High-throughput shoot imaging to study drought responses

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Abstract

Drought is a complex stress which elicits a wide variety of plant responses. As such, genetic studies of drought are particularly difficult. Elucidation of the genetic basis of components contributing to drought tolerance is likely to be more tractable than that of overall drought tolerance. Certain of the traits which contribute to drought tolerance in plants and the high-throughput phenotyping techniques available to measure those traits are described in this paper. On the basis of the dynamic nature of drought, plant development, and the resulting stress response, the focus is on non-destructive imaging techniques which allow a temporal resolution and monitoring of the same plants throughout the experiment. Information on the physiological changes in response to drought over time is vital in order to identify and characterize different drought-tolerance mechanisms. High-throughput imaging provides a valuable new tool which allows the dissection of plant responses to drought into a series of component traits.

Key words: Drought, imaging, infrared, phenotyping, RGB.

Introduction

Drought is one of the major limitations to food production worldwide (Pennisi, 2008). Maintaining a high yield in drought conditions has, therefore, become a priority, particularly when considering global environmental changes and the increase in world population (Takeda and Matsuoka, 2008). However, the physiological basis of yield maintenance under drought conditions remains poorly understood (Tuberosa and Salvi, 2006), due both to the numerous mechanisms that plants can use to maintain growth in conditions of low water supply and the complexity of the stress itself.

Breeders’ efforts are focused on minimizing the gap between yield potential and yield under stress (Cattivelli et al., 2008). Quantitative trait loci (QTL) or underlying genes conferring a yield benefit under drought conditions need first to be identified in phenotypic screens and then incorporated into elite germplasm using modern breeding technologies, such as marker assisted selection (Tester and Langridge, 2010). Despite some successes (Kumar et al., 2008; Venuprasad et al., 2008), the direct selection for yield in drought-prone environments has, however, proven to be difficult. Generally, the heritability of grain yield under drought is low compared with non-stress environments, due to many genotype×environment (GE) interactions (Cooper et al., 2001) resulting in numerous QTL×environment interactions (Piepho, 2000). Traits contributing to yield in the target environment may be less susceptible to GE interactions than yield itself (Fukai et al., 1999; Price and Courtois, 1999; Hammer et al., 2005), making these traits more suitable for a molecular breeding approach. Also, there is now evidence that selection for individual traits contributing to drought tolerance can improve grain yield in wheat (Richards, 2006) and maize (Edmeades et al., 1999).

The development of drought is dynamic in its nature and can occur at different periods of the crop cycle and with different intensities. Consequently, plants have developed various strategies in response to drought (Fig. 1) which can differ depending on species, genotypes, the type of drought, and the combination with other stresses, such as high temperature or evaporative demand.

Levitt (1972) classified plants growing in arid environments into drought-resistant plants (that can avoid or tolerate dehydration) and ‘escapers’, which avoid the terminal drought stress by shortening their growth cycle.
and/or by storing energy during the vegetative stage for grain filling during a later drought event (Bewley, 1979).

Dehydration avoidance is considered to be an adaptive strategy whereby plants decrease transpiration and modulate water extraction in order to retain water in the tissues and in the soil (Blum, 2009), thus avoiding the deleterious effects of later severe stress (Fig. 1). These processes are mainly co-ordinated by non-hydraulic signals, such as abscisic acid (Parent et al., 2009; Tardieu et al., 2010).

By contrast, dehydration tolerance is a conservative strategy and is specific to the type of damage that plants can suffer. In order to continue biomass accumulation under drought conditions, plants maintain photosynthesis and metabolism through low levels of senescence (stay-green phenotype), maintenance of growth and high stomatal conductance. At the same time, turgor is sustained through osmotic adjustment (Collins et al., 2008).

The final mechanism is the ability to survive and recover rapidly after a severe stress through protective mechanisms, such as cell wall folding, membrane protection, and the accumulation of antioxidants (Vicre et al., 2004; Barnabas et al., 2008).

In most temperate climates, dehydration tolerance is the only relevant mechanism (Chenu et al., 2008). However, in more severe conditions, such as in southern Australia and other Mediterranean climates, a combination of different mechanisms seems to come into play (Fig. 1). To determine the genetic basis of drought tolerance, therefore, it is necessary to separate variables implicated in hydraulic processes from others (Yue et al., 2006; Parent et al., 2010). Water relations themselves would determine whether the response is more conservative (tolerance) or adaptive (avoidance), and different strategies might be employed by lines bred for the same target environment (Izanloo et al., 2008).

