Review

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Feature Review

Phenomics – technologies to relieve the phenotyping bottleneck

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Global agriculture is facing major challenges to ensure global food security, such as the need to breed high-yielding crops adapted to future climates and the identification of dedicated feedstock crops for biofuel production (biofuel feedstocks). Plant phenomics offers a suite of new technologies to accelerate progress in understanding gene function and environmental responses. This will enable breeders to develop new agricultural germplasm to support future agricultural production. In this review we present plant physiology in an 'omics' perspective, review some of the new high-throughput and high-resolution phenotyping tools and discuss their application to plant biology, functional genomics and crop breeding.

Plant biology faces new challenges: a role for plant phenomics

Global agriculture and the plant biology underpinning it are facing major challenges which require new approaches to functional genomics and plant breeding. Global food security, the identification of appropriate and efficient plant-based biofuel feedstocks and coping with climate change are foremost in the minds of scientists, politicians and the general public. To address these issues, we need new high-yielding genotypes of agricultural crops adapted to our future climate. Agricultural crops fulfilling future food and fuel needs must display both high intrinsic yield and yield stability under abiotic stresses. Annual increases in yield achieved from traditional breeding programs worldwide are no longer sufficient to meet projected demand for all three major cereal crops: rice (*Oryza sativa*), maize (*Zea mays*) and wheat (*Triticum aestivum*) [1–3]. With the burgeoning world population, cereal grain yields alone must increase by at least 70% before 2050. In fact rice demand has already exceeded supply for the past 2 years [1]. Demand for biofuel feedstocks will also undoubtedly increase over the next decade [4], resulting in potential competition for arable land between food and fuel crops. At the same time the impacts of climate change on global temperatures and rainfall patterns are likely to lead to reduction in yields due to abiotic stress [3]. Although climate change can also have a positive impact on yields through the CO_2 fertilizer effect on photosynthesis [5,6], the benefit of high CO_2 on yield shows large inter- and intra-specific genetic variation and modern cultivars show particularly poor responses, which has stimulated interest in developing 'climate change-adapted' agricultural germplasm [5,6].

There has been a degree of confidence over the past decade that the genomics revolution and gene technology can provide solutions to the current challenges in plant breeding. Homozygous genome wide knockout lines are available in *Arabidopsis* (*Arabidopsis thaliana*) [7], 1001 *Arabidopsis* ecotypes are currently being sequenced to provide comparative genomic databases [8], the *Medicago* (*Medicago truncatula*) genome has been sequenced and genetic resources are publicly available [9] and the genome sequence of *Brachypodium* (*Brachypodium dystachion*), a model cereal, has also recently been reported [10]. The genome sequences of rice, maize, sorghum (*Sorghum vulgare*), barley (*Hordeum vulgare*) and many dicot and monocot crops are either sequenced or soon will be, as sequencing costs spiral downwards. 'Re-sequencing' of genomes of agricultural crops to assess allelic variation will become even more commonplace in the next few years.

To harness this wealth of genomic information for agricultural application, it has to be carefully and comprehensively linked to phenotype in a 'real world' environment. For model systems, linkage of genotype to phenotype has been often illusive. Phenotypic descriptions of genome-wide knockout collections are limited (see [11] for one of the most comprehensive examples). The frequency with which the annotation 'no visible phenotype' occurs is symptomatic of, among other things, a lack of capacity for the plant community to analyze the subtle and sometimes complex phenotypic effects of genetic modification. A combination of systematic 'industrial scale' phenotyping at high-throughput [12] to mine candidate germplasm for genes underlying traits of agricultural importance and high-resolution examination of subtle phenotypes of smaller subsets is clearly required. High-throughput phenotyping has become more common in laboratories in the commercial sector, but this information rarely enters the public domain, and then often after delay and with unknown completeness.

Marker-assisted selection (MAS) in plant breeding has become more common in recent years and is now used routinely for traits such as pathogen resistance conferred

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by single genes. Bi-parental recombinant inbred lines, doubled haploid sets or genetically characterized accessions for population genetics can be used to identify quantitative trait loci (QTLs) and ultimately clone genes of interest under these QTLs [3]. Although assembling the necessary genetic resources is in itself a challenge, phenotyping the populations is widely recognized as the most laborious and technically challenging part of this process. For a population to be screened for a valuable agricultural trait (such as grain size, abiotic stress tolerance, product quality or yield potential), replicated trials are necessary across multiple environments over a number of seasons. Phenotyping tools currently in common use require destructive harvests at fixed times or at particular phenological stages and are slow and costly. Furthermore, if a promising candidate gene is to be tested for allelic variation in a mapping population, this phenotyping work needs to be done precisely.

The labor-intensive and costly nature of conventional field phenotyping have meant that many crop breeding programs make a single measurement of final yield for replicated plots in contrasting environments over multiple seasons. However, yield itself is one of most poorly inherited traits in crop breeding (see [13] and references therein). The bottleneck in field phenotyping has driven intense interest over the past decade in applying remote sensing technologies to field crop monitoring and in this regard field phenomics is more advanced in many respects than controlled-environment, high-throughput analysis.

The 'phenotyping bottleneck' described above can now be addressed by combining novel technologies such as non-invasive imaging, spectroscopy, image analysis, robotics and high-performance computing. Phenomics could be described as simply 'high-throughput plant physiology'. As a result, field evaluation of plant performance is much faster, and facilitates a more dynamic, whole-of-lifecycle measurement less dependent on periodic destructive assays. Furthermore, application of these tools in dedicated high-throughput, controlled-environment facilities has the potential to improve precision and reduce the need for replication in the field. This brings us to the age of 'phenomics' (Box 1).

High-throughput phenomics of model systems: the phenomics–genomics pipeline

A clear goal of phenomics is to bridge the gap between genomics, plant function and agricultural traits. Particularly in the context of model systems, where availability of genomic sequence is burgeoning, there is a pressing need for a searchable phenotypic database linking gene sequence to plant structure, development, composition and performance, all measured in a clearly defined environment. To generate a meaningful database of phenotypic characters in a model species, the first requirement must be to have data objectively described, preferably in a mathematical, easily digitized and searchable format (using ontologies, or controlled vocabularies, for example, [14]). Second, the information on how the experiment was carried out, the plant material used and the growth conditions used must be recorded in a standardized format (the 'metadata'). Third, standardization or at least adequate reporting of the phenotyping techniques employed is desirable to allow comparison of data sets. The inadequacy of these descriptions and standardization of experimental treatments in the literature and in current databases has severely limited the utility of both transcriptional and metabolomic data (see [15] and references therein).

High-throughput phenotyping of model plants such as *Arabidopsis* using non-invasive imaging technologies is a rapidly advancing field (www.plantphenomics.org.au and [1,16–18]). Image analysis and mathematical treatment of imaging data to extract growth dynamics, morphological characters, and spatially described photosynthetic parameters are challenging and require sophisticated storage and linkage of primary images and calculated data. Utilization of digital imaging and image analysis to estimate growth rates from projected leaf area is relatively simple

Box 1. What is phenomics?

