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1 **A versatile phenotyping system and analytics platform reveals diverse temporal responses to water**
2 **availability in *Setaria***

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12

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17

18 **One-sentence Summary:**

19 A high-throughput image-based phenotyping platform with controlled watering and new open-source trait
20 extraction tools expose distinct responses to water availability in wild and domesticated *Setaria*.

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22

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10 accordance with the policy described in the Instructions For Authors (www.plantphysiol.org) is: Dr. Ivan
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12 ³ NF, MF, MAG, MSW and IB designed and collected high-throughput phenotyping experiment. NF, MF,
13 and MAG designed and wrote PlantCV image analysis tools. DWB, STH and CJM designed and wrote
14 PhenoFront database access tool. NF, MF, MAG, CS, SW, IK, TF, ST, KBG, and IB designed and
15 executed trait validation experiments. NF, MF, MAG, CS, and IB analyzed data. NF, MF, MAG, MSW,
16 CS, and IB wrote the manuscript. TPB, JCC, TCM and IB, supervised and reviewed the manuscript.

17

1 **ABSTRACT**

2

3 Phenotyping has become the rate-limiting step in using large-scale genomic data to understand and
4 improve agricultural crops. Here, the Bellwether Phenotyping Platform for controlled-environment plant
5 growth and automated, multimodal phenotyping is described. The system has capacity for 1,140 plants,
6 which pass daily through stations to record fluorescence, near-infrared, and visible images. Plant
7 Computer Vision (PlantCV) was developed as open-source, hardware platform-independent software for
8 quantitative image analysis. In a four week experiment, wild *Setaria viridis*, and domesticated *Setaria*
9 *italica* had fundamentally different temporal responses to water availability. While both lines produced
10 similar levels of biomass under limited water conditions, *Setaria viridis* maintained the same water-use
11 efficiency under water replete conditions, while *Setaria italica* shifted to less efficient growth. Overall,
12 the Bellwether Phenotyping Platform and PlantCV software detected significant effects of genotype, and
13 environment on height, biomass, water-use efficiency, color, plant architecture, and tissue water-status
14 traits. All ~79,000 images acquired during the course of the experiment are publically available.

1 **INTRODUCTION**

2
3 With growing and more affluent worldwide populations, agricultural crop yields will need to increase
4 significantly by 2050 (Ray et al., 2013; Gerland et al., 2014). Sustainably increasing crop yields in the
5 face of changing environments (IPCC, 2014), and doing so with a smaller environmental footprint, is one
6 of the most pressing global challenges of the 21st century. With changing climate, more prevalent
7 episodes of regional drought and damage due to precipitation extremes will limit agricultural productivity
8 (IPCC, 2014). Salination of agricultural lands and low soil fertility are also increasingly important
9 limitations to crop productivity in many regions.

10
11 Advances in DNA sequencing technology have facilitated rapid progress in plant genomics, which has
12 accelerated traditional breeding, molecular marker-assisted breeding, genome editing, and other
13 approaches for crop improvement. Application of genomic technologies, however, is limited by the ability
14 to reliably quantify plant traits (phenotypes). Traits can range in scale from gene expression to yield in the
15 field. Variation in most traits is quantitative, with contributions from multiple genetic loci. Use of
16 genetically defined populations, such as recombinant inbred lines (RILs), near-isogenic lines, or
17 accession/association panels, can be used to identify the underlying genetic bases for phenotypes, but only
18 when subtle changes in phenotype are accurately and consistently quantified (Benfey and Mitchell-Olds,
19 2008). Consequently, plant phenotyping is widely regarded as the rate limiting step in crop improvement
20 using genome-enabled approaches (Furbank and Tester, 2011; McCouch et al., 2013).

21
22 Abiotic stresses can have variable or gradual effects that depend on plant developmental stage. Therefore,
23 measuring phenotypes over time is necessary for in-depth understanding of plant stress responses
24 (Richards and Thurling, 1978; Schöffl, 1998; Mahfoozi, 2001). Destructive methods of assessing plant
25 phenotypes have been used in the majority of studies (Furbank and Tester, 2011), but such methods do
26 not allow traits of discrete individuals to be compared over time. Accordingly, destructive sampling
27 requires an exponential increase in sampling population size with each time-point added. Non-invasive
28 and non-destructive techniques permit temporal examination of traits in individual plants, reducing the
29 number of plants needed and permitting larger populations to be examined. The emerging field of plant
30 phenomics, the high-throughput, non-destructive examination of plant growth and development in field or
31 controlled-environment settings, provides new technologies that increase the accuracy and speed of data
32 collection and analysis (Furbank and Tester, 2011). Current non-destructive phenomics technologies
33 focus on a number of traits that, directly or indirectly, reflect chlorophyll content, carotenoid content,

1 photosynthesis, transpiration, plant water content, biomass, and height (White et al., 2012; Andrade-
2 Sanchez et al., 2014).

3

4 Phenotyping in the field has all the advantages and disadvantages associated with field-based research.
5 For example, field phenomics allows crop-sized plants to be examined in a natural environmental setting,
6 but field-grown plants are subject to discrete seasonal growing periods and uncontrollable environmental
7 conditions that increase experimental variability. Although a less natural setting, controlled-environment
8 phenomics platforms (Hartmann et al., 2011; Klukas et al., 2014) allow 1) precise control of
9 environmental variables and treatments, 2) experimental replication under reproducible conditions, and 3)
10 better control of instrument placement and functionality. Controlled environment phenotyping also
11 enables faster experimental turnover, which expedites the cyclical development of image processing
12 algorithms and proxy measurement models that are applicable to both field and controlled environment
13 phenomics.

14

15 The Bellwether Phenotyping Platform combines automated, controlled-environment plant growth with
16 high-throughput, non-destructive imaging and is representative of the next wave of phenotyping platforms
17 aimed at relieving bottlenecks of phenomics data collection. Following data collection, image processing
18 and trait analysis are the next barrier to understanding the underlying biology in high-throughput
19 phenotyping data. Standardized methods for processing image-based, high-throughput plant phenomics
20 data lag behind high-throughput sequencing analysis tools in part because of the variety of commercial
21 and non-commercial platforms used, the numerous research focus areas, and the variety of species and
22 treatments. There is a growing plant phenomics community (<http://www.plant-phenotyping.org/>) and an
23 excellent database of both commercial and open-source plant image processing software
24 (<http://www.plant-image-analysis.org/>; Lobet et al. 2013). However, we chose to develop a new trait
25 extraction software platform for daily high-throughput image analysis, Plant Computer Vision (PlantCV).
26 PlantCV is written in a scripting language that has been shown to be accessible to biologists (Mangalam,
27 2002; Dudley and Butte, 2009), is compatible with a variety of image types and sources, and has a
28 community contribution schema that has been successful for other bioinformatics resources (Oliphant,
29 2006; Hunter and Dale, 2007).

30

31 The utility of the system and software is demonstrated here using *Setaria* species and RILs grown under
32 different water conditions. The C4 model monocot *Setaria viridis* (green millet) and the drought-tolerant
33 domesticated crop *Setaria italica* (foxtail millet) (Zhang et al., 2007; Lata et al., 2010; Lata et al., 2011)
34 are closely related to each other and to important crops with similar architecture, such as maize, sorghum,

1 miscanthus and switchgrass (Brutnell et al., 2010; Li and Brutnell, 2011; Sage and Zhu, 2011). Given the
2 relatively limited understanding of monocot stress responses compared to *Arabidopsis* (Bray, 1997;
3 Wilkins et al., 2010), and the importance of cereal crops as sources of food (Ray et al., 2013), *Setaria* has
4 emerged as a useful model system to explore fundamental aspects of monocot biology (Nelson and
5 Dengler, 1997; Kellogg, 1999; Doust, 2007a). Concerted data collection with the Bellwether Phenotyping
6 Platform and analysis with PlantCV detected fundamentally different temporal responses to water
7 availability between the wild and domesticated *Setaria* species. The wild *Setaria* line maintains water-use
8 efficient growth while the domesticated line shifts to less water-use efficient growth in water sufficiency.
9 This publicly available *Setaria* dataset of ~79,000 images represents the next generation of big data to
10 query for phenotypes that impact important yield traits.

11

12 RESULTS AND DISCUSSION

13

14 Design of the Bellwether Phenotyping Platform

15

16 The design of the Bellwether Phenotyping Platform was key to the execution of the *Setaria* water-
17 limitation experiment described below. The Bellwether Phenotyping Platform is comprised of a Conviron
18 (Winnipeg, Canada) climate-controlled growth house integrated with a multi-camera digital imaging
19 system (LemnaTec Scanalyzer^{3D-HT}, Fig. 1). This automated, high-throughput platform allows for
20 repeated non-destructive image capture for multi-parametric analysis, and provides valuable information
21 on the physiological changes of the plants over time. The integration of Conviron and LemnaTec
22 technologies and other custom design decisions were motivated by the goal of tightly controlling the
23 environment while analyzing large numbers of plants. In comparison to greenhouse environments where
24 external factors such as cloud cover or extreme seasonal temperatures can greatly impact the
25 environmental conditions and introduce unpredictable variables into an experiment, the control of the
26 Bellwether Phenotyping Platform facilitates reproducible temporal phenotyping of plants.

27

28 A compact network of over 180 m of conveyor belts is used to store and transport 1,140 plants with a 4”
29 pot size diameter, to and from watering and imaging stations inside the chamber. The conveyor belts are
30 divided into four distinct modules (MOD1–4), which can be controlled independently or as a whole,
31 providing flexibility in experimental design (Fig. 1A). A custom engineered automated door maintains the
32 growth house environment while providing a route for plants to leave and enter the chamber (Fig. 1A).
33 The pots are moved in Radio-frequency identification (RFID) chipped carriers, which are associated with
34 plant barcodes that are used to organize collected image data and metadata, including water treatment

1 instructions. Each MOD contains a precision dual-tank watering station that is equipped with a scale,
2 which can be configured to water to a fixed volume or to a target weight, which makes a variety of
3 experimental watering schemes possible including periodic water stress. When watered to an
4 experimentally determined target weight, the real-time weight of each pot is used to calculate the amount
5 of water required to reach the pre-determined target.

6

7 The decision to install four watering stations inside the growth house (Fig. 1A) allows for tight control of
8 soil water status while maintaining imaging throughput. Due to the single-file movement of the carriers
9 through the closed-loop conveyor system, a single watering station would have limited the number of
10 water applications possible in a day and would have competed with imaging time. A single watering
11 station would have also introduced a significant temporal divide between the first and last watered plants,
12 adding variability into the treatment groups and confounding water usage analysis. Instead, four plants
13 can be watered simultaneously, improving efficiency and drastically reducing the time required to water
14 the entire population (less than two hours).

15 The digital imaging loop connected to the growth house consists of a dark adaptation tunnel and three
16 distinct imaging chambers: visible light (VIS), photosystem II (PSII) fluorescence, and near-infrared
17 (NIR) (Fig. 1A). The imaging stations are described in detail in the sections below. Visible light imaging
18 is used to capture and quantify morphological parameters such as plant shape, color, size and biomass.
19 PSII fluorescence is used for the analysis of photosynthetic efficiency, which can be used to estimate the
20 response to plant treatment or stress, or to measure photosynthetic differences among genetically diverse
21 lines (Maxwell and Johnson, 2000; Garg et al., 2002; Fernández-García et al., 2014; Talukder et al.,
22 2014). Near-infrared imaging is used to estimate the tissue water content (Carter, 1991; Peñuelas and
23 Filella, 1998; Seelig et al., 2008a; Seelig et al., 2009). The throughput of the system depends on
24 experimental design, the desired number of output images, the frequency of water treatment, and is
25 limited by the transport rate of the conveyor. At full capacity (1,140 plants), using all imaging chambers
26 to capture top-view and four side-view images (PSII top-view only), the entire population can be imaged
27 in approximately 30 hours, typically yielding between 25,000 and 37,500 images per week.

28 ***Setaria* Water-Limitation Experiment**

29 With target-weight water treatments possible on the Bellwether Platform, the water available to the plant
30 should be tightly controlled. To increase the precision of target-weight watering, pre-filled pots of soil
31 were used. The use of pre-filled pots also helped to ensure that soil density was similar between pots. To
32 test both the Bellwether Platform, and examine the response of *Setaria* to water-limited conditions, ten

1 *Setaria* lines were grown under four different water regimes (full water capacity: 100% FC, 66% FC, 33%
2 FC, and 0% FC) imposed 17 days after planting (DAP) and maintained for 17 days. Plants were watered
3 twice a day starting at ZT8 (Zeitgeber Time) and ZT23. For more experimental details see Materials and
4 Methods. The lines used were *S. viridis* (accession A10), *S. italica* (accession B100) and eight RILs
5 derived from a cross of *S. viridis* x *S. italica* (Devos et al., 1998; Wang et al., 1998; Bennetzen et al.,
6 2012). An average of 15 plants per line per water treatment were loaded onto the system. Additional *S.*
7 *viridis* and *S. italica* plants were also loaded for parallel destructive physiological tests.

8 The plants that received no water after 17 DAP died within 7 days of treatment and were removed from
9 the system leaving 33% FC as the severest water deficit treatment. Comparisons between 33% and 100%
10 FC were the focus of subsequent analysis. Figure 1B shows the volume of water added at each treatment
11 time-point for *S. viridis* plants watered to 100% and 33% FC. The differences between the two water
12 treatment set points can be seen clearly after the 33% pots complete their dry down from full water 17–19
13 DAP. As the plants grow and transpire, more water is needed to return the pots to their set points. More
14 water was added compared to surrounding days 18 DAP when the second water application was delayed,
15 and 24 DAP when the target-weight point was raised to account for increasing biomass of the plants (Fig.
16 1B). More watering treatments can be added if greater control over soil moisture content is desired.

17 ***Setaria* Image Acquisition**

18 During the experiment *Setaria* plants varied in height from 1.8 cm to 107.4 cm, so a scaled field of view
19 (adjusted optical zoom-level) was used during image acquisition to maximize the image pixels (px)
20 dedicated to plant material, and not background space. Consequently, plant traits measured in pixels from
21 images taken at different zoom levels are not directly comparable because of the difference in the field of
22 view. A reference object with known dimensions was imaged using the VIS side-view (SV) and top-view
23 (TV) cameras at different zoom levels. To compare digital traits across zoom levels, scaling factors were
24 calculated for both area and length traits using zoom-scaling functions (see Materials and Methods).

25 **PhenoFront Database Access Tool**

26 Image and water volume data from LemnaTec phenotyping systems are stored in a PostgreSQL database,
27 but images themselves are stored separately in raw format. To provide distributed access to image and
28 water treatment data an independent database interface tool (PhenoFront) was developed. It was necessary
29 to develop a new database access tool rather than use existing open-source interface tools (Klukas et al.,
30 2014) because the image storage structure of the LemnaTec version 4.0 database changed significantly
31 from previous versions.

1 PhenoFront has a convenient web-based user interface to the phenotyping database and provides an
2 experiment query-building tool that allows users to extract specific image and water treatment data.
3 Importantly, PhenoFront was built with diverse image types in mind (e.g. color vs. grayscale; 8-bit vs. 16-
4 bit) and allows access to images without conversion to a single file format. Queries can be directly
5 downloaded to the user's computer, or data can be downloaded to a remote server capable of high-
6 throughput image processing using utilities such as Wget (<http://www.gnu.org/software/wget/wget.html>)
7 or cURL (<http://curl.haxx.se/>). PhenoFront is available at <https://github.com/danforthcenter/PhenoFront>.

8 Data sets extracted with PhenoFront are structured by data acquisition events (snapshot:
9 weighing/watering, imaging) and a single metadata text file contains the experimental and system
10 parameters associated with each event. Image metadata from the experiment presented here includes:
11 experiment identification code; snapshot folder identification number; alphanumeric barcode encoded
12 with species identification, line identification, water treatment group, and unique plant number; unique
13 snapshot timestamp; weight and water volume information; individual image file names included in the
14 snapshot directory. The structured data sets can be easily read by analysis software and are convenient for
15 public data sharing.

16 Although strides have been made to create resources for image processing on the iPlant cyber
17 infrastructure (Kvilekval et al., 2010), a dedicated centralized database for sharing published plant
18 phenotyping image data has not been designated. Although a few plant phenomic datasets are publicly
19 available (Fahlgren et al., 2015), this is the largest publically available dataset of above-ground plant
20 tissue to date (79,200 images of 823 plants). Image data from this experiment is available via
21 <http://plantcv.danforthcenter.org/pages/data.html> in Portable Network Graphics (PNG) and Joint
22 Photographic Experts Group (JPEG) formats. JPEG format images with lossy compression consume
23 approximately 11-fold less storage size, but comparisons of image datasets in JPEG and PNG format
24 revealed significant differences in some analyzed traits (see Materials and Methods). Establishing a
25 dedicated centralized database that is designed to deposit and access large plant phenomic datasets and
26 standardized metadata is vital for aggregating and curating data for the community. The phenomics
27 community will need to collectively consider formats, data and metadata structure, minimal information
28 standards, ontologies, and other sharing issues. Implementing these phenotyping standards will expedite
29 tool development and alleviate barriers to crop improvement.

30 **Phenotype Extraction Using Plant Computer Vision (PlantCV) Software**

31

1 High-throughput plant phenotyping has the potential to improve modeling of genotype by environment
2 interactions and to expedite identification of germplasm that could increase yield and productivity of crop
3 plants. With numerous commercial and custom-built image-based phenotyping platforms in existence, it
4 is unlikely that standard hardware will be used to capture image-based phenomics data in the near future.
5 Lack of standard hardware thus requires each new platform to go through a significant initialization
6 period, and a gambit of validation experiments, many of which are described below in this study. Despite
7 different hardware platforms, image-analysis and trait-extraction is common ground between image-based
8 phenotyping platforms. Therefore, open-source trait-extraction software with mechanism for community
9 development will help to alleviate the phenotyping bottleneck on crop improvement.

10
11 Available commercial and open-source plant image processing software range in their capabilities,
12 flexibilities, and underlying programming languages and largely focus on analysis of single cells, leaves,
13 roots, or plants with rosette architecture (<http://www.plant-image-analysis.org>; Lobet et al. 2013). There
14 are analysis tools in the Plant Image Analysis database, such as LemnaGrid (<https://www.lemnatec.com>)
15 (Munns and Tester, 2008; Berger et al., 2010; Golzarian et al., 2011; Honsdorf et al., 2014), the ImageJ
16 (Abràmoff et al., 2004) plugin HTPheno (Hartmann et al., 2011), and the java-based open-source
17 Integrated Analysis Platform (IAP) (Klukas et al., 2014) that are capable of analyzing larger model plants
18 and crops with diverse architectures (Supplemental Table SI). We developed the open-source and open-
19 development PlantCV image analysis platform to emphasize the following features: flexible user-defined
20 analysis workflows; parallelizable image processing for fast throughput; and a scripting language
21 implementation that lowers the barrier to community contributions that extend functionality. It was
22 important to move away from commercial software for greater control and understanding of the image
23 processing and trait extraction algorithms used to process the data, as well as the freedom to expand
24 analyses at will. While some users may prefer graphical user interfaces for software, script-based
25 programs are easier to develop, and the precise workflows are detailed directly in the scripts themselves,
26 enabling reproducible research.

27
28 Extraction of temporal plant trait data from images occurs in three steps: 1) isolation of plant material
29 from background (Fig. 2); 2) identification of features (traits) from isolated plants (Fig. 2B); and 3)
30 analysis of traits across population, treatments, and time (Fig. 2C). PlantCV isolates plant material from
31 background, quantifies plant traits, and populates an SQLite (<https://www.sqlite.org/>) database that is
32 easily queried for further analysis across treatments, genotypes, and time. In debug mode, PlantCV
33 creates annotated intermediate images for each step of image analysis pipelines (e.g. outlining plant
34 perimeter), allowing users to verify that analysis steps are working as intended. Although *Setaria* images

1 from the high-throughput Bellwether Phenotyping Platform are analyzed here, PlantCV has been used to
2 analyze a variety of plant types (Fig. 2D–F), as well as images captured from non-commercial imaging
3 stations (Fig. 2E and F). For the *Setaria* experiment here, traits such as height, biomass, and plant
4 architecture, were manually measured and are highly correlated with traits computationally extracted by
5 PlantCV (Fig. 3, Fig. 4, and Fig. 5). Image datasets curated with manual measurements are available at
6 http://plantcv.danforthcenter.org/pages/data-sets/2013/setaria_burnin2.html and are a resource for
7 generating further trait measurement algorithms.

