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# COMPARATIVE FLOWER DEVELOPMENT IN *THEOBROMA CACAO* BASED ON TEMPORAL MORPHOLOGICAL INDICATORS

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To set the stage for studies on gene regulation in the cocoa plant, a detailed analysis of the sequence of floral development in *Theobroma cacao* L. is presented and compared with a well-studied model species *Arabidopsis thaliana*. Morphological measurements from time-lapse photography and LM and EM were used to create mathematical models of flower development. Comparison of the *T. cacao* floral developmental program to the rosid model system *A. thaliana* revealed that, although the final sizes and flower morphologies in the two species differ, their developmental programs are strikingly similar.

*Keywords: Theobroma*, cacao, flower, development, *Arabidopsis*, ABC genes.

## Introduction

The understanding of molecular mechanisms associated with the floral biology of angiosperms is expanding rapidly (for reviews, see Jack 2001, 2004; Boss et al. 2004; Ma 2005). This has been primarily achieved through the identification of developmental mutants in several model plant species, the subsequent characterization of genes associated with these mutations, and the pathways they control (Jack 2004; Ma 2005). It is now clear that flowers arise from a spatially and temporally controlled cascade of gene regulation; the gene products interact to determine the fate of cellular differentiation in various floral organs. This cascade, referred to as the ABC model, explains how cellular differentiation and morphogenesis of floral organs are temporally and spatially regulated. The ABC model has been shown to apply to a wide variety of both monocot and eudicot species (Bowman et al. 1991; Coen and Meyerowitz 1991; Meyerowitz et al. 1998; Ambrose et al. 2000; Theissen et al. 2000; Fornara et al. 2003). Deciphering such developmental control mechanisms has implications for our general understanding and may contribute to applications of plant biotechnology in the future.

The ABC genes are regulated by upstream factors such as the *LFY* gene, which acts as both an integrator of floral pathways (Blazquez et al. 1997; Nilsson et al. 1998) and an activator of floral organ identity genes (Weigel and Meyerowitz 1993; Parcy et al. 1998; Lenhard et al. 2001; Lohmann et al. 2001). *LFY* orthologs have also been identified in other species (Frohlich and Parker 2000; Gocal et al. 2001; Maizel and Weigel 2004) and are generally expressed across the entire apical meristem during the phase change from vegetative to inflorescence development and throughout the identity phase of organogenesis. The level of *LFY* mRNA expression is reduced once the organs are specified (Weigel et al. 1992).

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The use of molecular tools to investigate the complex pathways of floral development requires a detailed characterization of the morphological development of the flowers in question. Some key morphological landmarks and common terminology have been suggested for floral development (Buzgo et al. 2004). They studied three species occupying key phylogenetic positions and defined the following morphological landmarks: inflorescence formation, initiation of outermost perianth organs, initiation of inner perianth organs, first stamens, carpel initiation, microsporangia formation, ovule initiation 1 and 2, male and female meiosis, and flower opening.

*Theobroma cacao* L. is a Neotropical understory tree indigenous to the Amazon. The seeds of *T. cacao*, cocoa beans, are processed to create the main ingredients for chocolate, cocoa butter, and cocoa powder (Bartolome 1951; Wood and Lass 1985). The switch in development from juvenile to adult stages (phase change) occurs suddenly in *T. cacao* when the tree reaches ~1.5–2 m in height (~1.5 yr) and is marked by the formation of a four- or five-branched jorquette of near horizontally growing branches (Bartolome 1951). Shortly after this change, flowers form on the trunk and main branches of the plant, a developmental process called cauliflory. The initial flowers appear to arise above leaf scars in the axils of abscised leaves. Inflorescences arise continuously from the same spot and eventually flower cushions form, consisting of many compressed cincinnal cymes (Bartolome 1951). The flowers take ~1 mo to mature (Rajamony and Mohanakumarn 1991; Smith 1992) and are borne on long pedicels. The fully developed flowers have 5 free sepals, 5 free petals, 10 stamens (5 fertile and 5 nonfertile staminodes) and an ovary of 5 united carpels (Smith 1992). The petals are narrow at the base but extend into cup-shaped pouches. The petals are usually pink and white; the precise coloration and pattern may vary slightly and can indicate a given genotype. The stamens are arranged in two whorls; the outer consists of five nonfertile staminodes and the inner of five fertile stamens. The stamens have two anthers that lie in the pouch of the corresponding

petal. The style is twice as long as the ovary and consists of five parts around an axis (Wood and Lass 1985). The fused style has the appearance of being single but is divided at the tip into five stigmas (Wood and Lass 1985; Smith 1992). The floral organization of *T. cacao* is basically conserved across the genus *Theobroma* and its sister group *Herrania*. However, it is variable in the subfamily Byttnerioideae (Whitlock and Baum 1999; Whitlock et al. 2001).

Bayer and Hoppe (1990) described the order of events for flower development in *T. cacao* through analysis of a series of EMs, but no quantitative (size or time course) data were reported. Although it has been estimated that flower development in *T. cacao* takes between 21 and 30 d (Rajamony and Mohanakumarn 1991), previous studies did not characterize the stages of *T. cacao* flower development in the detail that they have been characterized for the model system *Arabidopsis thaliana* (Smyth et al. 1990). In addition, there have been no studies on the molecular mechanisms of flower development in *T. cacao* or on how the timing of key steps in floral development and gene expression compare between *T. cacao* and the *Arabidopsis* model.

The long-term goal of our research is to understand at the molecular level the degree to which the model plant *Arabidopsis* can be used to advance our understanding of the molecular mechanisms underlying the developmental pathways of *T. cacao*. For this study, we focused on one of the best-known pathways in *Arabidopsis*, flower development. The research described here has two specific aims. First, to determine precise timing of key morphological events in *T. cacao* flower development and, second, to compare floral development in *T. cacao* with *Arabidopsis*, in which flower development has been studied in depth. Our results suggest a very high degree of similarity exists in floral developmental sequence between these two species; therefore, our data suggest that *Arabidopsis* provides an excellent model system for "translational biology" to the important tropical crop species, *T. cacao*.

## Material and Methods

### Plant and Growth Conditions

Ramets of the *Theobroma cacao* L. PSU-"SCA6" clone were used in all experiments. The clone was sent to the Pennsylvania State University (PSU) from the USDA Tropical Research Laboratory in Miami, Florida. During the preparation of this article, DNA fingerprinting studies revealed that this accession is not identical to the authentic SCA-6 accession in the international germplasm collection at Trinidad (Cryer et al. 2006). The plants were grown in greenhouses at PSU in deep pots (6.35 cm diameter, 25.4 cm deep) with silica sand for adequate drainage. Humidity was maintained at 60%, and the photoperiod was set to 12 h light/29°C and 12 h dark/26°C. Natural light was supplemented with 430-W high-pressure sodium lamps as needed to maintain a minimum light level of 250 $\mu$mol $m^{-2}$ $s^{-1}$ PAR, while automatically retractable shading limited light levels to a maximum of 1000 $\mu$mol $m^{-2}$ $s^{-1}$ PAR. Irrigation with one-tenth-strength Hoagland's nutrient solution (160 ppm N) was applied daily at multiple times to maintain adequate moisture.

### Time-Lapse Photography and Morphometric Measurements

Time-lapse photography was performed using a statically placed Canon S40 digital camera controlled by an iMAC computer and Canon Remote Capture (version 2.7.3) software. Photos were taken every hour over two separate time periods. The first time period was from February 3 to February 18, 2003, consisting of 16 d; the second was from February 24 to March 31, 2003, consisting of 34 d. These photographs were interlaced using the Apple computer iMovie software and can be viewed at http://guiltinanlab.cas.psu.edu/Research/Cocoa/flowers.htm. Growth measurements were taken from the images obtained at 2:00 p.m. each day. Three measurements were taken for each flower: flower bud length (distal tip of the sepals to the base of the receptacle), flower width at its widest point, and the pedicel length (from the base of the flower bud to the tree). In total, six separate flower clusters were observed containing 65 flowers. Measurements were calibrated from the images using a ruler that was physically attached to the tree and included in each photograph. Using the Adobe Photoshop ruler tool, flower bud measurements were converted to millimeters. The measurements for each parameter were combined using Lowess curves (Chambers et al. 1983).