In order to incorporate these traits into molecular breeding programmes and to identify the underlying genes in a forward genetics approach, reliable phenotyping protocols are extremely important (Salekdeh et al., 2009). Whereas high-throughput techniques have made gene and marker identification faster than ever (Ponce et al., 1999; Seki et al., 2002), the phenotyping process currently limits forward genetics. With the availability of genetic resources, such as mutant populations or mapping populations, high-throughput phenotyping will be essential to close the gap between plant physiology and genetics (Finkel, 2009; Furbank, 2009). High-throughput phenotyping technologies will be particularly important for studies of drought tolerance. The highly complex responses to drought require dissection of the responses into a series of component traits, the measurements of which are most accurately and efficiently made using non-destructive imaging technologies.

Some of the shoot traits contributing to drought tolerance in plants and the high-throughput phenotyping techniques available to measure those traits are outlined here. Because of the dynamic nature of drought development and the resulting stress response, the focus is on non-destructive imaging techniques which allow a temporal resolution and monitoring of the same plants throughout the experiment. Information about the physiological changes in response to drought over time is vital in order to identify and characterize the different drought-tolerance mechanisms.

Root physiology and root morphology also play a crucial role in the response to drought stress and are, therefore, important factors to be considered in improving the drought tolerance of plants. Current techniques used for root phenotyping have been reviewed recently (Gregory et al., 2009) and future developments are likely to be technology driven (Jahnke et al., 2009; Tracy et al., 2010).

**Experimental set-up for phenotyping**

For various stress conditions, whether biotic or abiotic, the onset and intensity of the stress can be clearly defined and controlled during a phenotyping experiment. However, when studying drought tolerance in plants, the level and onset of water deficit is more difficult to control and monitor since it is a dynamic process and a combination of the available water in the soil and the plant’s water status. This makes continuous measurements even more important to link the level of drought experienced by the plant with the physiological changes occurring in response to it (Salekdeh et al., 2009). Consequently, acquired data must always be analysed with respect to the environmental or micro-climatic conditions (Rodriguez et al., 2005; Sadok et al., 2006).
et al., 2007; Parent et al., 2010). This means that drought monitoring and the acquisition of phenotypic data should be performed at regular time intervals throughout the life cycle of the plant, ideally in an automated manner. Consequently, field trials and greenhouse experiments present quite different challenges.

In a field situation, the water stress can be controlled to a certain extent with rain-out shelters and irrigation, but this will limit the size of the experiment. Furthermore, genotypic variation among plants will mean that water is taken up at different rates and depth leading to quite different levels of stress. For instance, large differences in drought response observed between rice genotypes in the different levels of stress. For instance, large differences in water uptake and shoot transpiration. A direct measurement of these variables is difficult and currently not possible in a high-throughput phenotyping approach. As a consequence, remote sensing has long been used in an attempt to measure the water status of individual plants or canopies (Cleghorn et al., 1970; Blum et al., 1982; Table 1). Probably the most commonly used technique in this context is thermal infrared imaging, or infrared thermography (IRT), to measure the leaf or canopy temperature. Due to its large cooling effect, evaporation is commonly the main determinant of leaf temperature, and there is a direct relationship between leaf temperature, transpiration rate, and stomatal conductance (Jackson et al., 1981; Jones et al., 2002, 2009).

Dehydration-tolerant genotypes and genotypes with a greater capacity for water uptake can maintain a higher stomatal conductance and also a higher rate of photosynthesis (Martin and Ruiztorres, 1992; Medrano et al., 2002). These genotypes could thus be identified as being the plants which have the cooler leaves.