8
9 PlantCV software is built upon the open-source libraries OpenCV (Bradski and Kaehler, 2008), NumPy
10 (Oliphant, 2006), and Matplotlib (Hunter and Dale, 2007), and contains pipelines built with modular
11 functions that are currently capable of automated analysis of color and grayscale images from VIS, PSII,
12 and NIR cameras. Separate pipelines are used to process images from different camera types due to
13 differences in image size and dimensions, spectral channels (e.g. color versus grayscale), special
14 processing steps (e.g. PSII imaging, see below), and other factors (e.g. lighting). PlantCV was written in
15 the Python programming language with hopes that the greater phenomics community will utilize and
16 extend its functionality. Although Python is not as efficient as compiled languages (e.g. C or Java) in
17 terms of memory usage and speed of execution (Fourment and Gillings, 2008), it is currently more widely
18 used by biologists, and is arguably easier to learn (Mangalam, 2002; Dudley and Butte, 2009). On a single
19 processor, PlantCV can process and extract data from 200–350 images per hour, depending on image size,
20 and the modular script-based architecture allows for easy parallelization. At the Danforth Center, PlantCV
21 is typically run on 10 CPUs. PlantCV is an open-source resource under a GNU General Public License
22 (GPLv2) share-alike license that can be leveraged for the further development of more complex trait
23 identification modules. PlantCV is also available via GitHub, providing a framework for community
24 contribution that has been successful for other science and Python-based projects such as NumPy (~284
25 contributors), and Matplotlib (~294 contributors) (Oliphant, 2006; Hunter and Dale, 2007). Community
26 contribution helps to maintain software permanence (Gentleman et al., 2004), which is often a problem
27 for bioinformatics tools maintained by individual labs (Gilbert, 2004). PlantCV, PlantCV documentation,
28 tutorials, parallelization scripts, downstream analysis scripts, and a contribution guide can be accessed at
29 <http://plantcv.danforthcenter.org>.

30

31 **VIS Image Processing and Traits**

32 The VIS imaging station in the Bellwether Phenotyping Platform captures visible light (400 – 700 nm)
33 images with two high-resolution (2454 x 2056 pixel) charge-coupled device (CCD) cameras. One camera
34 is mounted above the plant and the second camera is side-mounted for top- and side-view imaging.

1 respectively. The plant carrier is positioned on a piston lifter with a turner that can rotate the plant 360
2 degrees. During the *Setaria* experiment a single top-view and four side-view (0°, 90°, 180°, and 270°) VIS
3 images were acquired per plant per time point. In total, *Setaria* plants were imaged 6,399 times from 11–
4 33 DAP for a total of 31,968 VIS images (55 images per plant). The set of images collected for a single
5 plant in one imaging session is defined as a snapshot.

6
7 In general, PlantCV VIS image processing pipelines automatically identify background material within
8 the image, such as the carrier, pot, soil, and side paneling. To mitigate plant identification bias due to
9 genotype, developmental age, abiotic treatment, or other experimental parameters, plant material was
10 isolated after several stages of background removal, rather than first thresholding for plant ‘greenness.’
11 For a complete description of the VIS image processing steps, please refer to Supplemental Data and view
12 the PlantCV online documentation.

13
14 **Plant height:** Plant height was defined as the maximum vertical extent of the plant from the top of the
15 pot. For 173 randomly selected VIS side-view images, plant height (calibrated using the width of the
16 plant carrier) was manually measured using ImageJ (Abràmoff et al., 2004). For the same image set, plant
17 height was automatically estimated using PlantCV with height scaled to correct for the camera zoom level
18 (see Material and Methods). Ordinary least squares regression analysis confirmed that PlantCV measured
19 plant height was a good estimator of manually measured height and was robust across changes in camera
20 zoom level (adjusted $R^2 = 0.998$; Fig. 3A).

21
22 To analyze *Setaria* height more robustly at the population level, estimated plant height was averaged
23 between all four VIS side-view images for each snapshot and the effect of genotype and low water
24 availability on height was calculated. Intrinsic height differences under full water conditions were
25 observed for the *Setaria* genotypes, particularly from 23–33 DAP. *S. viridis* was slightly taller than *S.*
26 *italica* from 23–33 DAP with a mean height ranging from 50.3–101.2 cm compared to 43.0–98.6 cm,
27 respectively (Fig. 3B). The height of RIL102 was within the range of the two parent lines but the other
28 seven RILs were transgressive; RIL161 was shorter on average than the parents while the others were
29 taller (Supplemental Fig. S1). Height for the tallest lines was underestimated because the camera field of
30 view in this experiment was not zoomed out enough to completely capture the tallest plants. For example,
31 truncated plant height due to camera settings is noticeable from 15–20 DAP after which the field of view
32 was expanded and plants were fully imaged again (Fig. 3B and Supplemental Fig. S1). Although
33 thorough surveillance can be used to avoid lapses in coverage due to sub-optimal camera settings, the
34 large number of potentially diverse plants on high-throughput systems makes complete oversight

1 challenging. A better approach will be to add real-time analysis that raises alarm to potential framing
2 issues once preset threshold boundaries are reached. The parallelized rate of PlantCV analysis permits
3 future experiments to implement real-time boundary detection.

4

5 In addition to intrinsic height phenotypes, the impact of limited water availability on plant height was also
6 measured. *S. viridis* plants watered to 33% FC starting 17 DAP had a slower vertical growth rate than
7 plants at 100% FC from 21–33 DAP (4.1 and 5.0 g/day, respectively; Fig. 3B). In contrast, *S. italica*
8 plants from 100% and 33% FC groups did not have significantly different vertical growth rates,
9 suggesting that *S. italica* plant height is not tightly coupled to water availability (4.9 g/day; Fig. 3B).
10 Alternatively, these results could suggest that *S. viridis* responds to extra water availability with more
11 growth while *S. italica* does not. Of the ten lines analyzed, *S. viridis* has the largest difference in height
12 when comparing the 33% to 100% FC treatment groups (Supplemental Fig. S1). The difference in height
13 for the RILs was either similar to *S. italica* or was intermediate between the two parents (Supplemental
14 Fig. S1).

15

16 **Above ground biomass:** Throughout the experiment, 41 *S. viridis* and *S. italica* plants randomly selected
17 from the full capacity water group were collected and the above ground fresh- and dry-weight biomass
18 were recorded. The images acquired immediately prior to collection were analyzed with PlantCV to
19 measure plant traits that could be used to model biomass. Linear modeling was done with three PlantCV
20 measurements: Side-view area, the sum of the above ground plant pixel area from all four side-view VIS
21 images; Top-view area, the plant pixel area from the single top-view VIS image; and plant height, as
22 described above. The initial model included side-view area, top-view area and height, adjusted for zoom
23 level, and all pairwise interaction terms. A stepwise model selection procedure was done using the
24 Akaike's Information Criterion (Bozdogan, 1987), which resulted in the reduced model:

$$M_{fw} = 3.966 \times 10^{-5} A_{sv} - 0.3193$$

25 where M_{fw} is fresh-weight biomass and A_{sv} is side-view area (adjusted $R^2 = 0.981$; Fig. 4A). Dry-weight
26 biomass was also modeled efficiently with side-view plant pixel area (adjusted $R^2 = 0.975$; Supplemental
27 Fig. S2). It was hypothesized that residual variation in biomass could be explained by plants that were
28 partially out of frame or by genotype. PlantCV records when plants extend to or past the border of each
29 image, and the model including the out-of-frame status of each plant was accepted over the reduced
30 model ($p < 0.01$, F-test) but only slightly improved the model (adjusted $R^2 = 0.985$). In contrast, genetic
31 background did not improve the model significantly, suggesting that biomass estimation for *S. viridis* and
32 *S. italica* is robust to *Setaria* species differences, at least within the conditions tested.

33

1 **Growth rates and response to water availability:** Image-based biomass estimates were used to quantify
2 the impact of limited water availability on *Setaria* fresh-weight biomass (Fig. 4 and Supplemental Fig.
3 S3). Non-linear least squares regression was used to estimate the growth parameters (three-component
4 logistic model) for each group (genotype by treatment) (Fig. 4B and C). Under water deficit *S. italica* and
5 *S. viridis* grew at similar rates (maximum absolute growth rate at ~25 DAP was 1.6 and 1.7 g/day,
6 respectively; Fig. 4B and C). In contrast, under 100% FC treatment the maximum absolute growth rate of
7 *S. viridis* was 0.6 g/day more than *S. italica* (3 and 2.4 g/day, respectively) and was reached two days
8 earlier (Fig. 4C). As a result, the impact of water-limitation on fresh-weight biomass was larger and
9 observed more quickly in *S. viridis* compared to *S. italica*. *S. viridis* watered to 33% FC starting at 17
10 DAP accumulated significantly less biomass relative to the control group from 18 DAP through the end
11 of the experiment (95% confidence interval: 0.5–1.4 g; Fig. 4B). *S. italica* watered to 33% FC
12 accumulated significantly less biomass relative to the control group at 22 DAP through the end of the
13 experiment (95% confidence interval: 0.2–1.2 g; Fig. 4B). Plant growth and response to the environment
14 is a dynamic process and non-destructive phenotyping was key to understanding the differences between
15 *S. viridis* and *S. italica*. For example, although *S. viridis* grows faster and earlier than *S. italica* under
16 100% FC treatment, biomass at 33 DAP was similar, so endpoint biomass measurements would have
17 missed the growth differences between *S. viridis* and *S. italica* (Fig. 4B and C).

18
19 **Integrated water-use efficiency:** The ability to measure and record the volume of water applied to
20 individual plants (Fig. 1B) in combination with the estimated biomass of plants over time (Fig. 4B)
21 provides a framework for quantifying plant intrinsic water-use efficiency (WUE). Here intrinsic WUE is
22 operationally defined as the amount of biomass accumulated per ml of water applied (units: mg/ml). No
23 significant difference in WUE was observed for *S. viridis* plants watered to 100% or 33% FC (Fig. 4D).
24 Therefore, the amount of *S. viridis* accumulated biomass is proportional to the volume of water supplied.
25 In contrast, *S. italica* plants had lower WUE at 100% FC relative to 33% FC, with significant differences
26 observed from 22–27 DAP (Fig. 4D). This suggests that *S. italica* uses water less efficiently under water-
27 sufficient conditions.

28
29 **Plant architecture:** The traits discussed above do not require knowledge of specific plant architecture, but
30 architectural phenotypes such as tillering and leaf angle can have a significant impact on plant
31 performance (Warnasooriya and Brutnell, 2014). Known plant architecture differences between the wild
32 *S. viridis* and domesticated *S. italica* include the number of axillary branches and tiller number
33 (Darmency et al., 1987; Doust and Kellogg, 2006; Doust, 2007b; Doust et al., 2009). Computational 3D
34 reconstruction of plant architecture from 2D images would promote accurate estimates of tiller count, but

1 would have a significant impact on imaging throughput because of the increase in images per plant, per
2 time-point needed to enable 3D reconstruction (Phattaralerphong and Sinoquet, 2005; Paproki et al.,
3 2012). Modeling is an alternative approach that can be used to predict the number of tillers given one or
4 more morphological parameters that are more easily measured.

5

6 In a first attempt at generating a predictive model for *Setaria* architecture, tiller number, tiller angle (angle
7 between the two outermost tillers), and leaf angle (angle between the leaf and tiller of the first leaves on
8 the two outermost tillers) values were measured using ImageJ (Abràmoff et al., 2004) on 58 randomly
9 selected images of plants 25 and 26 DAP. The results were compared to two morphological traits that are
10 simple to calculate using PlantCV: height-width ratio (HW, height divided by extent-x; Fig. 5A) and
11 solidity (plant area divided by convex hull area). HW was significantly correlated ($p < 0.01$) with all three
12 manually measured traits, but the best model for HW, which accounted for 37% of the variance in an
13 ANOVA analysis, included only tiller angle and leaf angle (see Material and Methods). Solidity was not
14 significantly correlated with any of the manually measured traits. While HW ratio is not inherently a
15 measure of a single plant architecture trait, it can serve as an easily obtainable proxy for further analysis.
16 To generate a predictive model for tiller count, HW and several other traits were examined. Tillers were
17 manually counted for 195 randomly selected images. HW, solidity, fresh-weight biomass, width (extent-
18 x), and height were tested as potential explanatory variables for manual tiller counts. Ordinary least
19 squares regression was used to generate a model for the number of tillers:

$$TC = 0.2289M_{fw} - 2.165HW + 5.164$$

20 where TC is the number of tillers, and M_{fw} is fresh-weight biomass and HW is height-width ratio. The
21 tiller model explained 64.7% of the variation in tiller number (Fig. 5B). To further test the tiller model,
22 tillers were counted for a second set of 646 randomly selected images. The accuracy of the tiller model to
23 predict tiller number in the second set of 646 images was assessed, and the median difference between
24 predicted and manual tiller number was ± 1.29 tillers (Fig. 5C). Although there are clearly other variables
25 that contribute to the tiller model, the predicted tiller count model generated with spatial-independent
26 morphological characteristics can serve as a proxy measurement of *Setaria* architecture.

27

28 The tiller count model was used to predict tiller numbers in *S. viridis*, *S. italicica* and the eight *S. viridis* x
29 *S. italicica* RILs under 100% FC and 33% FC water treatments. For all *Setaria* genotypes, tiller number
30 decreased with reduced water treatment, but the intensity and speed of response varied (Fig. 5C and
31 Supplemental Fig. S4). *S. viridis* predicted tiller count is greater than *S. italicica* under both 100% FC and
32 33% FC water conditions (Fig. 5D), which is consistent with known architectural differences between the
33 two genotypes (Darmency et al., 1987; Doust and Kellogg, 2006; Doust, 2007b; Doust et al., 2009).

1 Predicted tiller number appears to be an introgressive trait for the RILs analyzed (Supplemental Fig. S4)
2 and can be genetically mapped in a larger *S. viridis* x *S. italica* RIL population.

3
4 **Color analysis:** Color is often used to gauge plant health in response to biotic and abiotic treatment, or to
5 identify lines that have possible defects in pigment development and thus changes in photosynthesis
6 (Albrecht-Borth et al., 2013; Kremnev and Strand, 2014; Kunz et al., 2014; Satou et al., 2014; Neilson et
7 al., 2015). For each identified plant pixel, PlantCV records intensity values of color data for each of the
8 three color channels of RGB (Red, Green, Blue), HSV (Hue, Saturation, Value), and LAB (Lightness,
9 Green-Magenta, Blue-Yellow) color space, yielding a set of three histograms for each color space. The
10 RGB data is presented here because images are captured in RGB color space. Results from interpolated
11 HSV and LAB color space are included in Supplemental Data, but interpretations of these results concur
12 with RGB color space (Supplemental Fig. S5, Supplemental Fig. S6, Supplemental Fig. S7, and
13 Supplemental Fig. S8). The VIS images obtained via PhenoFront are 8-bit color images, therefore each
14 pixel in each channel can have a maximum intensity value of 2^8 or 256. PlantCV was used to normalize
15 the *Setaria* color histograms by the number of plant pixels (plant size), and these values are averaged by
16 the four side-view angles.

17
18 Color data was evaluated to determine if water treatment effects could be distinguished 25 to 26 DAP in
19 *S. viridis*, where there is a significant difference in biomass under reduced water (Supplemental Fig. S5
20 and Supplemental Fig. S6). Principal Component Analysis (PCA) visibly separated full water from
21 reduced water treatment along PC2, which captures 16% of variation (Supplemental Fig. S5 and
22 Supplemental Fig. S6). Spearman correlation analysis found that PC2 was significantly negatively
23 correlated ($p < 0.01$; rho=-0.7056) with water treatment 25 to 26 DAP in *S. viridis*. Principal component
24 regression found that a two-component model of PC1 and PC2 covers 62% of the variation seen in
25 treatment 25 to 26 DAP (Supplemental Fig. S5 and Supplemental Fig. S6). Therefore, *Setaria* color can
26 be used to distinguish water treatments 25 to 26 DAP. PCA using all RGB color data as explanatory
27 variables from *S. viridis* 17 and 18 DAP did not visibly separate full water from 33% FC (Supplemental
28 Fig. S5 and Supplemental Fig. S6) and a two-component regression model included only 0.45% of the
29 variance explained by treatment. Consequently, *Setaria* color does not appear to as early an indication of
30 drought as biomass.

31
32 Color analysis was able to discriminate plant genotypes before treatment was applied, which was not the
33 case for estimated plant biomass (Supplemental Fig. S3). PCA of RGB color data prior to water treatment
34 (11 to 12 DAP) found that PC1 captures 41% of variation in color, and separates RIL161 (Fig. 6) from the

1 other genotypes. The RIL161 line was distinguishable from the other *Setaria* lines and appears to be pale
2 in pigmentation (Fig. 6B). The seven other RIL lines (Fig. 6A) grouped along PC1 and PC2 with the
3 parent lines, *S. viridis* (Fig. 6A, left) and the majority of *S. italica* (Fig. 6A, right). 17 out of 112 *S. italica*
4 plants clustered with RIL161 plants (Fig. 6A, right). Investigation of *S. italica* plants that clustered with
5 the RIL161 plants revealed that they are paler in color compared to *S. italica* that group with *S. viridis* and
6 the other RILs (Fig. 6B). The color PC1 trait appears to be a transgressively segregating phenotype in
7 RIL161 compared to the majority of parent phenotypes and may be genetically mapped in the larger *S.*
8 *viridis* x *S. italica* RIL population. The color PC1 trait may also be associated with a photosynthetic trait,
9 since RIL161 plants are not only pale but also shorter on average compared to other lines (Supplemental
10 Fig. S1).

11

12 **PSII Image Traits**

13 For more information on PSII image processing please refer to Supplemental Data. Comparison with
14 plant architecture traits from the VIS system revealed that F_v/F_m is strongly correlated with plant height in
15 a Spearman correlation analysis ($\rho=-0.852$, $p < 0.01$; Supplemental Fig. S9). To further test if plant
16 height significantly influences F_v/F_m measurements, a 3D-printed staggered platform was designed and
17 built to examine $\sim 4 \text{ cm}^2$ of excised *Nicotiana benthamiana* leaf tissue at fixed heights (Supplemental Fig.
18 S9B and C). *N. benthamiana* was used because leaves are large enough for tissue from a single leaf to be
19 used at all four platform levels. PSII analysis of the 3D-printed platform confirmed that height
20 significantly negatively correlates with F_v/F_m ($r=-0.976$; $p < 0.01$; Supplemental Fig. S9D). One
21 explanation for this correlation is that F_{\min} may be disproportionately underestimated relative to F_{\max} in
22 shorter plants resulting in an artificially inflated F_v/F_m value. Although PlantCV is still a useful tool for
23 analyzing photosynthetic efficiency, alterations in the physical configuration of the PSII imaging station
24 are necessary to prevent height differences from confounding photosynthetic efficiency measurements.

25

26 **NIR Image Processing and Traits**

27 The NIR imaging station captures near-infrared light (900–1700 nm) using two cameras (320 x 256 pixel)
28 mounted for top- and side-view imaging with a rotating lifter to allow for multiple side-view images. Four
29 side-view (0°, 90°, 180°, and 270°) NIR images were captured per plant per time point. The NIR top-view
30 camera was partially functioning during the experiment, so top-view images were not taken at all time
31 points. Additionally, the background signal of soil in the top-view NIR images makes plant signal
32 isolation difficult, so only side-view NIR images were used in the analysis. In total, *Setaria* plants were
33 imaged 6,399 times from 11–33 DAP for a total of 25,596 NIR side-view images.

34

1 PlantCV isolation of plant material from other background components in NIR grayscale images is
2 achieved through several image-processing techniques, including background estimation/removal and
3 object of interest sharpening to improve the effectiveness of binary thresholding. Once the plant is
4 identified, the grayscale NIR pixel-level intensity is summarized into a histogram containing 256 bins,
5 where each bin contains the proportion of plant pixels exhibiting the corresponding grayscale intensity
6 value. Shape attributes described above for VIS imaging are also recorded for each NIR image for quality
7 control purposes. Comparison of shape measurements between imaging stations allows for the detection
8 of outliers due to artifacts in the image processing pipelines. Similarity of NIR signal within snapshot sets
9 and image classes (genotype by treatment by DAP) using Chi-squared and Earth Mover's Distances were
10 also used to assess data quality. For more information on NIR image processing please refer to
11 Supplemental Data.

12

13 **NIR signal analysis:** The effects of camera zoom level can be clearly observed as the camera imaging
14 configuration changes from 3.1X to 1.4X optical zoom at 21 DAP (Fig. 7). Comparison of 100% and 33%
15 FC water treatments as the mathematical difference of treatment-averaged histograms illustrates a shift in
16 the distribution of NIR signal histogram toward increased NIR signal reflectance in drought treated plants
17 (Fig. 7B).

18

19 PCA was used to summarize the changes observed in the NIR signal histograms. Due to the shifts in the
20 NIR signal between zoom levels, PCA was done within each zoom level. While the first principal
21 components (PC1s) at each zoom level are not directly comparable, the loadings indicate that they are
22 driven by the same phenomenon: the two sides of the loading histograms with opposite signs indicate a
23 shift from higher absorbance pixels, which have been associated with higher water content in previous
24 studies, to lower absorbance pixels (Fig. 7C and D) (Carter, 1991; Peñuelas and Filella, 1998; Seelig et
25 al., 2008a; Seelig et al., 2009). PC1 captures a larger proportion of variation (33.5%) within NIR signals
26 at widest zoom level (1.4X), corresponding to later growth stages and more extended drought treatment,
27 than PC1s from earlier, narrower zoom levels (14.9% and 25.9% at 3.9X and 3.1X, respectively).

