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作品名稱　轉錄因子 bZIP16 參與阿拉伯芥開花途徑的分子機制研究

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關鍵字　轉錄因子、bZIP16、開花途徑

作者簡介

我是黃亦軒，目前就讀於台北市立建國高級中學。從小我對大自然就充滿好奇心，熱衷於做各種實驗來尋找答案。有時我覺得我就好像是一個偵探，不停的蒐集資料、線索、思考、推論、驗證，這個過程充滿樂趣，尤其是得到一些出乎意料的答案，更是精彩。進入中研院植微所做研究，讓我從過去僅能對物質表面現象的研究進入研究內在分子的領域，打開我的視野，更讚嘆大自然的奇妙。

此次能參與國際科學展覽會，特別感謝教授、老師熱心的指導和實驗室學姐、學長熱情的教導我操作各種實驗，更要感謝父母的支持，讓我放手去做我最喜愛的研究。

轉錄因子 bZIP16 參與阿拉伯芥開花途徑的分子機制研究

摘要

植物透過光受器和細胞內訊號分子來感知及反應環境變化，而轉錄因子為其中重要的細胞訊號分子。先前文獻證實阿拉伯芥轉錄因子 bZIP16 是一個整合植物荷爾蒙與光訊息傳導途徑的重要負向轉錄因子，促進種子的萌芽與幼苗的發育。然而對開花是否有影響並不清楚。本研究透過 bZIP16 在阿拉伯芥野生株不同組織的表現，發現 bZIP16 蛋白質在花苞和花具有高表現量。根據開花實驗顯示，*bzip16* 突變株不論生長在長、短日照下皆延遲開花。進一步透過微矩陣轉錄體 (transcriptome)分析與 qRT-PCR 分析其分子機制，發現 bZIP16 對吉貝素途徑、光週期途徑及春化途徑的基因沒有影響。然而，bZIP16 卻明顯抑制負調控開花因子 *FLC* 及促進 *SOC1* 和 *FT* 的表現。表明 bZIP16 藉由抑制 *FLC*，調控開花整合因子 *SOC1* 和 *FT* 的表現，進而促進植物開花。本研究證實 bZIP16 除了控制阿拉伯芥種子的萌芽與幼苗的發育之外，在開花途徑中具有正向調控開花的功能。此外，我們確認 bZIP16 是自主開花途徑基因的新成員。

Mechanistic Characterization of a Transcription Factor bZIP16 in Regulating Arabidopsis Flowering Pathways

Abstract

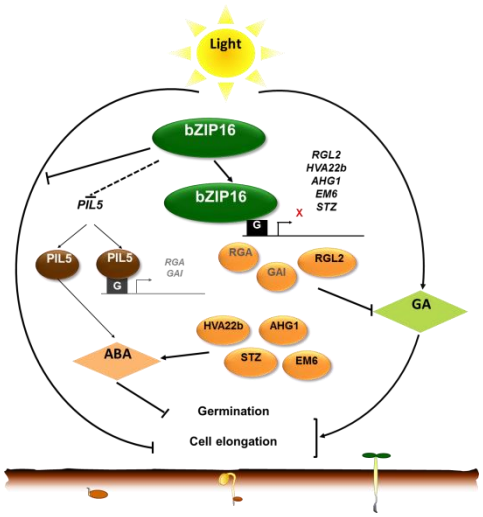
bZIP transcription factors exist in all eukaryotes. In plants, they are master regulators of many central developmental and physiological processes. In 2012. bZIP16 was reported to promote seed germination and hypocotyl elongation during the early seedling development by repressing *RGL2* and *PIL5*. Interestingly, a previous study has indicated that RGA and RGL2 both negatively regulate the floral transition. It inspired us to pursue whether bZIP16 also functions in Arabidopsis flowering time control. By counting the rosette leaf numbers at bolting, we found that *bzip16* mutants showed late-flowering phenotypes under long-day and short-day conditions in Arabidopsis. To clarify how the floral transition is regulated by bZIP16, we performed a transcriptomic study and qRT-PCR to analyze the expressions of genes regulating flowering time in both wild-type and *bzip16* mutant. Those results revealed that bZIP16 does not affect the expressions of genes in photoperiodic, gibberellin, autonomous and vernalization pathway. In contrast, bZIP16 significantly represses the expression of *FLC* and promotes the expressions of *SOC1* and *FT*. Our chromatin-immunoprecipitation assay indicated that bZIP16 directly binds to the *FLC* promoter harboring G-box motif. The expression repression of *FLC* by bZIP16 will de-repress the expressions of *SOC1* and *FT* to promote flowering. Our studies demonstrated that bZIP16 not only promotes seed germination and hypocotyl elongation in early seedling development but also plays a positive role in floral induction. Our research also revealed bZIP16 as a new floral regulator in controlling flowering time.

壹、 前言

一、研究動機和背景介紹：

轉錄因子存在於所有真核生物中,藉由與 DNA 序列進行專一性的結合來調控 DNA 轉錄至 mRNA 的過程。其利用單獨作用或結合其他蛋白質組成聚合體，來影響 RNA 聚合酶連接到特定基因上，以調節此基因的轉錄作用，此機制確保了特定的基因在生物體內能夠正確的開關。植物必須不斷的調節內部的生長機制以適應環境和氣候的變化，因此對植物發育生長而言，轉錄調控扮演一個相當重要的角色。

在阿拉伯芥野生株與其光形態突變株的基因轉錄組 (Transcriptome profiling) 中已經分析出上千個對光反應具有不同表現形式的基因 (Ma et al., 2001)。而植物中，bZIP 轉錄因子被報導參與許多的調控過程，包括病原體的防禦、光線與壓力的訊息傳導、種子成熟和花的發育。阿拉伯芥基因組序列包含 75 個不同的 bZIP 蛋白質，其中約有 50 個尚未被了解。bZIP16 被歸類於 basic region/leucine zipper motif 轉錄因子的 G 組 (Jakoby et al.,2002)。bZIP16 的功能已被證實是一個整合植物荷爾蒙與光訊息傳導途徑的重要負向轉錄因子，並控制阿拉伯芥種子的萌芽與幼苗的發育。bZIP16 扮演轉錄抑制者的角色，利用直接結合至植物荷爾蒙下游的關鍵基因，包括離層酸(ABA)下游基因和吉貝素(GA)訊息傳導相關的 RGA-LIKE2 (RGL2)之啟動子上，來抑制這些基因的表現，達成促進種子萌芽與下胚軸延長的生理作用(Hsieh et al, 2012)。



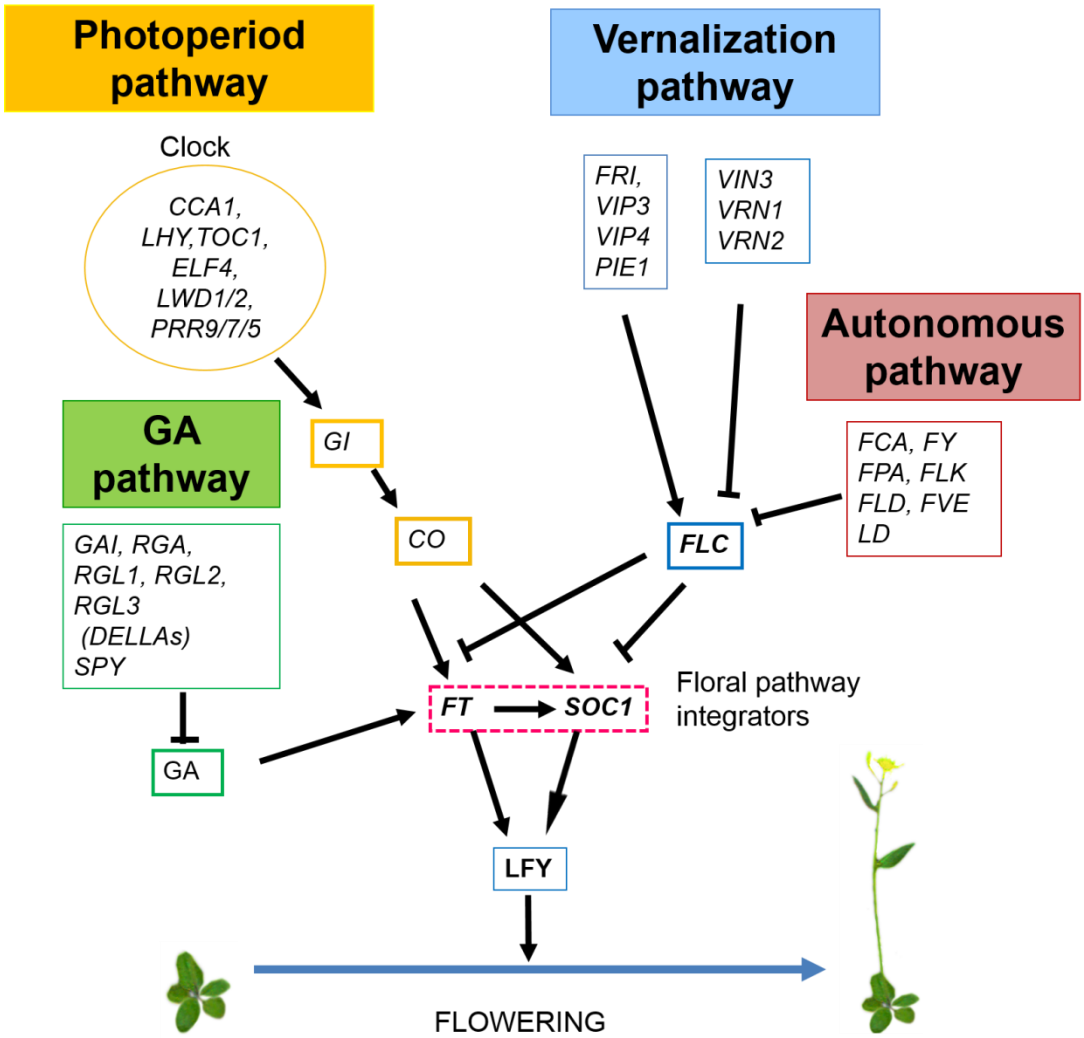
圖甲. bZIP16 在阿拉伯芥種子的萌芽與幼苗的發育之分子機制示意圖 (Hsieh et al, 2012)

bZIP16 藉由抑制 *RGA-LIKE2* (*RGL2*)，*PHYTOCHROME INTERACTING FACTOR 3-LIKE5* (*PIL5*)和 ABA 下游基因來促進種子萌芽及下胚軸延長作用。

吉貝素(GA)是促進植物生長和發育的重要賀爾蒙。它在種子萌發，生長和開花過程中扮演重要角色。缺乏吉貝素的突變體植株會生長遲緩和延遲開花，而對這些植物施予吉貝素則恢復正常。在光照或黑暗的環境下，吉貝素通過細胞分裂反應刺激莖伸長和葉細胞的膨脹和擴張 (Hauvermale et al., 2012) 。然而 DELLA 蛋白質則會抑制吉貝素各方面的作用，影響植物的生長 (Zentella et al., 2007)。DELLA 蛋白質包含 GA-INSENSITIVE(GAI)、REPRESSOR OF GA1-3(RGA)、RGA-LIKE1(RGL1)、RGL2 和 RGL3。先前文獻提及 RGL2 抑制種子萌發，RGA、RGL1 和 RGL2 參與調控植物的開花 (Tyler et al., 2004) 。

在阿拉伯芥中主要存在四條調控植物開花的途徑：光週期途徑、春化途徑、自主途徑和吉貝素途徑。這些途徑通過整合內在發育和外在環境兩方面的因素參與調控開花時間。光週期途徑負責感受和傳遞日照長短信號，而春化途徑則感受和反應環境溫度變化。另外兩條途徑上的基因能夠不依賴於環境信號而調控開花，其中自主途徑通過調控一些未知的植物內部因子來促進開花，而吉貝素能夠不依

賴於光週期促進開花。這幾條開花調節途徑通過幾個整合因子，如 *FT*、*SOC1* 和 *LFY* 互相聯繫。



圖乙. 阿拉伯芥中四條調控植物開花的途徑

根據前人的研究證實 RGA 和 RGL2 會影響植物的開花 (Tyler et al., 2004)。bZIP16 抑制 DELLA 基因之一的 *RGL2*，會促進吉貝素的作用(Hsieh et al, 2012)。

然而 bZIP16 是否參與植物的開花仍屬未知，因此激發我們去探討 bZIP16 蛋白質是否參與阿拉伯芥開花途徑的調控機制。

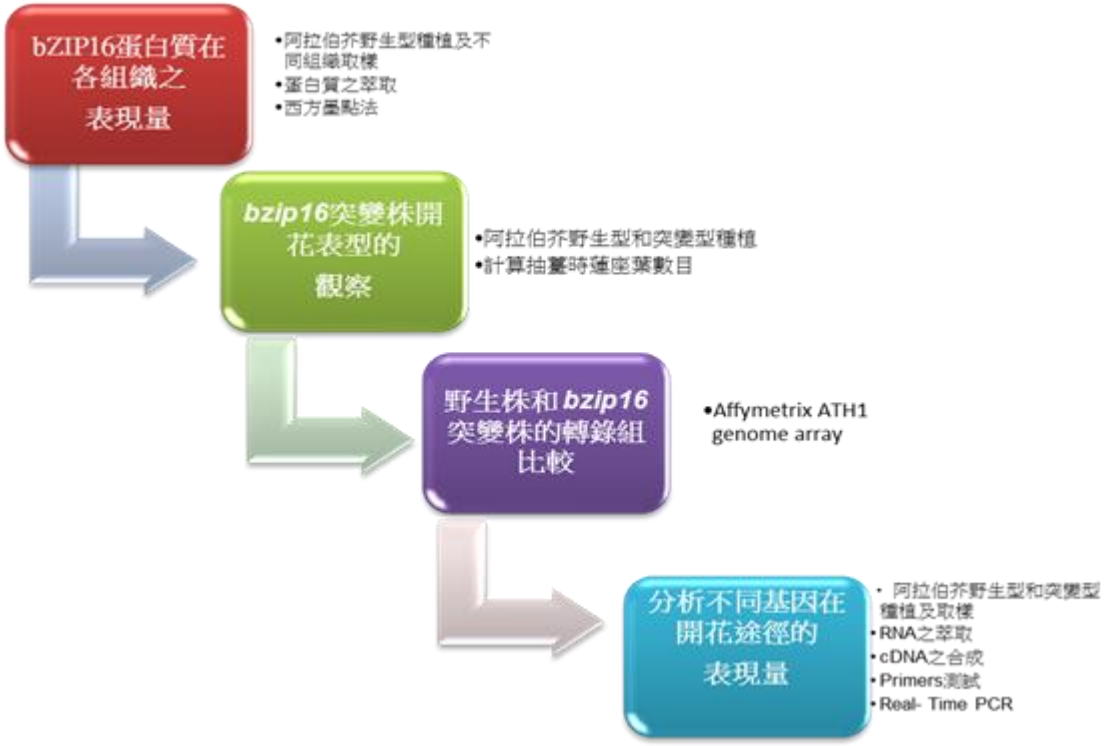
二、研究目的：

1. 探討 bZIP16 蛋白質在阿拉伯芥野生型植株中各組織之表現量

2. 研究 bZIP16 在阿拉伯芥開花途徑中的調控機制。

貳、 研究方法與過程

一、實驗流程



二、研究材料及設備

【阿拉伯芥野生株及突變株種植】

阿拉伯芥種子:

Landsberg erecta (Ler.野生型)

bzip16-2 突變株 GT9934 (剔除 bZIP16 基因突變型)

bzip16-2/ bZIP16 #2-8-1 (互補回復突變型)

Ler/35S- bZIP16-GFP#6-4 (大量表現 bZIP16 基因突變型)

試劑：

1. 70 % 酒精

2. 種子殺菌液（25% bleach；0.025% SDS）

3. 1/2 MS 培養基(0.5X Murashige and Skoog, 3%蔗糖(質量體積比)和 0.8%瓊脂(重量體積比)

【阿拉伯芥野生型植株不同組織 bZIP16 蛋白質之萃取】

材料：

阿拉伯芥野生型植株不同組織：種子、根、莖、葉、幼苗、花苞、花、果莢。

1. 蛋白質萃取液：

Working conc.	Stock	For 1ml
100mM Tris-HCL, pH7.8	1M pH8.0	0.1ml
4M Urea	MW60.6	0.24g
5% SDS	10%	0.5ml
15% glycerol	100%	0.15ml

2. 蛋白酶抑制劑：

Working conc.	Stock	For 1ml
2ng/ml aprotinin	1mg/ml	2μl
3ng/ml leupetin	1.5mg/ml	2μl
1ng/ml pepstain	1mg/ml	1μl
2mM PMSF	100mM	20μl
Protease inhibitor cocktail	50x	20μl

【西方墨點法】

材料:

SDS-PAGE 的電泳膠片, Bio-Rad protein assay kit , PVDF membrane, 甲醇(methanol), sample dye, β-ME, Bio-Rad, Mini trans-blot electrophoretic transfer cell, blocking reagent (5%牛奶, 0.1% Tween 20, 1XPBS 緩衝液), washing buffer (0.05% tween 20 in 1XPBS),acrylamide gel, coomassie blue, Tris-HCl(pH6.8 與 8.8)、一級抗體(bZIP16 antibody)、二級抗體(anti-rabbit,HRP)、底片、3mm 紙片

【阿拉伯芥野生型和各突變型植株總 RNA 之萃取】

試劑：

1. Pine tree extraction buffer（2% CTAB，2% PVP，0.5g/l Spermidine，2M NaCl，25mM EDTA, pH8.0，100mM Tris-HCl, pH8.0，2 % β-mercaptoethanol）
2. 10M LiCl
3. Chloroform:isoamylalcohol（24:1）
4. 10mM Tris-HCl，pH8.0
5. DEPC-dH2O（Diethylpyrocarbonate-H2O）

【阿拉伯芥野生型和突變種植株 cDNA 之合成】

材料：

1. DNase-treated RNA (8 µl, 2µg RNA (250µg/µl），0.6µl, RNase-free H_2O，2.4µl ,5x Superscript buffer (15mM $MgCl_2$)，1µl ,RQ1 RNase-free DNase (1U/µl, Promega)，Reverse transcription 反應混合液 (0.9µl, RNase-free H_2O，1.6µl ,5x Superscript buffer，2µl 0.1 M DTT，1µl ,10 mM dNTP，1µl RNasin(40U/µl)(final 2U, Promega)，0.5µl SuperScript II (Invitrogen)

設備：

微量滴管、加熱器、離心機、無菌操作檯、膠體電泳儀器、照相器、恆溫生長箱、PCR 儀器、鑷子、電磁加熱攪拌器、攪拌子、冰桶、20c.c.離心管、造膠臺、拭鏡紙、造膠玻璃、水噴瓶、微量吸管、微量離心管、酒精、計時器、齒梳、攝氏- 80 度冰箱、攝氏- 20 度冰箱、跑膠槽、電源供應器、Running buffer、vortex、刮膠器具、孔夾(黑與白)、玻璃棒、transfer buffer、轉印槽、剪刀、塑膠夾子、鐵夾子、壓片盒、暗房、顯影劑(developer)、定影劑(fixer)

三、實驗方法：

(一) 探討 bZIP16 蛋白質在阿拉伯芥野生型植株中各組織之表現量

實驗 1:阿拉伯芥野生型種植及不同組織取樣

實驗 1-1

方法：

1) 取阿拉伯芥野生株 Landsberg erecta 種子約 100 顆置於 1.5 ml 微量離心管中，並以 1 ml 70 % 酒精清洗 1 分鐘後，加水馬上將酒精去除，重複三次。
2) 以滅菌水清洗種子 3-5 次，即播種於土壤中。
3) 於 4℃暗房下預冷 3 天後，移至長日照(16 h 光照/8 h 黑暗), 22℃培養箱中。
4) 待阿拉伯芥開花結果莢時，關燈後第四小時分別取根、莖、葉、花樣本放
5) 入液態氮中，置入-80˚C 冰箱保存。

實驗 1-2

方法：

1) 取阿拉伯芥野生株 Landsberg erecta 種子和突變種 *bzip16-2* 共 150 顆，置於 1.5 ml 微量離心管中，並以 1 ml 70 % 酒精清洗 1 分鐘後。
2) 加種子殺菌液（25% bleach；0.025% SDS）消毒五分鐘。以滅菌水清洗種子 3-5 次，即可播種於 1/2 MS medium 上。
3) 於 4℃冷房黑暗中預冷 3 天後，留 50 顆在暗房中培養，50 顆移至 22˚C 長日照培養箱，50 顆移至 22 ˚C 全日照培養箱中。

4) 分別取樣如下：

長日照 *bzip16-2* 的花苞(對照組)

長日照野生株的花苞、花、果莢

野生株乾燥的種子野生株 4℃暗房三天種子

野生株 4℃暗房三天種子後 22℃ 全日暗一天幼苗

野生株 4℃暗房三天種子後 22℃ 全日暗四天幼苗

野生株 4℃暗房三天種子後 22℃ 全日照一天幼苗

野生株 4℃暗房三天種子後 22℃ 全日照四天幼苗

5) 將樣本放入液態氮中，置入-80 ˚C 冰箱保存。

實驗 2: 阿拉伯芥野生型植株不同組織蛋白質之萃取

方法:

1) 將蛋白質萃取液置於 100˚C 培養箱三分鐘後，加入蛋白酶抑制劑。
2) 分別加入 50μl 以上的蛋白質萃取液至各組織樣本，振盪混合均勻。
3) 加熱樣本到 100˚C　5 分鐘。
4) 以 1,3000 rpm 離心 5 分鐘。
5) 離心後所得上清液即為組織蛋白質萃取液，取出置於新的離心管中。
6) 以 Bio-Rad protein assay kit 測定蛋白質濃度。

實驗 3: 西方墨點法:

方法:

實驗 3-1: 蛋白質製備

1) 將已萃取的阿拉伯芥野生型植株不同組織的蛋白質取出。
2) 分別加入 5μl 樣品染劑和 2μlβ-ME 到 100μl 蛋白質萃取樣本中。
3) 置於 95˚C 培養箱中反應 90 秒,以 1,2000 rpm 離心 5 分鐘後，輸進丙烯酰胺凝膠。

實驗 3-2: SDS-聚丙烯醯胺凝膠電泳

1) 準備 10%-12% SDS-PAGE。
2) 將 5-10μg 蛋白質注入電泳槽尺狀凹槽中，放入電泳裝置中。
3) 加之以 70V 電壓 30 分鐘待蛋白質聚集，在以 100V 電壓 1.5 小時使其分離。

實驗 3-3: 轉漬

1) 以 100% methanol (甲醇) 稍微潤洗，活化 PVDF membrane。建構 the gel sandwich (by Bio-Rad, Mini trans-blot electrophoretic transfer cell) 將 transfer 夾鋪平，依次放上一片海綿、一張 3mm 濾紙、膠、PVDF
2) membrane、一張 3mm 紙片、一片海綿，最後將夾子夾起來，稍微擠壓將氣泡擠出來。
3) 放入 transfer 裝置以 100V 電壓 1 小時進行轉漬，即完成。

實驗 3-4: 阻斷和檢測

1) 將轉漬完的 membrane 浸泡在甲醇和水中。
2) 準備 blocking reagent (5%牛奶, 0.1%Tween 20, 1XPBS)。
3) 將 membrane 放入 10ml blocking reagent 中，在室溫下搖晃 blocking 1 小時。(60rpm)
4) 加入一級抗體 10ml(bzip16 antibody, 1:1000)後繼續搖晃 1 小時。(60rpm)
5) 使用 washing buffer (0.05% Tween 20 in 1X PBS) 沖洗 membrane，以 60rpm 搖晃 5 分鐘 5 次。
6) 加入 10ml 二級抗體 (anti-rabbit HRP, 1:5000) 在室溫下搖晃培養 1 小時。
7) 使用 washing buffer (0.05% Tween 20 in 1X PBS)沖洗 membrane，以 60rpm 搖晃 5 分鐘 5 次。

8) 將 substrate 加入 membrane 後，放入滅過菌的袋子。

9) 利用 substrate 將之顯影，顯影方式為將所附試劑 A: 500μl 加上試劑 B: 500μl，淋在袋子中，輕壓 membrane 使其完全浸在試劑中，將氣泡擠出後迅速封袋，放進壓片盒中。

10) 以冷光專用 X 光片於暗房中壓片，壓片 10 秒到 10 分鐘不等，洗出 X 光片。

11) 壓片完成後取出袋子中的 membrane，用 coomassie blue 將它染色以檢查 membrane 上的蛋白質。

(二) *bZIP16* 突變株表型的觀察

實驗: 阿拉伯芥野生型和各突變型種植與生長

方法：

1) 分別取阿拉伯芥野生型 Ler 種子、*bZIP16* 基因突變型（*bzip16-2*）、互補回復突變型(*bzip16-2/bZIP16*) 及大量表現 *bZIP16* 基因突變型（*bZIP16ox*）各 60 顆種子置於 1.5 ml 微量離心管中，並以 1 ml 70 % 酒精清洗 1 分鐘後，加水馬上將酒精去除，重複三次。

2) 以滅菌水清洗種子 3-5 次，即播種於土壤中。

3) 於 4℃下預冷 3 天後，分兩批各移至長日照(16 小時光照/8 小時黑暗) 和短日照 (8 小時光照/16 小時黑暗) 培養箱中，觀察他們抽薹時葉片數目及生長情形。

(三) 在野生株和突變株中各開花基因的轉錄組比較

實驗 1: 阿拉伯芥野生型和突變型種植及取樣

方法：

1) 取阿拉伯芥野生株 Ler 種子和 *bZIP16* 基因突變型(*bzip16-2*)種子各 360 顆置於 1.5 ml 微量離心管中，並以 1 ml 70 % 酒精清洗 1 分鐘。
2) 以滅菌水清洗種子 3-5 次，各播種於土壤中。
3) 於 4℃暗房下預冷 3 天後，移至短日照 (8 小時光照/16 小時黑暗)，22℃培養箱中 18 天後，在 ZT 12 小時取樣。

實驗 2: 阿拉伯芥野生型和 *bzip16* 突變型植株 RNA 之萃取

(For Affymetrix GeneChip)

方法：

1) 在液態氮中分別研磨之前備用的阿拉伯芥野生型和各突變型植株樣本至粉狀。
2) 加入 invitrogen Plant total RNA purification kit (500 µl) 劇烈振盪混合均勻。
3) 於 4℃下，以 13,000 rpm 離心 4 分鐘。
4) 取上層之澄清液，並加入 200µl 之 5M NaCl，混合均勻。
5) 加入 500µl 之 Chloroform:isoamylalcohol（24:1）混合均勻。
6) 於 4℃下，以 13,000 rpm 離心 10 分鐘。
7) 取上層之澄清液，並加入 900µl 之 isopropanol 混合均勻，等 10 分鐘，待其沉澱。
8) 於 4℃下，以 13,000 rpm 離心 10 分鐘。
9) 以 70% EtOH 沖洗 RNA，劇烈振盪直到其漂浮在溶液之中。
10) 於 4℃下，以 13,000 rpm 離心 10 分鐘。
11) 風乾 RNA 後, 再以 40 µL of DEPC-dH2O 回溶 RNA。
12) 以分光光度測定各樣本 RNA 濃度。

(四) 分析 RGA, RGL2, CO, GI, FT, LFY, FLC, SOC1 基因在阿拉伯芥野生型和各突變型植株中開花途徑的表現量

實驗 1: 阿拉伯芥野生型和突變型種植及取樣

方法：

1) 取阿拉伯芥野生株 Ler 種子和 *bZIP16* 基因突變型(*bzip16-2*)、互補回復突變型(*bzip16-2/bZIP16*)和大量表現 *bZIP16* 基因突變型(*bZIP16ox*) 種子各 270 顆置於 1.5 ml 微量離心管中，並以 1 ml 70 % 酒精清洗 1 分鐘。
2) 以滅菌水清洗種子 3-5 次，各播種於土壤中。
3) 於 4℃暗房下預冷 3 天後，移至短日照 (8 小時光照/16 小時黑暗) , 22 ℃培養箱中 18 天後。
4) 於開燈後三小時開始,每個時間點各種取樣本8至10株放入液態氮中。之後，每隔三小時取樣一次，共取樣本五次(連續 12 小時)，置入-80 ˚C 冰箱保存備用。

實驗 2：阿拉伯芥野生型和各突變型植株總 RNA 之萃取

方法：

1) 以 65℃水浴加熱 pine tree extraction buffer（5ml/g tissue）20 分鐘。
2) 在液態氮中分別研磨之前備用的阿拉伯芥野生型和各突變型植株樣本至粉狀，
3) 並分別分裝於 2 ml 之離心管中。
4) 加入 700μl 預熱過之 pine tree extraction buffer 劇烈振盪混合，於 65℃水浴加熱 5 分鐘。
5) 加入同體積 700μl 之 Chloroform:isoamylalcohol（24:1），劇烈振盪混合 20 秒。

6) 於 4℃下，以 12000 rpm 離心 15 分鐘。

7) 取上層之澄清液，並加入 1/4 體積 175μl 之 10M LiCl，混合均勻。於 4℃下沈澱 RNA 至隔夜。

8) 於 4℃下以 12000 rpm 離心 30 分鐘。

9) 去除上清液，倒置離心管於 kimwipes 上，以除去殘餘之 10M LiCl。

10) 加入 700μl 75%冰的 EtOH 以去除多餘的鹽類。

11) 10)於 4℃下以 12000 rpm 離心 30 分鐘。

12) 11)去除上清液，倒置離心管於 kimwipes 上。

13) 再以 10~50μl 之 DEPC-H2O 回溶 RNA。

14) 取 1μl 之 RNA，以分光光度分別測定各樣本 RNA 濃度。

15) 分別將所有 RNA 濃度稀釋到相同濃度後，放入-80 ℃冰箱中保存。

實驗 3：阿拉伯芥野生型和突變型植株 cDNA 之合成

方法：

1) 阿拉伯芥野生株 Ler、*bZIP16* 基因突變型（*bzip16-2*）及大量表現 bZIP16 基因突變型（*bZIP16ox*）各樣本的 RNA template 加入 DEPC-H_2O 稀釋至 250 ng/μl。

2) 將 2μg RNA template(8μl)、0.6μl 的 DEPC-H_2O、2.4μl 的 5xSuperscript buffer(15mM $MgCl_2$)、1.0μl 的 RQ1 RNase-free DNase (1U/μl, Promega) 分別加入各樣本之中。

3) 37˚C，反應 30 分鐘後，馬上置於冰上 1 分鐘。

4) 分別加入 1μl 的 oligo-dT(23)V（0.5μg/μl）。

5) 65℃，反應 10 分鐘後，馬上置於冰上 1 分鐘。

6) 分別加入 7μl 的 RT 反應混合液到 13μl 的 DNase-treated RNA 混合液，攪拌均勻。

7) 42℃反應 1 小時後，置於 72˚C 15 分鐘，儲藏於-20˚C 備用。

實驗 4：Primers 測試

方法：

1) 根據過去文獻的資料，找出 CO、GI、FT、FLC、LFY、SOC1、RGA、RGL2、bZIP16、UBQ10 等基因的專一性 primers。

Primers 序列如下：

qRT-PCR primer	Sequence (5'→3')	Reference
UBQ10-ABI-1 ABI-Ler-UBQ-2	AGAAGTTCAATGTTTCGTTTCATGTAA GAATGGAAACATAGTTGGAACAATTATTCA	Hsieh et al., 2012
AtbZIP16-F AtbZIP16-R	GCATGGACAATGACCACCAA TCTCTCTGCGGCACCTGTTT	Hsieh et al., 2012
CO-750-ABI-S CO-800-ABI-AS	CATTAACCATAACGCATACATTTCATC TCCGGCACAACACCAGTTT	Wu et al, 2008
FT-254-ABI-S FT-306-ABI-AS	ATCTCCATTGGTTGGTGACTGATA GCCAAAGGTTGTTCCAGTTGTAG	Wu et al, 2008
FLC-396-ABI-S FLC-550-ABI-AS	AGCCAA GAAGACCGAACTCA TTTGTCCAGCAGGTG ACA TC	Baurle and Dean, 2008
LFY-437-ABI-S LFY-549-ABI-AS	TTGATGCTCTCTCCCAAGAAG TTGACCTGCGTCCAGTAA	Ebine et al, 2012
SOC1-305-ABI-S SOC1-367-ABI-AS	AACAACTCGAAGCTTCTAAACGTAA CCTCGATTGAGCATGTTCCT	Ebine et al, 2012
RGA-687-ABI-S RGA-972-ABI-AS	AGAAGCAATCCAGCAGA GTGTACTCTCTTCTTACCTTC	Tyler et al, 2004
RGL2-977-ABI-S RGL2-1090-ABI-AS	CGGAGAATTCAGATTCGCTTCAAC CAAGATCCGATAAACTCTCAGCGG	Kang et al, 2011
GI-3513-ABI-S GI-3563-ABI-AS	ACTAGCAGTGGTCGACGGTTTATC GCTGGTAGACGACACTTCAATAGATT	Wu et al, 2008

2) primers 測試反應如下:

Fw primer : Rv primer 比例為 0.5, 1.5, 3 對 0.5,1.5, 3 。

MQ	6.5	5.5	4
Primer-ABI-S	0.5	1.5	3
Primer-ABI-AS	0.5	0.5	0.5
MQ	5.5	4.5	3
Primer-ABI-S	0.5	1.5	3
Primer-ABI-AS	1.5	1.5	1.5
MQ	4	3	1.5
Primer-ABI-S	0.5	1.5	3
Primer-ABI-AS	3	3	3

(9*3 repeats=27)

＊將這 9 組引子對利用此濃度比例體積為 7.5μl，加上 12.5μl 的 SYBR Green MasterMix (Applied Biosystem， at 4˚C)和 5μl 的 MQ，總體積為 25μl 的混合液中進行 qRT-PCR 反應。

1) 用 MQ 稀釋引子到濃度 100μM 後訂為標準溶液。

2) 再用 MQ 稀釋引子到濃度 5μM。

3) 測試 primers：

4) a)primers 濃度 100~300nM。

5) b)總共 27 個反應(一次反應 25μl，共重複 3 次)。

6) *UBQ10*、*CO*、*GI*、*FT*、*FLC*、*LFY*、*SOC1*、*RGA*、*RGL2*、*bZIP16* 個別的 primers 經過 9 種不同濃度組合的測試後得到的比例如下表:

5 μM primers	總體積為 25μl
UBQ10	Fw: Rv = 3.0: 3.0
bZIP16	Fw: Rv = 1.5 : 1.5
CO	Fw: Rv = 4.5 : 1.5
FT	Fw: Rv = 3.0 : 1.5
FLC	Fw: Rv = 0.5 : 0.5
LFY	Fw: Rv = 0.5 : 0.5
SOC1	Fw: Rv = 1.5 : 1.5
RGA	Fw: Rv = 1.5 : 1.5
RGL2	Fw: Rv = 0.5 : 0.5
GI	Fw: Rv = 3.0: 3.0

實驗 5： Real- Time PCR

Real- time PCR 反應混合液如下：

1) 分別將五個不同時段(3hr,6hr,9hr,12hr,15hr)採取的野生株和突變株樣本之 cDNA (0.5ng/5μl) 5μl+各自不同比例的 7.5μl primer 混合液+SYBR Green dye mixture 12.5μl 輸進 8 連排的離心管。
2) 分別以 *UBQ10*、*CO*、*GI*、*FT*、*FLC*、*LFY*、*SOC1*、*RGA*、*RGL2* 和 *bZIP16* 相應的引物進行 Real-Time PCR 反應，其混合液如下:

LFY, GI 和 *UBQ1* 的 primer 混合液:

	One reaction	70 reactions
MQ	1.5μl	105μl
Primer-ABI-S	3μl	210μl
Primer-ABI-AS	3μl	210μl
SYBR Green	12.5μl	75μl
cDNA (0.05ng/μl) final 0.25 ng/μl	5μl	
total	25μl	

bZIP16, RGA 和 SOC1 的 primer 混合液:

	One reaction	70 reactions
MQ	4.5μl	315μl
Primer-ABI-S	1.5μl	105μl
Primer-ABI-AS	1.5μl	105μl
SYBR Green	12.5μl	875μl
cDNA (0.05ng/μl) final 0.25 ng/μl	5μl	
total	25μl	

FLC 和 *RGL2* 的 primer 混合液:

	One reaction	70 reactions
MQ	6.5μl	455μl
Primer-ABI-S	0.5μl	35μl
Primer-ABI-AS	0.5μl	35μl
SYBR Green	12.5μl	875μl
cDNA (0.05ng/μl) final 0.25 ng/μl	5μl	
total	25μl	

CO 的 primer 混合液:

	One reaction	70 reactions
MQ	1μl	70μl
Primer-ABI-S	4.5μl	315μl
Primer-ABI-AS	1.5μl	105μl
SYBR Green	12.5μl	875μl
cDNA (0.05ng/μl) final 0.25 ng/μl	5μl	
total	25μl	

FT 的 primer 混合液:

	One reaction	70 reactions
MQ	3μl	210μl
Primer-ABI-S	3μl	210μl
Primer-ABI-AS	1.5μl	105μl
SYBR Green	12.5μl	875μl
cDNA (0.05ng/μl) final 0.25 ng/μl	5μl	
total	25μl	

3) NTC 則是 5μl 的 MQ + 各自不同比例的 7.5μl primer 混合液+SYBR GreenMasterMix 12.5 μl。

4) 加完後放入 qRT-PCR 儀器中執行 qRT-PCR。所使用之機體為 Applied Biosystem QuantStudio ™ 12K Flex Real-Time PCR System (http://www.appliedbiosystems.com）。

PCR 反應循環為:

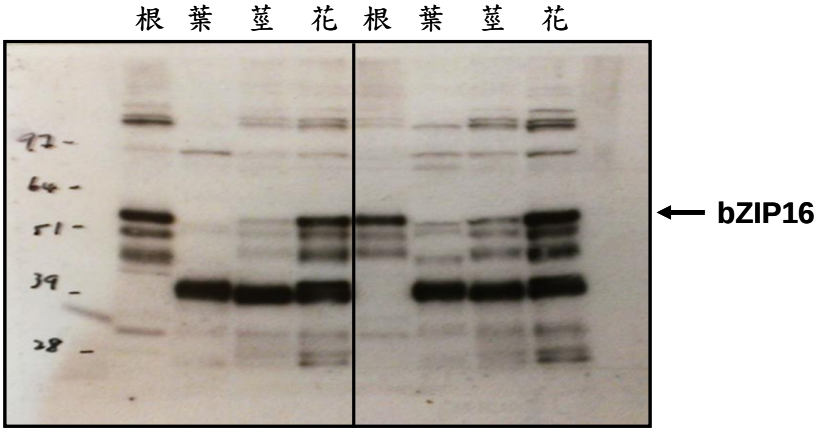
1) 50℃，2 分鐘 (去除 RNA)
2) 95℃，10 分鐘（活化 DNA Polymerase）
3) 95℃，15 秒（DNA 變性）
4) 60℃，1 分鐘（引子黏接及延長作用於一分鐘內同時進行）；其中步驟 3 及 4 共進行 40 個循環。

參、　研究結果與討論

(一) bZIP16 蛋白質在花苞與花中具有高表現量

針對 bZIP16 蛋白質在植物不同生長時期之表現量分析，首先萃取 *bzip16* 基因突變型和阿拉伯芥 Ler 野生型不同生長期之組織，包括種子、根、莖、葉、幼苗、花苞、花、果莢的蛋白質。再藉由西方墨點法執行四次實驗，發現 bZIP16 蛋白質在根、莖、幼苗、花苞、花、果莢中均有表現，其中以花苞和花的累積量最高。由於 bZIP16 蛋白質在花苞和花的高累積量，推測 bZIP16 可能也參與調控阿拉伯芥開花機制。（圖 1-1 至 1-3）

比較在 4℃ 處理三天後，放在 22℃ 黑暗環境中生長一天和四天的幼苗，顯示四天的幼苗中 bZIP16 蛋白質量增多。而比較在 4 天黑暗環境中生長的幼苗和在 4 天光照環境中生長的幼苗，顯示 4 天光照下幼苗中的 bZIP16 蛋白質量明顯減少。而在未經過 ˚4C 冷處理的乾燥種子及有經過 ˚4C 冷處理全光照 1 天環境中生長的幼苗中，則偵測不到 bZIP16 蛋白質。但經 4℃ 處理三天的種子，我們發現有 bZIP16 蛋白質的出現。顯示光照及冷處理對 bZIP16 蛋白質的表現量有密切關係。



（重複一）　　（重複二）

圖 1-1　bZIP16 蛋白質在根和花中表現量較多

阿拉伯芥野生型種植於 22℃ 長日照(16 h 光照/8 h 黑暗)生長箱，取 21 天成株的組織的樣本。圖中黑色箭頭指示的位置為 bZIP16 蛋白質，bZIP16 蛋白質的分子量約 51-55 kDa，從圖中此位置條帶的深淺可以代表 bZIP16 蛋白質的多少。