In a controlled environment, the experimental set-up can be optimized to combine the controlled irrigation and the phenotyping protocol. Cameras and the irrigation system can either be moved to the plants (Granier et al., 2006; Jansen et al., 2009), or the greenhouse can be fully automated with conveyor belts that will deliver the plants to watering, weighing, and imaging stations. This set-up is used in various phenotyping facilities (e.g. CropDesign, Ghent, Belgium; IPK Gatersleben, Germany; The Plant Accelerator, Adelaide, Australia) and has the advantage that the imaging stations can be optimized in terms of lighting conditions. Also, images can be acquired from different angles, which is important for cereal plants with a complex 3D structure. However, if plants are delivered to an imaging booth only one plant can be imaged at a time and, in order to permit a high-throughput, the time for image acquisition must be minimized. This is especially important when the circadian rhythm of the plant is likely to influence the imaging results, such as in the case of chlorophyll fluorescence measurements.

In a field situation, there is the choice of either moving the camera over the plants (e.g. mounted on a tractor: Moran et al., 1997; Montes et al., 2007), or taking images from a large enough distance to cover the entire field plot, for example, from a cherry picker or balloon (Moller et al., 2007; Jones et al., 2009). Use of a mobile camera close to the canopy has the advantage that images can be taken at high resolution and at exactly the same angle and distance for all field plots. However, it takes longer to acquire the images and climatic conditions such as photon irradiance or wind speed might change, which would be of particular concern for thermal images (Leinonen et al., 2006; Chaerle et al., 2009; Jones et al., 2009). If images are taken from a larger distance, many plots can be recorded simultaneously and the use of permanently mounted cameras (given that power supply and security are not an issue) allows for images to be recorded automatically at regular intervals.

Thermal infrared imaging to access transpiration rate

The integrator of drought is the plant water status (Jones, 2007), as determined by plant water content or water potential. It is the result of the equilibrium between root water uptake and shoot transpiration. A direct measurement of these variables is difficult and currently not possible in a high-throughput phenotyping approach. As a consequence, remote sensing has long been used in an attempt to measure the water status of individual plants or canopies (Jones et al., 1982). Probably the most commonly used technique in this context is thermal infrared imaging, or infrared thermography (IRT), to measure the leaf or canopy temperature. Due to its large cooling effect, evaporation is commonly the main determinant of leaf temperature, and there is a direct relationship between leaf temperature, transpiration rate, and stomatal conductance (Jackson et al., 1981; Jones et al., 2002, 2009). Dehydration-tolerant genotypes and genotypes with a greater capacity for water uptake can maintain a higher stomatal conductance and also a higher rate of photosynthesis (Martin and Ruiztorres, 1992; Medrano et al., 2002). These genotypes could thus be identified as being the plants which have the cooler leaves.

Canopy temperature has been used successfully as a selection criterion in breeding programmes for drought-prone environments (Blum et al., 1989; Fischer et al., 1998; Balota et al., 2008). Often, the selections were performed using infrared thermometers as these are less expensive and easier to handle than infrared cameras. However, thermal cameras offer several benefits compared with temperature sensors, most importantly the facility for spatial resolution. Most infrared cameras currently have arrays of 320×240 sensor elements, which means that more than 75 000 individual temperature readings are recorded in a single image. This allows more precise measurements in a fraction of the time needed to perform several replicate readings per plot with an infrared thermometer, which is also prone to error due to changing environmental conditions between measurements. Furthermore, a large number of plots in a field trial can be imaged at the same time, ideally allowing a comparison of differences in canopy temperature among genotypes without the need for normalization to determine the absolute leaf temperature (Jones et al., 2009). Nevertheless, it is important to keep in mind that even if all plots are imaged at the same time, plots at the edges or those surrounded by significantly taller plants might not be exposed to the same wind conditions.

The clear advantage in controlled conditions is the use of optimized imaging set-ups that facilitate an automated and
high-throughput data analysis, without the need to account for changing climate (Grant et al., 2006; Sirault et al., 2009). Using a temperature comparison under such conditions, individual mutant lines of barley and Arabidopsis with altered stomatal regulation were identified by imaging the mutant plants next to control plants (Raskin and Ladyman, 1988; Merlot et al., 2002; Xie et al., 2006). However, the plants or seedlings chosen in those studies were effectively two-dimensional structures. If plants with a more complex three-dimensional morphology are studied, both the orientation of the leaves towards the incident radiation and the camera angle used to record the images must be considered in the data analysis (Cohen et al., 2005; Kaukoranta et al., 2005; Grant et al., 2006; Jones et al., 2009). Colour images have been used successfully to identify sunlit and shaded leaves in a canopy and to determine the respective leaf temperature (Leinonen and Jones, 2004; Moller et al., 2007). The combination of colour and thermal imaging, therefore, has the potential to increase the information and precision of temperature measurements compared with thermal imaging alone, making this approach suitable for addressing issues of leaf orientation and canopy structure.

