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Applying high-throughput phenotyping to plant–insect interactions: picturing more resistant crops

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Through automated image collection and analysis, high-throughput phenotyping (HTP) systems non-destructively quantify a diversity of traits in large plant populations. Some platforms collect data in greenhouses or growth chambers while others are field-based. Platforms also vary in the number and type of sensors, including visible, fluorescence, infrared, hyperspectral, and three-dimensional cameras that can detect traits within and beyond the visible spectrum. These systems could be applied to quantify the impact of herbivores on plant health, to monitor herbivores in choice or no-choice bioassays, or to estimate plant properties such as defensive allelochemicals. By increasing the throughput, precision, and dimensionality of these measures, HTP has the potential to revolutionize the field of plant–insect interactions, including breeding programs for resistance and tolerance.

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Measuring plant defenses against insects

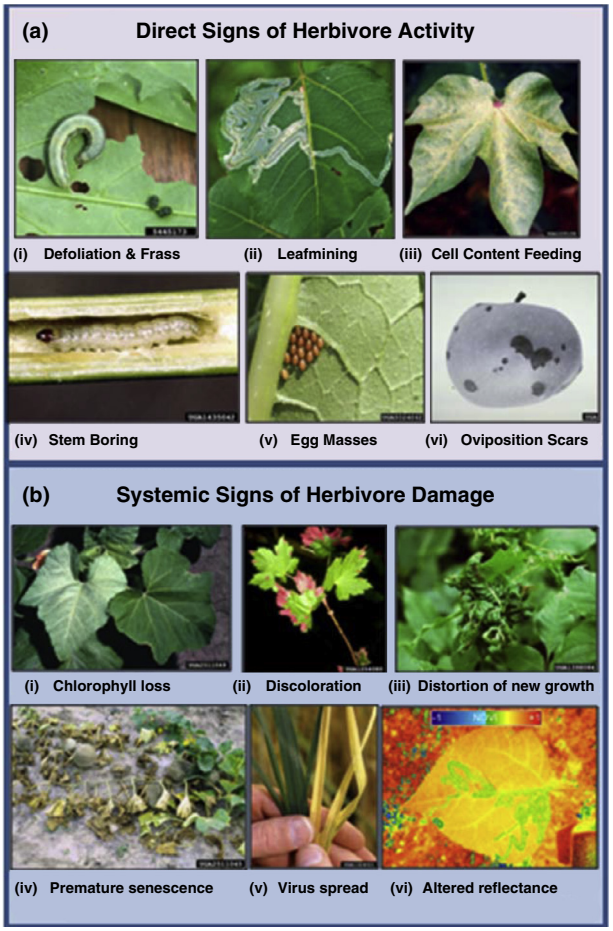
The importance of phenotyping

Plant phenotyping plays a critical role in developing crop varieties with enhanced insect resistance or tolerance, which is among the most effective, economical, and environmentally safe approaches to pest management. Host plant resistance to insects encompasses plant traits that suppress insect infestations; this includes antixenotic traits that repel or deter herbivory as well as antibiotic traits that reduce insect survival, reproduction, and/or development. Tolerance on the other hand does not directly impact insect population growth or feeding rates, but instead modifies plant responses to infestation, thereby limiting symptom development and yield [1]. Breeding for resistance or tolerance requires quantifying these traits in heterogeneous plant populations (i.e. phenotyping) and genotyping the plants to identify the traits' genetic bases. Genetic engineering for insect resistance also requires phenotyping to determine which transgenic lines display the strongest resistance and lack any negative impacts of the transformation process on growth and development [2]. In either case, plants can be phenotyped for defensive traits by measuring insect populations or their effects on plants. Indicators of infestation on plants include direct evidence of insect activity such as feeding scars, and also more systemic, indirect consequences such as chlorophyll loss (Figure 1). Since resistance and tolerance both limit the negative impacts of insects on plants but differ in their influence on insect populations, screening plant collections on the basis of systemic symptoms could represent a useful way to detect both resistance and tolerance in the same assay. However, for studies that are designed to identify the genetic loci responsible for plant defense, assays that measure highly specific components of defense may be more appropriate. Symptoms such as chlorosis or reduced yields are the net outcome of a multi-step infestation process that is influenced by many different plant traits (Box 1), each of which can have a different genetic basis. Lumping these traits together into a single parameter such as yield may result in a high proportion of missing heritability in association mapping studies [3]. Therefore, the detailed measurements of insect and plant performance that are used to discriminate antixenosis, antibiosis, and tolerance (Box 1) can also play a critical role in genome-wide association studies and other genetic analyses of plant defense.

