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Towards Measuring Plant Photosynthetic Heterogeneity

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ABSTRACT

Motivation: Photosynthesis is one of the most important biological processes on earth. Plant photosynthetic heterogeneity refers to a plant comprising multiple regions, many of which have significantly different photosynthesis properties, probably because of vastly different leaf developmental stage and tolerance level to environmental changes. Measuring plant photosynthetic heterogeneity enables biologists to interpret the sophisticated photosynthesis phonemics data, which is particularly important for plant primary productivity estimation and modeling.

Results: Taking advantage of the rapid developing non-invasive plant phenotyping technologies, we develop a new plant photosynthetic heterogeneity measurement called PlantPH to effectively identify and integrate the plant morphological and physiological features.

The application of PlantPH on more than 1,000 Arabidopsis chloroplast plants, including dozens of ecotypes and hundreds of chloroplast mutant strains, has successfully identified a group of genes that affect photosynthesis under simulated natural environments.

Availability: Software is available at XXX.

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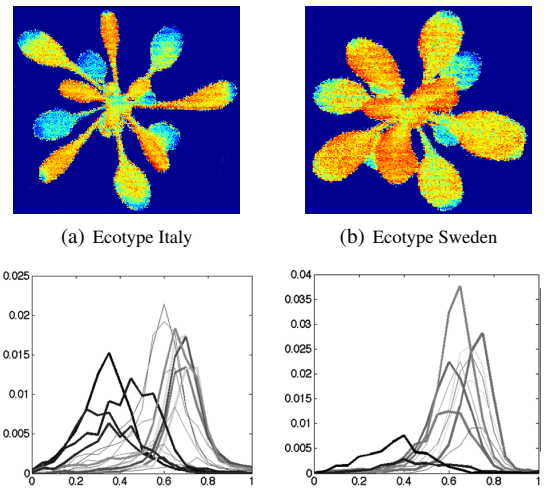
1 INTRODUCTION

By consuming water, light and CO_2, plants produce sugars and release O_2 with photosynthesis (Kramer and Evans, 2011). The process involves the formation of high energy intermediates capable of generating reactive oxygen species. The photosynthetic apparatus, chloroplast and surrounding leaf tissue is inherently susceptible to oxidative damage, especially under stress conditions when the supply of light energy exceeds the capacity to utilize it (Durrant *et al.*, 1990; Asada, 1996). Plants have evolved a number of mechanisms, such as photosynthetic apparatus damage and repair (Melis, 1999), to dissipate excess light energy to minimize the potential for damage at the expense of photosynthetic efficiency (Adams III *et al.*, 2006; Rochaix, 2014). However, these mechanisms are sensitive to leaf development and thus may change from one leave tissue to another, resulting in heterogenous photosynthetic patterns (see an example in Figure 1). The heterogeneous patterns also vary with the position, size and growth rate of leaves, since leaves at the same node is unique in age. By integrating plant morphological and physiological features, measuring plant photosynthetic heterogeneity aids interpretation of the sophisticated photosynthesis phonemics data, particularly important for plant primary productivity estimation and modeling (Meng *et al.*, 2007).



(a) Ecotype Italy (b) Ecotype Sweden

(c) Histogram of all leaves in (a) (d) Histogram of all leaves in (b)

Fig. 1. We demonstrate the photosynthesis heterogeneity using two Arabidopsis ecotypes (Italy and Sweden). Both plants were grown under the same stress conditions, but the false-color images of photosystem II activity (a,b) and the distributions of the leaf-level photosynthesis (c,d) show that the Italy ecotype is more suspectable to environmental changes.

Heterogeneity is a statistical concept relating to the uniformity in a substance (Hall, 2003). The granularity of the plant photosynthetic heterogeneity studies can range from cells to tissues, leaves, and even to the whole plant level. While in-leaf variability in photosynthetic activity has been well-studied for the understanding of the effects of stomatal conductance (Cheeseman, 1991; Buckley *et al.*, 1997), recent works show that photosynthetic capacity may decline with vertical gradient and leaf age (Kitajima *et al.*, 2002; Chen *et al.*, 2008), suggesting that leaf-based photosynthetic heterogeneity is a key towards processing and understanding photosynthesis phenotype images.