Visible and NIR imaging to assess the growth and water status of plants

Solar radiation occurs predominantly in the spectral range of the visible and near infrared (NIR) regions, with the wavelengths ranging from 400 nm to 2500 nm. The interaction of solar radiation with plant structure has been a major focus in remote sensing and in attempts to monitor the stress level of vegetation. Hyperspectral reflectance measurements (progressive scanning of very narrow wavebands) have enabled the identification of waveband signatures (spectral indices) indicative of plant stress levels. Even though hyperspectral reflectance measurements are not feasible for a high-throughput approach due to the time needed to acquire stable spectra, some aspects will be discussed here, since they underpin the basis for using visible and NIR imaging in assessing the drought response of plants.

In the visible spectrum, reflectance by single leaves or canopies is particularly low. This is due to absorption by leaf pigments, mainly chlorophyll, with a characteristic peak of reflectance in the green region of around 550 nm. With the transition from the visible to the NIR wavelengths, there is a sharp increase in reflectance, the so-called ‘red edge’. In the NIR, between 800 nm and 1300 nm, a large proportion of the incident radiation is reflected by leaves due to scattering within the leaf mesophyll (Knippling, 1970). Furthermore, NIR radiation can be transmitted from the upper leaves of the canopy to the lower leaves, which can reflect the photons back to the upper part of the canopy. As a consequence, leaf and canopy architecture, such as leaf thickness and growth habit, are the major determinants of the reflectance pattern in this part of the spectrum. With increasing wavelengths, beyond 1300 nm, reflectance decreases gradually due to an increased absorption by water present in the leaves with characteristic water absorption bands at 1450 nm, 1930 nm, and 2500 nm (Knippling, 1970): this is the basis for using NIR imaging to study water stress of plants.

However, incident sunlight is greatly influenced by atmospheric water vapour at about the same wavelengths leading to an extremely high noise-to-signal ratio (Sims and Gamon, 2003) and, therefore, largely restricting NIR measurements in the field to two minor water bands at 970 nm and 1200 nm. As a consequence, the most widely used reflectance indices using NIR reflectance focus on the area between 850 nm and 1200 nm, such as the Water Index or the Normalized Difference Water Index (Penuelas et al., 1993, 1997a; Gao, 1996). However, although these indices do allow the response of various plant species and crops to different water stress regimes to be monitored (Penuelas et al., 1993, 1997b; Babar et al., 2006; Seelig et al., 2008), this is more likely to reflect an overall change of plant and canopy architecture with a reduction in biomass and foliar density rather than a decreased relative water content (Knippling 1970). Indeed, various studies report a good correlation between NIR-based stress indices and the relative water content of the leaves only when severely drought-stressed samples were included (Eitel et al., 2006; Seelig et al., 2008; Table 1).

Table 1. Summary of non-destructive imaging methods to study Physiological changes occurring under drought

<table>
<thead>
<tr>
<th>Physiological changes occurring under drought</th>
<th>Non-destructive measurement method</th>
<th>Technical challenges</th>
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<tr>
<td>Decreased stomatal conductance</td>
<td>Thermal infrared</td>
<td>Influence of environmental conditions (air temperature, wind speed etc.)</td>
<td>Jones et al., 2009</td>
</tr>
<tr>
<td>Decreasing leaf water content</td>
<td>Near infrared</td>
<td>Orientation of leaves to light source and camera</td>
<td>Chaerle et al., 2009</td>
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<tr>
<td>Early onset of senescence</td>
<td>RGB / visible (or fluorescence if available)</td>
<td>Low sensitivity</td>
<td>Seelig et al., 2008</td>
</tr>
<tr>
<td>Decreased growth rate and biomass accumulation</td>
<td>RGB / Visible</td>
<td>Absolute measurements not possible at this stage, only monitoring of changes over time</td>
<td>Seelig et al., 2009</td>
</tr>
<tr>
<td>Decrease in photosynthetic activity (only under severe stress conditions)</td>
<td>Fluorescence</td>
<td>Pulsed imaging setups currently limited to about 100 cm² and only optimized for planophyll plants</td>
<td>Baker, 2008; Wo et al., 2008; Jansen et al., 2009</td>
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One way to increase the sensitivity is constantly to measure NIR reflectance over time, rather than at a single time point. Seelig et al. (2009) reported that even though an absolute measurement of the relative water content through remote sensing might not be possible at this stage, especially when working under mild drought stress conditions, they were able to monitor the onset of water stress in plants by performing NIR measurements at regular intervals after water withdrawal. This highlights the importance of continuous non-destructive phenotyping in drought-tolerance screens.

