

# The advantages of functional phenotyping in pre-field screening for drought-tolerant crops

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**Abstract.** Increasing worldwide demand for food, feed and fuel presents a challenge in light of limited resources and climatic challenges. Breeding for stress tolerance and drought tolerance, in particular, is one of the most challenging tasks facing breeders. The comparative screening of immense numbers of plant and gene candidates and their interactions with the environment represents a major bottleneck in this process. We suggest four key components to be considered in pre-field screens (phenotyping) for complex traits under drought conditions: (i) where, when and under which conditions to phenotype; (ii) which traits to phenotype; (iii) how to phenotype (which method); and (iv) how to translate collected data into knowledge that can be used to make practical decisions. We describe some common pitfalls, including inadequate phenotyping methods, incorrect terminology and the inappropriate use of non-relevant traits as markers for drought tolerance. We also suggest the use of more non-imaging, physiology-based, high-throughput phenotyping systems, which, used in combination with soil–plant–atmosphere continuum (SPAC) measurements and fitting models of plant responses to continuous and fluctuating environmental conditions, should be further investigated in order to serve as a phenotyping tool to better understand and characterise plant stress response. In the future, we assume that many of today's phenotyping challenges will be solved by technology and automation, leaving us with the main challenge of translating large amounts of accumulated data into meaningful knowledge and decision making tools.

**Additional keywords:** drought stress, drought tolerance, modeling, modelling, screening, stress physiology.

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## Introduction

A changing climate along with the projected increases in world population and consumption present a major challenge for food security (Tilman *et al.* 2011; Gerland *et al.* 2014). Many solutions have been suggested for dealing with this eminent problem, including better risk management by improving long-term weather forecasting, and improving agro-technical practices and crop breeding (Richards 1991; Vermeulen *et al.* 2012), particularly in under-yielding countries (Tilman *et al.* 2011); reducing food waste; better management of financial systems that would allow farmers in developing countries increased access to markets and the conversion of formerly unused areas to agricultural lands. These could include establishment of agriculture in open seas, large inland water bodies and land formerly uncultivated due to salinity and might be planted with new plant varieties (Godfray *et al.* 2010). Although better crop management and cultivation can help growers approach crop's maximal potential yields ('bridging the yield gap' in Godfray *et al.* (2010)), improved cultivation is still subject to the inherent limits of the genotype being cultivated. Since most agricultural production relies on rain and is exposed to varying environmental conditions, the number of growing seasons in

which maximal yield can be reached is limited even when advanced agro-technical methods are used (Passioura and Angus 2010).

Global climate change and the depletion of available soil (quality and quantity) for cultivation have driven crop-improvement efforts towards the development of cultivars that are more tolerant of abiotic stress. To date, crop breeding for improved performance under water-limited conditions has been almost exclusively based on field experiments rather than controlled conditions. However, this approach has yielded little successes. Moreover, only a few specific genes added to crops have been reported to have beneficial effects in this area (Richards *et al.* 2010; Passioura 2012). This can be attributed to the fact that single genes have little effect on quantitative traits such as yield and response to the environment, as well as the fact that only few of the genes tested under laboratory or controlled conditions have been tested in the field (Verslues *et al.* 2006; Passioura 2012). Consequently, the unpredicted outcome of genotype  $\times$  environment interactions ( $G \times E$ ) creates a major gap between successful breeding and yield improvement, especially under unfavourable and/or unpredicted conditions (Mifflin 2000; Moshelion and Altman 2015). The enormous

number of genes and germplasm candidates with the potential to improve yields only increases this gap. To date, the major obstacle in efforts to bridge this gap has been the absence of an efficient method for identifying and quantifying yield-related traits at early stages of plant growth across vast numbers of plants/genes, especially under unfavourable environmental conditions. Efficient pre-field phenotyping may save time and money and play a crucial role in the selection of candidates for inclusion in field tests to be conducted in a particular environment (Moshelion and Altman 2015).

In this review we suggest four key components (questions) that need to be considered when designing a screening procedure for phenotyping complex traits under conditions of uncertain water availability, with a focus on the identification of traits related to drought tolerance at the pre-field screening phase. These components are: (i) where, when and under which conditions should we phenotype (i.e. determining the conditions under which the experiment should be performed); (ii) what should we phenotype (i.e. clearly defining the trait comparative test in the screening process); (iii) how to phenotype (i.e. choosing the method of phenotyping); and (iv) how to translate the collected data into knowledge that can be used to make practical decisions. Each of these components will be discussed in turn below.

### Where and when to phenotype: selecting appropriate experimental conditions

In order to identify the right plant for the anticipated environment, the experimental treatment(s) should be well designed and relate, among other things, to two key questions raised before planning the experiment: *viz* where to perform the experiment (i.e. growth chamber, greenhouse or field), and when to perform the experiment (i.e. which part of the plant life cycle, at what frequency and at what time of day should data be collected). It is also critical to define the desired trait, as the term ‘drought response’ has many meanings – depending on the users’ point of view (Passioura 2007) – and the definition of this term will determine the experimental structure and conditions.