To determine the rate of flower opening, sequential 1-h photographic images were collected of eight individual flowers from four different flower cushions at five different starting times. These photographs were also used to determine the angle of flower opening by measuring the angle of the inside edges of the sepals with a protractor. Closed flowers had sepals at 0° while fully open flowers had sepals at 180°–220° angles.

### Microscopy

Flower samples were collected at various stages of growth and were fixed according to Zhao et al. (2001). Tissues with large airspaces were slit on one side using a razor blade to allow easier entrance of paraffin into the tissues during embedding. Tissues were embedded using the Shandon Citadel 2000 tissue processor (Shandon, Pittsburgh, PA) under the following 22-h program: 70% ethanol for 15 min, 80% ethanol for 30 min, 95% ethanol for 30 min, 95% ethanol for 8 h, 100% ethanol for 1 h, 100% ethanol for 30 min, three changes of Histosolve (ThermoShandon, Pittsburgh, PA) for 1 h each, and two changes of paraffin for 4 h each. The paraffin blocks with embedded tissues were hardened for 24 h at room temperature and then sectioned to 8-$\mu$m-thick slices using a Shandon Finesse Microtome (Thermo Electron Corporation, Pittsburgh, PA). The sections were placed on positively charged glass slides (cat. no. 6776214, Thermo Electron Corporation) and incubated overnight on a slide warmer at 42°C. The sections were stained using a solution of toludine blue (0.1%) for 15 s and rinsed in water for at least 1 h, after which they were dipped for 10 s each through the following ethanol and Histosolve series: 25% ethanol, 50% ethanol, 75% ethanol, 100% ethanol, and Histosolve and then covered with a glass coverslip. Sections were photographed at 500× magnification using an Olympus BX-60 compound microscope with an Automated Prior Stage (Prior, Rockland, MA) controlled by the ImagePro Plus (version 4.1; Media Cybernetics, Silver Spring,

MD) software. In total, for the measurements presented in this article, 12 flowers were photographed, encompassing all stages of flower development.

SEM was conducted by collecting 18 flower samples at various stages of growth. Samples were fixed in FAA (50% ethanol, 5% acetic acid, 3.7% formaldehyde, and 41.3% water) overnight (>16 h) under vacuum. The tissue was put through a series of ethanol washes of 50%, 50%, 60%, 70%, 85%, 95%, and three washes of 100% of at least 1 h each. The samples were then critical-point dried using bone-dry carbon dioxide with exchange conditions of 10°C and 3.4 atm at 31°C and 4.76 atm in the Bal-Tec CPD030 Critical Point Dryer (Technotrade, Manchester, MD). Tissues were then dissected, sputter-coated with gold, and viewed using a Jeol JSM-S400 SEM (Jeol, Peabody, MA). The images were calibrated using PGT Imix-PC V.10 software to obtain accurate size measurements.

### *Measurement of Floral Tissues*

Using all three sources of data, a large number of morphometric parameters were recorded (tables 1). In total, the internal organs of 32 flower buds were measured. To correct measurements for the level of tissue shrinkage during the fixation process, photographs of representative samples were taken before fixing, and the flower bud lengths was measured (FrL) using Adobe Photoshop. These same tissues were compared with the resulting fixed tissues used in LM and SEM (F × L). Using the two measurements FrL (length before fixation) and F × L (length after fixation), it was deduced that the average degree of shrinkage due to fixation was 14%, so all subsequent values were corrected by multiplication of F × L × 1.14 to give an estimated FrL.

## Results

### *Analysis of Floral Development Using Time-Lapse Imaging*

To precisely measure the rate of organ growth during floral development, time-lapse imaging was used to capture hourly images of flowers growing on *Theobroma cacao* trees under greenhouse conditions. The compiled images can be viewed as time-lapse movies and are available online (see "Material and Methods"). The 2:00 p.m. images were arbitrarily selected, and measurements from these images were compared for variation among flowers within floral cushions and between the time periods. No significant effect of time or flower cushion position on the individual growth rates of the flowers (fig. 1*A*, 1*B*) was observed. In other words, in this experiment, there were no positional effects or effects relating to the two time periods that the photographs were taken when compared with floral growth rate.

We combined the measurements for each parameter separately into several Lowess curves encompassing all of the data for bud length, bud width, and pedicel length, respectively. These curves were correlated to the raw data with an $r^2$ value of 0.96 (fig. 1*C*). By relating time with the length of the flower bud, we determined that the duration of flower development (from first meristem cell divisions to flower opening) in genotype PSU SCA-6 was 30 d. Although the length and width of the flower bud increased for the entire 30 d, the pedicel grew only between days 10 and 30. At the time of flower opening, the average length of the flower bud was 5.7 mm (SD = 0.4 mm), the average width of the flower bud was 3.6 mm (SD = 0.2 mm), and the average length of the pedicel was 13.2 mm (SD = 1.4 mm). Minor variation in mature flower size has been observed in the field and used as an indicator of genotype in *T. cacao* (M. Guiltinan, personal communication).

By calculating the rate of growth (size increment in millimeters per time in days) from the original data, then fitting Lowess curves to the rate data set, we were able to describe patterns in the relative rates of growth in the bud length, bud width, and pedicel length. From these calculations, we estimated that the pedicel had the highest terminal rate of growth (growth rate immediately preceding flower opening) of 0.54 mm $d^{-1}$, followed by bud length growth of 0.26 mm $d^{-1}$, and finally bud width growth of 0.16 mm $d^{-1}$ (fig. 1*D*). The rate of growth in pedicel length generally increased throughout the development of the flower bud. However, the greatest increase in growth rate was observed from day 17 to 30. The rate of growth of flower bud length and width actually decreased to a minimum growth rate (0.09 mm $d^{-1}$) at day 16, after which the growth rate increased for the remaining duration of growth (fig. 1*D*). When the flower initially forms, it is enclosed by two leafy bracts. By day 15, the flower bud emerges from the bracts, and the pedicel continues to elongate. At flower opening, the bracts have abscised, leaving a scar that was later observed to form just basal to

**Table 1**

**Growth Models of Percentage Growths of Various Organs during the Development of *Theobroma cacao* Flower Buds**

| Measurement vs. bud length | Model | Type | $R^2$ | Notes |
|---|---|---|---|---|
| % anther diameter | $.0895\ day^2 + .5545\ day - 7.1103$ | Linear | .92 | |
| % staminode length | $.1047e^{.2292 \times day}$ | Exponential | .85 | |
| % style length | $.6858\ day^2 - 24.748\ day + 221.55$ | Linear | .89 | After day 14 |
| % petal length | $3 \times 10^{-06}\ day^{5.104}$ | Power | .93 | |
| % ovary height | $1.7335e^{.1327 \times day}$ | Exponential | .76 | |
| % ovary diameter | $4.5929\ day - 39.247$ | Linear | .86 | |
| % filament length | $.6594\ day^2 - 25.225\ day + 242.7$ | Linear | .82 | After day 14 |
| % fresh length | $1.7783e^{.1355 \times day}$ | Exponential | 1 | |
| % fresh width | $2.6138e^{.1254 \times day}$ | Exponential | .96 | |
| % pedicel length | $.4077e^{.1865 \times day}$ | Exponential | .98 | |
| Length of flower bud | $.1223e^{.1355 \times day}$ | Exponential | 1 | |