28 Significant differences of PC1 values ($n=8$; $p < 0.01$) compared between treatments (100% and 33% FC)
29 were observed in both *S. viridis* and *S. italica* parental genotypes 23 DAP (Fig. 8). The NIR system
30 measures wavelengths of light that are absorbed by water, but signal transmittance and reflectance can
31 also change with differences in leaf thickness or tissue composition (Seelig et al., 2008b; Seelig et al.,
32 2009; Neilson et al., 2015). Therefore, shifts in NIR signal due to differences in leaf water content cannot
33 be distinguished from changes in leaf thickness or tissue composition in response to water deficit. More

1 advanced imaging analysis, perhaps integrating VIS data, or additional imaging hardware might be
2 utilized to distinguish between these two water-deficit responses.

3

4 **Integrated Analysis**

5

6 While each extracted trait can be treated individually, multimodal traits can be used to detect artifacts or
7 gain greater insight to biological responses. For example, correlations between plant height and F_v/F_m
8 signal were used to identify the physical limitations of the PSII imaging station, and examining biomass
9 with watering data expanded understanding of water use efficiency. Examining correlation between traits
10 may identify simple biometric relationships inherent to a biological system (such as the positive
11 correlation between plant biomass and height; Supplemental Fig. S10) or more intricate relationships that
12 are dependent on treatment or temporal factors (relationship between NIR PC1 and VIS color PC1 and
13 PC2; Supplemental Fig. S10). An important component of trait integration is an understanding of which
14 traits are precisely reporting on genotype or treatment effects. On four days that span the experimental
15 period, variance due to genotype, treatment, and genotype by treatment across the traits discussed was
16 examined using a simple linear model (Table I). Emphasizing the temporal dependence of the measured
17 traits, there were notable shifts in which factor accounted for the largest components of variation at
18 different time-points. In the six days from 19–25 DAP, as the cumulative water deficit became more
19 severe, the treatment term accounted for a much larger portion of the biomass, tiller count, and NIR PC1
20 variance, a trend that continues through 31 DAP. For WUE, treatment had the largest effect 19 DAP,
21 reflecting the dynamic and differing response to water availability between *S. viridis* and *S. italica* (Fig.
22 4). Temporal measurements allowed the identification of mechanistic differences in drought response,
23 which would be indistinguishable with only end-point measurements.

24

25 Genotype accounted for at least 17.9% of the variance at one or more time-point for all of the traits
26 described in Table I, including PCA traits from color and NIR signal, suggesting that these traits will be
27 tractable targets for temporal genetic and gene by environment analysis. Combined with the variance
28 accounted for by treatment, it is clear that traits measured by the VIS and NIR systems can be used to
29 investigate plant responses to a changing environment. An outstanding question is whether the distinct
30 image types are contributing independent insights to an experiment. While analysis of eight RIL lines was
31 sufficient to identify distinct responses in the two parents for multiple traits, it is not enough to determine
32 if the traits are correlated or segregating independently from each other. Analysis of larger genetic
33 populations is necessary to identify the underlying genetic loci for each trait and to determine if they
34 segregate independently.

1
2 It is important to note that the analysis routines presented here are just a starting point for the data that can
3 be extracted from the images collected. The most significant finding from the analysis is that wild *S.*
4 *viridis* and domesticated *S. italica* have different responses to water availability. This result comes from
5 analysis of pixel-estimated biomass that does not require integration of plant architecture for accurate
6 measure. However, integrating plant architecture, by including identification of leaf and stem pixels as
7 well as segmenting the plant by developmental age, could extract further information from VIS and NIR
8 images. Making the images and PlantCV software publically available is intended to encourage other
9 researchers to join in on addressing these questions.
10

11 CONCLUSIONS

12

13 There are three essential components of the Bellwether Phenotyping Platform: tightly-controlled and
14 recorded environmental variables, automated image capture, and scriptable analysis by PlantCV. With all
15 of the components in place, experiments can be conducted on a scale not previously possible. For
16 example, without the four automatic watering stations in the Bellwether Phenotyping Platform,
17 maintaining the moisture level of 1,140 pots in a high-light, high-temperature environment would require
18 considerable labor for the length of the experiment. The design decisions that led to the high capacity of
19 the Bellwether Phenotyping Platform permit repeated measurements of hundreds of lines with replicates
20 and multiple treatments. Analysis by PlantCV allows for flexible and timely image processing and
21 analysis. Open-source PlantCV software is platform independent, allowing it to scale from controlled-
22 environment growth chamber phenotyping to field phenotyping. PlantCV is currently used to process
23 images from four diverse custom-built imaging platforms at the Donald Danforth Plant Science Center.
24 The image dataset provided by this study can be utilized to extend PlantCV function by extracting further
25 architectural traits that are also important to crops with similar structure, such as maize and sorghum.
26

27 In this study, four different water regimes were imposed with multiple replicates for each line/treatment
28 combination. Although only ten *Setaria* lines were examined in the present study, this work showed that
29 *S. viridis* and *S. italica* have fundamentally different responses to water availability. The wild accession *S.*
30 *viridis*, adjusts its growth to utilize all of the available water, while the domesticated *S. italica* is less
31 efficient at converting water to biomass under high-water availability. Although, the domesticated crop *S.*
32 *italica* is known to be drought-tolerant from previous studies (Li and Brutnell, 2011), differences between
33 *S. viridis* and *S. italica* response to water availability would not have been detected if only end-point
34 biomass or WUE were examined, highlighting the importance of temporal examination of traits. The eight

1 randomly selected RILs displayed variation between the parents suggesting, unsurprisingly, that the
2 observed traits are controlled by multiple loci. Therefore, future phenotyping studies using the Bellwether
3 Phenotyping Platform that examine a larger population of *S. viridis* x *S. italica* RILs will likely be
4 successful in extracting multiple time-dependent drought-related QTL.

5

6 MATERIALS AND METHODS

7

8 Plant Growth

9

10 ***Setaria experiment growth details:*** 4-inch diameter white/gray pots from Hummert were prefilled with
11 ~473 ml of MetroMix360 soil, and 0.5 g of Osmocote Classic 14-14-14 fertilizer (Everris, US) was
12 manually distributed to the top of each pot. *S. viridis* (A10), *S. italica* (B10) and 8 RILs (RIL020,
13 RIL070, RIL098, RIL102, RIL128, RIL133, RIL161, RIL187) from the *S. viridis* x *S. italica* population
14 (Devos et al., 1998; Wang et al., 1998; Bennetzen et al., 2012) were planted, barcoded, then allowed to
15 germinate for 9 days in a Conviron growth chamber before being loaded into the Conviron growth area of
16 the Bellwether Phenotyping Platform. Barcoded information included genotype identification, water
17 treatment group, and a unique pot identification number. During germination, plants were grown under a
18 long day photoperiod (16h day/8h night) at 31°C day/21°C night. During the germination period the
19 maximum light intensity of the Conviron chamber was used (230 µmol/m²/s). At 9 DAP germinated
20 plants were loaded into the Conviron growth chamber of the Bellwether Phenotyping Platform using a
21 ‘random block’ design. In the phenotyping facility, plants were grown under a long day photoperiod (16h
22 day/8h night) at 31°C day/21°C night at a light intensity of 500 µmol/m²/s using metal halide and high-
23 pressure sodium lamps to deliver a broad spectrum of light for plant growth: 20.7% at 400-500 nm,
24 33.3% at 500-600 nm, 26.8% at 600-700 nm, 3.3% at 700-800 nm and 15.8% at 800-900 nm (measured
25 with Apogee Instruments Spectroradiometer PS-100).

26

27 ***Water treatments:*** The 4-inch pre-filled pots were dried down to remove excess moisture from the soil.
28 After dry down, the average dry pot, soil, and Osmocote weight was 73 g. To determine soil volume
29 water content, measured amounts of reverse osmosis water were added to the dry soil and allowed to
30 absorb for 1 hour. Volume water content (VWC) was determined by a Decagon Devices GS3 soil
31 moisture sensor and ProCheck Instantaneous data-logger. The full-capacity water treatment group was
32 assigned a target weight of 625 g. Given a carrier weight of 335 g and soil/pot dry weight of 73 g, the full
33 capacity treatment group corresponds to 217 ml of available water and a soil volume water content of
34 ~48%. Initially, five treatment group target weights were defined as 100%, 75%, 50%, 25%, and 0% of

1 the soil/pot wet weight, which corresponded to the target weights 625 g, 552.5 g, 480 g, 407.5 g, and 0 g.
2 However, accounting for only relative water availability the treatments were 100% FC (217 ml; 48%
3 VWC), 66.5% FC (144.5 ml; 31% VWC), 33% FC (72 ml; 14% VWC), and 0 ml for the remaining
4 groups.

5

6 **Hardware**

7

8 The LemnaTec Scanalyzer 3D^{H-T} is controlled using LemnaControl software. Image and water treatment
9 data from the phenotyping facility are transferred to a PostgreSQL database (LemnaDB) for storage.
10 PhenoFront (<https://github.com/danforthcenter/PhenoFront>) is used to access image data from the local
11 LemnaTec database before image analysis with PlantCV (<http://plantcv.danforthcenter.org/>).

12

13 **Conviron growth chamber specifications:** The 70 m² growth house combines a large growth area with
14 the precise climate control. The growth house temperature can be controlled from 12°C – 35°C with lights
15 off, to 15°C – 42°C with lights on while relative humidity is controlled by external chemical dryers. Metal
16 halide and high-pressure sodium lamps deliver a broad spectrum of light for plant growth with an
17 intensity of 0 – 800 μmoles/m²/s.

18

19 **VIS camera:** VIS imaging (400 – 700 nm) in the Bellwether Phenotyping Platform utilizes two 2/3"
20 Progressive Scan CCD cameras (Basler AG, Germany) with a frame-rate of 17 frame/s, and image
21 resolution of 2454x2053 pixels. The optics of the side and top-view VIS cameras feature a 3 motor lens,
22 focal length of 12.5-75mm, iris speed of 4.0s, focus motor speed of 5.0s, zoom speed of 5.0s, and
23 horizontal angle view of 6.7° to 38.8°. For the *Setaria* experiment imaging configurations please refer to
24 Supplemental Table SII for LemnaTec hardware control software settings.

25

26 **PSII camera:** PSII imaging (680-900nm) in the Bellwether Phenotyping Platform uses one top-view
27 CCD camera (Phenovation, Netherlands), with a frame-rate of 15 frames/s, image resolution of 1388x964
28 pixels, and field of view of 120X170mm. The red light pulse (630nm) can vary in intensity from 1000 to
29 6000 μmol/m²/s. Multiple top-view images are taken 30-500 μs from the initiation of the 1-2s light pulse.
30 The null (F₀), minimum (F_{min}) and maximum (F_{max}) fluorescence images are saved. For the *Setaria*
31 experiment imaging configurations please refer to Supplemental Table SII for LemnaTec hardware
32 control software settings.

33

1 **NIR camera:** NIR imaging (900 to 1700 nm) features two NIR-300/F (Allied Vision Technologies
2 GmbH, Germany) model cameras with InGaAs sensors. NIR camera image resolution is 320x256 pixels.
3 For the *Setaria* experiment imaging configurations please refer to Supplemental Table SII for LemnaTec
4 hardware control software settings.

5

6 **PlantCV Image Processing and Data Capture**

7

8 For a list of current measurements extracted by PlantCV please refer to Supplemental Table SIII. For
9 more information on image processing pipelines please refer to Supplemental Methods and for an
10 example pipeline workflow see Supplemental Fig. S11. Images were processed using 10 CPU (Intel Xeon
11 E7-8867L) on a Dell PowerEdge M910 blade server running CentOS 6.5.

12

13 **VIS images:** For *Setaria*, four levels of optical zoom were used to capture VIS side-view images (1.4X,
14 1.6X, 3.1X and 3.9X zoom). The same image processing strategy was applied to all zoom levels, except
15 that with 1.3X and 1.5X zoom configurations black background sidebars become visible in images, which
16 requires extra steps for processing. For VIS top-view images six levels of zoom were used (1.0X, 1.3X,
17 2.3X, 3.1X, 3.5X, and 3.9X zoom) and the image processing strategy was generally the same as side-view
18 images except that a pre-made mask was applied to remove brass pieces of the lifter that were visible
19 from the top-view. A Perl script parallelized the execution of PlantCV image analysis pipelines over a set
20 of images and also wrote resulting shape and color data to an SQLite database. Zoom-correction of shape
21 traits, calculation of multivariate traits (e.g. height-width ratio), and size normalization of plant color
22 histograms, was executed with secondary Python analysis scripts over the SQLite database (Supplemental
23 Table SIV).

24

25 **PSII images:** For the *Setaria* experiment, PSII images were captured at two lifter positions (LemnaTec
26 lifter positions 630 and 500). The same image processing strategy was applied to both lifter positions. For
27 more information on PSII image processing please refer to Supplemental Methods. A Perl script
28 parallelized the execution of PlantCV image analysis pipelines over a set of images and also wrote the
29 resulting PSII signal data to an SQLite database. Size normalization of PSII histograms was executed
30 with secondary Python analysis scripts over the SQLite database (Supplemental Table SIV).

31

32 **NIR images:** For the *Setaria* experiment, NIR images were captured at 3.9X, 3.1X and 1.4X zoom. The
33 same general image processing strategy was applied to all zoom levels. For more information on the NIR
34 image processing pipelines used please refer to the Supplemental Methods. A Perl script parallelized the

1 execution of PlantCV image analysis pipelines over a set of images and also wrote the resulting NIR
2 signal data to an SQLite database. Size normalization of NIR histograms was executed with secondary
3 Python analysis scripts over the SQLite database (Supplemental Table SIV).

4

5 Statistical Analysis and Modeling

6

7 All statistical analyses were done in R (R Core Team, 2014). Additional packages used include: analogue
8 (Simpson, 2007), car (Fox and Weisberg, 2011), emdist (Urbanek and Rubner, 2012), ggplot2 (Wickham,
9 2009), grid (R Core Team, 2014), gridExtra (Auguie, 2012), lattice (Sarkar, 2008), lme4 (Bates et al.,
10 2014), lubridate (Golemund and Wickham, 2011), MASS (Venables and Ripley, 2002), mvtnorm (Genz
11 and Bretz, 2009; Genz et al., 2014) nlme (Pinheiro et al., 2014) pls (Mevik et al., 2013), plyr (Wickham,
12 2011), qvalue (Dabney and Storey), rgl (Adler et al., 2014), scales (Wickham, 2014), and VennDiagram
13 (Chen, 2014). R scripts for all analyses are provided in Supplemental Table SIV.

14

15 **JPEG versus PNG format images:** The image datasets generated during an experiment have a relatively
16 large file storage footprint. An experimental image set extracted from the database with PhenoFront in
17 Portable Network Graphics (PNG) format with lossless compression is 200–300 gigabytes for a typical 4–
18 5 week experiment. To reduce the image storage footprint with a minimal loss of information content,
19 PhenoFront has the option to extract images in Joint Photographic Experts Group (JPEG) format with
20 lossy compression, resulting in an approximately 11-fold reduction in storage size. Individual image
21 compression ratios vary from ~7:1 to 20:1 depending on zoom level. The dataset presented here was
22 analyzed in both PNG and JPEG formats and a subset of plant phenotypes were compared to quantify
23 discrepancies between the image formats. Pearson’s correlation between JPEG and PNG traits extracted
24 by PlantCV was calculated using the cor.test function in the R stats package. Plant shape-based traits
25 (side-view area, top-view area and plant height from VIS images) were highly correlated ($r > 0.997$),
26 however the 2nd and 3rd PCs from RGB color analysis were less linear and less correlated ($r = -0.893$ and -
27 0.781, respectively; Supplemental Fig. S12). Therefore, JPEG images are sufficient for analysis when
28 only shape and morphological features are needed (e.g. real-time image configuration feed-back), but
29 images with lossless compression are preferable when signal/spectral data is analyzed.

30

31 **Camera zoom correction:** To compare digital measurements across camera zoom levels, scaling factors
32 for pixel area and length were calculated by imaging a 13.2 x 13.2 x 3.7 cm box at a series of zoom levels
33 in the VIS imaging cabinet (Supplemental Fig. S13). For images at each zoom level, box pixel area and
34 width was measured with ImageJ (Abràmoff et al., 2004). Pixel area was scaled relative to the 1X zoom

1 level area and pixel length was scaled relative to actual box dimensions. For both relative area and
2 pixels/cm scaling factors, regression analysis was done to estimate a function that would predict scaling
3 factors for each zoom level. Non-linear least squares regression was done to fit an exponential model and
4 ordinary least squares regression was done to fit a 2nd order polynomial model to both relative area and
5 pixels/cm data sets. For each scaling factor, the model that minimized the Akaike's Information Criterion
6 (Bozdogan, 1987) was chosen. For relative pixel area, the resulting model was an exponential function:

$$A_r = 0.8854e^{0.0007224z}$$

7 where A_r is relative area and z is the VIS camera zoom setting (Supplemental Fig. S13A). For pixel/cm,
8 the resulting model was a 2nd-order polynomial function:

$$s = 14.27 + 0.002077z + 0.00000217z^2$$

9 s is the px/cm scaling factor and z is the VIS camera zoom setting (Supplemental Fig. S13B).

10

11 **Height modeling:** To determine the accuracy of PlantCV-estimated plant height, a validation set was
12 created by manually measuring height for 173 randomly selected VIS side-view images from all zoom
13 levels using ImageJ (Abràmoff et al., 2004) where height in cm was calibrated using the width of the
14 plant carrier (12 cm wide). The manual height measurement was drawn as a straight vertical line from the
15 top of the pot to the highest point of the plant and was an average of three independent measurements.
16 Ordinary least squares regression was used to evaluate how well PlantCV estimated manually measured
17 height (Fig. 3A; Supplemental Table SIV).

18

19 **Biomass modeling:** To estimate fresh- and dry-weight above ground biomass with PlantCV, a training set
20 was created by harvesting the above ground tissue of 41 randomly selected *S. viridis* and *S. italica* plants
21 from the 100% FC group after imaging throughout the experimental period. After collection, fresh-weight
22 measurements were recorded and plant tissue was placed in polypropylene micro-perforated bags (PJP
23 MarketPlace #361001) and dried for ~3 days at 60°C before dry weight was measured. To generate a
24 model for predicting fresh- and dry-weight biomass, multiple linear regression analysis was done,
25 followed by model selection, to determine the best predictor variables of biomass. The initial full model
26 for fresh-weight biomass included terms for total VIS side-view pixel area, VIS top-view pixel area, plant
27 height, and all interactions. A stepwise procedure was used to test the effect of dropping terms from the
28 full model using the Akaike's Information Criterion (Bozdogan, 1987). The initial reduced model had
29 coefficients that were not significant, so a second reduced model that had total VIS side-view area as the
30 only explanatory variable was generated. Analysis of variance was done to compare the reduced models
31 and indicated that the residual error for the models was not significantly different, so the smaller model

1 was kept (see Results and Discussion; Fig. 4A). Total VIS side-view was used to predict dry weight
2 without model selection (Supplemental Fig. S2):

$$M_{dw} = 4.634 \times 10^{-6} A_{sv} - 5.295 \times 10^{-2}$$

3 For both fresh- and dry-weight models, adding an indicator variable for plant out-of-frame status
4 improved the models, but adding an indicator variable for genotype (*S. viridis* or *S. italica*) did not
5 improve the models.

6

7 **Growth-rate modeling:** *Setaria* plant growth patterns appeared to be asymptotic. Non-linear least squares
8 regression analysis was used to model a three-component logistic growth function for each group
9 (genotype by treatment) (Paine et al., 2012). Absolute growth rate over time (first derivative of the growth
10 function) was computed for each group (Paine et al., 2012). The growth models were used to estimate the
11 maximum absolute growth rate and the day it was reached for each group. Analysis of treatment effects
12 for *S. viridis* and *S. italica* were done by computing the differences in mean biomass between treatments
13 once treatment begins from 18–33 DAP using the logistic growth functions. For the difference in means
14 on each day, an adjusted 95% confidence interval was computed (Bonferroni correction), and intervals
15 that did not contain zero were considered significant. For the effect of treatment on *S. viridis* and *S. italica*
16 height, ordinary least squares regression was done using plant height from 21–33 DAP (after zoom
17 change put plants back in frame) to compare whether the vertical growth rates (g/day) for the 100% and
18 33% treatment groups were significantly different.

19

20 **Plant architecture modeling:** For 58 randomly selected VIS side-view images (25 and 26 DAP), tillers
21 were counted and ImageJ (Abràmoff et al., 2004) was used to manually measure tiller-angle and leaf
22 angle. Tiller angle was defined by the angle between two outermost tillers. Leaf angle was measured as
23 the average between first leaf angles of the two outermost tillers. All measurements were performed at
24 least three times and the average values were used for further analysis. The averaged results were
25 compared to height-width ratio (HW, height divided by extent-x) and solidity (plant area divided by
26 convex hull area) by cor.test function in R. HW was significantly correlated ($p < 0.01$) with all three
27 manually measured traits. A stepwise selection by Akaike's Information Criterion (Bozdogan, 1987) was
28 then used to generate a model that best explains HW:

$$HW = -0.017599A_{leaf} - 0.01298A_{tiller} + 2.696608$$

30 where HW is height-width ratio, A_{leaf} is leaf angle and A_{tiller} is tiller angle.