The leaf heterogeneity in photosynthesis was firstly been studied with computer simulation (Chen *et al.*, 2008). Due to the lack of high-throughput phenotyping technologies, the authors determined the effects of biochemical variability via the Farquhar model (a mechanistic, biochemical model widely used to describe steady-state CO_2 assimilation in leaves), incorporating defined degrees

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of spatial variability of its parameters (Sharkey, 1985; Farquhar *et al.*, 2001). Recently, with the advent of advanced technologies of biomedical imaging, directly measuring heterogeneity has recently assumed new importance (Tiihonen *et al.*, 1996; Wieneke *et al.*, 1999; Wang *et al.*, 1999; Cruz *et al.*, 2015). The rapid development of lighting and imaging techniques enables real-time non-invasive monitoring of photosynthesis (Houle *et al.*, 2010; Cruz *et al.*, 2015), resulting in vast amount of chlorophyll fluorescence images of plants (Wituszynska *et al.*, 2013). These images can be used to quantify photosynthetic behavior in genetically diverse populations, enabling to measure variability of photosynthetic parameters at high resolution across leaves, leading to better understanding of the underlying mechanisms that control the photosynthetic properties (Rascher *et al.*, 2011; Fiorani and Schurr, 2013).

Measuring leaf-based photosynthetic variability in large-scale phenotyping experiments requires automatical identification of most of the visible leaves in all fluorescence images and an appropriate measurement for the variation of photosynthesis parameters across leaves that takes geometrical (leaf position and size), temporal (leaf growth rate) and physiological (leaf age) information into consideration. However, there is currently no existing infrastructure to analyze such amount of phenotype information. In this paper, we present a new computational framework called *Plant Photosynthesis Heterogeneity* (PlantPH), and use the tool to analyze the leaf-level photosynthesis heterogeneity patterns of more than 100 Arabidopsis chloroplast mutant strains over dynamic lighting conditions, each with at least four replicates. The followed outlier detection process successfully identify mutants with distinct heterogeneity patterns under specific subsets of the environmental conditions. Overall, PlantPH has the following three advantages:

1. It is the first computational framework to measure plant leaf-level heterogeneity of photosynthesis with leaf size, position and growth rate.
2. It's performance is significantly better than the traditional heterogeneity tests that cannot fully use the spatial and temporal information in the fluorescence images.
3. It discovers multiple types of photosynthetic variabilities in a large scale experiment.

2 BACKGROUND

The general method of assessing whether the leaves of a plant are photosynthetically homogeneous or heterogeneous is by means of the Cochran's Q-test (Conover, 1999) and the I^2 statistic (Higgins and Thompson, 2002; Higgins *et al.*, 2003).

Cochran's Q-test is the classical measure of heterogeneity (Conover, 1999). In leaf-based photosynthetic heterogeneity, Q is calculated as the weighted sum of squared differences between the photosynthesis value of a individual leaf and the pooled photosynthesis value across all leaves with the weights being those used in the pooling method. The distribution of Q is a chi-square statistic with $k-1$ degrees of freedom, where k is the number of leaves. Cochran's Q-test has been widely used in biomedical studies. For example, heterogeneity in the aggressiveness of tumor cell populations has been adopted as an essential feature in predicting treatment success (O'Sullivan *et al.*, 2003). The I^2 statistic describes the percentage of variation across studies that is due to heterogeneity rather than chance (Higgins and Thompson, 2002; Higgins *et al.*, 2003). I^2 can be calculated as $I^2 = (Q-df)/Q$, where Q is Cochran's Q-test heterogeneity statistic and df is the degrees of freedom. A negative value indicates no observed heterogeneity, and larger values show increasing heterogeneity. I^2 is an intuitive and simple expression of the inconsistency. I^2 of leaf-based photosynthetic heterogeneity does not inherently depend upon the number of leaves, so that I^2 values of different plants become comparable. A confidence interval for I^2 can be constructed using either the iterative non-central chi-squared distribution method (Hedges and Pigott, 2001) or the test-based method (Higgins and Thompson, 2002).

Due to the nature of plant, leaf-based photosynthetic heterogeneity often includes a small number of leaves. For example, there are less than ten rosette leaves of 2-3 week old Arabidopsis thaliana (a model plant). Thus, the power of the traditional Cochran's Q-test and I^2 statistic in such circumstances could be low (Higgins *et al.*, 2003; Gavaghan *et al.*, 2000; Huedo-Medina *et al.*, 2006; Ioannidis *et al.*, 2007). Furthermore, leaves may have different locations and sizes, and varying growth rates (Van Lijsebettens *et al.*, 1991), but none of them has yet been taken into the computation of heterogeneity.

Based on the rapid development in the fields of bio-imaging and computer vision, we develop a new approach called PlantPH that quantifies the effect of heterogeneity of photosynthesis across leaves of the same plant, and compare the degree of inconsistency among mutant strains in varying environmental conditions.