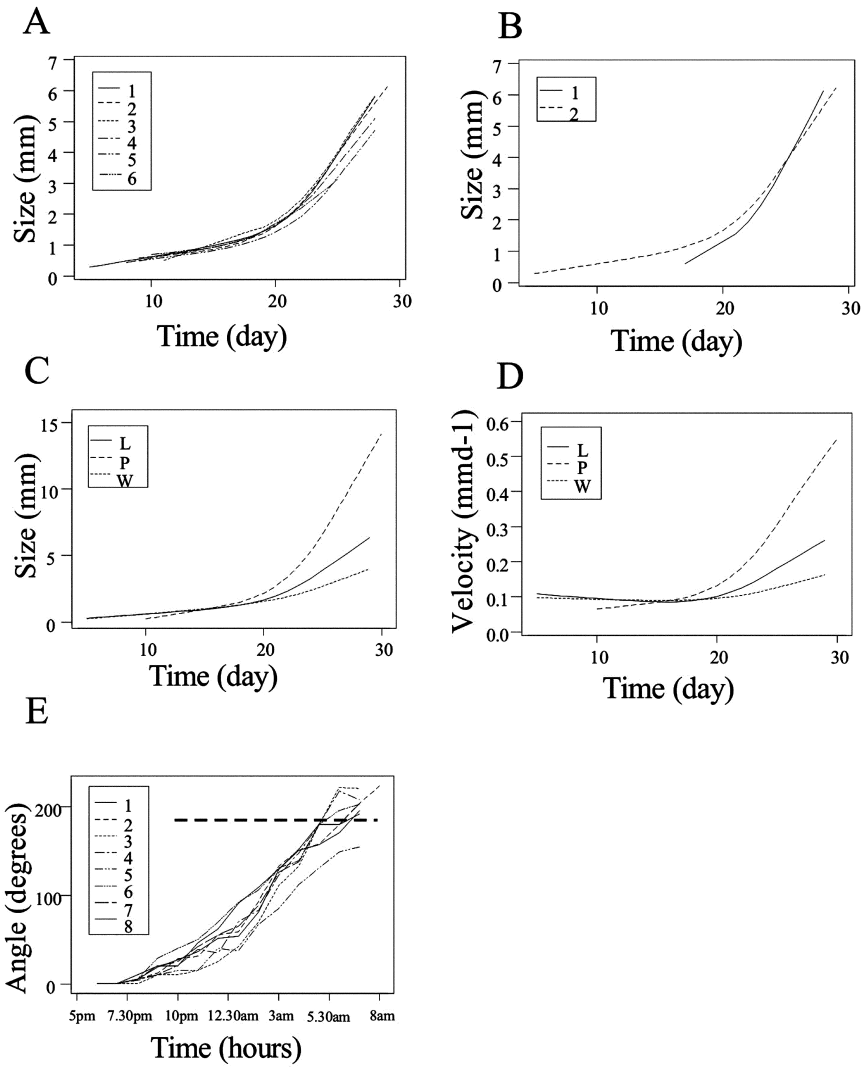


**Fig. 1** Growth, velocity, and opening angles of growing *Theobroma cacao* flower buds over time. *A*, Lowess curve showing the length of the bud (mm) versus time (day of growth) comparing flowers of six separate flower cushions representing 65 flowers. *B*, Lowess curves describing the length of the bud (mm) versus time (day of growth) comparing flowers growing between two separate time periods. *1* = 9 flowers measured February 3–18, 2003; *2* = 56 flowers measured February 24–March 31, 2003. *C*, Lowess curves describing size (length, width, length of pedicel, mm) versus time (day of growth) using combined data from all flower cushions and both time periods. *D*, Lowess curves describing velocity (length, width, and length of pedicel, mm $d^{-1}$) using data from combined flower cushions and both time periods. *E*, Opening angles (°) of eight flower buds over the time (h) required for the flowers to open (dashed line shows where the flowers are fully open; >180°).

the (200 $\mu$m in a 19-d-old flower bud) site of abscission of unfertilized flowers (fig. 2*A*).

### *Microscopic Analysis of Floral Development*

To assess in more detail the development of the internal structures of *T. cacao* flowers, 30 samples encompassing all stages of flower development were fixed and dissected (see "Material and Methods" for details). To allow us to compare size data from images taken of fresh flowers with those of fixed tissues, photographs were taken both before and after fixation to measure the level of shrinkage due to the fixation process. Between 7% and 20% shrinkage occurred (data not shown). No correlation occurred between tissue size fixed and the extent of shrinkage. There was also no difference in shrinkage between the tissues fixed for SEM versus LM. An average increase of 14% was used to correct all data.

Flowers falling into six general size categories were arbitrarily selected for observation by both EM and LM (fig. 3). Very small buds were extracted by dissecting the flower cushion from the tree while larger-sized flower buds could be removed without disturbing the flower cushion. The morphometric data were analyzed using linear regression to create models that described the particular mode of growth of each organ relative to the length of the total flower bud (e.g., linear, log, exponential). These growth models can be found in table 1. These models accurately describe the percentage growth of the organs relative to total percentage bud length contained in the four whorls of

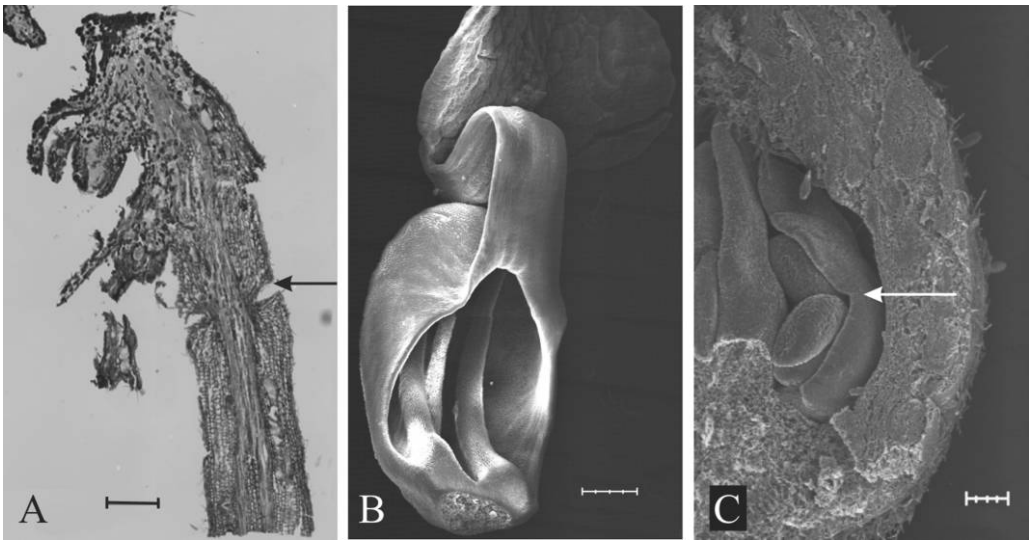


**Fig. 2** Micrographs showing important features of the *Theobroma cacao* pedicel (*A*) and features of the petal (*B*, *C*). *A*, SEM showing the pedicel of a stage-8 19-d-old flower bud. The arrow shows the restriction almost to the vasculature of the pedicel. This becomes the site of abscission for the flower. *B*, SEM of a stage-12 29-d-old petal of *T. cacao* showing the three sections; ligule, taenial-segment, and cucullate petal base, as well as the two "running tracks" used to guide the filament in the latter stages of elongation. *C*, SEM of a size-1 flower bud, 16 d old (stage 8). The arrow indicates the restriction appearing in the petal that will eventually form the taenial-segment. Bars = 200 $\mu$m (*A*), 100 $\mu$m (*B*), 500 $\mu$m (*C*).

the *T. cacao* flower. The models presented were expressed as percentages because it has been observed in both *Arabidopsis* and *T. cacao* that the absolute size of the flower buds can vary by up to 40% in *Arabidopsis* and 20% in *T. cacao*, depending on genotype and growth conditions (H. Ma and M. Guiltinan, personal communications).

