Review

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Cell to whole-plant phenotyping: the best is yet to come

Stijn Dhondt[1,2*], Nathalie Wuyts[1,2*], and Dirk Inzé[1,2]

[1] Department of Plant Systems Biology, VIB, Technologiepark 927, 9052 Gent, Belgium
[2] Department of Plant Biotechnology and Bioinformatics, Ghent University, Technologiepark 927, 9052 Gent, Belgium

Imaging and image processing have revolutionized plant phenotyping and are now a major tool for phenotypic trait measurement. Here we review plant phenotyping systems by examining three important characteristics: throughput, dimensionality, and resolution. First, whole-plant phenotyping systems are highlighted together with advances in automation that enable significant throughput increases. Organ and cellular level phenotyping and its tools, often operating at a lower throughput, are then discussed as a means to obtain high-dimensional phenotypic data at elevated spatial and temporal resolution. The significance of recent developments in sensor technologies that give access to plant morphology and physiology-related traits is shown. Overall, attention is focused on spatial and temporal resolution because these are crucial aspects of imaging procedures in plant phenotyping systems.

Plant phenotyping is a complex matter involving a plethora of systems and tools

'Phenomics' has been proposed as a novel discipline in biology and involves the gathering of high-dimensional phenotypic data at multiple levels of organization, to progress towards the full characterization of the complete set of phenotypes of a genome, in analogy with whole genome sequencing [1]. This ultimate aim will of course remain hypothetical; however, current and future developments in plant phenotyping and phenomics may benefit from the consideration of dimensionality, together with throughput and resolution, because our comprehension of plant processes in general, and the genotype–phenotype relationship in particular, is far from complete (Box 1). Plant phenotypes are inherently complex because they result from the interaction of genotypes with a multitude of environmental factors. This interaction influences on the one hand the developmental program and growth of plants, which can be described by means of structural traits, and, on the other hand, plant functioning, described by means of physiological traits (Figure 1). Both the structural and physiological traits eventually determine plant performance in terms of biomass and yield. Phenotypic traits at different organizational levels or in different categories may show high correlations (dependent variation) in one particular or in multiple environments. If robust, these may reduce the complexity of phenotyping (i.e., the number of different traits to be measured), but whether this is wanted depends on the biological question [2].

The biological question under investigation largely determines the phenotypic traits of interest and consequently the phenotyping system and tools. It may be exploratory in nature, meaning that the number of traits is limited by the phenotyping system itself and to what is

Glossary

Anisotropic cell expansion: extension of a cell along a particular axis or in a certain direction.
Confocal microscopy: or confocal laser scanning microscopy. A laser light is focused onto a very small and specific portion of a sample and the emitted fluorescence that passes through a detector pinhole is collected. The pinhole reduces the amount of out-of-focus light (i.e., fluorescence emitted by other points in the sample).
Displacement transducer: a measuring device that converts a linear or angular displacement into a signal (electric, mechanical, pneumatic, or other) suitable for recording, transmission, or conversion.
High-resolution X-ray computed tomography: a volume rendering technology in which X-ray projections of a rotating sample are combined into a 3D structure.
Hyponastic movement: upward bending of plant organs (e.g., leaves) because of differential extension across a plant axis (e.g., the petiole).
Kinematic parameters: describe the rate at which plant processes take place and their extent and duration in space and time.
Multiphoton microscopy: fluorescence microscopy in which the excitation of the fluorophore is achieved by the simultaneous absorption of two photons in the infrared. This is achieved by concentrating an ultrafast laser onto a specific point in the sample under the microscope.
Optical coherence microscopy: a non-invasive imaging technology which measures the inherent light-scattering properties of biological samples with an increased sample penetration depth compared to confocal microscopy [99].
Optical projection tomography: a volume rendering technology in which pictures of a translucent sample taken at different rotation angles are combined into a 3D structure.
Point cloud: a set of points with coordinates in 3D space (X, Y, Z). Each point contains data specific to its position in space, such as intensity, color, and orientation.
Rhizotron: a plant root observation chamber often constructed from transparent Plexiglas.
Segmentation: the operation of checking whether each individual pixel of an image belongs to an object of interest or not. Objects of interest are identified by finding suitable local features that allow them to be distinguished from other objects and from the background. Segmentation produces a binary image in which a pixel has the value one if it belongs to an object of interest; otherwise it is zero [130].
Segregating population: a population of plants, usually the progeny of a cross, showing variation in phenotypic traits.
Visible imaging: acquisition of images on digital sensors sensitive to light reflected in the visible part of the electromagnetic spectrum.

Corresponding author: Inzé, D. (dirk.inze@psb.vib-ugent.be)
* These authors contributed equally to this work.
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Box 1. Throughput, resolution, and dimensionality in plant phenotyping systems

Plant phenotyping

A plant phenotype is the set of structural, physiological, and performance-related traits of a genotype in a given environment. Plant phenotyping is the act of determining the quantitative or qualitative values of these traits. Given that a phenome consists in principle – of the set of all possible phenotypes of a given genotype, plant phenomics could be considered as the study of phenomes of multiple genotypes. Besides providing the tools to perform phenotyping itself, plant phenotyping systems usually comprise the means to grow plants in certain environments, which are either defined and controlled, or semi-controlled, or uncontrolled and measured. The characteristics of the phenotyping system determine its capacity in terms of the number of genotypes and the range of environmental conditions or treatments and, thus, its suitability for phenomics. Phenotyping systems can be described by means of throughput, resolution, and dimensionality.

Throughput

The throughput of a system is the amount of things it can do or deal with in a particular period of time (http://www.collinsdictionary.com/). In plant phenotyping systems, throughput refers to the number of individual units at particular organizational levels within plants, or at the plant or canopy level, that can be analyzed for a particular (set of) trait(s) at a given time.

Resolution

Resolution is the process or act of separating something into its constituent parts or elements (http://www.collinsdictionary.com/). In plant phenotyping systems, spatial resolution refers to the level of separation of plants or plant organs, tissues, and cells into their elementary or organizational units used for the measurement of plant traits (Figure I). Temporal resolution indicates the level of separation into elementary time periods significant for plant processes and used for the measurement of plant traits (Figure II).

