Advances in functional genomics for investigating salinity stress tolerance mechanisms in cereals

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INTRODUCTION

Abiotic stresses such as drought, salinity, extreme temperatures and nutrient deficiency, and toxicity are major causes of plant stress and agriculture yield losses. With an increase in the loss of arable land due to drought and salinity it is imperative that we increase the crop yield per hectare. In terms of worldwide production maize, rice, wheat, and barley are the four most important crops (844, 672, 650, and 123 MT, respectively, 2010). Wheat and barley are grown in increasingly hostile environments with soil salinity and drought both expected to increase this century, reducing the availability of arable land. Barley and wheat are classified as glycophytes (salt-sensitive), yet they are more salt-tolerant than other cereal crops such as rice and so are good models for studying salt tolerance in cereals. The exploitation of genetic variation of phenotypic traits through plant breeding could significantly improve growth of cereals in salinity-affected regions, thus leading to improved crop yields. Genetic variation in phenotypic traits for abiotic stress tolerance have been identified in land races and wild germplasm but the molecular basis of these differences is often difficult to determine due to the complex genetic nature of these species. High-throughput functional genomics technologies, such as transcriptomics, metabolomics, proteomics, and ionomics are powerful tools for investigating the molecular responses of plants to abiotic stress. The advancement of these technologies has allowed for the identification and quantification of transcript/metabolites in specific cell types and/or tissues. Using these new technologies on plants will provide a powerful tool to uncovering genetic traits in more complex species such as wheat and barley and provide novel insights into the molecular mechanisms of salinity stress tolerance.

Keywords: abiotic stress tolerance, functional genomics, spatial resolution, metabolomics, expression profiling

Complex traits such as an increased tolerance to abiotic stresses are multi-genic and thus are difficult to identify. The improvement of crops is largely dependent on exploiting genetic variation and this has been achieved in the past through traditional plant breeding methods (Langridge and Fleury, 2011). One approach to elucidate abiotic stress tolerance mechanisms is to identify naturally occurring genetic variation within a crop species by screening varieties, wild genotypes and landraces (Roy et al., 2011b). The use of forward genetics is a powerful approach to study traits contributing to abiotic stress tolerance, however, it is crucial that reliable and accurate quantitative phenotyping methods are employed to assist in the identification of these traits. In recent years, phenomics technologies have become much more advanced and high-throughput, non-destructive measurements can now be made to assist in accurate phenotyping (reviewed in Roy et al., 2011b). In conjunction, recent advances in high-throughput functional genomics technologies such as next generation sequencing (NGS), transcriptomics, metabolomics, and proteomics make it possible to use a systems biology approach to understand the response to environmental stress. In this review we will discuss the use of high-throughput functional genomics technologies to understand salinity stress tolerance mechanisms in plants focusing on cereal crops. Most functional genomics studies have used the model plant Arabidopsis and very few studies have been conducted on more genetically complex plant species such as wheat. However, with the rapid development of functional...
genetic variation was observed in their ability to exclude Na.

For example, in a study on durum wheat genotypes, a wide accumulation of toxic concentrations of Na later stage (usually after weeks or months) and is a result of the mechanisms that control Na exclusion (Munns and Tester, 2008).

Within a species there can also be naturally occurring genetic variation in salt tolerance and this can be exploited for breeding of salt-tolerant crops (Roy et al., 2011). It has been proposed that the temporal response of plant tissue to soil salinity occurs in two separate phases that have been termed as osmotic and ionic (Munns, 2002). The early phase (hours to days) is described as an osmotic stress, due to the low water potential around the roots, in response to saline soils and/or water deficit. Osmotic stress is described as a shoot ion independent stress and can result in cell dehydration and loss of cell turgor pressure, and can be characterized phenotypically by a reduction in root elongation, inhibition of photosynthesis and a reduction in shoot growth (Munns and Tester, 2005). In contrast, ionic stress occurs at a later stage (usually after weeks or months) and is a result of the accumulation of toxic concentrations of Na and Cl in the cell cytoplasm resulting in decreased growth and yield. In response to salt stress, crop plants have evolved the following three tolerance mechanisms – (1) osmotic stress tolerance: ability to maintain water uptake and growth, (2) Na+ exclusion: exclusion of toxic ions from the shoot tissues, and (3) tissue tolerance: compartmentalisation of toxic ions into the vacuole or specific tissues (Munns and Tester, 2008).