### *Description of Developmental Events*

To facilitate comparison of the morphological development of *T. cacao* and *Arabidopsis*, 12 morphological stages correlating to those found in *Arabidopsis* floral development were defined for *T. cacao* (tables 2, 3). Stages 1–6 primarily involved meristem development and organogenesis of the floral organs and were complete by day 10. Stages 7–12 were involved with elongation and further differentiation within individual organs to produce the fully developed flower by day 30.

#### *Stages 1–6*

Stage 1 is defined by the emergence of the floral buttress arising from the trunk in the initial flower of a specific flower cushion. This first stage was not observed, as this research only involved developed flower cushions, and no floral buttresses were observed directly in the cyme structure. We suggest that the floral buttress would occur only in the very first flower bud at each leaf axil. However, we did observe in young plants that primary floral meristems arose from the location of a leaf scar, most likely from axillary meristems that formed during vegetative development. Stage 2 begins with the formation of the flower primordium (fig. 3*A*). Other than the initial flower, all subsequent flowers initiate near the base of the pedicel of a maturing flower that is usually at stage 9 or above. The emergence of primordia was first observed as a dome of cells that was 140 $\mu$m wide.

The formation of sepal primordia marks the beginning of stage 3 and is apparent when the floral meristem is 0.18 mm in width (at about 3.5 d, 3% of the fresh tissue length). They initiate in a quincuncial sequence, meaning that if the sepals were numbered clockwise from 1 to 5, the sepals would initiate in the order 1, 3, 5, 2, and 4. The sepals continue to grow and overlie the floral meristem by day 5; this demarks stage 4. Stage 5 is defined by the emergence of petal primordia when the bud is 0.29 mm in length (6 d, 4% FrL; fig. 3*B*). Shortly after, the anther (0.35 mm, 6 d; fig. 3*B*), the staminodes (sterile stamenlike structures that also form part of this whorl; 0.32 mm, 6 d; fig. 3*B*), and five carpel primordia (0.35 mm in length, 6 d; fig. 3*B*) form on the meristem. The carpel primordia soon grow together and eventually fuse to form a tube.

Stage 6 occurs as the sepals continue to grow and to completely enclose the meristem (complete enclosure by day 8; fig. 3*A*). At this stage, the sepals are relatively thick and half of the total thickness of the developing flower (fig. 3*B*, 3*C*). Also at stage 6, the anthers form vertical splits down their centers, resulting in two lobed structures (0.44 mm, 8 d, 5.2% FrL). Concurrently, the staminodes begin to become pointed as the anthers form this vertical split. Finally, the pedicel begins to form at day 10 (6.9% FrL).

#### *Stages 7–12*

Stage 7 is marked by the formation of the anther filaments when the bud is 0.61 mm in length (11 d old, 7.9% FrL). Meanwhile the petals grow and form a triangular shape as they elongate from day 6 to day 14 (4%–11.9% FrL; fig. 3*C*). When the bud is 0.88 mm in length (14 d, 11.9% FrL),