In the literature, there are several different terms used to describe plant stress responses. For example, the terms ‘resistance’, ‘tolerance’, ‘avoidance’, ‘resilience’ and ‘survivability’ are all used in certain studies. This versatile terminology can lead to the selection of the wrong phenotyping components. We suggest

formulating clear definitions of agronomic crop tolerance, which is different from ecological tolerance to stress (Table 1). These definitions need to be plant- and aim-specific. For example, a study focussed on identifying drought tolerance in wheat should focus on grain yield or some other yield-related trait; whereas drought tolerance of non-breed genotypes, e.g. non-agricultural recreational pine forest, might focus on survivability.

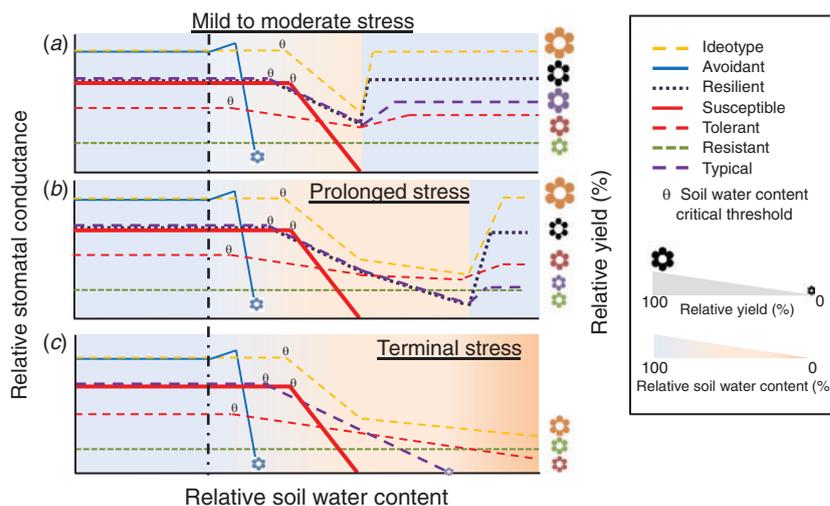
The differences between these trait definitions are fundamental, as survivability (a trait that exists in cacti, for example) has little effect on agronomic improvement of crop response to drought, as it slows growth and production (Morran *et al.* 2011). Moreover, there is no guarantee that a surviving ‘drought tolerant’ plant will not have severely reduced yields (Ghanem *et al.* 2015), as depicted in Fig. 1a–c.

This terminology disarray is also present in the literature. For example, the term ‘drought tolerant’ has been used in different ways across a variety of experimental procedures and assessment methods described in recent publications (from 2013), as shown in Table 2.

Even when the correct terminology is used, there remains a question of the conditions under which to search for the desired trait. The choice of treatment conditions should be based on the endogenous environmental conditions under which the crop is expected to grow. In the case of more basic research or unclear desired growth conditions, it is better to test several conditions as reported in Sade *et al.* (2009). In that work, transgenic tomato plants revealed significantly improved yield and harvest index when irrigated with 50% of the standard volume of water, as long as they were irrigated often (i.e. more irrigation pulses, but a smaller total amount of water supplied to the plants compared with the control). These same plants showed no improvement under lower irrigation frequency (longer drought periods between irrigations), even though under that treatment they were supplied with the same total amount of water as the control. Thus, the severity of the stress in question and the means of creating stress must be well defined. Researchers working with small pots must consider the fact that completely stopping irrigation causes rapid drying and might prevent the plant from adjusting to the new conditions. This, of course is different from field conditions, under which plants are more gradually exposed to water deficits. Other options include re-watering to a known soil water content, though this could discriminate to the advantage of faster

**Table 1. Trait terminology for plant response to drought stress (see Figs 1 and 2)**

Trait	Response of seasonal crop plants to drought stress
Drought tolerance	Minimum reduction in yield (or yield-related trait) when soil water content limits plant transpiration ( $\theta_{crit}$ , referred to as $SWC_{cr}$ in (Moshelion and Altman 2015)), as compared with well irrigated control plants. These plants might present a native low basal transpiration rate
Drought resistance	No reduction in yield, or yield-related trait, under terminal drought, as compared with well irrigated control plants. This trait might be non-existent, unless the native basal transpiration is extremely low, thus never reaching its soil water limitations. In such a case, plants will hardly produce even under well irrigated conditions
Survivability	The plant survives drought, but suffers major biomass loss. Productivity may be severely damaged
Drought avoidance	The plant avoids stress by completing its life cycle before the stress can affect it
Resilience	The plant presents high rate of recovery after stress, regaining its relatively high, whole-plant pre-stress capabilities (e.g. photosynthesis, transpiration) soon after the stress has ended