One approach to identify adaptive traits to abiotic stresses is to screen for genetic diversity in populations (Figure 1). Genetic variation in salt tolerance has been shown in many cereal crops including barley (Shavrukov et al., 2010) and wheat (James et al., 2008). Despite a number of recent phenotypic studies, the molecular mechanisms for osmotic stress tolerance remain unknown. Osmotic stress is initially detected by the roots upon exposure to a low water potential (either as a result of water deficit or salinity). There are few studies describing genetic variation in the root growth response to osmotic stress, mainly due to the difficulties associated with visualizing and phenotyping roots (Gewin, 2010; Richards et al., 2010; Zhu et al., 2011). One example of genetic variation in root growth has been shown by the measurement of root elongation rate of eight barley genotypes (including barley cultivars, a landrace and wild barley) in response to the early component of salinity stress. In this study the wild barley, Hordeum vulgare ssp. spontaneum, was identified as the most tolerant (Shelden et al., 2013). The identification of genetic variation in root phenotypes will provide important information for future genetic characterisation and molecular studies to elucidate the molecular pathways involved in the early phase of salinity tolerance.

UTILIZING FUNCTIONAL GENOMICS APPROACHES TO ELUCIDATE PLANT STRESS RESPONSES

Functional genomics approaches such as genomics, transcriptomics, metabolomics, proteomics, and ionomics, have been extensively used to evaluate abiotic stress tolerance mechanisms in plants (Cramer et al., 2011). These platforms can be utilized to improve our ability to discover the genes and pathways that control specific traits in response to abiotic stress (Figure 1).

Plants are complex organisms with many different tissue and cell types and the interaction between these tissues and cells requires complex regulation. To gain a greater understanding of the complexities of cell-specific regulation, an increase in the sensitivity of functional genomic tools is required. To date, functional genomic approaches have mainly been utilized for studies of whole organs such as leaves and roots (Widodo et al., 2009), thus it is possible that important changes in both metabolites and gene expression go undetected because they are diluted out by the surrounding tissue. Combining a forward genetics approach with spatially resolved ‘omics approaches may greatly improve our
FIGURE 1 | Proposed strategy for the integration of physiology and systems biology to gain insights into abiotic stress responses in cereals and the future development of abiotic stress tolerant crops.

chances of discovering gene candidates leading to the generation of abiotic stress tolerant crops.

ADVANCEMENTS IN FUNCTIONAL GENOMICS TECHNIQUES FOR SPATIAL RESOLUTION TRANSCRIPTOMICS

Microarrays were the first available method for genome wide transcript expression profiling and have been used extensively in plant biology. Microarrays have been extensively used to generate transcriptional profiles in response to abiotic stresses in a range of plant species including, but not limited to, *Arabidopsis* (Kreps et al., 2002), *Vitis vinifera* L. (grapevine; Cramer et al., 2007) and *Hordeum vulgare* L. (barley; Walia et al., 2006). Studies of the transcriptional response to salt stress have been conducted in barley (Ueda et al., 2004, 2006; Walia et al., 2006, 2007; Gruber et al., 2009) and the model cereal *Brachypodium* (Kim et al., 2012) but due to the polyploidy genome, studies of wheat are more limited (Kawaura et al., 2006, 2008; Mott and Wang, 2007; Jamal et al., 2011; Gang et al., 2013). As the technology of microarrays and cell separation techniques has become more advanced it has become possible to do spatial profiling of plant tissues and cell-specific studies (Brady et al., 2007; Dinneny et al., 2008; Spollen et al., 2008). Cell-type specific transcript studies have been conducted in many plant species including *Arabidopsis* and the cereals maize, rice, barley, and soybean using microarrays (Pu and Brady, 2010; Long, 2011; Rogers et al., 2012). Despite a large number of microarray experiments, very few have led to the identification of stress tolerance pathways (Deyholos, 2010).