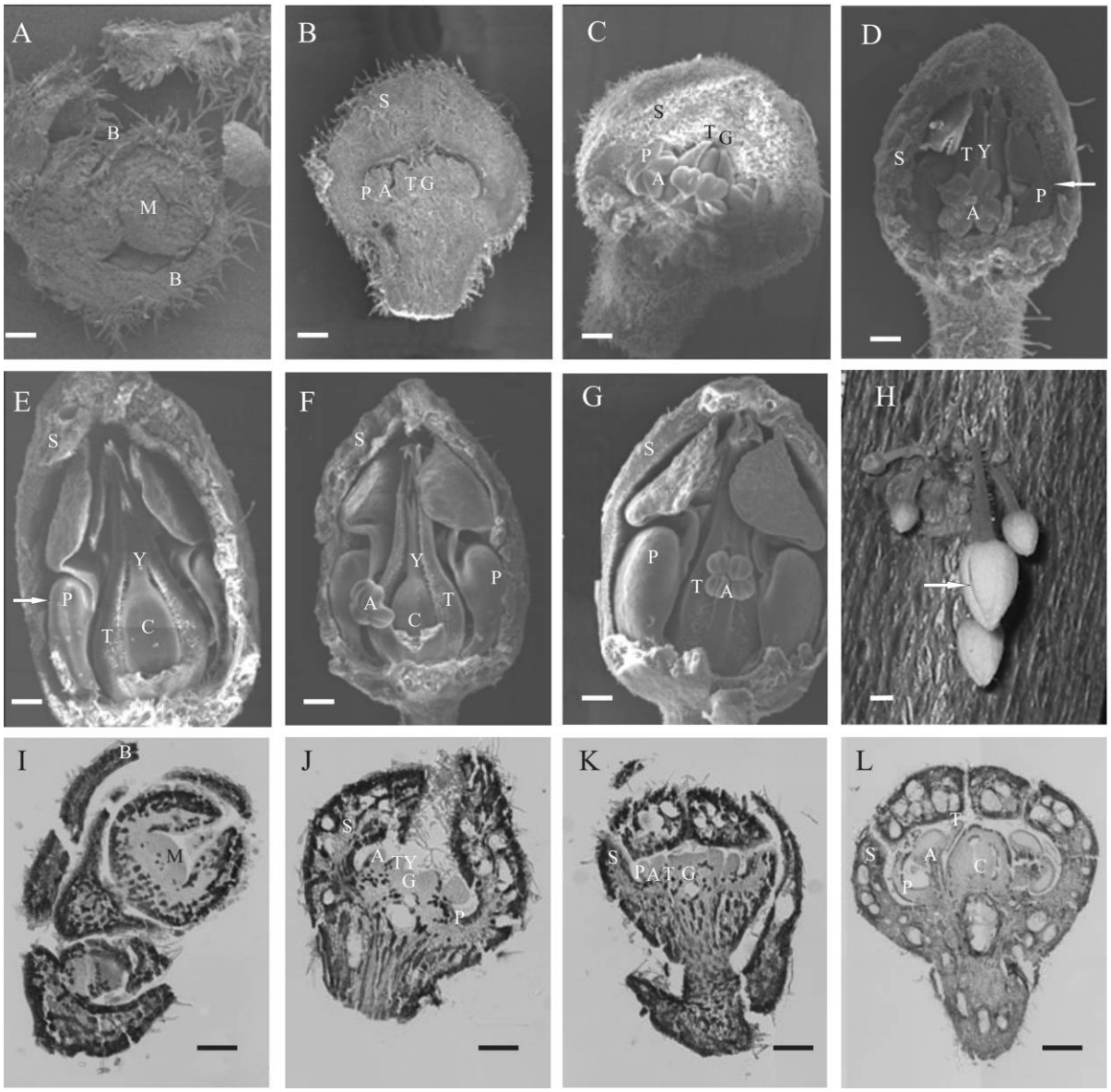


**Fig. 3** *A–G*, SEM photographs showing the ontogeny of the *Theobroma cacao* flower. *H*, Photograph of a flower cushion. *I–L*, LM of early *T. cacao* flower buds. *A*, Stage-3 4-d-old meristem emerged from the cyme and protected by leafy bracts. *B*, Stage-7 12-d-old flower bud showing the early formation of floral organs. The flower buds at this time have thick sepals relative to the thickness of the meristematic tissue. *C*, Early- to middle-stage-8 15-d-old flower bud showing that most organs are fully developed and are ready to expand. *D*, Late-stage-8 19-d-old flower bud showing the constriction of the petal that will eventually form the taenial-segment (arrow). *E*, Stage-9 22-d-old flower bud. The cucullate petal base has completely enclosed the anther whose filament has not yet begun to expand (arrow). *F*, Stage-11 27-d-old flower bud in which all organs are expanding. The style is not yet at the same length as the staminodes. *G*, Stage-12 29-d-old fully expanded flower bud just before flower opening. At this stage, all organs are fully developed and at their final relative lengths. The anther is housed in the cucullate petal base, the style is the equivalent length of the staminodes, and "bumps" have appeared on the carpel. *H*, Small flower cushion of *T. cacao*. The arrow shows the abscission zone where the sepals are beginning to separate in an opening flower. *I*, Stage-3 4-d-old meristem emerged from the cyme and protected by leafy bracts. *J*, Stage-7 flower bud just before the sepals enclose the bud. *K*, Stage-8 flower bud just after the sepals have enclosed the bud. *L*, A mid- to late-stage-9 bud clearly showing all organs are fully developed and are ready to expand. *A* = anther, *B* = bract, *C* = covary, *G* = gynoecium, *M* = meristem, *P* = petal, *S* = sepal, *T* = staminode, *Y* = style. Bars = 100 μm (*A*); 200 μm (*B*); 250 μm (*C*); 300 μm (*D*); 500 μm (*E–G*); 2 μm (*H*); 100 μm (*I*); 150 μm (*J*, *K*); 200 μm (*L*).

a constriction appears near the base of the petals (figs. 2*C*, 3*D*). The sections above the constriction form the ligule, while the sections below form the petal base. This constriction is indicative of stage 8. At this time, the anther primordia have separated from the meristem, and a horizontal split begins to form (0.85 mm, 14 d), giving anthers their characteristic tetrathecate morphology (fig. 3*D*, 3*F*, 3*G*). The staminodes are taller than the anthers at this stage. Moreover,

**Table 2**

**Comparison of Stages in *Theobroma cacao* and *Arabidopsis* Flower Development**

| *Arabidopsis* stage | *T. cacao* stage | Description | Time in *Arabidopsis* (d)[a] | Time in *T. cacao* (d)[a] | Ratio *T. cacao*/ *Arabidopsis* |
|---|---|---|---|---|---|
| 1 | 1[b] | Flower buttress arises | 0 | na | na |
| 2 | 2 | Flower primordium forms | 1 | 1 | 1 |
| 3 | 3 | Sepal primordia arise | 2.25 | 3.5 | 1.55 |
| 4 | 4 | Sepals overlie flower meristem | 3 | 5 | 1.66 |
| 5 | 5 | Petal and stamen primordial arise | 3.75 | 6 | 1.6 |
| 6 | 6 | Sepals enclose bud | 4 | 8 | 2 |
| 7 | 7 | Long stamen primordial stalked at base | 5.25 | 11 | 2.09 |
| 8 | 8 | Locules appear in long stamens | 6.25 | 14 | 2.24 |
| 9 | 9 | Petal primordial stalked at base | 7.25 | 20 | 2.75 |
| 10 | 10 | Petals level with short stamens (60% of growth) | 9.75 | 26 | 2.66 |
| 11 | 11 | Stigmatic papillae appear | 10.25 | 27 | 2.63 |
| 12 | 12 | Petals level with short stamens (100% of growth) | 11.5 | 29 | 2.52 |
| | | Flower opening | 13.25 | 30 | 2.26 |

Source. *Arabidopsis* stage from Smyth et al. (1990).

[a] Beginning of stage.

[b] This stage has not been observed in cacao because of the cyme structure, but it is thought to exist on the first flower produced in the flower cushion.

staminodes initially grow at the same rate as petals, eventuating in a longer final length than petals. The staminodes surpass the petals in length as the taenial-segments form (14 d).

Stage 8 continues with the flower buds expanding out of the bracts at day 15 (13.6% FrL). The carpels fuse at the top, and the style forms when the bud reaches a length of 1.17 mm, at 17 d (17.8% FrL). Another landmark of stage 8 is the initial formation of ovules when the bud is 1.21 mm in length (18 d, 20.4% FrL). The anthers follow a more logarithmic pattern of growth than the other organs. Because of this growth phase, anthers are one of the fastest-forming organs in the flower, completing 50% of their growth by day 19 (fig. 3*C*).

During stage 9, the petals form three distinct sections (figs. 2*B*, 3*B*). The cucullate petal base is proximal to the receptacle, and the ligule is most distal. The taenial-segment of the petal connects the ligule and petal base. The constriction that started in stage 7 begins to elongate when the bud reaches 1.58 mm in length (20 d, 26.7% FrL) and forms the taenial-segments. Pollen is fully formed soon after the horizontal split in the anther forms (1.87 mm, 21 d, 30.6% FrL). The average diameter of the pollen grains are 0.018 mm. The outside of the flower bud begins to change in color from light green to white as the flower bud expands (1.5 mm, day 19.5, to 3.5 mm, day 24, 46% FrL). Once filaments form in stage 7, they show little elongation until the petal bases cover the anthers, during which the filaments begin to accelerate in growth (4.47 mm, 25 d, 52.6% FrL; fig. 3*E*, 3*F*, 3*G*). The anthers are at 91% of final length when the filaments begin to expand. This filament growth is guided into the petal bases

**Table 3**

**Definitions and Abbreviations of Flower Measurements Made in *Theobroma cacao* Flowers**

| Name | Symbol | Measurement |
|---|---|---|
| Anther diameter | AntherD | Diameter of the anther from the face side |
| Filament length | FilL | Length of the filament from the connection to the anther to the receptacle |
| Staminode length | StamL | Length from the distal tip of the staminode to the receptacle |
| Ovary diameter | OvryD | Width of the ovary at its widest point |
| Ovary height | OvryH | Length of the ovary from the base of the style to the receptacle |
| Style length | StyleL | Length of the style from the top of the ovary |
| Sepal wall thickness | SepWlTh | Width of the sepal wall at its thickest point |
| Petal base length | CL | Length from the receptacle to the beginning of the connective "bar" (taenial-segment) |
| Taenial-segment length | TS | Length of the constrictive "bar" between the petal base and ligule |
| Ligule length | LL | Length of the blade of the petal from the distal end of the taenial-segment to the distal end of the petal |
| Inside | In | Inside of the flower bud from receptacle to the inside surface of the sepal |
| Outside | Out | Outside of the flower bud from the receptacle to the outside surface of the sepal |
| Pollen diameter | PolDia | Diameter of pollen |
| Female gametophyte | FG | Diameter of female gametophytes |
| Fixed length | FxL | Fixed total length from the beginning of the pedicel to the outside surface of the sepal |
| Fresh length | FrL | Fresh total length from the beginning of the pedicel to the outside surface of the sepal |
| Fresh width | FrW | Fresh total width of the flower bud at its widest point |
| Pedicel length | PedL | Length of the pedicel from the peduncle to the trunk |

