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Salt stress under the scalpel – Dissecting the genetics of salt tolerance

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Summary

Salt stress limits the productivity of crops grown under saline conditions, leading to substantial yield losses in saline soils and under brackish and saline irrigation. Salt tolerant crops could alleviate these losses while both increasing irrigation opportunities and reducing agricultural demands on dwindling freshwater resources. However, despite significant efforts, progress towards this goal has been limited, largely because of genetic complexity of salt tolerance for agronomically important yield-related traits. Consequently, the focus is shifting to studying traits that contribute to overall tolerance, thus dissecting salt tolerance into components that are more genetically tractable. Greater consideration for the plasticity of salt tolerance mechanisms throughout development and across environmental conditions furthers this dissection. The demand for more sophisticated and comprehensive methodologies is being met by parallel advances in high-throughput phenotyping and sequencing technologies that are enabling the multivariate characterisation of vast germplasm resources. Alongside steady improvements of statistical genetics models, forward genetics approaches for elucidating salt tolerance mechanisms are gaining momentum. Subsequent quantitative trait locus and gene validation has also become more accessible, most recently through advanced molecular biology and genomic analyses techniques, facilitating the translation of findings to the field. Besides fueling the improvement of established crop species, this progress also facilitates the domestication of

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naturally salt tolerant orphan crops. Taken together, these advances herald a promising era of discovery for research into the genetics of salt tolerance in plants.

Introduction

Salt stress is a major constraint to plant performance. Saline soils alone, which account for one twentieth of the global total and one fifth of irrigated lands, have been estimated to incur \$27.3bn in agricultural damages annually (Qadir et al., 2014). This is clear motivation for alleviating yield losses in saline soils, but the potential benefits from unlocking saline water (~98% of global water resources) to expand irrigated agriculture and relieve growing pressure on freshwater resources would likely be far greater. As such, addressing the issue of salt stress in plants offers a compelling contribution towards meeting the 50% increase in demand for food and freshwater required to sustain 10 billion people by 2050 (Gleeson et al., 2012; UN Water and Energy, 2014; Mekonnen and Hoekstra, 2016; FAO, 2017). Despite optimistic projections at the global scale, food scarcity will continue to persist regionally, particularly in Sub-Saharan Africa, the Middle-East and South Asia where population growth is highest and agricultural outputs are most limited (FAO, 2017). In these most exigent regions, the attainable yield gap (*i.e.* the difference between observed yields for a given area and attainable yields under a similar climate) is substantial and constitutes a major challenge to present and future agricultural production. But given that 80% of the required increase in agricultural production by 2050 is expected to arise from yield increases (Alexandratos, 2012), it also highlights an opportunity. For example, meeting attainable yields in rice, maize and wheat could increase production, respectively, by 230%, 135% and 224% in Sub-Saharan Africa, and 129%, 83% and 61% in South Asia (Mueller et al., 2012). An increase in irrigation alone could provide yield gains of over 100% for these major crops, yet access to freshwater poses a serious limitation (Mueller et al., 2012; Famiglietti, 2014; Rodell et al., 2018). The development of crops with enhanced performance under saline conditions could enable the use of saline water sources, such as brackish water and partially desalinated or diluted seawater, for irrigation, and contribute to the sustainable intensification of agronomic systems provided correct management.

Despite the potential of salt tolerant crops to significantly improve food production systems and the substantial efforts from the research community, relatively few salt tolerance genes or alleles have been identified and even fewer have led to real world applications. The genetic complexity of salt tolerance poses the greatest hurdle to progress. This review begins by revisiting how salt stress responses and tolerance are defined to provide a framework within which this complexity can be dissected to improve genetic resolution. We suggest that forward genetic approaches, genome-wide association studies (GWAS) in particular, provide unique insight “from the plant’s perspective” that can cut

through this complexity and drive the discovery of new salt tolerance genes and alleles. We focus on the major challenges that have hindered such approaches in the past decade, alongside recent advances that are emerging as promising solutions and facilitators to uncovering the genetic basis of salt tolerance.

Salt stress: effects, responses and phenotypes in plants

Salt stress can directly affect plant performance in several ways. High salinity causes a reduction of the osmotic and water potential of the growth medium, impeding water uptake. This, as well as the high levels of sodium ions themselves, can also affect nutrient absorption by disrupting native soil physico-chemistry and the function of uptake mechanisms. Sodium ions are also able to enter the root through constitutively expressed ion channels and transporters, eventually spreading throughout the plant via the vascular system. Over time, sodium ions can accumulate to such levels as to incur cytotoxic effects or to cause osmotic imbalances within tissues, cells and subcellular compartments. Accumulation, and therefore damage, tends to be greater in aerial tissues because sodium ions are delivered there as solutes in the transpiration stream – as water is lost through transpiration, sodium ions are deposited cumulatively. These combined effects lead to the impairment of vital biological processes.

Plants can respond in various ways to these challenges. Plant responses to salt stress are systemic in scope, occurring in both roots and shoots, at the organ, tissue, cellular and subcellular scales. Common responses include processes such as stress sensing and signalling, regulation of ion homeostasis, metabolism, stomatal aperture, photosynthesis, cell division and expansion, as well as changes in plant morphology and architecture, phenology and resource allocation (reviewed by (Munns and Tester, 2008) and (Hairmansis et al., 2014)). The responses employed, their nature, extent and the mechanisms that drive them can vary greatly between and within plant species. It is the interplay between the various direct effects of salt stress and the numerous potential plant responses that give rise to the wide diversity of salt stress-induced phenotypic effects observed across the plant kingdom.