31

32 To generate the tiller count model, tillers were counted from 195 randomly selected VIS images. The
33 number of tillers was counted at least three times and the average values were used for further analysis.

1 Tiller number was correlated with biomass, height-width ratio, solidity, extent-x, and height. Height-
2 width ratio was calculated based on zoom-corrected plant height and extent-x values generated by
3 PlantCV. A stepwise selection by Akaike's Information Criterion (Bozdogan, 1987) was then used to
4 generate a model that best explains tiller count. Tillers were counted for second set of 660 images and the
5 predictive power of the tiller count model was assessed.

6

7 **Color analysis:** 17 to 18 DAP and 25 to 26 DAP, histograms of red, green, and blue color signal were
8 extracted by PlantCV from *S. viridis* under 100% and 33% FC. Size-normalized data from each angle was
9 averaged by PlantCV. Histogram values that were zero in all samples were removed, and the remaining
10 data was used as explanatory variables in PCA. Principle component analysis was calculated using the
11 prcomp function in the R software stats package. The principal component regression function pcr from
12 the pls R package, regressed treatment group over the principal components of color data 17 to 18 DAP
13 and 25 to 26 DAP.

14

15 Color data was also assessed to see if *Setaria* genotypes could be separated before water treatment was
16 applied. Histograms of red, green, and blue color signal were extracted by PlantCV from all ten *Setaria*
17 genotypes under 100% and 33% FC 11 to 12 DAP. Size-normalized data from each angle was averaged
18 by PlantCV. Histogram values that were zero in all samples were removed, and the remaining data was
19 used as explanatory variables in PCA.

20

21 **F_v/F_m signal analysis:** An area-normalized histogram of F_v/F_m signal was calculated from PSII top-view
22 images using PlantCV. The Spearman correlation between median F_v/F_m signal value and estimated plant
23 height extracted by PlantCV was calculated with the cor.test function in the R stats package. To further
24 test the correlation between F_v/F_m signal and height a 3D-printed ‘plant’ was made. 5 cm tall rectangular
25 ‘stem’ and flat ‘leaf’ platform pieces were designed using openSCAD (<http://www.openscad.org/>) and 3D
26 printed using a Stratasys Fortus 250mc and ABSplus plastic. Four stem and leaf pieces were assembled
27 using Oatey all-purpose cement, so that each platform was visible from a top view angle. *N. benthamiana*
28 leaf tissue was excised and segmented into four ~4 cm² samples (avoiding the leaf mid-vein). Elmer’s
29 multipurpose spray adhesive was used to make platform pieces tacky and leaf samples were adhered to
30 each platform level. The bottom stem piece was positioned in a pot filled with blue non-fluorescent gravel
31 so the first leaf piece was flush with the gravel (referred to as 0 cm; Supplemental Fig. S9C). 3D plant
32 was PSII imaged and the process was repeated with a second *N. benthamiana* leaf. Resulting PSII images
33 were analyzed with PlantCV with masks to segment each platform level. Pearson’s correlation between
34 leaf height and median F_v/F_m was calculated using the cor.test function in the R stats package. Designs for

1 3D plant model pieces can be found at <https://www.thingiverse.com/thing:574287> and paths to analysis
2 scripts can be found in Supplemental Table SIV.

3
4 **NIR signal analysis:** Principle components were calculated using the prcomp function encoded by the R
5 software package. PCA was performed within each zoom level for *Setaria* with full and 33% FC water
6 treatments. The resulting PC scores for each of the four side view images were averaged. Significance
7 testing between treatments over time was applied to a rolling two day time period because plants were
8 imaged over two days. The number of replicates is not identical across genotypes and days. Therefore the
9 maximum equal number of individuals was chosen randomly from the larger set and used as a
10 representative sample for hypothesis testing. For each genotype on each experimental day, the
11 distributions of phenotypic values between treatments (100% FC versus. 33% FC) were compared using
12 the t.test function in R.

13
14 **Integrated trait analysis:** The contribution of experimental factors to phenotypic variance was assessed
15 using the lmer function encoded by the lme4 library in R (Bates et al., 2014). Variance partitioning was
16 estimated by linear modeling of phenotypic values as a function of genotype, treatment (100% and 33%
17 FC) and the interaction of genotype and treatment (specified as random effects) at four time-points (15-
18 16, 19-20, 25-26, 31-32 DAP). Variance estimates associated with each factor were extracted from the
19 resultant merMod model object using the VarCorr function and divided by total phenotypic variance to
20 estimate proportion of variance explained by each experimental factor. Trait correlation was assessed at
21 the same four time points and treatments using the cor function in R.

22
23 **SUPPLEMENTAL DATA**

24
25 The following materials are available in the online version of this article.

26
27 **Supplemental Methods.** Extended image-processing methods.

28
29 **Supplemental Figure S1.** Plant height estimated with PlantCV for ten *Setaria* genotypes.

30
31 **Supplemental Figure S2.** Above ground dry-weight biomass modeled using shoot and leaf pixel area
32 from four side-view VIS images.

33

- 1 **Supplemental Figure S3.** Modeled fresh-weight biomass estimated with PlantCV for ten *Setaria*
2 genotypes.
3
4 **Supplemental Figure S4.** Tiller count estimated with PlantCV for ten *Setaria* genotypes.
5
6 **Supplemental Figure S5.** Color can separate watering treatments 25 to 26 DAP, but not 17 to 18 DAP in
7 RGB, HSV and LAB color space.
8
9 **Supplemental Figure S6.** Loadings of color data 25 to 26 DAP and 17 to 18 DAP.
10
11 **Supplemental Figure S7.** Before water treatment is applied (11 to 12 DAP) color can distinguish *Setaria*
12 genotypes in RGB color (Fig. 6), which is also consistent in HSV and LAB color space.
13
14 **Supplemental Figure S8.** Loadings of color data 11 to 12 DAP.
15
16 **Supplemental Figure S9.** Height significantly correlates with median F_v/F_m .
17
18 **Supplemental Figure S10.** Matrices displaying Pearson's Correlation Coefficient calculated between
19 traits at four roughly equidistant time points.
20
21 **Supplemental Figure S11.** A visual illustration of the pipeline used to threshold plant tissue from
22 background within grayscale NIR images.
23
24 **Supplemental Figure S12.** JPEG images are sufficient for analysis when only shape and morphological
25 features are needed.
26
27 **Supplemental Figure S13.** Zoom-correction scaling factors for pixel area and pixel length.
28
29 **Supplemental Table SI.** Comparison of image-analysis software.
30
31 **Supplemental Table SII.** Camera configuration parameters.
32
33 **Supplemental Table SIII.** Description of measurements output by PlantCV.
34

1 **Supplemental Table SIV.** PlantCV image processing and analysis scripts.

2

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4

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Variance Type	Biomass	Height	Tiller Count	WUE	RGB.PC1	RGB.PC2	NIR.PC1	DAP
Genotype	14.8	61.3	53.2	20.6	75.1	65.8	38.9	15
Treatment	0	0	0	0	0	0	0	
Genotype x Treatment	5.2	2.4	0	5	0	1.8	2.5	
Residual	80	36.3	46.8	74.5	24.9	32.4	58.6	
Genotype	17.9	22.8	36.5	20.2	62.6	39.4	29.5	19
Treatment	4.8	0.4	2.8	27.8	0	0.3	6.7	
Genotype x Treatment	7.2	5.3	5.2	4.4	0	1	5	
Residual	70.1	71.5	55.5	47.5	37.4	59.4	58.8	
Genotype	14.4	65.4	11.5	22.2	66.3	60.9	30.5	25
Treatment	55.4	1.8	66.9	8.2	13.9	0	56.7	
Genotype x Treatment	4.6	4	6.7	5.6	0.9	7.3	4.1	
Residual	25.5	28.8	14.9	64	18.9	31.8	8.7	
Genotype	2.7	42.8	9.8	7.7	62.5	8.3	8.3	31
Treatment	83.7	7.3	78.9	8	7.7	1	75.5	
Genotype x Treatment	2.1	8	1.9	0	5.6	13.9	3.8	
Residual	11.5	41.9	9.4	84.3	24.1	76.8	12.5	

1

2 **Table I.** Proportion of variance explained by experimental factors. Variance associated with factors
3 genotype, experimental treatment, the interaction of genotype and treatment was assessed at four time
4 points (15, 19, 25, 31 DAP). Unexplained variance is denoted as residual variance.

1 **FIGURE LEGENDS**

2
3 **Figure 1.** Bellwether Phenotyping Platform at the Donald Danforth Plant Science Center. A, Diagram of
4 the Bellwether Phenotyping Platform. B, The volume of water added to *S. viridis* plants during morning
5 (ZT23) and evening (ZT8) water applications. The arrows indicate the day that the 33% FC water
6 treatment started.

7
8 **Figure 2.** PlantCV image analysis software developed at the Donald Danforth Plant Science Center. A,
9 Automated identification of plant material from background. Plant is identified (blue outline, right) from
10 the original image (left). B, Example of traits extracted by PlantCV. Shape characteristics such as convex
11 hull (left), and height (middle) are extracted from visible images. Differences in color can be visualized
12 by pseudocoloring by HSV, LAB or RGB color channels (right). *S. viridis* is pseudocolored based on the
13 value channel of HSV color space. C, PlantCV analysis of traits over time under 100% FC and 33% FC
14 water treatments. Traits such as biomass accumulation, growth-rate, and color can be tracked over time
15 under experimental conditions, such as drought, from data collected by PlantCV. *S. viridis* is shown
16 pseudocolored based on the value channel of HSV color space. D, Example of PlantCV extracted traits
17 from plant species other than *Setaria*. *Brachypodium distachyon*, *Arabidopsis thaliana*, and *Camelina*
18 *sativa* are pictured from left to right. Plants are pseudocolored based on the value channel of HSV. E,
19 Example of PlantCV analysis of an image of cassava captured by a cell phone camera. F, Example of
20 PlantCV analysis of an image of sweet potato captured by a digital camera.

21
22 **Figure 3.** Plant height estimated with PlantCV. A, Plant height estimated with PlantCV compared to
23 manually measured height. Data shown are for 173 randomly selected VIS side-view images at four
24 different zoom levels. The ordinary least squares linear model with standard error is plotted. B, Estimated
25 plant height for *S. viridis* and *S. italica* plants from 11–33 DAP. Plants watered to 100% or 33% FC are
26 shown. Local regression (LOESS) fitted curves with standard error are plotted for each genotype by water
27 treatment group. The arrows indicate the day that the 33% FC water treatment started.

28
29 **Figure 4.** *S. viridis* and *S. italica* respond differently to water-limited conditions. A, Above ground fresh-
30 weight biomass modeled using shoot and leaf pixel area from four side-view VIS images. Data shown are
31 for 41 plants where destructive fresh-weight biomass was measured throughout the experiment. The
32 ordinary least squares linear model with standard error is plotted. B–D, data shown are for *S. viridis* and
33 *S. italica* imaged from 11–33 DAP. Plants watered to 100% or 33% FC are shown. Arrows indicate the
34 day that the 33% FC water treatment started. B, Modeled fresh-weight biomass. Three-component logistic

1 growth curves with 95% confidence intervals are plotted. C, Absolute growth rates over time with 95%
2 confidence intervals. D, Water-use efficiency, accumulated fresh-weight biomass divided by cumulative
3 water added. LOESS fitted curves with standard error are plotted. C and D, see color key in B.

4

5 **Figure 5.** Tiller count modeled from spatial-independent morphological characteristics. A, Examples of
6 height-width ratio variation from randomly selected images of plants 25–26 DAP. B, Added-variable
7 plots of fresh-weight biomass and height-width ratio from 195 randomly selected images. C, Relationship
8 between manually measured tiller count from 646 randomly selected images and model-predicted tiller
9 count. D, Model-predicted tiller count for *S. viridis* and *S. italica* plants from 11–33 DAP. Plants watered
10 to 100% or 33% FC are shown. Arrows indicate the day that the 33% FC water treatment started.

11

12 **Figure 6.** Before water treatment is applied (11 to 12 DAP) color can distinguish *Setaria* genotypes. A,
13 PCA of RGB color for all *Setaria* genotypes before treatment is applied (11 to 12 DAP). PC1 and PC2 are
14 plotted for *S. viridis* (orange), *S. italica* (green), RIL161 (purple) and 7 other *S. viridis* x *S. italica* RILs
15 (gray). B, *S. italica* (yellow circle in A; yellow boxed image) found amongst R161 (blue circle in A; blue
16 boxed image) are more similar in color than *S. italica* (red circle in A; red boxed image) that groups with
17 other lines (gray). VIS images are pseudocolored based on the value channel in HSV color space.

18

19 **Figure 7.** Measurements derived from the NIR camera system. A, Average NIR signal distribution of
20 well-watered plants throughout the experiment. Bins corresponding to signal intensity levels 0 – 255 are
21 plotted along the x-axis, while experimental days are plotted along the y-axis. The proportion of pixels
22 that fall into each bin are expressed as a grayscale shade (0% black, larger percentages are increasingly
23 more white). Color bars along the y-axis illustrate zoom range (3.9X = light blue, 3.1X = blue, 1.4X =
24 dark blue). B, Difference of average NIR signal distribution between plants receiving 100% and 33% FC.
25 Signal bins shaded blue have an increased proportion of pixels in images of plants receiving 100% FC
26 water; signal bins shaded red have a greater proportion of pixels in images receiving 33% FC water; white
27 corresponds to no difference between images. C, A plot of PC1 loadings (3.9X = light blue, 3.1X = blue,
28 1.4X = dark blue). D, A plot of the average signal difference between water treatment levels 100% and
29 33% FC (3.9X = light blue, 3.1X = blue, 1.4X = dark blue).

30

31 **Figure 8.** NIR PC1 throughout time while zoom = 1.4X for *Setaria* genotypes *S. viridis* (A10) and *S.*
32 *italica* (B100). Plants watered to 100% or 33% FC are shown. Local regression (LOESS) fitted curves
33 with standard error are plotted for each genotype by water treatment group.

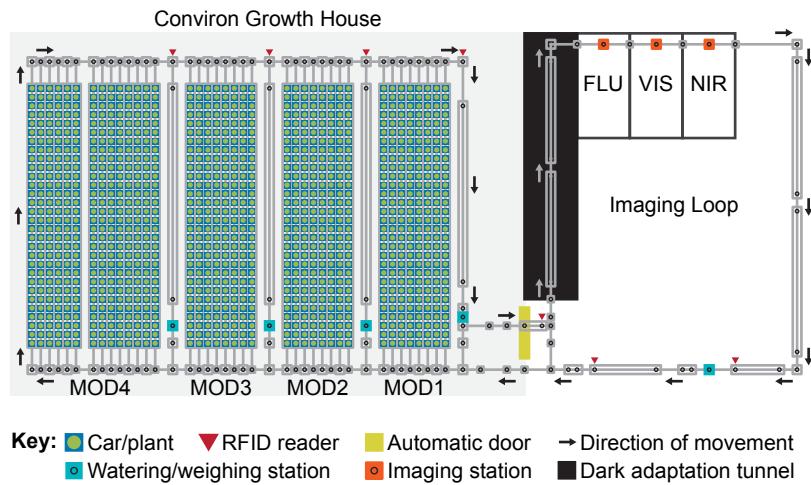
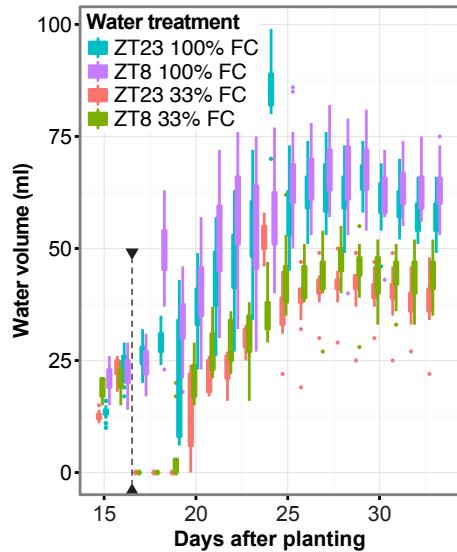
A**B**

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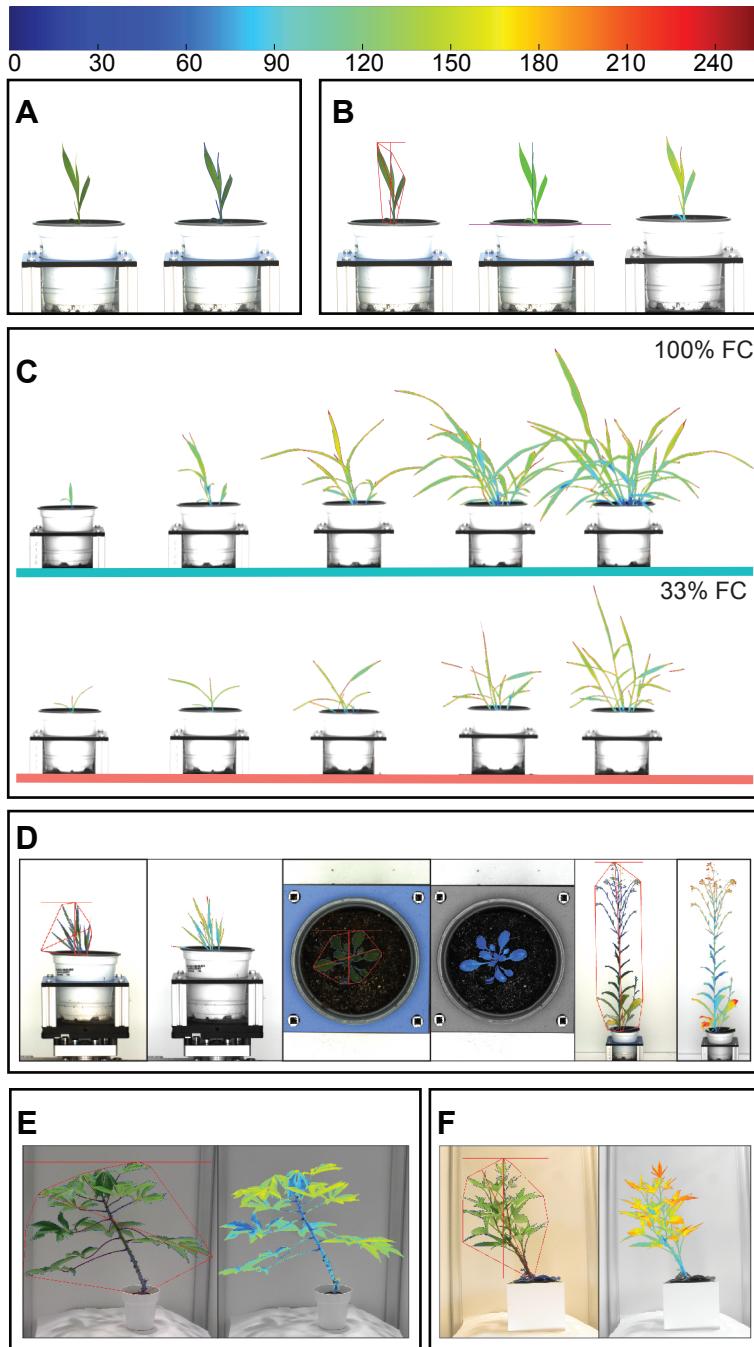


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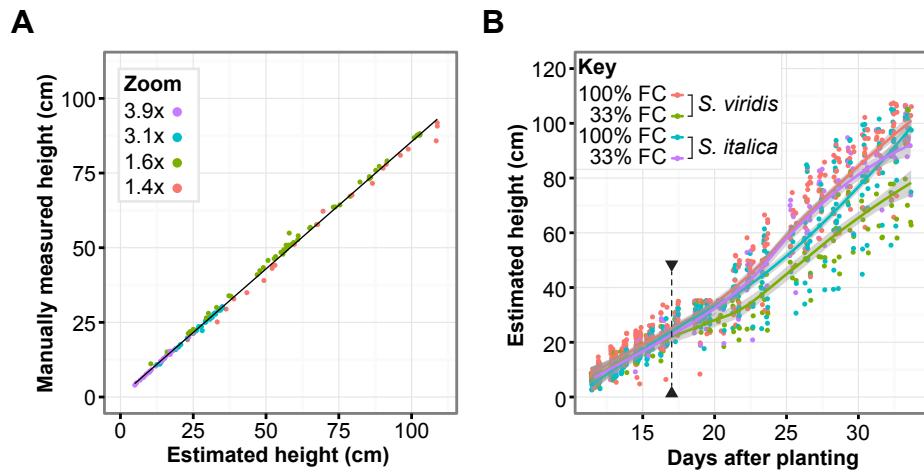