The phenotyping bottleneck

As genome sequencing and molecular breeding techniques have dramatically increased the speed at which large populations can be genotyped, phenotyping has in many cases become the rate-limiting step in breeding efforts [4–6]. Whether sampling insect infestations in the field or assaying plant–insect interactions in controlled environments, screening for variation in host plant defenses is a slow and labor-intensive process. Furthermore, assays to measure defoliation, chlorosis, or other markers of insect damage in plants often rely on visual estimation of the extent of the damage, and apply categorical rankings to parameters that show continuous variation. These visual rating systems do not allow

Figure 1



Signs of Insect Infestation. The direct effects of herbivore activity on their hosts **(a)** include feeding damage, which varies among different types of arthropods (a-i–a-iv), frass production (a-i), and oviposition (a-v–a-vi). More systemic physiological consequences of infestation **(b)** can include altered pigmentation (b-i–b-ii), malformation of new growth (b-iii), and premature senescence (b-iv). Insect can also transmit phytopathogens (b-v), and alter the spectral properties of plants within and beyond the visible spectrum (b-vi). Panel bvi presents the normalized difference vegetation index (NDVI) of a leaf with leafminer damage; NDVI is a graphical indicator of vegetation greenness that is calculated by comparing reflectance in the visible (red) and near-infrared regions. Photos of insect damage were kindly provided by Whitney Cranshaw, Colorado State University (a-i and a-v), Steven Katovich, USDA Forest Service (a-ii and b-iii), the Clemson University USDA Cooperative Extension Slide Series (a-iii and a-iv), the New York State Agricultural Experiment Station (a-vi), David Riley, the University of Georgia (b-i and b-iv), William M Ciesla, Forest Health Management International (b-ii), and Keith Weller, USDA Agricultural Research Service (b-v) (all from Bugwood.org). The estimate of NDVI based on an infrablue photograph (b-vi) is courtesy of Chris Fastie, Middlebury College, Vermont (Creative Commons).

precise quantification, and they can introduce subjectivity and inconsistency into the scoring process, all of which hinders efforts to identify the genetic bases of complex traits [7].

The selection of measures to quantify host plant defense

To overcome the phenotyping bottleneck in breeding for pest management, it is necessary to identify traits that are good measures of resistance or tolerance but that can be quantified quickly, consistently, and objectively. Some assays for resistance are based on monitoring insects; for example, Kloth and coworkers [3] propose to use automated video tracking of the green peach aphid (*Myzus persicae*) on leaf discs to screen hundreds of ecotypes of the model plant *Arabidopsis thaliana* for variation in levels of antixenosis and antibiosis. Alternatively, bioassays may measure the consequences of insect infestation for the plant. In sorghum, a rapid method for screening large numbers of accessions for greenbug (*Schizaphis graminum*) tolerance has been developed using a hand-held spectrophotometer (SPAD meter) to measure chlorophyll loss in intact, infested leaves [8]. In Arabidopsis, virus transmission by aphids has also been used as an indirect measure to screen for genetic variation in plant defenses against the green peach aphid. A collection of mutagenized Arabidopsis lines were exposed to viruliferous aphids and then the plants were screened for virus infection using a high-throughput antibody-based assay (ELISA) that is less labor-intensive than measuring aphid population levels. Plants that were negative for the virus were identified as candidates for aphid resistance, and this resistance was consequently validated with subsequent measurements of aphid infestation levels [9]. Lastly, another alternative to monitoring insects or their impacts on plants is to screen for quantitative differences in plant defenses or in traits that co-vary with these defenses. Assays to measure many plant metabolites are increasing in throughput and decreasing in cost, making it possible to screen large mapping populations for allelochemicals that contribute to insect resistance [10,11]. Moreover, even when the source of insect resistance has not yet been pinpointed or cannot be quantified in a high-throughput manner, it may be possible to identify spectral characteristics of the plant that co-vary with the source of resistance. For example, near-infrared (NIR) reflectance spectroscopy has been used as a high-throughput screening tool to select sugarcane cultivars with resistance to a stem borer, *Eldana saccharina* [12], because insect resistance in sugarcane is correlated with secondary metabolites that increase the plant's light absorbance in the NIR range [13]. This example highlights the utility of imaging technologies in plant phenotyping. In fact, although many lab-based assays such as metabolite profiling and ELISAs are widely used in plant phenotyping, image analysis is currently front and center in the emerging field of plant high-throughput phenotyping.

