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Opinion

Analysis of Stress Resistance Using Next Generation Techniques

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Abstract: Food security for a growing world population remains one of the most challenging tasks. Rapid climate change accelerates the loss of arable land used for crop production, while it simultaneously imposes increasing biotic and abiotic stresses on crop plants. Analysis and molecular understanding of the factors governing stress tolerance is in the focus of scientific and applied research. One plant is often mentioned in the context with stress resistance—*Chenopodium quinoa*. Through improved breeding strategies and the use of next generation approaches to study and understand quinoa's salinity tolerance, an important step towards securing food supply is taken.

Keywords: stress resistance; abiotic and biotic stresses; genetic diversity; *Chenopodium quinoa*; omics

1. Introduction—Strategies to Improve Crop Yield

How to feed the world's population is still one of the most challenging questions. A United Nations report expects the current world population of 7.6 billion to reach 8.6 billion in 2030, 9.8 billion in 2050, and 11.2 billion in 2100 [1]. At the same time, the size of arable land is dramatically reducing. Erosion rates from ploughed fields are, on average, 10 to 100 times greater than rates of soil formation, with the result that the world has lost nearly a third of its farmable land to erosion or pollution in the last 40 years [2]. Crops are also exposed to different biotic and abiotic stresses: Biotic stresses include pathogen infection and herbivore attack [3], while abiotic stresses are environmental factors that compromise plants and reduce their productivity. These factors include extreme changes in temperature, water, nutrients, gases, wind, radiation and other environmental conditions. Rapid climate change also intensifies abiotic stresses and limits the time for a crop to adapt to new environmental conditions. For breeding programs, improvements in tolerance to drought, salinity and heat as well as the analysis of water economy in plants are most important [4]. Besides traditional breeding approaches, next generation techniques are also used to identify and study stress-tolerant plants. This includes "omics" approaches, which are very effective molecular methods to investigate biochemical, physiological and metabolic strategies of plants exposed to biotic and abiotic stresses. These include genomics (study of genome), transcriptomics (structural and functional analysis of coding and non-coding RNA), proteomics (protein and post-translational protein modification) and metabolomics (analysis of metabolites). Together, omics provide a powerful tool to identify the complex network of stress tolerance [5]. New insights promise to deliver new breeding targets for the stress adaptation of traditional crops by exploiting the allelic but also core- and pan-genomic reservoir. Finally, precision editing tools promise tailored adaptation of molecular circuits in future crop generations. In this article, influences of abiotic and biotic stresses are summarized, next generation analyses are introduced, and potential routes to increase food production are discussed.

2. Abiotic and Biotic Stresses and Responses

Both biotic and abiotic stresses affect crop plants and severely reduce their yield. As plants are sessile organisms, they are not able to escape these stresses but developed a range of strategies to adopt. Depending on the respective stress factors, these defense mechanisms can include morphological, biochemical and molecular modifications, such as altering certain signaling pathways, changes in cell wall structure, etc. A complete and in-depth understanding of these mechanisms is seen as important contributor for future breeding targets and for sustainable agriculture [3,6,7].

Biotic stress is triggered by interactions with other organisms like pests, parasites and pathogens, which are responsible for plant diseases [8]. To withstand, plants use different strategies such as passive barriers and active recognition systems. They produce chemical compounds against herbivores and pathogens, and use thickened cuticles and waxy layers as physical defense against intruders [9]. Plants have established an effective immune response to counteract biotic stress. By so-called pattern recognition receptors (PRRs), they can recognize microbial- or pathogen-associated molecular patterns (MAMPs or PAMPs) like flagellin, inducing PAMP-triggered immunity (PTI). Furthermore, plants possess disease resistance or R genes, which are encoding NB-LRR (nuclear binding—leucine rich repeat) proteins. These NB-LRR proteins recognize pathogen effectors and induce the effector-triggered immunity (ETI) [10]. PTI and ETI induce a first response against biotic stress which leads to an increase in cytoplasmic calcium concentration, the production of reactive oxygen species (ROS) and the activation of mitogen-activated protein kinases (MAPKs). Both PTI and ETI also induce several downstream signaling pathways in which phytohormones, mostly salicylic acid (SA), jasmonic acid (JA) and ethylene (ET), play an essential role [6].

