Title: Tasselyzer, a machine learning method to quantify anther extrusion in maize, based on PlantCV

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Summary

Male fertility in maize is controlled by development and genetic programming and is directly impacted by environmental factors such as light, temperature, water, and nutrient availability; the control of this trait has substantial agronomic utility. Maize anthers emerge from male florets, which are clustered to form the tassel at the top of the plant separated from the female ear. Quantification of anther extrusion is one important aspect in the determination of male fertility. To address the lack of an automated method to measure anther extrusion on a large scale, we developed 'Tasselyzer', a quantitative, image-based color trait analysis pipeline for tassel image segmentation, based on the existing PlantCV platform, and we applied it to determine the proportion of anther extrusion. We evaluated Tasselyzer in maize during the seven-day period of pollen shedding as well as in the temperature-sensitive male sterile mutant *dcl5*. With tassel images obtained with a smart phone camera, we show that the anther scores positively correlate with anther extrusion, and such methods can be used to measure environmental impacts on the *dcl5* mutant. Altogether, this work establishes an automated and inexpensive method to quantify anther extrusion in maize, which would be useful for research and breeding.

Keywords: anther, tassel, maize, male fertility, PlantCV, machine learning

Significance Statement

Tasselyzer is a novel image-based segmentation tool, to address the lack of an automated method to measure anther extrusion, and the impact of genetic and environment variation on male fertility in maize.

Introduction

Male fertility of maize tassels is a key determinant of the final crop yield, but it depends on natural variation in genetic stock, as well as on biotic and abiotic stressors. The ability to

quantify male fertility helps us to understand the impacts of various genetic and environmental conditions. One way to quantify male fertility is to measure numbers of viable pollen grains in the air using a passive pollen sampler device, because maize is a wind-pollinated plant and produces and sheds enormous amounts of aerial pollen grains (Hofmann *et al.*, 2014). Because shed pollen is also deposited on the leaf surfaces, maize male fertility may also be estimated by measuring maize leaf pollen density over their flowering period; however, these methods are either indirect or hard to apply to individual maize plants (Hofmann *et al.*, 2016). Thus, improved methods of assessing maize male fertility are needed.

Maize tassel morphology could serve as another indicator of male fertility. The tassel is a cluster of male maize florets on the plant top (Bonnett, 1954). It normally has one main spike and a varied number of side branches; a spike or a branch contains a number of spikelets; a spikelet contains one upper and one lower floret; and each floret contains three anthers that are developmentally at roughly the same stage. When an anther is mature, its attached filament elongates which allow the anther to emerge – the emergence is called anther “extrusion” or “exertion”. A tassel takes 3 to 7 days to finish developing flowers, depending on the genetic context and growth conditions. For example, in a W23 inbred under optimal yield conditions, the day that the first anthers emerge from the upper florets in the center of the main spike is designated as day 1; more anthers emerge from florets on the main spikes on day 2, including the lower florets in the center and upper ones above and below; on day 3, more anthers are extruded from lower florets on the main spike, plus upper florets on the side branches, and so on until anthers emerge from the lowest branches (Egger and Walbot, 2015). Assuming there is no gametophytic defect, the number of extruded anthers positively correlates with the amount of pollen production.

In previous work, the length and diameter of tassel branches were shown to positively correlate with pollen production (Fonseca *et al*., 2003). Another study suggested that the total number of spikelets may be the best architectural variable related to pollen production (Ricci *et al*., 2012). However, both methods apply only to a fully fertile tassels and exclude the consideration of impacts of genetic and environmental variation on tassel or anther development. As far as we know, there currently is no automated method to measure anther extrusion in maize, likely because anthers are too numerous to quantitate for each tassel. Previous work focused primarily on completely male sterile plants, which fully lack anther extrusion, without significant analysis of the degrees of anther extrusion in partially or fully male fertile plants. In addition to the historic unstable cytoplasmic male sterile mutants in maize (Weider *et al*., 2009), additional environment-sensitive male sterile mutants have been discovered including the exotic *Ms45* expression material (Unger *et al*., 2002), *dcl5* (Teng *et al*., 2020) and *ocl4* (Yadava *et al*, 2021). A robust automated quantification method for anther extrusion would facilitate the study of this agronomically important trait across a variety of maize lines.

In this study, we propose that the degree of anther extrusion is an excellent indicator of male fertility and can be assessed using “Tasselyzer", a quantitative image-based color trait analysis pipeline based on the PlantCV platform (Fahlgren *et al*, 2015; Gehan *et al*., 2017). In this method, the degree of anther extrusion of individual maize plant can be scored from two-dimensional tassel images using a computational segmentation algorithm. We applied the Tasselyzer pipeline in an analysis of the maize A632 inbred lines during the standard seven-day period of pollen shedding, as well as in the temperature-sensitive male sterile mutant *dcl5-mu03* (Teng *et al*., 2020). This algorithm can also be applied to quantify pollen viability based on conventional staining methods. Furthermore, the pipeline can be programmed automatically for high-throughput studies. Altogether, we demonstrate that maize male fertility can be assessed in an automated manner by measuring anther extrusion and pollen viability using Tasselyzer.

Results

Tasselyzer, an image-based quantification of the degree of anther extrusion

Anther extrusion is the first criterion of male fertility and occurs when anthers complete the sporophytic and meiotic stages; the second criterion is pollen number and viability. Maize anthers with sporophytic or meiotic defects typically halt in development prior to anther extrusion (Wan *et al*., 2019). While a completely male sterile maize mutant will have no anther extrusion, the morphology of tassels can vary depending on its genetic and environmental context. We could find no established method to quantify anther extrusion in maize. This process is challenging because hundreds to thousands of anthers emerge for seven consecutive days, and the architecture of tassels can vary. A quantitative method is required to effectively and reliably compare degrees of anther extrusion.

In most cases, anthers are easily distinguished from the remainder of the tassel based on color; anthers are normally yellow or pink, while the rest of the tassel, including the branch and glum, are green. These distinct color features can help distinguish anthers from other tassel components using a red-green-blue (RGB) tassel image on a dark background (Figure 1a). Then, anther extrusion levels can be estimated by the proportion of anthers in the image. Here we describe “Tasselyzer”, the non-destructive and automated image-based quantification pipeline we developed based on PlantCV (Supporting Figure S1; Fahlgren *et al*, 2015; Gehan *et al*., 2017). Tasselyzer employs a Naïve Bayes classification method for pixel segmentation to identify the two-class tassel and non-plant background. Depending on the variability of colors, RGB information was sampled using ImageJ 2 (Schindelin *et al*., 2015; Rueden *et al*., 2017) from several hundred to several thousand defined pixels for each class (anther, tassel branch and glum, and background), and used to generate a tab-delimited table (Figure 1b). Then, this RGB information table was used as input to generate probability density functions (PDFs),

which are used to classify the probability a pixel in a tassel image belongs to each class. Binary masks of each classified pixel were generated with white foreground (targeted class) and black background (non-targeted classes), merged in pseudo-colors and overlaid onto the original tassel images (Figure 1c). Pixel numbers in each class were summarized and an anther ratio was calculated; the anther ratio represents the ratio of the pixels classified as extruded anther to the total classified as tassel (anther plus branch and glum) (Figure 1d). To assess the accuracy of Tasselyzer, a high-resolution overlaid tassel image was magnified to examine individual pixels (Figure 1e). As a test case, we assessed a tassel image from a Mo17 fertile inbred line and determined the anther ratio to be 0.45 when anthers were fully emerged throughout the entire tassel (Figure 1c). Overall, Tasselyzer could accurately differentiate anthers from the remainder of the tassel, on a dark background, in a two-dimensional smart phone acquired image, and calculate the proportion of anther pixels relative to branch pixels.