High-throughput phenotyping

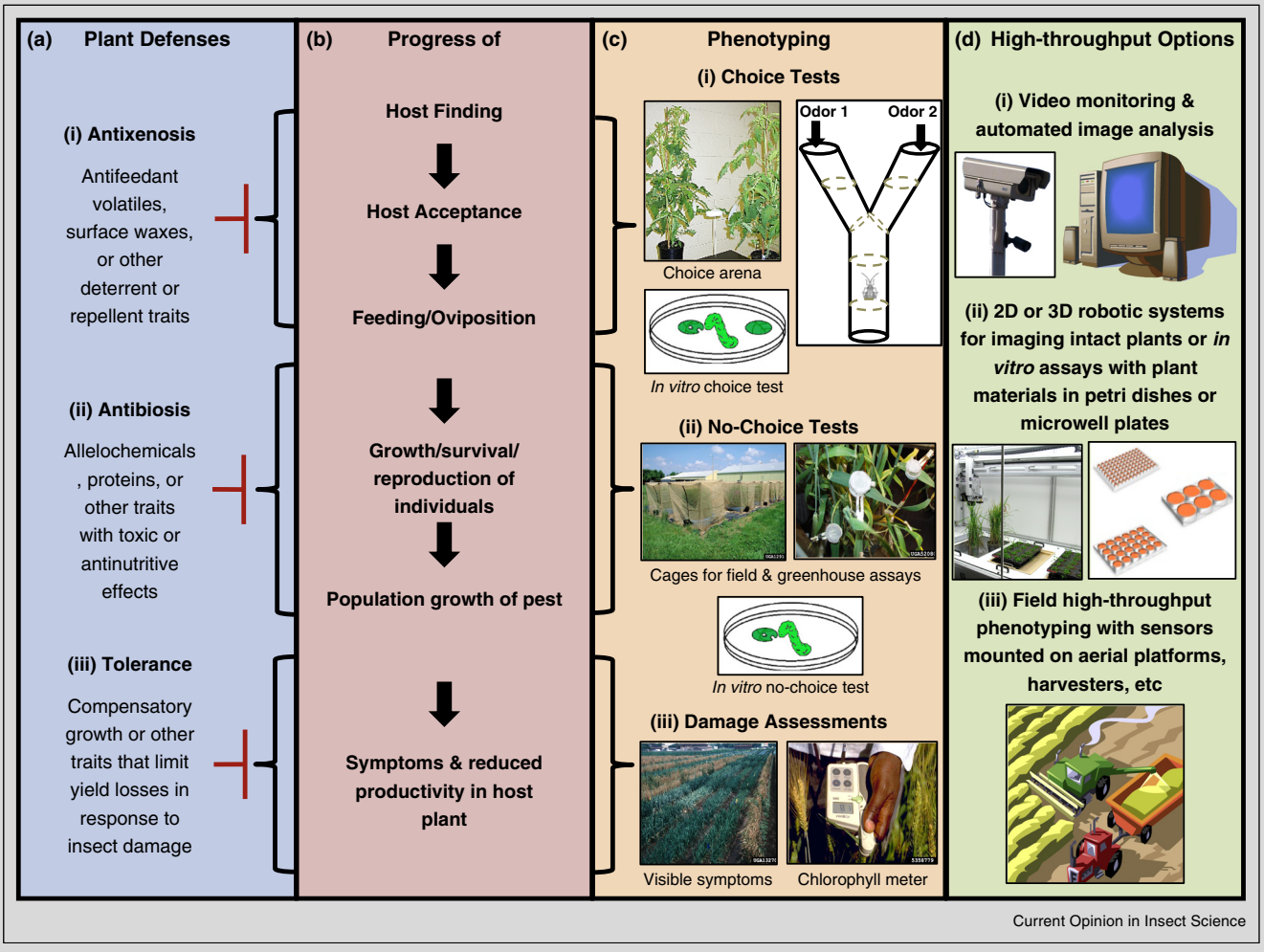
High-throughput phenotyping defined

There is presently a major emphasis in the plant biology community to develop better methods for high-throughput phenotyping (HTP). By definition, HTP utilizes