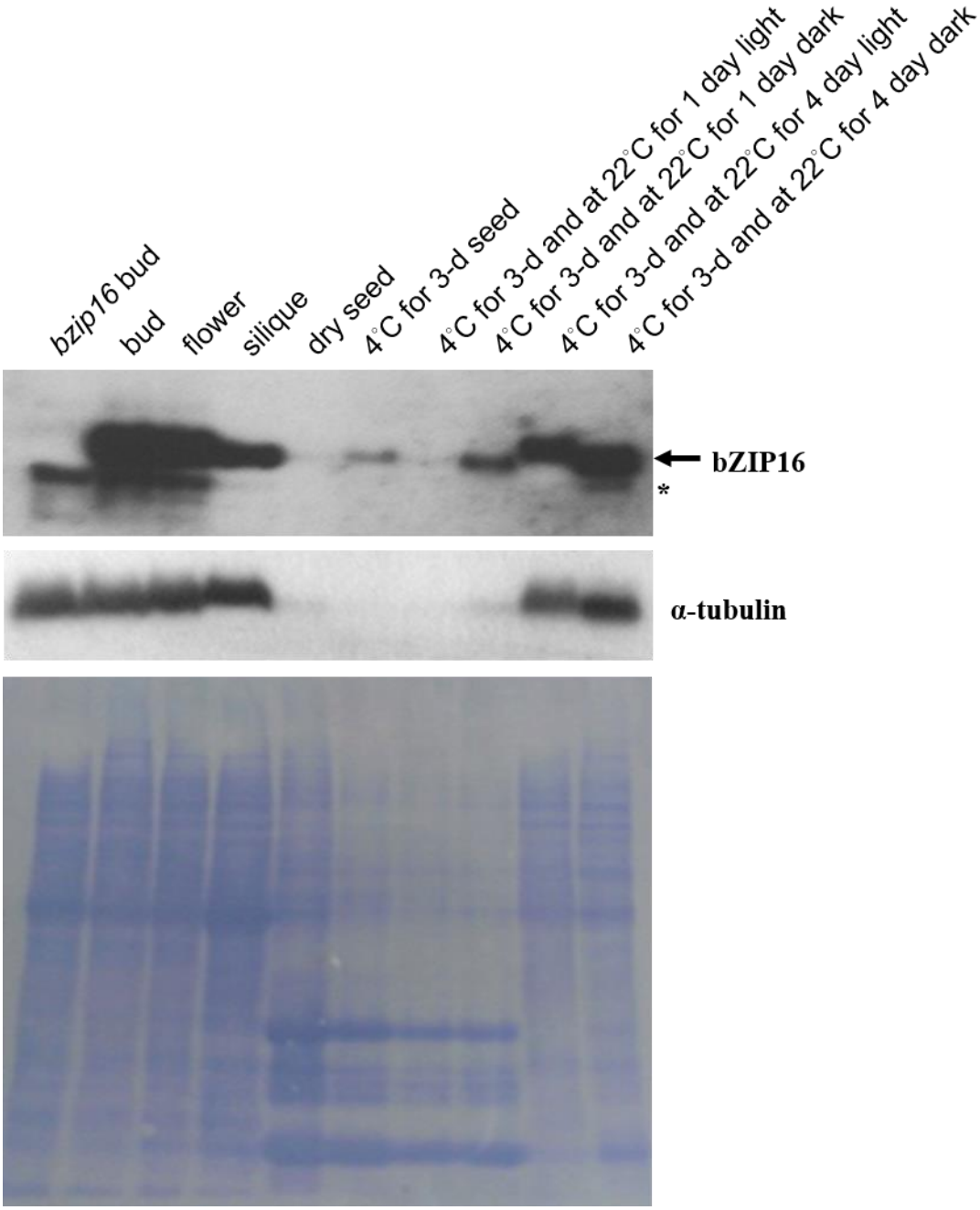


圖 1-2　bZIP16 蛋白質在花、花苞和在黑暗環境中生長 4 天的幼苗表現量較多。

萃取 *bzip16* 基因突變型和阿拉伯芥 Ler 野生型不同生長期之組織，包括種子、根、莖、葉、幼苗、花苞、花、果莢的蛋白質。以 α-tubulin 蛋白質做為 loading control。下圖利用 coomassie blue 染色以確認 membrane 上的蛋白質。

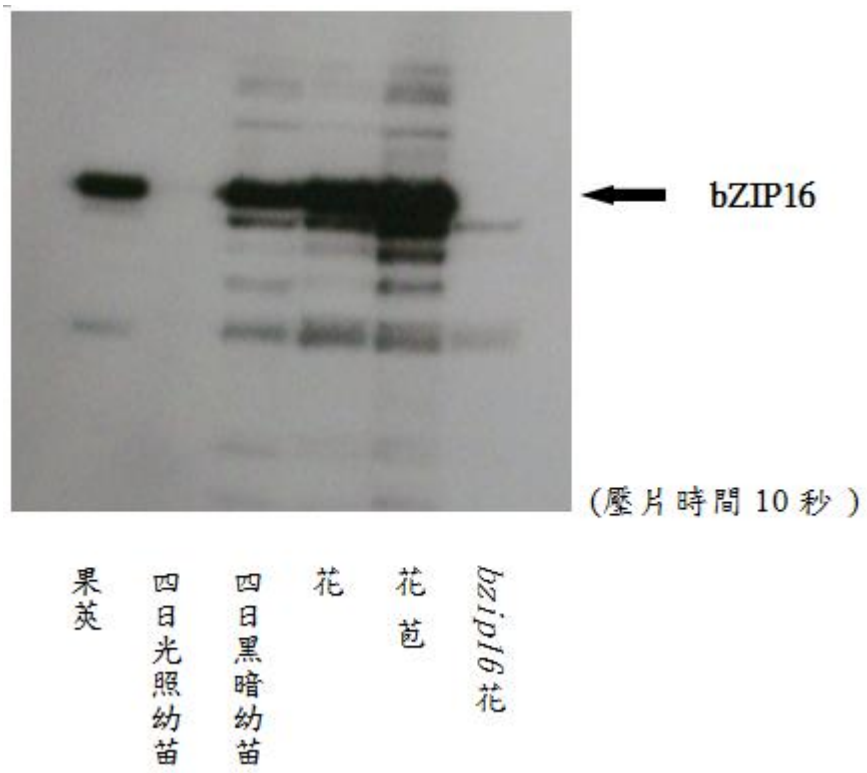


圖 1-3　bZIP16 蛋白質累積在果莢、花、花苞和在黑暗環境中生長 4 天的幼苗表現量較多。萃取 *bzip16* 基因突變型和阿拉伯芥 Ler 野生型不同生長期之組織，包括四日光照幼苗、四日黑暗幼苗、花苞、花、果莢的蛋白質。

(二) *bzip16* 突變株在長日照與短日照下皆具有延遲開花的現象

為了解 bZIP16 在植物中之生理功能及特性，藉由觀察及比較阿拉伯芥野生型植株 Ler、*bzip16* 突變株（*bzip16-2*）互補回復突變型(*bzip16-2/bZIP16*)及大量表現 bZIP16 基因之植株（*bZIP16ox*）之外觀型態，來推測 bZIP16 可能扮演的角色。將野生型和突變型分別種植於不同光照處理（長日照：16 小時光照/8 小時黑暗；短日照：8 小時光照/16 小時黑暗）之生長箱，並於生長其間觀察其外觀性狀。比較野生型植株 Ler 和 *bzip16* 突變株於長日照生長環境下之性狀。

結果顯示在長日照下，第一次開花實驗中，*bzip16* 突變株的蓮座葉平均數目比野生型多一片，而野生型在長出 4 片蓮座葉後便抽苔開花。而 *bzip16* 突變株在開花前平均產生 5 片的蓮座葉（圖 2-2A）。而長日照下第二次開花實驗，野生型平均長出 4.5 片蓮座葉後便抽苔開花，但 *bzip16* 突變株平均長出 5.5 片的蓮座葉後才抽苔開花，顯示 *bzip16* 突變株在長日照下會延遲開花。在短日照下，野生型平均

長出 23.55 片蓮座葉後便抽苔開花，而 *bzip16* 突變株平均產生 31.36 片的蓮座葉後才抽苔開花(圖 2-4)。顯示由營養生長到生殖生長的轉變明顯延遲。綜合上述結果顯示 bZIP16 會促進開花。

Ler　　***bzip16-2***　　***bzip16-2/bZIP16***　　***bZIP16ox***

圖 2-1 長日照下，*bzip16* 突變株蓮座葉較多

消毒每種品系的種子後，泡水一小時，種在泥土中，置入 4℃ 冷房三天，之後移至 22℃ 之長日照下(16 小時光照/8 小時黑暗)的植物生長箱，光照強度為 70~100μmol m^{-2} sec^{-1}。

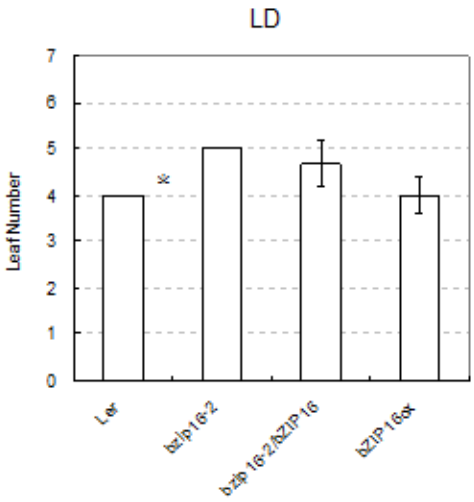


圖 2-2A

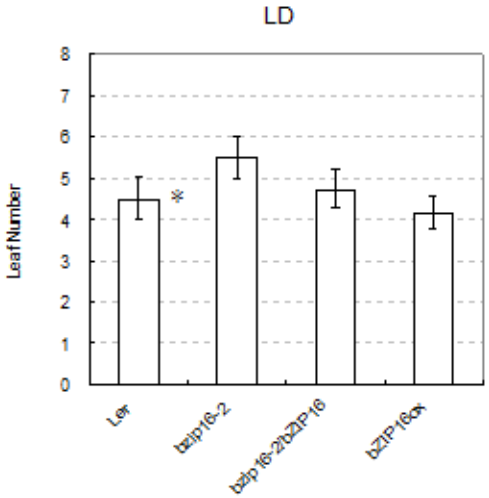


圖 2-2B

圖 2-2　*bzip16* 突變株在長日照下會延遲開花。

圖 2-2A 長日照下，野生型平均長出 4 片蓮座葉後便抽苔開花，而 *bzip16-2* 突變株平均長出 5 片的蓮座葉後才抽苔開花。圖 2-2B 野生型平均長出 4.5 片蓮座葉，但

bzip16-2 突變株平均長出 5.5 片的蓮座葉。互補回復突變株(*bzip16-2/bZIP16*) 平均長出 4.72 片蓮座葉，大量表現 *bZIP16* 株(*bZIP16ox*）長出 4.16 片蓮座葉。圖 2-2A 和圖 2-2B 為分別兩次不同實驗。*表示 *bzip16* 突變株明顯較野生株延遲開花。計算其開花前蓮座葉的數目(Student's t test; P<0.001, n ≥ 10)

bzip16　　Ler　　*bzip16-2/bZIP16*　*bZIP16ox*

圖 2-3　在短日照下，*bzip16* 突變株蓮座葉較多

消毒每種品系的種子後，泡水一小時，種在泥土中，置入 4℃ 冷房三天，之後移至 22℃ 之短日照(8 小時光照/16 小時黑暗)的植物生長箱，光照強度為 80~110μmol m^{-2} sec^{-1}。

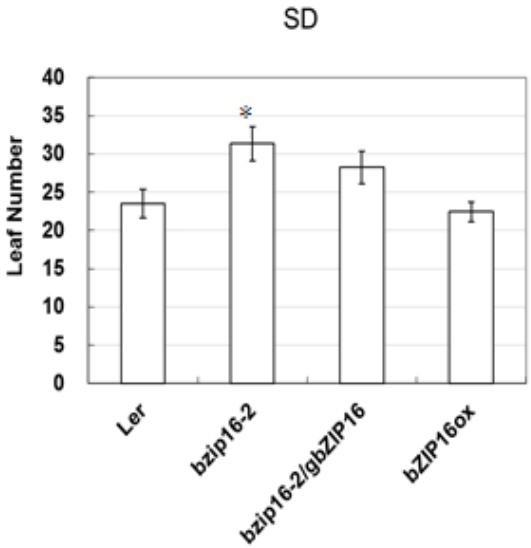


圖 2-4　*bzip16* 突變株在短日照下明顯延遲開花。

野生型平均長出 23.55 片蓮座葉後便抽苔開花，而 *bzip16-2* 突變株平均產生 31.36 片的蓮座葉後才抽苔開花。*表示 *bzip16* 突變株明顯較野生株延遲開花。計算其開花前蓮座葉的數目(Student's t test; P<0.001, n ≥ 10)

(三) bZIP16 明顯抑制 *FLC* 表現量

為了解 bZIP16 如何調控開花，我們利用 Affymetrix ATH1 GeneChip 分析，從檢測的 24000 個基因找出四十三個調控開花的基因，比較開花途徑中參與調控開花的基因於短日照下生長十八天的野生株和 *bzip16* 突變株中轉錄的變化。第一類是屬於自主途徑的基因，包含 *MULTICOPY SUPPRESSOR OF IRA1 4 (FVE), FLOWERING LOCUS D (FLD), FLOWERING LOCUS KH DOMAIN (FLK), LUMINIDEPENDENS (LD), FCA, FY* 及開花整合因子 *SOC1* 和 *FLC*。第二類是屬於吉貝素途徑的基因，包含 *REPRESSOR OF GA* (*RGA), GIBBERELLIC ACID INSENSITIVE(GAI), SUPPRESSOR OF PHYA-105 (SPY), RGA-LIKE 1 (RGL1), RGA-LIKE2 (RGL2)* 和 *RGA-LIKE3* (*RGL3*)。第三類是屬於光週期途徑的基因，包含 *CIRCADIAN CLOCK ASSOCIATED1(CCA1), LATE ELONGATED HYPOCOTYL (LHY), EARLY FLOWERING3 (ELF3), EARLY FLOWERING4 (ELF4), LOV KELCH PROTEIN2(LKP2), ,GIGANTEA(GI), FLAVIN-BIDING, KELCH REPEAT, F-BOX1(FKF1), PSEUDO-RESPONSE REGULATOR3 (PRR3), PRR5, PRR7, PRR9, ZEITLUPE(ZTL), TIMING OF CAB EXPRESSION1 (TOC1),CYCLING DOF FACTOR1 (CDF1), CONSTANS (CO), ARRHYTHMO/PHYTOCLOCK1 (LUX), LIGHT-REGULATED WD1 (LWD1), LIGHT-REGULATED WD2 (LWD2), (CCA1 HIKING EXPEDITION, TCP DOMAIN PROTEIN 21) CHE,*及開花整合因子 *FLOWERING LOCUS T (FT)*。第四類是屬於春化途徑的基因，包含 *VIRE2-INTERACTING PROTEIN 1* (*VIP1), VIRE2 INTERACTING PROTEIN 2 (VIP2), VERNALIZATION INDEPENDENCE 3 (VIP3),VERNALIZATION INDEPENDENCE 4 (VIP4), VERNALIZATION INDEPENDENCE 5 (VIP5), PHOTOPERIOD-INDEPENDENT EARLYFLOWERING 1 (PIE1), FRIGIDA (FRI), REDUCED VERNALIZATION RESPONSE 1 (VRN1),* 和 REDUCED VERNALIZATION RESPONSE 2 (*VRN2*)。

微矩陣轉錄體(transcriptome)分析顯示，在四十三個調控開花的基因中，以 *FLC* 的 mRNA 表現量變化最大，其它基因的 mRNA 表現量變化很小。在野生株中，*FLC* 的 mRNA 表現量下降；反之，在 *bzip16* 突變株中，*FLC* 的 mRNA 表現量升高。(圖 3-1)而 bZIP16 對吉貝素途徑、光週期途徑、春化途徑和自主途徑的基因似乎沒有影響。

此外，檢查 24000 個基因在野生株和 *bzip16* 突變株中的轉錄差異變化多於兩倍的基因共有 30 個，其中受 bZIP16 上調控的基因有 11 個(圖 3-2)，受 bZIP16 下調控的基因有 19 個 (圖 3-3)，除了 *FLC* 是抑制開花基因外，其它基因都不屬於開花途徑基因。以上結果證實 bZIP16 抑制 *FLC* 的表現，bZIP16 有可能是自主途徑基因。

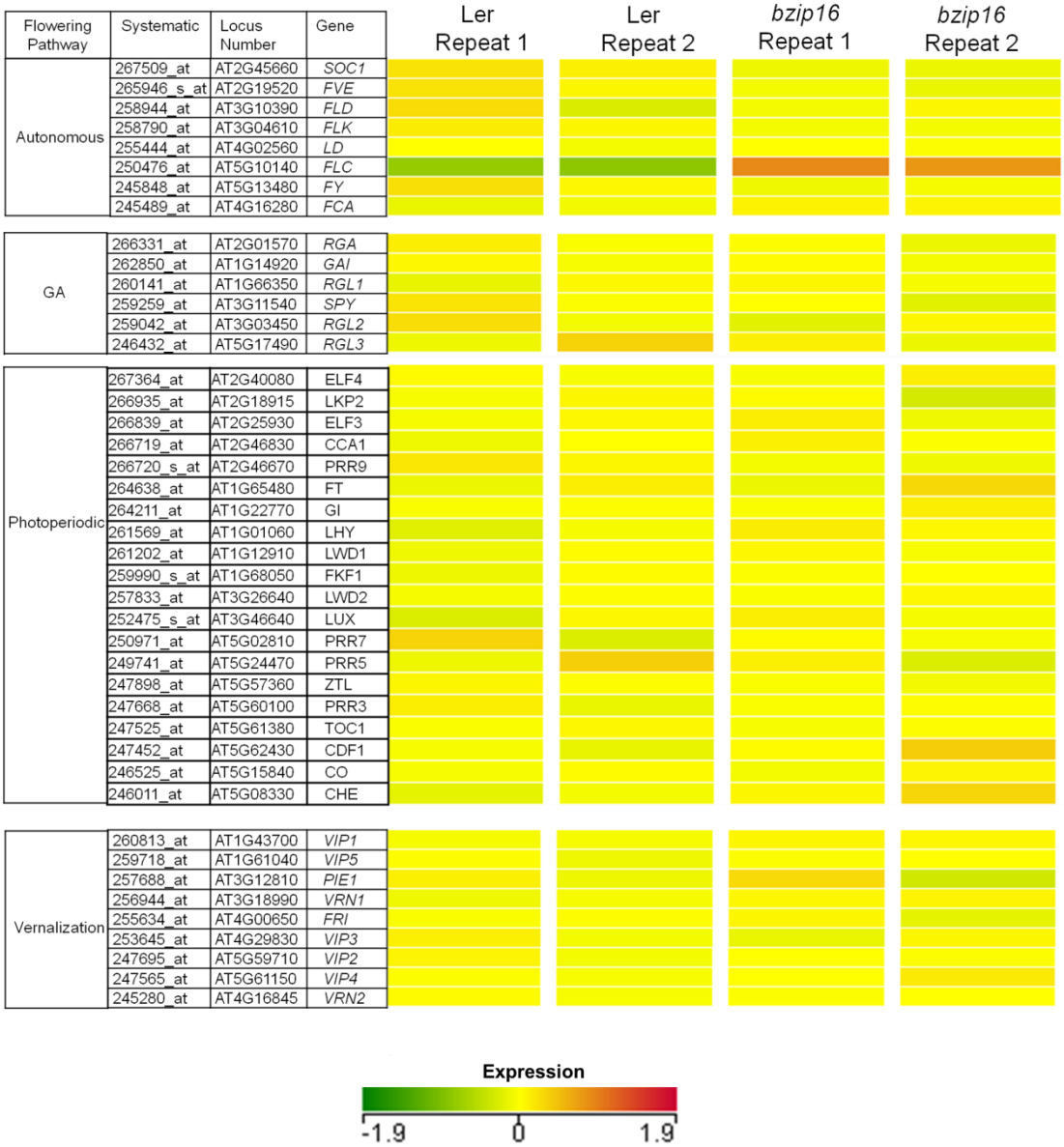


圖 3-1.　*FLC* 的 mRNA 表現量在 *bzip16* 突變株中明顯升高

比較基因轉錄組，分析 18 天大在(ZT) 12 小時的野生株和 *bzip16* 突變株中 43 個在開花途徑中的基因。每個基因的平均表達顯示為黃色。平均相對表現量上升為紅色，表現量下降為綠色。共執行兩次不同實驗。在野生株中，*FLC* 的 mRNA 表現量下降；反之，在 *bzip16* 突變株中，*FLC* 的 mRNA 表現量升，bZIP16 對吉貝素途徑、光週期途徑和春化途徑的基因似乎沒有影響。

Probe Set ID	Ler R1	Ler R2	*bzip16-2* R1	*bzip16-2* R2	expression fold	Unigene (Avadis)	Gene Symbol	AGI	Target Description
266634_at	1.04	1.17	-1.28	-1.04	4.8	At.37663		AT2G35530	putative G-box binding bZIP transcription factor
262634_at	2.24	2.48	-2.29	-2.24	23.0	At.26590		AT1G06690	unknown protein
262636_at	0.71	0.79	-0.71	-0.96	3.0	At.20810	NIH	AT1G06670	DEIH-box RNA/DNA helicase
260835_at	2.35	2.42	-2.42	-2.35	27.2	At.12093		AT1G06700	protein kinase interactor
260412_at	1.35	0.99	-1.99	-0.99	6.3	At.24555	AMY3	AT1G69830	putative alpha-amylase
257076_at	1.13	0.65	-0.69	-0.65	2.9	At.8144		AT3G19680	unknown protein
256245_at	1.4	0.65	-0.82	-0.65	3.4	At.47608	HSP70	AT3G12580	heat shock protein 70
255064_at	0.74	0.6	-0.6	-0.74	2.5	At.22399	EXO	AT4G08950	putative phi-1-like phosphate-induced protein
254452_at	2.49	2.15	-2.15	-2.15	22.1	At.32663	DDB1B	AT4G21100	UV-damaged DNA-binding protein-like damage-specific DNA binding protein 1
253942_at	0.59	0.49	-0.49	-0.59	2.1	At.54525		AT4G27010	putative protein
250304_at	0.92	0.89	-0.89	-0.93	3.5	At.699		AT5G12110	elongation factor 1B alpha-subunit