High-throughput, deep sequencing technology (RNA-Seq) provides a new strategy to detect and accurately quantitate changes in the transcriptome. NGS is still in its infancy in plant biology but is expected to supercede microarray based approaches as the technology becomes more developed. NGS technology is a powerful tool for gene expression profiling and has been used successfully in a number of organisms including mammalian systems (Mortazavi et al., 2008), yeast (Nagalakshmi et al., 2008) and plants (Lister et al., 2008) to show transcriptional activity. The use of NGS technology for expression studies is becoming more popular with studies reported in the model plant *Arabidopsis* (Weber et al., 2007) and several cereal species including *Glycine max* L. (soybean; Fan et al., 2013), *Lolium perenne* L. (perennial ryegrass; Studer et al., 2012), *Triticum aestivum* (wheat) endosperm (Gillies et al., 2012),
Zeae mays (maize; Litt et al., 2010), Sorghum bicolor (sorghum; Dugas et al., 2011), Panicum virgatum L. (switchgrass; Wang et al., 2012) and the extremeophile Thellungiella parvula (Dusanazaze et al., 2011). Studies now show that RNA-Seq provides better quantitation and accuracy than microarrays (Jain, 2012). Thrase of RNA-Seq in plant biology has been comprehensively reviewed and will therefore not be reviewed here (Jain, 2011, 2012). There are only very few studies published utilizing NGS to study abiotic stress responses in plants (Deyholos, 2010). These include studies on salt stress (Molina et al., 2009; Fan et al., 2013), cold stress (Tanamura and Yonemaru, 2010) and drought stress (Dugas et al., 2011; Dong et al., 2012; Vidal et al., 2012).

With the availability of a number of cereal genomes including Barley Sequencing Consortium (2012), sorghum (Paterson et al., 2009), maize (Schnable et al., 2009), rice (Project, 2005), and the cereal model Brachypodium (Vogel et al., 2010) it is now possible to use this information to conduct large scale NGS transcriptomic experiments in cereal crops. For example, in a study on Sorghum bicolor, RNA-Seq technology was utilized in combination with the sorghum genome sequence to analyze the root and shoot transcriptome in response to osmotic stress and abscisic acid (ABA; Dugas et al., 2011). The study identified over 28,000 unique genes that were transcriptionally regulated in response to osmotic stress and ABA. A collection of drought-regulated gene networks and transcription factors were identified that can provide a basis for further studies. The data in this study was also compared to other sequenced transcriptomes including maize, rice and Arabidopsis, identifying more than 50 differentially expressed drought responsive gene orthologs. The lack of fully sequenced genomes in plants with larger, more complex genomes, such as wheat (hexaploid), present many difficulties for these types of ‘omics studies. Utilizing these new technologies in combination with the completed genome sequences of cereals will provide powerful tool to uncovering genetic traits in more complex species such as wheat.