Quantification of anther extrusion during a seven-day period of pollen shedding

A tassel contains hundreds of individual spikelets on a main spike, and a few side branches, with each spikelet containing one upper and one lower floret. Each floret contains three anthers roughly at the same developmental stage. Under optimal greenhouse conditions, anthers of A632 fertile inbred tassels first emerge from the upper florets at the center of the main spike, then from the lower florets, and progress upward, downward and outward over five days; most of the extruded anthers remain, with none, or small numbers, of the older anthers detaching before the filaments break (Figure 2). We were able to quantify anther ratios in whole tassel images over seven days using Tasselyzer to reflect the anther extrusion process described above (Figure 2a, 3a and Supporting Figure S2); we showed that the anther ratio gradually increases from day 1 to day 4 (0.11 ± 0.04 to 0.57 ± 0.06), and peaks around day 5 (0.63 ± 0.06) and slightly declines on day 6 (0.61 ± 0.03).

Quantification of the sections of the main spike (Figure 2b and 3b) or lower side (Figure 2c and 3c) branches alone showed similar trends but with a greater deviation when compared to whole tassels. This especially varied for the main spike on day 2 (Figure 3b and 3c, 0.59 ± 0.13) and side branches on day 2, 4 and 5 (0.32 ± 0.10, 0.49 ± 0.12, and 0.51 ± 0.18). Since anthers emerge first from the center of the main spike on day 1 and start emerging from the lower side branches on day 2, ranges of anther extrusion ratio also varied comparing whole tassel (0.11 ± 0.04 to 0.63 ± 0.06), main spike (0.45 ± 0.03 to 0.77 ± 0.07), and side branches (0.02 ± 0.04 to 0.60 ± 0.03) from day 1 to day 6 (Figure 3); and it was most substantial on day 1. When anthers emerged from all branches, the anther ratio peaks at 0.76 ± 0.06 for main spikes on day 4, 0.63 ± 0.06 for whole tassels on day 5, and 0.60 ± 0.03 for side branches on day 6. Together, anther ratio could reflect the degree of anther extrusion of a developing tassel over a seven-day period with whole or partial tassel images; while whole tassels yield the best results, which is comparable to partials.

Tasselyzer can quantify anther extrusion altered by genetic and environmental variation

Deficiency of *Dicer-like 5* (*Dcl5*) in maize results in male sterility under a typical optimal growth regime for maize (Teng *et al.*, 2020); this sterility is partially rescued by permissive lower day and night temperatures. Here, the optimal temperature for normal maize growth is the restrictive temperature for *dcl5* mutants. In our previous work, we applied a prototype of Tasselyzer to well-controlled greenhouse grown *dcl5-1* to measure the impacts of restrictive (28° C/ 22° C) temperatures versus permissive temperatures (22° C/ 20° C), at 14 h day/10 h night; our quantitative results supported the phenotypic observations mentioned above (Teng *et al.*, 2020). In this study we assessed an additional allele, *dcl5-mu03*, grown in a CONVIRON PGC20 chamber with precisely-controlled restrictive and permissive temperatures. The temperature

treatments were applied during the approximate meiotic to post-meiotic stages that are most relevant to the sterility phenotype, and images were taken of whole tassels for quantification by Tasselyzer (Supporting Figure S3). The anther extrusion ratio of *dcl5-mu03* under restrictive temperature was 0.05 ± 0.03 (28° C/ 22° C, 14 h day/10 h night temperatures), which could be considered completely male sterile as compared to their heterozygous fertile siblings (0.62 ± 0.05) under these conditions (Figure 4a and Fig.5); the anther ratio of *dcl5-mu03* under the permissive temperature was 0.38±0.05 (22° C/ 20° C, 14 h day/10 h night temperatures), which was almost two-thirds of their heterozygous fertile siblings at 0.58±0.02 (Figure 4b and Fig.5). Assessing the enlarged main spike, anthers of *dcl5-mu03* plants grown under permissive temperature were slender but in comparable number compared to their fertile siblings (Figure 4d); no major impact could be observed for fertile siblings in the two temperature regimes (Figure 4c and 4d). Together, Tasselyzer could quantify the degree of anther extrusion with pseudo-colored images in the temperature-sensitive male sterile maize mutant *dcl5-mu03*.

Discussion

There was no existing, well-known, automated method to measure anther extrusion in maize, likely because the anthers are too numerous to easily quantify: there are hundreds of spikelets and almost six-times that number of anthers on each tassel. Most previous studies of male sterility in maize focus on mutants demonstrating complete sterility, lacking anther extrusion, but few discuss degrees of anther extrusion in male-fertile or partially fertile plants. Furthermore, existing methods were typically destructive, laborious, descriptive, and qualitative. For example, *Ms45* encodes a strictosidine synthase-like enzyme that functions during the vacuolate stage in maize anther, and it is essential for male fertility (Cigan *et al*., 2001). In another study focused on male fertility, different types of promoters were used to transactivate *Ms45* and were transformed into male sterile mutant *ms45* plants to determine their role in rescuing male fertility

(Unger *et al.*, 2002). Their transgenic T_0 plants were roughly classified as “fertile”, “shedder” and “sterile”, depending on how well *Ms45* functioned in the transgenic plant. Both “fertile” and “shedder” plants exhibited anther extrusion but produced differing amounts or quality of mature pollen grains. However, in that work, these phenotypes weren't quantified with respect to the number of pollen grains produced or the anthers that emerged. In a more recent study (Yadava *et al.*, 2021), the maize *ocl4* mutant was reported to be male fertile under cooler conditions, but anther numbers were reduced with heat waves (≥ 32° C) in the field or greenhouses. In this study, degrees of anther extrusion were visually assigned on a scale of 0 to 5 comparing to a fully fertile plant, but no quantification method was introduced (Yadava *et al.*, 2021). Similarly, for a previous study of cytoplasmic male sterile hybrid maize tassels (Weider *et al.*, 2009); the difference between partially and fully fertile tassels were not indicated. To more effectively study anther extrusion-related male fertility, a quantitative method of anther extrusion is needed.

Although one might measure anther extrusion as the total number of extruded anthers on a tassel, such a metric is difficult to determine due to the dynamics of tassel development; further, it is unreliable to compare absolute anther number between individuals when considering the variation of tassel architecture or genetic background. Instead, quantifying the proportion of anthers that have been extruded, or in other words, the degree of anther extrusion, could be a practical and comparable measurement. One case would be to count the total number of anthers and spikelets there are on a tassel, and the anther extrusion degree could reflect the number of anthers that emerge from a single spikelet. For example, results could be categorized on a scale from 0 to 6 with 0 corresponding to complete male sterility and 6 corresponding to full fertility. While accurately measuring the number of extruded anthers, or even spikelets, is not easy, using currently-available imaging techniques and computational technology, measuring the ratio of anther to non-anther material on a two-dimensional tassel image is simpler. Producing the anther ratio relies on the correlation between the quantity of anther material and

the number of anthers, and the correlation between the quantity of tassel branches with the number of spikelets. The degree of anther extrusion is thus reflected in the anther ratio, which is the anther area divided by the sum of the areas of anthers and branches. The more fertile the tassel, the more anther material will be present, while the less fertile the tassel, the less anther material there will be, when compared to the branch area in an image.

