Epi-illumination microscopy coupled to in situ hybridization and its utility in the study of evolution and development in non-model species

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Abstract:

Evolutionary developmental biology often combines methods for examining morphology (e.g., scanning electron microscopy, SEM) with analyses of gene expression (e.g., RNA in situ hybridization). Due to differences in tissue preparation for SEM and gene expression analyses, the same specimen cannot be used for both sets of techniques. To aid in the understanding of morphological variation, it would be particularly useful to have a high- magnification image of the very same sample in which gene expression is subsequently analyzed. To address this need, we developed a method that couples extended depth of field (EDF) epi-illumination microscopy to in situ hybridization in a sequential format, enabling both surface microscopy and gene expression analyses to be carried out on the same specimen. We first created a digital image of inflorescence apices using epi-illumination microscopy and commercially available EDF software. We then performed RNA in situ hybridizations on photographed apices to assess the expression of two developmental genes: *Knottedl*(*Knl*) in *Zea mays* (Poaceae) and a *PISTILLATA* (PI) homolog in *Musa basjoo* (Musaceae). We demonstrate that expression signal is neither altered nor reduced in the imaged apices as compared with the unphotographed controls. The demonstrated method reduces the amount of sample material necessary for developmental research, and enables individual floral development to be placed in the context of the entire inflorescence. While the technique presented here is particularly relevant to floral developmental biology, it is applicable to any research where observation and description of external features can be fruitfully linked with analyses of gene expression.

Article:

INTRODUCTION

The analysis of gene expression patterns in non-model species is a major component of research on the evolution of developmental mechanisms. Spatial and temporal patterns of gene expression are best assessed using RNA in situ hybridization. In this technique, a labeled probe is hybridized to endogenous mRNA and detected through either auto- radiography or a chromogenic reaction, depending on the label used (Jackson 1991; Kramer 2005). In situ hybridization results are often published in conjunction with scanning electron microscope (SEM) images of external morphology (e.g., Kim et al. 2003). Unfortunately, once a specimen is fixed and prepared for SEM, it is no longer suitable for in situ hybridization. In alternative

techniques to SEM, for example, cryo-SEM, environmental SEM, and laser scanning confocal microscopy, the tissue preparation methods themselves are not inherently damaging or incompatible with downstream gene expression analyses. The microscopy itself, however, is often destructive to tissue, precluding downstream RNA in situ hybridization (Blancaflor and Gilroy 2000; Lemon and Posluszny 1998). Because tissue fixation and preparation for epi-illumination microscopy is similar to the initial stages used for RNA in situ hybridization (Kramer 2005; Sattler 1968), it is possible to photograph a specimen using epi-illumination and continue with gene expression analysis on the same specimen. This can be advantageous in light of the paucity of material often available for developmental studies in non-model organisms.

Epi-illumination, or incident light microscopy, is a form of light microscopy in which the light source is above the object being viewed. It is used for the examination of opaque objects illuminated by reflected light (Locquin and Langeron 1983). Epi-illumination microscopy has been used in the biomedical sciences, forensics, metallurgy, micropaleontology, and botany (Leroy and Crane 1964; Locquin and Langeron 1983; Nickolls 1937; Sattler 1968; Tanaka 2006). The technique became widely used in biology when Leitz designed an incident light illuminator—the Ultropak—and a series of 15 objectives with magnifications ranging from ×3.8 to ×100 for use with the Leitz Laborlux, Ortholux, Orthoplan, and Panphot microscopes. Through the use of an annular condenser and an angled annular mirror, the Ultropak illuminator and objectives allow for epi-illumination microscopy of irregular surfaces by separating the illuminating light rays from the image-forming light rays, thus reducing reflection (Leroy and Crane 1964; Locquin and Langeron 1983).