3 METHOD

In this article, we present a new leaf-based heterogeneity measurement, followed with a framework to efficiently use the measurement on large-scale experiments.

3.1 PlantPH measurement

We introduce a new leaf-based heterogeneity measurement called *PlantPH* as follows. First, let $T = \{T_1, T_1, \ldots, T_n\}$ be the set of effect estimates of a plant p with n leaves. The effect estimate T_i of leaf l_i in plant p is defined as the difference between the averaged photosynthetic values of the leaf and the whole plant. Mathematically, in each effect estimate T_i, let u_i and u_p be the averaged photosynthesis values of leaf l_i and the whole plant p, respectively. Under the assumption of normal distribution and homoscedasticity, the effect estimate T_i is the standardized mean difference (Hedges and Vevea, 1998), which can be estimated by:

$$T_i = \frac{c(l_i)\,(\mu(l_i) - \mu(p))}{S(l_i, p)} \tag{1}$$

where $c(l_i)$ is a correction factor for the positive bias suffered by the standardized mean difference with small sample sizes, which can be estimated by $c(l_i) = 1 - 3/(4(|l_i| - 1) - 1)$ (Hedges and Olkin, 1985). This adjustment will reduce the effect estimate of small leaves that only have a few pixels, and thus increase the robustness of the heterogeneity model (Huedo-Medina *et al.*, 2006). The pooled estimate of the within-group standard deviation $S(l_i, p)$ can be computed with (Hedges and Vevea, 1998):

$$S(l_i, p) = \sqrt{\frac{(|l_i| - 1)std^2(l_i) + (|p| - 1)std^2(p)}{|l_i| + |p| - 2}} \tag{2}$$

where $std^2(l_i)$ is the variance of leaf l_i, $std^2(p)$ is the variance of the averaged values of all leaves in plant p, and $|p|$ are the total number of pixels of leaf l_i and the whole plant p, respectively.

Then the Cochran's Q-test statistic for determining whether there is true leaf-based photosynthetic heterogeneity among the leaves is defined as:

$$Q = \sum_{i=1}^{n} w_i \left(T_i - \frac{\sum_{j=1}^{n} w_j T_j}{\sum_{j=1}^{n} w_j} \right)^2 \tag{3}$$

where n is the total number of leaves of the plant p, the right part of the equation is the weighted mean of effect estimates of all the leaves of the

plant p, and $w_i = 1/(\tau^2 + \delta_i^2)$, where δ_i^2 is the sampling variance of the effect estimate T_i, and τ^2 is the between-study variance of all the effect estimates (Huedo-Medina *et al.*, 2006).

To estimate δ_i^2, the sampling variance of each effect estimate T_i, we use the photosynthetic value of every pixel in the high-resolution florescence images, in which the total number of samples of a plant is much greater than 1000. According to (Huedo-Medina *et al.*, 2006), δ_i^2 is close to 0. Hence, by ignoring the computation of δ_i^2 and replacing it with 0, we have:

$$Q = \frac{1}{\tau^2} \sum_{i=1}^{n} \left(T_i - \frac{\sum_{j=1}^{n} T_j}{n} \right)^2 \tag{4}$$

Since both the between-study variance τ^2 and the Q statistic represent the true heterogeneity among the distributions of the leaf-level photosynthesis, we move τ^2 to the left of the equation and define a new measure called *PlantPH*:

$$PlantPH(f) = \sum_{i=1}^{n} \frac{\left(nT_i f(l_i) - \sum_{j=1}^{n} T_j f(l_j) \right)^2}{n^3} \tag{5}$$

In Equation 5, $PlantPH(f)$ is a measure for determining whether there is true heterogeneity among all leaves of a plant. It is independent to the total number of leaves, allowing for being comparable among different plants. We take plant leaf morphology into the plant heterogeneity test by defining $f(.)$ as a function to measure the morphological properties (e.g., the area, position or growth rate) of a piece of leaf.

Specifically, in $PlantPH(area)$ we have $f(l_i) = area(l_i)$, which measures the leaf surface area (Boyes *et al.*, 2001; Tessmer *et al.*, 2013), and in $PlantPH(growth)$ we define $f(l_i) = growth\_rate(l_i)$ by adopting a three-parameter nonlinear growth model to compute both the absolute growth rate (AGR) and the relative growth rate (RGR) (Richards, 1959; Hunt, 1982; Tessmer *et al.*, 2013). Moreover, in $PlantPH(position)$, we define $f(l_i) = position(l_i)$ to be 0 if l_i is at the center of the plant, and 1 otherwise, in order to measure whether the leaves with a similar developmental stage have heterogeneous photosynthetic values.