Observable phenotypic effects of salt stress tend to be divided into rapid shoot ion-independent effects (previously referred to as osmotic effects) and delayed ionic effects. Shoot ion-independent effects typically include rapid reductions in turgor, stomatal closure, and reduction of transpiration, photosynthetic and growth rates. Ionic effects develop gradually as sodium accumulation progresses and generally involve a gradual decline in photosynthetic, metabolic and growth rates, photosystem damage and early senescence.

It can be difficult to differentiate between the direct effects of salt stress and plant responses to them in establishing the salt stress phenotype. Plant responses *per se* could be viewed as those processes that lead to a deviation from the theoretical base effects of

salt stress, rather than the observable phenotype as a whole. For example, a reduction in transpiration rates can be expected as a direct consequence of the reduced water potential of the growth medium, but can also be a plant response that limits sodium accumulation. This response can be considered adaptive, but it bears noting that plant responses are not necessarily adaptive. For instance, deliberate, prolonged reduction of transpiration rates can prove a fatal plant response, as it usually implies reduced growth rates that in turn could jeopardise reproductive success. Depending on the context, it can be more advantageous to maintain high transpiration and growth rates, pushing through the reproductive cycle quickly to ensure seed production.

Investigation of plant responses to salt stress involves comparing genotypes with distinct phenotypes under salt stress, by studying either mutants or natural variation. Identifying the underlying genetics can remain highly challenging given the wide array of available plant responses that can give rise to a particular phenotypic effect. The issue is further exacerbated for phenotypic effects that relate to complex traits. To address this issue, the recommended strategy is to dissect the phenotypic effects of salt stress (*e.g.* effects on photosynthesis, effects on growth, effects on transpiration) and study the plant responses and their genetic basis for each trait separately, as these are likely to arise from distinct mechanisms.

Dissecting plant salt tolerance into contributing traits and mechanisms

In its broadest sense, salt tolerance is a measure of the maintenance of desirable plant performance traits under salt stress relative to control conditions. In practice, there is no single measure of salt tolerance as there are various indices by which maintenance of performance can be expressed, each with a slightly different focus (examples presented in Table 1). For instance, the S/C index is the ratio of trait values under salt and control conditions, while the TOL index is the absolute difference between the two values. Generally, salt tolerance is measured using S/C index due to its widespread use and broad relevance. There are also numerous plant performance traits that can be considered when evaluating salt tolerance. For application-oriented research, salt tolerance should ultimately be assessed based on economically or societally relevant traits in crops. Yield is typically the most important, but biomass, fruit or grain quality and others may be valid, depending on the species and market demands. Other traits (*e.g.* transpiration, photosynthetic activity, metabolite profiles) should be considered, not as measures of salt tolerance in themselves, but rather as potential contributing factors to salt tolerance in terms of these traits of real world relevance.

Yield, for example, is a highly complex trait with many contributing traits. As a result of this complexity, it can be challenging to study the genetics of salt tolerance for yield *in toto*. Instead, it is more effective to study the genetics of the downstream traits that contribute to salt tolerance for yield. These contributing traits, their responses to salt stress and how they contribute to salt tolerance for yield can be dissected hierarchically (Figure 1). As such, salt tolerance for yield can arise from the maintenance of harvest index, *i.e.* the yield fraction of overall biomass, which itself depends on maintained growth rates, in turn determined by traits such as transpiration, radiation and nutrient use efficiency (TUE, RUE, NUE), each of which are influenced by traits such as stomatal aperture and photosynthetic activity – and so on, down to the gene level. Each downstream component can contribute to salt tolerance for yield; thus, constituting a salt tolerance mechanism. With a sufficiently comprehensive phenotyping methodology, insight into the eventual contribution of component traits to salt tolerance in terms of yield can be gained using correlation analyses. Though each of these components may remain complex themselves, this disambiguation helps to dissect salt tolerance for yield into constituent parts that are more genetically tractable than their agglomerate.

The mechanisms that contribute to salt tolerance for yield, and subcomponents thereof, can vary between and within species, and also throughout development and across different environmental conditions. It is therefore important to account for factors such as genotype, salt stress concentration and distribution (e.g. temporal dynamics, topography), developmental stage at which stress is imposed, weather conditions, cultivation practices, soil type and water availability (Greenway and Munns, 1980; Tal and Shannon, 1983; Mano and Takeda, 1997; Foolad, 1999; Yamaguchi and Blumwald, 2005; Munns and Tester, 2008; Negrão et al., 2017). For instance, salt tolerance in terms of relative growth rate can vary across the seedling, young and mature plant stages, with different tolerance mechanisms contributing significantly throughout development. The same can apply for different environmental conditions. Salt tolerance can therefore be further dissected and become more genetically tractable by refining experimental methodologies to account for these details. As such, measures of salt tolerance for yield under different conditions at different developmental stages, for example, can be considered in isolation; contributing traits and underlying genetics can then be determined independently. The same framework can be applied to dissect salt tolerance using any trait of interest.

The perspective is therefore shifting from viewing salt tolerance as a complex trait to a complex of traits that can be dissected through the use of different indices, traits and conditions. Moreover, implementing this deconstruction is becoming ever more practicable with the emergence of new phenotyping technologies and robust methodologies that allow

the non-destructive, simultaneous and high-throughput measurement of numerous traits including many that were previously intractable.