One of the first processes affected by water deficit is leaf growth, as its decrease usually occurs before any reduction of stomatal conductance or photosynthesis (Boyer, 1970; Saab and Sharp, 1989). The sensitivity of leaf growth to water deficit results in a decrease in final leaf area and final biomass accumulation (Monteith, 1977), and it can be highly variable even within the same species (Granier et al., 2006; Welcker et al., 2007). In addition, leaf growth seems to be genetically related to the sensitivity of reproductive growth (Welcker et al., 2007; Fuad-Hassan et al., 2008) and can, therefore, have a strong impact on yield under drought. In controlled environments, images of individual plants taken with digital colour cameras have been used to estimate biomass and to quantify relative growth rates (Granier et al., 2006; Tackenberg, 2007; Wiese et al., 2007; Rajendran et al., 2009; Table 1), and hence have the potential to be used for screening for genotypic differences in leaf elongation under drought conditions.

In addition, the colour information can be used to identify and quantify the degree of senescence (Rajendran et al., 2009). Senescence of older leaves during drought can be seen as an escape process, where the energy and nutrients in the older leaves are used to fill grain before the plant dies, or as an avoidance mechanism to decrease the evaporative surface. However, a stay-green phenotype can be equally advantageous under drought (Foulkes et al., 2007), allowing maintenance of photosynthesis.

In a field situation, greenness and biomass accumulation were traditionally estimated using reflectance measurements in the visible and NIR wave range (Penuelas and Filella, 1998; Carter and Knapp, 2001; Sims and Gamon, 2002; Campos et al., 2004; Harris et al., 2007). SPAD readings and the Normalized Difference Vegetation Index (NDVI) are possibly the most widely used vegetation indices to assess plant health and predict yield in the field (Markwell et al., 1995; Raun et al., 2001; Richardson et al., 2002), and specifically designed SPAD and NDVI spectrometers are commercially available. An advantage of these instruments is the ease of use and their wide application in the assessment of plant health in response to stress conditions, such as drought, salinity or nutrient deficiency (Penuelas et al., 1997b; Rodriguez et al., 2005; Perry and Roberts, 2008). At the same time, however, this means that these spectral indices measure a general stress level of the plant canopy rather than a stress-specific trait (Rodriguez et al., 2005). Even if they allow the identification of plants with an overall tolerance to a specific stress, they are unlikely to help in dissecting overall tolerance into genetically tractable traits. Hence, the question arises as to whether spatial resolution using colour and NIR cameras might provide more information than classical vegetation indices in plant phenotyping, helping to dissect stress responses into component traits. Attempts to replace spectral vegetation indices with colour image-derived indices are promising and these have been used to estimate traits, such as grain yield under drought conditions (Casadesus et al., 2007), the rate of senescence of wheat (Adamsen et al., 1999), and the early identification of wilting symptoms in tomato (Takayama et al., 2009). Furthermore, imaging has the advantage that it can resolve heterogeneity occurring at the canopy, plant, or leaf level (Chaerle and Van der Straeten, 2001; Lenk et al., 2007; Chaerle et al., 2009) and also makes it possible to separate plants from background soil, which is important in early and late stages of growth when the plants do not entirely cover the ground.

While digital colour cameras are widely used due to their steadily decreasing prices, the application of NIR cameras still lags behind and reports of the use of NIR imaging in plant phenotyping are few, the focus remaining on technical development. Kobori and Tsuchikawa (2009) used an NIR camera fitted with a band-pass filter with a peak transmittance at 1450 nm to specify the region of a water absorption band. Individual leaves of Ligustrum japonicum with largely differing water contents were imaged, but how sensitive the set-up would be to subtle changes in plant water content remains to be seen.