**Fig. 1.** Graph describing hypothetical  $g_s$  responses of seasonal crops exhibiting different types of drought tolerance, in the presence of three types of drought (the change in  $g_s$  is described as in the use of gravimetric systems). (a) Mild to moderate stress; (b) prolonged stress; and (c) terminal drought stress. The light blue background represents high soil water availability (field capacity) and the broken vertical line represents the last point at which water enters the soil (i.e. precipitation or irrigation). The orange background represents the gradual depletion of soil water content (light to dark, respectively):  $\theta$  ( $\theta$  crit) indicates the point at which  $g_s$  begins to be affected by limited soil water availability. (Under well irrigated conditions, the trend line for  $g_s$  as a function of soil water content would be straight with no  $\theta$ )  $\theta$  is mostly correlated with the level of risk taken by the plants, with earlier  $\theta$  points correlating with more conservative behaviour (i.e. plants that are sensitive to changes in soil water and, upon sensing those changes, immediately close their stomata to limit water loss). The ideotype represents ideal crop plant behaviour, which may not be possible under certain conditions (see Fig. 2 for further ideotypic behaviours). The avoidant plant reacts to stress by initiating its reproductive stage, which may involve increasing  $g_s$  and early flowering, thus producing seeds regardless of the duration of the stress. This behaviour is also referred to as 'drought escape' and is correlated with early flowering regardless of stress conditions (Blum 1988; Araus *et al.* 2002). The resilient plant decreases its  $g_s$  similar to the typical plant under stress, however, upon resuscitation, it returns to its previous  $g_s$  more quickly than the typical plant. The resistant plant does not change its stomatal conductance in response to drought. Note that this model suggests that under mild stress, higher yields are still reached by the typical plant, as compared with the tolerant, resistant and avoidant plants due to the typical plant's initially higher  $g_s$ . However, during prolonged stress, the typical plant's yield is similar to that of the resistant and avoidant plants and is lower than that of the tolerant plant. Under terminal drought stress, the typical plant's yield is lower than that of all of the other plants, except for the susceptible.

growing plants, or watering with a fraction of the control water volume (Passioura 2012).

### What to phenotype: defining the desired trait

Field trials are the best way to evaluate drought tolerance through direct measurement of the optimal trait, for example, grain yield, under drought conditions. To date, it has been extremely difficult and expensive to perform these measurements in a high-throughput manner. One of the ultimate goals of agronomic drought-tolerance research is to find effective methods for predicting yield. Namely, to find yield-related traits that are easy to measure as early as possible in the plant's life cycle, to enable the selection of the best-performing candidates for inclusion in further evaluations. This approach will allow the testing of more candidates with fewer field trials and improve the efficiency of the drought-tolerance breeding process

(Moshelion and Altman 2015). The method should screen as many candidates as possible in a high-throughput manner and avoid selecting artefacts and/or plants that behave well only under stress (i.e. show negative effects under well-irrigated conditions). Below, we will elaborate on the advantages and disadvantages of the methods and traits used today.

In our examination of recent works that searched for drought tolerance in crops (see Tables 2, 3), we noted that aside from direct yield evaluation, the following traits were commonly evaluated: plant survival following drought treatment and resuscitation, wilting or 'stay green' phenotypes following drought treatment, stomatal conductance ( $g_s$ ), transpiration ( $E$ ), carbon assimilation ( $A_N$ ), water-use efficiency (WUE), transpiration efficiency using carbon isotope composition (TE), relative water content (RWC), biomass before harvest, plant weight, plant height, root length and flowering time. Many studies have found that high  $g_s$  is the trait best correlated with high yield

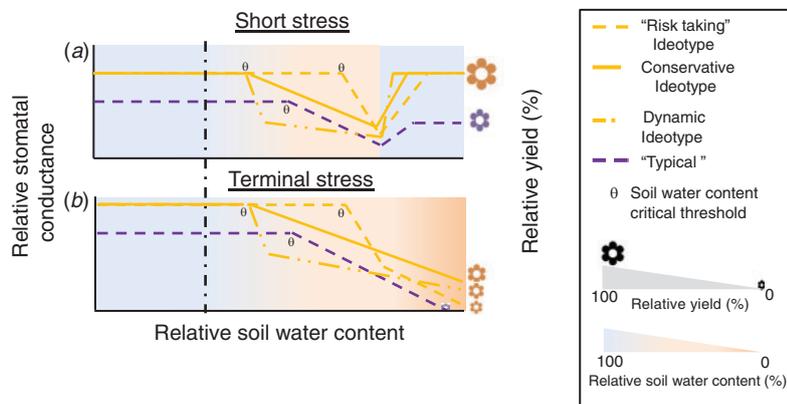
(de Wit 1958; Tanner and Sinclair 1983; Sinclair *et al.* 1984; Lu *et al.* 1994; Fischer *et al.* 1998; Richards 2000; Kemanian *et al.* 2005; Blum 2009). Nevertheless, although this correlation holds true under non-stressed conditions, high  $g_s$  can be harmful under severe stress conditions due to the hazardous water loss rate. That is, plants exhibiting high  $g_s$  under all conditions will perform poorly under severe drought and, on the other hand, a plant that always exhibits low  $g_s$  will be drought tolerant, but will not produce maximal yields under optimal conditions (as exemplified by drought-tolerant and sensitive pearl millet lines (Kholová *et al.* 2010)). We suggest that the ‘drought-tolerance’ traits should be dynamic traits controlled by the plant as part of ‘risk management’ mechanism optimising yield related response to changing environmental conditions.