Abiotic stresses are caused by non-living factors that have an impact on growth conditions. Already in 1982, it was suggested that environmental factors limit crop production by 70%. A quarter century later, in 2007, it was reported that 96.5% of worldwide land area is influenced by abiotic stress [11]. Plants have developed different strategies to face these environmental changes. The stress responses can be both elastic (reversible) and plastic (irreversible), and are mostly very complex. Plant cells are able to sense environmental changes, which are subsequently reflected by specific changes in their gene expression, metabolism and physiology. Until today, only a few sensors have been identified, maybe due to functional redundancy in sensor protein encoding genes or to their essentiality, meaning mutations in these genes are lethal [3]. The phytohormone abscisic acid (ABA) seems to play a central role as endogenous messenger in abiotic stress responses. It was shown that especially under drought and salinity stress, increased ABA levels in combination with highly altered gene expression are detectable. In 2009, a small protein family, which is able to bind ABA, was identified as ABA receptors. These findings initiated the analysis of ABA pathways and ABA-induced gene transcription. Furthermore, the understanding of stomatal closure regulation due to ABA signals, which are controlling ion channels in guard cells, was improved [12].

In addition, abiotic stresses have an effect on the occurrence and spread of biotic stressors. Alterations of environmental conditions also directly influence pest-plant interactions by affecting physiological and defense responses. Apparently, the combination of stress factors is more harmful, but not always additive. Plants are able to pyramid responses to combined stress factors. The identification and development of crops with enhanced stress tolerance to combined biotic and abiotic stresses is in the focus of research [13].

3. Drought and Salinity—The most Affecting Abiotic Stresses

During the next decades, climate change will impose increased abiotic stresses—mainly drought, heat, and salinity. It is expected, that drought will be most influential on crop productivity, as by the end of the twentieth century, 30% of land will be extremely dry [4]. Salinity rises constantly since irrigation with brackish water increases the worldwide area of salt-damaged arable land. Every minute, three hectares of land become unusable for crop production [14,15].

More than 50 years ago, plant drought responses were grouped in three categories—drought escape, drought avoidance and drought tolerance. Plants often combine these strategies and their viability depends on how effective the composition of these changes is [4]. Drought escape is an adaptive mechanism including faster development to complete the plant's life cycle before the drought period starts, such as through early flowering. Drought avoidance describes better water uptake due to deeper roots and decreased water evaporation through thicker waxy layers. Drought tolerance is induced after stress occurrence and enables the plant to grow under water deficiency due to biochemical changes. The decrease in osmotic potential by osmolyte accumulation is defined as osmotic adjustment (OA), a typical physiological mechanism against dehydration [4,16,17]. Typical osmolytes, also often called osmoprotectants, are betaine, proline and fructans. These substances do not take part in biochemical responses but influence the osmotic behavior of cells. The accumulation of osmolytes affects gene expression in order to regulate the production of relevant enzymes [18].

In land plants, different strategies evolved to handle high salt (NaCl) concentrations in the soil. Non-salt tolerant glycophytes actively transport salt from the roots back into the environment. This is only effective when facing low salt concentrations. Only about 2% of all plant species are halophytes with high salt tolerance. Halophytes can tolerate NaCl concentrations comparable to seawater and developed two strategies to cope with increased salt concentrations. Succulent halophytes have large internal vacuoles in which they store sodium (Na^+) in order to protect the core plant from toxic salt loads. Another possibility to exclude NaCl from sensitive tissues is the ability to sequester large quantities of salt to so-called epidermal bladder cells (EBCs), which are present in 50% of all halophytes. The diameter of EBCs is about 10-times larger than normal epidermal cells resulting in a 1000-times larger storage volume for Na^+ compared to vacuoles of normal leaf cells [15].