Box 1 Phenotyping host plant resistance and tolerance

Insect damage is the net result of a multistep infestation process, and of the plant's response to infestation. Plant defenses are classified as antixenosis, antibiosis, or tolerance based on the stage at which they intercept the progress of damage **(a)**. In order to discriminate among these three forms of plant defense, investigators must observe multiple steps in the infestation process **(b)** using more than one type of bioassay **(c)**. To detect *antixenosis*, insect behavior is monitored in choice tests (c-i) in response to intact plants, detached plant parts such as leaf discs, or plant-derived cues such as volatiles presented through an olfactometer. The behaviors most commonly tracked in these assays include directed flight, walking, sampling, feeding, and oviposition. Alternatively, for insects that leave quantifiable signs of feeding or oviposition on their hosts, the incidence or magnitude of this damage can be measured after the fact in lieu of tracking behavior. To detect *antibiosis*, investigators measure the growth, survival, and reproduction of individuals or populations in caged no-choice tests in the field, greenhouse, or laboratory (c-ii). *In vitro* assays that chart insect growth and development relative to food intake and excretion can be particularly useful in characterizing antibiotic effects. Lastly, measuring *tolerance* requires measuring the impact of the insect on plant health or productivity (c-iii) in addition to quantifying insect populations so that the relationship between insect pressure and insect damage can be compared among different plant genotypes. Plant productivity is most readily quantified under field conditions, but in some cases tolerance can be measured in greenhouse or laboratory assays, particularly if early indicators of damage such as chlorophyll loss can be used as predictors of potential yield losses. HTP approaches to automate data collection and analysis (d-i through d-iii) have the potential to increase the throughput, sensitivity, and accuracy of all of these assay types. Photos kindly provided by David Voegtlin, Illinois Natural History Survey (c-ii, left), Juan Manuel Alvarez, University of Idaho (c-ii, right), Alton N Sparks Jr., University of Georgia (c-iii, left), and JS Quick, Colorado State University (c-iii, right) (all from Bugwood.org).

Figure



methods of describing a plants' phenotype that are designed to speed up the phenotyping process and maximize the number of plants that can be processed per experiment. This emphasis on throughput is driven by the fact that the probability of detecting valuable traits and identifying their genetic basis increases with the size of the population that is screened. In fact, mapping populations and diversity panels of thousands of recombinant inbred lines have recently been developed for molecular breeding [14]. To facilitate the rapid screening

of large numbers of plants, HTP typically involves mechanization of data collection and automation of data analysis. Another characteristic of HTP protocols is that they usually utilize non-destructive sampling methods. This allows investigators to collect seed after phenotyping; also, it allows the same plants to be sampled over time to track their development and measure responses to changing variables such as insect infestation. Lastly, many (but not all) HTP approaches are designed to maximize the 'dimensionality' of the data, or the number of different plant characteristics that can be measured at one time. Enhanced dimensionality has several benefits. From an applied standpoint, it allows scientists to: firstly, screen for many known traits of interest at one time; secondly, analyze the interactions among traits; and finally, utilize *post hoc* analyses to identify variables that correlate well with desirable traits like insect resistance, and that could have predictive value in future studies. From the perspective of basic science, HTP methods with high dimensionality are also critical to the emerging field of plant phenomics, which aims to help decode the relationship between genotype and phenotype by describing all aspects of a plant's structure and function (i.e. its 'phenome') over its lifetime [4,6].