Deploying emerging phenotyping technologies for comprehensive salt stress phenotyping

Phenotyping the effects of salt stress in plants has traditionally been a relatively manual and laborious process. The focus has been on traits such as direct biomass measurements (e.g. shoot and root, fresh and dry), yield-related traits (e.g. total seed mass, 1000-grain weight, grain number), basic morphometric and architectural parameters (e.g. plant height, root length, number and length of side shoots, tillers, lateral roots etc.), visual scores of general plant health status (e.g. survival, senescence) and physiological measurements (e.g. ion contents, stomatal conductance, chlorophyll content), as well as laboratory-based analyses of biochemical traits (e.g. metabolite and hormone levels) and gene expression (Campbell et al., 2017; Klem et al., 2017; Tschiersch et al., 2017; Ubbens et al., 2018). These methods have served well, particularly for reverse genetics approaches that typically deal with relatively small numbers of genotypes and thus sample sizes. However, for practical reasons, they have proven difficult to apply to forward genetics experiments that generally deal with large numbers of genotypes and sample sizes. Certainly, forward genetics salt tolerance screens have been widely performed on staggering numbers of genotypes, yet these have relied on a very small number of very simple traits such as survival at the young seedling stage. The recent emergence of high-throughput phenotyping (HTP) platforms is rapidly closing this gap, allowing the capture of large numbers of highly informative plant traits across vast experimental populations. Moreover, many of these new technologies are deployable in the field, offering much needed capabilities for studying key yield-related traits that are necessary for application-oriented research. Indeed, the vast majority of research into salt tolerance has thus far been extensively performed using model plants such as *Arabidopsis thaliana*, primarily due to practical constraints and the resulting focus of resources (methodological, genomic and genetic) towards this convenient organism. Yet, while *Arabidopsis* has without a doubt been the single most important species for the advancement of plant science over the past decades, its contributions to application-oriented research into salt tolerance are somewhat constrained by its inherently low level of salt tolerance and lack of agronomically relevant yield-related traits (Moller and Tester, 2007).

The past decade has seen the emergence of numerous HTP systems, which overwhelmingly rely on frequent, non-invasive, automated imaging to phenotype tens, hundreds, even thousands of plants in a short time frame (Furber and Tester, 2011; Ghanem et al., 2015; Junker et al., 2015). These systems facilitate the phenotyping of large numbers of genotypes in both controlled and field environments. HTP platforms typically use

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red green blue (RGB), thermal infrared (TIR), chlorophyll fluorescence (ChlF) and, more recently, multispectral and hyperspectral imaging (see reviews by (Humplik et al., 2015; Fahlgren et al., 2015)). In RGB imaging, pixels are captured and subsequently analysed to inform on plant morphometric and colorimetric parameters enabling the quantification of differences in growth rates or senescence, for example, under stress compared to control conditions (Rajendran et al., 2009; Awlia et al., 2016; Al-Tamimi et al., 2016). TIR imaging is used to measure canopy and plant heat signatures to provide indirect assessments of stomatal conductance and transpiration, which negatively correlate with leaf temperature (Merlot et al., 2002; Sirault et al., 2009). Measuring the photosynthetic performance of plants directly by measuring steady-state values upon light incidence or by observing the quenching kinetics of chlorophyll using ChlF imaging provides valuable insight into plant photosynthetic capacity and performance under stress (Baker, 2008; Jansen et al., 2009); (Campbell et al., 2015). Furthermore, multi- and hyperspectral imaging, which can capture narrow wavelength bands within and beyond the visible spectrum, show potential for the detection of stress responses (Fahlgren et al., 2015). Multi- and hyperspectral reflectance data is typically used to derive various vegetation indices that provide different estimations of the radiative properties of plant leaves or canopies. For example, the normalised difference vegetation index (NDVI) has been developed to assess plant health, while more complex spectral profiles afforded by hyperspectral imaging can be used to extrapolate the content of specific compounds, such as water, chlorophyll and even secondary metabolites like flavonoids and terpenoids (reviewed by (Klem et al., 2017)). The application of hyperspectral imaging for salt stress research is still in its infancy with only a few examples that focus on the estimation of vegetation indices as a proxy for crop stress (Syta et al., 2017; Naumann et al., 2009; Behmann et al., 2014; Romer et al., 2012). Although hyperspectral imaging is more expensive than other imaging techniques and requires higher expertise for operation, image processing and data analysis and interpretation, it is expected to provide useful insights into the structural, biochemical and physiological traits related to salt stress responses. Lately, HTP methods have become widely employed to measure plants in the field by using these imaging tools with either ground-based or aerial imaging to monitor crop responses to stress (Baluja et al., 2012; Grieder et al., 2015). Unmanned aerial vehicles (UAV), such as drones and fixed-wing aircraft, can measure plant traits at relatively high spatial and temporal resolutions. Moreover, they are becoming more affordable, accessible and common in crop research (reviewed by (Araus and Cairns, 2014)). However, data captured by UAVs requires substantial computational power and highly trained personnel to analyse, which poses challenges to the deployment of UAV-based phenotyping platforms (Banan et al. 2018).

The major challenge of these imaging technologies, and in HTP in general, is the massive amount of data generated by these systems, which requires sophisticated methods for data management, storage and analysis. To overcome this challenge, machine learning has become the leading method to facilitate data assimilation and trait identification for stress phenotyping (reviewed by (Singh et al., 2016)). More low-cost and open-source phenotyping technologies are continuously being developed, increasing their accessibility and usability for scientists and farmers alike (Tsafaris and Noutsos, 2009; Fahlgren et al., 2015). For example, the recently developed PlantCV is a low-cost HTP platform using low-cost Raspberry Pi microcomputers and cameras to acquire plant image data together with an open source analysis software (Tovar et al., 2018; Gehan et al., 2017).