Dimensionality

Dimensionality indicates the number of different aspects of something or the quality of having depth and richness (http://www.collinsdictionary.com/). Dimensionality in plant phenotyping refers to the diversity of phenotypic traits measured at different spatial and temporal resolutions and in different categories, such as plant structure, physiology, and performance. In phenomics, dimensionality additionally includes the number of genotypes and the diversity of environmental conditions and treatments taken into account upon phenotyping.

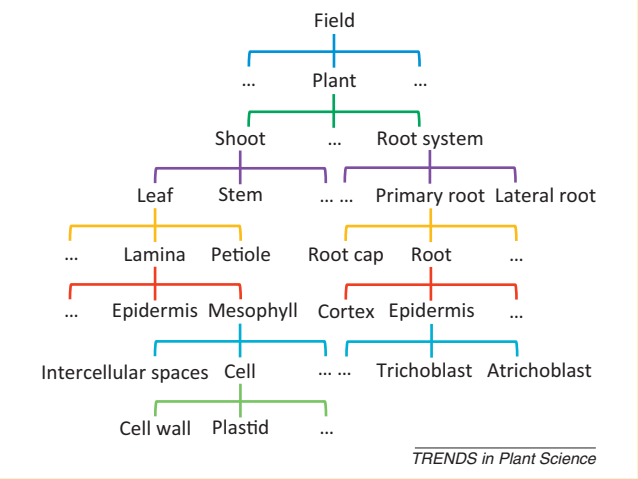

Figure I. Spatial resolution in plant phenotyping.

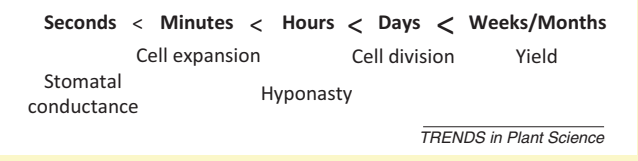

Figure II. Temporal resolution in plant phenotyping.

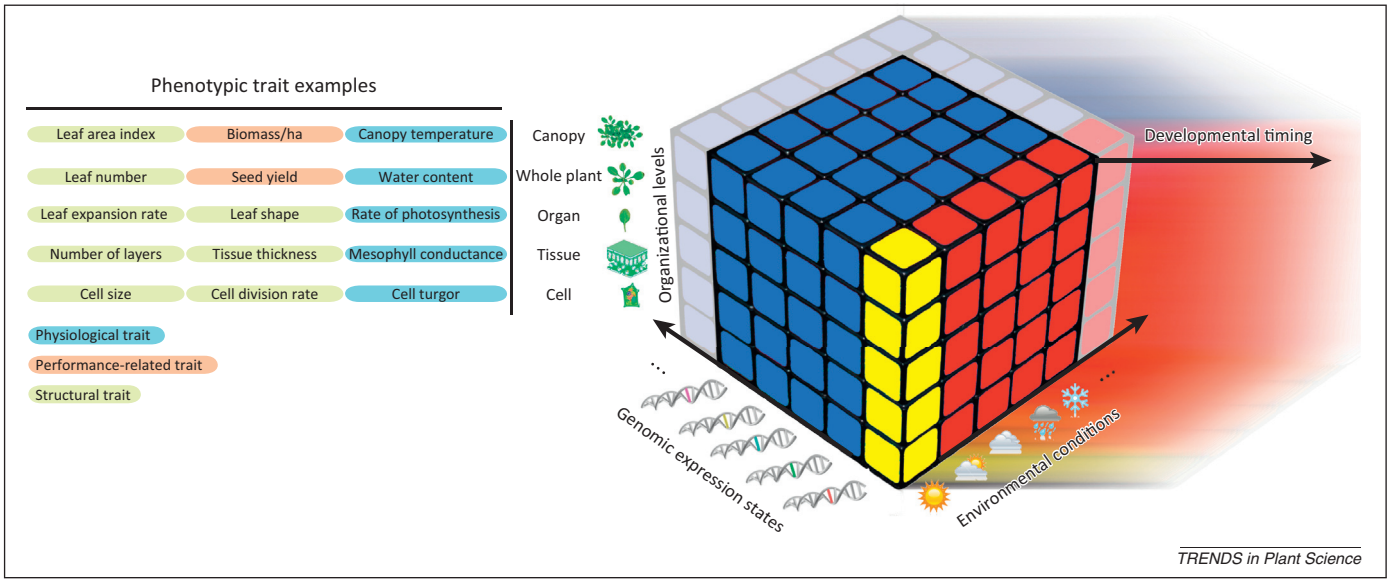

Figure 1. From plant phenotyping to phenomics. Plant phenotyping can be performed at multiple organizational levels, ranging from the field and canopy, to the whole-plant, organ, tissue, and cellular level (and eventually subcellular level). Phenotypic traits of interest can be categorized as physiological, structural, or performance-related. Plant phenotyping is the quantitative or qualitative investigation of these traits at any organizational level, in a given genomic expression state and a given environment. This is shown as a single column of yellow cubes, which could be positioned anywhere in the overall cube. A phenome corresponds to all possible phenotypes under different environmental conditions of a given genotype, represented by the combination of yellow and red cubes. Genomic expression states cover the complete range of available plant genetic resources (e.g., overexpresssion lines, mutants, natural accessions, and segregating populations). Plant phenomics could be considered as the study of phenomes of multiple genomic expression states, represented by the combination of yellow, red, and blue cubes. Light-colored cubes illustrate the (in principle) infinite possibilities of environmental conditions and genomic expression states. Notably, plant phenotypes can be assessed at specific times during development, or alternatively in a dynamic manner.

practically feasible. In the case of explanatory phenotyping, traits are typically restricted to what is required for hypothesis testing.

Plant growth conditions within phenotyping systems include both *in vitro* cultures in artificial substrates and *in vivo* growth in soil in a variety of containers, ranging from Petri dishes to pots, rhizotrons (see Glossary) and hydroponic or aeroponic containers, and *in situ* in field plots. The environmental conditions are controlled in incubators and growth chambers, semi-controlled in greenhouses, and uncontrolled in field trials. Phenotyping tools range from the manual measurement of leaf length to complex robotic systems with automated acquisition and measurement workflows. They comprise destructive or non-invasive measurements, *in* or *ex situ*, at a spatial resolution stretching from the subcellular level (nm-scale precision) to canopy stands (meter-scale precision), and temporal resolutions ranging from seconds to entire growing seasons.