Epi-illumination microscopy was first employed in the investigation of floral development by Sattler (1968) and became widely used thereafter for many developmental studies in plant biology. In the 20 years following its initial publication, Sattler's paper was cited 63 times (BIOSIS). The technique has lost favor in recent years, with only three citations since 2000, presumably due to the reduced depth of field as compared to scanning electron microscopy and the difficulty in obtaining the now discontinued Ultropak system. With the advent of digital photography and specialized extended depth of field software such as Nikon Imaging System (NIS) Elements, MediaCybernetics Image- Pro, and Reindeer Graphics Focus Extender, increasing depth of field digitally has become relatively simple. The use of readily available metallurgical lenses in biology has also reduced the need for the Leitz system (Lacroix and Macintyre 1995).

Many minor adjustments and additions to Sattler's (1968) original method have been proposed and implemented over the years. Epi-illumination microscopy has been used in combination with serial sectioning and histology (Posluszny et al. 1980); depth of field has been increased by combining photographs at various focal depths in Adobe® Photoshop® (Wilson et al. 2006) and staining schedules and dissecting techniques have been revised (Charlton et al. 1989). This investigation, however, is the first to couple epi-illumination microscopy, newly available extended depth of field software, and in situ hybridization.

Our work has three goals: (1) to investigate the use of the NIS Elements software to increase depth of field, (2) to verify that the epi-illumination preparation techniques do not affect the in

situ results, and (3) to use epi-illumination and in situ hybridization to assess the expression of a floral organ identity gene in a non-model organism.

MATERIALS AND METHODS

The use of the NIS Elements software was explored with inflorescence apices of *Costus cylindricus* (Costaceae), a species in which a number of flower primordia are clustered near the apex and which is thus difficult to photograph with conventional methods. The effect of epi-illumination preparation techniques on in situ hybridizations was investigated in maize with *Knottedl*(*Knl*), a well-characterized gene expressed in meristems (Jackson et al. 1994). The combination of these techniques was then tested in a non-model organism; the expression of *PISTILLATA* (PI), a floral organ identity gene (Coen and Meyerowitz 1991), was assessed in inflorescences and attached flowers of *Musa basjoo* (Musaceae).

Floral material

Maize (*Zea mays* var. mays) recombinant inbred line B73 seeds were grown in the greenhouses of the Department of Plant and Microbial Biology at UC Berkeley. Female inflorescences (ears) were harvested when they first became evident at approximately 6 weeks. *Costus cylindricus* (USBGH 2002-127) inflorescences were also dissected from greenhouse-grown plants. Entire inflorescences were harvested at an early developmental stage and bracts were sequentially removed to expose the early stages of floral development. In *C. cylindricus*, a single flower is enclosed within each bract. *Musa basjoo* inflorescences were obtained from the collection of the University of California Botanical Garden at Berkeley (UCBG 89.0873). As with Costus, bracts were serially removed from young inflorescences to expose the youngest cincinni (‘hands’).

Fixation and staining

Following dissection, all floral materials were immediately fixed in freshly prepared cold formalin-aceto-alcohol (Jackson 1991). Each specimen was then dehydrated from 50% to 100% ethanol using an adjusted microwave technique that decreases the time at each stage of the dehydration series, enabling the entire series to be completed in 1.5 h (Schichnes et al. 1998). Subsequent to dehydration, apices were stained for 72 h at 4°C in a solution of 1 % w/v fast green FCF in 100% ethanol (Charlton et al. 1989). Apices were destained in 100% ethanol for 2 h prior to photography.