PROTEOMICS

Proteomic studies of the response to salt stress have been conducted in many plant species including the cereals rice (Oryza sativa), wheat (Triticum durum and Triticum aestivum), barley (Hordeum vulgare), and maize (Zea mays; reviewed in Zhang et al., 2012). A number of comprehensive reviews describing proteomic responses to abiotic stress are available and therefore will not be reviewed here (Kosova et al., 2011; Roy et al., 2011a; Zhang et al., 2012). Single cell proteomic studies have been successful in mammalian systems where cells can be cultured to increase the starting material (Schröl et al., 2013; Dikn and Peppelensbolsh, 2004), but there are relatively few examples of single cell proteomic studies in plants, and especially in cereals, due to the difficulties in obtaining enough material. Single cell-type proteomic analyses have been reported for Arabidopsis guard cells (Zhao et al., 2009a), trichomes (Wienkoop et al., 2004; Kryvych et al., 2011) and pollen (Holmes-Davis et al., 2005), soybean (Glycine max) root hairs (Wan et al., 2005) and tobacco (Nicotiana tabacum) trichomes (Amzser et al., 2005). Tissue specific proteomic studies of the response to salinity include a comprehensive proteomics study of rice anthers (Sarbahi et al., 2012) wheat root seedlings (Guo et al., 2012) and rice plasma membranes (Cheng et al., 2009). Kamal et al. (2012) analyzed wheat chloroplast protein abundance only and correlated their salt-responsive behavior to numerous physiological parameters. However, in most of these studies only a small number of salt-responsive proteins were identified (Zhao et al., 2008).

METABOLICOMICS

Metabolites are the building blocks for structural and enzymatic molecules and carry the energy required for growth and maintenance of living cells. Metabolites are a key link between genetic information and a phenotype and are a measure of the physiological state of an organism. In the past decade enormous efforts have been made to develop high throughput and untargeted methodologies to analyze as many metabolites as possible. The resulting relatively new field of metabolomics uses a set of sophisticated analytical biochemistry tools for the identification and quantification of metabolites. The application of bioinformatics and statistical tools allows the extraction and analysis of multivariate data sets supporting biological interpretation. An inherent challenge of metabolomics derives from the fact that the metabolome is a simple descriptor for what is, in fact, huge chemical diversity. Hundreds and hundreds of compounds with different physical and chemical properties such as molecular weight, molecular size, polarity, stability, volatility, solubility, and many more, require the development of comprehensive and/or complementary extraction, separation, detection, and quantification techniques (Beckles and Roessner, 2012). A number of analytical technologies, including chromatographic separation techniques, such as liquid and gas chromatography, coupled to mass spectrometry and nuclear magnetic resonance (NMR) spectroscopy have been successfully used to analyze metabolites in many different organisms, tissues and biofluids (Roessner and Beckles, 2009).

Metabolomic analysis has been used extensively to study abiotic stress responses in plants (Usano et al., 2010; Ohata and Ferrise, 2012). Due to the importance of salinity and drought stress in agriculture, many metabolomic studies have been conducted on agriculturally important crops to gain insight into the effect of such stress on metabolic activity. Analyzing genotypes that have differing phenotypic responses to abiotic stress (sensitive vs. tolerant) will help elucidate the specific metabolic changes that are contributing to the increased tolerance of a genotype. For example, in a study conducted on two genotypes of barley that differ in salt tolerance, it was found that the landrace Sahara accumulates higher levels of metabolites involved in cellular protection in the leaves, corresponding with higher leaf Na+ concentrations compared to the less tolerant genotype, Clipper (Widodo et al., 2009).

To date, most metabolomics analyses have been carried out on bulked tissues (e.g., whole roots or leaves) due to sensitivity levels of the technologies employed. This means that the metabolic profile of different tissue and cell types, each likely to be characterized by a specific metabolite profile or a particular response to environmental or genetic stimuli, have to be measured together. Instrumentation with increased sensitivity will help substantially, but a major problem remains, that it is often difficult or even impossible to separate and isolate tissue types or single cell types.
from plant tissues. The first success of a single cell metabolomics approach has been reported in a study of Arabidopsis where cryo-sectioning was used to preserve cellular structures. Specific cell types were cut and collected using laser micro-dissection to obtain a sufficient number of cells to allow the detection of about 68 major metabolites by gas chromatography/mass spectrometry (GC-MS; Schad et al., 2005). In future, it may be possible to develop cell-type specific protoplasts that can be cultured to increase the amount of tissue for analysis (Kersberg et al., 2011). However, one issue in any such metabolomics analysis is that protoplasts are cell-wall free cells and their generation and culture may affect the physiological state of the cells and therefore the range and concentration of metabolites.