Plant phenotyping and phenomics are often associated with high-throughput phenotyping systems in plant breeding and biotechnology for crop improvements relating to current and future demographic and climatic scenarios. Here, automated workflows enable several hundreds of plants to be processed daily by means of non-invasive imaging and image processing [3]. Most of this phenotyping is performed at the organism level (i.e., shoots or root systems), which means at a limited spatial resolution and dimensionality in terms of plant processes and the phenotypic traits that describe these processes. At the research laboratory scale, a substantial amount of effort is spent on phenotyping in relation to gene function and mode-of-action analysis, and aimed at unraveling the fundamental developmental and physiological processes involved in growth, reproduction, and responses to environmental factors in both model and crop species [4]. In-depth, high-dimensional phenotyping is mostly performed at the organ and cellular level of plant organization and often involves destructive and manual measurements of structural and physiological traits. Compared with whole-plant phenotyping systems, throughput is rarely high, but spatial and temporal resolution tends to be increased.

Overall, the plant phenotyping community seems somewhat divided between high-throughput, low-resolution phenotyping and in-depth phenotyping at lower throughput and higher resolution. The divergence is currently most prominent in the phenotyping of the vegetative stage of plant development. This review focuses on the technological aspects of vegetative shoot and root system phenotyping, covering the organism (whole plant) to cellular level. Phenotyping systems and tools are discussed by examining three key characteristics – throughput, dimensionality, and resolution. Novel technologies that may improve dimensionality in high- and low-throughput systems are highlighted. For phenotyping of plant reproductive organs and performance, the reader is referred to other recent work [5–7].

Automation in whole-plant phenotyping

In plant mutant collections, exploratory phenotyping is used to detect 'extra-ordinary' traits (the ordinary being the wild type phenotype) [4], whereas natural and segregating populations are evaluated for desirable traits, potentially in response to stressful environmental conditions such as drought or low temperatures [8,9]. The phenotyping of large collections of genotypes enables the identification of trait-associated genomic regions and ultimately gene cloning, and the establishment of genetic markers that may assist in marker-based selection of germplasm [10]. Screening purposes typically require large numbers of plants to be processed, preferably by means of high-throughput systems, for the measurement of phenotypic traits, usually at the whole-plant or organism level (i.e., either shoots or root systems). In general, automated workflows enable increased throughput when time-consuming or repetitive manual interventions and analyses can be replaced by all or part of the following means: (i) non-invasive sensors, (ii) automated data processing to obtain phenotypic traits of interest, (iii) robotized delivery of plants to sensors or vice versa, (iv) robotized plant culturing, and (v) automated analysis of processed data in a data management pipeline (Figure 2).

Visible imaging of plants has revolutionized plant phenotyping by enabling the non-destructive measurement of plant morphological traits and, therefore, the dynamic aspects of shoot and root system development. Plant-associated pixels can be distinguished from the image background by several segmentation methods, using the red, green, and blue (RGB) or the hue, saturation, and value (HSV) color spaces [11–13]. This is then followed by the scaling of relevant pixels to metric values. *Arabidopsis* (*Arabidopsis thaliana*) rosettes and the vegetative shoots of some other dicots are usually imaged from above for the measurement of projected leaf surface area (Figure 3A) [11,14–16]. Two side views, at 0° and 90°, are used for monocots [17]. The projected leaf surface area of monocots shows linear variation with total leaf surface area, and with shoot biomass in early vegetative stages, or over the complete duration of vegetative shoot establishment when plant age is taken into account [18]. In essence, it is the combination of robotics and dedicated image processing that has boosted throughput in plant phenotyping (Figure 2). Automated imaging of the aboveground parts of plants has been incorporated in robotized platforms in growth chambers [12,16,19,20] and greenhouses [21,22], where either plants are transported to cameras or, vice versa, cameras get positioned in line with plants. In some cases, these platforms include automated weighing and irrigation of plants to establish soil water stress conditions [16,19,21,22]. Imaging and image processing tools have been developed for the analysis of *in vitro Arabidopsis* rosette growth in many research environments, for example, the 'in vitro growth imaging system' (IGIS) (http://www.yieldbooster.org/resources/methods/154-in-vitro-growth-imaging-system-igis). Likewise, several sophisticated tools enable the assessment of hypocotyl growth rate, apical hook opening and photo- and gravitropic bending with a high spatiotemporal resolution [23–25].

In the case of roots, automation of phenotyping systems is mainly limited to the level of image analysis. Some rhizotron systems have robotized delivery of plants to the imaging sensor [17]; however, in general, root systems are manually positioned in front of a camera or on a flat-bed scanner, and imaged either once or continuously