The role of imaging in HTP

Because of the need for automation and non-destructive sampling in HTP, a majority of HTP platforms rely on imaging to capture plant phenotypes. Moreover, because of the emphasis on high dimensionality, many HTP systems use multiple modalities for image capture in order to increase the number and diversity of phenotypic traits that are recorded [15,16]. Visible (a.k.a. RGB) cameras are used to acquire high-resolution images that allow the characterization of plant pigments and the quantification of plant size, architecture, chlorosis, and necrosis. Fluorescence cameras most commonly measure chlorophyll fluorescence, which is in turn used to assess the functioning of the photosynthetic machinery [17]. Multicolor fluorescence imaging can also compare levels of chlorophyll fluorescence with fluorescence from other compounds such as cinnamic acids in the cell walls. The ratios of these different sources of fluorescence can be used to estimate chloroplast abundance and detect plant stress responses [18]. In addition, near-infrared and far-infrared sensors are used to estimate water content, leaf temperature, and stomatal conductance, and laser scanners are used for 3D mapping of plants and observations of leaf movement [19]. Certain HTP systems also include hyperspectral cameras, which, along with multispectral imaging and near-infrared reflectance spectroscopy (NIRS), are widely used in the field of remote sensing. These remote sensing techniques characterize plants' reflectance in a broad range of bands within and beyond the visible spectrum and then compare this spectral data to other measures of plant health or productivity to identify unique spectral signatures that are predictive of important plant traits [14,19]. Hyperspectral imaging or NIRS could also be applied in HTP to screen for numerous traits such as biotic and abiotic stresses, nutrient status, or protein content. In addition to the laser scanners, RGB, fluorescent, infrared, and hyperspectral cameras that are available on commercial HTP systems, custom-built HTP platforms are experimenting with a wide range of other modalities, such as time-of-flight cameras [19] and light curtain arrays [20]. Together, all of the imaging modalities used in HTP enable plant scientists to detect important phenotypes that are not always visible to the naked eye, and to measure both visible and cryptic plant traits in quantitative units rather than in purely comparative terms subject to human biases. Moreover, these sensors can capture multiple traits within a single image, and images can be stored and reanalyzed when new research questions or new improvements in image processing and analysis arise.

Platforms for HTP data capture

During the last decade, multiple automated or semi-automated image-capture systems for plant HTP have been developed in both the academic and private sectors [21–27], as reviewed in [19,28]. While RGB and fluorescence cameras are the most common features of these systems, HTP platforms can include varying numbers of additional modalities such as laser scanners, near-infrared or far-infrared sensors, and hyperspectral cameras (described above). Automated HTP platforms also vary in the portion(s) of the plant that are phenotyped, the spatial scale and resolution at which plant traits are measured, and the degree to which growth and imaging conditions can be controlled. In general, most HTP systems are designed to phenotype the above-ground portions of intact plants. However, several platforms for phenotyping roots have also been developed using growth conditions that allow the roots to be examined [29–32], tomographic techniques that can penetrate soil [33,34], or destructive sampling of root systems (i.e. 'shovelomics') [35•]. In addition, certain HTP systems can phenotype seeds or other detached plant parts in multiwell plates. To optimize the spatial resolution of the images and to maximize the investigators' ability to control experimental conditions, most HTP systems visualize plants at close range in environmentally controlled imaging chambers, to which the plants are manually or mechanically transported from adjacent growth chambers or greenhouses. Some of these systems generate two-dimensional (2D) images taken from a single perspective, while others photograph plants from more than one angle or use scanners to generate three-dimensional (3D) data on plant architecture. A limitation of these systems, however, is that controlled environments cannot fully replicate field conditions, and the results of phenotyping in greenhouses or growth chambers are not always predictive of plant performance in real-world settings [14,36,37•]. Therefore, field-based imaging HTP systems are also emerging, and can utilize

sensors mounted on harvesters or other ground-based 'phenomobiles', stationary 'phenotowers', small airplanes, blimps, or unmanned aerial vehicles [14,19]. Investigators have far less control over imaging conditions or plant growth conditions in field experiments; however, outdoor managed environment facilities for plant phenotyping can be designed to control for variation in fixed factors like soil type, and to monitor continuously for changing variables like weather [38•]. Moreover, water conditions can be controlled in the field using a combination of irrigation and coverings that exclude rainfall (so-called drought simulators or rainout shelters). These approaches enable at least some sources of environmental variation to be incorporated into the design and analysis of field phenotyping experiments.