An important aim of the characterization of metabolites in plants is to understand the spatial distribution of single metabolites. Initial such analyses have included indole-3-acetic acid (IAA) in Arabidopsis roots (Petersson et al., 2009) or ATP in Vicia faba embryos (Borsjuk et al., 2003), but these studies are now being extended to as many metabolites as possible. A very promising new approach to the spatial analysis of both proteins and metabolites is to raster across thin tissue slices and ionize any compound (peptides following proteolytic digestion or metabolites) using matrix-assisted laser desorption ionization (MALDI) coupled with analysis of every ionized molecule in a mass spectrometer (Kaspar et al., 2011). This technique, although not having cellular resolution, allows the determination of tissue-type metabolite distributions. This approach has been well developed for lipids (Vikolas et al., 2010; Hurn et al., 2012) since they are easily ionized, while efforts are now being made to optimize this approach for proteins/peptides (Grassl et al., 2011) and small molecules (Jun et al., 2010; Lee et al., 2012; Peukert et al., 2012). However, there are currently no reports in the literature on applications of metabolomics to the spatial analysis of salinity responses in plants. Therefore this approach provides the potential to monitor the cell and tissue specific adaptation mechanisms in cereals and provide novel ideas for the development of more tolerant crop genotypes. Techniques such as laser micro-dissection (Yi et al., 2012), fluorescence-activated cell sorting (FACS; Borges et al., 2012; Evrard et al., 2012) or MALDI based mass spectral imaging (Kaspar et al., 2011) may pave the way for such highly spatially resolved analyses.

IONOMICS

The high-throughput analysis of elemental compositions is called ionomics and has been extensively used in the plant sciences for screening mutant collections, forward and reverse genetics approaches and investigating mechanisms of elemental or ion uptake, transport, compartmentalisation and exclusion (Baxter, 2009). In the area of abiotic stress research, elemental profiling provides a way to investigate a plant’s response to salinity, nutrient deficiencies and toxicities or hyper-accumulation. There are 13 minerals essential to all plants, including macronutrients, such as N and P, and micronutrients such as Na, K, B, Mn, Fe, and Ca. Several mineral deficiencies lead to dramatic growth retardation and possibly death, but excess amounts of some minerals can also be toxic. In both cases, plant metabolism is dramatically affected; plants are able to develop mechanisms in order to cope with either deficiency or toxicity. Analysis of the elemental composition is not only important when studying effects of high salt on a plant but also when identifying mechanisms of adaptation and tolerance to any osmotic stress such as drought. Some ions have hygroscopic properties and therefore are utilized to retain water in the cell in order to sustain turgor pressure upon osmotic stress such as water deficiency. There have been many reports on the effects of the expression of ion transporters in plants; studies aimed at determining the function of those transporters (Horie et al., 2009; Jiang et al., 2010; Xue et al., 2011) or increasing tolerance to either salinity or nutrient deficiencies or toxicities (Hauser and Horie, 2010; Plett and Moller, 2010; Horie et al., 2011; Mian et al., 2011).

The simultaneous analysis of the major macro-elements and many of the microelements can be achieved using inductively coupled plasma spectroscopy or optical emission spectroscopy coupled with mass spectrometry (ICP-MS and OES-MS, respectively). With appropriate sample preparation methods, these techniques allow the analysis of between 12 and 20 elements and ions simultaneously (Baxter, 2009). However, in most studies bulk tissue (e.g., leaves or roots) is extracted for elemental analysis, thus giving no information about spatial distribution of elemental composition. Emerging technologies that allow image distributions of elemental composition of biological tissues have now become available for plant tissue sections. Laser ablation inductively coupled plasma mass spectrometry (LA-ICP-MS) is a very sensitive and efficient technique that produces three-dimensional images of spatial element distributions in thin biological tissue sections (Becker, 2010). This well advanced technique for the analysis of elemental distributions in human or animal tissue sections allows the reconstruction of three-dimensional atlases of spatial concentrations of elements (Horie et al., 2012). In the plant sciences this technique has not been utilized extensively but it is easy to envisage using it in numerous productive applications.