As HTP systems can be employed for measuring large numbers of complex traits, they have great potential to measure the physiological effects of salt stress. For example, monitoring plant growth daily upon salt stress allows the distinction between tolerant and sensitive genotypes (Rajendran et al., 2009). HTP protocols have also been described in *Arabidopsis* using RGB and ChlF imaging, which identified a set of robust traits, such as growth rate, greenness and non-photochemical quenching for phenotyping the early responses to salt stress (Awlia et al., 2016). The use of HTP has allowed the dissection of the genetic components of temporal salt stress responses in crops, such as rice and barley (Hairmansis et al., 2014; Schilling et al., 2014; Campbell et al., 2015; Al-Tamimi et al., 2016). The availability of reference genomes and genotypic datasets for several plant species has facilitated the association of genomic and phenotypic data using mapping methods to detect more quantitative trait loci (QTL) that can significantly increase crop yield and stress tolerance in future breeding initiatives. The use of next-generation phenotyping with new imaging technologies and novel traits is also paving the way for modelling genotype-phenotype interactions to reflect processes affecting crop growth and yield (Cobb et al., 2013). Moreover, the advances in monitoring environmental conditions, with the use of sensor technology, should be exploited to correctly characterise salt tolerance in terms of its conditional factors, e.g. soil salinity, water availability, or climate (Figure 1). As such, genotype x environment (GxE) effects can be considered in the association and phenotypic modelling of traits contributing to salt tolerance.

As these new HTP platforms become increasingly sophisticated and accessible, our ability to screen large numbers of genotypes for forward genetics approaches grows steadily. Yet, to fully seize the opportunity of these new capabilities, there is a clear need for expansive germplasm resources to characterise and mine the genetic components of salt tolerance. Fortuitously, the phenotyping revolution has been accompanied by another technological breakthrough that is unlocking vast genetic resources: the explosion of DNA sequencing technologies.

Harnessing the genetic diversity of exotic germplasm

Current agricultural systems overwhelmingly rely on a small number of highly productive crops. A mere 20 plant species are used to fulfill 90% of the world's calorie requirements, with just three of these – rice, maize and wheat – supplying approximately 60% of the total (Massawe et al., 2016; Buchanan-Wollaston et al., 2017). Domestication of these food crops from wild species began more than 10,000 years ago, over the course of which the performance and genetic architecture of the original progenitors were radically transformed (Tanksley and McCouch, 1997; McCouch, 2004). The selective breeding of a small number of wild varieties carrying beneficial traits, such as compact plant stature, non-brittle rachis and loss of germination inhibition, produced landraces with superior performance, but also gradually eroded the genetic diversity in successive populations (Dempewolf et al., 2017). The contraction of crop genetic diversity has been further exacerbated by modern plant breeding whereby high-yielding elite cultivars are developed by crossing productive landraces, while the wild ancestors with greater genetic variation but poor agronomic value are ignored (Gascuel et al., 2017). Such genetic bottlenecks have been confirmed experimentally (Abbo et al., 2014; Haudry et al., 2007). The inclination of farmers to switch from growing local varieties and landraces to genetically uniform and high-yielding varieties has led to the loss of approximately 75% of genetic diversity in crops (Buchanan-Wollaston et al., 2017). Importantly, as modern breeding tends to be carried out in optimal agricultural settings, the genetic components of disease and pest resistance, as well as tolerance to abiotic stresses such as salt stress, are often amongst the lost genetic fraction. As a result, attempts to identify genes conferring salt tolerance within commercial crop varieties have yielded limited results.

Several sources of genetic variation are available to complement the genetic paucity of modern elite cultivars. Genetic diversity generated artificially through mutagenesis has proven a valuable resource for various crop species, as variants can be directly produced in commercial germplasm (Tu Anh et al., 2018; Gulfishan et al., 2016; Caldwell et al., 2004; Wang et al., 2017) (Mba, 2013; Çelik and Atak, 2017) (Nikam et al., 2015; Pando and Deza, 2017). However, given that salt tolerance is most likely to arise from the concerted effects of numerous mechanisms, the potential to artificially create salt tolerant variants is surely limited. On the other hand, these are quite likely to emerge through natural selection in plants exposed to harsh environmental conditions, as observed in landraces or wild relatives of crops (Mayes et al., 2012). Therefore, it is essential to study the naturally diverse germplasm available. Global genebanks have collected approximately two million distinct plant accessions of which a high percentage are landraces and wild relatives of crops (Commission on Genetic Resources for Food and Agriculture, 2010). The prospect of using

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such germplasm repositories as sources of natural variation for tolerance to environmental stresses such as salt tolerance has been widely discussed (Ali et al., 2017; Dwivedi et al., 2017; Buchanan-Wollaston et al., 2017; Zhang et al., 2017; Hanin et al., 2016; Gascuel et al., 2017; Tsujimoto et al., 2015; Cheng, 2018; Mabhaudhi et al., 2017; Massawe et al., 2015). Assessments of genetic diversity have been performed for various crop germplasm collections, such as maize (Whitt et al., 2002; Prasanna, 2012; Patto et al., 2004; Zheng et al., 2013; Kuhn et al., 2014; Warburton et al., 2008; Laborda et al., 2005; Liu et al., 2003), wheat (Nielsen et al., 2014; Laido et al., 2013), rice (Cho et al., 2000; Temnykh et al., 2001; McCouch et al., 2002; Garris et al., 2005; Xu et al., 2012; Thomson et al., 2017), barley (Struss and Plieske, 1998; Parzies et al., 2000; Fernandez et al., 2002; Moragues et al., 2010) and tomato (Bauchet and Causse, 2012; Albrecht et al., 2010; Aflitos et al., 2014; Pailles et al., 2017). Furthermore, various studies using forward genetics approaches have already demonstrated the value of utilising diverse germplasm in practice by detecting loci associated with various measures of salt tolerance in established crops, for example in maize, rice, wheat, barley, soybean, rapeseed and alfalfa (Al-Tamimi et al., 2016; Cui et al., 2015; Wan et al., 2017; Liu and Yu, 2017; Oyiga et al., 2018; Zeng et al., 2017).