An alternative to visible and NIR imaging with simple digital cameras is the use of more sophisticated set-ups that combine spectral and spatial resolution. Tran and Grishko (2004) used an NIR camera fitted with an acousto-optic tunable filter to monitor changes in leaf water content of detached olive leaves over time. Unfortunately, the system described was not used for further plant phenotyping. Technical advances in precision agriculture appear to have followed a similar path in using cameras with tunable filters (Fitzgerald, 2004; Calpe-Maravilla et al., 2006) or line scanning cameras to gain both spectral and spatial information (Tarabalka et al., 2009). The 2D array of the latter has spectral specific sensor lines and the spatial information is gained by either moving the object in front of the camera (achivable in a greenhouse) or by moving the camera along the object (e.g. mounted on a tractor or a plane to screen field plots).

Fluorescence imaging to complement reflectance imaging

Imaging of chlorophyll fluorescence is used as a diagnostic tool in many areas of plant physiology (Baker, 2008), such as the early detection of stress symptoms induced by pathogen attack or herbicide treatment, where a spatial resolution of the heterogeneity is important (Chaerle et al., 2007a, b, 2009; Lenk et al., 2007; Konishi et al., 2009). Changes in fluorescence may also accompany increased photosynthesis in a developing leaf, helping to develop a ‘life
history’ for a particular leaf. Even though water deficit can affect photosynthesis, this is linked primarily to a decrease in stomatal conductance (Martin and Ruiztorres, 1992; Medrano et al., 2002; Jansen et al., 2009). Only under severe water stress conditions is there a notable change in photosynthetic efficiency (Flexas et al., 2002a, b; Woo et al., 2008). Hence, fluorescence imaging on its own does not seem suitable for the early detection of water stress, but it has the potential to complement other imaging techniques or to provide information about responses to severe drought. While colour imaging can be used to measure leaf area and growth rate, this can also be achieved with steady-state chlorophyll fluorescence, with the advantage of easier image processing (foreground-background separation; Jansen et al., 2009). Fluorescence images can also help with the interpretation of colour images, in particular for defining senescing areas of the leaf.

High-throughput image and data analysis

Even though the focus of this review is on image acquisition, it must be appreciated that this is only the first step in a high-throughput phenotyping process. High-throughput analyses of images and data are also essential components of a functional work flow. Image analysis in plant phenotyping can build on expertise already present in other areas and take advantage of existing software, such as MatLab® (MathWorks Inc.) or the free-ware package ImageJ (Abramoff et al., 2004). However crucial is the engagement of experts in image analysis and computer vision and to convince the plant science community of the validity of using image-derived measurements as a valuable alternative to more traditional methods. A single image has the potential to yield a large number of measurements or phenotypic descriptions, adding further to the complexity of the subsequent data analysis. This makes high capacity computing and data storage essential. In a first step, the type of information that can be extracted from the images should be explored, and information that is useful in describing the plant phenotype selected. Not all geometric parameters that can be used to describe the identified region of interest in an image (e.g. single plant, part of a canopy) will necessarily be biologically relevant. However, morphological descriptions, such as ‘compactness’ or ‘eccentricity’ are likely to be useful, and it will be important to establish and clearly define a common terminology which should be organized at an international level. The International Plant Phenomics Network (www.plantphenomics.com) could provide the appropriate vehicle for establishing this agreement. This will be crucial when phenotypic data is being shared and made commonly available. A non-expert user will need to understand and be able to interpret image-derived phenotypic data and be able to access this information without prior knowledge of image analysis. Hence, the generation of publicly available databases is important and they have to be carefully designed to be useful for the broader plant science community.

In conclusion, we contend that high-throughput imaging provides a valuable new tool which allows the dissection of plant responses to drought into a series of component traits. Elucidating the genetic basis of these dissected traits will be significantly more tractable than is the case for whole plant tolerance to drought. In fact, given the complexity of both drought and plant responses to drought, trait dissection effected by high-throughput phenotyping could provide a significant new opportunity in furthering the understanding of plant responses to drought, elucidating the genetic bases for these responses, and then introgressing these traits into appropriate combinations to improve yield maintenance under a variety of drought conditions in crops.

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