EXAMPLES OF SPATIALLY RESOLVED ‘OMICS FOR IDENTIFYING ABIOTIC STRESS TOLERANCE MECHANISMS IN CEREALS

There are very few examples of using spatially resolved functional genomics technologies to identify abiotic stress tolerance mechanisms in cereal crops. Utilizing emerging high resolution technologies to profile changes in a defined region of the plant could lead to novel insights into the molecular mechanisms of abiotic stress tolerance. The Arabidopsis root has been used as a model for understanding the mechanisms of cell-specific processes and has been extensively reviewed by Deumens (2010). The development of green fluorescent protein (GFP) reporter lines enabled the characterization of 14 different cell types in the Arabidopsis root, revealing a complex pattern of spatial control of biological functions (Brady et al., 2007). The advancement of these technologies and our current understanding from model systems such as Arabidopsis can now be utilized to increase our understanding of more complex plant systems such as the cereal crops. This will allow development of crops with enhanced tolerance mechanisms to environmental stresses.

The importance of using spatial resolution for ‘omics studies of abiotic stress has been shown in a study on maize roots in response to water deficit (Yamaguchi and Sharp, 2010). The maize primary root has been extensively studied as a model crop...
system for understanding the root growth response to water deficit. Physiological studies demonstrated that the response to osmotic stress is not uniform in the apical region of the root; the root elongation rate varies spatially in the elongation zone in response to both water deficit (Sharp et al., 1988) and salinity (Bernstein and Kafkafi, 2002). Physiological studies were combined with transcriptomic and proteomic profiling of the root zone (Yamaguchi and Sharp, 2010). Transcriptomic analysis was conducted on four regions of the root tip, designated as regions 1–4, defined by their differing spatial growth response to water deficit (Spollen et al., 2008). Only 7.5% of the differentially expressed transcripts were found to be the same between regions 1 and 2, indicating that the transcriptomic response was dependent on the position along the root apex. A proteomic analysis of the cell wall proteins identified an increase in cell wall extension proteins and apoplastic reactive oxygen species in the most apical region of the root where root elongation rate is maintained (Zhu et al., 2007). This raises the possibility that these compounds regulate extension properties contributing to the maintenance of root elongation in response to water deficit. This work has led to important advances in understanding the regulation and adaptation of root growth to water deficit and highlights the importance of studying specific regions of the root. These studies clearly demonstrate the importance of combining physiological data with functional genomic data as well as emphasizing the importance of using spatial resolution.

Other studies integrating spatially resolved ‘omics in cereals are limited. Using laser-capture microdissection (LCM) spatially resolved transcriptomics was utilized to gain insight into the development of the maize leaf (Li et al., 2010). Along the developmental gradient, 64% of genes were differentially expressed, and 21% were differentially expressed between the bundle sheath and mesophyll cells. This study utilized the completed maize genome sequence and high-throughput Illumina sequencing for spatial profiling of the maize leaf, providing novel information for understanding photosynthetic development in C4 plants. This information serves as a foundation for systems biology approaches and provides strong evidence for the importance of spatial profiling in plant studies.

CONCLUSIONS AND FUTURE PERSPECTIVES

To increase our understanding of a plant’s response to abiotic stress and identify the mechanisms involved in abiotic stress tolerance it is important to study both the physiology and molecular networks that are involved. The use of functional genomic tools in abiotic stress research has substantially extended our knowledge about the basic biology and physiology of abiotic stress responses, however, it has not yet allowed the identification of reproducible markers that could be used to determine if, and to what extent, the plant was under stress. By utilizing advances in both phenomics and functional genomics technologies it is becoming possible to identify cell and tissue specific changes in the molecular and biochemical networks in response to abiotic stress. This will lead to an increased understanding of the molecular mechanisms responsible for abiotic stress tolerance and the generation of abiotic stress tolerant crops.

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