Phenome = Gene × Environment or the expression of the genome as traits in a given environment.

Plant phenomics

Plant phenomics is the study of plant growth, performance and composition (Figure I). Forward phenomics uses phenotyping tools to 'sieve' collections of germplasm for valuable traits. The sieve or screen could be high-throughput and fully automated and low resolution, followed by higher-resolution, lower-throughput measurements. Screens might include abiotic or biotic stress challenges and must be reproducible and of physiological relevance. Reverse phenomics is the detailed dissection of traits shown to be of value to reveal mechanistic understanding and allow exploitation of this mechanism in new approaches. This can involve reduction of a physiological trait to biochemical or biophysical processes and ultimately a gene or genes.

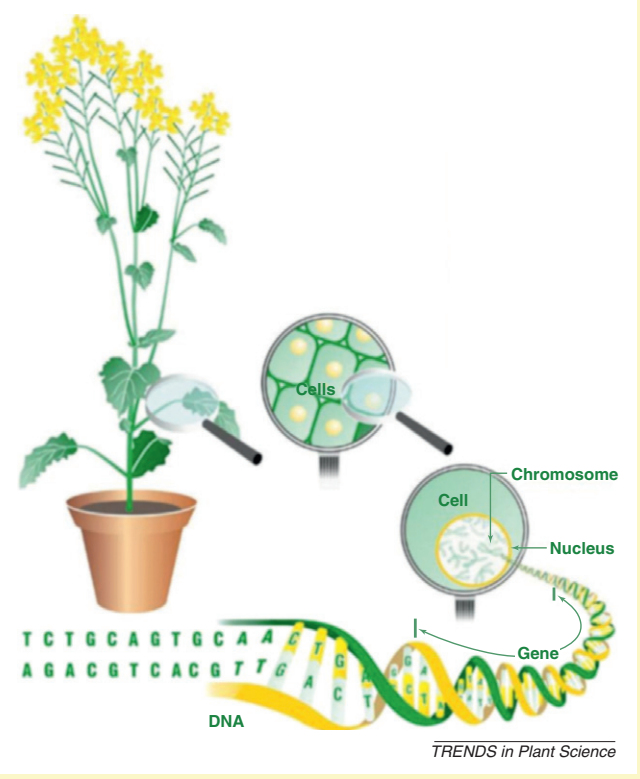

Figure I. Plant phenomics is the study of plant growth, performance and composition.

and accurate for rosette plants such as *Arabidopsis* and can be achieved in high-throughput using trays of 20 or more individuals [19,20]. Such a growth-imaging approach has already been used to screen for drought tolerance in *Arabidopsis* accessions [19] and for QTLs linked to biomass increases induced by heterosis in more than 400 recombinant inbred lines of *Arabidopsis* [21]. Recently, this technique has been extended to include pulse-modulated chlorophyll fluorescence imaging as a tool for examining photosynthetic responses to drought stress in *Arabidopsis*, in addition to growth rate response [17]. Morphological descriptors for herbarium identification and plant development are well established and based on vectorization of 2-D digital images [22].

Three-dimensional plant models, more appropriate to cereals and larger dicots, have been developed using mathematical approaches known as 'L-systems' [23,24], which simulate plant development with a series of generative rules for plant organs. Although such generative models have been used successfully to describe floral development [25] and to generate realistic rendering of trees in 3-D [26], utilization of L-systems approaches for quantitative high-throughput phenomics, functional genomics and plant breeding is in its infancy.

Geometric descriptors of plant organs were primarily developed for leaf shape analysis and comprise terms such as projected area, center of mass, eccentricity or symmetry, and statistical moments, which allow accurate mathematical representation of plant shape without the need to store large images for comparison [22]. This approach has recently been adapted in the analysis software of a commercial imaging device (MAT or Morphological Analysis Tool; LemnaTec Scanalyser, www.lemnatec.com) and similar information can also be extracted from images collected using simple imaging systems and public domain software [27]. The potential utility of this shape characterization for phenomics of *Arabidopsis* is illustrated in Figure 1. In this experiment, the heterotic behavior of progeny of a cross between the *Arabidopsis* ecotypes C24 and L*er* was examined by digital growth analysis using projected leaf area (derived from a series of top camera images over time) and, using the mathematical shape analysis tools described above, by extraction of rosette morphology at fixed time points. (In this case, the means for 20 plants at 14 days after planting are shown; R.T. Furbank and X. Sirault, unpublished.) Typical images of the parent plants and the cross are also shown after a thresholding algorithm was applied. Even this simple experiment can provide insights into the genetics controlling rosette morphology if carried out across large collections of genetic material. In Figure 1, shape analysis is plotted in a 'radar' graph where data for each ecotype are normalized against the largest value for a given character in the entire data set, to give values between 0 and 1. The parents of the cross are clearly different, with L*er* having both a greater projected area and much greater rosette eccentricity. The progeny of the inter-ecotype cross obviously has a much larger projected leaf area at this developmental stage than both parents and considerably reduced eccentricity compared to L*er*. If such an analysis were to be carried out over a large number of ecotypes with sequenced genomes, recombinant inbred lines or genome-wide gene inactivation lines grown under controlled conditions, considerable progress could be made in understanding the genetics of plant growth and morphology.

As discussed above, a database of phenomics information requires comprehensive metadata description and agreed ontologies. It is unlikely that large-scale standardization of experimental conditions and techniques will be possible (although an International Plant Phenomics Initiative has been established to address these issues; www.plantphenomics.com). Well-described metadata mitigates the issue of standardized experimental conditions to some degree and recently there have been two attempts to provide ontology-based solutions to combining metadata repositories with phenotypic databases and search tools.