Therefore, the ideal stress response behaviour (trait) is plastic behaviour, specifically, dynamic anisohydric–isohydric behaviour. Isohydric behaviour is characterised by conservative water use, which helps plants maintain a nearly constant leaf water status (minimal daily leaf water potential ( $\Psi_{\text{leaf}}$ ) and RWC). However, anisohydric behaviour is characterised by ‘riskier’ water use under stress conditions, with  $\Psi_{\text{leaf}}$  allowed to decrease as evaporative demand rises (Tardieu and Simonneau 1998; Attia *et al.* 2015). Behaviours regulated by environmental conditions and the plant’s developmental stage might be favourites (Moshelion and Altman 2015). Such a plant would be able, for example, to have high  $g_s$  under non-stressed conditions and respond quickly to stress by lowering its  $g_s$ , followed by a quick return to maximal values upon resuscitation (Fig. 2*a, b*). In order to screen for such traits, Continuous high-throughput measurement of plant behaviour is needed and may become very helpful in screening for such traits. Other studies have involved experimental set-ups whose relevance to drought treatment is debatable. For example, some studies monitored

water loss from cut *Arabidopsis* rosettes (to estimate short-term water loss avoidance) and involved transferring seedlings from agar plates to plates supplemented with polyethylene glycol (PEG), which reduces the agars’s osmotic potential and simulates drought stress (Verslues *et al.* 2006).

### How to phenotype: choosing the best method

The need to improve drought tolerance in crops highlights the bottleneck faced in many breeding programs, namely, phenotyping large quantities of plants (Fiorani and Schurr 2013). As field experiments are notoriously difficult, especially when genetically modified plants are involved, many trait-screening processes are done under controlled, fixed conditions. However, these conditions can be very different from the dynamic and unpredicted conditions in the field. In order to fill the need for accurate, pre-field, phenotyping, a multiple-tier system has been suggested in which beneficial traits are first screened using high-throughput systems. This approach aims to enable the elimination of unfitting plants rather than the direct selection of the best-performing ones. For these initial screens, selection can focus on the elimination of plants that have lost previously cultivated traits, such as flowering time, disease resistance, coleoptile length or growth vigour (Richards *et al.* 2010). These screens, when done under controlled conditions, are highly correlated with results in field tests (Richards *et al.* 2010). Nevertheless, further phenotyping cycles and stages are necessary, in order to reduce the number of candidate plants to an amount appropriate for field trials. Furthermore, phenotyping performed under desired stress conditions (such as drought or salinity treatment) is essential. Pre-field screening for pre-determined phenotypes has the potential to substantially reduce the number of plant candidates that are not likely to contain the



**Fig. 2.** Different suggested models for ideotypic behaviour of seasonal crop plants. The definition of ideal plant behaviour depends on agronomic needs and the level of environment-related risk. (a) Three ideotypic behaviours under short-duration drought stress. The risk-taking ideotype (anisohydric) maintains a high  $g_s$  as much as possible, followed by a steep reduction once there is no more water available (late  $\theta$ ). The conservative (isohydric) ideotype responds early to stress, and reduces  $g_s$  gradually. Dynamic ideotype responds early to stress by a steep reduction of  $g_s$  followed by very moderate reduction. The ideotypes superiority is presented in relation to the ‘typical’ plant behaviour (‘standard’). We assume that under short stress the relative advantage of each ideotype at different parts of the stress period will be diminished. Nevertheless, yields under terminal drought stress (b) are expected to be higher for those ideotypes that exhibit earlier  $\theta$  points.

**Table 2. Drought stress applications and drought-tolerance terminology in recent reports**

Note these are some representative examples of recent studies in which 'drought tolerance' was examined. The methods used to create the stress in the different studies are listed in the 'drought treatment' column; measurements performed in order to evaluate 'drought tolerance' are listed in the 'phenotype screened for' column. Abbreviations:  $A_N$ , carbon assimilation; RWC, relative water content