Dissection and photography

One inflorescence from each species, *C. cylindricus*, Z. mays, and M. basjoo, was dissected and photographed with epi-illumination microscopy. Black silicone gasket sealant that releases acetic acid (DAP, Baltimore, MD, USA) (Sattler 1968) was prepared as a surface for photography by placing a small amount of the silicone in a small glass Petri dish, stirring it vigorously, letting it sit for 5–20 min, and then flooding the dish with 100% ethanol. Samples were placed in this medium both to hold the specimen during dissection and to provide a black background for photography. Dissection was performed in one prepared silicone dish, photography in another. The use of two dishes keeps the black background for photography free of plant debris generated during dissections. To increase depth of field in individual photographs, it was important to position the objects of interest parallel to the focal plane of the camera. To keep the samples cool and to reduce streaming in the 100% ethanol, the Petri dishes

were placed in a rectangular container filled with ice which was changed approximately every 15 min. Photographs at various focal distances—from the top of the specimen to the bottom—were taken using the ×3.8 Leitz objective on a Leitz Orthoplan microscope equipped with a Nikon Digital Sight 5M digital camera. The first photograph was taken with the flowers closest to the objective in focus; the next photograph had lower flowers, but not the lowest, in focus. This process of changing the focal depth and taking a photograph was continued until focused images of all of the flowers at all positions on the visible face of the inflorescence had been captured in a sequentially numbered series of photographs.

Generation of focused images

Photographs were merged to create a single focused image using the extended depth of focus function (purchased as an add-on) of the NIS Elements D software package (Nikon). We used two of the different ways of creating focused images: smoothing and local. The smoothing function relies heavily on the quality of the first photograph and blends all lower images into the first. The local function stitches together areas that are in focus in each photograph (Nikon 2006). For maize ear primordia, the 'smoothing' function achieved better results, whereas for taller objects (Costus and Musa inflorescences) the 'stitching' function achieved better results. In the case of maize where the inflorescence was too large to fit into a single field of view, focused images of the length of the ear were stitched together using Adobe® Photoshop® CS2 (version 9.0.2).

RNA in situ hybridization

To ensure that neither the heat generated during photography nor the staining and mounting needed for dissection and photography interfere with RNA in situ hybridization, hybridizations were performed first on maize. Experiments with maize included the following controls: (1) eliminate staining in fast green, (2) eliminate dissection in black silicone medium, and (3) eliminate photography (i.e., potential heat damage from illumination). Once it was ascertained that the dissection and photography did not negatively affect in situ results, we performed in situ hybridizations on a photographed *M. basjoo* inflorescence. In both the maize and Musa in situs, one pair of slides was probed with sense probe as a negative control. All in situs were performed as described below, modified from Jackson (1991) and Kramer (2005).

Probe development Probe for *Knl*corresponded to bp 364– 999 (59%) of the *Z. mays Knl*coding sequence (GenBank accession number NM_001111966). The *M. basjoo* PI probe (GenBank accession number EU433562) corresponded to 68% of the coding sequence of *MADS4* (PI homolog) from *Oryza sativa* (GenBank accession number L37527) and spanned the MADS, I, and K domains of the gene. For both *Knl*and PI, sequences were maintained in pBluescript SK vectors. Polymerase chain reaction (PCR) was performed using M13 primers; the vector containing the transcript was used as template. The amplified region included a T7 RNA polymerase start site and a T3 RNA polymerase start site. Probe was labeled through in vitro transcription from the PCR products using DIG-labeling mix (Roche) and T7 (antisense probe) or T3 (sense probe) RNA polymerases (Invitrogen). Probe was quantified by comparing it to dilutions of DIG-labeled control RNA (Roche). The 642-bp *Knl*probe was hydrolyzed to 150-bp stretches using sodium carbonate and sodium bicarbonate hydrolysis (Kramer 2005). The PI probe was only 431 bp in length and consequently was not hydrolyzed.

Microtechnique and hybridization Following photography but before infiltration with paraffin, a final change of 100% ethanol was performed to remove any contaminating water that could have entered the solution from the ice bath. Samples were infiltrated using a tissue-processing microwave oven (Microwave Research and Applications, MRA BP111RS) following the protocol of Schichnes et al. (1998). Paraffin blocks containing the apices were trimmed and sectioned at 8 µm on a Micron retracting rotary microtome. Sections were mounted on positively charged ProbeOn Plus slides (Fisher Scientific) by incubation at 42°C overnight.