Overall, investigations into natural variation for salt tolerance remain scant considering the potential and the available germplasm resources. This is true for major crop species, but even more so for underutilized or orphan crops. Despite showing great potential in terms of salt tolerance, these valuable genetic sources have remained comparatively untapped. This is mainly due to the relative intractability of these germplasm. For instance, it can be challenging to rigorously phenotype highly diverse and undomesticated germplasm panels. Limited genetic and genomic resources for these germplasm as well as difficulties in effectively transferring beneficial alleles into elite varieties have also proved significant obstacles (Mayes et al., 2012; Wang et al., 2017). Nevertheless, the recent technological breakthroughs in the fields of phenotyping, DNA sequencing, and molecular-assisted breeding are increasingly facilitating the use of these genetic resources for crop improvement. For example, quinoa (*Chenopodium quinoa*), an undomesticated pseudo-cereal that has gained popularity in developed countries due to its nutritional potential (Cheng, 2018), displays substantial genetic and phenotypic diversity (Figure 2), including accessions with high tolerance to salt stress (Jacobsen et al., 2003). The recent publication of two high-quality quinoa genomes, alongside genomic data for a growing number of accessions and close relatives, provides great promise for detecting the genetic components underlying this species' elevated salt tolerance (Jarvis et al., 2017; Zou et al., 2017). Interestingly, attempts are currently being made to domesticate these already salt-tolerant orphan crops, ahead of transferring this tolerance into major crops.

The accelerated development of DNA sequencing and genome assembly capabilities over the past decade is increasingly facilitating the genomic characterisation of new and uncharacterised germplasm – potential repositories of salt tolerance alleles and genes. Reference genome sequences are now available for over 300 plants species (as of March 2018; www.plabipd.de), with chromosome-level assemblies for several major crops including rice, maize, and wheat as well as less established species such as quinoa (Jackson, 2016; Schnable, 2012; Mayer et al., 2014; Jarvis et al., 2017). While the ever-decreasing cost of short-read sequencing platforms have driven much of this progress, it is the emerging long-read, real-time, linked-read, and single-molecule sequencing techniques that are allowing researchers to overcome the intrinsic challenges of plant genomes, namely their large size, frequently complex ploidy levels, and high repeat content (Bevan et al., 2017). The resulting high-quality reference sequences provide the basis for genome analyses towards identifying structural variants responsible for species-specific traits (e.g. genes involved in fleshy fruit evolution in *S. lycopersicum*, or triterpenoid saponin biosynthesis in *C. quinoa*) (Sato et al., 2012; Jarvis et al., 2017). Such analyses have also provided insight into the potential genomic basis of drought and salt tolerance in the wild tomatoes *S. pennellii* and *S. pimpinellifolium*, respectively (Bolger et al., 2014)(Razali et al., 2018).

The greatest value of these genomic resources lies in their ability to empower the capture and characterisation of genetic variation. Indeed, high-quality genomes beget high-quality genotyping, from well-established genotyping chips to more recent genotyping-by-sequencing methods, including various reduced representation sequencing and whole genome resequencing approaches (Bevan et al., 2017; Scheben et al., 2017; Dwivedi et al., 2017). Using these approaches, vast numbers of accessions can now be genotyped relatively quickly and cheaply, with the potential to discover millions of SNPs. Extensive genotypic resources are thus available for all sequenced major crop species, a number of wild relatives and increasingly for aforementioned orphan crops, including diversity panels and mapping populations (e.g. bi-parental, nested association) (reviewed by (Rasheed et al., 2017)). These growing resources will facilitate continued and improved investigations into the genetic basis of salt tolerance.

Beyond improvements for cost and throughput, increases in coverage, accuracy, and the ability to discriminate haplotypes between homologous chromosomes offer growing insight into structural variations such as copy number variants (CNVs), presence-absence variants and chromosomal rearrangements (insertions, deletions, inversions, translocations) that are typically not captured by SNP-based approaches. Our greater understanding of these structural variants, in particular, CNVs that are widespread in plant genomes where they have been shown to contribute significantly to phenotypic variation, will provide much-needed progress for the improvement of crop salt tolerance. Furthermore, the increased

potential for *de novo* genome assembly for large numbers of accessions from a given species or closely related species facilitates the compilation of pangenomes, which summarise the structural variation observed across a large number of genotypes, providing a more comprehensive insight into structural variation (Golicz et al., 2016). Pangenomes have been compiled for many major crop species such as maize, rice, hexaploid wheat, soybean, and *Brassica oleracea*, but have yet to be fully explored in terms of connecting genetic and phenotypic variation, particularly with regards to salt tolerance (Li et al., 2014; Zhao et al., 2018; Golicz et al., 2016; Montenegro et al., 2017; Hirsch et al., 2014).

The increasing availability of reference genomes and genotypic resources for exotic plant species, alongside the continuous development of tools to rigorously phenotype various salt tolerance components, will enable the scrutiny of these exotic germplasm resources for salt tolerance, the underlying mechanisms and their genetic basis. Such applications will continue to rely heavily on existing and emerging statistical approaches that detect associations between genetic markers and phenotypic data through GWAS.