圖 3-2 bZIP16 正向調控的基因，11 個基因都不屬於開花途徑基因。

Probe Set ID	Ler R1	Ler R2	*bzip16-2* R1	*bzip16-2* R2	expression fold	Unigene (Avadis)	Gene Symbol	AGI	Target Description
266693_at	-0.67	-0.58	0.58	0.67	2.4	At.12894	MIOX2	AT2G19800	unknown protein
264824_at	-0.90	-0.80	0.80	0.80	3.1	At.69970	2A6		unknown protein
264826_at	-0.61	-0.68	0.61	0.77	2.5	At.19920	2A6	AT1G03410	putative 1-aminocyclopropane-1-carboxylate oxidase
263268_at	-0.93	-1.13	0.94	0.93	3.9				unknown protein
262010_at	-0.97	-1.32	0.97	1.10	4.5	At.47239			hypothetical protein
262019_s_at	-1.11	-0.80	0.80	0.82	3.4				hypothetical protein
260011_at	-0.60	-0.70	0.60	0.84	2.6	At.19366		AT1G68110	hypothetical protein
259620_s_at	-1.26	-1.43	1.30	1.26	6.2				Tam3-like transposon protein
257306_at	-0.84	-0.79	0.79	1.15	3.4	At.53591		AT3G30200	hypothetical protein
256940_at	-2.85	-2.85	2.85	2.85	52.0	At.36724	QQS	AT3G30720	unknown protein
256300_at	-0.83	-0.43	0.43	0.68	2.3	At.24919	NAP	AT1G69490	unknown protein
256166_at	-1.03	-1.01	1.08	1.01	4.2	At.49945		AT1G36920	hypothetical protein
255414_at	-2.01	-1.95	1.95	2.10	16.0	At.54106		AT4G03156	hypothetical protein
254343_at	-0.88	-0.55	0.55	0.80	2.6	At.2106	APR3	AT4G21990	PRH26 protein
250476_at	-0.79	-0.85	0.89	0.79	3.2	At.75671	FLC	AT5G10140	MADS box protein FLOWERING LOCUS C
248969_at	-0.61	-0.55	0.60	0.55	2.2	At.27375		AT5G45310	unknown protein

248676_at	-0.66	-0.60	0.60	0.97	2.7	At.29820	ATSDI1	AT5G48850	similar to unknown protein
245032_at	-0.80	-0.74	0.74	0.92	3.0				En/Spm-like transposon protein

圖 3-3 bZIP16 負向調控的基因

除了 *FLC* 是抑制開花基因外，其它 18 個基因都不屬於開花途徑基因。

(四) *FLC* 在 *bzip16* 突變株的表現量會增高而 *SOC1* 和 *FT* 表現量則會下降

為了進一步探討是否 *bZIP16* 是屬於自主途徑的基因, 我們利用即時定量反轉錄 PC 技術(quantitative real- time RT- PCR)，藉由 RNA 的抽取，反轉錄合成 cDNA，再以此 cDNA 為模板，依據特定目標的 primer，進行後續 qPCR 的實驗。分析 8 個調控開花時間的基因 *RGA, RGL2, CO, GI, LFY, FT, FLC* 和 *SOC1* 在阿拉伯芥野生型植株 Ler、*bzip16* 突變株（*bzip16-2*）、互補回復突變株(*bzip16/bZIP16*)及大量表現 *bZIP16* 的植株（*bZIP16ox*）mRNA 的表現量。為得到精確的資料，分析了各基因在不同時刻的表現量，以選擇最佳取樣時間。選定取樣時 間為於開燈後三小時開始收第一次樣本，之後，每三小時收一次樣本，至十五小時止共收取五個不同時間點的樣本來進行分析。

首先先分析 *bZIP16* 在不同植株中的表現量。結果顯示 *bZIP16* 在突變株中並不存在，反之在 *bZIP16ox* 中則會大量表現。證明所使用的植株正確無誤(圖 4-1)。接著分析 *bZIP16* 對不同調控開花時間基因之表現的影響。結果顯示 *FLC* mRNA 的表現量在 *bzip16* 突變株明顯高於野生型(圖 4-2)。而受 *FLC* 負調控的調節開花的重要整合因子 *SOC1* 和 *FT* 在 *bzip16* 突變株中的 mRNA 表現量則明顯下降。反之，在大量表現 *bZIP16* 的植株（*bZIP16ox*）中，*FLC* 的 mRNA 表現量明顯下降，而 *SOC1* 和 *FT* 的 mRNA 表現量明顯上升(圖 4-3,4-4)。

然而, 比較野生型和突變型各株，吉貝素途徑的 *DELLA* 基因 *RGL2* 和 *RGA* 的表現量沒有明顯差異(圖 4-5, 4-6)。光週期途徑的 *CO* 和 *GI* 基因的 mRNA 表現量也沒有明顯變化(圖 4-7,4-8)。另一個調節開花的整合因子，花序分生組織特徵基因 *LFY* 的 mRNA 量太少，無法偵測出數據。

上述結果表明，bZIP16 對阿拉伯芥開花的調控，是通過抑制 *FLC* 的表現，從而促進 *SOC1* 和 *FT* 的表現而最終促進植物開花。

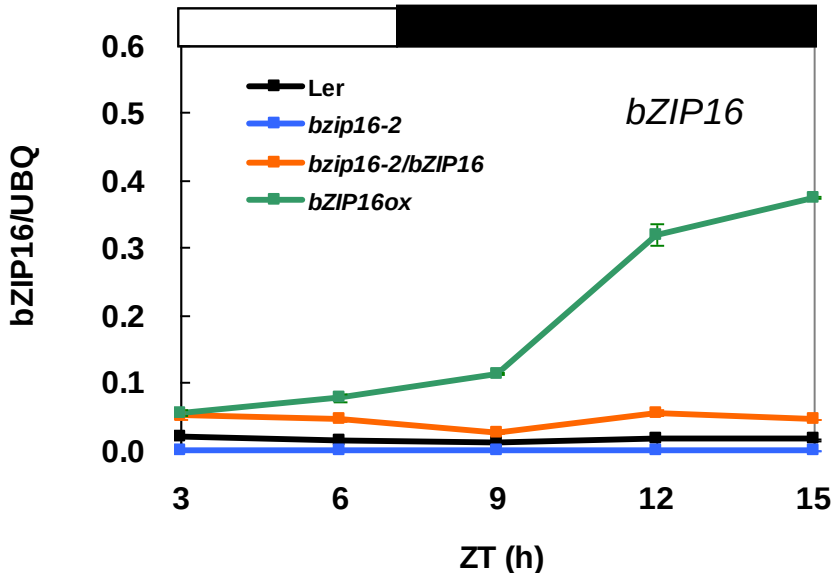


圖 4-1　*bZIP16* 在不同植株中的表現

bzip16 突變株（*bzip16-2*，藍色)，在野生型 Ler(黑色) (*bzip16/bZIP16*，及大量表現 *bZIP16* 的植株(*bZIP16ox*，綠色)。植物生長於短日照下(8 小時光照/16 小時黑暗)，種植 18 天大，於開燈後三小時開始取樣，每隔三小時取樣一次(連續 12 小時)，共取樣本五次。抽取 RNA 進行 qRT-PCR。圖上白色橫條表示光照期，黑色橫條表示黑暗期。All values in (Fig.4-1~ 4-8) are means (±S.E.) from three technique repeats

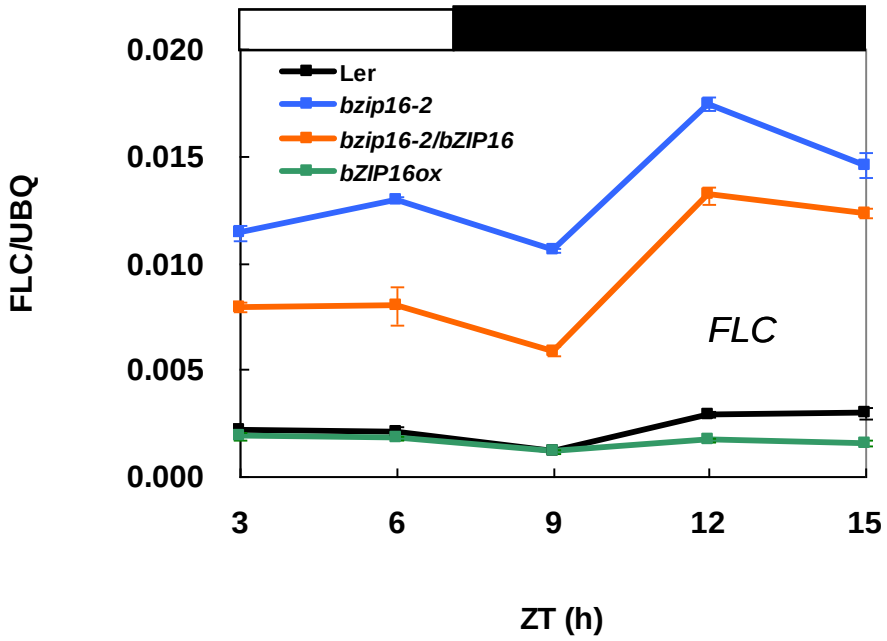


圖 4-2　*FLC* 的 mRNA 表現量在 *bzip16* 突變株中明顯高於野生型。

執行二次實驗，結果相似。

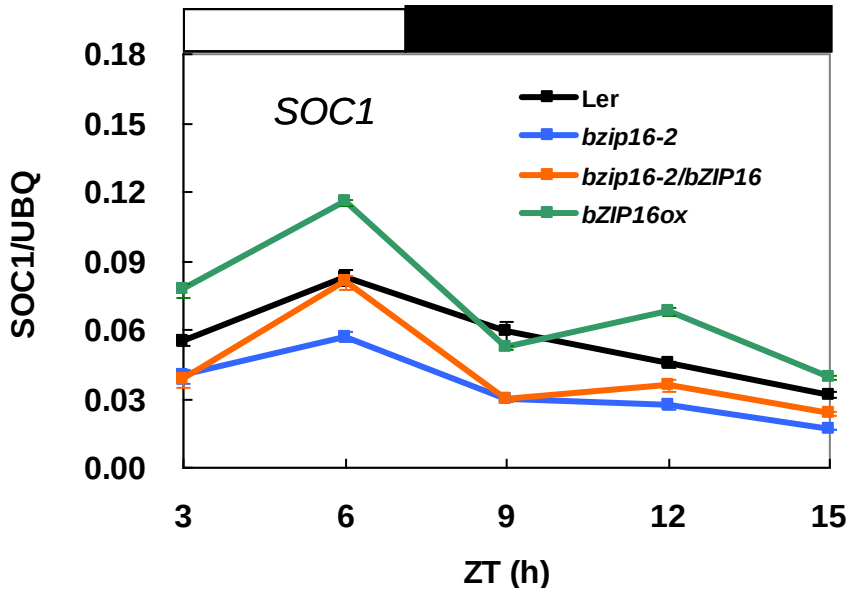


圖 4-3　*SOC1* 的 mRNA 表現量在 *bzip16* 突變株中明顯低於野生型。
執行二次實驗，結果相似。

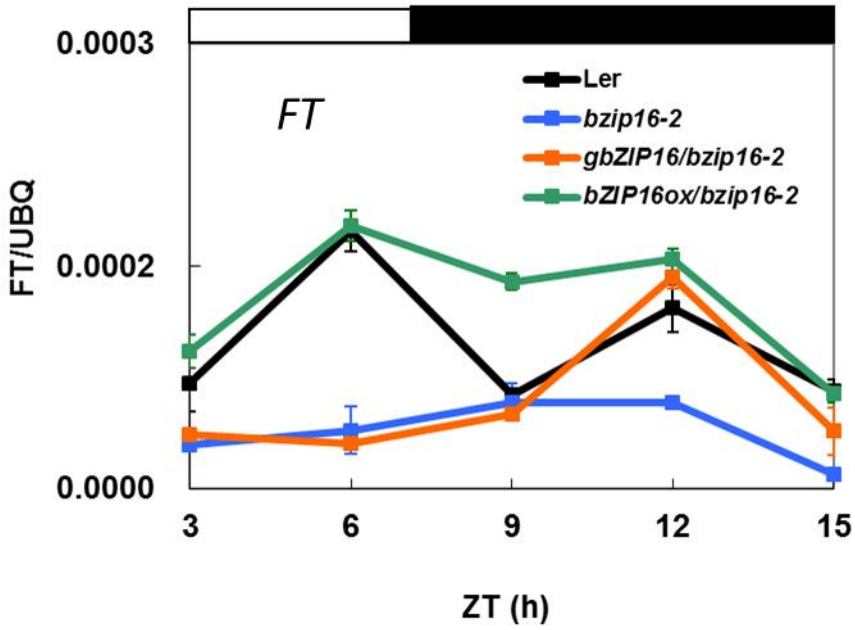


圖 4-4　*FT* 的 mRNA 表現量在 *bzip16* 突變株中明顯低於野生型。
執行二次實驗，結果相似。

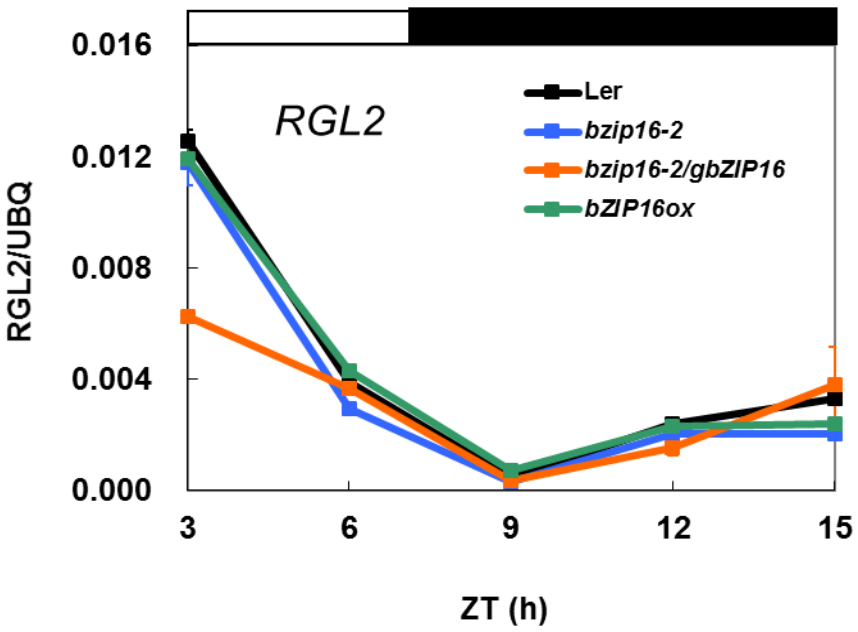


圖 4-5　*RGL2* 的 mRNA 的表現量在不同植株中，沒有明顯差異。
執行二次實驗，結果相似。

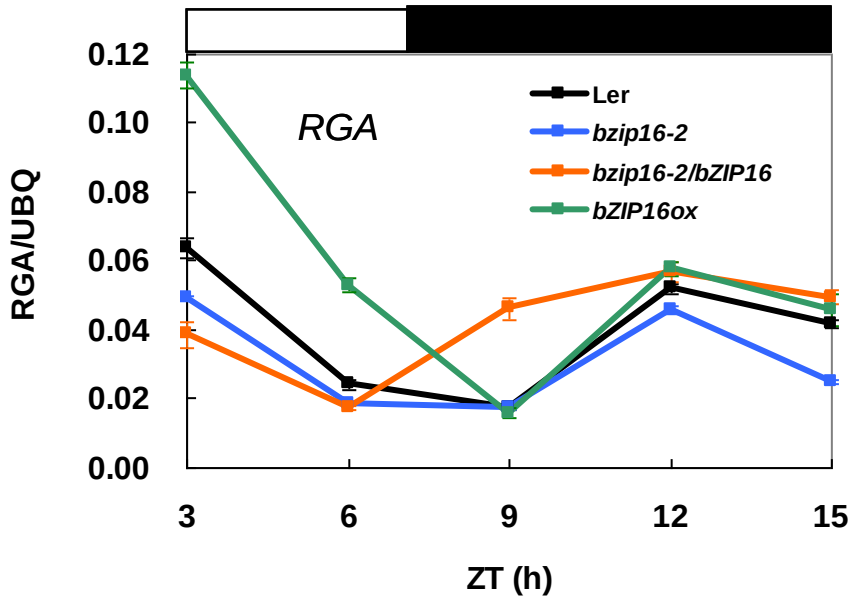


圖 4-6　*RGA* 的 mRNA 量在 *bzip16* 突變株中比野生型略為減少。
執行二次實驗，結果相似。

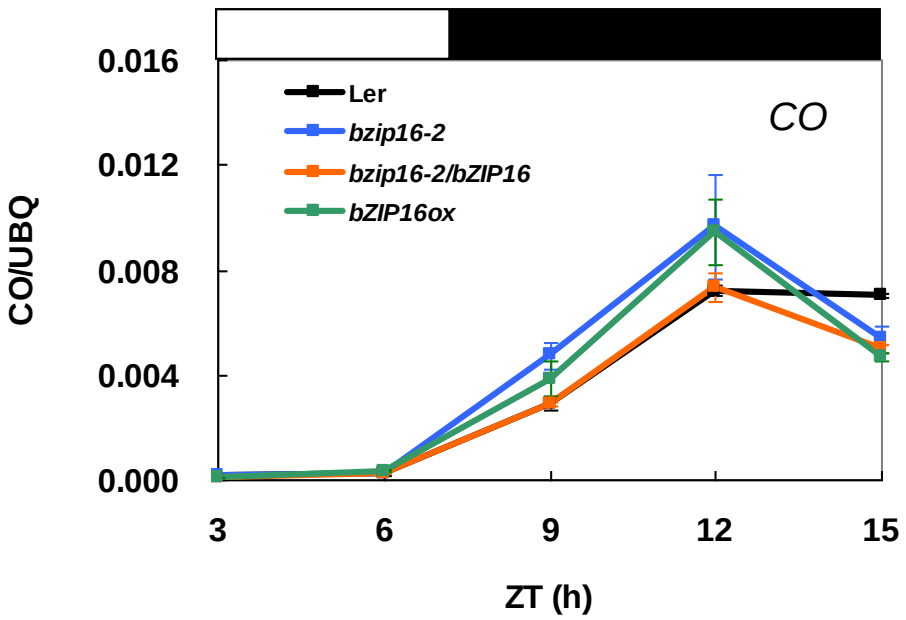


圖 4-7　*CO* 的 mRNA 表現量在不同植株中沒有明顯差異。

執行二次實驗，結果相似。

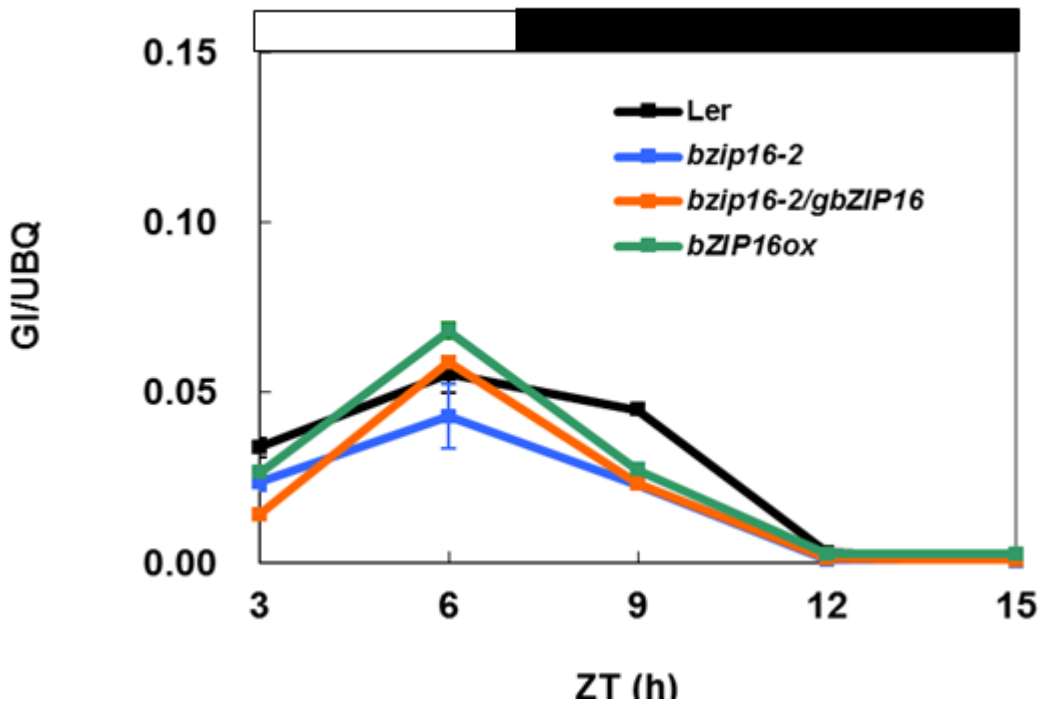


圖 4-8　*GI* 的 mRNA 表現量在不同植株中沒有明顯差異。

執行二次實驗，結果相似。

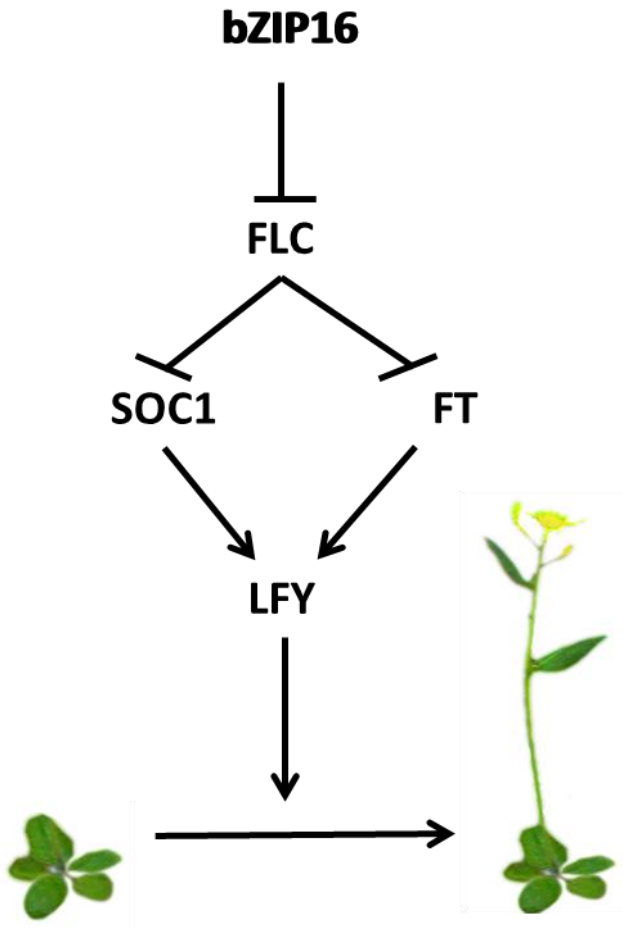
肆、 討論

檢測 bZIP16 蛋白質在種子、幼苗、花苞、花、果莢的表現量時，發現經過 4C°冷處理三天後，放在 22℃ 全黑暗 4 天環境中生長的幼苗所含的 bZIP16 蛋白質比在相同處理後，全光照 4 天環境中生長的幼苗多。而在未經過°4C 冷處理的乾燥種子及有經過°4C 冷處理全光照 1 天環境中生長的幼苗中，則偵測不到 bZIP16 蛋白質（圖 1-1 至 1-3)，顯示冷處理和光影響 bZIP16 的表現量。此外，bZIP16 蛋白質在花苞和花的高表現量和 *bzip16* 突變種在短日照和長日照下都延遲開花，顯示 bZIP16 可能參與調控阿拉伯芥開花機制。