Reference	Plant species	Gene/process affected	'Drought' treatment	Phenotype screened for	Trait definition in the article	Actual trait tested
(Okamoto <i>et al.</i> 2013)	<i>Arabidopsis</i>	ABA signalling	Leaves cut and left to dry	Weight loss	Drought tolerance	Water loss/Transpiration
(Okamoto <i>et al.</i> 2013)	<i>Arabidopsis</i> /soybean ( <i>Glycine max</i> )	ABA signalling	Cessation of irrigation followed by resuscitation	Wilting	Drought tolerance	Transpiration
(Ni <i>et al.</i> 2013)	<i>Arabidopsis</i>	<i>GmNFYA3</i>	Cessation of irrigation followed by resuscitation	Plant survival	Drought tolerance	Survivability
(Jeong <i>et al.</i> 2013)	Rice ( <i>Oryza sativa</i> )	<i>OsNAC5</i>	Cessation of irrigation in pots followed by resuscitation	Chlorophyll reflectance	Drought tolerance	Survivability
(Jeong <i>et al.</i> 2013)	Rice	<i>OsNAC5</i>	Cessation of irrigation in field conditions followed by resuscitation	Yield	Drought tolerance	Drought tolerance
(Nakabayashi <i>et al.</i> 2014)	<i>Arabidopsis</i>	Antioxidant flavonoids	Cessation of irrigation followed by resuscitation	Plant survival	Drought tolerance	Survivability
(Luo <i>et al.</i> 2013)	<i>Arabidopsis</i>	<i>WRKY20</i>	Cessation of irrigation followed by resuscitation	Plant survival	Drought tolerance	Survivability
(Martinez <i>et al.</i> 2015)	<i>Arabidopsis</i>	<i>CHSR1</i> (from yeast)	Cessation of irrigation followed by resuscitation	Rosette fresh weight	Drought tolerance	Survivability
(Ruiz-Lozano <i>et al.</i> 2016)	Tomato ( <i>Solanum lycopersicum</i> , cv. Reimlams Rhums)/lettuce ( <i>Lactuca sativa</i> , cv. Romana)	Arbuscular mycorrhizal symbiosis	Soil maintained at 75% of well-watered control (moderate stress) or 55% of well watered control (severe stress)	Plant biomass over the course of the drought	Drought tolerance	Drought tolerance, though this was shown only partially for tomato
(Ramiro <i>et al.</i> 2016)	Sugarcane	<i>At1F1</i>	Cessation of irrigation	Lesion formation, $A_N$ , RWC, stomatal conductance	Drought resistance/drought tolerance	Drought tolerance
(Ahmad <i>et al.</i> 2015)	Rice ( <i>Oryza sativa Japonica</i> )	<i>OsTPKb</i>	Soil water content maintained at 50% of field capacity (compared with 100% control)	Fresh weight after 6 weeks of drought	Drought tolerance	Drought tolerance
(Metz <i>et al.</i> 2015)	<i>Biscutella didyma</i>	Parental effects	Plants irrigated with 6 different water volumes, the lowest of which was 1.1% of control	Number of seeds produced, ratio of survival to reproduction, biomass of survivors, time to first flowering and height at that time, mass of single-seeded fruit	Drought tolerance	Drought tolerance

**Table 3. Phenotyping methods for assessing drought tolerance**

Abbreviations:  $A_N$ , carbon assimilation;  $E$ , transpiration;  $g_s$  stomatal conductance; RWC, relative water content; SPAC, soil–plant–atmosphere continuum; WUE, water use efficiency;  $A_{max}$ , maximal  $A_N$  as measured according to previously performed light curves

Reference	Plant species	Measuring technique	Trait directly measured	Trait evaluated	Measurement frequency	Growth environment	Stress-induction method
(Sade <i>et al.</i> 2009)	Tomato ( <i>Solanum lycopersicon</i> )	Gravimetric high-throughput system (load cells lysimeters). Confirmed by yield measurements	Pot weight, SPAC, leaf RWC	Transpiration, photosynthesis, fruit yield, plant biomass, harvest index	Continuous	Net-house and greenhouse with commercial growth conditions	Irrigation with 50% recommended volume, in three pulses a week followed by irrigation to saturation at week's end, or irrigation once a week to saturation instead of three times a week Peg 6000
(Kang <i>et al.</i> 2013)	Wheat ( <i>Triticum aestivum</i> )	Destructive measurements	Height, root length, fresh and dry biomass	–	Once, 72 h after treatment	FPG-300C-30D incubator	
(Timmusk <i>et al.</i> 2014)	Wheat	Destructive measurements, gas exchange	Survival rate, root and shoot biomass, $g_s$ , $E$ , $A_N$ emission of stress-related volatiles	Water use efficiency	Once for destructive measurements, five times in 10 days for gas exchange and volatile emission	MLR-35 IH (Phanasonic, IL, USA) growth chamber	Cessation of irrigation, followed by resuscitation
(Mammathan <i>et al.</i> 2013)	Wheat	Destructive measurements	Fresh + turgid + dry biomass, plant vigour, wilting phenotype	RWC, WUE	Once, 11 days after treatment began	Growth room	Soil kept at 50% field capacity
(Honsdorf <i>et al.</i> 2014)	Barley ( <i>Hordeum vulgare</i> )	High-throughput phenotyping using RGB photography	Three photographs per plant, one from above and two side views.	Plant biomass and growth rate	Once, a day after treatment began	Plant accelerator greenhouse	Soil kept at 15% or 12% gravimetric water content (compared with 22% control)
(Al Abdallat <i>et al.</i> 2014)	Barley	Visual assessment, destructive measurements	Greenhouse: RWC, survival; Field: number of tillers, spikes and seeds, biomass, yield	Spike/seed ratio	Once	Greenhouse, field in drought-stricken region	In greenhouse: cessation of irrigation, followed by resuscitation; in field: natural low rainfall
(Kapanigowda <i>et al.</i> 2014)	Sorghum ( <i>Sorghum bicolor</i> )	Gas exchange	$E$ , $A_N$ , vapour pressure deficit	$A_N:E$	Four times on successive days.	Greenhouse, field condition without drought treatment	In greenhouse, soil kept at 40% of field conditions, relative to 80% control
(Avramova <i>et al.</i> 2016)	Corn ( <i>Zea mays</i> )	High-throughput photography, destructive measurements	Image acquisition of shoots and roots, length of fourth leaf	Traits correlated with drought tolerance under field conditions, seedling root and shoot biomass	Once a day for imaging and length of the fourth leaf, once at the end of the experiment for transpiration and destructive measurements	Rhizotrons (in greenhouse)	Soil pre-dried to 34% soil water content compared with 54% control (not irrigated after initial irrigation) or allowed to dry to same degree in growth-room experiment
(Mao <i>et al.</i> 2015)	Corn	Visual assessment	Survival rates	–	Once	Cultivation pool	Irrigation stopped when plants reached three true leaves.