Adapting HTP to the study of plant defenses against insects: opportunities and obstacles

Applications of HTP to measure insect damage

Given that HTP systems are designed to measure plant health and productivity, they have obvious applications to measure feeding damage caused by herbivorous insects. Defoliation by caterpillars, chlorosis and necrosis caused by aphid infestation, and feeding scars caused by thrips have all been quantified digitally using images captured with RGB cameras or flat bed scanners [39–41]. HTP platforms could be used to automate and standardize both the capture and analysis of these RGB images. Furthermore, the additional modalities available on many HTP systems could detect symptoms of infestation that are not visible to the naked eye. Physiological studies indicate that insect infestations can influence stomatal conductance and plant water balance [42,43], which could be measured with near-infrared and far-infrared cameras. Herbivores also alter photosynthetic efficiency, chlorophyll content, and the relative abundance of other fluorescent compounds, all of which could be detected with fluorescence cameras [18,42–44]. For example, multicolor fluorescence imaging can be used to detect mite infestations on plants because mites cause a strong increase in the ratio of blue (F440) to red (F690) autofluorescence [18]. Similarly, HTP systems with hyperspectral cameras could be used to visualize changes in plant reflectance that result from arthropod infestation. Multispectral and hyperspectral imaging allow remote sensing of numerous insect pests, such as aphids [45]. If these diagnostic spectral signatures can be correlated with the intensity of pest damage, they could be used to quantify symptom development and possibly even estimate pest abundance in phenotyping studies; moreover, changes in plants' spectral properties could potentially be used to detect cryptic herbivores such as stem borers that hide within plant tissues (Figure 1a-iv). Root phenotyping systems could also be deployed to study plant interactions with major root pests such as corn rootworms (*Diabrotica* spp.) or grape phylloxera (*Daktulosphaira vitifoliae*).

Other applications of HTP

Besides quantifying the symptoms of insect infestation, high-throughput imaging could also be applied to compare base-line performance of different plant genotypes in the absence of herbivory, monitor pathogen transmission by insects, visualize plant defenses, and quantify herbivore behavior and performance. Many studies of plant defenses utilize mutant plants to assess the effects of specific plant genes on infestation levels. However, if the mutations have pleiotropic effects, any observed differences in insect infestations might be mediated by differences in overall health or development rather than by differences in plant defenses. HTP is therefore an invaluable tool to confirm that the mutants used to study plant–insect interactions have equivalent growth and development with wildtype controls [46]. HTP can also be used to monitor insect transmission of plant viruses, since many plant pathogens induce diagnostic visible symptoms or changes in fluorescence in their host plants [47•]. This approach could expedite attempts to identify the factors in insects and pathogens that control pathogen acquisition and transmission [48]; it could also facilitate high-throughput screens for insect resistance that use virus transmission as an indirect measure of insect feeding [9]. In addition, HTP systems can reduce the need for time-consuming bioassays by screening for spectral traits that co-vary with plant defenses, similar to the way in which NIR reflectance spectroscopy has been exploited to select for insect-resistant sugarcane varieties [12]. High-throughput imaging could also be applied to decrease labor and processing time for measurements of insect behavior, survival and development — bioassays that are critical to the characterization of host plant resistance (Box 1). Certain 2D HTP platforms can image detached plant materials in multi-well plates or petri dishes, and can be programmed to scan the same samples repeatedly at specified time points. These systems could be used to quantify insects' positions, food consumption, and growth in choice and no-choice bioassays if the resolution of the imaging system is adequate for the size of the insect and if the assay arena allows the insect to be visible at all times. More refined behavioral assays could also be achieved by incorporating video cameras into a high-throughput platform, similar to Kloth and coworkers' video analyses of aphid feeding on leaf discs [49]. Ethovision and other video analysis software help investigators automate the time-consuming process of identifying behaviors and quantifying their durations and frequency [50]. The field of host-plant interactions could also exploit new tools for high-throughput behavioral monitoring that ethologists have developed for the fruit fly *Drosophila melanogaster* [51,52•,53]. Together, these high-throughput approaches would allow scientists to screen much larger plant populations for insect resistance, and also to characterize resistance with more precision, depth, and comprehensiveness than has ever been possible before.