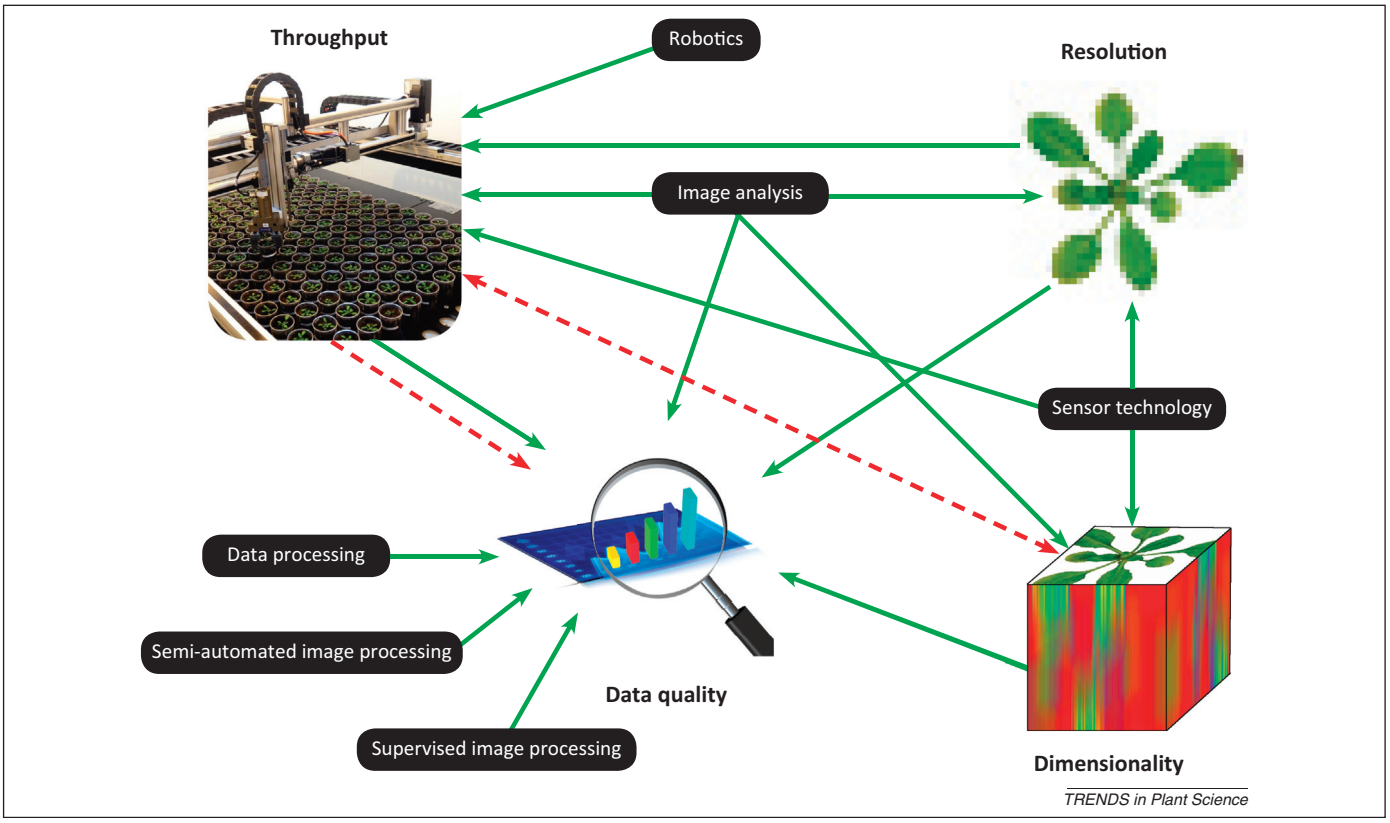

Figure 2. Throughput, resolution, and dimensionality are pivotal aspects of plant phenotyping systems with consequences for data quality. Advances in phenotyping systems are led by robotics, sensor technology, image analysis, and the way that data and image processing is handled. A dashed red arrow indicates a negative influence that could disappear in the future, and a solid green arrow shows a positive influence. The throughput in plant phenotyping systems is positively affected by the implementation of robotics, sensors that are able to acquire data more rapidly, automated image processing, and increased image resolution, enabling more plants to be monitored simultaneously. Throughput can have a negative effect on dimensionality in phenotyping systems when only a limited number of phenotypic traits is measured. This may change in the near future when novel sensor technologies are put into practice. However, throughput may be negatively influenced by increased dimensionality if plant processing is slowed down in phenotyping systems. Image resolution could be increased by the development of larger sensors. Image analysis may assist in increasing the effective resolution of, for example, thermal images, and dimensionality in general by extracting multiple traits. Likewise, the introduction of new sensors to the field of plant phenotyping positively affects dimensionality. Increased throughput can have a positive as well as a negative impact on data quality because, although it may improve the statistical power of the analysis, it often affects the quality of the individual measurement in a negative manner. This may be resolved by higher dimensionality, enhanced image resolution, improved image analysis, and human intervention. 'Human intervention' refers to the processing of the produced data, assistance in image analysis steps in the case of semi-automated image processing, and supervision of imaging results.

during growth in time-lapse sequences [26–31]. Because of the necessity of throughput in screening experiments aimed at retrieving the genetic basis of root system architecture, the growth of plants and their root systems in artificial, but accessible and standardized environments is still justifiable [32,33]. Some root image processing tools have been developed for specific growth environments, including transparent gel-based media in Petri dishes in the case of *Arabidopsis* root systems [26] or bigger containers for crop species [30,31]. Other software tools accept images of root systems grown under a variety of conditions, including hydroponic and aeroponic systems, and in paper pouches and soil [28,29]. Besides total root system length, several traits describing root system architecture can be derived from images, including individual root length and diameter, lateral root number, insertion angle and density, and dynamic changes in these traits. A non-exhaustive set of calculated traits has recently been added to further describe and compare the spatial distribution of roots [30,31]. Similarly, for a near-planar vegetative shoot axis, such as the *Arabidopsis* rosette, traits describing the roundness or compactness of the rosette have been calculated based on its dimensions (surface area, perimeter, and convex hull) [34,35]. These examples illustrate that automated image analysis often encourages the extraction of more data from the same sample. Segmentation is often the most crucial and difficult step in image processing, but once succeeded, the extraction of extra size, shape, and color features is in general more straightforward, particularly compared with additional manual measurements. Nevertheless, one should always carefully consider the biological relevance of the recorded measurements (Figure 2).

A final avenue for automation in plant phenotyping systems is the development of platform-associated data management pipelines, including dedicated and annotated databases, and standardized analyses of phenotypic trait data [15,36]. Furthermore, proper consideration of data management is necessary because of the acquisition of gigabytes of data on a daily basis in many robotized platforms, including images, phenotypic trait values, data generated by environmental sensors, and experimental metadata. Initiatives to stimulate the general adoption of data management into phenotyping systems have been taken [37,38], because this may advance developments in genotype–phenotype maps and plant models, and enable meta-analyses [36,38–40].

Lately, there has been much talk about the 'phenotyping bottleneck' in the high-throughput screening of genetic