3.2 PlantPH workflow

The input to the PlantPH measurement is leaf-level photosynthesis, which relies on leaf alignment and tracking (Yin *et al.*, 2014a). Leaf alignment is a recent developed algorithm to identify leaf boundaries from a serial of florescence images. XXX

There are two obstacles preventing us from directly using the leaf alignment algorithm for leaf heterogeneity test. First, the leaf alignment process may fail when the leaf overlap rate exceeds 50%, and the leaf boundary is not precisely identified because of the use of predefined leaf templates. Subsequently, the leftover of leaf alignment process on a plant image consists of partial leaves that are highly overlapped and leaf boundaries of the successfully recognized leaves (Fig. XX). Leaves covered by the other leaves are usually old leaves with diverse photosynthesis activities. Simply deleting them may cause inaccurate heterogeneity scores. Second, the leaf alignment process is time consuming. Processing a serial of 100 images each containing about 20 plants may take up to 5 hours. It is not practical to process all the phenotype images, which has occupied 30T space in our hard drive, array using leaf alignment.

In this article, we introduce an efficient workflow for leaf heterogeneity test. We observe that the heterogeneous plant images are rare in the whole dataset. And the distribution of a homogenous plant usually falls into one normal distribution with a relative narrow range of phenotype values compared to the distribution of a heterogenous plant (Fig 1). Therefore, we screen all the plant images and only select those with relatively wide range of values (Equation XX) and pass them to the leaf alignment process. This process is efficient since it is very simple. Even though, it can quickly eliminate about 85% of all the plant images, according to the results on the real data. Note that we do not use standard deviation of a similar measure because the distribution of a heterogeneous plant consists of multiple distributions and each of them may not be normal.

$$PlantRange(p_i) = max(p_i) - min(p_i) \tag{6}$$

where p_i is plant i, and max and min return the maximal and the minimum values of a plant photosynthetic parameter. Note that we delete top and bottom 2% of the values to exclude outliers.

$$P - value(PlantRange(p_i)) = \tag{7}$$

where the p-value is

4 RESULTS

4.1 Arabidopsis Images

4.1.1 Data Acquisition and Preprocessing In the photosynthesis phenotyping experiment, hundreds of Arabidopsis thaliana plants (wild type and genetic variations with gene knockout) were grown side-by-side under three different light conditions (constant, sinusoid, fluctuate), for in total three days. Top-view fluorescence images were collected every 15 minutes in order to observe the photosynthesis activity of all of the plants simultaneously. Each fluorescence image is a grey-scale image with a resolution of 1M pixels at 12-bit intensity.

To accurately capture the photosynthesis activities of plants from fluorescence images, a image segment method is applied to remove the background, identify every piece of leaf (Yin *et al.*, 2014a), measure the intensity of pixels on leaves, and finally convert the intensity values to the measure of four kinds of photosynthesis parameters. The extracted measurements of photosynthesis parameters are presented in the form of multi-dimensional time-series, one dimension for every photosynthesis parameter.

4.1.2 PlantPH on Arabidopsis data In this experiment, wild type (col-0) and more than 100 Arabidopsis chloroplast mutant strains were grown side by side in a dynamic light condition for 3 days from 10 days old from seedling. A top-view fluorescence image was taken every 15 minutes during the day time, in order to observe the photosynthesis activity and the growth of the plants simultaneously. The overview of the experimental results is shown in Figure ???. In total, XXX fluorescence images were collected, preprocessed and fed to *PlantPH*. Note that the averaged leaf cell size of Arabidopsis is about $6000\mu m^2$ (Gegas *et al.*, 2014) and our image resolution is 3600 pixels per squared inch. Therefore, each pixel in an image is sampled from about 1000 leaf cells.

The architecture of the whole process is shown in Figure **??**. First of all, we apply a leaf alignment and tracking method that we recently developed to identify most of the leaves from the top-view fluorescence images (Yin *et al.*, 2014b,a), and then compute the leaf-based photosynthesis value for every leaf. In addition, we add a leaf by drawing a small circle in the center of every plant to represent the young leaves that are difficult to identify. All the pixels not contained in any leaf boundary are considered as an extra leaf. A leaf is ignored if its area is smaller than the plant center circle. Next, we apply *PlantPH* to compute the heterogeneity value for every plant at every snapshot, resulting in a heterogeneity matrix H. Finally, we recognize heterogeneity patterns in H with an outlier detection method, and visually explore them with the L'Abbe plot (Song, 1999).