We previously determined a whole tassel anther ratio of 0.45 from another inbred hybrid, Mo17, which likely differs from the A632 inbred hybrid due to variation of tassel architecture and arrangement of spikelets (Figure 1). Further, we found that a permissive temperature condition could significantly rescue male fertility in *dcl5-mu03*, a temperature-sensitive male sterile mutant, without obvious impact on their fertile siblings, *dcl5-mu03//Dcl5* (Figure 4 and 5). Our prior work demonstrated that *dcl5* anthers have fewer bi-nucleated tapetal cells at 1.5 and 2.5 mm in optimal conditions comparing to fertile siblings, suggesting its tapetal development is delayed or arrested (Teng *et al*., 2020). The tapetum is known to supply nutrition, enzymes and pollen wall components (Ariizumi and Toriyama, 2011), therefore it is possible these functions are altered in the *dcl5* tapetum under optimal conditions. While under permissive temperatures, rescued *dcl5-mu03* anthers seem smaller compared to anthers of heterozygous plants, suggesting the tapetum of *dcl5-mu03* could partially but not fully function. Again, this is consistent with the observation that the proportion of bi-nucleated tapetal cells in *dcl5* mutant anther was similar or normal comparing to the heterozygous anthers at 1.5 and 2.5 mm (Teng *et al*., 2020). Therefore, the anther ratios produced through Tasselyzer are closely correlated with the degree of anther extrusion and are comparable even if the tassel architecture is variable. When comparing variable tassels, measurements of whole tassels yielded more consistent results compared with sampling individual sections of main spikes and side branches; further, ratios are only comparable within the same architectural level, i.e., they aren't fully comparable between sections of main spikes and side branches. Our work suggests that Tasselyzer could

be applied to more sophisticated studies that would benefit from quantifying anther extrusion, such as detecting quantitative trait loci (QTL) for anther extrusion-related male fertility under environmental impacts.

Beside its primary use to quantify anther extrusion in maize grown in chambers or greenhouses, Tasselyzer may be useful to evaluate pollen grain viability using Alexander solution-stained pollen images taken by a fluorescent microscope (Supporting Figure S4). Such a sub-functionality of Tasselyzer could enable the pipeline to better evaluate individual male fertility. A limitation of Tasselyzer is that it is optimized to distinguish anthers that are yellow or pink in hue, which are distinct in color from the green branches. However, Tasselyzer could not effectively process tassel images when the colors of anthers and branches were too similar, for example, when assessing anthers with a green hue (Duangpapeng *et al*., 2018); therefore, an alternative method will be required in these cases. In the future, a highly automated imaging and analysis system could be developed to evaluate individual maize male fertility based on Tasselyzer to meet the requirements of larger-scale field research.

Conclusion

In this study, we demonstrate an approach for analyzing the process of maize tassel flowering by using Tasselyzer, a PlantCV-based machine learning pipeline. The degree of anther extrusion at its peak could be quantified and used to evaluate male fertility of maize tassels. The impacts of genetic and environmental factors on anther extrusion could be measured and distinguished by using Tasselyzer. Thus, Tasselyzer is a useful tool to study male fertility in maize.

Experimental Procedures

Plant material and growing conditions

Maize (*Zea mays*) A632, Mo17 inbred, and *dcl5-mu03* seedlings (Teng *et al*., 2020) were grown in an optimized greenhouse for maize (28° C/ 22° C, 14 h day/10 h night temperatures, approximately 500 □mol/m^2/s). The established 1:1 segregating line of temperature-sensitive male sterile *dcl5-mu03* and their heterozygous siblings were grown in the greenhouse first, and then were treated under two temperature regimes in a CONVIRON PGC20 chamber during the phenotype critical stages, and finished their cycle in the optimized greenhouse. The phenotype critical stages refer to plants with anthers between meiotic to post-meiotic stages (anthers approximately 1.5 to 3.0 mm in length). *dcl5-mu03* is male sterile under the restrictive temperature regime (28° C/ 22° C, 14 h day/10 h night temperatures, approximately 300 μmol/m^2/s), and its male fertility partially could be partially restored under permissive temperature regime (22° C/ 20° C, 14 h day/10 h night temperatures, approximately 300 μmol /m^2/s).

Determination of anther extrusion

Tassel RGB images were obtained using smart phones with digital cameras. Each image contained one whole tassel on a dark background and any excess maize leaves were removed or covered by a dark cloth. Each tassel image contains three classes of pixels for downstream analysis including anther, other tassel parts (branch and glum), and dark background. Training pixels of the three classes were collected by using the pixel inspection tool of ImageJ 2 (Schindelin *et al*., 2015; Rueden *et al*., 2017). Each cell in the resulting table contained a comma-separated RGB value, and each column contained values of a class in a tab-limited table. Multiple images were sampled for generating the training table, as an input for 'plantcv-train.py' script to generate probability density functions (PDFs) for each class. Then, Tasselyzer segmented images into classes, generated the pseudo-colored images and calculated anther

ratios by using the PDFs to parameterize the naïve Bayes classifier function. Two-tailed Student's *T*-test was applied for statistics for Figure 5.

Microscopy and imaging

Alexander's staining solution (Alexander, 2009) was used to test the viability of *dcl5-mu03* pollen grains in the permissive condition. Stained pollen samples were imaged using a Zeiss Axiocam 512 color CCD camera on a ZEISS Axio Zoom with green- and red-light filters. In the original images, the viable pollen grains are round and orange, and the dead pollen debris shrinks with only green pollen wall remaining. Tasselyzer pseudo-colored the original images and assigned fuchsia to pollen, and green to pollen walls. A ratio of pollen viability was calculated from the sum of viable pollen pixels (fuchsia) divided by the sum of total pollen pixels (fuchsia and green).

Code and Data Availability

Tasselyzer is based on PlantCV and developed by integrating Matplotlib v 3.2.1, PlantCV v3.8.0, OpenCV v 3.4.9, NumPy v 1.18.1, Python v 3.7.7, and skimage v 0.14.3 modules. The code, the original and pseudo-colored images in this study will be made available in an open-access folder with the title of this publication within the GitHub of https://github.com/danforthcenter/plantcv-tasselyzer-tutorial; also within Zenodo of https://doi.org/10.5281/zenodo.5524971 (Teng *et al*., 2021). The full image sets were used in this study are available within the Zenodo of https://doi.org/10.5281/zenodo.5525073 (Teng *et al*., 2021).

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Author contributions

C.T., N.F., and B.C.M. conceived of the project, N.F. programmed the scripts, and C.T. performed and interpreted experiments, C.T. wrote the manuscript with editing by N.F., B.C.M.

Conflict of Interest

The authors declare no competing interests.

Supporting Information

Supporting Figure S1. Tasselyzer workflow outline.

Supporting Figure S2. Additional examples of fertile tassels over seven days.

Supporting Figure S3. Pollen viability analyzed by Tasselyzer.