Obstacles to adoption of HTP

Although HTP has the potential to revolutionize the field of plant–insect interactions, there are several significant obstacles that must be overcome before it can be widely adopted in this field. First of all, despite the widespread adoption of HTP in industry, due to high equipment costs there are few public sector HTP facilities in the US (e.g. the Phenotyping Facility at Arkansas State University [http://www.astate.edu/a/abi/about] and the Bellwether Foundation Phenotyping Facility in St. Louis, MO [http://www.danforthcenter.org/scientists-research/core-technologies/phenotyping]) or worldwide (e.g. the Australian Plant Phenomics Facility in Canberra [http://www.plantphenomics.org.au/], the National Plant Phenomics Center in the United Kingdom [http://www.plant-phenomics.ac.uk/en/], and the Laboratory of Plant Ecophysiological responses to Environmental Stresses in France [http://www1.montpellier.inra.fr/ibip/lepse/english/]). Moreover, controlled environment HTP facilities typically have high fees, long waiting lists, and restrictions against bringing insects into their facilities. HTP systems also generate large data sets that pose unique challenges for processing, analysis, and storage. Data processing methods vary among different imaging modalities, but typically require steps to: normalize variation in background levels within and among images; distinguish the sample of interest from the background (i.e. segmentation); extrapolate the 3D structure of the sample from a 2D image; and identify and measure features of interest (e.g. plant organs, morphological features, etc.) [15]. Commercial 2D and 3D HTP systems utilize expensive proprietary softwares to perform these functions, and so laboratories that outsource image collection to centralized HTP facilities typically also rely on these facilities for data processing. The high-dimensionality of HTP data also requires multivariate and function-valued statistical methods in order to identify the key sources of genetic and environmental variance that shape the observed phenotypes [54–56]. Lastly, unlike genomics or transcriptomics, the field of phenomics does not yet have well-established community standards for the design, analysis, and reporting of experiments, nor are there centralized data repositories to make large HTP data sets available for data mining and meta-analysis [57].

Potential solutions

Fortunately, there are solutions to these problems on the horizon. Public research networks and consortia such as the International Plant Phenotyping Network (IPPN: http://www.plant-phenotyping.org/), the European Plant Phenotyping Network (EPPN, www.plant-phenotyping-network.eu/eppn/structure) and the Plant Imaging Consortium (PIC, http://plantimaging.cast.uark.edu/index.php/home) are a promising mechanism to offer training in HTP, broaden access to shared-usage HTP facilities, develop data repositories, and promote community standards for the design, analysis, and reporting of large HTP data sets. Several recent reports also suggest that HTP systems can be built at a significantly lower cost than the leading commercial systems [58–60]. Moreover, the 'Maker' movement is ushering in a revolution in do-it-yourself science and engineering projects (maker.danforthcenter.org). Fueled by cheap microprocessors (Rasberry Pis and Arduinos) and affordable 3D printers, phenotyping tools can be custom-built on a modest budget and deployed in field plots or controlled environment facilities that allow insects. Since most insect bioassays requires cages or other containment that can obstruct visibility (Box 1), the ability to develop customized sensors and cages that are compatible with each other will be critical. In addition, resources for automated image analysis are rapidly proliferating. Multiple academic groups have recently developed free image processing and analysis pipelines for 2D and 3D commercial HTP systems [27,61], and published the most critical factors that need to be optimized for proper processing and analysis of HTP data [37•]. The Plant Image Analysis website (http://www.plant-image-analysis.org/) matches users' needs to a list of over 120 free plant image analysis tools [62•], and the iPlant Bisque Image Analysis Environment provides an online platform to use existing software or to integrate new customized tools (http://www.iplantcollaborative.org/ci/bisque-image-analysis-environment). Public domain, open architecture image analysis programs like ImageJ (http://imagej.nih.gov/) and open-source libraries of algorithms for automated image analysis such as Point Cloud Library (pointclouds.org) and OpenCV (opencv.org) also enable maximum customization for each application. Furthermore, even for investigators who do not have access to automated HTP systems for data acquisition, these image analysis tools can be applied to images taken with commodity webcams, video cameras, cell phones, or even gaming consoles [63•]. For example, Green and coworkers [7] developed an open-source software for plant image analysis that can be applied to any image source as long as it contains a reference color chart that is photographed with the plants. They demonstrated that this web-based software could be used to quantify the leaf areas consumed by cabbageworm larvae (*Pieris rapae*) on Arabidopsis plants or beet armyworm larvae (*Spodoptera exigua*) on detached soybean leaves; moreover, they found that automated image analysis was more accurate than manual damage rankings at estimating very high or very low amounts of damage.

Conclusions

HTP vastly increases the population sizes that can be screened for desirable traits like plant defenses, and the precision and accuracy with which these traits can be measured. By quantifying multiple physiological parameters in parallel, HTP could also advance our understanding of plant responses to insect infestation, and the influence of these changes on levels of host plant resistance, tolerance, or susceptibility. In short, HTP is a

critical technology for the future of basic and applied studies of plant–insect interactions.

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