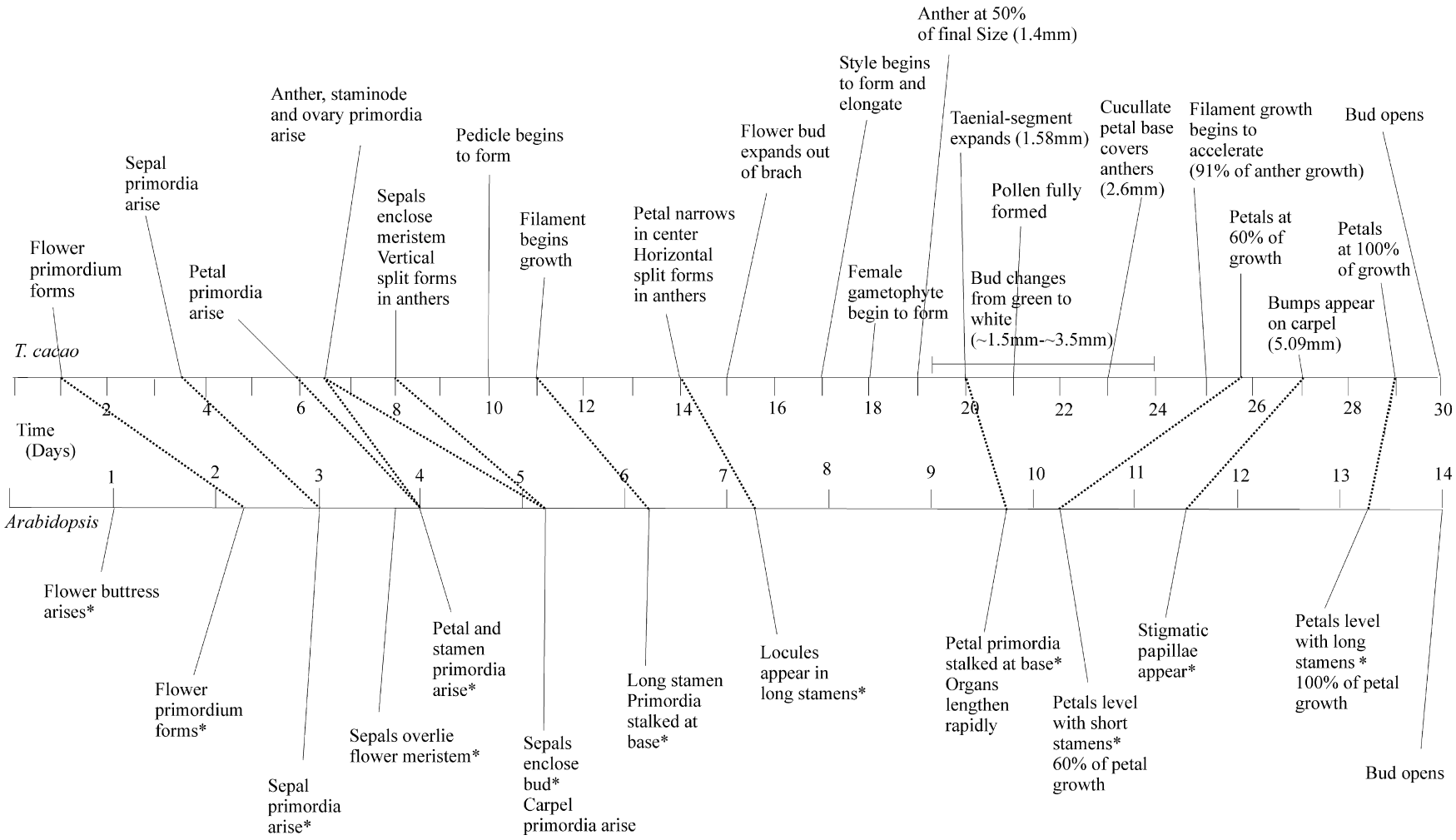


**Fig. 4** Comparison of *Theobroma cacao* and *Arabidopsis* flower development. The major morphological events in the growth of *T. cacao* (top) and *Arabidopsis* (bottom) plotted along an axis of 30 and 14 d, respectively. An asterisk indicates *Arabidopsis* stages as defined by Smyth et al. (1990). The dotted lines connecting the two diagrams represent our proposed linkage between *T. cacao* and *Arabidopsis* flower development stages as defined by Smyth et al. (1990).

by two tracklike grooves on the adaxial surface (fig. 3*B*). These "tracks" appear to guide the filaments to position the anther in the center of the petal base. We speculate that these tracks and the precise positioning of the anthers within the enclosing chamber of the petal base may facilitate efficient contact of pollinating insects with the mature pollen grains.

Stage 10 is reached when the petals obtain 60% of their final length (at day 26, 60.2% FrL). Stage 11 is observed as the final morphological structures appear as "bumplike" lobes on the surface of the carpel and hairlike trichomes on the staminodes (fig. 3*F*, 3*G*). The staminodes completely enclose the style of the flower (fig. 3*F*, 3*G*). Stage 12 is reached when the petals obtain 100% of their final length (day 29). At this point, the taenial-segments are curved and represent ribbonlike structures (fig. 3*E*–3*G*). As the flower continues to grow and lengthen, the sepal walls do not grow any thicker. They lengthen but remain at the initial thickness of 0.25–0.35 mm until flower opening (fig. 3*D*–3*G*).

### *Flower Opening and Flower Abscission from the Tree*

Marking the initiation of flower opening, the sepal tissues, which initially develop as one contiguous whorl, form a series of five longitudinal abscission zones from the tip to the base of the floral bud. These longitudinal abscission zones separate (fig. 3*H*), forming the five individual sepals, which then expand and open outward. As the sepals open outward, the petals also open. Our measurements reveal that flower opening takes place over a 12-h period, beginning in the evening of day 29. Under our greenhouse conditions, on average, the first observable splits (average of 4.8° from the original closed position, SD = 2.6°) in petals occur between 6:00 p.m. and 7:00 p.m. of the twenty-ninth day and concluded with the flower completely open (an average of 197° from the original closed position, SD = 20.7°) by 6:00 a.m. on the thirtieth day, shortly before sunrise.

Interestingly, flower opening is well synchronized between the cohort of mature flowers opening each night. The flowers open at almost exactly the same time and rate, irrespective of their position on the trunk (fig. 1*E*), as shown dramatically in the online video images (http://guiltinanlab.cas.psu.edu/Research/Cocoa/flowers.htm). Of eight individual flowers located in different flower cushions, seven were observed to begin opening during the same hour interval (6:00 p.m. to 7:00 p.m.). Observations show that flowers all opened at similar rates, with an average standard deviation among the opening angle of eight flowers over the entire period of flower opening of only 12.8°. The velocity associated with the opening of the flowers was also fairly constant among the flowers studied (average of 14.7° $h^{-1}$), with the fastest rates occurring between 1:00 a.m., 3:00 a.m., and 4:00 a.m. (23°–33° $h^{-1}$, SD = 15° $h^{-1}$).

Unfertilized flowers were observed to abscise from the trunk ~22–24 h after flower opening. Abscission of the unfertilized flower is estimated to occur at the earliest between 3:00 a.m. and 5:00 a.m. the day after flower opening. Normally, a small physical disturbance is required to shake the flower loose (e.g., wind, physical contact), and, as a result, in absence of contact, some flowers do not fall until several days after flower opening. We also noticed that the site for abscission is at the bract scars.

## Discussion

### *Morphogenesis of* Theobroma cacao *Flowers*

Under the conditions used in our studies, flowers of *Theobroma cacao* take 30 d to form from an initial floral meristem containing a few cells to a fully developed receptive flower. This is consistent with several previous reports (Wood and Lass 1985; Bayer and Hoppe 1990; Smith 1992) but longer than the time of 21–24 d reported by Rajamony and Mohanakumaran (1991). This latter discrepancy could have resulted from (1) Rajamony and Mohanakumaran (1991) not recording the earliest stages of development, (2) differences in genotype, or (3) environmental effects, none of which could be determined from their report. The flower forms from a compressed cincinnal cyme structure and is cauliflorous in nature, growing from the main trunk and branches. The specific site of inflorescence has been observed by others to be from the periderm of old stems and axils of abscised leaves in lateral branches (Bartolome 1951; Zamora et al. 1960). In young flowering plants, we observed that the primary floral meristem arises from the location of a leaf scar, most likely from axillary meristems formed during vegetative development. The flower bud grows to an average length of 5.7 mm and an average width of 4 mm immediately before opening. The pedicel grows to an average length of 14.1 mm.

