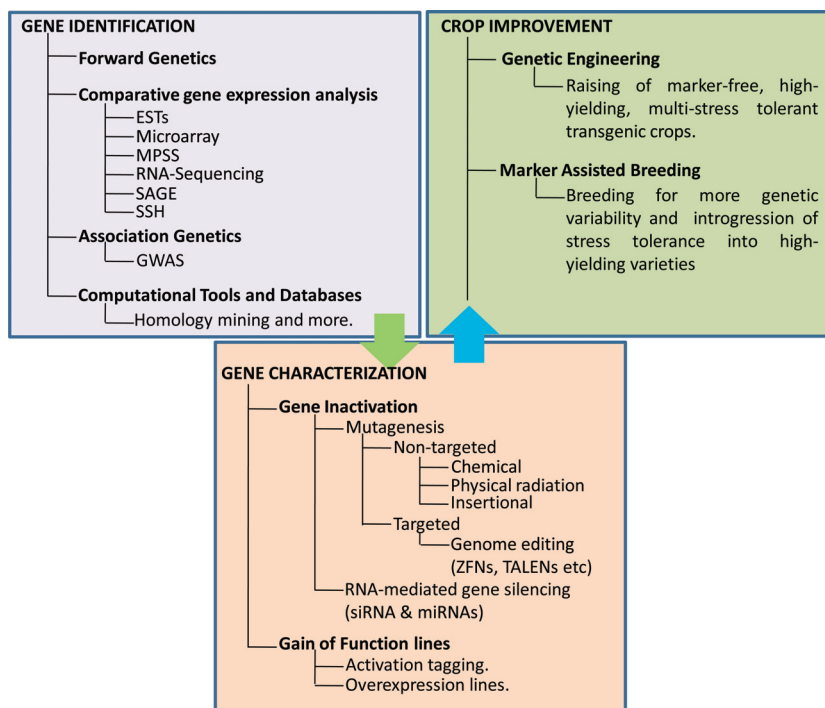
Fig. (3). Genomics approaches for improving salinity tolerance in crop plants. Genomics aims to, first, identify genes/QTLs responsible for tolerance and, after thorough characterization of these genes/QTLs, utilize them for crop improvement through genetic engineering or marker assisted breeding or a combination of both.

homeostasis. Other hormones like cytokinins, jasmonic acid (JA), gibberellic acid (GA), and ethylene also play a less prominent, yet, equally vital role. Cytokinins play a negative regulatory role in the salinity stress response of Arabidopsis through activation of type-A response regulator proteins which not only represses *ABI5* expression but also interferes with the ABA signaling through physical interaction with *ABI5* [125]. Giberellic acid (GA) also acts as a negative regulator of the salinity stress response. GA binds gibberellin insensitive dwarf 1 (*GID1*), and facilitates the formation of the *GID1-GA-DELLA* complex, which triggers *DELLA* proteolysis [127]. *DELLA* proteins act as a positive regulator of salinity stress response by interacting with XERICO, which is an inducer of ABA biosynthesis. *DELLA* proteins also play a role similar to JA by inhibiting JAZ proteins, which are negative regulators of salinity stress response [127]. JA, on the other hand, stimulates *DELLA* by inhibiting the binding to *GA-GID1* and, thus, prevents proteolysis of *DELLA* [127]. Ethylene also plays a positive regulatory role in salinity stress response by inactivating its receptors, *ETR1/ETR2/EIN4*, which are negative regulators of salinity tolerance. Ethylene receptor inactivation leads to the activation of EIN2, which positively regulates salinity stress responses [126]. The hormonal signaling crosstalk illustrates the complexity of the salinity stress response in plants. There is much more which we are yet to discover and perhaps with advancements in genomics we might come closer to achieving a complete understanding of this phenomenon. Fig. **2** provides us with an overview of the signaling pathways involved in the salinity stress response. It is beyond the scope of this article to mention all the known salinity stress response related genes. A few representative genes/gene families are provided in Table **1**.

6. CONCLUSION

The recent developments in genomics have certainly revolutionized our approach towards not only understanding salinity tolerance in plants but also paved the way for faster and more efficient ways to develop salinity tolerant crops which has been summarized in Fig. **3**. The traditional breeding methods however still find a place, especially when it comes to breeding with exotic wild species. It should be noted that genomics as a tool serves mainly to improve existing technologies and not replace them. The data acquired is very extensive and has yet to be used to its full potential. We are only at the initial stages of the genomics era for crop improvement and the future looks promising. Of course, with new technologies, there is a need for precaution. Regulatory measures need to be established and crops need to be thoroughly tested before they are distributed. The main objective is to be able to maintain a continuous increase in agricultural production which could lead to global food security. This increase, however, should not be at the expense of existing natural ecosystems.

CONFLICT OF INTEREST

The author(s) confirm that this article content has no conflict of interest.

ACKNOWLEDGEMENTS

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