Wax was removed from slides with xylene and sections were hydrated through a graded ethanol series and incubated for 20 min in 2 µg/ml proteinase K solution to digest cell walls and improve probe penetration. After the destabilizing proteinase K treatment, sections were re-fixed in 4% formaldehyde in phosphate-buffered saline (PBS) for 10 min. To reduce background, excess positive charges were acetylated using a triethanolamine—acetic anhydride treatment. Slides were dehydrated through a graded ethanol series, following which 200 µl of probe in hybridization solution (Kramer 2005) was placed on slides. The *Knl*probe was used at a concentration of 1.33 ng µl-1 kb-1; the PI probe was used at a concentration of 2.5 ng µl-1 kb-1. Slides were sandwiched together in pairs with the probe inserted between the pairs. The slide sandwiches were elevated above 50% formamide-wet paper towels in a slide box, which was, in turn, placed in a sealed plastic bag. The slides were incubated overnight in an oven preheated to 53°C.

The following day, slides were separated and washed twice in 0.2× sodium chloride—sodium citrate buffer (SSC) for 30 min at 53°C and twice in 1× sodium—Tris—EDTA buffer (NTE) for 5 min at 37°C. An RNase A treatment (20 µg/ml RNase A in 1× NTE, 30 min at 37°C) was performed to digest single-stranded RNA and reduce background. This was followed by two 5-min washes in 1× NTE (37°C), one 60-min wash in 0.2× SSC (53°C), and 5 min in PBS (4°C). Slides were blocked using 0.5% w/v Boehringer block in 1 × Tris-buffered saline (45 min, room temperature) and washed in buffer A (1.0% bovine serum albumin, 100 mM Tris pH 7.5, 150 mM NaCl, and 0.3% Triton X-100). Alkaline phosphatase-conjugated anti-DIGantibody was diluted 500× in buffer A and 200 µl of antibody solution was used to make slide sandwiches as described above. Slides were incubated with antibody above water-wet paper towels in a slide box at room temperature for 1 h. They were then separated and washed in detection buffer (0.1 M Tris pH 9.6, 0.1 M NaCl, and 0.05 M MgCl2). Finally, slide sandwiches were made again, this time using 200 µl of detection buffer plus substrate (1.6 µl 5-bromo-4-chloro-3-indolyl phosphate and 2.2 µl nitro blue tetrazolium chloride per milliliter of detection buffer) and incubated in a slide box, in a drawer to prevent light contamination. Slide sandwiches were periodically assessed for color development. Once signal was evident, the reaction was stopped by dipping the slides in water. The slides were dehydrated through a graded ethanol series, washed twice in xylene to remove any residual ethanol, and coverslipped using Cytoseal-60 mounting medium (Richard Allen Scientific). Sections were photographed using a Zeiss Axiophot 381 microscope equipped with a QImaging color camera.

RESULTS AND DISCUSSION

EDF epi-illumination microscopy

The epi-illumination technique with NIS Elements EDF software was able to deliver a single high-quality image of the *C. cylindricus* inflorescence with relative ease (Fig. 1). This result was achieved in considerably less time than the method suggested by Wilson et al. (2006) in which a focused image was created by stitching together 6–22 photographs in Adobe® Photoshop®.