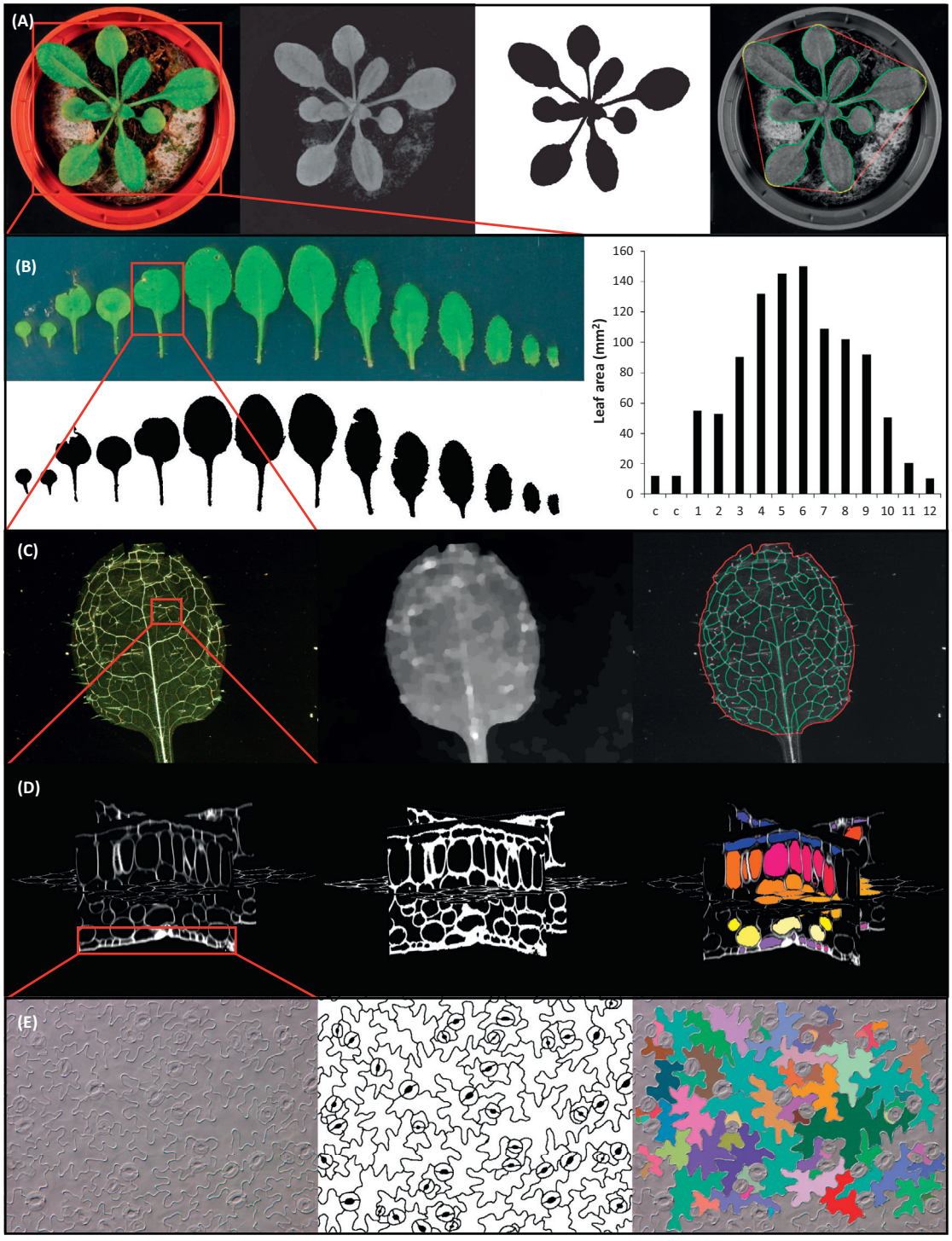

Figure 3. Plant phenotyping at multiple organizational levels. **(A)** Image color channel manipulation followed by thresholding is used for the extraction of the *Arabidopsis* rosette from the background, and the subsequent measurement of the projected rosette area, perimeter, and convex hull. **(B)** Leaf series are created by dissecting the rosette and arranging the individual leaves on an agar plate. Leaves are segmented by means of automated image processing and their individual sizes can be plotted in a leaf area profile. **(C)** Dark-field image of a cleared *Arabidopsis* leaf. The LIMANI tool extracts several venation parameters by consecutive segmentation of the leaf and the vascular pattern [62]. **(D)** Orthogonal view of a confocal image stack of an *Arabidopsis* leaf. Volumes of individual epidermal and mesophyll cells are obtained by 3D image processing [55]. **(E)** Differential interference contrast picture of the *Arabidopsis* abaxial epidermis. A microscopic drawing aids in the extraction of size and shape characteristics of segmented epidermal pavement cells [47].

resources for leads to improve crop yield [3]. However, phenotyping also constitutes a bottleneck in the sense that the wealth of the already available sequence information has not been adequately exploited. A significant proportion of *Arabidopsis* genes remains annotated with 'unknown function', and functional analyses often report 'no visible phenotype' [4]. Hence, besides the adoption of automated workflows to intensify throughput in phenotyping systems, there is a need for increased dimensionality or 'data richness' to better comprehend plant phenotypes and their

subtleties. Whole-plant growth traits constitute only a small portion of the matrix of structural, physiological, and performance-related traits (Figure 1). One way of improving dimensionality is to consider traits at multiple levels of organization (i.e., organs, tissues, and cells), at a higher spatial and temporal resolution, although this may still be at the expense of throughput (Figure 2).

Plant phenotyping at the organ and cellular level

Shoot and root system growth is the cumulative result of development in individual organs, leaves, and roots, which themselves integrate two fundamental processes, cell division and expansion. 'No visible phenotype' situations may occur when identical plant or leaf surface areas are obtained via different, compensating developmental pathways, for example, by means of coinciding variances in leaf or cell number and size, respectively. Final leaf size is generally highly correlated with cell number, but reductions in cell number may be made up for by enlarged cells [41]. Moreover, the duration of processes needs to be taken into account because an increase in cell number may be obtained by either higher cell division rates or an extended cell proliferation phase. Similarly, organ and cell expansion may occur at reduced rates, but for a longer time [42,43]. In addition, these growth processes are influenced by environmental conditions, either directly, for example, the negative effect of soil water deficit on the leaf elongation rate [2], or indirectly via effects on the plant developmental stage, such as the effect of daily accumulated light on floral transition, and its consequences for leaf number and individual leaf development [42]. Other phenotypic differences may even be more subtle in the sense that they may only become discernible under certain environmental conditions [44].

During the past decade, transcriptome, proteome, and metabolome profiling techniques have increasingly been applied to unravel the genetic basis of growth phenotypes. However, they often focus only on the molecular level and lack details on processes that occur at the cellular level, and at the organ and whole-plant level. To understand growth regulatory mechanisms fully, profiling experiments should be combined with integrated growth analyses so as to be able to link regulatory processes at the molecular level to plant phenotypes [45,46]. In well-characterized phenotypes, it is possible to target a specific developmental stage, such as the transition from cell division to expansion [47,48], whereas in other cases, the dynamic aspect of phenotypic traits may need to be determined before robust conclusions can be drawn [12,49]. Organ and cellular size measurements at single time points, such as a particular time after sowing, may be problematic in the comparison of genotypes if no attention is focused on the difference in timing of germination between genotypes. Likewise, genotypes may differ in the rate of leaf initiation, which has consequences for the interpretation of differences in leaf area. Overall, the temporal resolution required to perform dynamic analyses depends on the biological question, the relevant spatial resolution, and the phenotypic traits of interest. Organ growth rate may be studied by means of time-lapse sequences of one to several days, whereas intervals of minutes may be required if one is interested in diurnal growth patterns [50]. Cell division is a discrete event occurring at day intervals, whereas cell expansion can be measured in second(s) intervals at high spatial resolution [51–53].