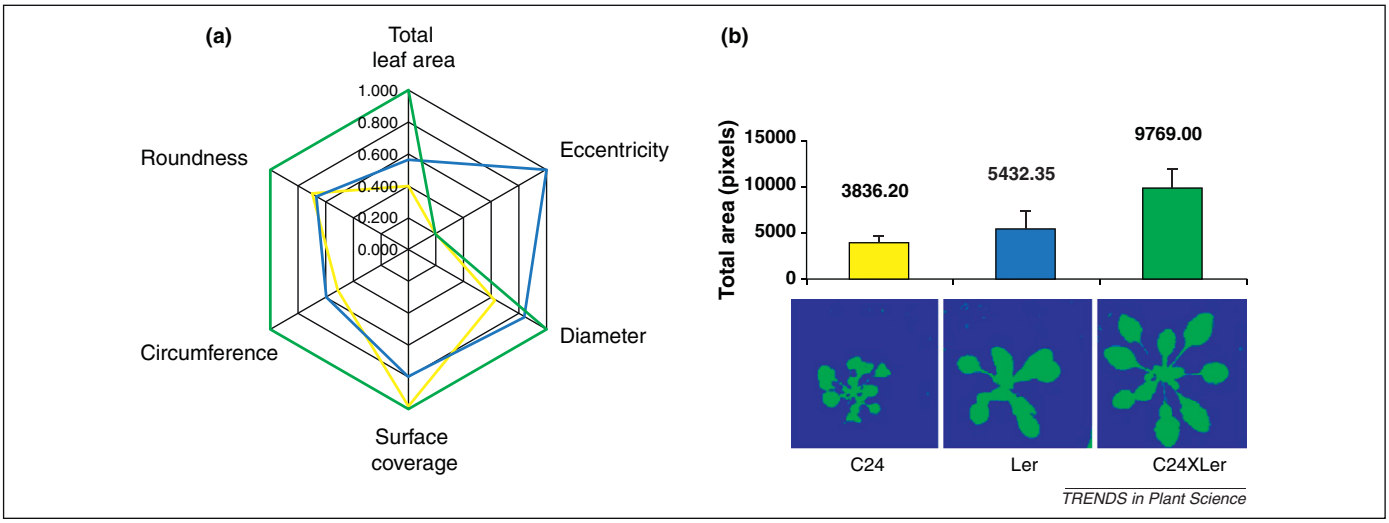

Figure 1. Shape analysis of C24, L*er* ecotypes of *Arabidopsis* and a C24 × L*er* cross. An example of the extraction of morphological features from images of *Arabidopsis*. Plants were grown at 300 μmol m^{-2} s^{-1} quanta, 10-h photoperiod and 21 °C for 14 days in trays of 20 individual pots and imaged from above daily using a LemnaTec Scanalyser 3-D (LemnaTec, Wuerselen, Germany). Images were analyzed for morphological descriptors [61] using the algorithms in the LemnaTec Morphological Analysis Tool (www.lemnatec.com; LemnaLauncher and Miner manual 24.07.2010.pdf). **(a)** Radar plot of five morphological characters of ecotypes C24, L*er* and an F_1 cross between the two ecotypes, derived from images of 20 plants at 14 days after planting. Values were the mean of 20 values, normalized to the largest value of the data set. **(b)** Mean and standard errors of projected rosette area of plants and an example of images after thresholding to remove pixels containing soil and pot.

The first of these, Xemlab [15], was conceived primarily to deal with metabolomic data and associated metadata, but has the potential to form a basis for wider application and is still under development. Also under development at the Australian Plant Phenomics Facility (APPF) is PODD, a Phenomics Ontology Driven Database (http://www.plant-phenomics.org.au/PODDProject).

PODD is intended to service both plant and animal phenomics and, although similar in concept to Xemlab in focusing on metadata and ontologies linked to a web-based graphical user interface, it utilizes different software solutions to achieve these aims. For plant applications, it is intended to provide a mechanism for archiving and retrieval of phenotypic data produced from imaging, spectral analysis and a vast array of physiological (phenomics) data produced from the Australian Plant Phenomics Facility. There is substantial interest in integrating such databases with the genomic information currently available via international databases such as TAIR, TIGR and NCBI, and with other 'omics' information such as metabolomic, proteomic and transcriptomic data.

Phenotyping for abiotic stress tolerance in crop plants

As discussed above, the challenges agriculture currently faces require significant advances in both yield potential and yield stability. For yield stability, both abiotic and biotic stress tolerance are crucial traits. Many of these traits can be screened for at the seedling stage in single pots in controlled environments or in the field, and in many cases the same phenomics tools can be used across all these scales of phenotypic screening.

Two important abiotic stress tolerance traits in many environments are drought and salinity tolerance [28,29]. In many respects, these stresses produce quite similar phenotypic effects and the phenomics approaches to screening show a high degree of crossover. One of the first effects of exposing a crop to salinity (hours to days) is stomatal closure, induced, at least in part, by the deleterious osmotic effect of solutes on the ability of roots to take up water from the soil (reviewed in [28]). This osmotic stress, which is similar in nature to drought stress and salinity stress, has been termed 'chemical drought' [29]. The effect of stomatal closure is a reduction in photosynthesis, but screening based on photosynthetic parameters or stomatal conductance measurements are generally slow and often have low reproducibility [29]. As is the case with many plant phenomics tools, a surrogate measurement can be used to screen for stomatal or photosynthetic responses under osmotic stress.

One of the best examples of using a phenomics approach with a 'surrogate' measurement is the success of carbon isotope discrimination (termed CID in plant breeding) as a reproducible indicator of transpiration efficiency in crop physiology and plant breeding [13]. This technique is derived from observations made more than 25 years ago that plants discriminate against the heavy isotope of carbon (^{13}C) naturally present in atmospheric CO_2, both in the process of CO_2 diffusion into the leaf and in the metabolic processes of photosynthesis [30]. This isotopic discrimination is reflected in the isotopic signature of plant dry matter and in C_3 crops, CID values are strongly related to stomatal conductance and transpiration efficiency for a given photosynthetic capacity [13,30]. Not only has this proven to be a useful research tool, it has also been successfully used to find genetic variation in transpiration efficiency in wheat and to breed commercial varieties with greater water-use efficiency and yield [30]. The utility of this approach is that samples can be collected at the end of the growing season and the isotopic composition reflects the integrated effect of the entire growing season, avoiding the issues of measuring leaves and plant organs at key phenological stages. The disadvantages of CID are expense (up to US$30 per sample), and the need to normalize data to photosynthetic capacity or yield potential to obtain varieties which are both good performers in terms of growth and yield and conservative consumers of water. It is not known whether CID measurements can be scaled up to the field from seedling measurements in controlled environments.

Recently, infrared thermography has been successfully used at the young seedling stage in wheat and barley to select genotypes capable of maintaining stomatal conductance under osmotic stress (Box 2; [31]). In this case, salt was used to induce osmotic stress, but this technique is also applicable to high-throughput seedling screening for drought tolerance in the vegetative stages of crop development. Such screens early in plant development allow many thousands of lines to be assessed rapidly and at low cost relative to techniques requiring measurements across the whole lifecycle. Although traits phenotyped on seedlings in isolated pots might not always hold up when scaled to the field, in this case identical ranking of genotypes for salinity tolerance was observed both in the seedling and the adult plant stage [31]. The respective pros and cons of pot versus field experiments are likely to depend on the traits being measured – some will be robust to the reduced reality of the pot in a controlled environment and others will not. It is likely that traits such as yield and maintenance of yield on reduced water supply will be more greatly affected by growth in pots than less complex traits such as the osmotic component of salinity tolerance.

Infrared thermography

Infrared thermography or even simple automated spot canopy temperature measurements also have great potential for low-cost, high-throughput field phenotyping. Carrying out porometry in the field to assess stomatal response to low soil water potential is laborious, even with modern tools [29]. Canopy temperature has been widely used to infer crop water use and photosynthesis and in some cases to predict yield for close to 30 years [32,33]. Handheld thermopile-based infrared thermometers or canopy temperature 'guns' can now be purchased cheaply but are still not routinely used in crop breeding programs. One reason for this is the long time taken to walk hundreds of plots logging canopy temperature, and the associated changes in environment and physiological state of the crop during the period of measurement. Furthermore, the inability to differentiate between signals originating from the plant and those from the surrounding soil restricts use to phenological stages after canopy closure. Mounting of multiple sensors on a tractor boom and passing over the crop can improve the

Box 2. Thermography screening for osmotic tolerance in cereals

Thermal imaging or 'thermography' can be used to extract leaf or canopy temperature at the single plant level (Figure I) or the plot level (Figure II). Figure I shows thermal images of durum wheat seedlings treated with salt for 3 days before measurement compared to a control (adapted from [31]). The approximate differential in temperature for the control and treated seedlings is 1 °C. With careful control of environmental conditions, the magnitude of this differential can be used to screen for tolerance to osmotic stress and has been validated for a range of durum wheat genotypes using traditional screening based on biomass accumulation [31].