Going beyond conventional mapping

In forward genetic studies, the population size, phenotyping accuracy and linkage/association populations need to be carefully considered (Zhao et al., 2007). Striking a balance between the number of screened plant accessions, duration and temporal resolution time, traits to be measured and phenotyping precision are essential parameters to consider when planning an experimental setup that can reliably detect plausible loci with high statistical power. For marker-trait association mapping, GWAS can provide insight into complex trait architecture, highlighting potential QTLs affecting traits that contribute to salt tolerance depending on the model used (Korte and Farlow, 2013). The basic and original approach in GWAS is to evaluate the association between each genotyped marker and a phenotype of interest that has been scored across a large number of individuals. The widely used conventional mixed linear model (MLM) association using the efficient mixed-model association (EMMA) algorithm, creates a linear mixed model that corrects for population structure using a marker-based kinship matrix (Kang et al., 2008). In this section, available GWAS methods and models are summarised, presenting the advantages and disadvantages of each, for easy reference for the plant science community (Table 2).

There is a large number of software packages that facilitate essential GWAS analyses for estimating the genotype-phenotype associations, but very few published comparisons of the available methods by which an informed selection can be made. The most commonly used for applications in plant science are TASSEL, GAPit and PLINK, with a growing number of studies being carried out using the R statistical computing software, which allows more flexibility in the development of complex genetic association models.

However, no available software or R package has been able to provide the ideal balance between advanced statistical methods, time efficient simulations, accessibility and user-friendliness. With the major developments in phenotypic technology and exploration in analytic approaches, GWAS has become an even more effective and efficient technique to study the genetics underlying trait variation. The field of plant genetics has recently proposed many improved statistical methods to increase statistical power, reduce false-negative rates, and incorporate biological context in GWAS results. Moreover, advanced forward genetics experimental setups, such as those that measure many traits over time for a large number of plants, are computationally demanding, adding to the requirement of more efficient methods for analysing high-throughput phenotyping data. For this purpose, longitudinal genome-wide association models have been developed to elucidate the genetic basis of the dynamic rice growth responses to salt stress (Campbell et al., 2015). The model used accounts for both genetic relationships between genotypes due to the rice subpopulation structure and the inherent non-independent nature of daily observations.

Mixed models and Bayesian approaches are being explored to take into account environmental and treatment effects while other models account for the effects of genetic background, interactions between multiple phenotypes, as well as confounding phenotypes. Studies have used phenotypic covariates to address relationships among traits. An alternative GWAS interaction model was developed which integrates control and saline conditions. This interaction model incorporates the 'main effects' of the marker (SNP effect) and treatment (control or salt) as well as the marker-by-treatment interaction (SNP effect in response to the treatment). The use of this interaction model enabled the identification of significant loci specifically associated with salt stress (Al-Tamimi et al., 2016). Another approach can be used to manage correlated phenotypes through deriving a fully parameterised multi-trait mixed model (MTMM) that considers both the within-trait and between-trait variance components simultaneously for multiple traits. This GWAS approach was first used for an Arabidopsis dataset for flowering measurements in two different locations, identifying loci whose effects are specifically determined by the environment (Korte et al., 2012). A study in rice controlled for the confounding effects of flowering time on panicle architecture, by eliminating SNPs associated with the former, making it easier to investigate loci associated uniquely with panicle phenotypes and their effects on yield (Crowell et al., 2016). Such approaches can greatly improve the efficacy of GWAS approaches and are strongly encouraged towards elucidating complex phenotypes such as salinity tolerance.

The conventional EMMA method is based on single-locus tests (Kang et al., 2008) but as traits can be controlled by many loci with broader effects, as is the case for salt tolerance, this model does not produce a good estimate of marker effects. Numerous multi-

locus methodologies, which assume several loci contribute to the phenotype of interest, have been suggested, including penalised regressions (Hoggart et al., 2008), Lasso (Waldmann et al., 2014; Wang et al., 2011), (Yi and Xu, 2008), and even the elastic net (Waldmann et al., 2014). Another multi-locus mixed model method involves the EMMA eXpedited (EMMAX) algorithm, which approximates the genetic and residual variance components and uses the same variance for all SNPs, re-evaluating the genetic and residual variances at each step of the algorithm to avoid repetitive variance component estimation for each SNP (Segura et al., 2012). An example of multi-locus mixed linear models used for salt tolerance traits in rice showed 20 QTN (quantitative trait nucleotides) associated with 11 traits including 6 QTN affecting salt tolerance at the germination stage and 14 QTN at the seedling stage (Naveed et al., 2018). Bayesian approaches have also extended to genetic association studies due to shown practical and theoretical advantages in recent papers (Marchini et al., 2007; Servin and Stephens, 2007; Wakefield, 2007; Verzilli et al., 2008; Fridley, 2009). A number of software packages (e.g. SNPTEST (Marchini et al., 2007) and BIMBAM (Servin and Stephens, 2007)) enable simple genome-wide Bayesian analyses to be done easily and quickly on any standard desktop computer. Additionally, (Wang et al., 2012) proposed new GWAS approaches called GWAS by single-step genomic BLUP (ssGBLUP) and single-step GWAS (ssGWAS). The ssGWAS method provides the most comprehensive information for genomic evaluation. The ssGWAS model allows all the available data, including genetic markers, phenotype records and pedigree information to be examined simultaneously in one step. Lately, many studies have validated this method and effectively implemented ssGWAS in pigs (Wu et al., 2018; Howard et al., 2015) and other species (Silva et al., 2017), achieving a greater power and more precise estimate values than other models. This single step GWAS is currently being tested in plant species by exploring the use of raw data (individual replicates) as opposed to standard approaches that use the means (across replicates), which leads to error inflation for the association analysis (unpublished data). Thus, genetic markers, the replicates measurements of each trait, spatial and design factors, and marker-treatment interaction can be simultaneously considered in a single model. Results from this approach are expected to increase in statistical power as each individual plant is considered as the input data instead of using the means of each genotype. This approach could be beneficial for complex quantitative salt tolerance traits that have various components, which can be dissected effectively by a single step GWAS model, as we are able to detect significant loci specifically associated with salt stress due to the incorporation of the interaction model to the ssGWAS.