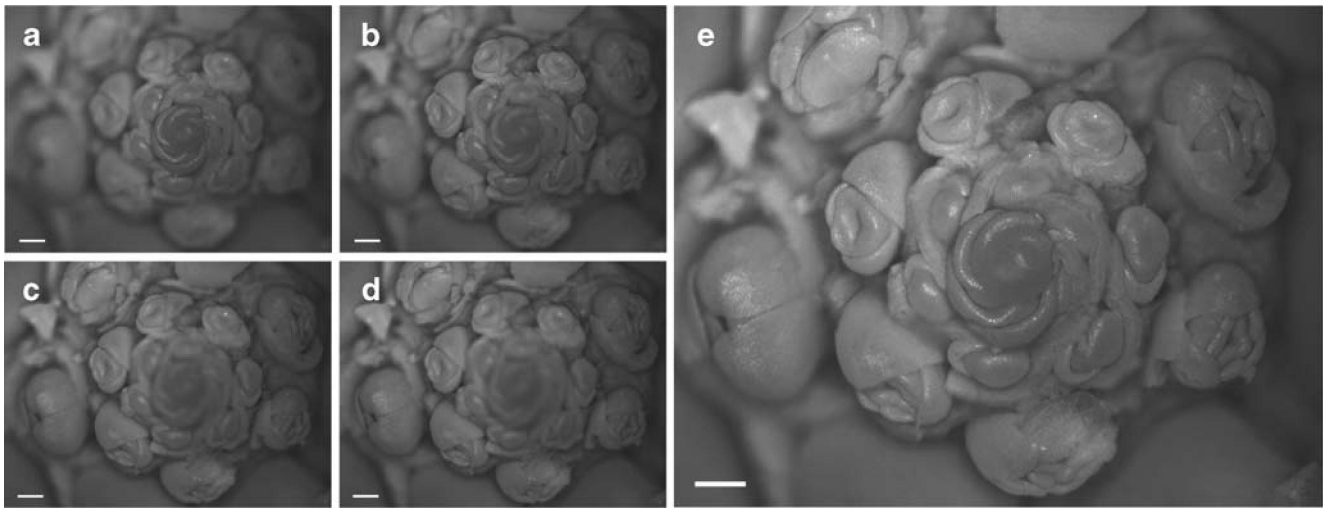

Fig. 1 Extended depth of field (EDF) epi-illumination microscopy of *C. cylindricus*. The four unfocused images (a—d) were combined using the NIS Elements software to produce a single focused image (e). Scale bars, 200 µm

The combined image of *C. cylindricus* shows all of the developing flowers on the floral apex in focus. The path of floral development can be traced using this single information-rich image (Fig. 1e). The youngest flower is closest to the inflorescence apex; the next oldest flower is to the right of the previous flower, moving in a right- handed spiral around the floral apex. The basic steps in Costus floral development, as described for *Costus scaber* (Kirchoff 1988), can be discerned in this single image. Development proceeds from initiation of the first sepal through development of the common stamen—petal ring primordium, through differentiation of the petals, stamen, and labellum, and ending with gynoecium development.

Although the cost of the microscope, accessories, and camera necessary to produce EDF images is high, it is trivial compared to electron microscopy and, perhaps most importantly, the technique is manageable at the laboratory rather than at the institutional level. The technique can be implemented even at institutions where there is no infrastructure or funding for establishing a SEM facility.