Thermography can also be used in the field as a remote sensing tool to capture canopy temperature data for a large number of plots. Figure II shows a thermal image of a wheat trial imaged from a cherry picker, comprising 5 m × 2 m plots per genotype, arrayed in a grid (Deery, Sirault and Furbank, unpublished). Differences in water extraction or stress tolerance can be detected by comparing average temperatures of each plot following image processing to remove soil signals and correction for solar fluxes and heat balance [29].

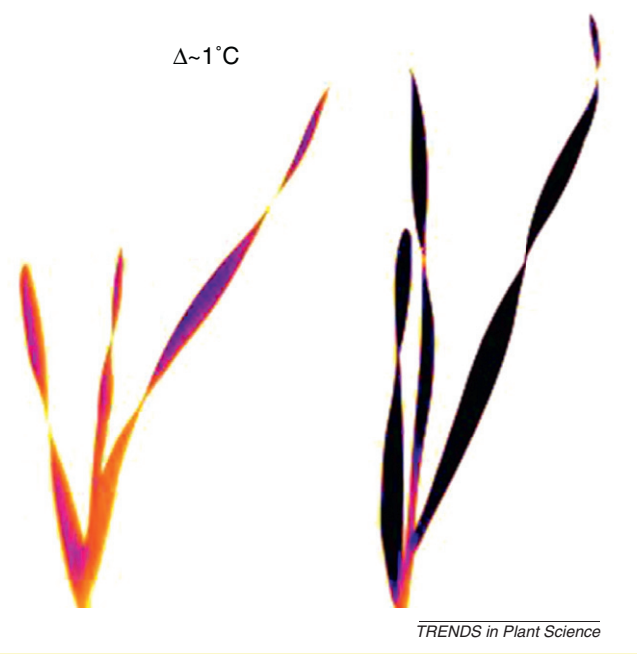

Figure I. Thermal images of durum wheat seedlings treated with salt.

Figure II. Thermal image of a wheat trial imaged from a cherry picker.

speed of deployment, but an imaging sensor allows a much larger number of plots to be assessed simultaneously [29]. Microbolometer-based thermal imaging sensors are improving rapidly in spatial resolution (cameras capable of imaging 640 by 480 elements are now affordable) and these can be mounted on platforms above the crop on model aircraft, helium balloons or manned aircraft; however, the speed of acquisition gained by raising the height of the imaging sensor above the crop obviously reduces spatial resolution [29]. The complexities of diurnal variation of the radiation load on the crop, angle of view and solar angle must still be accounted for to obtain biologically meaningful and reproducible results [34]. Recently, the use of distributed sensor networks which continuously monitor canopy temperature with wireless communication has also improved the utility of such measurements by increasing the temporal resolution throughout the growing season [35].

Chlorophyll fluorescence analysis

Chlorophyll fluorescence has also been used as a surrogate measurement for maintenance of photosynthetic function under stresses such as drought. The most easily measured, and hence the most commonly used, fluorescence parameter in stress studies is dark-adapted Fv/Fm (a measure of the intrinsic photochemical efficiency of light harvesting in photosystem II; [36]). This measurement is now possible using affordable commercial instrumentation designed for imaging of whole leaves or small plants using pulse amplitude-modulated (PAM), fluorometry [36]. It is feasible in high-throughput to obtain whole-plant average measurements or to target leaves at the same developmental stage if the commercial systems and software are adapted to this purpose. This is particularly applicable to high-throughput studies of stress response in species which grow predominantly in the horizontal plane in the seedling stage [model plants such as *Arabidopsis* and tobacco (*Nicotiana tabacum*) or seedlings of dicots such as canola (*Brassica napus*) or cotton (*Gossypium* ssp.)]. For high-throughput and to minimize the costs of plant culture, plants can be grown in large trays or, for small seedlings, in microtitre plates or similar vessels. Fluorescence imaging also allows the determination of projected leaf area and hence the growth rate if measurements are made regularly over time [37]. Fv/Fm has recently been measured in two drought studies with *Arabidopsis* using systems scalable to high-throughput screening [17,38]. One study reported the use of a system for analyzing drought tolerance which measures pulse-modulated chlorophyll fluorescence and digital growth analysis from projected leaf area in compartmented trays [17]. Comparisons of the time course of relative growth rate (RGR) and Fv/Fm in the *Arabidopsis* ecotype C24 and PARP mutants after withholding of water clearly show that Fv/Fm is relatively insensitive to drought stress, whereas RGR falls off rapidly after watering is ceased. The

drought studies show that dark-adapted Fv/Fm is useful mainly as an osmotic stress 'survival' screen, which is of limited utility for many annual crops but might be of more use in perennials or where sporadic rain events are experienced through the growing season. A similar result was found for salinity stress, with Fv/Fm showing no more stress sensitivity than measurement of leaf chlorophyll content [39]. In the case of tissue tolerance to salt, fluorescence imaging might be able to provide valuable leaf level information on the pattern of accumulation of sodium and its effects on the photosynthetic apparatus.

Alternative quenching parameters which can be derived from chlorophyll fluorescence analysis are electron transport rate (ETR) or non-photochemical quenching (NPQ). Chlorophyll fluorescence has not been widely used as a surrogate for ETR under abiotic stress, although this can be calculated from chlorophyll fluorescence quenching if the incident light intensity and the absorption properties are known [40]. Although ETR is challenging to estimate on a whole-plant basis owing to the requirement for light interception to be quantified, it should prove much more sensitive than Fv/Fm in stress tolerance studies. NPQ has frequently been used as an indicator of stress in both model plants and crop species [36]. This parameter is related to the dissipation of energy from the photosynthetic apparatus as heat and is a sensitive measure of photo protection through the xanthophyll cycle [36]. Under stress conditions, NPQ is often seen to rise significantly, either because of photo protection of photosystem II or due to feedback on electron transport from inhibition of carbon metabolism [36].

For cold, heat and UV stress, where the photosynthetic light-harvesting apparatus is often the first point of damage, chlorophyll fluorescence can be a useful early screening system for stress tolerance [17]. In the case of cold and heat stress, effects on photosynthesis and even changes in membrane lipid properties can lead to immediate effects on chlorophyll fluorescence [41], whereas UV stress can result in oxidative damage to the photosystems, once again perceived as a loss of efficiency of light harvesting, useful as a screening tool for tolerance to UV-B exposure [42].