These rapidly evolving genome-wide association models in crops are promising and highly useful tools for allele and marker discovery.

Future directions

To provide new insights into genotype-phenotype relations in terms of salt stress responses and tolerance in plants, the focus lies on the rapid advances of DNA sequencing technologies and HTP. This exciting era of omics tools promises to deliver powerful genetic and phenotypic resources that will enable a new generation of salt tolerant crops. The knowledge generated by these resources is expected to improve: i) longitudinal GWAS combining HTP and new association methods (similarly to what has been used in other research areas (Ning et al., 2017; Vujkovic et al., 2016); ii) the genetic power to pinpoint the causal gene underlying a specific locus – for example, in Arabidopsis, the availability of 10 million SNPs has allowed the mapping of significant SNPs resulting from GWAS and to specifically mark them by saturating the genome browser, thus uncovering the causal genes; and iii) breeding programs through identification of accessions carrying causal SNPs as donors for crossing schemes, and the use of causal SNPs to drive marker-assisted selection. Moreover, speed breeding methods (Watson et al., 2018), which can achieve up to six generations per year instead of the typical 2-3, is expected to contribute to the fast delivery of salt tolerant crops.

Rapid advances in breeding strategies, together with the availability of new genomic tools, have paved the way for the use of genomic selection (Crossa et al., 2017). Genomic selection uses a training population of individuals that have been phenotyped for the trait of interest, and genotyped with genome-wide markers that predict the breeding value of these individuals, to enable the selection of superior lines and predicting a genotype performance (Jannink et al., 2010). For example, different genomic models were tested to facilitate the selection of superior genotypes and candidate genes for breeding drought tolerance in subtropical maize germplasm (Shikha et al., 2017). The conjunction of genomic selection with the information provided by population structure, GWAS and the known genetic architecture of specific traits has successfully led to an increase of breeding efficiency in crops such as rice (Spindel et al., 2015). To our knowledge, genomic selection has not yet been used for breeding for salt tolerance in any plant species. However, we anticipate that decreasing costs of genotyping and improved genomic models to predict breeding values will greatly contribute to accelerating the delivery of superior breeding lines with salt tolerance.

Moreover, the adoption of genome editing tools will enable the validation of causal gene function. For example, CRISPR-Cas9 is expected to elucidate causal relationships between candidate genes identified by GWAS and phenotypic traits for the implementation of targeted molecular-assisted breeding programs (Yin et al., 2017). However, genome editing will not only assist the functional validation of loci of interest, but will also enable the simultaneous

modification of multiple genetic loci in elite crops, thus advancing breeding programs and the translation of findings to the field.

Genetic diversity is a key asset to improve yield stability and stress tolerance. To improve agriculture, researchers use germplasm of crops and their wild relatives adapted to different environments as genetic resources (Mickelbart et al., 2015; Tanksley and McCouch, 1997). The use of wild relatives should not be confined to their use for increasing the genetic diversity of elite crops. One may consider the hypothesis of domesticating minor and orphan crop species with higher salt tolerance directly. Quinoa, for example, has a high potential of development as it exhibits a high tolerance to several abiotic stresses including salt tolerance (Bazile et al., 2016; Jarvis et al., 2017; Jacobsen et al., 2003), and thus breeding for important agronomic traits, such as shorter plants with fewer branches and more compact seed heads, will encourage commercial production in sub-optimal environments where salinity is a major obstacle.

Here we have emphasised the importance of field HTP using UAVs. QTL mapping using traits collected by UAVs has enabled the identification of different alleles contributing to the rapid selection of high yielding varieties in rice (Tanger et al., 2017). We anticipate that machine learning algorithms will help leverage the outputs from emerging imaging technologies such as hyperspectral and thermal imaging to drive advances in the identification of genomic regions controlling salt tolerance components in important crop species.

Finally, it is worth highlighting that all the strategies discussed in this review paper can certainly be expanded beyond research into salt tolerance. For example, harnessing new genetic resources such as understudied crops or taking advantage of new imaging techniques using HTP along with new statistical association models will accelerate the discovery of new mechanisms of stress tolerance for any given stress. By establishing the connection between genotype and phenotype, we can advance the selection of high-yield stress tolerant crops and improve agricultural production to address the growing pressures on food security.

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Conflict of interest

All authors declare that there are no conflicts of interest.

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Table 1. Examples of stress tolerance indices.

Y_c and Y_s denote the value of the trait selected for assessing salt tolerance for a particular experimental unit, under control and stress conditions, respectively. $Y_{\bar{c}}$ and $Y_{\bar{s}}$ denote the population-wide yield under control and stress conditions, respectively.

Index	Described in
$S/C = \frac{Y_s}{Y_c}$	Salt tolerance index (Munns, 2002)
$TOL = Y_c - Y_s$	Tolerance index (Fox and Rosielle, 1982; Fernandez, 1992)
$MP = \frac{Y_c + Y_s}{2}$	Mean productivity index (Rosielle and Hamblin, 1981)
$GMP = \sqrt{Y_c \times Y_s}$	Geometric mean productivity (Fernandez, 1992)
$SSI = \frac{\frac{Y_c - Y_s}{Y_c}}{\frac{Y_{\bar{c}} - Y_{\bar{s}}}{Y_{\bar{c}}}}$	Stress susceptibility index (Fischer and Maurer, 1978)
$STI = \frac{Y_c \times Y_s}{(Y_{\bar{c}})^2}$	Stress tolerance index (Fernandez, 1992)
$SWP = \frac{Y_s}{\sqrt{Y_c}}$	Stress weighted performance (Saade <i>et al.</i> , 2016)

Table 2. Comparison of available GWAS methods and models.