Organ level phenotyping

Although it is already possible to obtain (semi-)automated organ size measurements from images of root systems, such as individual root length, images of plant shoots in general do not lend themselves to the measurement of individual leaf dimensions, because leaves may not be (completely) visible or they may overlap because of complex, non-planar arrangements. Moreover, in the earliest phase of development, leaves are either too small to be visualized by conventional imaging or they remain hidden within leaf sheaths until emergence. *Arabidopsis* leaf primordia have been visualized and measured at the shoot apical meristem by means of histological or optical sectioning followed by 3D reconstruction [54,55], and a stereomicroscope can be used to dissect the shoot and measure the smallest leaves directly [56]. Monocot leaf length and elongation rate are still measured manually or by means of a displacement transducer [9,57]. Dicot leaf surface area is usually determined destructively by physically dissecting shoots and arranging emerged leaves before imaging (Figure 3B). Most of the tools processing these images provide leaf surface area, height, width, and perimeter, whereas others have been specifically developed to express leaf shape and the extent of leaf serrations in a quantitative manner [15,58,59]. Besides size and shape, there is an interest in determining leaf vein patterning to investigate the relationship between growth and the establishment of the hydraulic structure of leaves; in this case, leaves are fixed, cleared, and imaged by means of dark-field microscopy (Figure 3C) [60–62]. In contrast to leaves, dynamic analyses of elongating roots to determine root growth rate, and the responses of roots to stimuli such as gravitropic bending, are non-destructive and have been automated in plants grown on transparent media in Petri dishes [63–66].

The existence of growth zones in both leaves and roots has motivated the development of image acquisition systems and software tools that provide spatially resolved data on local relative growth rates. Both particle-tracking and optical flow-based methods, quantifying the deformation of applied markers or local intensity features, respectively, have been applied to time-lapse sequences of root and shoot images. The temporal resolution in these systems ranges from several hours to minutes in organ level analyses, and to seconds at higher spatial resolution [53,67,68]. The technical difficulties in applying this type of analysis on the earliest stages of leaf development have now been overcome by the use of microscopic fluorescent markers and a fluorescence macroscope, enabling *in situ* time-lapse imaging of small leaves (<500 μm) and non-planar leaves, whereby growth is tracked in three spatial dimensions [69].

Leaves not only expand in surface area but also in thickness. Physiological processes such as gas exchange and photosynthesis take place in, and are determined by, the overall 3D anatomy of the leaf, including the epidermis, stomata, mesophyll, and veins. Leaf thickness and

tissue-specific traits have therefore been measured on histological and optical sections (Figure 3D) [55,70–72].

Cellular level phenotyping

Cellular level analyses in vegetative shoots have so far mainly focused on epidermal tissues because these are more accessible and can reasonably be considered as planar, which makes surface area a good quantitative measure of cell dimensions. Moreover, the epidermis is considered to be the major growth-driving tissue layer [73]. Epidermal peels, varnish imprints, fixation and clearing of leaf samples in combination with direct microscopic measurements, imaging or microscopic drawings are used to determine cell size and number [57,74,75]. Image analysis scripts have been developed to distinguish automatically between leaf epidermal cell types and to extract individual cell sizes starting from microscopic drawings (Figure 3E) [47]. The combination of these measurements with mathematical modeling has, for example, revealed distinct division and expansion patterns for pavement and guard cells [76], and functional relationships between cell and organ level phenotypic traits [75].

The delineation of meristematic, expansion, and maturation zones in monocot leaves and roots in general involves the determination of kinematic parameters at the organ level and number and size measurements at the cellular level [48,57,77]. Software tools have been developed for either automated root cell segmentation and measurement or as an aid in cell length measurement in confocal images [78–80]. Confocal imaging in *Arabidopsis* roots enables the non-destructive acquisition of cellular features at any chosen focal plane in the root, which means that the analysis is not limited to the epidermis. Moreover, if fluorescent protein expression is localized in structural features, such as the plasma membrane and nucleus, and appropriate plant culture conditions and imaging setups are chosen, cellular dynamics, including division and expansion, can be studied at high temporal resolution and in a spatially resolved manner [52,53]. Another advantage is that growth analyses can then easily be correlated with protein localization and gene expression dynamics [81,82]. This type of 'live imaging' has also provided novel insights in shoot meristem development [83] and intracellular processes associated with anisotropic cell expansion [84], but its use in roots of crop species and leaves in general is still limited because of tissue thickness and cellular content. Tissue fixation and rigorous clearing in combination with staining are required to access the mesophyll and vascular tissues in leaves by confocal or multiphoton microscopy [55,85]. Software tools have been developed to measure the volumes of cells in 3D reconstructions of leaf tissue, which then enable a dynamic analysis of cell expansion in both the epidermis and mesophyll (Figure 3D) [55,72].

Phenotyping at higher spatial and temporal resolutions requires more complex procedures, including extensive sample preparation and advanced microscopic acquisition, which decreases throughput at the sampling, measurement, imaging, or image-processing stage depending on the level of automation. Image processing is not a major bottleneck in organ and cellular level phenotyping. However, novel developments in imaging sensors and setups may increase throughput and concurrently dimensionality (Figure 2).

Increasing dimensionality for a better comprehension of the phenotype

Phenotypes are composed of structural, physiological, and performance-related traits, and their mutual interaction at multiple levels of organization. Increases in dimensionality to better comprehend the phenotype may thus be achieved by including physiological and, at the same time, structural traits that help to interpret physiological traits [86]. Technologies aimed at extracting the inherent 3D structure, development, and functioning of plants are described first, followed by novel sensors that have the potential to report on the physiological status of the plant.