The flowers of *T. cacao* undergo opening over a 12-h period. In our study, this took place at night between ~6:00 p.m. to 7:00 p.m. and 6:00 a.m. (under greenhouse conditions). Additionally, the flowers, irrespective of their position on the tree, opened in a synchronous fashion at similar rates over this period. This implies that there is a very strong regulation controlling the initiation and the rate of flower opening. This high degree of synchronicity is likely the result of selective pressure to ensure the optimal receptivity of flowers upon opening at the time of maximal insect activity in the early morning hours. This hypothesis is supported by anther dehiscence studies where it was observed that maximal pollen release occurred early at night (8:00 p.m.–midnight), with most insect pollination occurring in the early morning (Young et al. 1984). Additionally, we observed that the opening of the flower was not a smooth motion but rather discontinuous, with the rates of opening consistently increasing and decreasing. These changes in opening rates were reproducible from flower to flower, suggesting that the plant has mechanisms in place to control the rates that flowers open. The mechanism and its physiological nature remain to be investigated.

The flowers normally remain open on the tree for 1 d after which they abscise. A constriction close to the base of the pedicel, distal from the flower, was observed first by Cheeseman (1932). He did not describe the function of the constriction but did note that it extended almost to the vascular strands of the pedicel. We have also observed the constriction (fig. 2*A*) and postulate that this constriction is the site for flower abscission.

To validate our models relating organ growth to bud length, we reanalyzed the electron micrographs from Bayer and Hoppe (1990) in the same way that we analyzed our own EM (data not shown). There was little difference observed between our data versus the Bayer and Hoppe (1990) data in terms of the actual sizes and the models produced.

This provides further validation of the universality of the models presented here. Therefore, our observations of the development of the internal organs of the *T. cacao* flower bud generally agree with those made by Bayer and Hoppe (1990) and Zamora et al. (1960). However, there is some disagreement in the literature on developmental sequence. Zamora et al. (1960) infer that the internal whorls (androecium and gynoecium) both begin to form together, whereas Bayer and Hoppe (1990) state that the flower forms in an acropetal sequence, sequentially initiating in the order of sepals, petals, androecium, and gynoecium. In our studies, we also observed that the flower forms in an acropetal sequence. However, the internal two whorls initiate within 24 h of each other, very near the limit of the temporal resolution of the models presented in this study.

Our time-lapse data and the resulting Lowess curves allowed us to relate temporal data to the size of the observed flower buds. This provides predictive power to relate subsequent size data to age of the bud. By sampling over the entire spectrum of floral development, we were able to use models describing the size or shape and growth rate of different organs. The models allow experimental manipulations to be performed on specific stages of development without having to dissect flowers each time to determine their stage of development.

### *Comparison of the Floral Developmental Programs of* T. cacao *and* Arabidopsis *Indicates a High Degree of Conservation between the Two Species*

*Arabidopsis thaliana* is the most well-studied model system for investigating the mechanisms regulating flower development (Meinke et al. 1998; Simpson and Dean 2002). *Arabidopsis* flowers are less than half the size of *T. cacao*, with a bud length just before flower opening of ~2.8 mm. The structure of the *Arabidopsis* flower includes a calyx of four sepals, a corolla of four petals, an androecium with four medial (long) stamens, and two lateral (short) stamens. The gynoecium comprises two carpels (Smyth et al. 1990). Smyth et al. (1990) divided *Arabidopsis* flower development into 12 temporal stages. They reported that *Arabidopsis* buds took 13.25 d to form and develop. Evolutionarily, *T. cacao* and *Arabidopsis* are related, with both species belonging to Eurosids II. Here, we compared the relative rates and duration of *Arabidopsis* and *T. cacao* flower development in relation to developmental events. The total duration was defined as the time that elapsed between meristem formation and flower opening, against which we compared timing of the developmental stages of flowers of both species (fig. 4). Despite very different final morphologies of *T. cacao* and *Arabidopsis*, we were able to see many similarities in their developmental processes.

These stages may also be compared with those defined by Buzgo et al. (2004). This staging series is important to consider because it allows for developmental comparisons across many more species and not just *Arabidopsis*. Briefly, Buzgo et al. (2004) defined flower development in eight stages. Stage 1 involves flower and bract formation. Stage 2 refers to the initiation of outer bracts and tepals. Stages 3, 4, and 5 refer to the respective initiation of the inner perianth organs, stamens, and carpel. Stage 6 refers to the development of sporophylls and microsporangia, while stages 7 and 8 refer to initiation of the ovules and male meiosis, respectively. Because our focus is to directly compare the floral stage timing and sequencing between *Arabidopsis* and *T. cacao*, all stages, unless otherwise stated, refer to those defined by Smyth et al. (1990). However, where appropriate, stages are defined by Buzgo et al. (2004) and will be indicated by B-"stage number."

*Stages 1–6.* Stage 1 (B-1) is defined in *Arabidopsis* as the appearance of the flower buttress. We did not observe this stage in *T. cacao* flower development as our studies started with previously formed flower cushions in which the buttress is hypothesized to already exist. This stage was not included in our analysis because of the difficulty in capturing this very rare event. Stage 2 (B-1) is defined as the formation of the flower primordium. For *T. cacao*, this occurs at day 1 and lasts for 3.5 d, and in *Arabidopsis*, this stage also begins at day 1 but lasts for 2.25 d. Stage 3 (B-2) is defined in *Arabidopsis* as when the sepal primordia arise, which begins at day 2.25 and lasts for 18 h. In *Arabidopsis*, the abaxial sepal arises first followed by the adaxial and two lateral sepals (Smyth et al. 1990). In *T. cacao*, this stage begins at day 3.5 and lasts for 1.5 d. The sepal primordia arise in a quincundaler sequence in both species. Sepals overlying the meristem demarcate the beginning of stage 4. This occurs in *Arabidopsis* at day 3 and lasts for 18 h compared with *T. cacao* where it occurs at day 5 and lasts for 24 h.

Stage 5 (B-3, 4, and 5) in *Arabidopsis* is defined as the formation of petal and stamen primordia and is the first major deviation in development between the two species. In *Arabidopsis*, both petal and stamen primordia form simultaneously at day 3.75, and the stage lasts for 6 h. However, in *T. cacao*, petal primordia form (B-3) initially at day 6 followed 12 h later by the formation of the anther (B-4), staminode, and carpel primordia (B-5) sequentially. Stage 5 lasts for 2 d in *T. cacao*. Stage 6 is marked in *Arabidopsis* by the sepals completely enclosing the bud. This occurs at day 4 and lasts for 1.25 d. Another important event at stage 6 for *Arabidopsis* is the initial formation of the carpel primordia (B-5). This is another difference between the two species, as *T. cacao* has already formed carpel primordia by the end of stage 5. Stage 6 in *T. cacao* begins at day 8 and lasts for 3 d. Important events that occur in *T. cacao* stage 6 that are not seen in *Arabidopsis* are the vertical splits that form down the length of the anthers and the initial formation of the pedicel.

*Stages 7–12.* The initial filament formation defines the start of stage 7 in *Arabidopsis* and occurs at day 5.25, lasting for 1 d. In *T. cacao*, stage 7 occurs at day 11 and lasts for three days. Stage 8 is marked by the formation of the locules in *Arabidopsis*; formation occurs at day 6.25, and lasts for 1 d. This can be identified by the long stamens forming visible convex protrusions on the inner surface. In *T. cacao*, the locules occur once the horizontal split forms on the anther, causing its four-lobed structure. Stage 8 in *T. cacao* begins at day 14 and lasts for 6 d. Another important difference between the two species is that *Arabidopsis* has four stamens, two long and two short. *Theobroma cacao* has five fertile stamens that all grow at identical rates to one another and grow to the same length. In *T. cacao*, other landmark events include the expansion of the flower from the bract, the formation of the style and beginning elongation, and the initiation of the female gametophytes (B-7). At 1.4 mm (19 d), the anther has already formed 50% of its total final diameter.