High-throughput chlorophyll fluorescence screening of crop plants after the seedling stage is somewhat problematic. Cereals and dicot crops with complex vegetative structure are difficult to accurately image without construction of a full 3-D model, which requires images to be acquired from multiple viewing angles [43]. Using pulse-modulated fluorescence imaging to acquire this information is also challenging, because a saturating pulse of light, usually produced by LED panels in commercial instruments, must be applied to the entire plant. Obtaining a 'whole-plant' Fv/Fm for a mature crop plant is possible without full 3-D reconstruction, but the value of this is yet to be demonstrated. The technical challenges of field phenotyping using chlorophyll fluorescence are even greater. PAM fluorometry is at present limited to single leaf measurements in the field using either handheld sensors or 'monitoring' fluorometers which can be left attached to a single leaf over a period of weeks or months. An interesting development for crop application is the LIFT (laser induced fluorescence transient) system, which does not require a saturating pulse of light to be applied in order to deconvolute the chlorophyll fluorescence signal [44]. Developed for remote sensing applications and not an imaging system, this instrument has, however, been shown to provide reproducible results similar to those obtained with PAM both for whole-plants and single leaves [44].

Another established optical technique related to chlorophyll fluorescence with utility in stress-related phenomics is leaf spectroscopy or hyperspectral reflectance spectroscopy using radiometric or, more recently, imaging sensors [34]. Leaves of crops absorb incoming light strongly in the region 400–700 nm but reflect light strongly in the near-infrared (700–1100 nm) and shortwave infrared (to 2500 nm). Well-established indices such as NDVI (normalized difference vegetation index) have been developed to relate leaf chlorophyll content and crop biomass to spectral reflectance features and are widely used in remote sensing, crop physiology and precision agriculture [34]. Similarly, an index known as PRI (photosynthetic reflective index) has been proposed as a crop stress indicator based on the absorption features of the photoprotective pigments known as xanthophylls in leaves [45]. Leaf reflectance can also be used to detect steady-state chlorophyll fluorescence in the field [46] using spectral features between 699 and 710 nm. Also, since reflectance in the short-wave infrared region is strongly influenced by tissue water content, there might be opportunities to use this region of the reflectance spectrum as a surrogate for relative leaf water content [47]. There is also scope for developing indices in the short-wave infrared for other plant tissue components such as protein nitrogen and carbohydrate.

Hyperspectral reflectance spectroscopy has not commonly been used in plant breeding, although reflectance spectrometers in the visible/near-infrared region are now very affordable and commercial LED-based instruments measuring NDVI are in common agricultural use. A major limitation to the utility of hyperspectral data, in common with the difficulties in interpreting canopy temperature data, is variability in environmental conditions during measurements [34]. Most spectrometers are passive in nature, relying on solar radiation as the light source, leading to major difficulties in quantitatively comparing results between plots and genotypes owing to cloud cover and changes in solar angle and measurement angle during the photoperiod [34]. The availability of more reasonably priced imaging spectrometers might reduce this problem, particularly if combined with low-level aerial observation of crops in the field [34].

Digital growth analysis

As discussed above, one of the least complicated but useful methods for quantitatively determining stress tolerance is digital growth analysis. Simple analysis of projected leaf area in model plants has proven useful and the availability of commercial systems for carrying out quasi '3-D' digital growth analysis on crop species have meant that, such approaches are becoming more popular for *in situ* crop phenotyping in controlled environment facilities (www.plantphenomics.com and www.plantphenomics.org.au). This technique uses multiple viewing angles (usually two side views and a top view) to extract a mathematical relationship between these three digital images and

biomass or leaf area [48]. The correlation between digital estimation of leaf area and that obtained for destructive harvest can exhibit an r^2 value of greater than 0.9 [48]. This technique will have greater accuracy early in plant development and will reduce in utility as occlusions become problematic, such as after tillering in cereals.

Digital imaging of growth over a period of plant development allows assessment of the sum of stress response mechanisms and offers the opportunity to tease apart many of these responses. For example, shortly after application of salt, just as stomata shut (discussed above), inhibition of plant growth also occurs rapidly, which is independent of the accumulation of the salt. This provides an opportunity to separate salt-dependent and salt-independent components of plant responses to salinity. After longer exposure to salinity, leaf senescence can be quantified by separating yellow and green areas of the leaf, and this can be related to the tolerance of tissues to accumulated salt [28,48]. With non-destructive image analysis, these components of salinity tolerance can be measured on a single plant. Furthermore, they can be measured rapidly and accurately, so these components can be measured in large populations – such as mutant populations, or mapping populations – which enables a genetic approach to be undertaken to identify genes underlying variation in these respective components of tolerance.

Digital imaging in visible wavelength regions provides information not only on plant size, but also on the color of the plants, thus enabling quantification of senescence arising from, for example, nutrient deficiencies or toxicities, or pathogen infections. This approach has recently been validated in an experiment where digital imaging was used to quantify toxicity of germanium (as a toxic analogue of boron) in a mapping population of barley [49] to identify a QTL at the same locus as previously identified for boron tolerance using a visual score of symptoms [50].

Transpiration of plants can also be measured over time by automatically monitoring water consumption gravimetrically. When combined with the measurement of plant growth using digital imaging, water-use efficiency (WUE) can be monitored through the life of the plant, and effects of environment and genetic make-up on WUE can be tested. The first attempts at this have been recently published [51].

Phenomic screening for biotic stress tolerance

Non-destructive techniques such as digital imaging in the visible spectrum and imaging of chlorophyll fluorescence have been used to monitor the progress of disease symptoms in leaves for some years [52]. Foliar and stem fungal pathogens such as rusts and mildews produce large-scale reprogramming of metabolism soon after infection, often reflected by persistent changes in ETR and NPQ of chlorophyll fluorescence, calculated from chlorophyll fluorescence images of the affected area of the leaf [52]. This technique allows the early detection of symptoms (before symptoms are visible to the eye), quantification of the area of infected tissue and potentially the quantification of the susceptible and resistant response to pathogen attack, at least in the case of mildew on barley leaves [53,54].

Digital imaging in just the visible region offers no advantage in sensitivity over the detection of symptoms by eye, but it provides a high-throughput technique to quantify lesions or chlorotic areas on leaves. Using a combination of careful image capture, image analysis and color classification, it is possible to follow the progression of lesions over time quantitatively. However, this approach has not commonly been used to date in screens for pathogen resistance in crop plants.

One of the reasons that application of plant phenomics to pathology has only recently developed might be that the genetics of resistance to major pathogens such as rusts are relatively simple. Rust resistance genes of the NBS-LRR type, or R-genes [55], act in a gene-for-gene specific manner with respect to virulence and avirulence genes and are easily scored and followed in crosses of crop breeding germplasm using MAS. Recently, however, there has been intense interest in 'slow rusting' or adult plant resistance genes for rust [56], particularly with the occurrence of 'super strains' of rust such as UG99 which have overcome the current subset of R-genes [57]. The adult plant resistance genes represent greater phenotypic challenges in scoring for disease symptoms owing to the need to examine plants at multiple time points during the progression of symptoms, in addition to genotypic challenges, because these traits are quantitative and can be non-race-specific rather than 'gene-for-gene'. Non-destructive imaging using fluorescence and hyperspectral reflectance offers great promise in quantitative scoring of such adult plant resistance phenotypes.