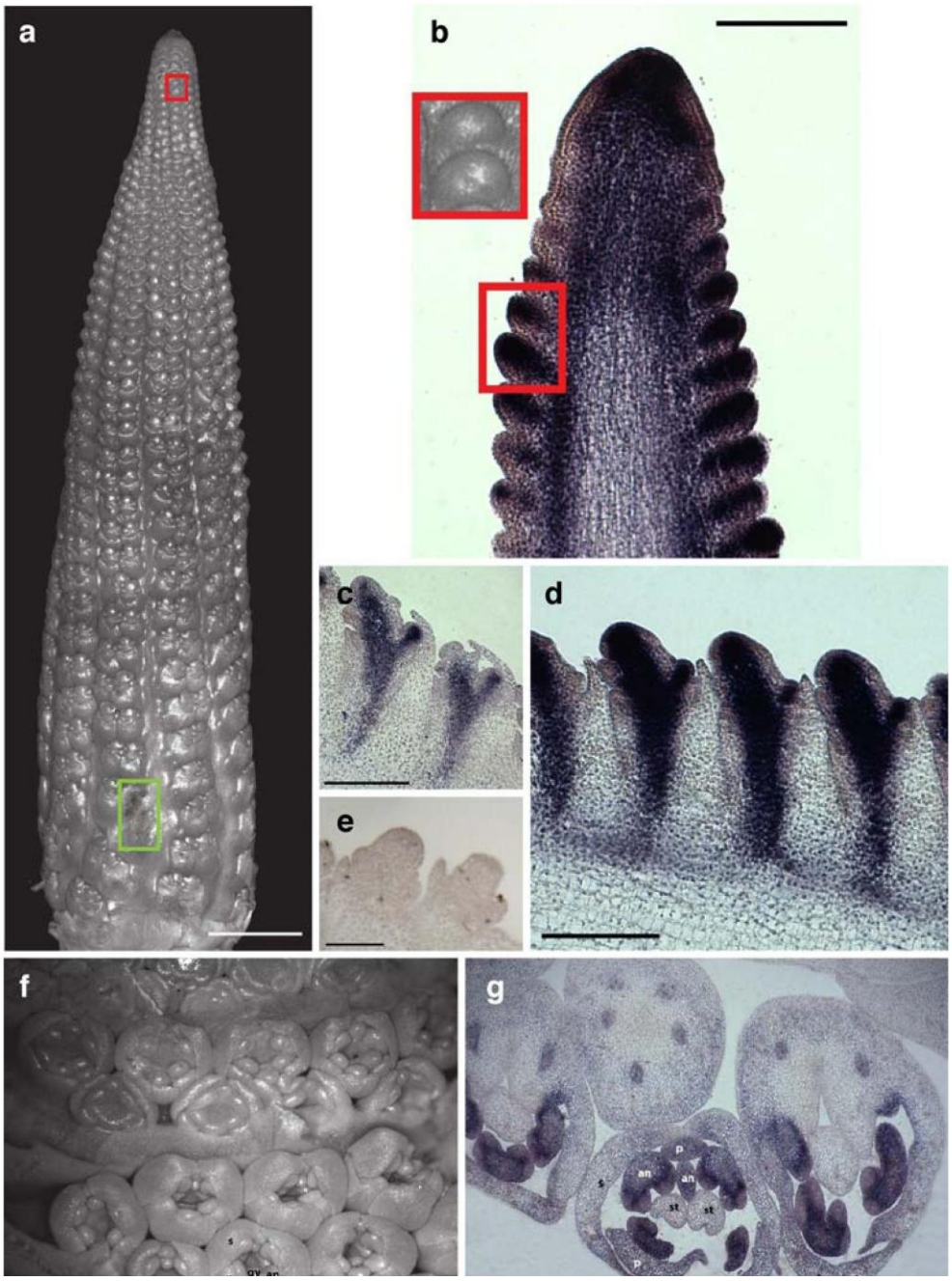

Fig. 2 Epi-illumination microscopy coupled to in situ hybridization of *Z. mays* and *M. basjoo*. **a–d** *Z. mays*. **a** EDF epi-illumination image of a female inflorescence. **b** *Kn1* expression in the same inflorescence and enlargement of two spikelet meristems from the inflorescence. The *red boxes* surround identical spikelet meristems. The *green box* highlights the damage used to orient sections and locate individual flowers (see text). **c** *Kn1* expression in an apex that was not exposed to the silicone dissecting material and stain or photographed with epi-illumination. The signal is comparable to that observed in the photographed inflorescence (see **a**). **e** Negative in situ control. The sections were probed with a sense probe. **f, g** *M. basjoo*. **f** EDF epi-illumination image of two hands of flowers. Androecium (*an*), petals (*p*), sepals (*s*), and developing gynoecium (*gy*) indicated. **g** *PI* expression in slightly older flowers. Expression is seen in the androecium and petals but not in the sepals or style (*st*). All *scale bars* 200 μm, except in **a** (1 mm)

Unfortunately, it is increasingly difficult to obtain the equipment used by Sattler (1968) since Leitz, now Leica Microsystems, has ceased the manufacture of the Ultropak. Recently, however, similar results have been achieved using conventional and readily available metallurgical objectives (Lacroix and Mcintyre 1995).