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Figures and Figure Legends

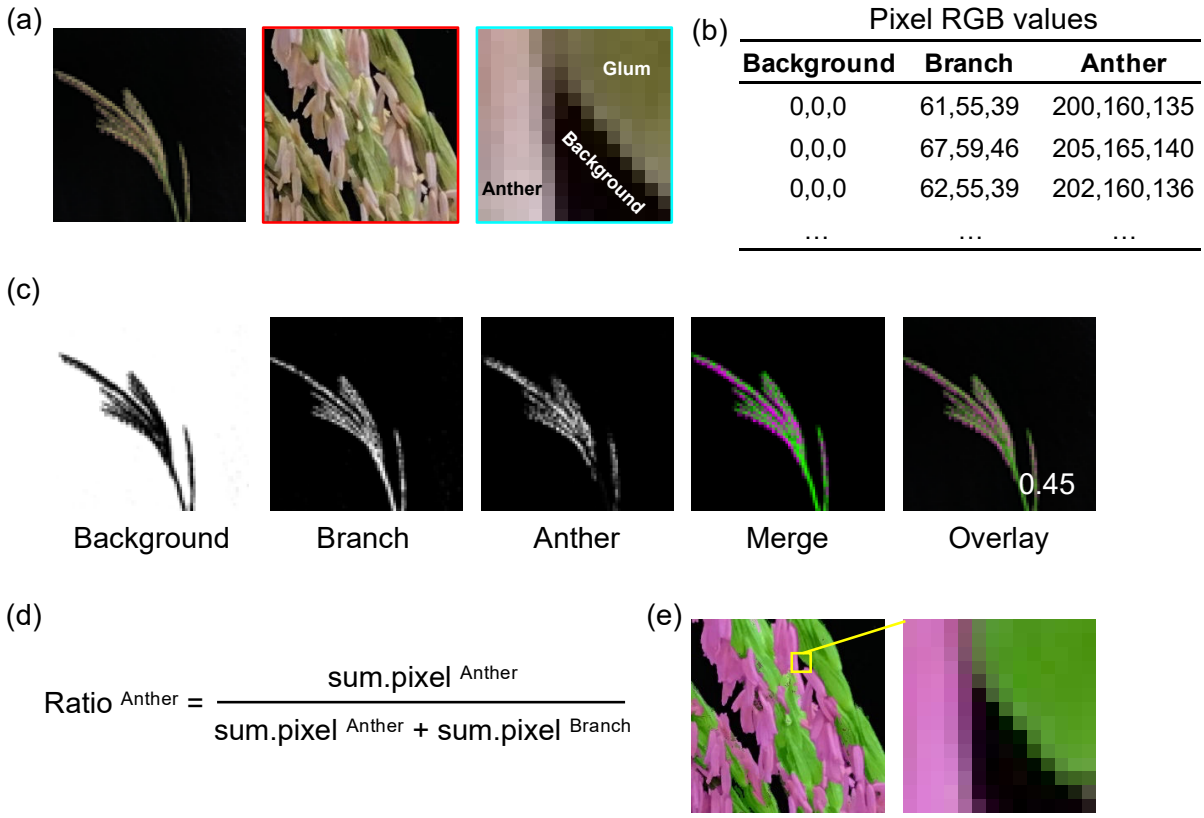


Figure 1. Tasselyzer anther ratio quantification based on PlantCV.

(a) An example fertile Mo17 inbred maize tassel image (left); a part of branches was enlarged (red box, middle); and a small cluster of pixels from anther, glum and background was further enlarged (cyan box, right).

(b) Pixels of anther, branch (or glum), and background have distinct RGB values, which were sampled by using ImageJ 2.

(c) These RGB values were used to classify the background, branch and anther pixels (in white) of this example tassel image by using Naïve Bayes approach; next, pseudo-colored images with pixels classified as branch or glum (green) and anthers (fuchsia) were merged and overlayed on the original tassel image. Anther ratio was determined for this example Mo17 inbred tassel (number in white, bottom right), and the formula is shown in (d).

(d) Formula of anther ratio is determined by sum of anther pixels (fuchsia) dividing to sum of tassel pixels (anther and branches, fuchsia and green).

(e) The pseudo-colored enlarged tassel branches (left) and further enlarged cluster of pixels (yellow box, right) in (a).

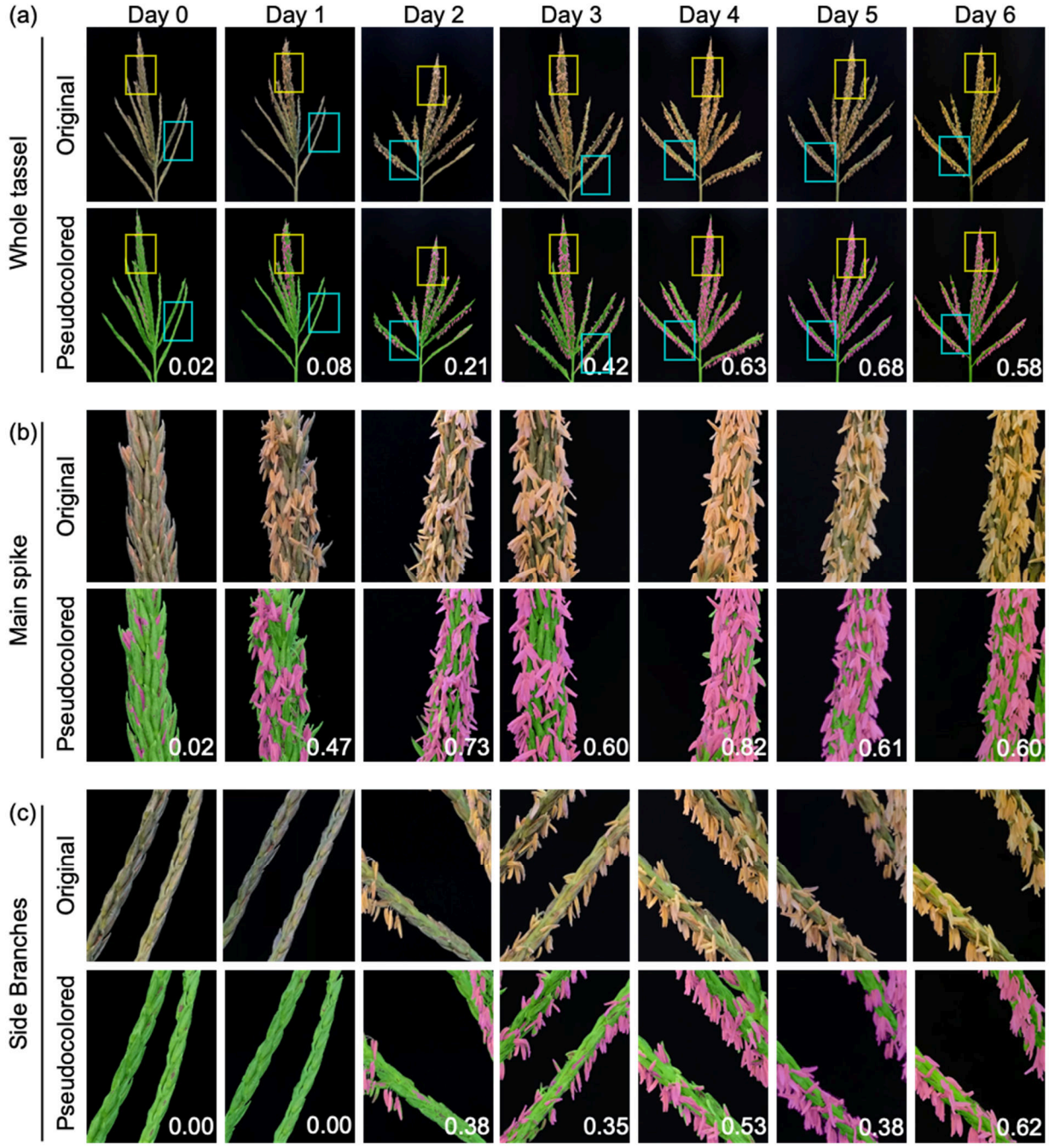


Figure 2. Anther extrusion of a male-fertile tassel over seven days.