在阿拉伯芥中主要存在四條調控植物開花的途徑：光週期途徑,春化途徑,自主途徑和吉貝素途徑(Mouradov et al., 2002)。這些途徑通過整合內在發育和外在環境兩方面的因素參與調控開花時間。感受和傳遞日照長短信號屬於光週期途徑，而感受和應答環境溫度變化則在春化途徑上。另外兩條途徑上的基因能夠不依賴於環境信號而調控開花，其中自主途徑通過感應一些未知的內部因子來促進植物開花，而吉貝素能夠不依賴於光週期促進開花。這幾條開花調節途徑通過幾個整合因子,如 *FT*，*SOC*1 和 *LFY* 互相聯繫。

以 Affymetrix GeneChip 分析，證實 bZIP16 抑制 *FLC* 的表現，而對吉貝素途徑、光週期途徑和春化途徑的基因似乎沒有影響。進一步以即時定量反轉錄 PCR（qRT- PCR）分析 8 個開花途徑的基因 *RGA*、 *RGL2*、 *CO*、 *GI*、*LFY*、 *FT*、 *FLC* 和 *SOC1*。已知 *bZIP16* 在阿拉伯芥種子的萌芽與幼苗的發育中，藉由抑制 *RGL2* 的作用來控制離層酸，促進吉貝素作用來促進幼苗的發育。但本研究發現 *bZIP16* 在開花途徑中，對 *RGL2* 卻沒有影響(圖 3-5)。而光週期途徑的基因 *CO* 和 *GI*，其 mRNA 表現量亦無明顯變化(圖 3-7)。表示 *bZIP16* 不是透過此二路徑調控開花。然而在 *bzip16* 突變株中 *FLC* 的 mRNA 表現量顯著高於野生型(圖 3-2)。而受 *FLC* 負調控之調節開花的重要整合因子 *SOC1* 和 *FT* 在 *bzip16* 突變株中的 mRNA 表現量

則明顯低於野生型(圖 3-3、3-4)。這些結果表明 *bZIP16* 在 *FLC* 的上游抑制其表現，隨後 *FLC* 又負調控 *SOC1* 和 *FT* 的表現，促進阿拉伯芥的開花 (圖丙)。自主途徑基因的主要功能在於抑制 *FLC* 的表現。目前所知在自主途徑基因 *fca, fld, fpa, fve, fy* 及 *ld* 突變株中，*FLC* 表現都會上升(Michaels and Amasino, 1999)。光週期途徑基因的突變株只在長日照下才延遲開花，但自主途徑基因的突變株不論在長日照或短日照下都會延遲開花。(Mouradov et al., 2002)。本研究證實 *bzip16* 突變種在短日照和長日照下都延遲開花並抑制負調控開花基因 *FLC* 的表現量，結果顯示 bZIP16 是自主途徑基因的一員。



圖丙 bZIP16 參與阿拉伯芥開花途徑的分子機制

箭頭代表著促進 T 字型代表抑制

伍、 結論與未來展望

先前文獻證實阿拉伯芥轉錄因子 bZIP16，藉由抑制 *RGL2* 與 *PIL5* 的表現來控制離層酸，並且促進吉貝素作用，來促進阿拉伯芥種子的萌芽與幼苗的發育(Hsieh et al, 2012)。本研究發現 bZIP16 蛋白質在花苞和花的表現量最高，在開花途徑中，bZIP16 藉由抑制 *FLC*，調控開花整合因子 *SOC1* 和 *FT* 的表現進而促進植物開花。此外，先前文獻證實自主途徑基因的突變株不論在長日照或短日照情況下都會延遲開花且都具有 *FLC* 高表現的特徵 (Michaels and Amasino, 1999 ; Mouradov et al., 2002)。 *bzip16* 突變株和自主途徑基因突變株的特徵相同，進一步透過微矩陣轉錄體(transcriptome)分析與即時定量反轉錄 PCR 分析，發現 bZIP16 對吉貝素途徑、光週期途徑及春化途徑的基因沒有影響，顯示 bZIP16 透過自主開花途徑調控開花時間。

本研究證實 bZIP16 除了控制阿拉伯芥種子的萌芽與幼苗的發育之外，在開花途徑中具有正向調控開花的功能。*bZIP16* 為阿拉伯芥新的開花調控基因，並且發現 bZIP16 是自主開花途徑基因的新成員。

研究科學最刺激的一部分就是我們永遠無法預測研究的成果是什麼，而專注的研究總得到意想不到的驚喜。當初我們以為 bZIP16 可能會透過吉貝素開花途徑的 *RGL2* 促進開花，結果，很幸運的發現 bZIP16 的另一功能－調控開花，找到一個新的開花基因。不過也意外的發現，bZIP16 在成長階段並不會抑制 *RGL2*，可見植物內部分子機制的複雜超過我們可以想像。希望未來可以進一步研究 bZIP16 如何抑制 *FLC*，探討它是否和其它八個自主開花途徑基因有相同的作用機轉。

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I. Introduction

A. Literature Review and Motivation

Transcription factors which exist in all eukaryotes are essential for the control of gene expression. These proteins bind to DNA regulatory sequences and control the transcription of genetic information from DNA to messenger RNA, promoting or blocking the recruitment of RNA polymerase to specific genes. They affect the transcription of specific genes, essentially determining whether a particular gene will be turned "on" or "off" in an organism. Plants are sessile, so they have to modulate the underlying developmental program accordingly to cope with the variation of environments. Therefore, transcriptomic adjustment plays a crucial role in plants.

Transcriptome profiling has revealed hundreds to thousands of genes with differential expression patterns in response to light in wild-type Arabidopsis and photomorphogenic mutants (Ma et al., 2001). In plants, basic region/leucine zipper motif (bZIP) transcription factors regulate processes including pathogen defense, light and stress signaling, seed maturation and flower development. The Arabidopsis genome sequence contains 75 distinct members of the bZIP family, of which ~50 are not described in the literature. bZIP16 is a basic region/leucine zipper motif (bZIP) transcription factor and is classified in group G (Jakoby et al.,2002). bZIP16 is primarily a transcriptional repressor of abscisic acid (ABA) responsive genes, could directly target ABA-responsive genes and RGA-LIKE2(RGL2), a DELLA gene in the gibberellin (GA) signaling pathway. bZIP16 has G-box-specific binding activity. It functions to promote seed germination and hypocotyl elongation during the early stages of Arabidopsis seedling development (Hsieh et al., 2012, Fig.A). The diverse roles of

bZIPs inspire us to explore whether bZIP16 regulates other developmental stages in Arabidopsis.

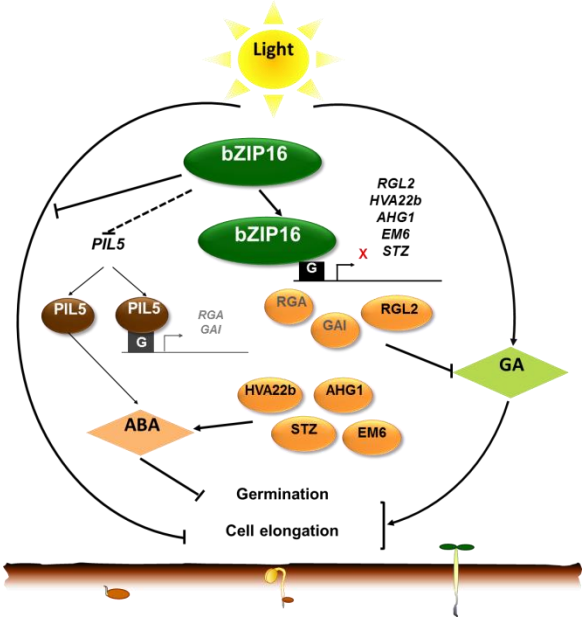

Fig.A Working model illustration molecular actions of bZIP16 in Arabidopsis early seedling development (Hsieh et al., 2012). bZIP16 positively regulates seeds germination and hypocotyl cell elongation by repressing *RGA-LIKE2* (*RGL2*), *PHYTOCHROME INTERACTING FACTOR 3-LIKE5* (*PIL5*) and ABA-responsive genes.

Gibberellins (GAs) are plant hormones that promote important processes of plant growth and development, such as seed germination, growth through elongation, and floral transition. Hence, mutant plants that are deficient in GA exhibit a dwarf and delayed flowering phenotype, and treating these plants with GA restores normal growth. GA also stimulates stem elongation and leaf expansion through cell expansion and cell division in response to light or dark (Hauvermale et al., 2012). DELLA proteins are conserved growth repressors that modulate all aspects of GA responses. (Zentella et al., 2007). The Arabidopsis genome encodes five DELLAs including GA-INSENSITIVE (GAI) REPRESSOR OF GA1-3 (RGA) RGA-LIKE1 (RGL1), RGL2 and RGL3 that play distinct but also overlapping functions in repressing GA responses. RGL2 inhibits

seed germination. RGA, RGL1, and RGL2 are all involved in modulating floral development (Tyler et al., 2004).

There are at least four flowering pathways in Arabidopsis, namely photoperiod, autonomous, vernalization, and gibberellins dependent pathways (Mouradov et al., 2002). The photoperiod pathway refers to regulation of flowering in response to day length and quality of light perceived. The autonomous pathway refers to endogenous regulators that are independent of the photoperiod and gibberellin pathways. (Koornneef M,et al.,1991) The vernalization pathway refers to the acceleration of flowering on exposure to a long period of cold. *FLOWERING LOCUS C (FLC)* delays flowering by blocking the transcription of genes in the photoperiodic flowering pathway. Vernalization inhibits transcription of *FLC*. The *FLOWERING LOCUS T (FT)*, *LEAFY (LFY)*, and *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS1 (SOC1)* genes act as central floral integrators (Blazquez et al., 1998; Nilsson et al., 1998; Samach et al., 2000).The gibberellin pathway refers to the requirement of gibberellic acids for normal flowering patterns. *SOC1* integrates the GA-mediated flowering-time signal with these environmental cues (Moon et al., 2003). The FT protein and mRNA appear to be the flowering stimulus that moves from leaves into the shoot apical meristem region, where it evokes the transition from vegetative to reproductive meristem identify.(Corbesier et al.,2012)

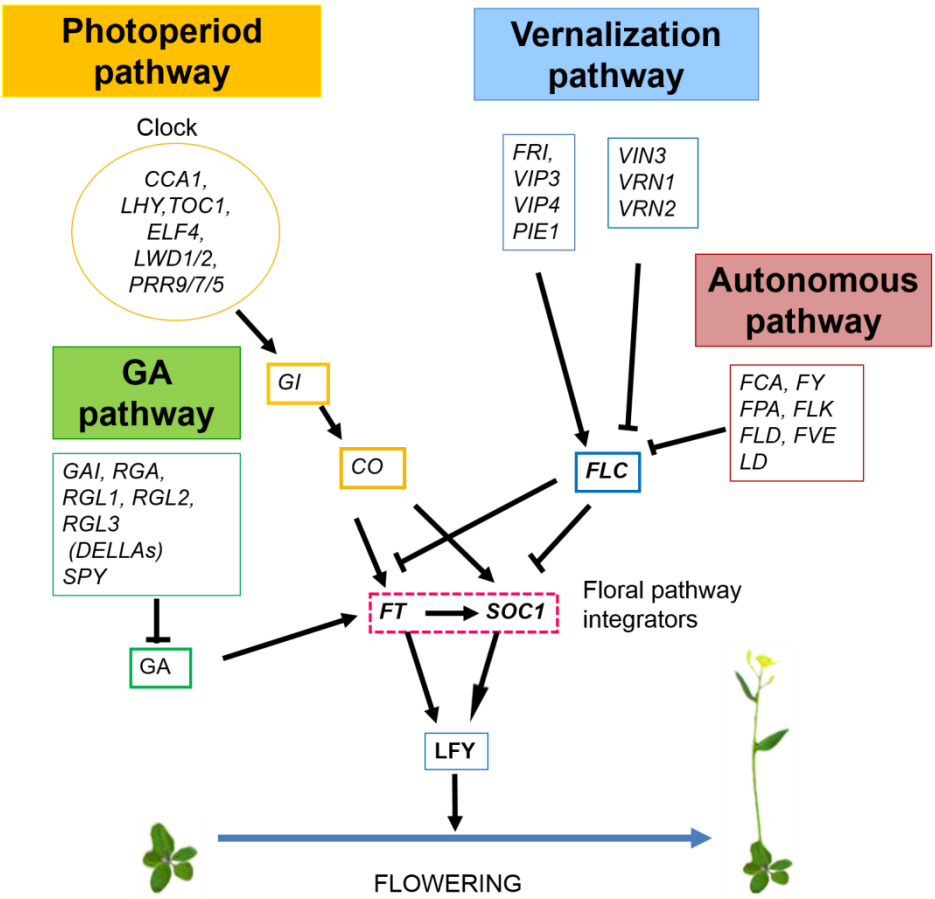

Fig. B Flowering pathways

B. Hypothesis

A previous study has indicated that RGA and RGL2 both negatively regulate the floral transition (Tyler et al., 2004; Fig.B). In early seedling development, bZIP16 is the repressor of *RGL2/RGA*. We thus hypothesize that bZIP16 also represses *RGL2/RGA* to promote floral transition. Our goals are to test this hypothesis and to understand the regulatory mechanisms imposed by bZIP16 in Arabidopsis flowering pathways.

II. Materials and Methods

A. Research Flow

1. Investigating bZIP16 protein level in different growth and development stages of Arabidopsis by western blot analysis to understand whether bZIP16 accumulates higher level in flowers.

2. Counting the rosette leaf numbers at bolting for wild-type (Landsberg *erecta*), bZIP16 knock-out line (*bzip16* mutant) and bZIP16 overexpression line (*bZIP16ox*) to understand whether bZIP16 regulates flowering time

3. Performing transcriptomic comparison between wild-type plants and *bzip16* mutants by Affymetrix ATH1 GeneChip to reveal genes regulated by bZIP16

4. Analyzing the expressions of different genes in flowering pathways by quantitative reverse transcription-PCR to elucidate the role of bZIP16 in the flowering pathways.

5. Using 18d-old Zeitgeber time 12 hr wild-type and bZIP16ox to perform chromatin immunoprecipitation quantitative PCR assay to test whether bZIP16 directly binds to the *FLC* promoter

B. Materials and Method

1. Investigating bZIP16 protein level in different growth development stages of *Arabidopsis*.

Experiment 1:

Planting Wild-type Arabidopsis, *bzip16* mutants and sampling different tissues seeds:

Landsberg erecta (Ler, wild type)

bzip16-2 mutant, GT9934 (null allele)

Ler/35S- bZIP16-GFP#6-4 (overexpression line in a Ler background)

Materials:

70 % of ethanol, half-strength

Murashige and Skoog (MS) medium with 0.8% agar and dd$H_2$0

Exp.1-1

Procedures:

1) Take 100 seeds of Ler into a 1.5ml eppendorf. Add 1 ml of 70 % ethanol and rinse for 1 min.
2) Use dd H_2O to wash the seeds 3 to 5 times and plant them in soil. Put plants into 4℃ cool room at dark for 3 days to synchronize the germination.
3) Transfer them to LD growth chamber (16 hours light/8 hours dark) at 22℃.
4) When plants flower, collect roots, stems, leaves and flowers as samples. And put them into liquid nitrogen and store them at-80 ˚C. (In darkness collect all the samples at the fourth hour after truning off light.)

Exp.1-2

Procedures:

1) Take 150 seeds of Ler and bzip16-2 mutant into a 1.5ml eppendorf, respectively. Add 0.5 ml of 70 % ethanol for 1 min.
2) Add 0.5 ml of 25% bleach for 5 min.
3) Use ddH20 to clean the seeds several times and plant them on half-strength MS plates with 0.8% agar for 3 days at 4℃ dark chamber.
4) Transfer plates to growth chambers under LD, constant white light and constant dark at 22℃.
5) Take samples as below:

   bzip16 mutants (LD) : flowers (for controlling)

   Ler. (LD): buds, flowers and siliques

   Ler: Dry seeds

   Seeds at 4℃ for 3 days under dark condition

   Seeds at 4℃ for 3 days and at 22℃ under dark condition for one day

   Seeds at 4℃ for 3 days and at 22℃ under light condition for one day

   Ler: Seedlings (at 4℃ for 3 days and at 22℃ under dark condition for 4 days)

   Seedlings (at 4℃ for 3 days and at 22℃ under light condition for 4 days)
6) Harvest and put the samples into liquid nitrogen and store them at-80 ˚C.

Experiment 2: Un-stable Protein Extraction

Materials:

Extraction buffer:

Working conc.	Stock	For 1ml
100mM Tris-HCL, pH7.8	1M pH8.0	0.1ml
4M Urea	MW60.6	0.24g
5% SDS	10%	0.5ml
15% glycerol	100%	0.15ml

Protease Inhibitors

Working conc.	Stock	For 1ml
2ng/ml aprotinin	1mg/ml	2μl
3ng/ml leupetin	1.5mg/ml	2μl
1ng/ml pepstain	1mg/ml	1μl
2mM PMSF	100mM	20μl
Protease inhibitor cocktail	50x	20μl

Procedures:

1) Prepare the protein extraction buffer and heat at 100˚C for 3 min and add protease inhibitors.
2) Add 50μl of extraction buffer to the samples and mix well by vortex.
3) Denature all samples at 100˚C for 5 min.
4) Centrifuge at 1,3000 rpm for 5 min.
5) Transfer the supernatant to a new eppendorf.
6) Measurement of protein concentration by protein assay DC (Bio-Rad)

(The processes were handled as soon as possible.)

Experiment 3: Western Blot Analysis

A. Total Protein preparation

1) Protein was extracted from samples
2) Quantified by BCA method (Bio-Rad protein assay kit)
3) Add 5μl of sample dye and 2μl of β-ME into 100μl sample.
4) Heat at 95˚C for 90sec, then spin 12,000 rpm for 5min.

B. Electrophoresis

1) Prepare 10%-12% SDS-PAGE,
2) Load 5-10μg total protein to each lane,
3) Run 70V for 30min for stacking and 100V for 1.5hr for separating,

C. Blot transfer

1) Activate PVDF membrane by 100% methanol,
2) Buildup the gel sandwich (by Bio-Rad, Mini trans-blot electrophoretic transfer cell),
3) Run 100V for1hr with Western transferring buffer,

D. Blocking and detection

1) Activated membrane by 100% methanol and rinse with H_2O.
2) Prepare blocking reagent (5% milk, 0.1% Tween 20, 1X PBS),
3) Preblock membrane with 10ml of blocking reagent for 1 hr shaking at room temperature (60rpm),
4) Block with primary antibody (bZIP16 antibody, 1:1000) within 10ml of blocking reagent for 1hr with shaking at RT (60rpm).
5) Wash membrane by shaking with washing buffer (0.05% Tween 20 in 1X PBS) 5min for 5 times.

6) Block with 10ml of anti-secondary antibody (anti-rabbit HRP, 1:5000) blocking reagent 1 hr shaking at room temperature.
7) Wash membrane by shaking with washing buffer (0.05% Tween 20 in
8) 1X PBS) 5min for 5 times.
9) Add substrate to the membrane and seal the membrane to plastic bag.
10) Detect signals by exposing to a X-ray film.
11) Use the coomassie blue to stain the membrane and detect the protein loading on each sample.

2. Observing phenotypes of *bzip16* mutants

Materials: Ler and *bzip16* mutants and *bZIP16ox*

Procedures:

1) Take 240 seeds of Ler, *bzip16-2* mutant and *bZIP16ox* into a 1.5ml eppendorf, respectively. Wash them by 1ml of 70 % ethanol for 1 min.
2) Use dd H_2O to wash the seeds 3 to 5 times and plant them in soil. Put plants into 4 ˚C cool room at dark for 3 days to synchronize the germination.
3) Transfer 12 seedlings for each line to LD (16 hours light/ 8 hours dark) and SD growth chamber (8 hours light/16 hours dark) at 22˚C.
4) Observe their phenotypes and count the rosette leaf number at bolting.