The results show ...

4.1.3 Leaf alignment and tracking The chlorophyll fluorescence images are false-color images, where the light intensity of every pixel is proportional to photosynthetic efficiency (Toet and Walraven, 1996) (see Figure 1). Differences between individual leaves with similar photosynthetic efficiency can be subtle, making the boundaries between them difficult to define and creating a significant challenge for subsequent shape analysis. The difficulty even arises when individual leaves overlap and occlude one another in these false-color images.

We have developed a framework based on the well-known Chamfer Matching algorithm (Yin *et al.*, 2014a). Multi-leaf alignment aims to segment all leaves with pre-defined leaf templates and estimate the two tip points of each leaf. The tracking algorithm consists of two steps. First, a set of leaf templates are applied to the target image to generate the same amount of leaf candidates. Second, we adopt multi-objective optimization to select a subset of leaf candidates. The objective is to select a minimal number of leaf candidates with smaller Chamfer distances to cover the test image mask as much as possible.

Multi-leaf tracking is an extension of the leaf alignment algorithm. Given a serial of fluorescence images taken over time, we first apply the alignment algorithm to the last frame, and then continuously apply template transformation to the current leaf candidates in order to fit to the previous frame. A new objective function considering the Chamfer Matching distances, target image mask, and the rotation angles of all leaves is adopted. Both the leaf alignment and leaf tracking directly benefit the study of leaf behavior in plant biology, such as leaf growth, leaf-level photosynthesis, leaf-level variations in plant mutant, etc.

4.2 Refining leaf alignment with density-based clustering

Given that practically any algorithm for leaf tracking and alignment will fail in some cases, we have selected an alignment method that will typically fail by ”missing” leaves (parts of the plant will be excluded from alignment results) rather than giving false positives (parts of the plant are over-selected or the background is selected as a leaf). This sort of failure allows us to add another step after the initial alignment in which we use DB Scan (Kriegel *et al.*, 2011) to intelligently cluster the non-segmented plant pixels into separate leaves. DB Scan was chosen for being practical, having relatively few parameters, and not depending on the pixel intensities. Using a clustering algorithm that relies heavily on pixel intensities would defeat the purpose of identifying leaves for our application: further down the pipeline we want to consider leaves that have large amounts of variation in intensity. DB Scan is widely understood and used for analysis of spatial information with physical constraints (Zaïane and Lee, 2002), and allows fine-tuning of the minimum thickness of the resulting clusters. Avoiding very thin clusters is also imperative to our application, given that a nearly-correct leaf segmentation may result in a thin band of non-segmented pixels around the edge of a leaf. Considering these pixels as a separate leaf would not be appropriate - setting a minimum-thickness guarantees that any such pixels would not be clustered. Similarly, a leaf with very few pixels is statistically insignificant and often misleading even if the leaf is genuine. By setting a minimum number of pixels per cluster we discard very small clusters even if they meet the minimum thickness requirement.

4.3 PlantPH on synthetic data

A set of synthetic data were generated to test whether *PlantPH* is properly designed.

5 DISCUSSION

A consensus view of the data is that the photosynthesis ability of a plant is not uniform across the whole area (Charles 2008, Meng 2007). The photosynthetic properties of plants can vary dramatically across cells, tissues, and organs (), reflecting differences in development, stress responses, regulation of processes such as stomatal conductance (), photodamage (), and storage of photosynthate () and contribute substantially to productivity ().

For example, we observed that the acclimation of photosynthesis in response to cold temperatures appears to be more rapid and robust in younger or emerging than older leaves, and ecotypes isolated from different latitudes show distinct heterogeneity patterns, implying that these responses are important for adaptation of photosynthesis to fluctuating temperatures. In other cases, exposure of plants to fluctuating light resulted in loss of photosynthetic capacity or increased photoinhibition in specific sets of leaves or leaf sectors. In many cases, older leaves are preferentially affected, suggesting that resources for maintenance or acclimation responses are preferentially directed to younger leaves. However, we have also identified mutant lines where younger leaves are preferentially affected, which presumably affect the development of photosynthetic robustness.

In order to systematically study the leaf level photosynthesis phenotypes, especially in a high-throughput screen manner, we developed a novel computational tool to automatically conduct statistical analysis on leaf based photosynthesis.

In the future, we will consider more plant-specific constraints including leaf shape similarity and symmetry.

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