Anther extrusion of a fertile A632 inbred tassel takes approximately five days in greenhouse with optimized growth condition for maize. No anthers emerged on day 0; anthers emerged from the upper florets of main spike on day 1; anthers from the lower florets of main spike and upper

florets of the side branches emerged on day 2; upper florets on the lowest side branch on day 3; anther extrusion peaked on day 4 and day 5 with more anthers emerged from lower florets on the side branches; anther extrusion was maintained or slightly declined on day 6. Anther ratio was determined for each image (number in white, bottom right of pseudo-colored images).

(a) Original (upper) and pseudocolored (lower) images from an example fertile A632 inbred tassel from day 0 to day 6. Pseudocolored images with pixels classified as branch (green) and anthers (fuchsia) overlay on the images.

(b) Enlarged original (upper) and pseudo-colored (lower) images of main spike in (a, yellow boxes).

(c) Enlarged original (upper) and pseudo-colored (lower) images of side branches in (a, cyan boxes).

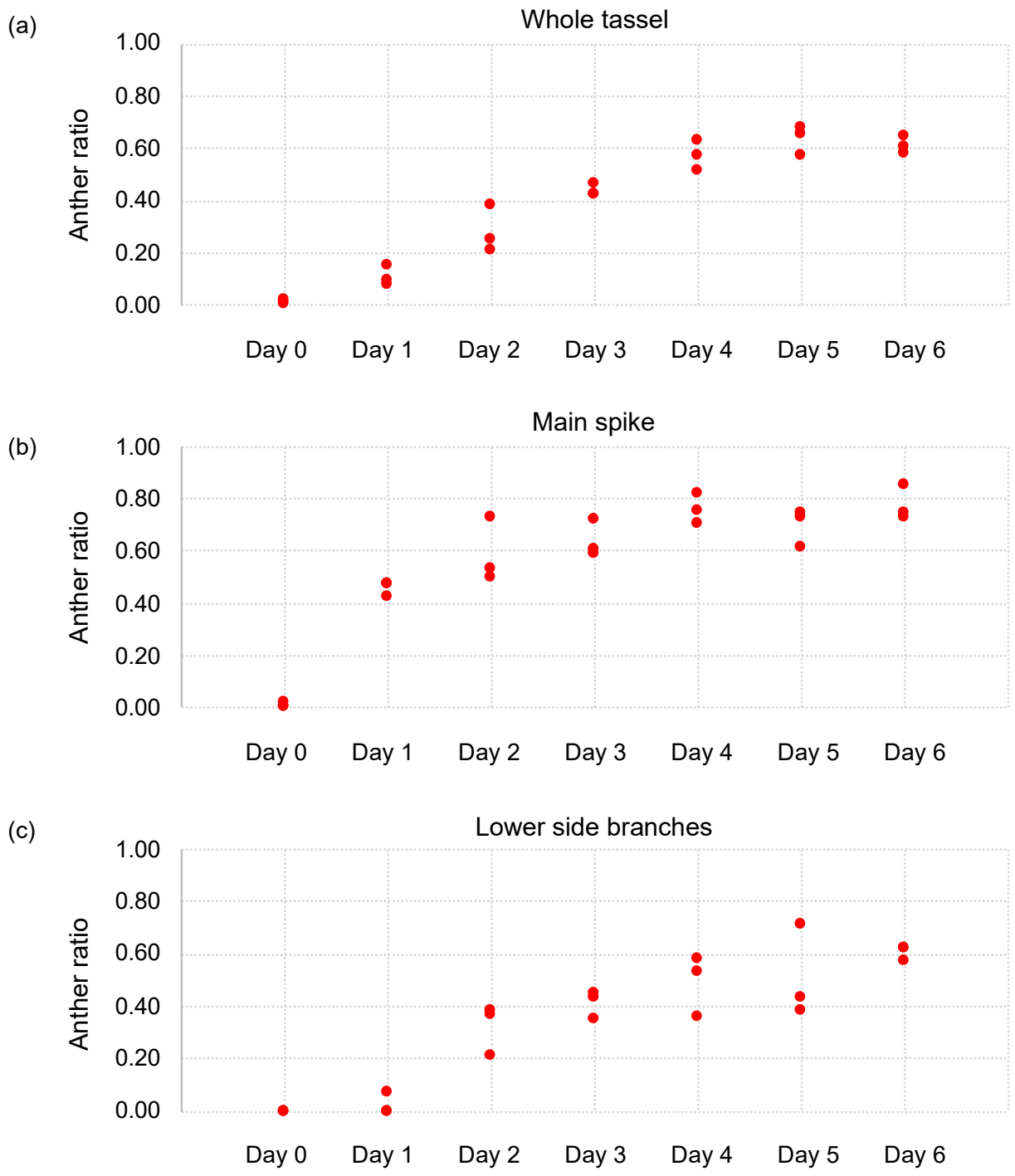


Figure 3. The anther ratio of fertile tassels over a seven-day period.

(a) Anther ratio dot plot of whole tassels from three example fertile A632 inbred plants over a seven-day period of anther extrusion.

(b) Anther ratio dot plot of main spikes from three plants in (a), over a seven-day period of anther extrusion.

(c) Anther ratio dot plot of side branches from three plants in (a), over a seven-day period of anther extrusion.