Although the application of leaf- and shoot-level phenomics to pathogen resistance is in its infancy, at least this screening has a well-developed basis in mechanistic research on foliar symptoms. High-throughput phenomics of root pathogens has received little attention thus far and the methods employed to achieve uniform infection and scoring of resistance are laborious for most root pathogens. One example of this is the root fungal pathogen *Fusarium oxysporum*, a pathogen of many crop species but notably of cotton [58]. This pathogen is soil-borne, infects through the root and is xylem-contained, but the phenotypic effect is blockage of the xylem tissue, stunting and seedling death [58]. Currently, disease resistance is scored by percentage survival of seedlings, wilting or stunting of growth at a fixed time after germination or via scoring of visible symptoms in the seedling conductive tissue [58]. Disruption of xylem tissue causes reduced transpiration, stomatal closure and thus potentially, hotter canopies, offering an opportunity for a rapid non-invasive screening technique for tolerance or resistance using the thermography screen for transpiration described above [31]. Pathogens such as 'verticillium wilt' (*Verticillium dahliae*) and 'black root rot' (*Thielaviopsis basicola)* exhibit similar phenotypes and might also be amenable to this approach. Direct phenotyping of roots for pathogenic effects might also be feasible by adaptation of imaging techniques to measure root elongation such as developed for screening for aluminum (Al) tolerance [59].

Application of plant phenomics to trait-based physiological breeding

The challenge for comprehensive and quantitative analysis of traits for physiological breeding has been the application

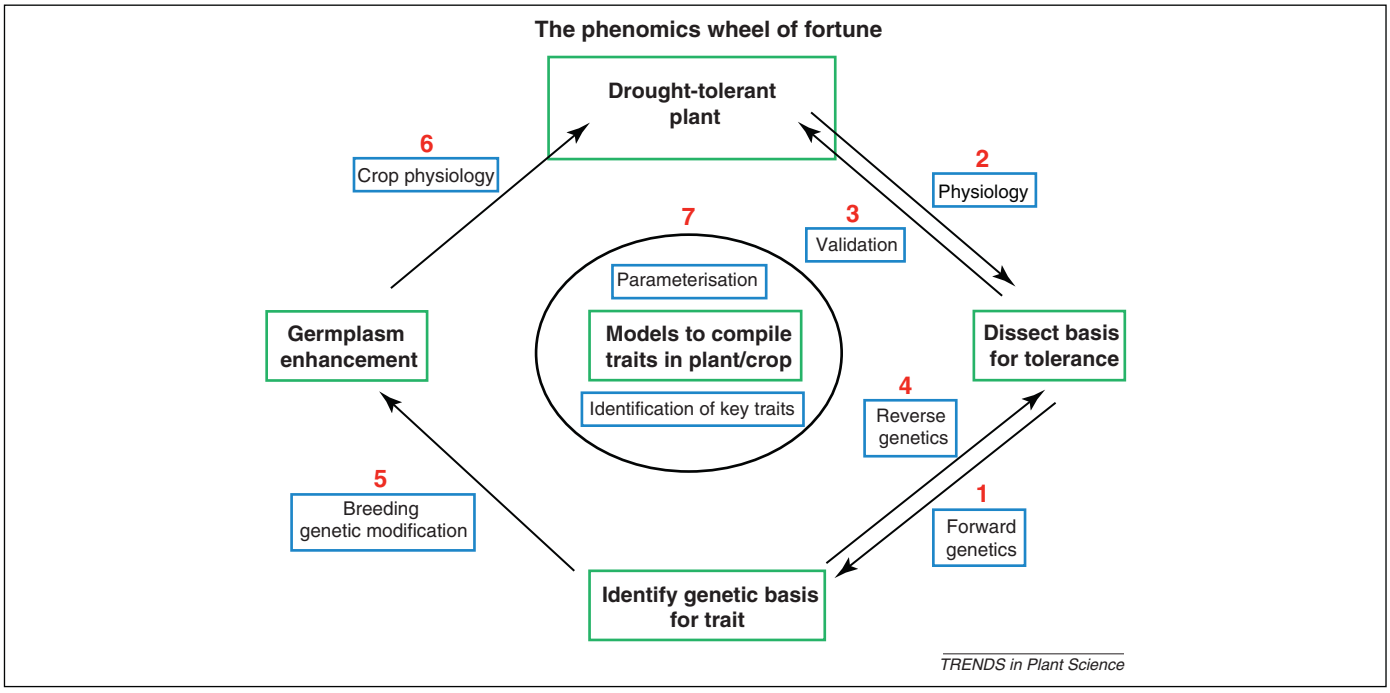

Figure 2. Closing the gene to genotype loop with phenomics.

of appropriate non-invasive tools to directly measure these traits or their surrogates, as indicated above. Once important traits or 'yield components' underpinning a superior crop variety are identified (by what we might now term 'reverse phenomics'), either a genomic region needs to be identified to select for this trait by MAS in breeding or, in the case of multigenic traits commonly encountered in quantitative physiological breeding, a robust phenotypic marker is pivotal.

Digital imaging can be used for obtaining measures of biomass and growth, and for probing some aspects of physiological function. Specific traits of particular interest to breeders, such as tillering, early vigor, coleoptile length and biomass at anthesis [13], can also be obtained from analyzed images. These traits are currently scored manually, and this can be improved in both speed and accuracy using phenomics tools. Proxies for some traits can be obtained by relatively simple properties of shape and texture, but the aim of image analyses is to develop 3-D models through time of the growing plants to enable extraction of more detailed, specific information, such as the life history of each leaf or even each tiller. This will allow the automated quantification of properties of particular interest to breeders working to improve traits underlying characteristics such as stress tolerance. For example, such models would allow the longevity of specific leaves to be quantified, which, in a salt-stressed plant, would allow the determination of the tolerance of the leaf to accumulated sodium ions (Na^+).

The advantage of direct measurement of the trait of interest by imaging technologies rather than proxies is that it should be possible to exploit all the genetic variation responsible for a trait rather than one component. For example, one component of early seedling vigor, an important trait for conserving soil moisture, has been shown to be related to embryo size and is currently scored phenotypically by the width of the fully expanded leaves at young vegetative stage [13]. Whether there are valuable sources of variation in vigor unrelated to this parameter has not been extensively examined because growth rate itself has not been amenable to high-throughput screening until recently. Obviously, some simple surrogate measurements are still valuable and will continue to be used owing to their rapidity and ease of measurement.

Using plant phenomics to close the 'gene to genotype' loop

An impressive array of tools is now available for high-throughput phenotyping and the approaches described above can be used in many different ways to facilitate the process of trait identification, gene identification and genotype development necessary to produce a new crop variety. Examples of how phenomics can be used to develop a crop genotype or variety with tolerance to a particular type of drought are presented in Figure 2.