EDF epi-illumination microscopy coupled to in situ hybridization

A single composite photograph was created for the *Z. mays* female inflorescence (Fig. 2a). RNA in situ hybridization on this same inflorescence using antisense *Knl*probe (Fig. 2b) demonstrates *Knl*expression in spikelet meristems and vasculature. Expression patterns and

levels are in keeping with published expression patterns of *Knl*in maize (Jackson et al. 1994). Expression levels are also comparable to those in the control that was not subjected to any staining with fast green, dissection, photography, or exposure to the silicone dissection media (compare Fig. 2c and d).

One potential disadvantage of the epi-illumination technique is that the inflorescence apex is subjected to tissue damage because of the dissection necessary for photography. Typically, protective bracts are not removed from inflorescences prior to fixation and in situ hybridization (Jackson et al. 1994). Minor damage, however, can be used as an indicator of location in an inflorescence when interpreting sections. The damage that occurred close to the base of the Zea inflorescence during the initial dissection (Fig. 2a, green box) was used to orient the sections. The inflorescence was oriented in the paraffin wax so that the sections were made in the same plane as the damaged flower. The damaged flower was then located in the sections as a means of determining their orientation on the slides. Using both measurement and information about orientation, we can link florets visible on the epi-illumination image with florets showing *Knl*expression in the in situ results. The two spikelet meristems highlighted in the epi-illumination image are the same pair of meristems as those highlighted in the in situ results (red boxes, Fig. 2a,b). This process of floret identification can be repeated for the entire inflorescence. This is particularly useful in the study of non-model organisms where material is often limited. In addition, this technique reduces the dependence on developmental series with defined developmental stages. Gene expression patterns can be precisely referenced back to single flowers rather than to a generalized developmental stage as defined by examining flowers of another inflorescence or individual.

In order to demonstrate the utility of these techniques in a non-model organism, we repeated epi-illumination photography and in situ hybridization in *M. basjoo* with a gene for which there are no published expression patterns in this species. *PISTILLATA* (PI) is a B-class MADS box gene expressed in petals and stamens (Coen and Meyerowitz 1991). We analyzed the expression of a PI homolog in *M. basjoo* flowers that had previously been photographed (Fig. 2f). Expression was observed in the entire androecium and in the petals (Fig. 2g).

In inflorescences such as those of Costus, Zea, and Musa, many floral developmental stages can be captured through sectioning a single inflorescence, thereby providing gene expression data across a developmental series. The positions of the flowers within the inflorescence and the relationships between flowers are retained in the sections and can be traced back to the epi-illumination micrographs. This is particularly useful when studying inflorescences with complex structure where tying a particular flower back to its position in the inflorescence is necessary for interpretation of development. Removal, dissection, and probing of individual flowers would result in the loss of positional and possibly developmental information. For example, the homologies of *Heliconia* (Heliconiaceae) floral organs can only be understood in the context of the entire inflorescence (Kirchoff 2003). Finally, the technique provides a method whereby the variability between specimens (and in the case of floral development within an inflorescence, e.g., Bateman and Rudall 2006) is no longer of as much concern as when working with individual flowers or when using SEM micrographs of similar but not identical flowers.

CONCLUSION

The study of evolution and development in non-model plant species is often hampered by a paucity of material available for observation and experimentation, and the inability to investigate variability within an individual or species with sequential high-magnification visualization and gene expression analyses. The technique presented here makes it possible to perform both surface microscopy and gene expression analyses on the same specimen, thereby reducing the amount of fresh material required and enabling detailed study of within-species developmental variation. This technique has particular relevance to the investigation of floral development and evolution but has the potential to be used in any research where observation and description of external features can be fruitfully linked with studies of gene expression.

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