3. Transcriptomic comparison between wild-type plants and *bzip16* mutants

Experiment 1: Planting Ler and *bzip16* mutants and sampling

Procedures:

1) Take seeds of Ler and *bzip16* mutants.

2) Plant 360 seeds per line to soil at 4˚C cool room at dark for 3 days to synchronize the germination.
3) Transfer to SD growth chamber (8 hours light/16 hours dark) at 22 ˚C for 18 days and then collect samples at ZT 12hr.

Experiment 2: RNA extraction for Affymetrix ATH1 genome array

Material: Invitrogen kit for plant RNA extraction

1) Pulverize tissue in eppendorf with a blue pestle pre-cooled in liquid nitrogen.
2) Add in the Invitrogen Plant total RNA purification kit (500 µL powder) into the ground tissue and immediately mix by vortexing until well mixed.
3) Centrifugate 13,000 rpm for 5 min at 4℃.
4) Save supernatant and add 200 µL 5M NaCl, mix well.
5) Add 600 µL Chloroform:isoamyl alcohol (24:1), mix well.
6) Centrifugate 13,000 rpm for 10 min at 4℃.
7) Save supernatant, add 900 µL isopropanol, mix well. Wait for 10 min until it precipitates.
8) Centrifugate 13,000 rpm for 10 min at 4℃.
9) Wash pellet with 70% EtOH (Vortex the pellet until it floats).
10) Centrifugate 13,000 rpm for 10 min at 4℃.
11) Briefly dry the pellet until it turns transparent, and suspend it in 40 µL DEPC-dH2O.
12) Measure RNA concentration (OD260/280) using the Nanodrop spectrophotometer.

4. Analyzing the expression of different genes in flowering pathways

Experiment 1: Planting Ler and *bzip16* mutant and sampling

Procedures:

1) Take seeds of Ler, *bzip16* mutant and *bZIP16ox*.
2) Plant 270 seeds per line to soil at 4˚C cool room at dark for 3 days to synchronize the germination.
3) Transfer them to SD growth chamber (8 hours light/16 hours dark) at 22 ˚C for 18 days.
4) Collect 8-10 plants each line at ZT 3 hr, 6 hr, 9 hr, 12 hr and 15 hr after light on, respectively. Quickly and carefully put samples into labeled 2mL eppendorf without physical damage. Immediately dip the tube into liquid nitrogen and then store at -80˚C.

Experiment 2: RNA Isolation

Materials:

1. Pine tree extraction buffer（2% CTAB, 2% PVP, 0.5g/l Spermidine, 2M NaCl, 25mM EDTA pH8.0, 100mM Tris-HCl pH8.0, 2% β-mercaptoethanol）
2. 10M LiCl
3. Chloroform:isoamylalcohol（24:1）
4. 10mM Tris-HCl, pH8.0
5. DEPC-dH_2O（Diethylpyrocarbonate-H_2O）(Add 0.1% DEPC into H_2O and mix thoroughly. Incubate at 37 ˚C for overnight and autoclave.)

Procedures:

1) Pre-cool centrifugation and set 65˚C water bath.
2) Calculate pine tree extraction buffer require (5ml/g tissue). Add β-ME into pine tree extraction buffer and heat in 65˚C water bath for 20 min.
3) Pulverize tissue in Eppendorf with a blue pestle pre-cooled in liquid nitrogen.
4) Add extraction buffer in the ground tissue and immediately mix by vortexing until well mixed.
5) Heat sample in 65˚C water bath for 5min.
6) Add equal 700µlof chloroform: isoamyl alcohol, mix by vortexing for 20 sec.
7) Centrifugate at 4˚C, 12,000 rpm, until the phases are well-separated.
8) Transfer aqueous layer into a new tube and repeat chloroform extraction for 1-2 times, until the interface is clear.
9) Add 1/4 volume (175µl) of 10M LiCl and mix by pipetting. Precipitate RNA at 4˚C overnight.
10) Harvest RNA by centrifugation at 4˚C, 12,000 rpm for 30min and immediately remove supernatant and invert on kimwipe to drain.
11) Add 700µlof cold 75% EtOH to wash away excess salt.
12) Centrifugation at 4˚C, 12,000 rpm for 30min.
13) Immediately remove supernatant and invert on kimwipe to drain.
14) Resuspend pellet with 10-50µl of DEPC-H_20, and then transfer into a new eppendorf.
15) Measure RNA concentration by OD260/280, using DEPC-H_20 for blank.
16) Store RNA at -80 ˚C

Experiment 3: Reverse transcription

Material: 2μg RNA template in DEPC-H_2O,

Filter tips, RT mix (0.9μl of RNase-free H_2O, 1.6μl of 5x Superscript Buffer, 2μl of 0.1 M DTT, 1 μl of 10 mM dNTP, 1μl of RNasin (40U/ml, final 2U, Promega), 0.5μl SuperScript II (Invitrogen)

Procedures:

1) Dilute RNA template to 250 ng/μl by using DEPC-HO.
2) Add 2μg of RNA template (8μl) with 0.6μl of DEPC-H2O, 2.4μl of5X Superscript buffer (15mM MgCl2) and 1.0μl of RQ1 RNase-free DNase (1U/μl, Promega).
3) Incabate 30min at 37˚C to remove contaminating genomic DNA, then return to ice for 1 min.
4) Add 1μl of oligo-dT(23)V(0. 5μg/μl), incabate at 65˚C for 10min, then return to ice for 1 min.
5) Add 7μl of RT mix to 13μl of DNase-treated RNA.
6) Mix well, and incubate 1 hr at 42˚C, 15min at 72˚C and 4˚C, then store at -20˚C.

Experiment 4: Primers test

According to previous literatures, find out the primers of *CO, GI, FT, FLC, SOC1, RGA, RGL2* and *bZIP16*.

The list of primers as below:

qRT-PCR primer	Sequence (5'→3')	Reference
UBQ10-ABI-1 ABI-Ler-UBQ-2	AGAAGTTCAATGTTTCGTTTCATGTAA GAATGGAAACATAGTTGGAACAATTATTCA	Hsieh et al., 2012
AtbZIP16-F AtbZIP16-R	GCATGGACAATGACCACCAA TCTCTCTGCGGCACCTGTTT	Hsieh et al., 2012
CO-750-ABI-S CO-800-ABI-AS	CATTAACCATAACGCATACATTTCATC TCCGGCACAACACCAGTTT	Wu et al, 2008
FT-254-ABI-S FT-306-ABI-AS	ATCTCCATTGGTTGGTGACTGATA GCCAAAGGTTGTTCCAGTTGTAG	Wu et al, 2008
FLC-396-ABI-S FLC-550-ABI-AS	AGCCAA GAAGACCGAACTCA TTTGTCCAGCAGGTG ACA TC	Baurle and Dean, 2008
SOC1-305-ABI-S SOC1-367-ABI-AS	AACAACTCGAAGCTTCTAAACGTAA CCTCGATTGAGCATGTTCCT	Ebine et al, 2012
RGA-687-ABI-S RGA-972-ABI-AS	AGAAGCAATCCAGCAGA GTGTACTCTCTTCTTACCTTC	Tyler et al, 2004
RGL2-977-ABI-S RGL2-1090-ABI-AS	CGGAGAATTCAGATTCGCTTCAAC CAAGATCCGATAAACTCTCAGCGG	Kang et al, 2011
GI-3513-ABI-S GI-3563-ABI-AS	ACTAGCAGTGGTCGACGGTTTATC GCTGGTAGACGACACTTCAATAGATT	Wu et al, 2008

Materials: Filter tips, MQ, 0.05ng/µl cDNA, 5µM of real-time primer, SYBR Green Master Mix (Applied Biosystem) at 4˚C, Applied Biosystem QuantStudio™ 12K Flex Real-Time PCR System (http://www.appliedbiosystems.com).

Procedures:

1) Using Filter tips to dissolve real-time primer with MQ to 100µM for stock.

2) Dilute the primer with MQ to final 5μM.

3) Primer test:

a) Primer concentration: final 100~300nM

b) Total 27 reactions (25μl per reaction).

c) 5μl of cDNA (0.05ng/μl)+7.5μl primer mixture + 12.5μl SYBR Green Master Mix and 5μl of MQ+7.5μl primer mixture + 12.5μl SYBR Green Master Mix for No Template Control (NTC)

d) The reactions of primer test is as below:

MQ	6.5	5.5	4
Primer-ABI-S	0.5	1.5	3
Primer-ABI-AS	0.5	0.5	0.5
MQ	5.5	4.5	3
Primer-ABI-S	0.5	1.5	3
Primer-ABI-AS	1.5	1.5	1.5
MQ	4	3	1.5
Primer-ABI-S	0.5	1.5	3
Primer-ABI-AS	3	3	3

(9*3 repeats=27)

Experiment 5: Real-Time PCR

1) Take 5μl cDNA (0.05ng/μl) of the different samples which were collected at ZT 3, 6, 9, 12, 15hr after light on each line and add 20μl of primer mixture (7.5μl individual primer mixture and 12.5 μl SYBR Green Master Mix), respectively.

2) NTC: 5μl of MQ + 20μl primer mixture.

3) qRT-PCR programs: 50˚C for 2 min; 95˚C for 10 min; 40 cycles of 95˚C for15 sec and 60˚C for 1 min.

Primer mixtures of *UBQ10, CO, GI, FT, FLC, SOC1, RGA, RGL2* and *bZIP16* as below :

Primer mixtures of *GI*, and *UBQ10*:

	One reaction	70 reactions
MQ	1.5µl	105µl
Primer-ABI-S	3µl	210µl
Primer-ABI-AS	3µl	210µl
SYBR Green	12.5µl	875µl
cDNA (0.05ng/µl) final 0.25 ng/µl	5µl	
Total	25µl	

Primer mixtures of *bZIP16, RGA* and *SOC1*:

	One reaction	70 reactions
MQ	4.5µl	315µl
Primer-ABI-S	1.5µl	105µl
Primer-ABI-AS	1.5µl	105µl
SYBR Green	12.5µl	875µl
cDNA (0.05ng/µl) final 0.25 ng/µl	5µl	
Total	25µl	

Primer mixtures of *FLC* and *RGL2*：

	One reaction	70 reactions
MQ	6.5µl	455µl
Primer-ABI-S	0.5µl	35µl
Primer-ABI-AS	0.5µl	35µl
SYBR Green	12.5µl	875µl
cDNA (0.05ng/µl) final 0.25 ng/µl	5µl	
Total	25µl	

Primer mixtures of *CO*:

	One reaction	70 reactions
MQ	1µl	70µl
Primer-ABI-S	4.5µl	315µl
Primer-ABI-AS	1.5µl	105µl
SYBR Green	12.5µl	875µl
cDNA (0.05ng/µl) final 0.25 ng/µl	5µl	
Total	25µl	

Primer mixtures of *FT*:

	One reaction	70 reactions
MQ	3µl	210µl
Primer-ABI-S	3µl	210µl
Primer-ABI-AS	1.5µl	105µl
SYBR Green	12.5µl	875µl
cDNA (0.05ng/µl) final 0.25 ng/µl	5µl	
Total	25µl	

5. Performing chromatin immunoprecipitation quantitative PCR assay

Experiment 1: Planting Ler and bZIP16 overexpression line and sampling

Procedures:

1) Take seeds of Ler and *bZIP16ox* (*Ler/35S-bZIP16-GFP*)
2) Plant 360 seeds per line to soil at 4˚C cool room at dark for 3 days to synchronize the germination.
3) Transfer to SD growth chamber (8 hours light/16 hours dark) at 22 ˚C for 18 days and then collect samples at ZT 12hr

Experiment 2. Fixation

Fresh prepare Cross-linking buffer

	Stock	Volume of 150 ml	Final
Cross-linking	2M Sucrose (5x) 1M Tris-HCL pH8.0 (100x) 37% Formaldehyde (37x) MQ	30 ml 1.5 ml 4.05 ml 114.45 ml	0.4 M 10 mM 1%
Stop solution	2M Glycine		125 mM

1) Collect 0.2g tissue would use for ChIP.
2) Rinse the tissue with 50 ml water to remove soil
3) Add 37 ml cross-linking buffer and place tissue under vacuum for 20 min (5min x 4).
4) Add 2.4 ml 2M (final 0.125M) glycine and place tissue under vacuum for 5 min.
5) Rinse tissue two times with 50 ml water to remove formaldehyde. Remove as much water as possible after second wash by kitchen paper.
6) In this step, plant materials can be shock-frozen in liquid nitrogen. Grind tissue to a fine power and store in -80 ˚C

Experiment 3. Extraction and sonication (all steps are on ice)

Prepare Nuclei isolation buffer, Nuclei lysis buffer, Miracloth, ice, Centrifuge 4℃

Buffer	Reagent	Final	Stock	x	50 ml
Nuclei Isolation buffer	PIPES (pH 6.8)	15 mM	150mM	10 x	5 ml
	MgCl2	5 mM	1 M	200 x	250µl
	KCL	60 mM	200 mM	3.3 x	15ml
	Sucrose	0.25 M	2 M	8 x	6.24ml
	NaCl2	15 mM	3 M	200 x	250µl
	CaC2	1 mM	1 M	1000 x	50µl
	TritonX-100	0.9%	20%	22.2 x	2.27ml
	PMSF	0.1mM	100mM	1000 x	50µl
	Protease inhibiter (Roche)	1 X	50x	50x	1 bullet
	MQ				23.9ml
Nuclei lysis buffer	HEPES pH7.5	50mM	1 M	20x	300µl
	Sodium	0.1%	10%	100 x	60µl
	deoxycholate(DOC)	0.5%	10%	20 x	300µl
	SDS	150 mM	3 M	200 x	30µl
	NaCl	1%	20%	20 x	300µl
	TritonX-100	0.1mM	100 mM	1000 x	6µl
	PMSF	1x	50 x	50 x	120µl
	Roche PI				to 6ml
	MQ				

1) Add 500 µl of cold nuclei lysis buffer into samples.
2) Divid the samples into two aliquots of 330 µl each in tubes. Filter the solution through two layers of Miracloth into fresh 50 ml falcon tube on ice.
   - Set aside 10µl from each sample for the control of sonicated chromatin.
3) Shear the DNA into 500～1000 bp fragments by Bioruptor (power high, 15s ON, 15s OFF). Add ice very 5 min, total sonication time is 30 min
4) Check the fragment by electrophoresis - 10µl sample + 10µl MQ for loading
5) Centrifuge samples for 10 min at 13000 rpm at 4℃ to pellet debris.
6) Collect samples by combining the supernatants. (sonicated chromatin)

Experiment 4. Immunoprecipitation

Prepare pre-equilibrated salmon sperm DNA/protein A agarose beads

a) 200μl protein A sepharose 50% slurry (GE71-5017-54 AD) in the spin column, centrifuge at 750g for several seconds.

b) Wash with protein A sepharose with 200μl lysis buffer for 3 times and centrifuge at 750g to discard supernatant.

c) Add 100μl lysis buffer and mix with 0.2mg sonicated salmon sperm DNA and 0.2 mg BSA.

d) Pre-incubate mixture at 4℃ with rotation for 4 hrs, and store at 4℃ before using.

Procedures

1) Wash prepared salmon serm/protein A-sepharose beads with 800μl lysis buffer lysis buffer for 3 times and centrifuge at 750g to discard supernatant. Resuspend resin with lysis buffer (=bed volume)
2) Add 60 μl washed salmon sperm/protein A-sephaarose bead to 300μl sonicated chromatin for 1 h at 4℃with gentle rotation.
   - Take 30μl (1/10 volume) of supernatants as DNA input control
   - Take 20μl as protein input control.
3) Add 1μl anti-GFP antibody (Ab290, rabbit polyclone) and incubated 3hr at 4℃with gentle rotation.
4) Centrifuge samples at 750g for 2 min at 4℃.
   - Collect flow through 20μl for SDS-PAGE and western to check the binding efficiency

5) Wash

	Reagent	Final	Stock	x	V of 10ml	Wash volume
Nuceli lysis buffer	Same as the buffer for experiment3					800µl x3 times
LNDET	LiCl NP40 DOC EDTA MQ	0.25 M 1% 1% 1 mM	10 M 100% 10% 0.5M	40 x 100 x 10 x 500 x	250µl 100µl 1 ml 20µl 9630µl	800µl x3 times
TE	Tris pH8.0 EDTA MQ	10 mM 0.1M	1 M 500 mM	100 x 500 x	100µl 20µl 9880µl	800µl x3 times

6) Take samples of every two washes for western blot to check the quality of samples.

7) Elute the immuno-complex by adding 60µl of freshly prepared elution buffer by incubating at room temperature for 5 min.

	Reagent	Final	Stock	x	V of 1 ml	Wash volume
Elution buffer	SDS NaHCO3 MQ	1% 0.1 M	10% 0.5 M	10 x 5 x	100µl 200µl 700µl	60µl x 2 times

8) Centrifuge samples at 750g for 2 min at room temperature and transfer the supernatant into a new tube. Repeat the elution step 9 again.

9) Combine two elutions

- Take 1/10 volume of elution for western to check IP

10) Add 1% SDS and 0.1M $NaHCO_3$ into 80µl DNA input control to adjust the same concentration with elutions.

11) Add 1.5µl proteinase K (Invitrogen, 20 mg/ml) into 120µl elutions and input solution(final concentration 0.25 mg/ml), incubate overnight at 65℃).

12) Extract the DNA by using the PCR purification kit (QIAGEN). Elute in 120 and 60μl MQ.

13) Measure the DNA content by Nanodrop.

Primers of ChIP:

qRT-PCR primers	Sequence (5'→3')
pFLC-ABI-a-Fw	AAGTAGCAAAGACGCTCGTCA
pFLC-ABI-a-Rv	GGTAAACGAGAGTGATGCAAA
FLC-ABI-b-Fw	TGTCATTTTCAATCTGCCGA
FLC-ABI-b-Rv	CCAATGAATTATGTGGGCTAAC
UBC21-ABI-c-Fw	TCCTCTTAACTGCGACTCAGG
UBC21-ABI-c-Rv	GCGAGGCGTGTATACATTTG

III. Results

1. bZIP16 protein accumulates at higher expression levels in buds and flowers.

To examine bZIP16 protein level in different growth developmental stages of Arabidopsis, we first performed the western blot to measure the bZIP16 protein expression in different tissues. We found that bZIP16 protein expressed in roots, stems, seedlings, buds, flowers and siliques. But bZIP16 protein was not detected in dry seed and 1-day-old seedlings at 22℃ under light conditions. And the results showed higher protein levels in buds and flowers (Fig. 1-1~1-3). In addition, bZIP16 protein was detected more in 4-day-old seedling under dark conditions than in 4-day-old seedling under light conditions. The high levels of bZIP16 protein in flower buds and flowers (Fig. 1-1~1-3) suggest that bZIP16 may be important for floral development.