In this scheme, phenomics features at a number of levels. 'Forward' phenomics can be used to identify phenotypic, and thus genetic, variation in particular traits of interest (**1** in Figure 2), for traits indicated as important and validated to be important by physiological studies ('reverse phenomics') of plants with differing drought tolerance (**2** in Figure 2). This genetic approach can take the form of a large genotype screen using a bi-parental or multi-parent population, or by direct analysis of a 'diversity panel' of lines for analysis by association genetics. As discussed above, accurate, cost-effective[1], high-throughput phenotyping is pivotal to fine mapping of traits, regardless of the genetic approach for producing allelic recombination or assessing variation by re-sequencing technologies.

[1] For an idea of the cost of high-throughput screens, see some example pricing at The Plant Accelerator website at http://www.plantaccelerator.org.au/services/pricing/.

Phenomics is also essential for good quality reverse genetic studies, to test hypotheses regarding the role of particular genes in the function of a plant (**4** in Figure 2), and to test the effects of altering patterns, levels or alleles of target genes on the traits of germplasm (**5** in Figure 2) and the drought tolerance of the resultant crop (**6** in Figure 2).

Reverse phenomics (**2** and **3** in Figure 2) allows the dissection of a trait to elucidate mechanisms and inform the process of identifying gene candidates through a hypothesis-driven rather than a high-throughput screening approach. Reverse phenomics utilizes a suite of new tools applied to a limited set of germplasm to elucidate common strategies responsible for stress tolerance or yield potential, for example. By drilling down to mechanisms, such an approach can be used to elucidate the next generation of traits for the breeding toolbox. Crop modeling approaches are centrally important (**7** in Figure 2) to inform this process [60]. Whole-of-lifecycle measurements of plant performance are valuable for obtaining parameters for crop models such as APSIM [60] and crop models can inform phenomics approaches by giving estimates of trait 'value' under different environments, allowing the construction of plant ideotypes.

The schema presented in Figure 2 is drawn in a circle because phenomics both facilitates the discovery of genes responsible for drought tolerance (in this example)and helps to increase drought tolerance by alteration of the genes that contribute to tolerance. The process of discovery and use of the discovery are, ultimately, circular.

Concluding remarks

Plant phenomics can, in fact, be considered as simply plant physiology in 'new clothes', but it promises to bring physiology up to speed with genomics by introducing the incredible recent advances made in computing, robotics, machine vision and image analysis to the wider field of plant biology. A multidisciplinary team in plant phenomics crosses biology, physics and mathematics, not 'just' genetics, biochemistry, physiology and plant breeding. This trans-disciplinary approach promises significant new breakthroughs in plant science. Phenomics provides the opportunity to study previously unexplored areas of plant science, and it provides the opportunity to bring together genetics and physiology to reveal the molecular genetic basis of a wide range of previously intractable plant processes. The challenges ahead in plant-based agriculture will require the scale of quantum advances we have seen in information technology in the past 20 years and we need to build on these advances for security of global food, fiber and fuel.

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References

1 Furbank, R.T. *et al.* (2009) C_4 rice: a challenge for plant phenomics. *Funct. Plant Biol.* 36, 845–856
2 Reynolds, M. *et al.* (2009) Raising yield potential in wheat. *J. Exp. Bot.* 60, 1899–18918
3 Tester, M. and Langridge, P. (2010) Breeding technologies to increase crop production in a changing world. *Science* 327, 818–822
4 Sticklen, M.B. (2007) Feedstock crop genetic engineering for alcohol fuels. *Crop Sci.* 47, 2238–2248
5 Ziska, L.H. and Bunce, J.A. (2007) Predicting the impact of changing CO_2 on crop yields: some thoughts on food. *New Phytol.* 175, 607–618
6 Leakey, A.D.B. *et al.* (2009) Elevated CO_2 effects on plant carbon, nitrogen, relations: six important lessons from FACE. *J. Exp. Bot.* 60, 2859–2876
7 O'Malley, R.C. and Ecker, J.R. (2010) Linking genotype to phenotype using the *Arabidopsis* unimutant collection. *Plant J.* 61, 928–940
8 Weigel, D. and Mott, R. (2009) The 1001 Genomes Project for *Arabidopsis thaliana*. *Genome Biol.* 10, 107
9 Cannon, S.B. *et al.* (2009) Three sequenced legume genomes and many crop species: rich opportunities for translational genomics. *Plant Physiol.* 151, 970–977
10 Vogel, J.P. *et al.* The International Brachypodium Intitiative (2010) Genome sequencing and analysis of the model grass *Brachypodium distachyon*. *Nature* 463, 763–768
11 Miyao, A. *et al.* (2007) A large-scale collection of phenotypic data describing an insertional mutant population to facilitate functional analysis of rice genes. *Plant Mol. Biol.* 63, 625–635
12 Lu, Y. *et al.* (2008) New connections across pathways and cellular processes: Industrialized mutant screening reveals novel associations between diverse phenotypes in *Arabidopsis*. *Plant Physiol.* 146, 1482–1500
13 Richards, R.A. *et al.* (2010) Breeding for improved water productivity in temperate cereals: phenotyping, quantitative trait loci, markers and the selection environment. *Funct. Plant Biol.* 37, 85–97
14 Ilic, K. *et al.* (2007) The plant structure ontology, a unified vocabulary of anatomy and morphology of a flowering plant. *Plant Physiol.* 143, 587–599
15 Hannemann, J. *et al.* (2009) Xeml Lab: a tool that supports the design of experiments at a graphical interface and generates computer-readable metadata files, which capture information about genotypes, growth conditions environmental perturbations and sampling strategy. *Plant Cell Environ.* 32, 1185–1200
16 Finkel, E. (2009) With 'phenomics' plant scientists hope to shift breeding into overdrive. *Science* 325, 380–381
17 Jansen, M. *et al.* (2009) Simultaneous measurement of leaf growth and chlorophyll fluorescence via GROWSCREEN FLUORO allows detection of stress tolerance in *Arabidopsis thaliana* and other rosette plants. *Funct. Plant Biol.* 36, 902–914
18 Berger, B. *et al.* (2010) High throughput imaging to study drought responses. *J. Exp. Bot.* 61, 3519–3528
19 Granier, C. *et al.* (2006) PHENOPSIS, an automated platform for reproducible phenotyping of plant responses to soil water deficit in *Arabidopsis thaliana* permitted the identification of an accession with low sensitivity to soil water deficit. *New Phytol.* 169, 623–635
20 Walter, A. *et al.* (2007) Dynamics of seedling growth acclimation towards altered light conditions can be quantified via GROWSCREEN: a set up and procedure designed for rapid optical phenotyping of different plant species. *New Phytol.* 174, 447–455
21 Meyer, R.C. *et al.* (2010) QTL analysis of early stage heterosis for biomass in *Arabidopsis*. *Theor. Appl. Genet.* 120, 227–237
22 Costa, L. and Cesar, R.M. (2001) *Shape Analysis and Classification: Theory and Practice*, CRC Press
23 Prusinkiewicz, P. *et al.* (1996) L-systems: from the theory to visual models of plants. In *2nd CSIRO Symposium on Computational Challenges in Life Sciences* (Michalewicz, M.T., ed.), CSIRO Publishing
24 Prusinkiewicz, P. *et al.* (2002) The use of positional information in the modeling of plants. *SIGGRAPH'01,* 289–300 DOI: 10.1145/383259.383291
25 Cui, M-L. *et al.* (2010) Quantitative control of organ shape by combinatorial gene activity. *PLoS Biol.* 8, e1000538
26 Kang, S.B. and Quan, L. (2010) Image-based modeling of plants and trees. In *Synthesis Lectures on Computer Vision #1* (Medioni, G. and Dickinson, S., eds), pp. 1–73, Morgan and Claypool
27 Hartmann, A. *et al.* (2011) HTPheno: An image analysis pipeline for high-throughput plant phenotyping. *BMC Bioinform.* 12, 148
28 Munns, R. and Tester, M. (2008) Mechanisms of salinity tolerance. *Annu. Rev. Plant Biol.* 59, 651–681