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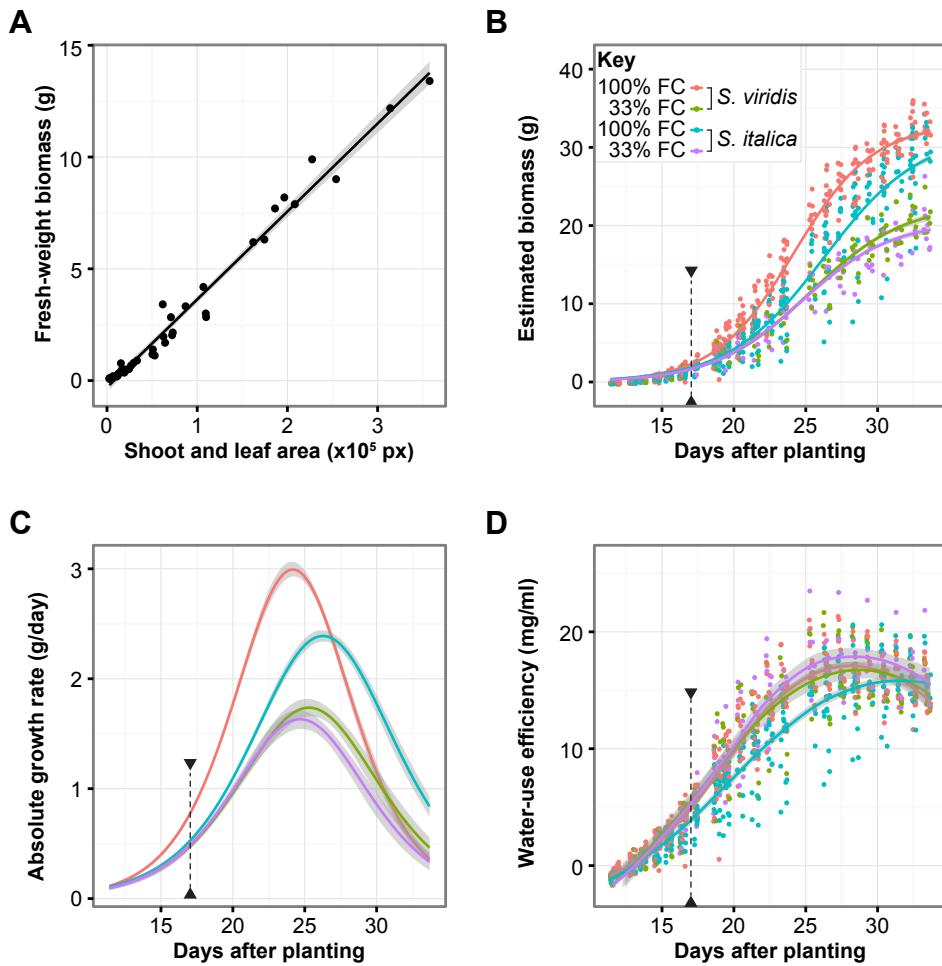


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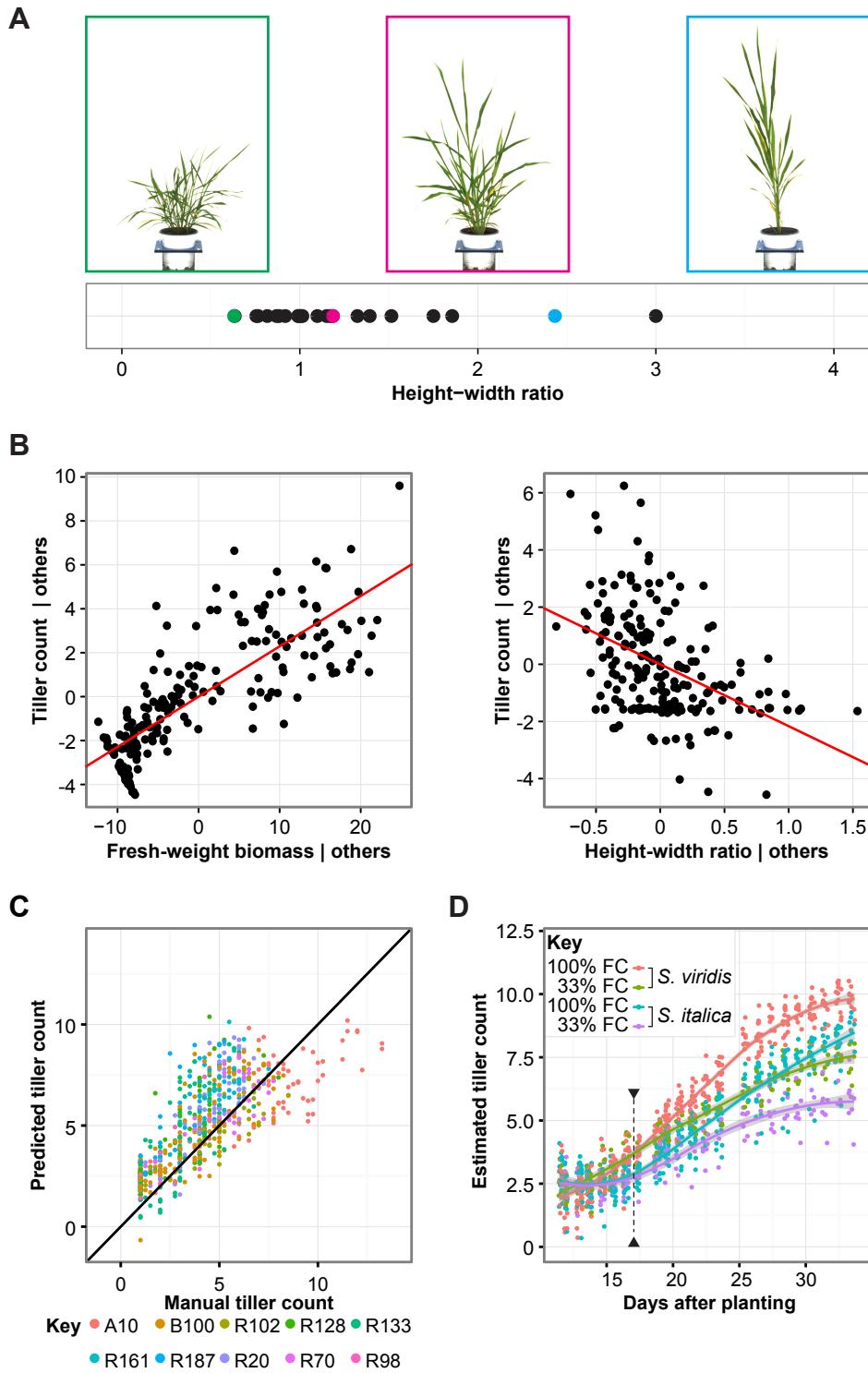


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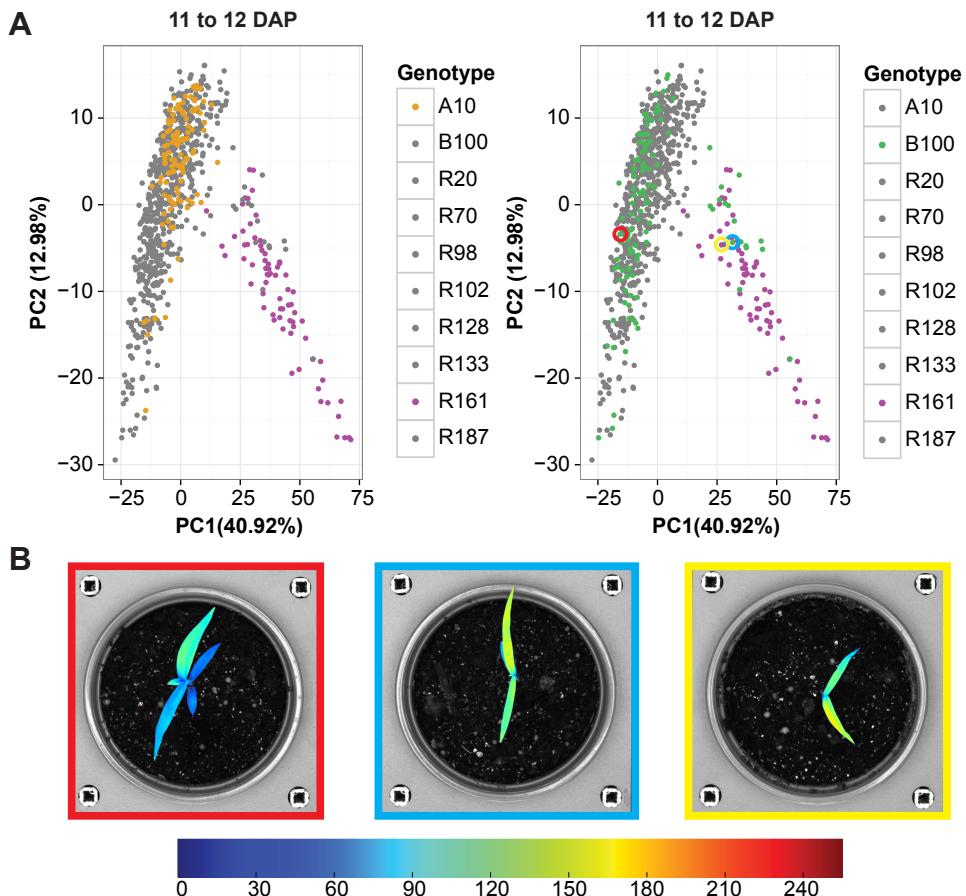


Figure 6. Before water treatment is applied (11 to 12 DAP) color can distinguish *Setaria* genotypes. A, PCA of RGB color for all *Setaria* genotypes before treatment is applied (11 to 12 DAP). PC1 and PC2 are plotted for *S. viridis* (orange), *S. italica* (green), RIL161 (purple) and 7 other *S. viridis* x *S. italica* RILs (gray). B, *S. italica* (yellow circle in A; yellow boxed image) found amongst R161 (blue circle in A; blue boxed image) are more similar in color than *S. italica* (red circle in A; red boxed image) that groups with other lines (gray). VIS images are pseudocolored based on the value channel in HSV color space.

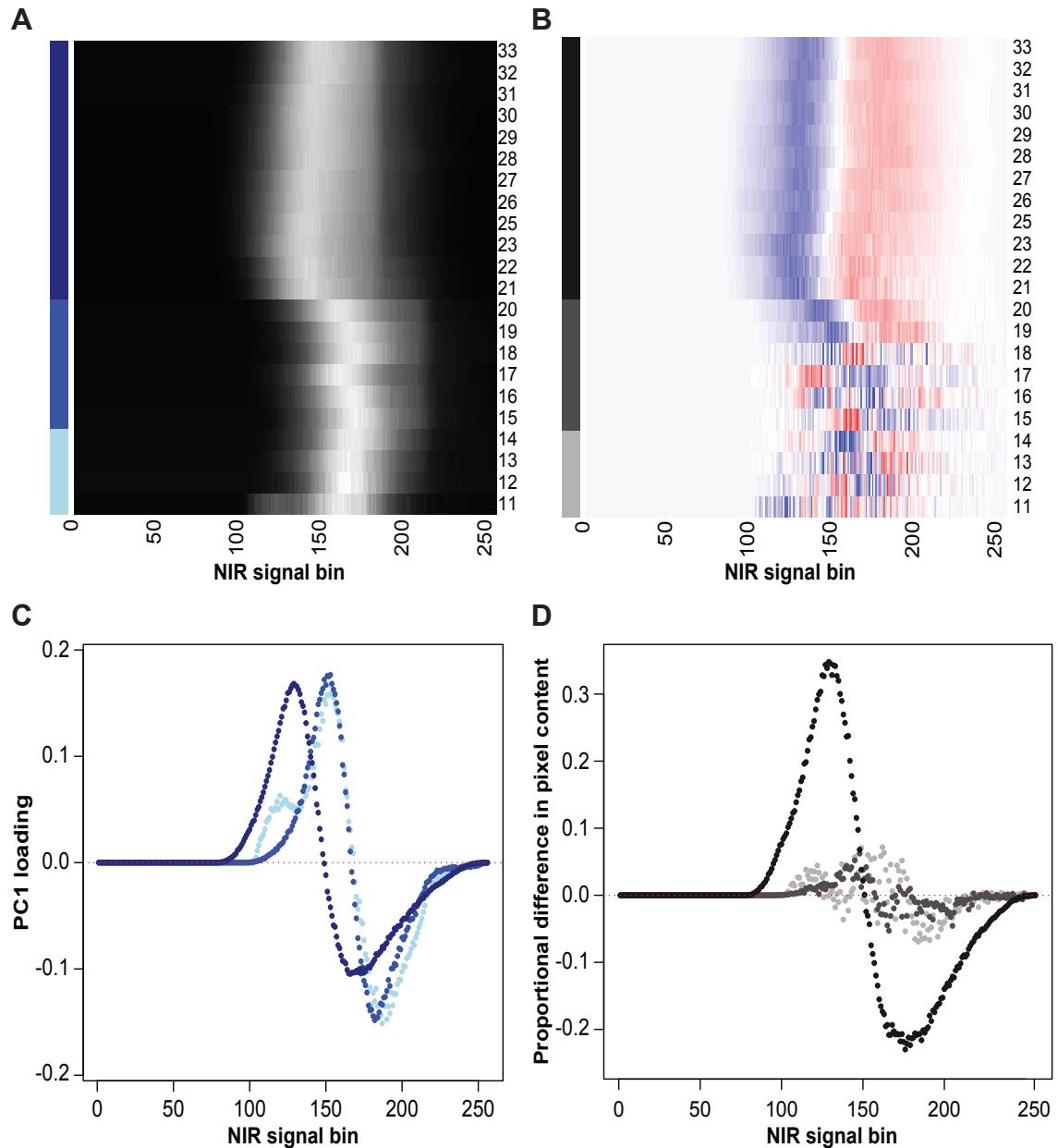


Figure 7. Measurements derived from the NIR camera system. A, Average NIR signal distribution of well-watered plants throughout the experiment. Bins corresponding to signal intensity levels 0 – 255 are plotted along the x-axis, while experimental days are plotted along the y-axis. The proportion of pixels that fall into each bin are expressed as a grayscale shade (0% black, larger percentages are increasingly more white). Color bars along the y-axis illustrate zoom range (3.9X = light blue, 3.1X = blue, 1.4X = dark blue). B, Difference of average NIR signal distribution between plants receiving 100% and 33% FC. Signal bins shaded blue have an increased proportion of pixels in images of plants receiving 100% FC water; signal bins shaded red have a greater proportion of pixels in images receiving 33% FC water; white corresponds to no difference between images. C, A plot of PC1 loadings (3.9X = light blue, 3.1X = blue, 1.4X = dark blue). D, A plot of the average signal difference between water treatment levels 100% and 33% FC (3.9X = light blue, 3.1X = blue, 1.4X = dark blue).

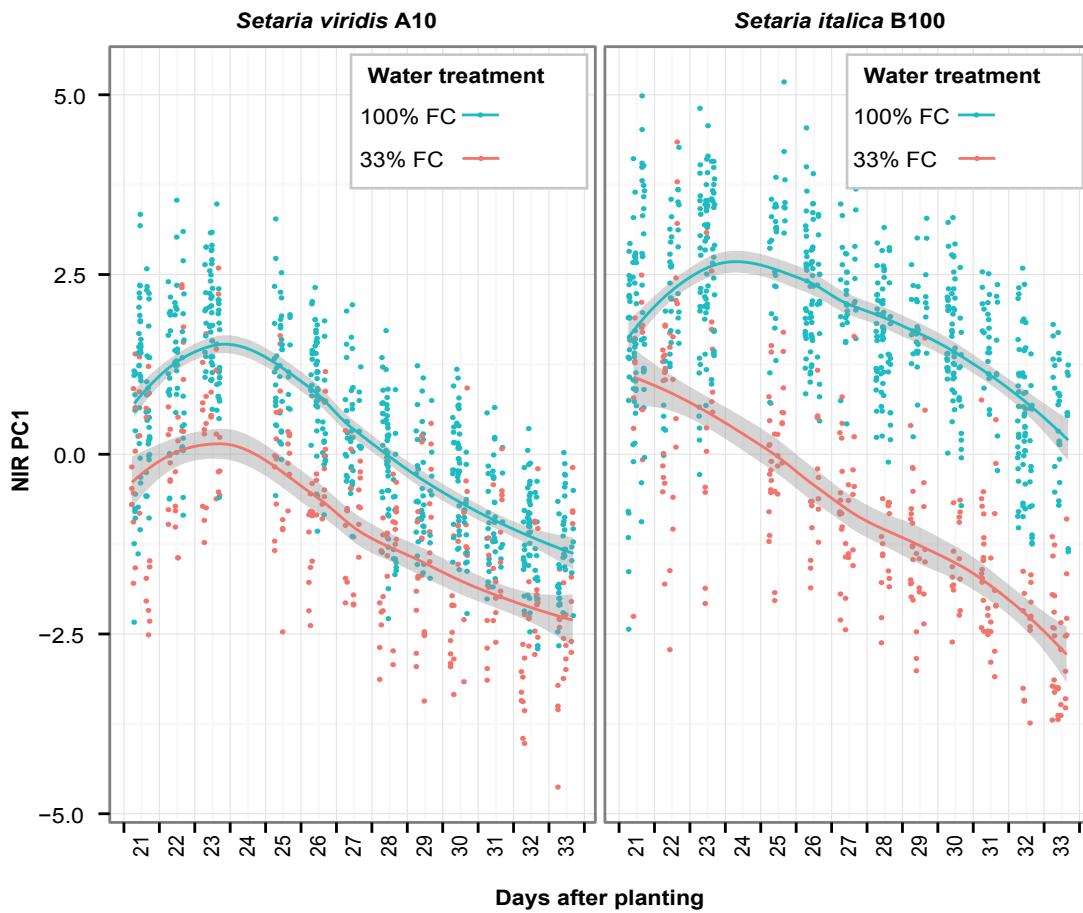


Figure 8. NIR PC1 throughout time while zoom = 1.4X for *Setaria* genotypes *S. viridis* (A10) and *S. italica* (B100). Plants watered to 100% or 33% FC are shown. Local regression (LOESS) fitted curves with standard error are plotted for each genotype by water treatment group.

1 **SUPPLEMENTAL DATA**

2

3 **SUPPLEMENTAL METHODS**

4

5 **Image Processing**

6

7 The following describe the image processing pipelines and outlier flagging algorithms used to process
8 this *Setaria* experiment. Paths to image processing and trait extraction pipelines can be found in
9 Supplemental Table SIV. For further details on individual functions please refer to the documentation at
10 <http://plantcv.danforthcenter.org/>.

11

12 **VIS image processing:** For 1.4X and 1.6X side-view zoom configurations, the RGB image first was
13 converted to HSV color-space and the saturation channel was isolated. To remove most of the pot from
14 the image, a threshold was applied to the isolated saturation channel, which converted the image to
15 binary, with white pixels as objects. A median blur was then used to remove some of the noise from the
16 binary image.

17

18 The original RGB image was then converted to LAB color-space and the blue-yellow channel was
19 isolated. To remove most of the blue pot carrier from the image, a threshold was applied to the isolated
20 blue-yellow channel, which converted the image to binary, with white pixels as objects. The two binary
21 images from the saturation and blue-yellow channels were then joined together so that only pixels that
22 were white in both images were isolated. The joined binary image was then used to mask the original
23 RGB image so the pot and pot carrier were removed. This new masked image contained plant material as
24 well as residual background from the black sidebars in the background. By masking a portion of the non-
25 plant material from the original image a less stringent threshold could be applied to the masked image to
26 better isolate plant material.

27

28 The masked RGB image was converted to LAB color-space and both the blue-magenta, and green-yellow
29 channels were isolated and thresholded to produce two binary images that extract pixels containing plant
30 material from background. The two binary images were combined so object pixels from either image
31 were kept and small areas of noise were filled. The resulting binary image was used to mask the
32 previously masked image that had removed the pot and pot carrier.

33

1 In the resulting masked image, areas that contain black bars were isolated with a region-of-interest tool,
2 and plant material overlapping the black bars was separated with a series of thresholding fill, and masking
3 steps. After thresholding and masking, a binary image with the white object pixels overlapping the
4 sidebars was saved. The region of interest tool was used to define an area that encompasses everything
5 except the black background bars in the image, and object pixels were detected and combined with the
6 object pixels in the sidebars. The final binary object mask was used for object detection and the detected
7 objects were grouped together for extraction of shape characteristics. The plant color analysis function
8 also used this final mask to isolate plant pixels for color analysis. VIS shape and color traits currently
9 extracted by PlantCV are described in more detail in Table 3 and the documentation of PlantCV software
10 (<http://plantcv.danforthcenter.org/>). For analysis of images at 3.1X and 3.9X magnification, background
11 sidebars were no longer visible in images so sidebar steps were not included in plant identification
12 pipelines.