(Reguera <i>et al.</i> 2013)	Rice ( <i>Oryza sativa Japonica</i> )	Visual assessment, gas exchange	Growth rate, $A_N$ , $g_s$ , RWC, maximum carboxylation, electron transport and triose phosphate utilisation rates, chlorophyll fluorescence	Rubisco activity, ribulose biphosphate regeneration, and phosphate limitations	Once, 3 days after stress began	Greenhouse	Soil water content allowed to drop to 70% or 58%, as compared with 98% control; plants re-watered after wilting pre-anthesis and plants resuscitated when visual stress symptoms appeared
(Tang <i>et al.</i> 2012)	Rice	Visual assessment	Survival rate, rate of water loss from detached leaves		Once, after resuscitation	Greenhouse	Seedling irrigation halted for 7 days at the 4-leaf stage (~3 weeks old), followed by resuscitation; during the reproductive stage, irrigation was stopped at the panicle-development stage and resumed at the flowering and seed maturation stages
(Kissel <i>et al.</i> 2015)	Banana ( <i>Musa</i> spp.)	Non-destructive measurements, gravimetric measurements, isotope discrimination	Morphological measurements of leaf and pseudostem, pot weight loss, carbon isotope discrimination	Biomass, transpiration, transpiration efficiency (TE)	Every 2 weeks for biomass, every 2 days for gravimetrics, once for isotope discrimination	Screen-house	Volumetric water content was kept between 27–28%, or between 19–25%, as compared with 30–33% in the control
(Tugendhaft <i>et al.</i> 2016)	Olive ( <i>Olea europaea</i> )	Non-destructive measurements, gas-exchange, pressure chamber.	Stem diameter, leaf water potential, $A_N$ , $g_s$ , PSII electron transport rate (ETR)		Twice, 11 and 18 days after drought start (33% and 10% of field capacity, respectively)	Greenhouse	Irrigation withheld until pots reached 33% of field capacity and then 10% of field capacity compared with 100% control
(Sehgal <i>et al.</i> 2015)	Pearl millet ( <i>Pennisetum glaucum</i> )	Non-destructive measurements, harvest measurements	Grain yield, panicle yield, biomass yield, stay green, leaf rolling	Panicle harvest index, grain harvest index, grain number per panicle (GNPP), grain number per m <sup>2</sup>	Once for harvest measurement, ongoing until wanted trait found for some non-destructive measurements	Irrigated field	Terminal drought stress initiated either at 50% flowering by withholding irrigation 1 week before flowering or initiated during early grain-filling by withholding irrigation at 50% flowering
(Thu <i>et al.</i> 2014)	Soybean ( <i>Glycine max</i> )	Destructive measurements, visual assessment	Survival, root and shoot length and biomass, RWC	Drought tolerance index constructed by multiplying non-wilted plant percentage over the course of the drought treatment and resuscitation	Once for destructive measurements, every 2 days for wilting and resuscitation evaluation	Net-house	Irrigation halted for 15 days at 12 days after planting, followed by 15 days of resuscitation

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Table 3. (continued)