29 Munns, R. *et al.* (2010) New phenotyping methods for screening wheat and barley for water stress tolerance. *J. Exp. Bot.* 61, 3499–3507
30 Condon, A.G. *et al.* (2004) Breeding for high water-use efficiency. *J. Exp. Bot.* 55, 2447–2460
31 Sirault, X.R.R. *et al.* (2009) A new screening method for osmotic component of salinity tolerance in cereals using infrared thermography. *Funct. Plant Biol.* 36, 970–977
32 Pinter, P.J. *et al.* (1983) Infrared thermometry: a remote sensing technique for predicting yield in water stressed cotton. *Agric. Water Manag.* 6, 385–395
33 Fischer, R.A. *et al.* (1998) Wheat yield progress associated with higher stomatal conductance and photosynthetic rate and cooler canopies. *Crop Sci.* 38, 1467–1475
34 Jones, H.G. and Vaughan, R.A. (2010) *Remote Sensing of Vegetation: Principles, Techniques and Applications*, Oxford University Press
35 O'Shaughnessy, S.A. *et al.* (2011) Evaluation of a wireless infrared thermometer with a narrow field of view. *Comput. Electron. Agric.* 76, 59–68
36 Baker, N.R. (2008) Chlorophyll fluorescence: a probe of photosynthesis *in vivo*. *Annu. Rev. Plant Biol.* 59, 89–113
37 Barbagallo, R.P. *et al.* (2003) Rapid, non-invasive screening for perturbations of metabolism and plant growth using chlorophyll fluorescence imaging. *Plant Physiol.* 132, 485–493
38 Woo, N. *et al.* (2008) A rapid non-invasive procedure for quantitative assessment of drought survival using chlorophyll fluorescence. *Plant Methods* 4, 27
39 James, R.A. *et al.* (2002) Factors affecting CO_2 assimilation, leaf injury and growth in salt-stressed durum wheat. *Funct. Plant Biol.* 29, 1393–1403
40 Genty, B. *et al.* (1989) The relationship between the quantum yield of photosynthetic electron transport and quenching of chlorophyll fluorescence. *Biochim. Biophys. Acta* 990, 87–92
41 Berry, J.A. and Björkman, O. (1980) Photosynthetic response and adaptation to temperature in higher plants. *Ann. Rev. Plant Physiol.* 31, 491–543
42 Jansen, M.A. *et al.* (2010) Variations in constitutive and inducible UV-B tolerance; dissecting photosystem II protection in *Arabidopsis thaliana* accessions. *Physiol. Plantarum* 138, 22–34
43 Konishi, A. *et al.* (2009) 3D monitoring of spatio-temporal effects of herbicide on a whole plant using combined range and chlorophyll a fluorescence. *Funct Plant Biol.* 36, 874–879
44 Pieruschka, R. *et al.* (2009) Optical remote sensing and laser induced fluorescence transients (LIFT) to quantify the spatio-temporal functionality of plant canopies. *Nova Acta Leopoldina* 96, 49–62
45 Gamon, J.A. *et al.* (1992) A narrow waveband spectral index that tracks diurnal changes in photosynthetic efficiency. *Remote Sensing Environ.* 41, 35–44
46 Zarko-Tejada, P.J. *et al.* (2003) Steady state chlorophyll *a* fluorescence detection from canopy derivative reflectance and *double-peak* red-edge effects. *Remote Sensing Environ.* 84, 283–294
47 Curran, P.J. (1989) Remote-sensing of foliar chemistry. *Remote Sensing Environ.* 30, 271–278
48 Rajendran, K. *et al.* (2009) Quantifying the three main components of salinity tolerance in cereals. *Plant Cell Environ.* 32, 237–249
49 Schnurbusch, T. *et al.* (2010) Boron toxicity tolerance in wheat and barley: Australian perspectives. *Breeding Sci.* 60, 297–304
50 Jefferies, S.P. *et al.* (1999) Mapping of chromosome regions conferring boron toxicity tolerance in barley (*Hordeum vulgare* L.). *Theor. Appl. Genet.* 98, 1293–1303
51 Harris, B.N. *et al.* (2010) A water-centred framework to assess the effects of salinity on the growth and yield of wheat and barley. *Plant Soil* 336, 377–389
52 Scholes, J.D. and Rolfe, S.A. (2009) Chlorophyll fluorescence imaging as a tool for understanding the impact of fungal diseases on plant performance: a phenomics perspective. *Funct. Plant Biol.* 36, 880–892
53 Swarbrick, P.J. *et al.* (2006) The metabolic consequences of susceptibility and the activation of race specific or broad spectrum resistance pathways in barley leaves challenged with the powdery mildew fungus. *Plant Cell Environ.* 29, 1061–1076
54 Chaerle, L. *et al.* (2009) Multi-sensor plant imaging: towards the development of a stress-catalogue. *Biotechnol. J.* 4, 1152–1167
55 Ellis, J.G. *et al.* (2000) The generation of plant disease resistance gene specificities. *Trends Plant Sci.* 5, 373–379
56 Krattinger, S.G. *et al.* (2009) A putative ABC transporter confers durable resistance to multiple fungal pathogens in wheat. *Science* 323, 1360–1363
57 Xu, S.S. *et al.* (2009) Evaluation and characterization of seedling resistances to stem rust Ug99 races in wheat-alien species derivatives. *Crop Sci.* 49, 2167–2175
58 Hillocks, R.J. (ed.) (1992) *Cotton Diseases*, CAB International
59 Famoso, A.N. *et al.* (2010) Development of a novel aluminum tolerance phenotyping platform used for comparisons of cereal aluminum tolerance and investigations into rice aluminum tolerance mechanisms. *Plant Physiol.* 153, 1678–1691
60 Hammer, G. *et al.* (2006) Models for navigating biological complexity in breeding improved crop plants. *Trends Plant Sci.* 11, 587–593
61 Costa, L.D.F. and Cesar, R.M., eds (2001) *Shape Analysis and Classification. Theory and Practice*, pp. 421–426, CRC Press