4. Sequence Diversity in Crops—Searching for Tolerant Plants

Structural gene variants like presence/absence variants (PAVs) and copy number variants (CNVs) are contributing to the diversity genepool [19]. Often different crop varieties are adopted and optimized for growth in different habitats. The optimal development of these ecotypes is influenced by their allelic diversity, which is reduced in elite cultivars as a consequence of intense breeding and selection for particular characteristics often ignoring others [20]. Consequently, the complete gene and allele pool diversity cannot be captured by an individual variant, but requires the analysis of a broader set of cultivars. To include all existing genes, contribution to phenotypic and agronomic trait diversity, the construction and analysis of pan-genomes is necessary. This pan-genome contains the complete gene set, including the core-genome, in which all genes are present in all members of a species, and variable genes, which occur only in some variants [21]. Since one reference genome represents only one variety, increasing awareness is put towards the fact that a range of genomes need to be sequenced completely to generate high resolution pan-genomes. For a number of species, including wheat, maize, rice, soybean and cabbage, pan-genomes have been analyzed. The analysis of the cabbage pan-genome, for example, revealed that 20% of genes are affected by presence/absence variation. Some of these were related to important agronomical factors like stress resistance, flowering time and vitamin biosynthesis [19,20].

The genomes of many traditional crops, such as tomato, barley, wheat, sorghum and wild emmer, were sequenced in the last years [20,22–26]. Several consortiums are working on the assembly of additional genomes to gain insights into the pan-genomes of all important crops. The allelic diversity in the gene pool will aid in analyzing different stress resistances in detail. However, also non-traditional crops with high stress tolerances need to be sequenced to study their (pan-) genomes, in order to understand their particular molecular mechanisms to eventually learn and profit for adjusted breeding goals and solutions. Quinoa (*Chenopodium quinoa*), a plant reputed for its high salinity tolerance, has yet a relatively unimportant role compared to traditional crops. Quinoa has the potential to serve as model plant for stress resistance. Besides the recently published reference genome, a variety of quinoa ecotypes exist that seem to grow under nearly all climate conditions.

5. *Chenopodium quinoa*—A Salinity Tolerant Crop

A halophyte plant often used to study salt tolerance is *Chenopodium quinoa*. Quinoa is a highly nutritious crop and is supposed to have been domesticated more than 7000 years ago by pre-Columbian cultures in the Andean region. It was called "mother grain" during the Incan Empire. It is a pseudocereal crop of the family *Amaranthaceae*, also including the important economic plants *Beta vulgaris* (sugar beet), *Spinacia oleracea* (spinach) and *Amaranthus hypochondriacus* (amaranth) [15,27]. Quinoa has become a plant of interest: It is called a "superfood" as its seeds contain a high amount of essential amino acids and vitamins but no gluten. This makes quinoa an alternative to replace wheat-based products in cases of celiac disease. The seeds are rich in several minerals and, in comparison to other grains, have an excellent ratio of proteins, lipids, fiber and carbohydrates. Because of these characteristics, the United Nations Food and Agricultural Organization (FAO) declared 2013 as the "International Year of Quinoa", an award which plants only received three times. In its origin, the Andean region, quinoa is used to grow in several harsh environmental conditions. It adapted to the salty coast as well as to the highlands 3500 m above sea level, with extreme differences in abiotic factors like temperature, precipitation and salt concentrations. Due to this broad adaptation combined with the nutritious characteristics, the number of quinoa-growing countries has increased 10-fold during the last 30 years [28–30].

6. Omic Approaches Using Quinoa

C. quinoa has not only reached public attention as food of the future but also its salinity tolerance is in the focus of several research groups. Next to traditional growth experiments, in order to decipher the salt concentration tolerated by quinoa, the studies were supported by omic approaches, namely genomics, transcriptomics, proteomics and metabolomics.

The quinoa genome was published recently [28,29] and the evolution of quinoa and its salt tolerance were studied. The analysis of ABA-related genes showed that the key factors of ABA biosynthesis, transport and perception were expanded in the quinoa genome, contributing to salinity tolerance [28,29]. In further studies, these genomes were used for resequencing approaches [31,32]. One example is the detection of genomic variations, like single nucleotide polymorphisms (SNPs) and insertions/deletions (InDels), to distinguish among different ecotypes which can be used in further breeding programs [31].