Reference	Plant species	Measuring technique	Trait directly measured	Trait evaluated	Measurement frequency	Growth environment	Stress-induction method
(Li <i>et al.</i> 2013)	Soybean	Pressure chamber, destructive measurements, gas exchange, harvest measurements	Detached leaf water loss, RWC, leaf water potential, transpiration rate and biomass; yield in field tests		Once at the end of the drought treatment and 2 days after resuscitation for survival in growth chamber	Growth chamber and field with rain shelter	Growth chamber: irrigation of 21 day old seedlings stopped for 10 days, followed by resuscitation. for RNA extraction, two levels of drought (60% and 40% of control volumes) were examined. Field drought conferred by rain shelter set up at the R1 stage for 21 days, followed by irrigation Plants grown after the rainy season had ended
(Vaishney <i>et al.</i> 2014)	Chickpea ( <i>Cicer arietinum</i> )	Semi-automated high-throughput phenotyping.	Root length, root length density, root dry weight, rooting depth, root surface area, root volume, plant dry weight, plant height, plant stand, plant width, 100-seed weight, yield, biomass, and carbon isotope discrimination	Ratio between root and plant dry weight, harvest index, TE	Once	Semi-automated high-throughput precise phenotyping facility, field	
(Singh <i>et al.</i> 2013)	Lentil ( <i>Lens culinaris</i> )	Destructive and non-destructive measurements, visual assessment	Survivability, categorised assessment of wilting, growth inhibition, root and shoot length, fresh and dry biomass		Once	Hydroponics and soil in phytotron	Hydroponics: seedlings roots exposed to air for 5 hours daily for 6 days. Soil: saturated soil allowed to dry for 6 weeks until 2% of field capacity compared with 60–70% control. An additional experiment included a 12 day resuscitation after drought Pots were saturated and pre-weighed. During treatment, pots were weighed every 48 h to assess water loss. To water volume as that which was lost was added. To treatment pots 50% lost volume was added
(Rolando <i>et al.</i> 2015)	Potato ( <i>Solanum tuberosum</i> )	Harvest measurements, pressure chamber, SPAD, gas exchange RGB photography	Plant dry biomass, tuber dry biomass, leaf water potential, chlorophyll concentration, leaf N concentration, $g_s$ , $A_{max}$ , $E$ , plant coverage	Intrinsic WUE ( $A_{max}/E$ ), drought susceptibility index (DSI)	Once a week for chlorophyll and gas exchange measurements, six times during experiment and one time at experiment end for harvest measurements	Air conditioned glasshouse	

traits later searched for in field conditions. In this manner, it can improve the ratio of genes found to have beneficial effects under field conditions, as compared with commercial cultivars.

In recent years, technological advances have been applied in attempts to bypass phenotyping bottlenecks. These include remote-sensing methods such as RGB (visible light) imaging, near infrared spectroscopy (NIR), spectral reflectance and thermal imaging (Fiorani *et al.* 2012; Fiorani and Schurr 2013; Fahlgren *et al.* 2015), which can be used to measure chlorophyll fluorescence, starch content, biomass, transpiration and nitrogen (N) content, used as indicators of the plant's physiological status (Montes *et al.* 2007); multi- or hyper-spectral imaging based on equipment that can be mounted on multiple platforms such as satellites, drones, tractors and towers; and light detection and ranging (LIDAR) imaging used for plant coverage analysis (Araus and Cairns 2014). These techniques allow the evaluation of a wide variety of plant traits in a non-destructive manner, enabling multiple measurements throughout the growing season. However, wavelengths used may vary between growing seasons and plants' physiological state (Osborne *et al.* 2002), leaf angle, and atmospheric conditions. For this reason, system calibration before measurements is important.

In our examination of phenotyping methods used in experiments performed on crops in recent years (since 2012) and listed in Table 3, we mainly saw the use of low-throughput methods such as destructive measurements, in addition to visual evaluation. The two types of high-throughput phenotyping methods used (not including visual evaluation) were RGB photography of plants or semi-automatisation of destructive measurements.

Most of the studies listed in Table 3 included some of the core elements used to assess drought tolerance. The majority of tests within Table 3 were based on physiological measurements, but many of them did not actually point to drought tolerance. For example, though WUE seems to be a good trait for predicting drought tolerance, it is often reached, at least partially by lowering stomatal conductance (Iuchi *et al.* 2001; Blum 2005; Robredo *et al.* 2007; Cattivelli *et al.* 2008) thereby lowering transpiration, but also photosynthesis and productivity (Çakir 2004). Aside from having lower productivity under non-stressed conditions, such a plant might survive drought, but produce insignificant grain yield (cacti can serve as an example for extreme embodiment of high WUE and low yield; see Fig. 1, the 'resistant' plant describes a similar behaviour pattern to cacti, though less extreme).

In addition, some valuable data could be missed due to low sensitivity or low measurement frequency. For example, Yoo *et al.* (2009) demonstrated how a small reduction in stomatal conductance can bring the carbon assimilation rate to the linear rise on the graph of its relation to  $C_i$  (instead of the plateau where it was previous to this reduction). The reduction in  $A_N$  up to this point is small compared with the large reduction seen in transpiration (Yoo *et al.* 2009). This demonstrates that under mild stress, there is room to improve plant water use without causing major damage to productivity parameters. That is, substantial drought tolerance may be reached without any reduction in grain yield under mild stress conditions. Phenotyping for delayed response to stress for several days (e.g. reduction in  $g_s$ ), might be a good idea in areas in which the probability of

rain before the onset of drought response causes a negative yield effect is relatively high (Rivero *et al.* 2007). This approach could be supported by the fact that, unlike salinity tolerance, extreme drought tolerance without any reduction in yield is extremely rare in crops, if not impossible.