Phenotyping in three spatial dimensions

The 3D characteristics of plant structure can be captured by various means; here, 3D rendering based on surface data is distinguished from volume rendering. Vegetative shoot-level traits of interest at the laboratory and field scale that can be extracted from surface renderings include canopy structure, plant height, leaf number, individual leaf length, position, and angle. Leaf position and angle are determined to either quantify hyponastic movement in response to light and circadian rhythms [87,88], or to estimate the capacity of canopies or individual leaves to capture incident radiation, which determines their heat content and contributes to the modeling of radiation absorption [86]. The acquisition of point cloud data for surface rendering, based on height or depth, relative to the ground or sensor, respectively, has been achieved by laser scanning in laboratory setups [88,89] and by a portable scanning lidar (light detection and ranging) instrument applied on scales ranging from forest and canopy structure down to individual plants [90,91]. Long acquisition times have motivated developments in 'depth imaging', whereby light is projected onto a scene and its reflection from the scene is used to build a depth image by measuring either the time of flight between emission and reception, or the deformation of a spatially structured light pattern [92–94]. Both approaches have great potential because of their simplicity and large community support in the development of processing tools. The drawbacks include low precision (cm-scale), disturbance by direct sunlight and small imaging sensors. Further development of these types of sensors will most likely be directed in the first instance towards larger sensors instead of higher precision (Figure 2). Nevertheless, in combination with visible imaging, depth imaging sensors may aid the 3D reconstruction of plants by indicating the coarse position of structural features such as leaves in 3D space [94]. Stereoscopic approaches have been used to calculate surface structure morphology from matching object patterns on images taken by multiple cameras or camera positions at slightly different angles. Height and total leaf area of single wheat (*Triticum aestivum*) seedlings [95], spatial orientation of individual leaves in canopy stands [96], and diurnal growth of isolated leaf discs [97] have been estimated by means of stereoscopic imaging. Finally, the full 3D reconstruction of plants, for example, by multiple view imaging, may provide

quantitative data on whole-plant surface area or root system architecture, and the surface area, length, position, and angle of individual elements, such as leaves or lateral roots [32,98]. A single 3D model may thus deliver measurements of both whole-plant and organ level structural traits.

High-resolution X-ray computed tomography (HRXCT), optical coherence microscopy (OCM), and optical projection tomography (OPT) are examples of volume rendering technologies that have been applied to record 3D morphological and anatomical traits of shoots, but with an overall trade-off between sample size, spatial resolution, and acquisition time [99–102]. *In vivo* imaging using OCM does provide a tool for monitoring developmental changes at the organ level during leaf growth [99], whereas OPT was introduced to capture 3D data of primarily *ex vivo* plant specimens. OPT enables the visualization of gene expression patterns from whole-mount RNA *in situ* hybridization or β-glucuronidase staining patterns [101]. HRXCT enables the rapid extraction of 3D morphological traits from *in vivo Arabidopsis* seedlings and the visualization of the cellular organization of *ex vivo* plant tissue samples at submicron resolution [102]. This same technology can be applied for in-soil phenotyping of root system architecture [103,104], which offers exciting opportunities to study root–soil interactions.

3D structural as well as physiological traits have been acquired non-invasively by positron emission tomography (PET) and magnetic resonance imaging (MRI). PET scanning is used to measure the distribution of products labeled with unstable isotopes, such as ^{11}C-labeled photoassimilates [105], and MRI is applied to map and quantify water flows in xylem and phloem vessels by the nuclear magnetic resonance of water protons [106]. An exciting development in this area is the downscaling of MRI to portable devices that are usable under greenhouse and field conditions [107].

Physiology-related trait measurement

Under field conditions, high-throughput refers to the screening of an extensive surface area, and, thus, potentially a broad array of plants of different genetic backgrounds and phenotyping at a phenomics level. However, field phenotyping is still of relatively low dimensionality because only a few traits are recorded at low spatial and temporal resolution, mainly because of laborious and time-consuming manual measurements. Nevertheless, phenomics is evolving from the investigation of mainly morphological traits (e.g., plant height, number of leaves, number of tillers), developmental stages (e.g., emergence, male and female flowering), and performance-related traits (e.g., seed number and weight) towards physiological traits, which are recorded by means of remote sensing to enable high-throughput acquisition and processing of data [3]. The motivation for this change lies in the understanding that traits in the different categories interact, examples of which can be found in drought avoidance and tolerance strategies [108,109]. Non-invasive sensors are brought to plants by various means at increased heights (e.g., moving platform, tower or crane, unmanned aerial vehicle, or airplane), but with decreasing spatial and temporal resolution. Some of these techniques have been or are being adapted for greenhouse and laboratory-level screening, where they can be used at higher spatiotemporal resolution. The goal is to remotely quantify traits such as disease incidence, water and metabolite content, evapotranspiration, and photosynthesis [3,108,110–113]. The most promising systems include thermal infrared, multispectral, and hyperspectral imaging, providing spatially resolved data in high-throughput systems, as opposed to point measurements obtained by instruments such as thermal infrared spectrometers, porometers, portable photosynthesis systems, and gas analyzers.

Infrared thermography is the most established technique and has been used historically to determine crop water stress for irrigation scheduling [114]. More recently, thermography has been used to compare evapotranspiration between genotypes at the canopy and plant level, and for the comparison and measurement of stomatal conductance in individual plants and leaves [108,111,115–117]. In both applications, the dynamic diurnal aspects of plant water use behavior need to be taken into account. In field phenotyping, images may be acquired at daily intervals, at a specific time of the day, at crucial developmental stages, or when the environment imposes significant constraints. At the laboratory scale, it is more likely that temporal resolution can be increased to minute(s) and hourly intervals for part of or the complete photoperiod to enable the estimation and comparison of stomatal conductance in the context of its dynamic behavior.

Multispectral imaging involves the simultaneous collection of narrow-band plant reflectance at selected wavelengths, whereas hyperspectral imaging covers a spectral region in the visible and near infrared (Vis-NIR) and the short wave infrared (SWIR). A significant number of existing spectral indices developed in the context of remote sensing (satellite images) are now being tested at the field and canopy level, whereas to a lesser extent novel characteristic spectra are searched for under laboratory conditions [113,118]. Indices relate to biomass, canopy structure, pigment activity, light-use efficiency, and water content [119].