Method	Description	Tools	Literature	Benefits	Limitations		
Single locus method	Exact methods	EMMA	Efficient Mixed-Model Association	TASSEL (http://www.maizegenetics.net/tassel/), EMMA package	Kang et al. (2008)	Polygenic variance is re-estimated with each marker analyzed	Computationally intensive, only allows single effect (samples taxa) to be tested. Random effect. All other effects are treated as fixed
		GEMMA	Genome-wide Efficient Mixed-Model Association	GEMMA software (http://stephenslab.uchicago.edu/software.html)	Zhou and Stephens (2012)		
		FaST-LMM	Factored Spectrally Transformed Linear Mixed Models	FaST-LMM (python)	Lippert et al. (2011)		
Single locus method	Approximate methods	EMMAX	Efficient Mixed-Model Association Expedited	TASSEL	Kang et al. (2010)	Scales linearly with cohort size in both run time and memory use, substantially increased speed	Do not involve re-estimating polygenic variance, less accuracy, systematic and appreciable underestimation of the most significant values
		CMLM	Compressed Mixed Linear Model	TASSEL and GAPIT (http://zzlab.net/GAPIT/)	Zhang et al. (2010)		
		P3D	Population Parameters Previously Determined	TASSEL	Zhang et al. (2010)		
Multi-locus methods		MLMM	Multi-Locus Mixed Model	Python (https://github.com/bvihal/mixmogam/), (https://github.com/Gregor-Mendel-Institute/mlmm/)	Segura et al. (2012)	Identify evidence for allelic heterogeneity, well interactions, unbiased analysis for interactions within the selected set of SNPs, to some extent handle the confounding usually attributed to population structure	Forward-backward inclusion, SNPs limits exploring the huge model space
		LMM-Lasso	Least absolute shrinkage and selection operator	Python (https://github.com/BorgwardtLab/LMM-Lasso)	Rakitsch et al. (2013)		
		BSLMM	Bayesian Sparse Linear Mixed Model	GEMMA software (http://stephenslab.uchicago.edu/software.html)	Zhou et al. (2013)		
Multi-trait methods		MTMM	Multi-Trait Mixed Model	R software (https://github.com/Gregor-Mendel-Institute/mtmm)	Korte et al. (2012)	Considers both the within-trait and between-trait variance components	Difficult to implement in natural population-based mapping owing to computational complexity, cannot control for population
		Interaction model	Marker-by-treatment interaction model	Asreml (http://datadryad.org/doi:10.5061/dryad.3118)	Al-Tamimi et al. (2016)		
Bayesian methods		SBL	Sparse Bayesian Learning regression model	V2 sparse Bayes software for Matlab*, NPTEST, BIMBAM, SAS programs (BAYES, SVS and ENAL)	Tipping (2001)	Ability to estimate PE which is the total proportion of variance in response explained by relevant covariates, combine prior beliefs of marker effects, which are expressed in terms of prior distributions	Computationally intensive as it requires Markov chain Monte Carlo (MCMC)
*One-step GWAS		ssGBLUP	single-step Genomic BLUP	BLUPF90 software (http://nce.ads.uga.edu/software/)	Wang et al. (2012)	Greater power and precise estimate value	Computationally demanding, not implemented in plant species yet.
		one-step interaction	one-step marker-by-treatment interaction model (unpublished)	Asreml	Al-Tamimi (unpublished)	Greater power and precise estimate value	Computationally demanding, cannot work with missing data

Figure 1. A plant scientist's guide to dissecting salt tolerance.

For application-oriented research, salt tolerance should be assessed according to the final trait of interest, most commonly yield – these are typically highly complex. Overall salt tolerance can be hierarchically dissected to identify downstream traits that are more genetically tractable. Traits that are seen to correlate with overall salt tolerance point towards processes that contribute to overall salt tolerance, *i.e.* salt tolerance mechanisms. A non-exhaustive list of salt tolerance-related traits and mechanisms is presented with a broad sense of their hierarchical organisation. This top-down approach can eventually lead to the identification of underlying genetic components. Importantly, salt tolerance can be calculated using various stress tolerance indices, each of which provides a different perspective and a distinct focus on different aspects of salt tolerance. Moreover, any measure of tolerance can be influenced by the conditions under which it is assessed (examples are illustrated in circles) and the causal mechanisms underlying each measure can be distinct. HI, harvest index; RMR, root-mass-ratio; RGR, relative growth rate; TUE, NUE, RUE, transpiration-, nutrient-, and radiation-use efficiency, respectively; S/C, Salt tolerance index (Munns, 2002); TOL, tolerance index (Fox and Rosielle, 1982; Fernandez, 1992); MP, mean productivity index (Rosielle and Hamblin, 1981); GMP, geometric mean productivity (Fernandez, 1992); SSI, susceptibility index (Fischer and Maurer, 1978); STI, stress tolerance index (Fernandez, 1992).

Figure 2. The phenotypic diversity of *Chenopodium quinoa* germplasm.

The genetic diversity of quinoa germplasm can be clearly seen through the wide variety of panicle shape, size and colour displayed in these field-grown specimens (30 distinct accessions grown in the same season and field) (Photo credits: Gabriele Fiene).