Modern crops, bred under non-stressed conditions, use immense amounts of water (de Wit 1958). Much of the improvement in crop yields in past years was reached by increasing plant water conductance (i.e. transpiration (Lu *et al.* 1994; Fischer *et al.* 1998; Richards 2000) and hydraulic conductance (Sack and Holbrook 2006)), thereby increasing photosynthesis, and thus making plants more susceptible to drought. It is particularly difficult to breed for drought tolerance simply due to the fact that many modern crops have high water-use levels (a direct outcome of non-stressed breeding for maximal yields). The question still remains whether we are willing to sacrifice potential yield, under non-stressed conditions, in breeding for higher drought tolerance. If we look at the history of breeding, as well as traits currently sought by today's breeders, the answer to this question is no (Passioura 2012).

Another conclusion from the articles listed in Tables 2 and 3 is that although high-throughput systems have been used to some extent in recent years for phenotyping drought-tolerance traits, the use of remote-sensing techniques such as NIR, LIDAR and hyper-spectral methods though present in theory, is not yet a real option in most cases. This may be due to technical difficulties, the lack of fitting models for converting raw data into clear phenotypes or low measurement resolution.

One possible way to bridge the gap between the need for high-throughput phenotyping and technical difficulties in the utilisation of platforms based on remote sensing is the use of physiology-based gravimetric systems that enable direct measurement of the soil-plant-atmosphere-continuum (SPAC; see Figs 1, 2, which depict hypothetical graphs of data obtained through such systems). In these systems, plants are placed on weighing lysimeters that measure changes in pot weight at high frequency. This data is then combined with measurements of environmental parameters in the greenhouse, including radiation, humidity and temperature, as well as soil water conditions. Using pre-measured data including soil weight and initial plant weight, a great deal of phenotypic data can be extracted including data on stomatal conductance, growth rates, transpiration and soil water content and plant dynamic behaviour such as the critical  $\theta$  point, which is the soil water content at which plants start to respond to stress by reducing their stomatal conductance (see Figs 1, 2). This phenotypic data can then be used to characterise the dynamic plant-environment interaction (Sade *et al.* 2010; Kelly *et al.* 2013; Lugassi *et al.* 2015). The continuous data acquired by these systems aids the evaluation of plant behaviour throughout the plant life cycle, as opposed to data collected at only one or several points in time. These data can then be used to predict the plant's plastic response to different environmental conditions.

### Translating the data into knowledge for practical decision-making

High-throughput systems produce vast amounts of data, especially when continuous measurements are performed. This mass of

data has created new problems of data-handling and analysis (Houle *et al.* 2010; Fiorani and Schurr 2013), in particular, the translation of data to knowledge.

In recent years there has been a realisation that along with new high-throughput phenotyping systems there needs to be a focus on the implementation of the data collected from these systems through the development of supporting hardware and software (Fahlgren *et al.* 2015; Minervini *et al.* 2015). Phenomic data analysis can benefit from public phenomic datasets (those existing today were reviewed by Fahlgren *et al.* 2015), which are similar to existing sets of sequencing data (Fahlgren *et al.* 2015). However, translation of raw data into meaningful information, such as green pixels into plant biomass, is only the first stage in the realisation of the potential of high-throughput phenotyping systems and the translation of such information into real knowledge may emerge as the next phenotyping bottleneck. In the future (though this future may be further off than expected, as shown in Table 3), automated systems will probably be able to supply an almost complete set of a plant's physiological data. The challenge then will be to translate that data into meaningful knowledge that will help understand dynamic plant behaviour in relation to a particular environment, and aid in the selection of promising candidates for field trials. Figs 1 and 2 show hypothetical  $g_s$  behavioural models for different plants with different drought-stress response patterns. This data could be collected using a gravimetric system (e.g. weighing lysimeters). This type of comparative-behavioural data regarding plant responses to changes in the soil water content and the duration of the drought period can serve as an example of the conversion of mass quantities of data into knowledge. In this case, data regarding pot weight and environmental conditions are converted into pot soil water content and  $g_s$  and then a model is constructed to identify the 'theta crit.' point at which soil water content becomes a limiting factor. In this manner, we can see how each plant (pending its morphological, anatomical, biochemical and physiological status) responds to the stress in its own particular way.

## Conclusion

Exact phenotyping constitutes a significant bottleneck in crop breeding for stress tolerance. The use of correct terminology, experimental planning and the choice of phenotyping methods can all help to optimise the application of experimental results for the development of commercial crops. Though major technological advances in high-throughput phenotyping have been made in recent years, the use of these systems remains limited and confined to robotic measurements in the greenhouse or gravimetric systems. Comparative and continuous SPAC measurements of numerous plants simultaneously, supported by algorithms that correlate data with practical decisions, may provide a relatively simple way to evaluate plant behaviour and select the optimal behaviour for particular environments. Nevertheless, our biggest challenge is to develop better tools and algorithms to unclog the bottleneck that currently limits the translation of collected data into meaningful knowledge.

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