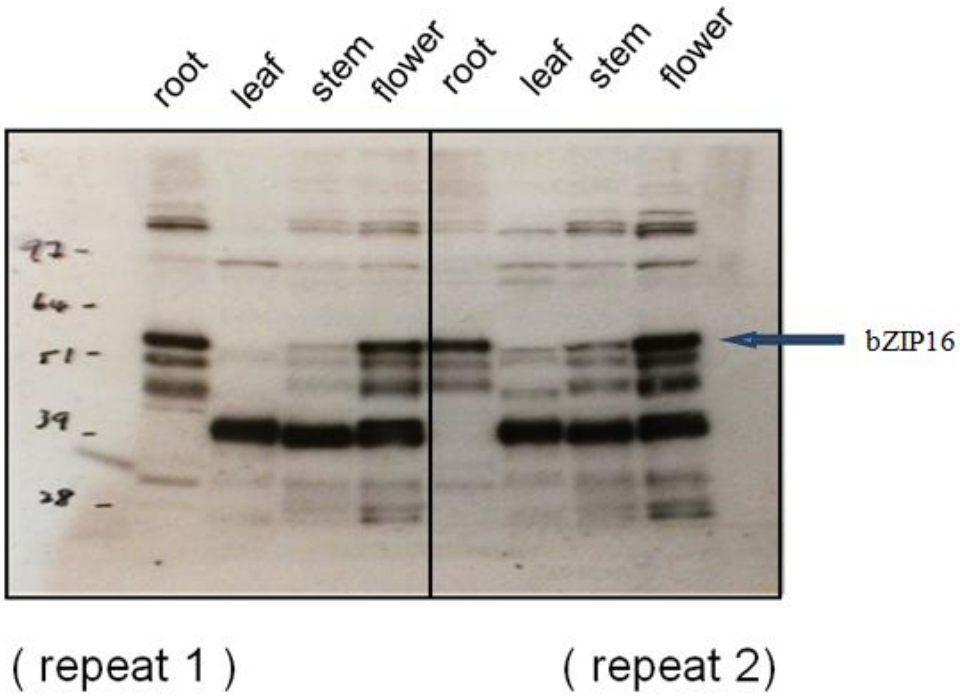

Fig. 1-1 bZIP16 protein higher accumulation in roots and flowers.
Amount of bZIP16 protein loaded was as marked. The molecular weight of bZIP16 protein is about 51-55 KDa

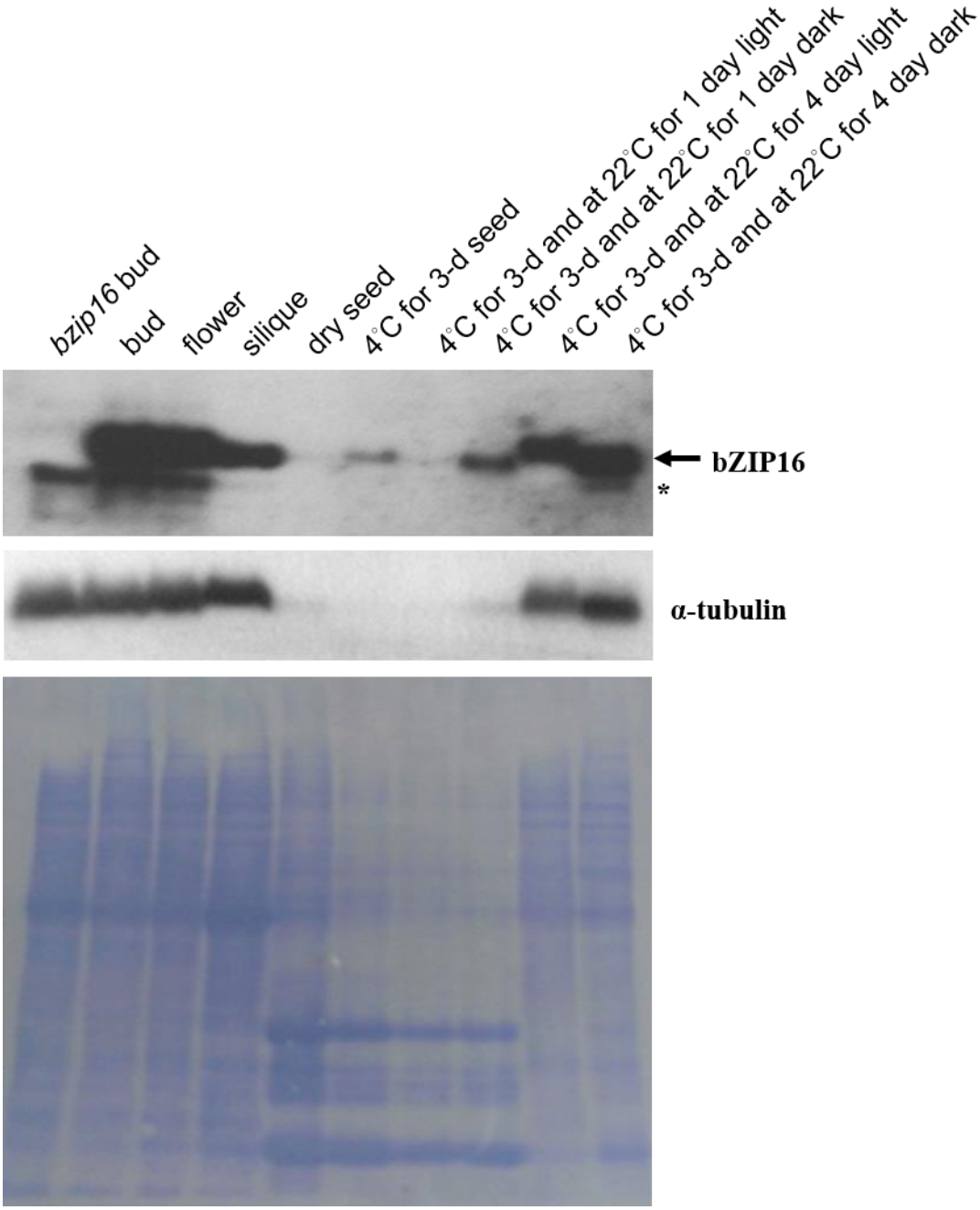

Fig.1-2 bZIP16 protein higher accumulation in buds, flowers, and 4- day-old seedling under dark condition.

The tissues and developmental stages tested were, from left to right in the graph, buds of *bzip16* mutant, buds, flowers, siliques, dry seeds, 4℃-treated-3d seeds, 4℃-treated-3d and constant light-treated-1d, 4℃-treated-3d and constant dark-treated-1d, 4℃-treated-3d and constant light-treated-4d, and constant dark-treated-4d of wild-type. α-tubulin was also detected in these samples to show equal loading of protein from each sample (exposure for 3 min). The asterisk indicated the non-specific bands. The coomassie blue stained gel was included as loading controls.

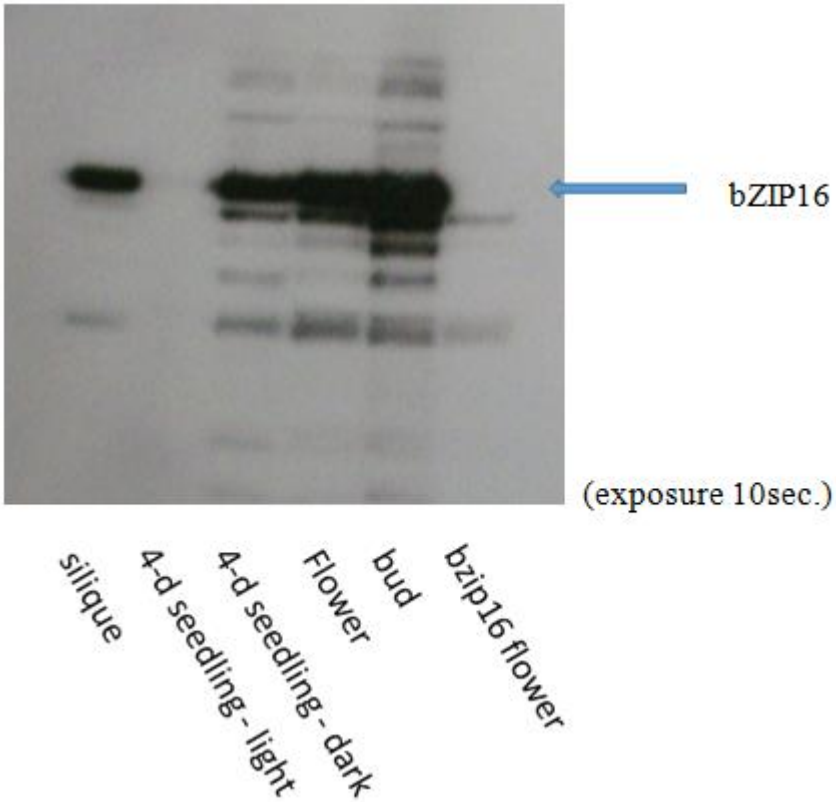

Fig. 1-3 bZIP16 protein higher accumulation in siliques, flowers, buds and 4-day-old seedling under dark condition.

The tissues and developmental stages tested were, from left to right in the graph, siliques, 4℃-treated-3d and constant light-treated-4d seedlings, 4℃-treated-3d and constant dark-treated-4d seedlings, flowers, buds and flowers of the *bzip16* mutants

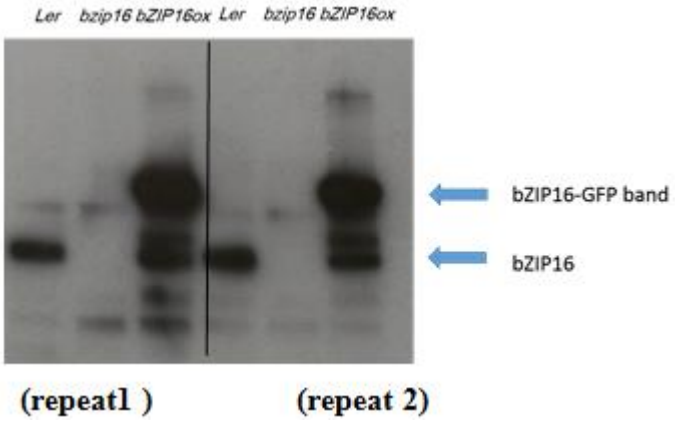

Fig. 1-4 The seeds of Ler, *bzip16* and *bZIP16ox* are correct.

Twelve-day-old wild-type, *bzip16-2* and *bZIP16ox* line grown under short-day conditions (8-h light/16-h dark) were harvested.

2. *bzip16-2* mutants showed late-flowering phenotypes under long-day and short-day conditions.

To investigate the other roles of the *bZIP1*6 in Arabidopsis, in this study, Ler, bZIP16 knock-out line (*bzip16-2*), and *AtbZIP16-GFP* which was driven the 35S promoter of *bZIP16* overexpression line (*bZIP16ox*), were planted to observe the phenotypes. The results revealed that under SD conditions, the plant size of *bzip16-2* mutants was shorter than wild-type plants.(Fig.2-3)

To further evaluate the contribution of bZIP16 in flowering pathways, we calculated the rosette leaf number at bolting. The number of rosette leaves will not increase after bolting; thus it can represent the physiological age at flowering. Under long-day (LD) conditions, in the first experiment, the *bzip16* mutants and wild-type plants began flowering with an average of 5 and 4 total rosette leaves, respectively (Fig.2-2A). In the second experiment, the *bzip16* mutants and wild-type plants began flowering with an average of 5.5 and 4.5 total rosette leaves, respectively. *bZIP16* overexpression lines had 4.16 leaves at bolting, indicating that *bZIP16* promoted flowering (Fig.2-2B). Under short-day (SD) conditions, in the first experiment, the bzip16 mutants forming 27.91 leaves compared to 23.17 leaves for wild-type (Fig. 2-4A). In the second experiment, the *bzip16* mutants also flowered late comparing with wild-type plants. *bzip16* mutants forming 31.36 leaves compared to 23.55 leaves for wild-type (Fig. 2-4B). These results demonstrated that bzip16 mutants showed late-flowering under both LD and SD conditions.

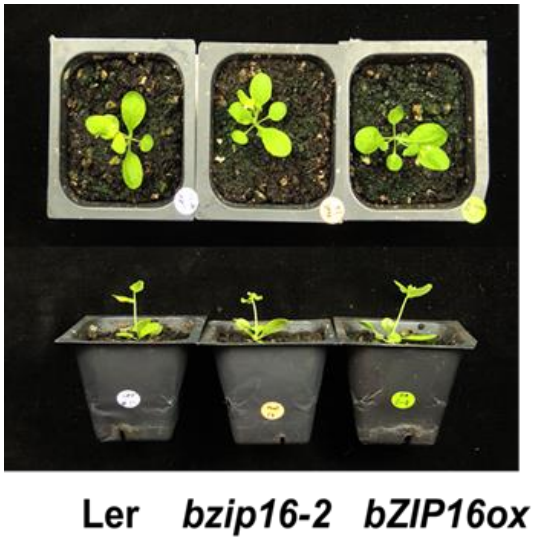

Fig. 2-1 *bzip16-2* mutants showed more rosette leaves

Seeds for each line were surface-sterilized, imbibed for 1 hour at 22℃ and then planted in soil at 4℃ for 3 days. Afterwards, plants were transferred and grown at 22℃ under long-day (16-h light/8-h dark) conditions at cool white fluorescent lights (70 to 100 μmol $m^{-2}sec^{-1}$)

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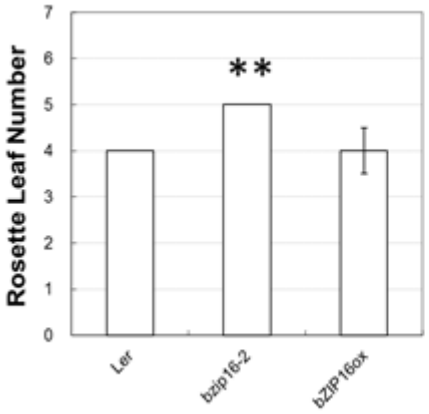

Fig.2-2A

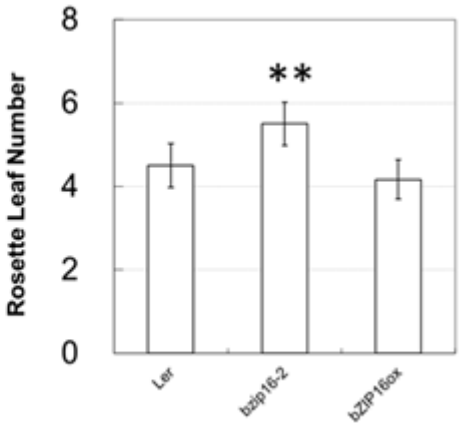

Fig. 2-2B

Fig.2-2 *bzip16-2* mutant plants showed late-flowering under long-day conditions

Flowering time indicated by numbers of rosette leaves at bolting for wild-type, *bzip16-2* and *bZIP16* overexpression line under LD (16- h light/8- h dark) conditions. Error bars indicate ± SD. Asterisks indicate that *bzip16-2* mutant plants flowered significantly later than wild-type plants (Student's t test; *:P<0.05, **:P<0.001, n ≥ 10)..

Fig. 2-3 *bzip16-2* mutants showed more leaf numbers.

Plants were grown under SD (8-h light/16-h dark) conditions at cool white fluorescent lights 80~110μmol $m^{-2}sec^{-1}$).

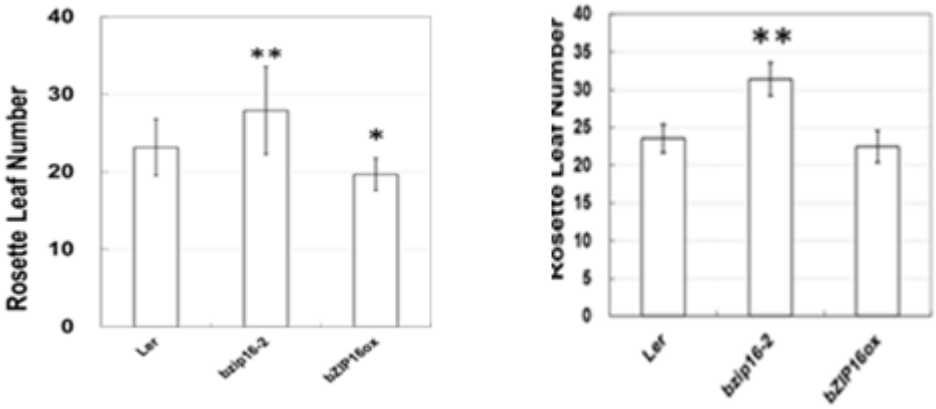

Fig.2-4A Fig. 2-4B

Fig.2-4 *bzip16-2* mutant plants showed late-flowering under short-day conditions

Flowering time indicated by numbers of rosette leaves at bolting for wild-type, *bzip16-2* and *bZIP16* overexpression line under SD (8- h light/16- h dark) conditions. Error bars indicate ± SD. Asterisks indicate that *bzip16-2* mutant plants flowered significantly later than wild-type plants (Student's t test; *:P<0.05, **:P<0.001, n ≥ 10).

3. bZIP16 Repressed Significantly the Expression of *FLC.*

To clarify how the floral transition is regulated by bZIP16, we performed a transcriptomic comparison of 18-d-old wild-type plants and *bzip16* mutants grown under SD conditions using Affymetrix ATH1 GeneChip. 43 flowering-related genes

were retrieved for hierarchical clustering analysis. There are four representative expression clusters. Cluster I is the autonomous pathway genes *MULTICOPY SUPPRESSOR OF IRA1 4 (FVE), FLOWERING LOCUS D (FLD), FLOWERING LOCUS KH DOMAIN (FLK), LUMINIDEPENDENS (LD), FCA, FY* and the floral integrators *SOC1, FLC*. Cluster II is the GA pathway genes *REPRESSOR OF GA* (*RGA), GIBBERELLIC ACID INSENSITIVE (GAI), SUPPRESSOR OF PHYA-105 (SPY), RGA-LIKE 1 (RGL1), RGA-LIKE2 (RGL2)* and *RGA-LIKE3* (*RGL3*). Cluster III is the photoperiodic pathway genes *CIRCADIAN CLOCK ASSOCIATED1 (CCA1), LATE ELONGATED HYPOCOTYL (LHY), EARLY FLOWERING4 (ELF4), LOV KELCH PROTEIN2 (LKP2), EARLY FLOWERING3 (ELF3), GIGANTEA (GI), FLAVIN-BIDING, KELCH REPEAT, F-BOX1 (FKF1), PSEUDO-RESPONSE REGULATOR3 (PRR3), PRR5, PRR7, PRR9, ZEITLUPE (ZTL), TIMING OF CAB EXPRESSION1 (TOC1), CYCLING DOF FACTOR1 (CDF1), CONSTANS (CO), ARRHYTHMO/PHYTOCLOCK1 (LUX), LIGHT-REGULATED WD1 (LWD1), LIGHT-REGULATED WD2 (LWD2), (*CCA1 HIKING EXPEDITION, *TCP DOMAIN PROTEIN 21) CHE* and the floral integrators *FLOWERING LOCUS T (FT)*. Cluster IV is the vernalization pathway genes *VIRE2-INTERACTING PROTEIN 1* (*VIP1), VIRE2 INTERACTING PROTEIN 2 (VIP2), VERNALIZATION INDEPENDENCE 3 (VIP3), VERNALIZATION INDEPENDENCE 4 (VIP4), VERNALIZATION INDEPENDENCE 5 (VIP5), PHOTOPERIOD-INDEPENDENT EARLY FLOWERING 1 (PIE1), FRIGIDA (FRI), REDUCED VERNALIZATION RESPONSE 1 (VRN1),* and *REDUCED VERNALIZATION RESPONSE 2 (VRN2)* (Fig.3).

Transcriptome analysis revealed the impact of the mutation in *bZIP16* was significant on the expression of *FLC*. The expression of *FLC* transcripts was reduced in wide-type plants. In contrast, the expression of those genes involved in photoperiodic

pathway, GA pathway, vernalization pathway and autonomous pathways almost were unaffected in the *bzip16* mutant (Fig.3).

We have also checked the expression of the 24,000 genes represented on the ATH1 GeneChip. By applying a 2-fold expression changes between wild-type plants and the *bzip16* mutants, we found that 11 genes were up-regulated and 19 genes were down-regulated by bZIP16. Among them, only *FLC* is previously reported to be a flowering gene, while most of the other genes encoding unknown proteins. (Table1, 2). Possible functions of these bZIP16-regulated genes remain to be clarified.

These results imply that *bZIP16* represses the expression of *FLC* and it does not affect the expression of genes in photoperiodic pathway, GA pathway, vernalization pathway and autonomous pathways, suggesting *bZIP16* may be a new component of flowering control in Arabidopsis.

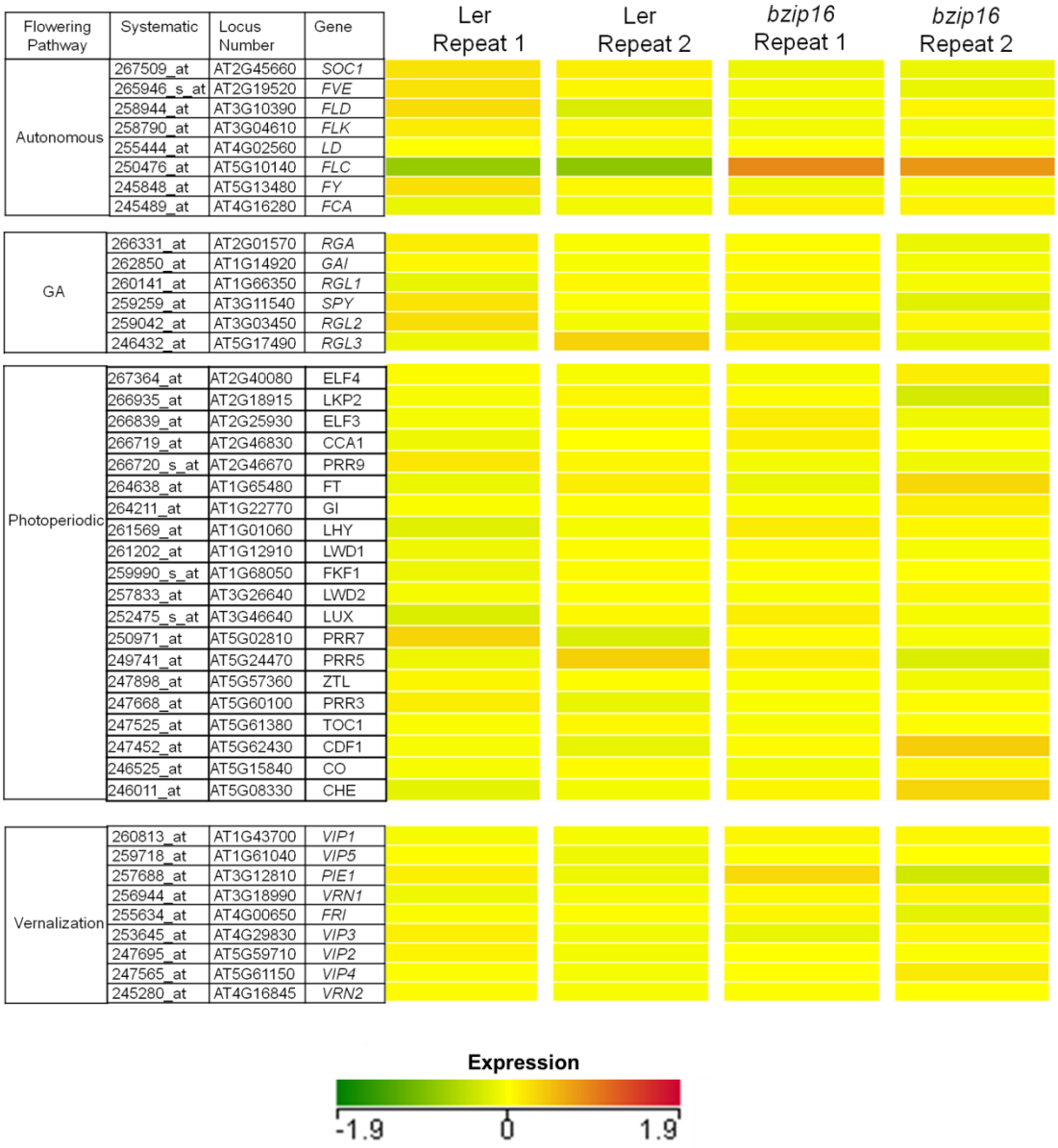

Fig.3 The expression of FLC transcripts was significantly increased in the *bzip16* mutant

Transcriptomic comparison was performed to analyze genes in the four flowering pathways in 18-day-old at Zeitgber time (ZT) 12 hr of wild-type plants and *bzip16-2* mutants. Hierarchical clustering of bZIP16-regulated genes differentially expressed in Ler and *bzip16-2*. Expression data are shown as relative expression of each gene to the median of 4 samples. The median expression of each gene is shown in yellow. Relative up- or downregulation from median expression is red and green respectively. Two biological replicates were performed for each treatment.