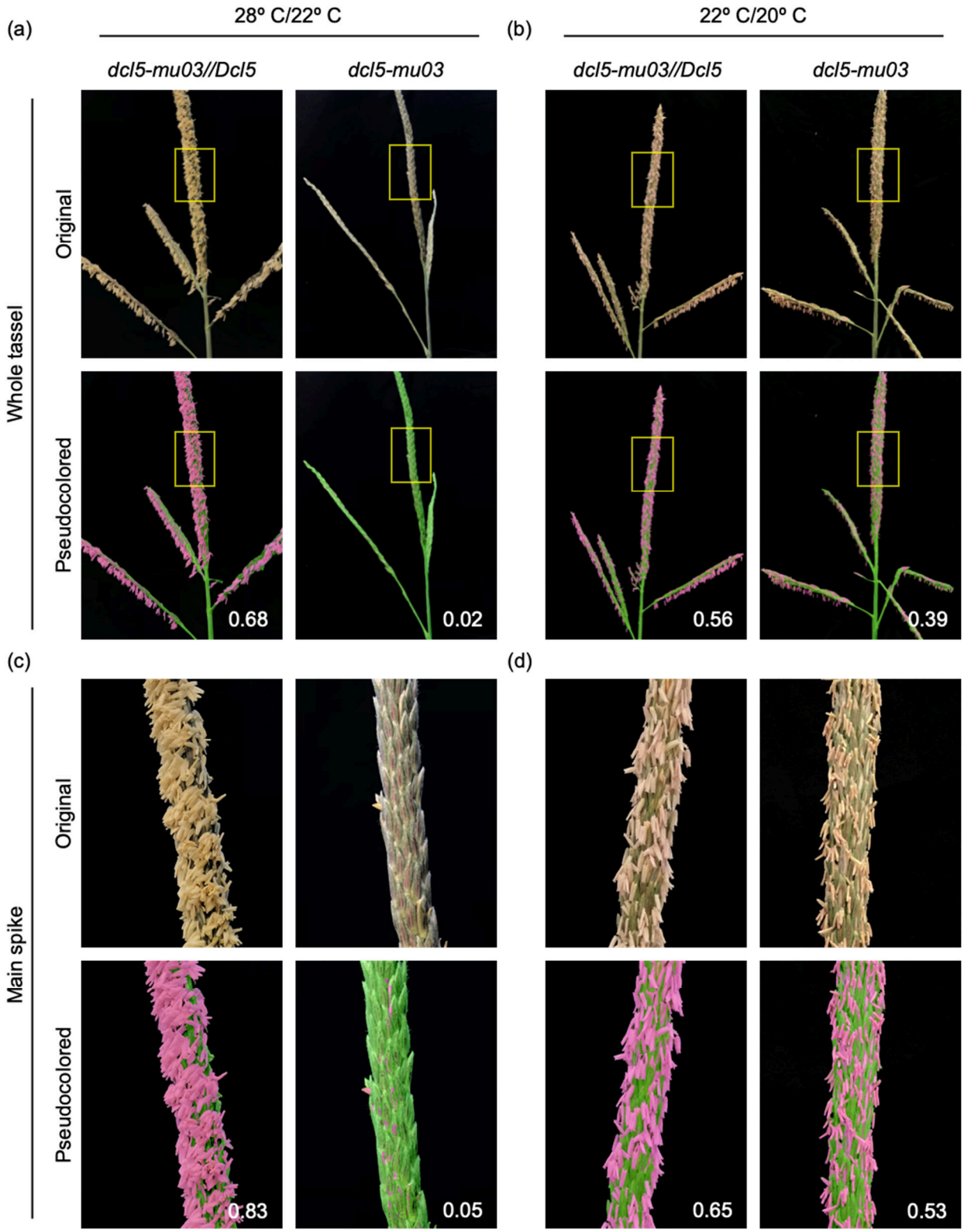


Figure 4. Anther extrusion in the temperature-sensitive male sterile *dcl5-mu03* mutant.

Anther extrusion of the temperature-sensitive male sterile mutant *dcl5-mu03* in chambers with restrictive (28º C/22º C, 14 h day/10 h night) or permissive (22º C/20º C, 14 h day/10 h night) temperature for maize, comparing to fertile sibling.

(a) Original (upper) and pseudocolored (lower) tassel images from an example *dcl5-mu03* or heterozygous *dcl5-mu03//Dcl5* fertile sibling grow under the two regimes. Pseudo-colored images with pixels classified as branch (green) and anthers (fuchsia) overlay on the images.

(b) Enlarged main spike (yellow boxes). Anther ratio representing pixels of anthers (fuchsia) to pixels of tassel (fuchsia and green, i.e. anthers + branches) was determined for whole tassel images (number in white, bottom right).

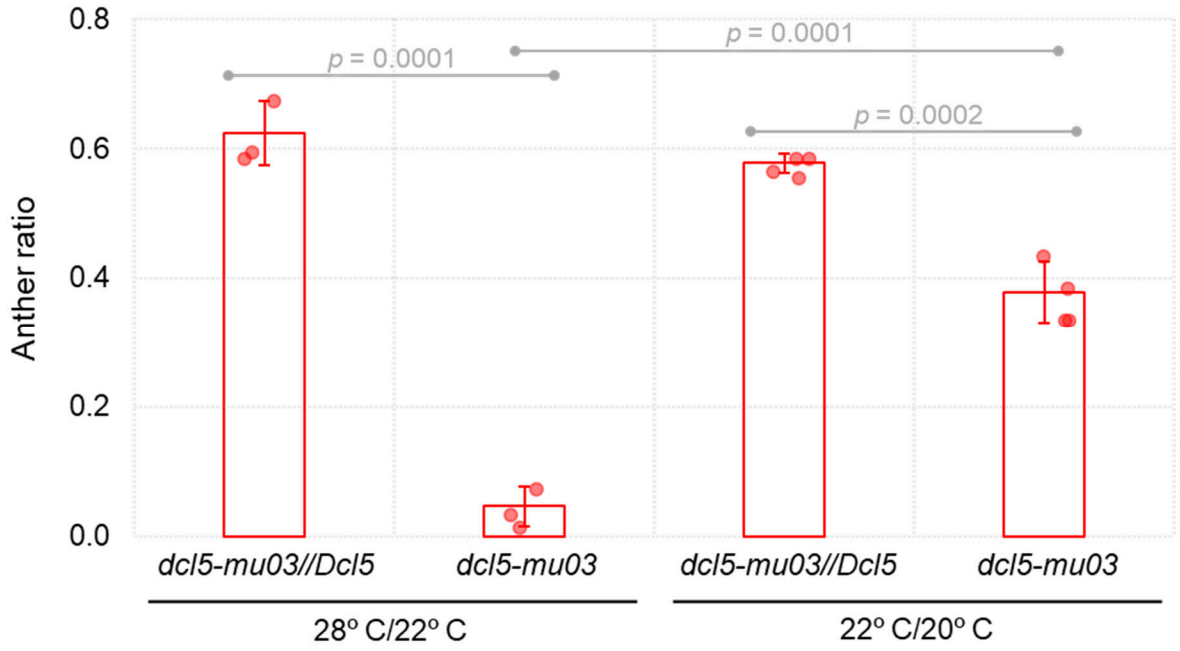
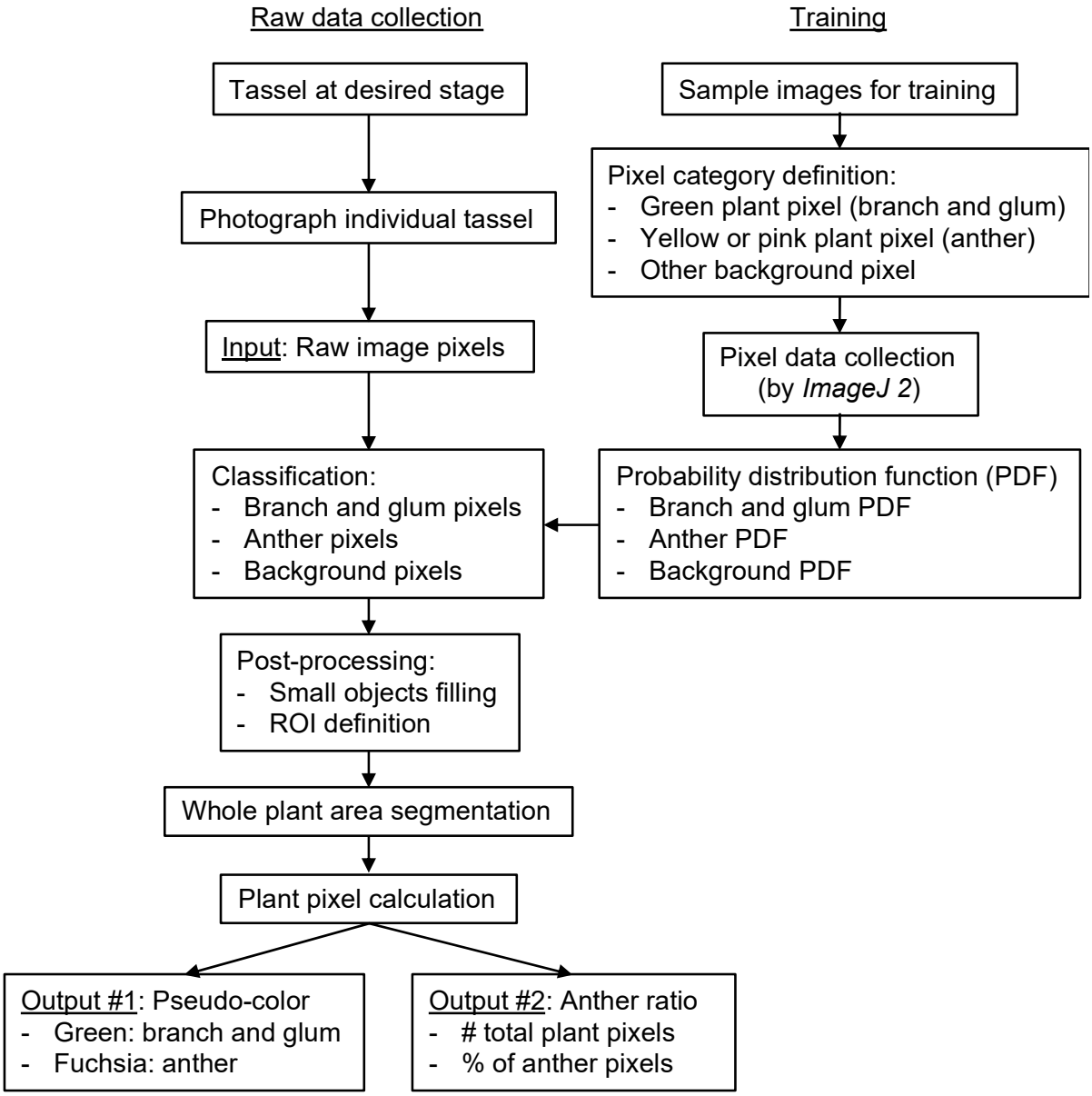