13

14 **PSII image processing:** First a pre-made mask for the pot carrier screws that auto-fluoresce was
15 converted to a binary image and applied to an 8-bit color version of the 16-bit grayscale F_{max} image. The
16 8-bit color F_{max} image was used to create a mask to isolate plant pixels, which was later applied to the 16-
17 bit grayscale images. A binary threshold was applied to the 8-bit F_{max} image with masked screws and a
18 median blur and fill step was used to reduce some of the noise. Objects (white pixels) were identified
19 within the resulting image. The region of interest tool was then used to define a large area that
20 encompasses the plant (centered on the pot carrier position that is consistent in all images). Objects that
21 were within or partially within the region of interest were kept. A binary image with the kept objects was
22 saved as an image mask. The kept objects were also grouped together for shape analysis. Shape data was
23 used as a quality control of plant detection in image processing. The mask of the kept objects was then
24 applied to the F_0 , F_{min} and F_{max} images and F_v/F_m measurements were calculated over a matrix of
25 identified plant pixels.

26 The F_0 image was captured before the fluorescence inducing light-pulse and was therefore used as a null
27 standard in each image set. If the maximum signal in the null image surpasses a threshold, the image set
28 was flagged so the user may further examine if there were problems during image capture. In this data set
29 no F_0 images were flagged as outliers. F_v/F_m , was calculated for each plant pixel in F_{min} and F_{max} images
30 ($F_{max}-F_{min}/F_{max}$), and a histogram of F_v/F_m values was built using 1000 bins (values from 0 to 1).

31 **NIR image processing:** NIR images derived from pots without plants captured throughout the experiment
32 were averaged within each zoom level and were used to calculate background signal. These zoom specific
33 averages of background signal intensity were then subtracted from the image matrix of interest (plant-

1 containing). Binary thresholding was then performed on the background-subtracted matrix, isolating plant
2 material from image background components.

3
4 Further refinement in plant capture was achieved using an image sharpening approach similar to the
5 detection methods developed to enhance visualization of skeletal systems in x-ray images (Gonzalez and
6 Woods, 2007). Laplacian filtering (second derivative) was applied to the original grayscale image to
7 detect the boundary between plant material and background components. Edges between plant and
8 background were subsequently sharpened by subtracting the Laplacian-filtered image from the original.
9 Other, more ambiguous boundaries and texture changes within the image were then detected using Sobel
10 filtering (first derivative) applied across both the x and y-axis of the original image. The resulting Sobel
11 filtered (both x and y-axis) images were then combined through addition and subsequently smoothed
12 using a median filter to remove noise artifacts generated during application of the derivative filter. The
13 resulting smoothed, Sobel filtered image was then subtracted from the Laplacian filtered image; further
14 sharpening the contrast between plant material and background components. Image sharpening enhanced
15 the ability to isolate plant material from background using binary thresholding. After thresholding, the
16 resultant binary images then underwent four parallel erosion steps (each step removed any focal pixel
17 with less than two adjacent non-zero pixels) to remove remaining background noise.

18 This binary image was combined with the binary image obtained from the background-subtraction routine
19 described above using a logical ‘OR’ join statement. Finally, problematic background components such
20 as the cart/pot, and image chamber paneling were excluded from the image through application of image
21 masks. Grayscale NIR pixel-level intensity of plant material was summarized into a histogram containing
22 256 bins, where each bin contains the proportion of plant pixels exhibiting the corresponding grayscale
23 intensity value.

24 To isolate pots with *Setaria* plants that did not germinate, values of side-view area and extent y were
25 calculated at each zoom level with pots known to be empty. NIR images with side-view area and extent-y
26 values equal to or less than the maximum value observed in empty-pot NIR images were removed from
27 the dataset. In total 1083 images were filtered out of the *Setaria* dataset using NIR side-view area and
28 extent-y criterion (691 at 3.9X zoom; 36 at 3.1X zoom; 356 at 1.4X zoom).

29 To identify potential NIR image plant detection problems, plant area ($r = 0.992$), extent y ($r = 0.971$) and
30 perimeter ($r = 0.962$) were correlated between VIS and NIR images. Images flagged for growing outside
31 of the field of view in VIS images, but not outside the field of view in NIR images, were omitted from the
32 shape outlier detection routine. NIR images where measurement residual error was greater than two

1 standard deviations from the predicted model values were flagged as potential outliers. Chi-square
2 distance and Earth Mover's Distance metrics were used to compare histograms of NIR signal between
3 image angles, and sample groups (genotype x treatment x date). Chi-square and Earth Mover's Distances
4 were calculated in R using the distance function encoded by the analogue package (Simpson, 2007) and
5 the emd2d function in the emdist library (Urbanek and Rubner, 2012), respectively. Image histogram
6 distance values that were greater than two standard deviations from the mean were flagged as potential
7 outliers. Manual examination of pseudo-colored (colored by plant area identified) NIR images flagged as
8 outliers found that plant capture was adequate in these images so no additional images were removed
9 from the dataset.

10 **SUPPLEMENTAL REFERENCES**

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12 Upper Saddle River.
13
14 **Simpson GL** (2007) Analogue Methods in Palaeoecology: Using the analogue Package. J Stat Softw **22**:
15 1–29.
16
17 **Urbanek S, Rubner Y** (2012) emdist: Earth Mover's Distance.
18
19

Feature	PlantCV	IAP	HTPheno	EBIImage	LemnaTec***
Image Types*	Color, Grayscale, PSII	Color, Grayscale	Color	Color, Grayscale	Color, Grayscale, PSII
Community Contribution	Via GitHub	Contact Authors	Contact Authors	Contact Authors	--
Database Type	SQLite	MongoDB	--	--	--
Extendibility	Python Modules**	IAP Plugin	ImageJ Plugins	R Packages	--
Image Analysis Functions	48	51	12	32	111
Interface	Command Line	GUI or Command Line	ImageJ/GUI	R (GUI or Command Line)	GUI
Language	Python	Java	Java	R	--
License	GPLv2	GPLv2	GPLv2	LGPL	Commercial
Scalability/ Parallelization	Multi-thread	Grid	--	--	Grid

1 **Supplemental Table SI.** Comparison of image-analysis software. *Color (e.g. VIS cameras), Grayscale
2 (e.g. NIR, IR, FLU, IR, or thermal-infrared cameras), PSII (saturating-pulse FLU camera system).
3 **Examples of Python libraries to extend PlantCV function include OpenCV (Python), SciPY, NumPy,
4 and MatPlotLib. ***LemnaLauncher v4.x.
5

1 **A VIS Image Configurations**

2

Days After Planting	Camera	Exposure	Gain	Iris	Focus	Zoom Setting	Optical Zoom	Lifter
11 to 14	side-view	110	0	1500	1900	3500	3.9X	2
15 to 16	side-view	110	0	1500	1900	2500	3.1X	2
17 to 20	side-view	110	0	1500	1900	2500	3.1X	2
21	side-view	110	0	1500	1900	500	1.4X	1
22 to 31	side-view	110	0	1500	1900	700	1.6X	1
32 to 33	side-view	110	0	1500	1900	500	1.4X	1
11 to 14	top-view	110	0	1500	800	3500	3.9X	2
15 to 16	top-view	110	0	1500	800	3000	3.5X	2
17 to 20	top-view	110	0	1500	800	2500	3.1X	2
21	top-view	110	0	1500	800	1500	2.3X	2
22 to 31	top-view	110	0	1500	800	300	1.3X	2
32 to 33	top-view	110	0	1500	800	1	1X	2

3

4 **B PSII Image Configurations**

5

Days After Planting	Camera	Gain	Focus	Pulse Power	Shutter	Lifter
11 to 31	top-view	100	4800	1200	500	630
32 to 33	top-view	100	4800	1200	500	500

6

7 **C NIR Image Configurations**

8

Days After Planting	Camera	Exposure	Gain	Iris	Focus	Zoom Setting	Optical Zoom	Lifter
11 to 14	side-view	90	0	2000	1200	3500	3.9X	2
14 to 20	side-view	90	0	2000	1200	2500	3.1X	2
21 to 33	side-view	90	0	2000	1200	500	1.4X	1

9

10 **Supplemental Table SII.** Camera configuration parameters. A, LemnaTec control software VIS image
 11 configuration settings for *Setaria* experiment. B, LemnaTec control software PSII image configuration
 12 settings for *Setaria* experiment. C, LemnaTec control software NIR image configuration settings for
 13 *Setaria* experiment.

14

1 **PlantCV Output Measurements**

2

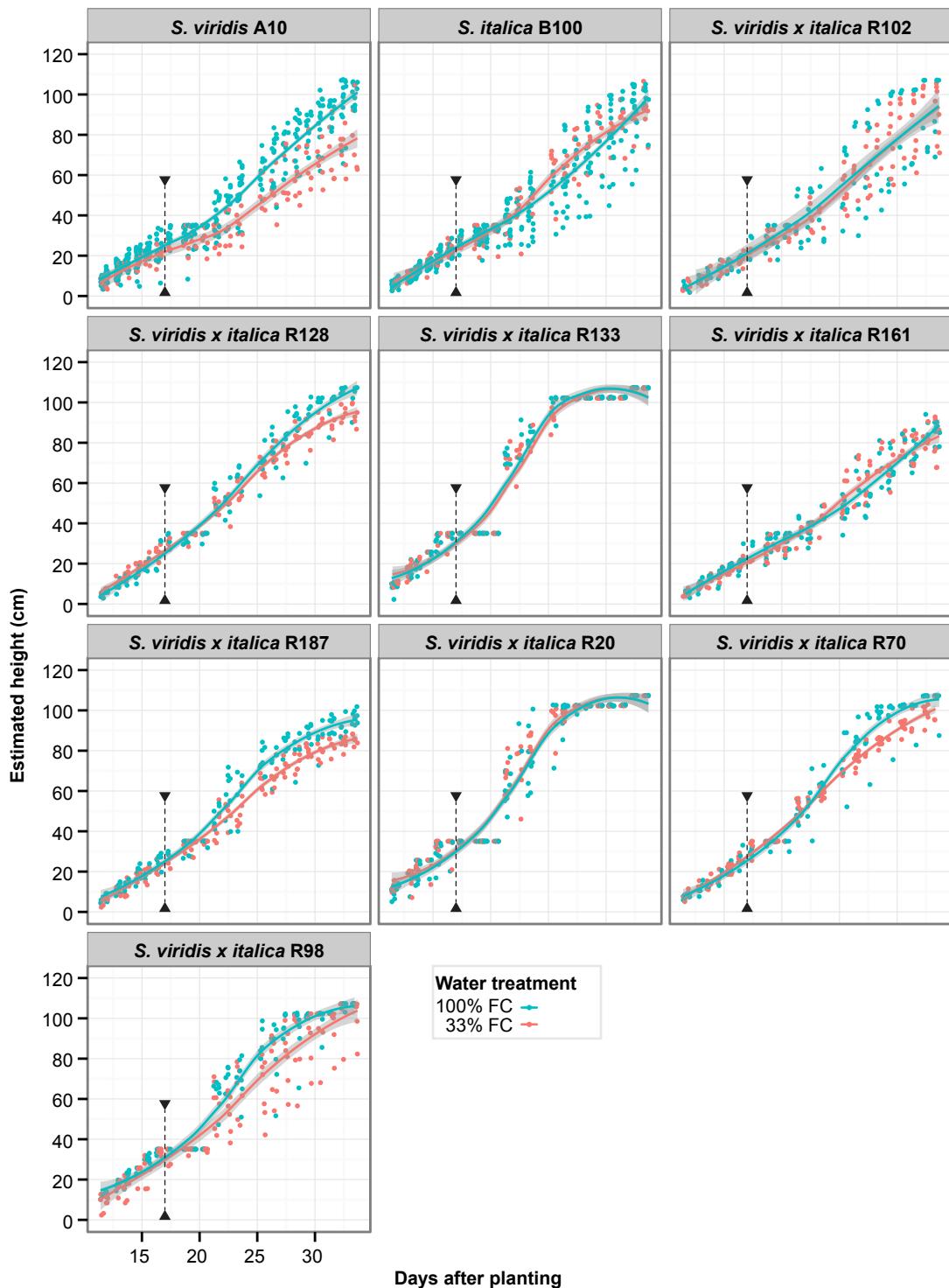
Camera	Measurement	Function Name	General Function Description
VIS	Area Above Bound	analyze_bound	object area above a preset boundary line
VIS	Area Below Bound	analyze_bound	object area below a preset boundary line
VIS	Centroid Height	analyze_object	y position of object centroid to preset boundary line
VIS	Centroid X Position	analyze_object	x position of object centroid
VIS	Centroid Y Position	analyze_object	y position of object centroid
VIS	Color	analyze_color	splits image into RGB, HSV, and LAB color space and outputs histogram of each color channel
VIS	Exent X	analyze_object	total span of object pixels in along x axis
VIS	Exent Y	analyze_object	total span of object pixels along y axis
VIS	Height Above Bound	analyze_bound	total span of object pixels along y axis above a preset boundary line
VIS	Height Above Bound	analyze_bound	total span of object pixels along y axis below a preset boundary line
VIS	Height-Width Ratio	analyze_object	total span of object pixels along y axis above a preset boundary line, divided by total span of object pixels in along x axis
VIS	Long Axis	analyze_object	total span of pixels from convex hull point furthest from the centroid, through the centroid to the intersecting point on convex hull
VIS	Perimeter	analyze_object	total length of pixels around object convex hull
VIS	Side View Area	analyze_object	total number of pixels in object
VIS	Solidity	analyze_object	total number of pixels in object divided by convex hull area
VIS	Top View Area	analyze_object	total number of pixels in object
PSII	Maximum FLU Signal	fluor_fvfm	maximum bin value of Fv/Fm histogram
PSII	Fv/Fm Signal	fluor_fvfm	histogram of Fv/Fm (fmax minus fmin, divided by fmax) values for each pixel
PSII	Median Fv/Fm Signal	fluor_fvfm	median bin value of Fv/Fm histogram
NIR	NIR Signal	analyze_NIR_intensity	histogram values of NIR signal

3

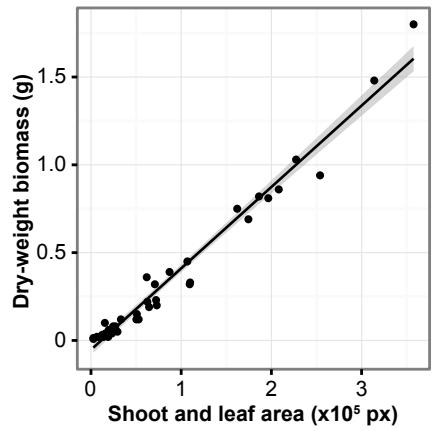
4 **Supplemental Table SIII.** Description of measurements output by PlantCV. For more information on
5 each function please refer to PlantCV documentation (<https://plantcv.danforthcenter.org>).

Camera	Description	Script Type	Notes	Path
PSII	Lifter 630 FLU TV	Image Processing and Output	N/A	/plantcv/scripts/image_analysis/flu_tv/flu_z630_L1.py
PSII	Lifter 500 FLU TV	Image Processing and Output	N/A	/plantcv/scripts/image_analysis/flu_tv/flu_z500_L1.py
NIR	3.9X Zoom NIR SV	Image Processing and Output	N/A	/plantcv/script/image_analysis/nir_sv/nir_sv_z3500_L1.py
NIR	3.1X Zoom NIR SV	Image Processing and Output	N/A	/plantcv/script/image_analysis/nir_sv/nir_sv_z2500_L1.py
NIR	1.4X Zoom NIR SV	Image Processing and Output	N/A	/plantcv/script/image_analysis/nir_sv/nir_sv_z500_L1.py
VIS	1.4X Zoom VIS SV	Image Processing and Output	N/A	/plantcv/scripts/image_analysis/vis_sv/vis_sv_z500_L1.py
VIS	1.6X Zoom VIS SV	Image Processing and Output	N/A	/plantcv/scripts/image_analysis/vis_sv/vis_sv_z700_L1.py
VIS	3.1X Zoom VIS SV	Image Processing and Output	N/A	/plantcv/scripts/image_analysis/vis_sv/vis_sv_z2500_L1.py
VIS	3.9X Zoom VIS SV	Image Processing and Output	N/A	/plantcv/scripts/image_analysis/vis_sv/vis_sv_z3500_L1.py
VIS	1.0X Zoom VIS TV	Image Processing and Output	N/A	/plantcv/script/image_analysis/vis_tv/vis_tv_z1_L1.py
VIS	1.3X Zoom VIS TV	Image Processing and Output	N/A	/plantcv/script/image_analysis/vis_tv/vis_tv_z300_L1.py
VIS	2.3X Zoom VIS TV	Image Processing and Output	N/A	/plantcv/script/image_analysis/vis_tv/vis_tv_z1500_L1.py
VIS	3.1X Zoom VIS TV	Image Processing and Output	N/A	/plantcv/script/image_analysis/vis_tv/vis_tv_z2500_L1.py
VIS	3.5X Zoom VIS TV	Image Processing and Output	N/A	/plantcv/script/image_analysis/vis_tv/vis_tv_z3000_L1.py
VIS	3.9X Zoom VIS TV	Image Processing and Output	N/A	/plantcv/script/image_analysis/vis_tv/vis_tv_z3500_L1.py
ALL	Parallelization	Paralellization	N/A	/plantcv/scripts/image_analysis/image_analysis.pl
PSII	Extract FLU signal	Data Extraction and DB Query	N/A	/plantcv/scripts/db/flu_fvfm_exporter.py
NIR	Extract NIR Signal	Data Extraction and DB Query	N/A	/plantcv/scripts/db/nir_signal_exporter.py
VIS, NIR	Extract Shape Data	Data Extraction and DB Query	N/A	/plantcv/scripts/db/vis_measurement_exporter.py
VIS	Extract Color Data	Data Extraction and DB Query	N/A	/plantcv/scripts/db/vis_color_exporter.py
None	Watering	Statistical Analysis Script	Fig. 1	/plantcv/scripts/manuscripts/manuscript_1/ vis_analysis.Rmd
ALL	Vis Shape and Zoom	Statistical Analysis Script	Fig. 3,4,5,12	/plantcv/scripts/manuscripts/manuscript_1/vis_analysis.Rmd
VIS	Color	Statistical Analysis Script	Fig. 6	/plantcv/scripts/manuscripts/manuscript_1/color_early_late_21115.R
VIS	Color	Statistical Analysis Script	Fig. 7	/plantcv/scripts/manuscripts/manuscript_1/color_r161_a10_b100_21115.R
PSII	PSII	Statistical Analysis Script	Fig. 8	/plantcv/scripts/manuscripts/manuscript_1/PSII_setaria_3315.R
NIR	Analyze NIR Signal	Statistical Analysis Script	Fig. 9, 10	/plantcv/scripts/manuscripts/manuscript_1/NIR_image_analysis_burnin2.R
NIR	Outlier Identification	Statistical Analysis Script	N/A	/plantcv/scripts/manuscripts/manuscript_1/NIR_image_QCQA_burnin2.R
VIS	JPEG vs. PNG	Statistical Analysis Script	Fig. 11	/plantcv/scripts/manuscripts/manuscript_1/color_pngvsjpg_2-20-15.R
ALL	Variance	Statistical Analysis Script	Table 1	/plantcv/scripts/manuscripts/manuscript_1/variance_partitioning_script_burnin2.R

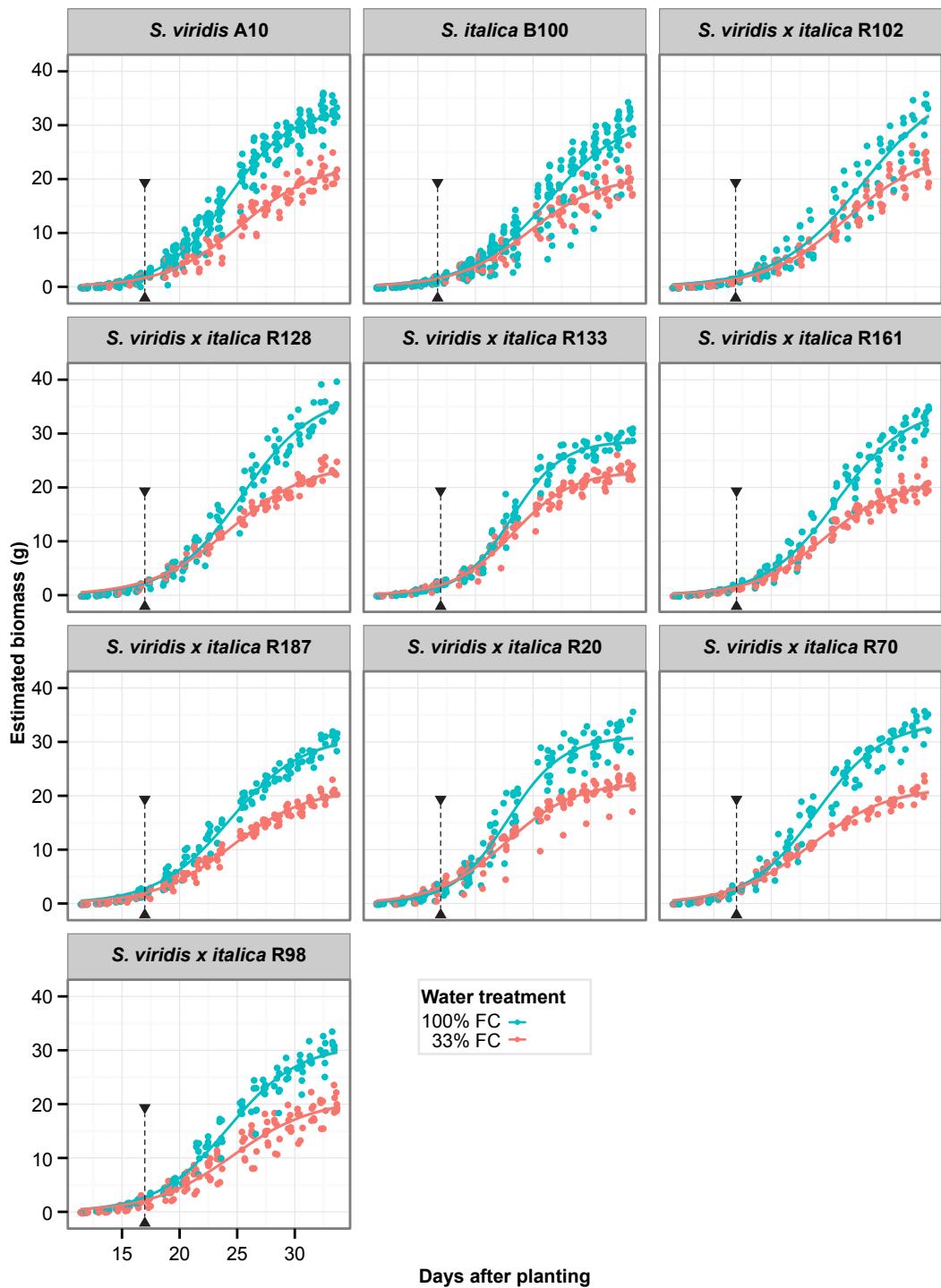
1 **Supplemental Table SIV.** PlantCV image processing and analysis scripts. For more information on image processing procedures and analysis
2 methods please refer scripts and documentation at <https://plantcv.danforthcenter.org>.