For transcriptomics, the gold standard approach is RNA sequencing (RNAseq). This method is applied in several approaches, e.g., to identify differentially expressed genes (DEGs) between different conditions, tissues or ecotypes. Several studies on salt tolerance and its sequestration in EBCs were performed [28,33,34]. In combination with the quinoa reference genome, Zou and colleagues analyzed the transcriptome of EBCs and leaves under salt-treated and non-treated conditions. Out of totally 54,438 protein-coding genes present in the quinoa genome, they identified 8148 DEGs between bladder and leaf cells. In EBCs, genes involved in abiotic stress response and cell wall synthesis were upregulated, while those related to photosynthesis were downregulated. These findings underlined the functions of bladders being cells that sequester salt but are inactive in anabolic functions. Furthermore, several ion transporters were found to be upregulated in EBCs which seem to be involved in salt secretion [28]. Another study analyzed RNAseq data from several closely related *Chenopodium* species (*C. quinoa*, *Chenopodium berlandieri*, *Chenopodium hircinum*) differing in tolerated salt concentration. Investigating DEGs between the species, the group identified 15 genes encoding for putative transmembrane proteins that potentially contribute to a higher salinity tolerance [27].

Several analyses of quinoa seeds using proteomics approaches were performed. Aloisi and colleagues investigated the changes in the amino acid and protein profiles of seeds from several quinoa ecotypes grown under salt-treatment. They were able to show that salinity influences proteins which belong to functional categories like stress-protein, metabolism and storage [35]. Another group analyzed 16 grains from different crops, including quinoa. The comparison of the different proteomes showed that over 90% of detected proteins from extensively studied cereals like wheat, barley, maize and rice are registered in the Uniprot database. In the case of the quinoa proteome, only 3% of detectable proteins showed an entry in this database. Thus, quinoa opens a so far largely unexplored territory also with respect to the proteome and protein composition of the seeds [36].

Since plants contain the largest metabolome of all life forms, metabolomics is an important part of the omic approaches. Under normal conditions and especially under stress conditions, plants produce a high and diverse amount of primary and secondary metabolites. While primary metabolites directly regulate growth, development and reproduction, secondary metabolites have other important ecological functions like protecting the plant from stresses. In quinoa and other halophytes, metabolites contributing to salinity tolerance were studied [37]. More than half of all metabolites were significantly affected by salinity—e.g., the osmoprotectant proline was about 17-fold increased. As a next experimental step, quinoas EBCs were mechanically removed prior to the salt treatment resulting in the loss of plant's salt tolerance. This procedure dramatically altered the metabolite composition demonstrating that EBCs also serve as metabolite storage [33].

7. Conclusions and Perspectives

While the world population is massively growing, farmers will increasingly face harsh environmental conditions affecting agricultural productivity, accelerated by the rapid climate change. Biotic and abiotic stresses are associated with crop loss and are in the focus of active research and breeding programs. The analysis of plant's stress response revealed that phytohormones play a key role as messengers in downstream signaling pathways [6,38,39]. A lot of studies were performed to unravel all details of hormone production, transport and its characteristics. Their impact in stress response is still not completely understood but further discoveries will reveal new possibilities to increase tolerance of traditional crops. Available data on completely sequenced plant and crop genomes increase continuously, and pan-genomes of crops become available for the detection of allelic variants, stress-associated alleles and tolerant phenotypes.

As about 22% of worldwide agricultural land is saline, tolerant plants and detailed knowledge about the ability to grow efficiently in salt-contaminated environments are urgently needed. The investigation of halophytes has advanced the knowledge about salinity tolerance as these plants are able to grow even when watered with seawater. In the recent years, the halophyte *C. quinoa* was studied intensively by the use of next generation omic approaches. New insights in the function of quinoa's EBCs were gained, also demonstrating the storage capacity for metabolites in EBCs and a model of how ions are transported into these salt dumpers. These findings and further studies will help to understand the molecular mechanisms of salt tolerance and the engineering of salt-tolerant crops. Additional genome sequences of quinoa varieties and close relatives will trigger insights into the pan-genome. Combining the study and understanding of stress resistance, targeted breeding, potential application and engineering in other crops as well as the use of tolerant ecotypes in areas useless for traditional crop production, a step towards securing food supply will be undertaken.

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