Figure 5. Determination of anther ratios of *dcl5-mu03* tassel under temperature regimes. Anther ratio dot plot of whole tassels of temperature-sensitive male sterile mutant *dcl5-mu03* in grown in chambers with restrictive (28º C/22º C, 14 h day/10 h night) or permissive (22º C/20º C, 14 h day/10 h night) temperature for maize, comparing to heterozygous *dcl5-mu03//Dcl5* fertile sibling. Statistics: two-tailed Student's *T*-test. At least three replicates were used, and standard deviation was plotted.

Supporting Information

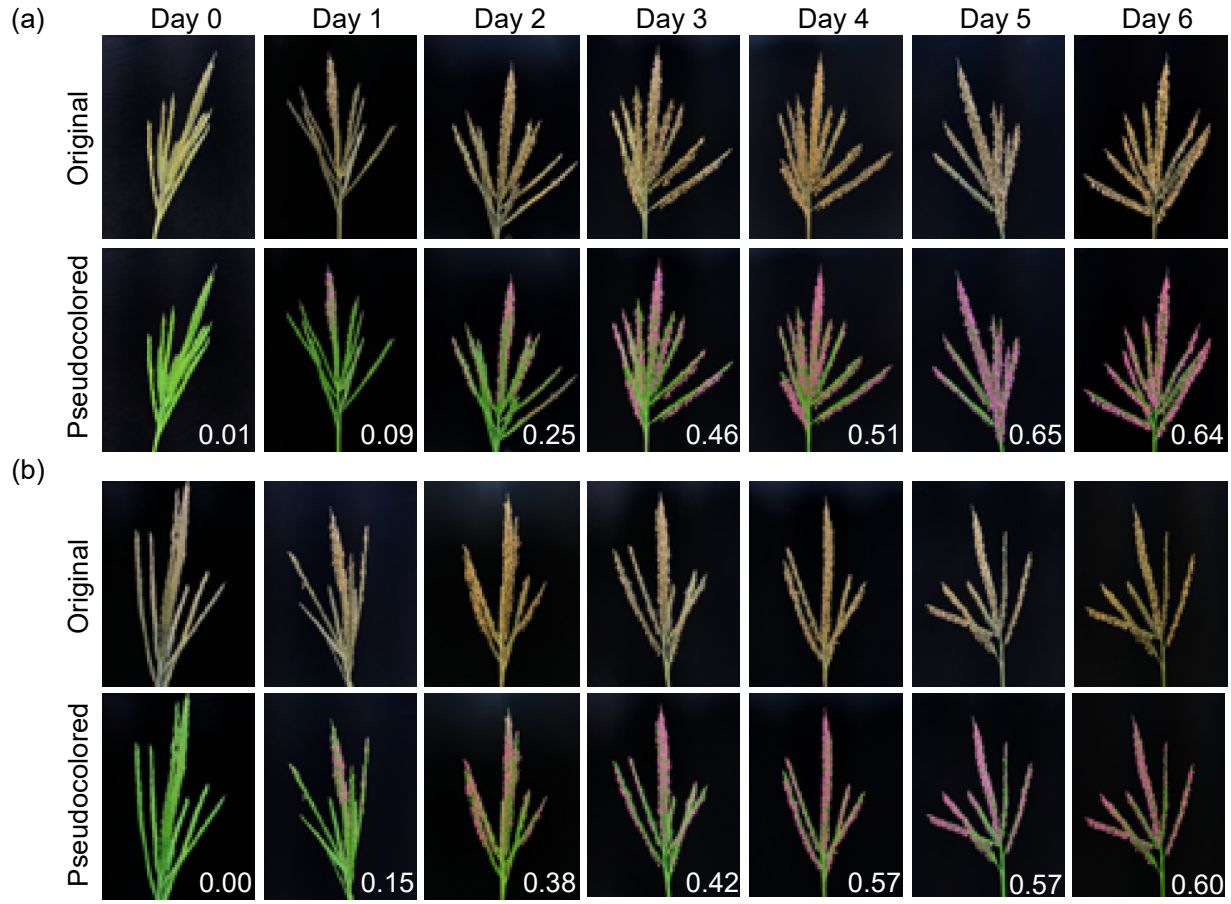
Supporting Figure S1-3.



Supporting Figure S1. Tasselyzer workflow outline.

The overall workflow is similar as reported previously (Zheng *et al*., 2019). For image collection, individual tassel was photographed at desired stages at described in the Experimental Procedures; then multiple sample images were used to collect pixels for each classes. Three pixel-classifiers were identified and RGB information of pixels in each class were collected to build up the training datasets. The Probability distribution functions (PDFs) for each category