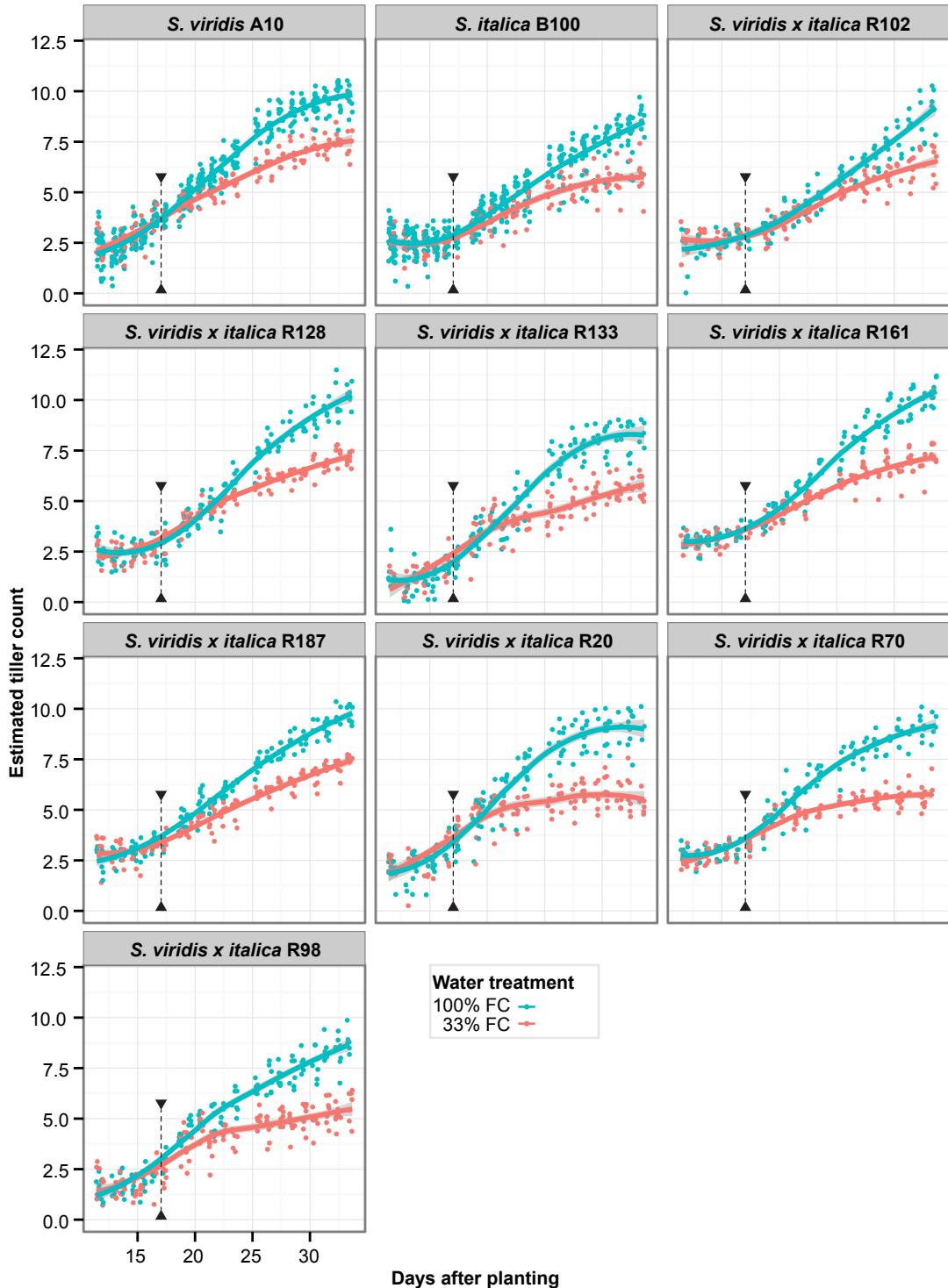
Supplemental Figure S1. Plant height estimated with PlantCV for ten *Setaria* genotypes. Estimated plant height for *S. viridis*, *S. italica*, and eight RIL plants from 11–33 DAP. Plants watered to 100% or 33% FC are shown. Local regression (LOESS) fitted curves with standard error are plotted for each genotype by water treatment group. The arrows indicate the day that the 33% FC water treatment started.



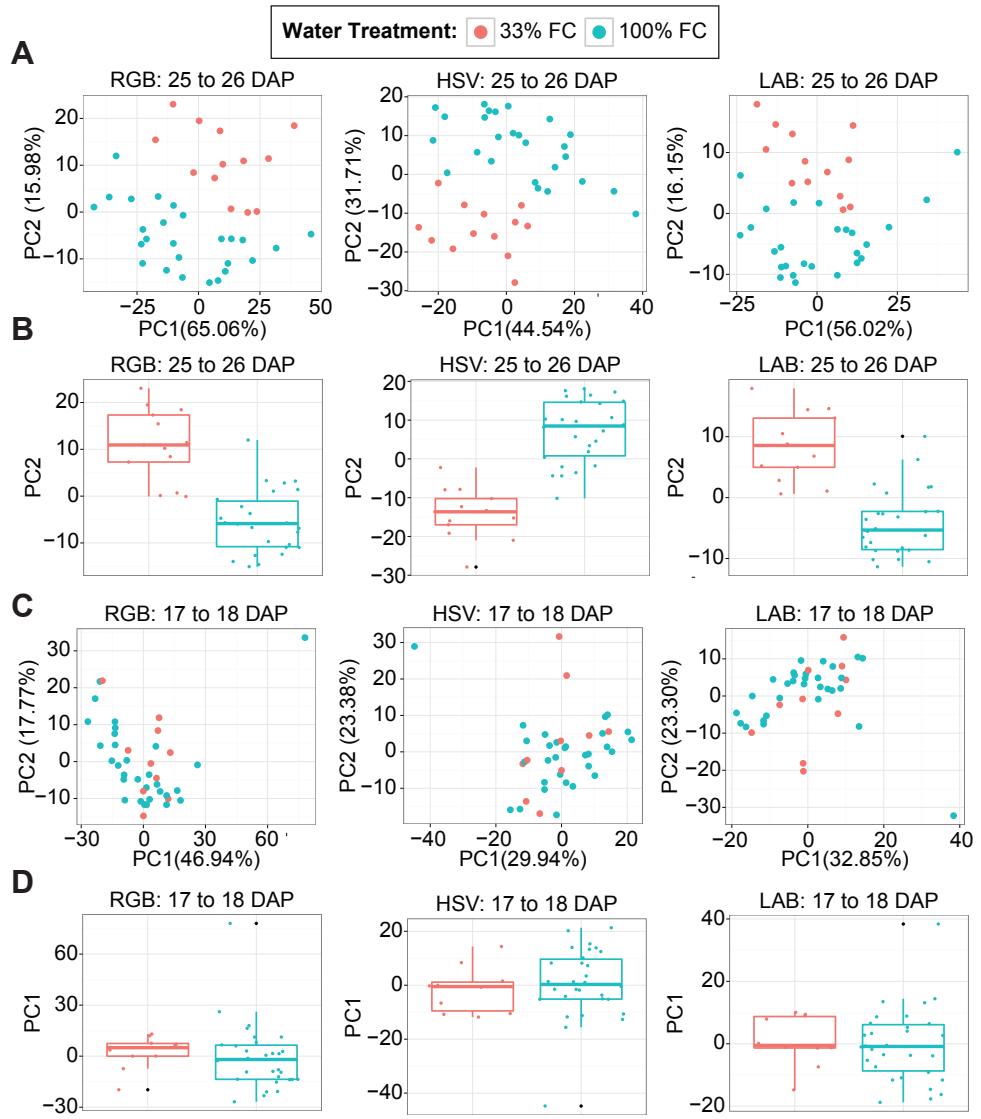
Supplemental Figure S2. Above ground dry-weight biomass modeled using shoot and leaf pixel area from four side-view VIS images. Data shown are for 41 plants where destructive dry-weight biomass was measured throughout the experiment. The ordinary least squares linear model with standard error is plotted.



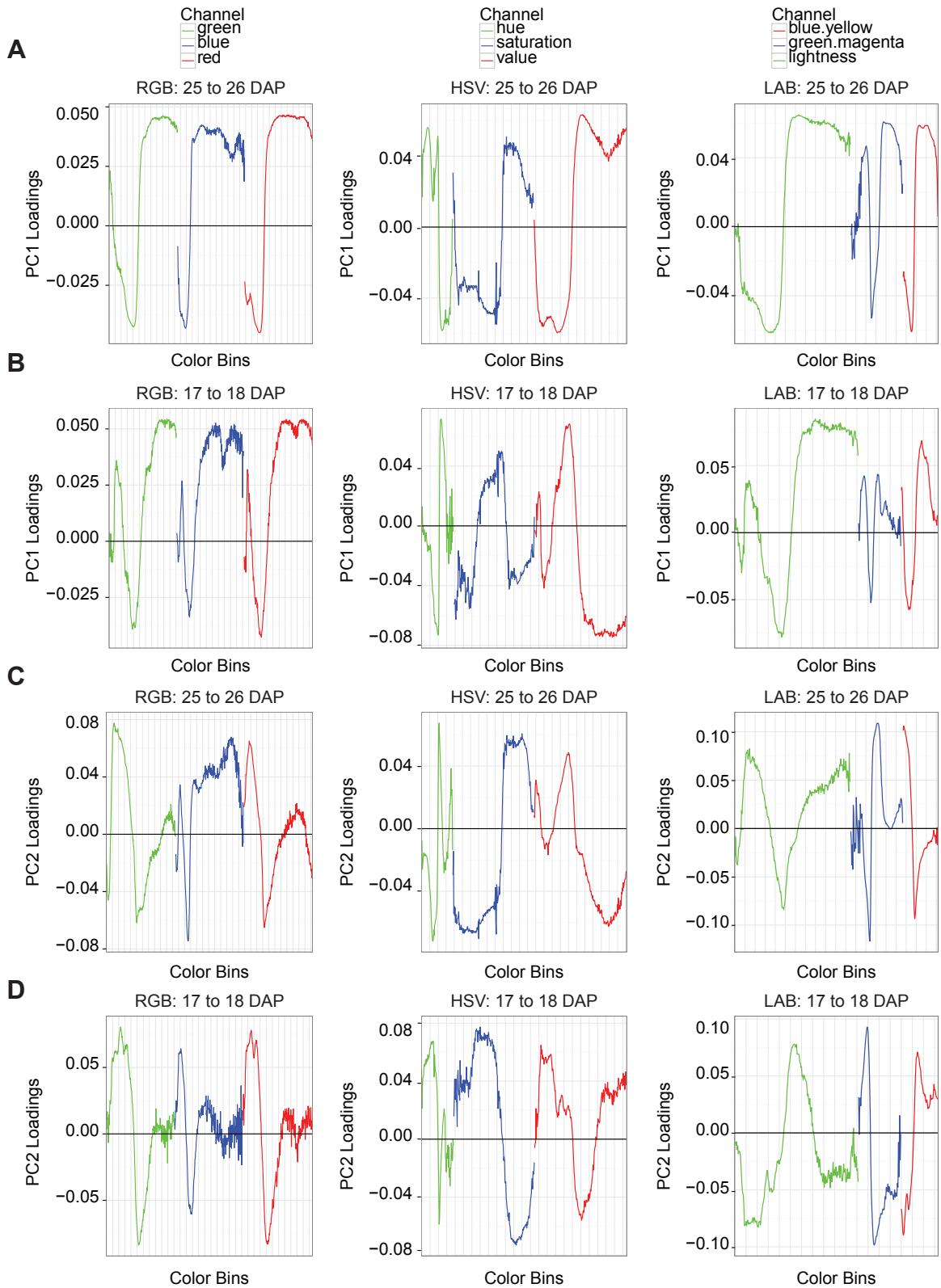
Supplemental Figure S3. Modeled fresh-weight biomass estimated with PlantCV for ten *Setaria* genotypes. Plants watered to 100% or 33% FC are shown. Arrows indicate the day that the 33% FC water treatment started. Three-component logistic growth curves with 95% confidence intervals are plotted.



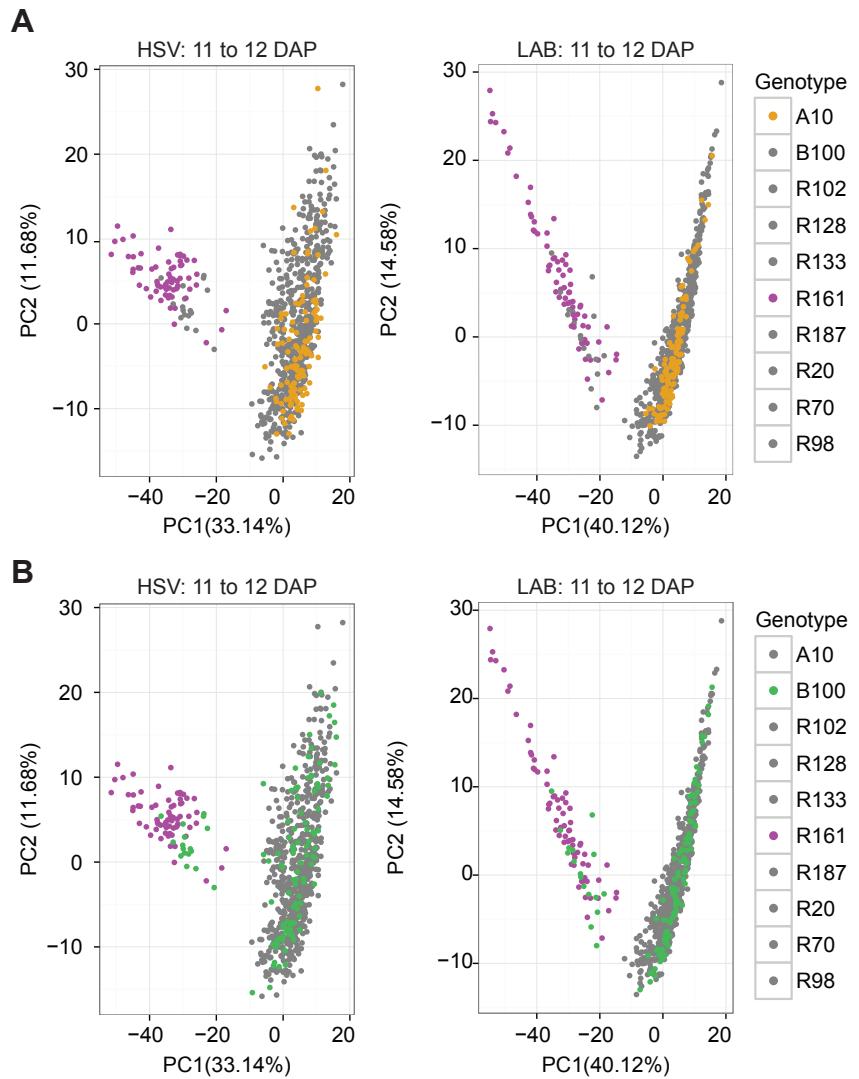
Supplemental Figure S4. Tiller count estimated with PlantCV for ten *Setaria* genotypes. Estimated tiller count for *S. viridis*, *S. italica*, and eight RIL plants from 11–33 DAP. Plants watered to 100% or 33% FC are shown. Local regression (LOESS) fitted curves with standard error are plotted for each genotype by water treatment group. The arrows indicate the day that the 33% FC water treatment started.



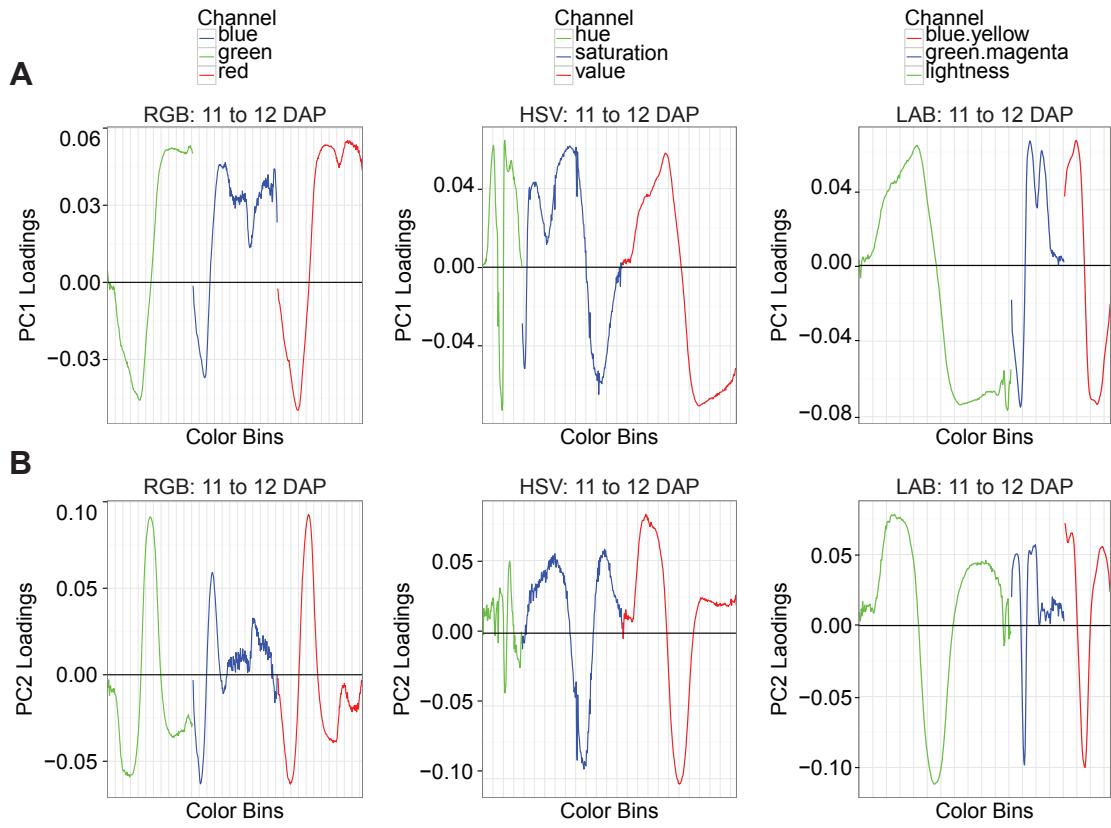
Supplemental Figure S5. Color can separate watering treatments 25 to 26 DAP, but not 17 to 18 DAP in RGB, HSV and LAB color space. A, PC1 and PC2 of *S. viridis* color PCA 25 to 26 DAP under 100% FC (blue) and 33% FC (red) water treatment. RGB PC1 accounted for 65.06% of variation and PC2 accounted for 15.98% of variation. B, Principal component regression showed that a two component model including PC2 separates a significant proportion of variation due to 100% FC (blue) and 33% FC (red) water treatments 25 to 26 DAP in RGB, HSV and LAB color space. C, PC1 and PC2 of *S. viridis* color PCA 17 to 18 DAP under 100% FC (blue) and 33% FC (red) treatment. RGB PC1 accounted for 46.94% of variation and PC2 accounted for 17.77 % of variation. D, 17 to 18 DAP, PC1 of RGB, HSV, or LAB color did not separate 100%FC (blue) and 33% FC (red) water treatments.