Whereas thermal and spectral imaging sensors passively acquire radiation and reflectance data, fluorescence is actively recorded at specific wavelengths after induction by laser or light-emitting diode (LED) light and related to photosynthesis parameters, such as the dark-adapted F_v/F_m, which is a measure of maximum quantum efficiency of photosystem II photochemistry [120]. Its use in plant phenotyping is controversial because of its relative insensitivity to certain types of stress, such as drought and osmotic stress, and because of the required imaging conditions (i.e., homogeneously distributed high-intensity light pulses, which are difficult to obtain at the whole-plant and canopy level) [3,121]. In laboratory cultures, where individual reasonably sized plants and seedlings can be positioned in close proximity to this type of light source, chlorophyll fluorescence imaging has been applied with success in a high-throughput phenotyping system [34].

Overall, extensive 'ground truthing' is required to test and validate the correlation of remotely obtained data with data originating from established point sensors or destructive sampling procedures. Another option would be to

include well-characterized or designed phenotypes, or known effects of specific environmental conditions. Temporal resolution, as for thermal imaging, also needs to be considered for hyperspectral and fluorescence imaging because observed parameters may display a dynamic behavior. Moreover, spatial resolution needs to be in line with the biological question under investigation and the required throughput (Figure 2). Under field conditions, the most prevalent imaging mode aims to include as many plants as possible to enable a direct comparison of canopy features between genotypes under identical environmental conditions. The sensor is then positioned at a considerable height and many mixed pixels are obtained because of the presence in the image of multiple plants, together with background features such as soil and sky. Moreover, canopy structure may significantly affect physiological trait measurement because of the instant effect of solar radiation on the heat content and light-use efficiency of the leaves. When thermal imaging is used at the single plant level and even more so at the individual leaf level, it is crucial to take into account the difference in heat content because of leaf orientation towards the incident radiation [108]. A solution has been offered by a combination of thermal and visible imaging of canopies, whereby the canopies are segmented into shaded and sunlit leaves [122]. This illustrates the positive effect of image processing on resolution and subsequent data quality (Figure 2). Furthermore, environmental conditions such as air temperature, vapor pressure deficit, wind speed, and light intensity have been shown to affect leaf temperature [123]. When performing these types of remote sensing, image processing and even more so the interpretation of the resulting data are challenging, but at the same time promising because of the large potential for multidimensional data acquisition in high-throughput systems. Developments towards larger sensors, in analogy with visible imaging, may aid in increasing spatial resolution, improving the performance of image analysis, and improving data quality (Figure 2).

Perspectives on throughput, resolution, dimensionality, and quality

Robotic hardware and automation in workflows have boosted plant phenotyping, particularly at the whole-plant level (Figure 2). The adoption of remote sensing in its historical sense (i.e., image acquisition by satellites and analysis based on specific wavelength-derived indices) to field, greenhouse and laboratory scales is likely to deliver a significant amount of data to be published in the coming years. Moreover, dimensionality should be boosted because plant physiology-related traits will be included. However, important progress can still be made in the 3D recording and reconstruction of shoots, and the dynamic, quantitative analysis of structural traits at the shoot as well as the individual leaf level. Quantitative 3D analysis is important because throughput could be improved for leaf-level traits, as simple as length and area, or more complex such as leaf position and angle, which to date have been measured manually, destructively or not at all. At the same time, shoot and leaf traits would be determined simultaneously, thereby refining comprehension of the phenotype. At the cellular level, improvements could be situated at the level of imaging by developments in microscopic systems driven by specific research problems or questions. Indeed, high-throughput microscopic imaging is finding its way into plant research [124,125].

On the downside, high-throughput phenotyping systems and automated workflows may increase the risk of data quality deterioration if appropriate checkpoints are not implemented at various stages, such as imaging and image processing (Figure 2). Supervised and semi-automated image processing tools tend to unjustly have a negative connotation compared with fully automated tools and batch jobs because some form of human intervention is required. However, supervised image processing offers the possibility to review and correct errors and imperfections in automatically obtained data (e.g., [62,80]), and in the case of semi-automated tools, the user assists in image processing steps that cannot (yet) be fulfilled at an acceptable level by computer vision (e.g., [28,55]).

Remaining issues: the 'bigger picture'

The *in situ* characterization of root system architecture and root growth dynamics remains a hard-to-overcome hurdle in the assessment of genomic effects on whole-plant fitness for survival and performance in demanding or fluctuating conditions in the surrounding soil and atmosphere. Adding to this, comprehensive whole-plant phenotyping, including both shoots and roots, is only occasionally practiced, and the influence of shoot–root interactions on structural and physiological traits on both levels is hardly ever considered to its full extent.

Because of the definition of phenotypes, the simple extrapolation of valuable traits from *in vitro* to soil conditions, or from growth chamber and greenhouse environments to fields is not expected. Either exhaustive comparative data are required to elucidate the 'predictive powers' or robustness of traits and their genetic background obtained in particular phenotyping systems, or modesty about their extent should remain in place given that traits assume values relevant to the conditions in which they are determined. This further points to the importance of data quality and annotation, and more specifically to the quantity or specificity of recorded and reported experimental metadata.

Dimensionality as well as raw data quality may be improved by novel developments in imaging, across the spectrum, and in sensors suitable for high-precision, spatially and temporally resolved measurements of structural and physiological traits such as leaf thickness and elongation rate, transpiration rate, internal carbon dioxide concentrations, and incident light at the leaf level. Progress in these areas will require the active involvement of applied physicists. Moreover, improved dimensionality will increase the call for modeling approaches to manage and interpret the extensive array and diversity of gathered data [39].

Finally, computer scientists and biologists, separately or in joint efforts, have created a large number of software tools for the automated or assisted processing of raw image data, aimed at extracting an increasingly large number of phenotypic traits. These are often specific with regard to

the requisites imposed on image input data and to the collected phenotypic traits. Here, significant progress may also be achieved by an improved interaction between disciplines, or, alternatively, the translation of algorithms on the one hand, and specific plant phenotyping needs on the other hand, into a format readily understandable by biologists and computer scientists or mathematicians, respectively [126]. A community effort towards the sharing and re-use of plant phenotyping software tools or algorithms, similar to ImageJ [127], R (http://www.R-project.org/), and the 'Internet Analysis Tools Registry' [128] is needed to improve tool design and stimulate their use in general. The first modest step in this direction has been taken (http://www.root-image-analysis.org/) and now awaits upscaling to the level of ImageJ, R, and the 'Internet Analysis Tools Registry' and similar initiatives such as OpenAlea [129] and iPlant [40].

Overall, as long as preserving data quality is kept high on the agenda and it can be shown with sound data that seeing is believing (i.e., image processing is delivering), plant phenotyping should continue to improve.

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