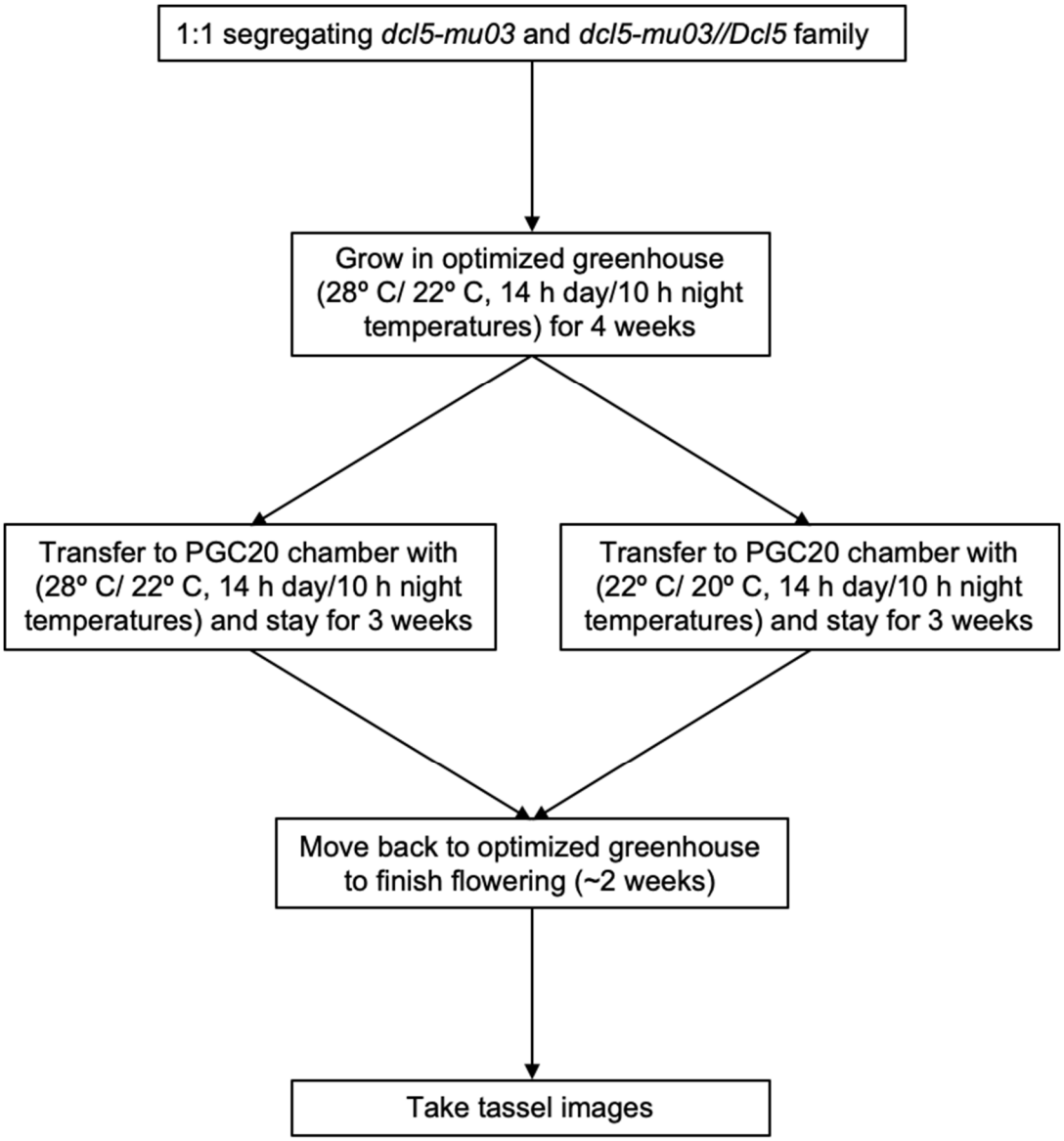
were calculated based on the training datasets, and then these PDFs were used to classify pixels in the tassel images. After post-processing steps, the pipeline produced pseudo-colored images to merge with original image, and summaries of pixel numbers and anther ratio.



Supporting Figure S2. Additional examples of fertile tassels over seven days.

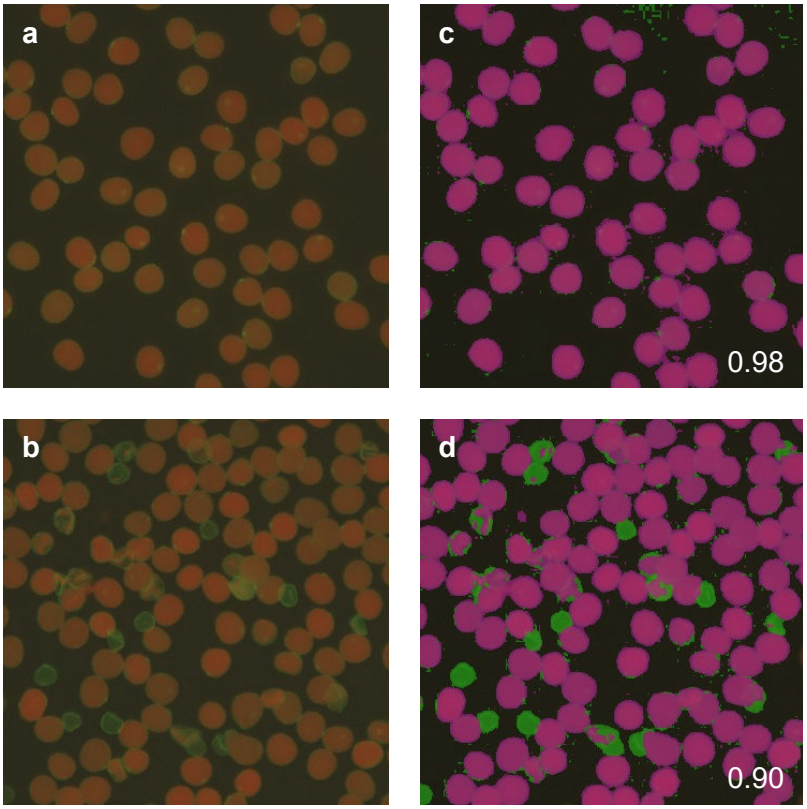
(a) Original (upper) and pseudocolored (lower) images from a second example fertile A632 inbred tassel from day 0 to day 6. Pseudocolored images with pixels classified as branch (green) and anthers (fuchsia) overlay on the images.

(b) Original (upper) and pseudocolored (lower) images from a third example fertile A632 inbred tassel from day 0 to day 6. Anther ratio representing pixels of anthers (fuchsia) to pixels of tassel (fuchsia and green, i.e. anthers + branches) was determined for each image (number in white, bottom right).



Supporting Figure S3. Segregating 1:1 *dcl5-mu03* family growth under temperature regimes. About twenty seeds from a 1:1 segregating *dcl5-mu03* and *dcl5-mu03//Dcl5* family were initiated in the optimized greenhouse with temperature at 28º C/22º C as indicated in the Experimental Procedures. The seedlings were genotyped at week two after sowed. After four weeks after sowed, six plants of each genotype were transferred to the CONVIRON PCG20 chamber with 14 h day/10 h night temperature at 28º C/22º C or 22º C/20º C. At this point, the plants just developed baby tassels before the phenotype critical stages. Then they grew in the chamber for

another three weeks, and then were moved back to the optimized greenhouse to finish flowering for tassel images.



Supporting Figure S4. Original and pseudo-colored images from *dcl5-mu03* pollen grains under permissive condition.

To determine pollen grain viability of *dcl5-mu03* under permissive growth condition (22 ℃ /20 ℃ day/night), pollen grains from fertile *dcl5-mu03//Dcl5* heterozygous and *dcl5-mu03* homozygous plants were stained by using Alexander's staining method, and then imaged under an epi-fluorescence microscope.

(a) Original image of pollen grains (n=57) from *dcl5-mu03//Dcl5*, which were all viable (orange).

(b) Original image of pollen grains (n=122) from *dcl5-mu03*, with 22 dead pollen grains (green) which are smaller in size and empty inside.

(c) Pseudo-colored images of pollen grains from *dcl5-mu03//Dcl5* in (a), with pixels enhanced in green (dead) and fuchsia (viable). A ratio representing pixels of viable (fuchsia) to total pollen (viable+dead, fuchsia+green) was determined (numbers in white, bottom right).

(d) Pseudo-colored images of pollen grains from *dcl5-mu03* in (b). A ratio of viable pollen was determined.