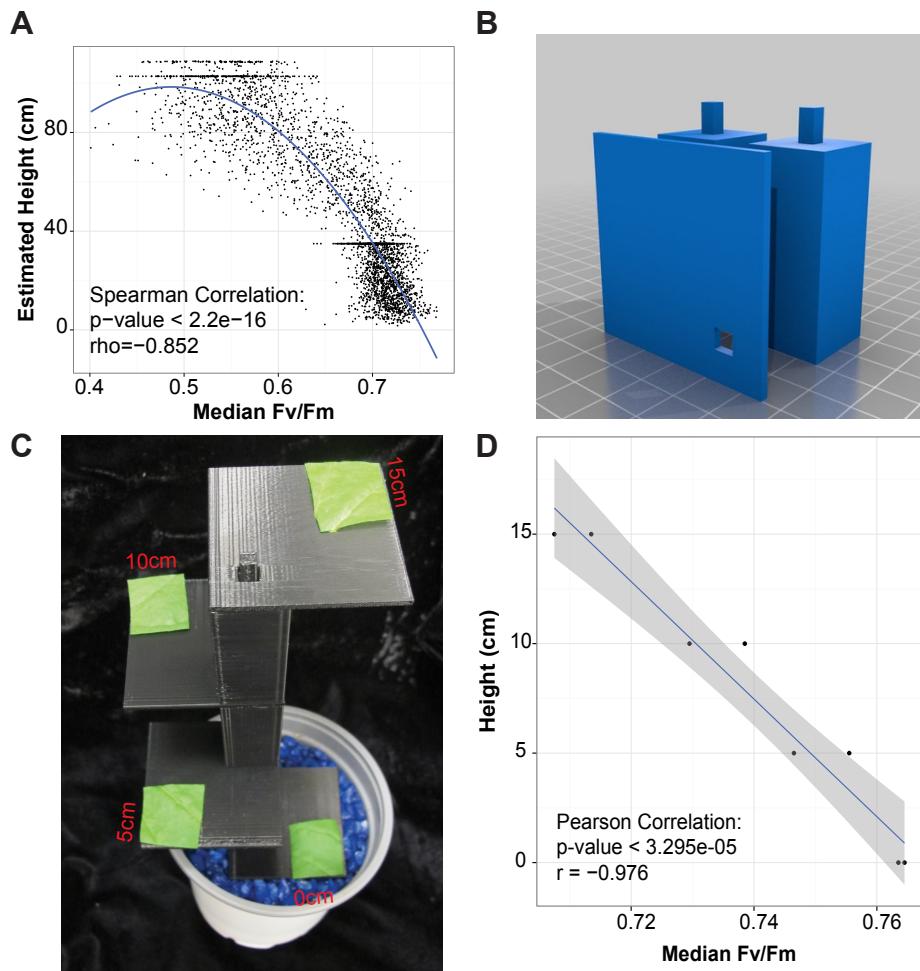
Supplemental Figure S6. Loadings of color data 25 to 26 DAP and 17 to 18 DAP. A, PC1 loadings of *S. viridis* color data PCA 25 to 26 DAP. B, PC1 loadings of *S. viridis* color data PCA 17 to 18 DAP. C, PC2 loadings of *S. viridis* color data PCA 25 to 26 DAP. D, PC2 loadings of *S. viridis* color data PCA 17 to 18 DAP.



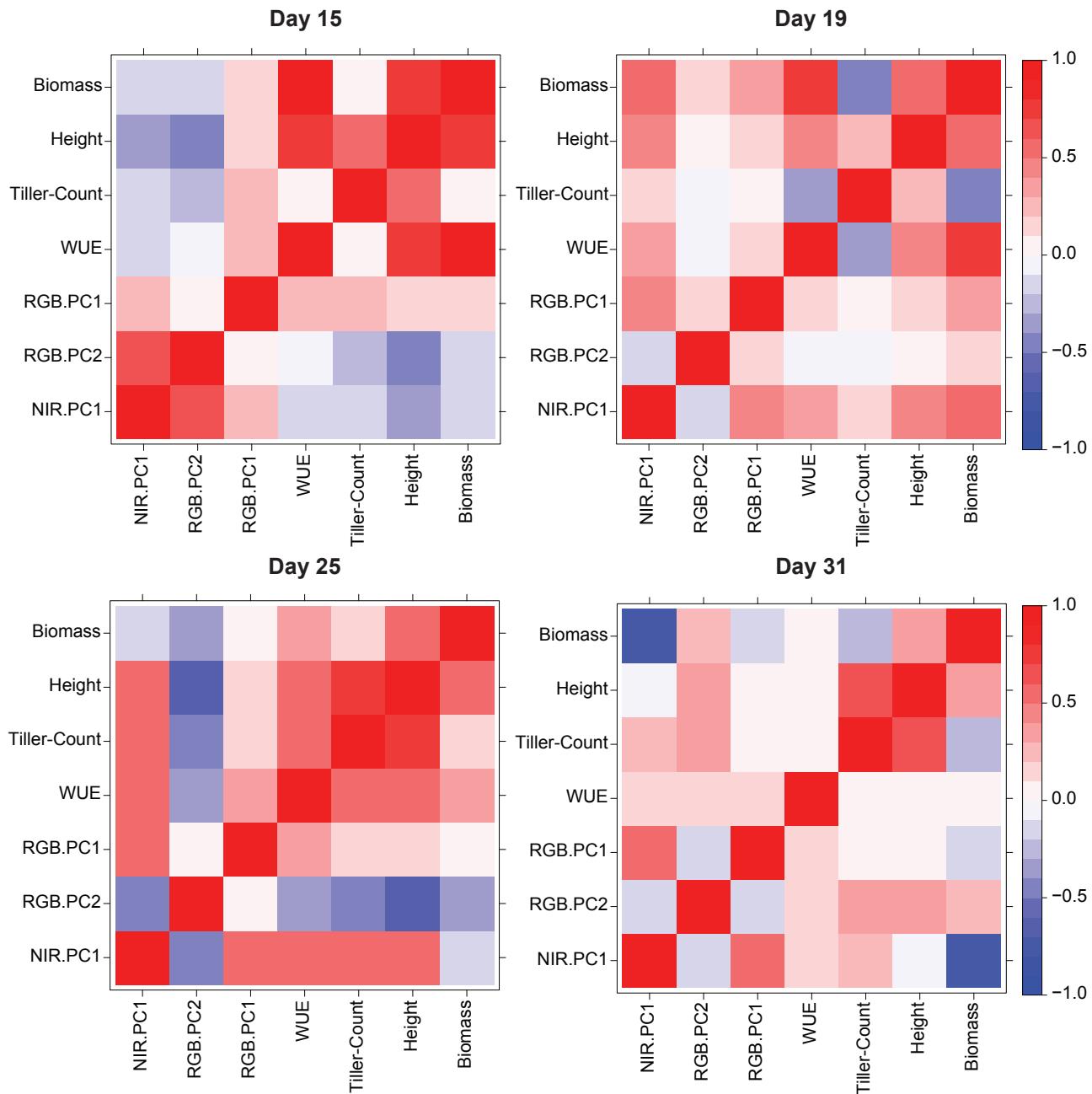
Supplemental Figure S7. Before water treatment is applied (11 to 12 DAP) color can distinguish *Setaria* genotypes in RGB color (Fig. 6), which is also consistent in HSV and LAB color space. A, PCA of HSV (left) and LAB (right) color for all *Setaria* genotypes before treatment is applied (11 to 12 DAP). PC1 and PC2 are plotted for *S. viridis* (orange), *S. italica* (gray), RIL161 (purple) and 7 other *S. viridis* x *S. italica* RILs (gray). B, PCA of HSV (left) and LAB (right) color for all *Setaria* genotypes before treatment is applied (11 to 12 DAP). PC1 and PC2 are plotted for *S. viridis* (gray), *S. italica* (green), RIL161 (purple) and 7 other *S. viridis* x *S. italica* RILs (gray).



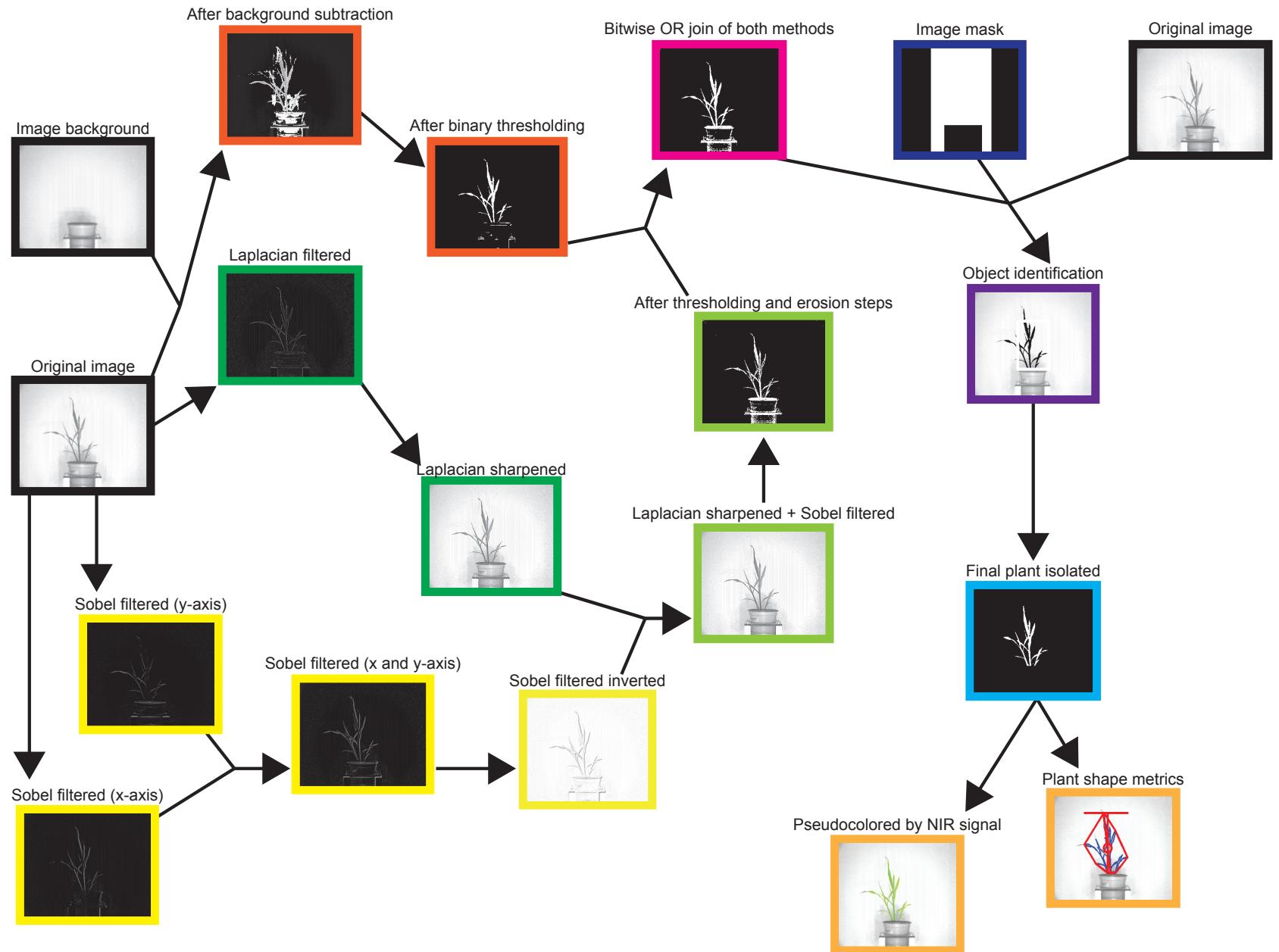
Supplemental Figure S8. Loadings of color data 11 to 12 DAP. A, PC1 loadings of *S. viridis* color data PCA 11 to 12 DAP. B, PC2 loadings of *S. viridis* color data PCA 11 to 12 DAP.



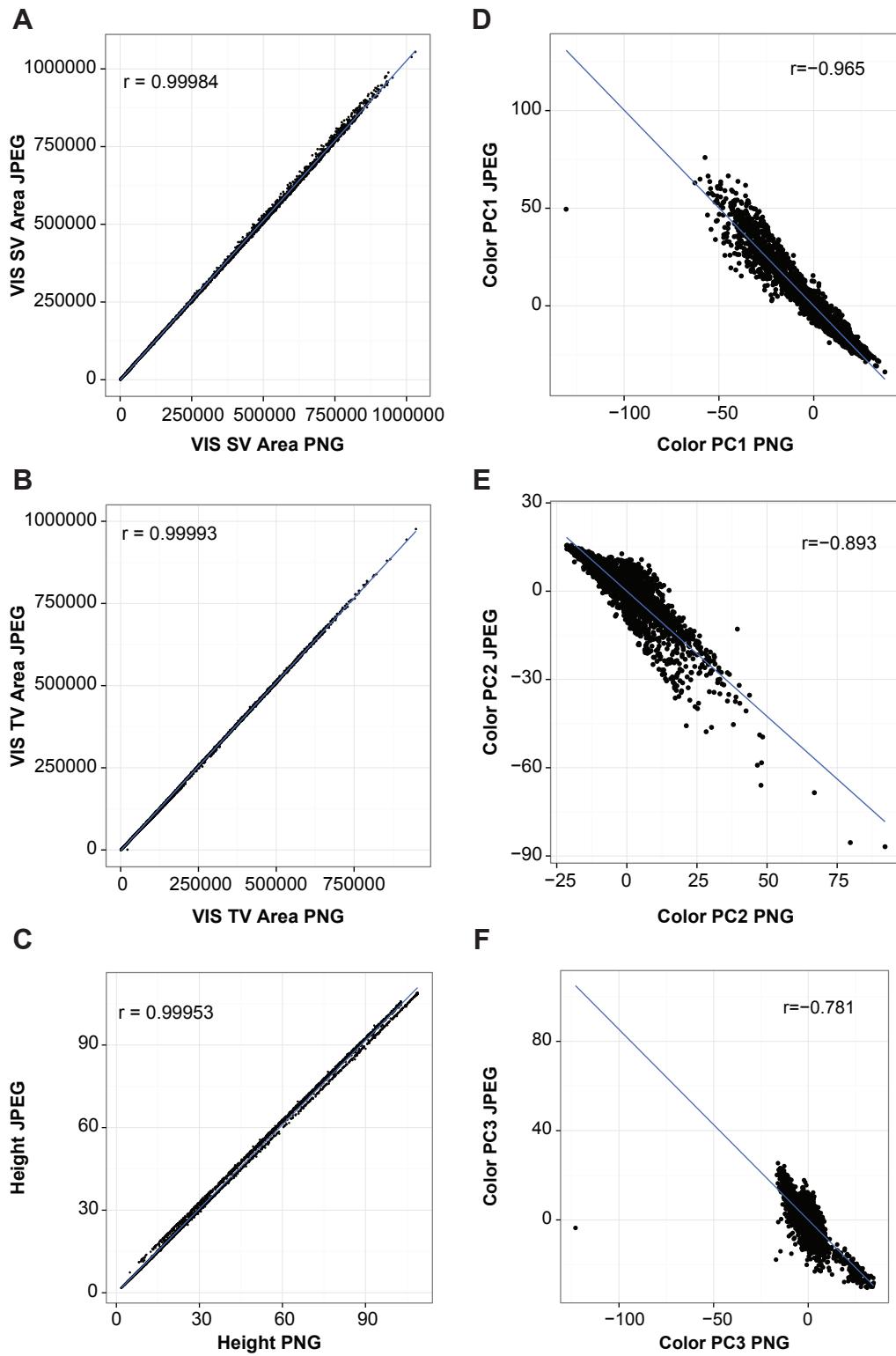
Supplemental Figure S9. Height significantly correlates with median Fv/Fm. A, Median Fv/Fm is significantly negatively correlated ($p < 0.01$; $\rho = -0.852$) with estimated plant height. Average estimated height was extracted from four side-view VIS images by PlantCV from all ten *Setaria* genotypes over time under both 100% FC and 33% FC water treatments. Fv/Fm values for each plant pixel was calculated by PlantCV from F0, Fmin and Fmax PSII images. Median Fv/Fm is the median bin value in the Fv/Fm per pixel histogram for each plant. B, 3D-printed ‘plant’ components consist of two 5 cm ‘stem’ pieces and one flat ‘leaf’ platform piece. Designs for 3D-printed ‘plant’ components are available at <https://www.thingiverse.com/thing:574287>. C, Assembled 3D-printed plant with excised *N. benthamina* leaf tissue. *N. benthamina* leaf was positioned at 0 cm (flush with gravel in pot), 5 cm, 10 cm, and 15 cm. D, Median Fv/Fm is significantly negatively correlated ($p < 0.01$; $r = 0.976$) with height in the 3D-printed plant. 3D-printed plant with excised *N. benthamina* leaf tissue was imaged by the PSII imaging station two times (tissue from a second leaf during second imaging). Fv/Fm values for each plant pixel at each platform level was calculated by PlantCV from F0, Fmin and Fmax PSII images. Median Fv/Fm is the median bin value in the Fv/Fm per pixel histogram calculated for plant tissue at each platform level.



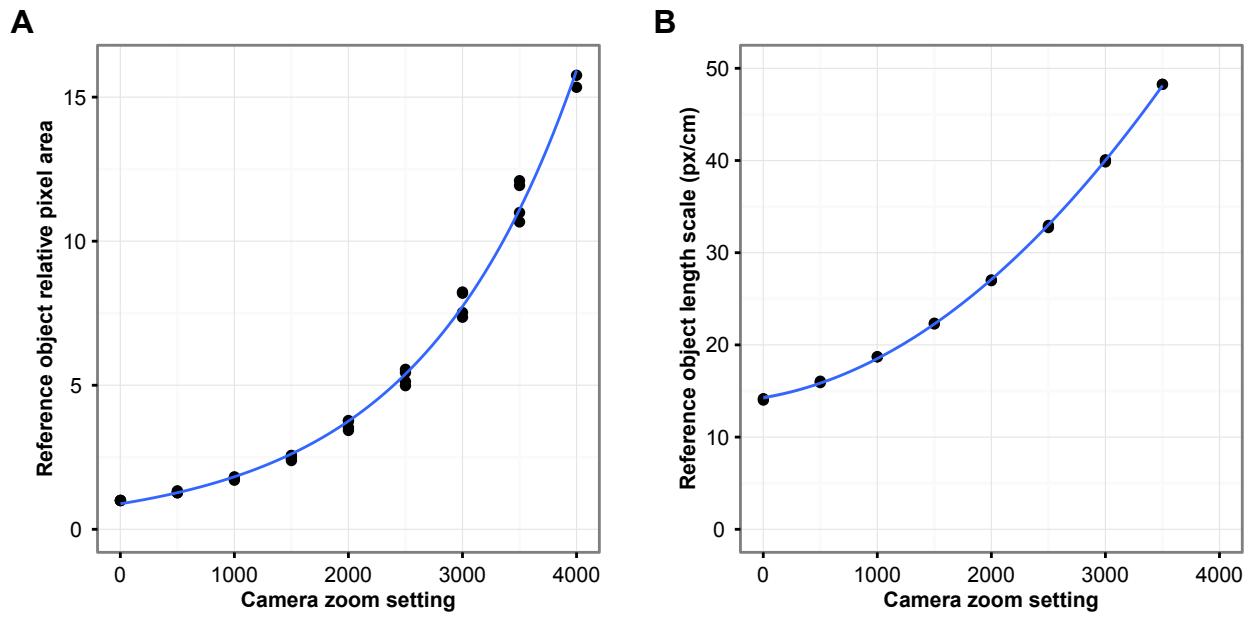
Supplemental Figure S10. Matrices displaying Pearson's Correlation Coefficient calculated between traits at four roughly equidistant time points. Darker red shading indicates a stronger positive correlation, whereas increased blue shading indicates stronger negative correlation.



Supplemental Figure S11. A visual illustration of the pipeline used to threshold plant tissue from background within grayscale NIR images. Color boxes group together sets of related processes (Original images = dark blue; Original images processed with background subtraction = orange; Laplacian filtering = dark green; Sobel filtering = yellow; Combined derivative filtering = light green; bitwise OR join = fuchsia; image masking and object identification = purple; final processed images = light blue).



Supplemental Figure S12. JPEG images are sufficient for analysis when only shape and morphological features are needed. PNG format images are preferred when signal/spectral data is analyzed. A–F, Correlation analysis included all *Setaria* genotypes and the 100% and 33% FC water treatment groups. A, VIS side-view plant pixel area. B, VIS top-view plant pixel area. C, Estimated plant height. D, PC1 of RGB color PCA. E, PC2 of RGB color PCA. F, PC3 of RGB color PCA.



Supplemental Figure S13. Zoom-correction scaling factors for pixel area and pixel length. A, Zoom-correction scaling factor model for pixel area calculated from a reference object of known area. B, Zoom-correction scaling factor model for pixel length calculated from a reference object of known length.