Stage 9 occurs in *Arabidopsis* when the petal primordia form stalks at their base. It also marks a period where all organs lengthen rapidly. Stage 9 begins in *Arabidopsis* at day 7.25 and lasts for 2.5 d. *Theobroma cacao* also undergoes a period of rapid expansion during stage 9 (day 20 and lasts for 6 d). The initiating event is the lengthening of the taenial-segment, which is followed by the completion of pollen formation (B-8). The chonchas cover the anthers in stage 9 and the filaments elongate pushing the anthers into the chonchas using the previously described "running track"–like structures. Stage 10 is defined as the petals in *Arabidopsis* being level with the short stamens; it begins at day 9.75 and lasts for 12 h. Because *T. cacao*'s flower petals do not grow straight, we measured petal length from micrographs in Smyth et al. (1990) and determined that when the petals are level, that is, the same height as the short stamens, they are at 60% of their final length. We therefore redefined stage 10 as when the petals are at 60% of their final length. In *T. cacao*, this occurs at day 26 and lasts for 1 d.

Stage 11 is defined in *Arabidopsis* with the formation of stigmatic papillae that grow from the end of the stigma. This begins at day 10.25 and lasts for 1.25 d. Stigmatic papillae are the final morphological characteristic that appears on the *Arabidopsis* flower before opening. *Theobroma cacao* does not form stigmatic papillae; however, "bumplike" lobes appear on the surface of the ovary and are the last observed morphological characteristic before flower opening. This occurs at day 27 in *T. cacao* and lasts for 2 d. Stage 12 is defined by the petals being level with the long stamens and is the last morphological event before flower opening. In *Arabidopsis*, it occurs at day 11.5 and lasts for 1.75 d at which time the flower opens. For the same reasons as described for stage 10, we have defined the equivalent stage in *T. cacao* as when the petals reach 100% of their length. In *T. cacao*, this occurs at day 29 and concludes with flower opening after 1 d.

### *Comparison of Timing between* Arabidopsis *and* T. cacao

The developmental stages of flowering in *T. cacao* and *Arabidopsis* were compared in a time-corrected scale as presented in figure 4. In real time, the *T. cacao* flower developmental process takes roughly 2.26 times longer than *Arabidopsis*. When related to overall bud length obtained, it can be seen that the overall relative rate of growth is also similar in the two species. This indicates that although *Arabidopsis* flowers may develop faster than *T. cacao* in absolute terms the relative growth rates of various organs are in fact similar. For example, the ratio of final bud length in *T. cacao* (5.7 mm) to final bud length in *Arabidopsis* (2.8 mm) and the ratio of flower bud development time in *T. cacao* (30 d) and *Arabidopsis* (13.25 d) are identical, at 2.03. *Arabidopsis* and *T. cacao* flower buds develop at the same rate if measured in unit length per time (0.211 mm $d^{-1}$ for both). This relationship involving the duration of the stages between the two plants indicates that although the two species are morphologically different their relative morphological programs are similar.

### *Staminode Development*

The flowers of *T. cacao* have several features that are quite different from *Arabidopsis*. Other than size and number of organs, the most obvious feature is the formation of sterile, stamenlike structures called staminodes. Staminodes occur in other plant species and have been used as a morphological tool to understand plant evolution (Walker-Larsen and Harder 2000). These are interdigitated with the stamens in whorl 3 and begin development at the same time as the stamens. The function of the staminodes is unknown. Nectaries have often been observed at the base of staminodes in other staminode-containing species, suggesting a role in attracting insect pollinators. However, these nectaries are absent in *T. cacao* (Young et al. 1984). Therefore, it seems reasonable to speculate that the staminode barrier between the anthers and the stigmatic surfaces may reduce the incidence of self-pollination by midges and other small insects and thus contributes to increase outcrossing in self-compatible genotypes of *T. cacao*. Although, some evidence indicates a correlation between pollinator size and bristle staminode in *Penstemon* (Dieringer and Cabrera 2002). However, *Penstemon* has very different floral morphology compared with *T. cacao*, so this hypothesis remains to be tested (Dieringer and Cabrera 2002).

### *Molecular Mechanisms Underlying* T. cacao *Flower Development*

Analysis of the literature comparing the expression of the ABC genes *AP1*, *AP3*, *AG*, and *PI* as well as the floral integrator gene *LFY* in other species reveals that there is a high degree of conservation of gene expression patterns across a wide range of plants (Mandel et al. 1992; Weigel et al. 1992; Weigel and Meyerowitz 1993; Gustafson-Brown et al. 1994; Jack 2004; Ma 2005). These results imply that although there is much diversity among final individual floral morphologies among species the fundamental unifying principles stated in Jack (2004) are probably correct in that all flowers require, to some degree, the underlying mechanisms described by the ABC model and *LFY*. This in turn indicates that genes underlying meristem formation and subsequent organogenesis have similar expression programs.

Preliminary gene expression work in our laboratory indicated that the genes *AP1*, *AP3*, *AG*, *PI*, and *LFY* in *T. cacao* seem to be, at least initially, expressed in accordance with the current literature for model systems (data not shown; Jack 2004). As further evidence, Chaidamsari et al. (2006) have identified a *T. cacao AGAMOUS* homologue and have transformed the gene into *Arabidopsis*. Under the control of a constitutive promoter, this *AG* homologue produces results similar to what is observed with overexpression of the native *Arabidopsis AG* gene (Chaidamsari et al. 2006). Together with our detailed morphological development comparisons and our preliminary molecular data, it can be tentatively suggested that *T. cacao* most likely follows some variation of the ABC model of flower development.

However, for floral tissues in cacao that do not exist in *Arabidopsis*, additional gene expression studies and comparisons will be required. Interestingly, our preliminary gene expression data (not shown) suggest that the B class genes *AP3* and *PI* express in regions where staminode primordia develop in *T. cacao*, an organ that *Arabidopsis* does not possess. Work by Kramer et al. (2007) in *Aquilegia*, a species that produces staminodes, has revealed that three homologues exist for AP3 (*AqvAP3-1*, *AqvAP3-2*, and *AqvAP3-3*) and a single homologue

for *PI* (*AqvPI*). Expression studies with these homologues indicate that *AqvPI* is expressed in petal, stamen, and staminode primordia. Of the three *AqvAP3* homologues, only *AqvAP3-1* seems to have sustained expression in staminodes (Kramer et al. 2007). It will be important in future work to establish whether similar mechanisms are indeed at work in the *T. cacao* system.

As the study of flower development advances, especially with the abundance of molecular data becoming available through genomics projects, it is becoming increasingly important to have a good model of normal or typical flower development (Brukhin et al. 2003). It is obvious from the literature that there is not consistency among the different morphological landmarks used by researchers among different species, nor on the basis of comparison. Furthermore, there is not enough data from comparable studies on multiple plant species to assess how generic the developmental features of flowers in the model species such as *Arabidopsis* are. With the application of genomics to the study of evolution and development, it will be important that the development and morphology of the plants under study be standardized (i.e., described in similar terms so that the molecular data can be applied and compared across species in a consistent manner).

We have described the flower developmental program in *T. cacao* and have compared it with other species in the literature in terms of relative developmental time and size. Although both of these perspectives show that flowering is indeed complex, we were able to define developmental benchmarks that are in agreement with Buzgo et al. (2004) for comparisons among species. These benchmarks will be useful as a "springboard" to compare molecular data in studies on conservation of flower development among all flowering plant species and especially for further research on productivity and biotechnology applications in *T. cacao*.

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