Table 1. The up-regulated genes by bZIP16

Probe Set ID	Ler R1	Ler R2	*bzip16-2* R1	*bzip16-2* R2	Expression fold	Unigene (Avadis)	Gene Symbol	AGI	Target Description
266634_at	1.04	1.17	-1.28	-1.04	4.8	At.37663		AT2G35530	putative G-box binding bZIP transcription factor
262634_at	2.24	2.48	-2.29	-2.24	23.0	At.26590		AT1G06690	unknown protein
262636_at	0.71	0.79	-0.71	-0.96	3.0	At.20810	NIH	AT1G06670	DEIH-box RNA/DNA helicase
260835_at	2.35	2.42	-2.42	-2.35	27.2	At.12093		AT1G06700	protein kinase interactor
260412_at	1.35	0.99	-1.99	-0.99	6.3	At.24555	AMY3	AT1G69830	putative alpha-amylase
257076_at	1.13	0.65	-0.69	-0.65	2.9	At.8144		AT3G19680	unknown protein
256245_at	1.4	0.65	-0.82	-0.65	3.4	At.47608	HSP70	AT3G12580	heat shock protein 70
255064_at	0.74	0.6	-0.6	-0.74	2.5	At.22399	EXO	AT4G08950	putative phi-1-like phosphate-induced protein
254452_at	2.49	2.15	-2.15	-2.15	22.1	At.32663	DDB1B	AT4G21100	UV-damaged DNA-binding protein-like damage-specific DNA binding protein 1
253942_at	0.59	0.49	-0.49	-0.59	2.1	At.54525		AT4G27010	putative protein
250304_at	0.92	0.89	-0.89	-0.93	3.5	At.699		AT5G12110	elongation factor 1B alpha-subunit

Table 2. The down-regulated genes by bZIP16.Among them, only *FLC* is a flowering gene.

Probe Set ID	Ler R1	Ler R2	*bzip16-2* R1	*bzip16-2* R2	expression fold	Unigene (Avadis)	Gene Symbol	AGI	Target Description
266693_at	-0.67	-0.58	0.58	0.67	2.4	At.12894	MIOX2	AT2G19800	unknown protein
264824_at	-0.90	-0.80	0.80	0.80	3.1	At.69970	2A6		unknown protein
264826_at	-0.61	-0.68	0.61	0.77	2.5	At.19920	2A6	AT1G03410	putative 1-aminocyclopropane-1-carboxylate oxidase
263268_at	-0.93	-1.13	0.94	0.93	3.9				unknown protein
262010_at	-0.97	-1.32	0.97	1.10	4.5	At.47239			hypothetical protein
262019_s_at	-1.11	-0.80	0.80	0.82	3.4				hypothetical protein
260011_at	-0.60	-0.70	0.60	0.84	2.6	At.19366		AT1G68110	hypothetical protein
259620_s_at	-1.26	-1.43	1.30	1.26	6.2				Tam3-like transposon protein
257306_at	-0.84	-0.79	0.79	1.15	3.4	At.53591		AT3G30200	hypothetical protein
256940_at	-2.85	-2.85	2.85	2.85	52.0	At.36724	QQS	AT3G30720	unknown protein
256300_at	-0.83	-0.43	0.43	0.68	2.3	At.24919	NAP	AT1G69490	unknown protein
256166_at	-1.03	-1.01	1.08	1.01	4.2	At.49945		AT1G36920	hypothetical protein
255414_at	-2.01	-1.95	1.95	2.10	16.0	At.54106		AT4G03156	hypothetical protein
254343_at	-0.88	-0.55	0.55	0.80	2.6	At.2106	APR3	AT4G21990	PRH26 protein

250476_at	-0.79	-0.85	0.89	0.79	3.2	At.75671	FLC	AT5G10140	MADS box protein FLOWERING LOCUS C
248969_at	-0.61	-0.55	0.60	0.55	2.2	At.27375		AT5G45310	unknown protein
248676_at	-0.66	-0.60	0.60	0.97	2.7	At.29820	ATSDI1	AT5G48850	similar to unknown protein
245032_at	-0.80	-0.74	0.74	0.92	3.0				En/Spm-like transposon protein

4. *FLC* is highly induced and *FT, SOC1* are repressed in *bzip16* mutants *under short-day conditions*

Floral regulatory signals generated in the photoperiod, *FLC*-dependent, and GA-dependent pathways are integrated by a group of genes called floral integrators. *FLOWERING LOCUS T (FT)*, *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS1 (SOC1)*, and *LEAFY (LFY)* have been identified as floral integrators. *FT* and *SOC1* integrate floral regulatory signals generated in the photoperiod and *FLC*-dependent pathways. *SOC1* is also involved in the integration of GA-dependent floral promotion signals (Moon et al., 2003). *LFY* integrates photoperiodic and GA-dependent signals through discrete cis elements in the promoter (Blázquez and Weigel, 2000) And *RGA*, *RGA-LIKE2 (RGL2)* are members of DELLA proteins which repress GAs in GA pathway.

To elucidate the role of *bZIP16* in the flowering pathways, we employed the quantitative real-time PCR (qRT-PCR) technique using gene-specific primers to quantify transcript levels of the major flowering-time regulators *CONSTANS (CO)*, *GIGANTEA (GI)*, *RGA*, *RGL2*, *FT*, *SOC1*, *LFY* and *FLC* genes in 18-day-old plants of wild-type plants, *bzip16* mutants and *bZIP16* overexpression lines under SD conditions. According to web-based tool, DIURNAL, we found that time points of the high expressions of the mentioned genes above is between 4 to 16 hour after light on. Therefore, we collected the samples of each line at ZT 3, 6, 9, 12 and 15hr after light on, respectively

The *bZIP16* transcripts were not detected in *bzip16* mutants. By contrast, the level of *bZIP16* transcripts in *bZIP16* overexpression lines was significantly higher than in wild type plants. These data demonstrated that all lines in this study were correct (Fig. 4-1). The *bzip16* mutants showed the high level of *FLC* transcripts, low level of *SOC1* and *FT* transcripts. In addition, in *bZIP16* overexpression line, the level of *FLC* transcripts was reduced and levels of *SOC1* and *FT* transcript were increased. These data are consistent with the late-flowering phenotype of *bzip16* mutants (Fig. 4-2~4-4). Furthermore, there were minor different expressions of *RGL2* and *RGA* in the *bzip16* mutants, demonstrating that bZIP16 didn't have an effect on the expression of *RGL2* and *RGA* in GA pathway (Fig. 3-5, 3-6). The transcript levels of *CO* and *GI* in all lines were similar, indicating that *bZIP16* didn't regulate flowering by photoperiodic pathway (Fig. 4-7, 4-8). The *LFY* expression was too low to be detected (data not shown). We have performed two independent experiments and the results are similar.

In flowering pathways, *FLC* is in upstream negative regulator of *SOC1* and *FT*. Our studies demonstrated that *bZIP16* repressed the expression of *FLC* and promoted the expression of *SOC1* and *FT* to regulate flowering positively.

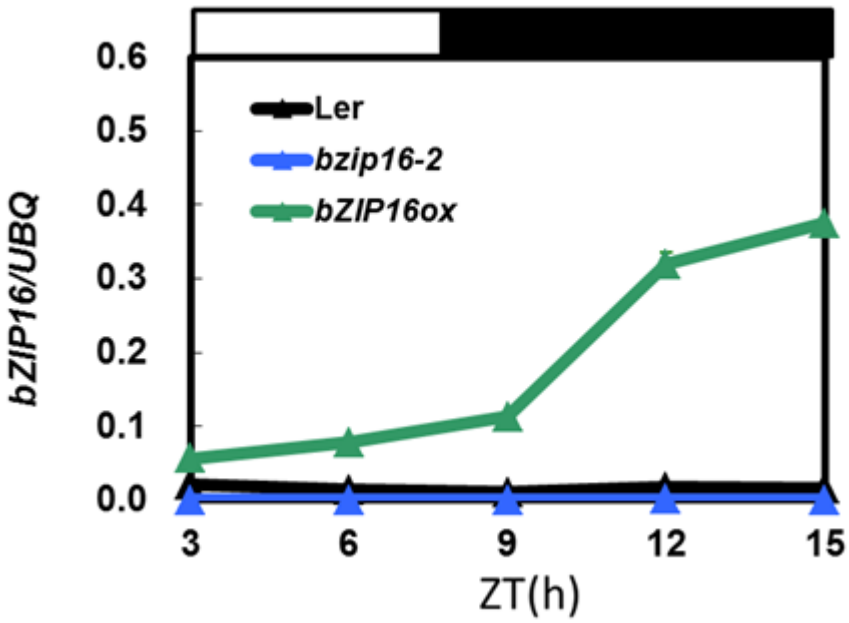

Fig4-1 The transcripts of *bZIP16* in different plants.

Wild-type (black), *bzip16-2* mutant (blue), and *bZIP16ox* overexpression (green). Eighteen-day-old wild-type, *bzip16-2* and *bZIP16ox* line grown under short- day conditions (8-h light/16-h dark) were harvested at zeitgber time (ZT) 3hr, 6hr, 9hr, 12hr and 15hr after light on for total RNA isolation. White bar denotes the light intervals, and black bar denotes darkness. qRT-PCR was used to monitor the expression of genes and *UBQ10* specific primers used as a control for input RNA in the RT reaction. All values in (Fig.4-1~ 4-8) are means (±S.E.) from three technique repeats.

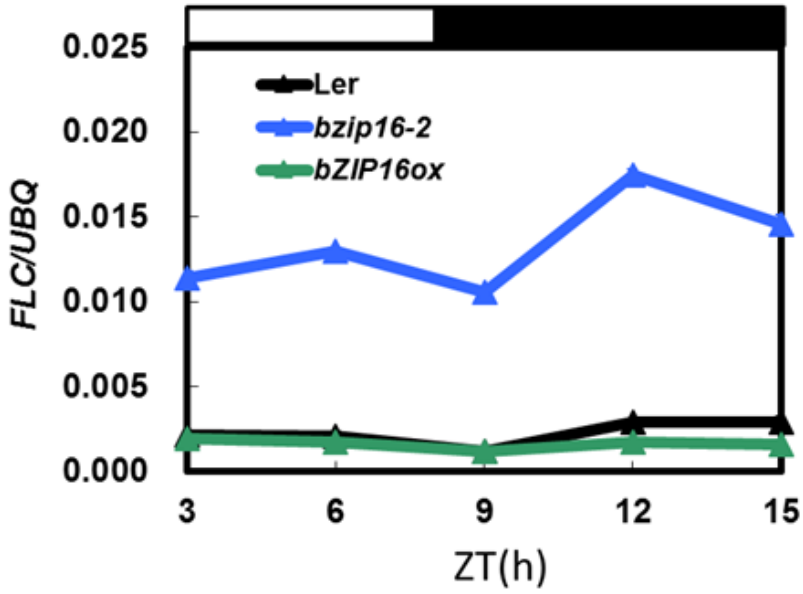

Fig.4-2 The level of *FLC* transcripts was significantly high in *bzip16-2* mutant.

Two independent experiments were performed and showed similar results.

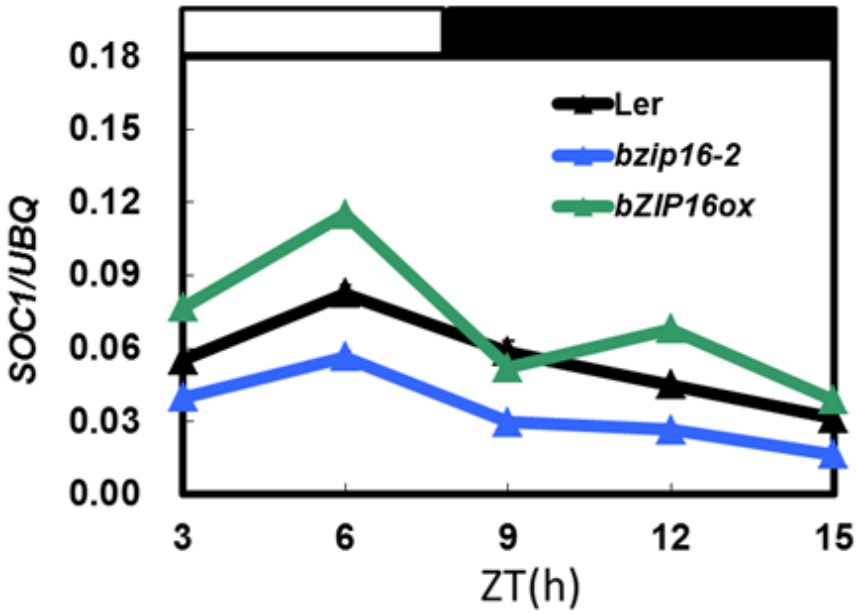

Fig.4-3 The level of *SOC1* transcripts was low in *bzip16-2* comparing with the levels of wild-type and *bZIP16* overexpression line.

Two independent experiments were performed and showed similar results.

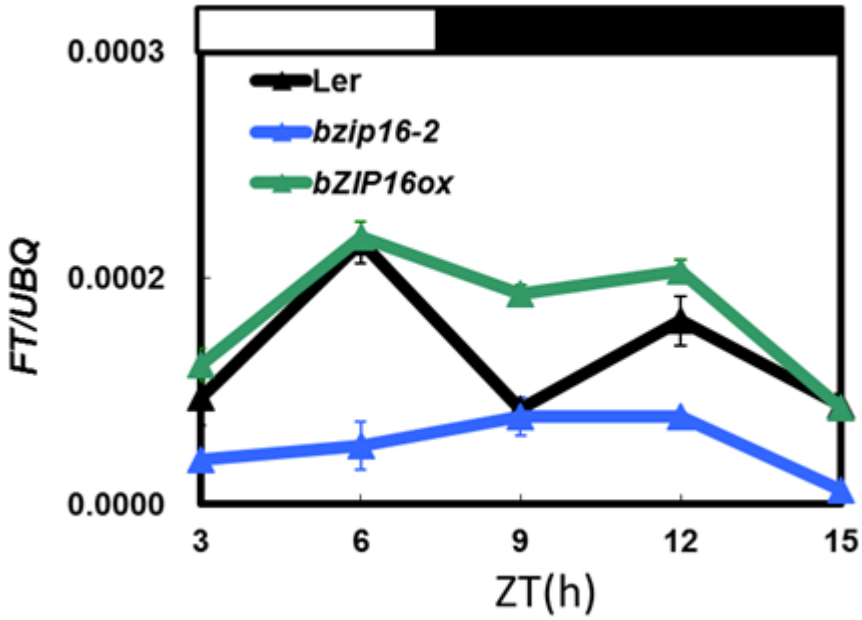

Fig.4-4 The level of *FT* transcripts was low in *bzip16*-2 mutant comparing with the levels of wild-type and *bZIP16* overexpression line.

Two independent experiments were performed and showed similar results.

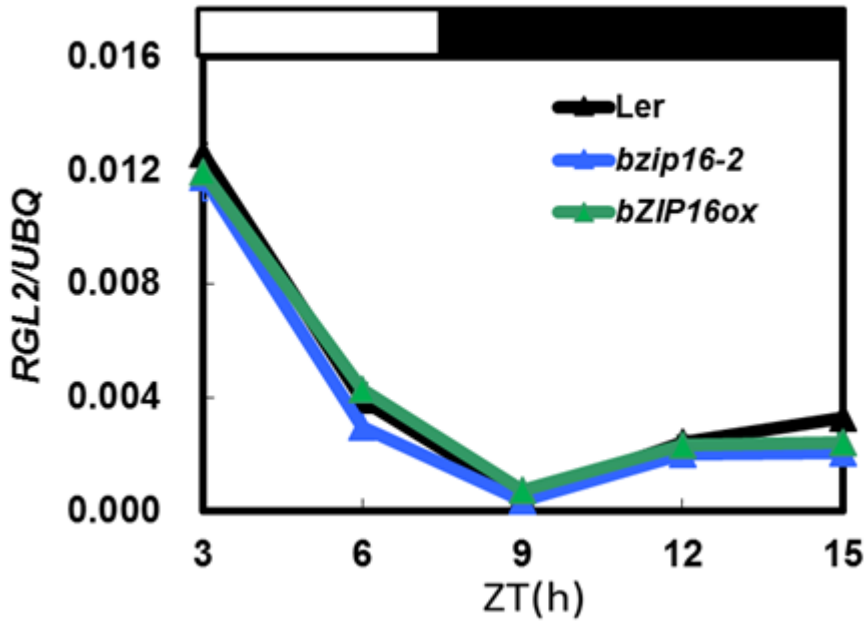

Fig.4-5 Except for *bzip16-2/bZIP16* at ZT3, the levels of *RGL2* transcripts were comparable in all lines among time points examined.

Two independent experiments were performed and showed similar results.

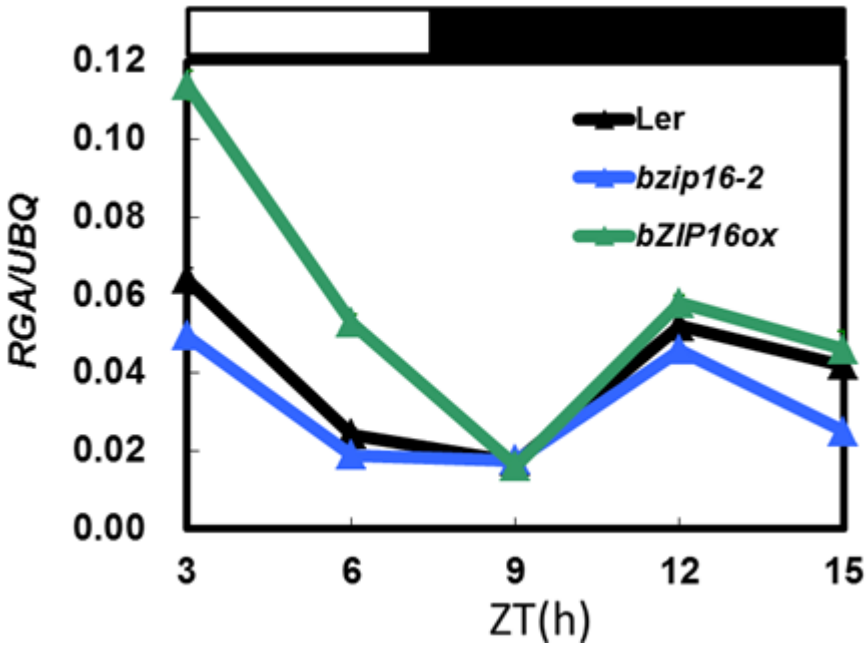

Fig.4-6 The level of *RGA* transcript was reduced slightly in *bzip16*-2 mutants.

Two independent experiments were performed and showed similar results.

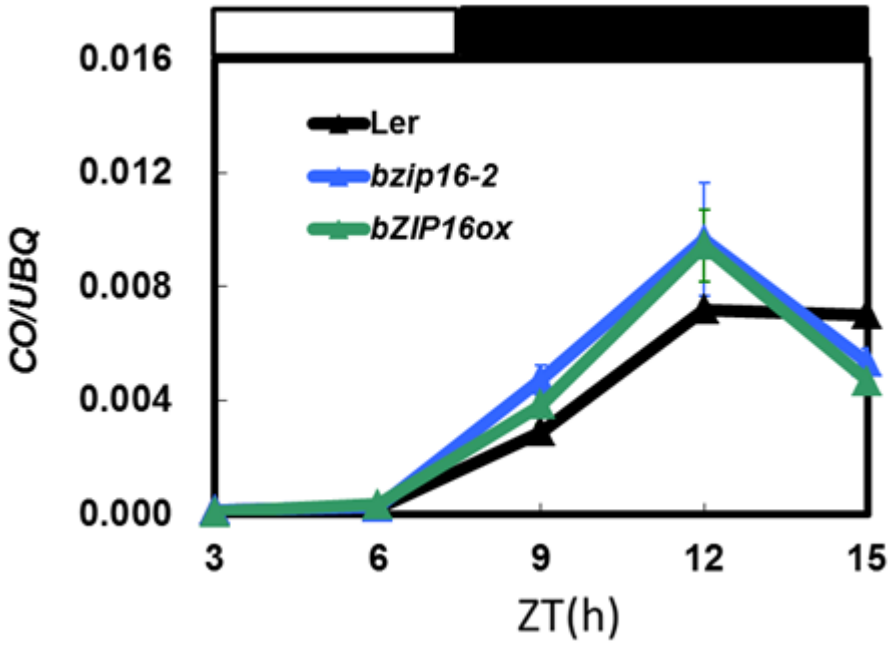

Fig.4-7 The transcript levels of *CO* in all lines were similar.

Two independent experiments were performed and showed similar results.

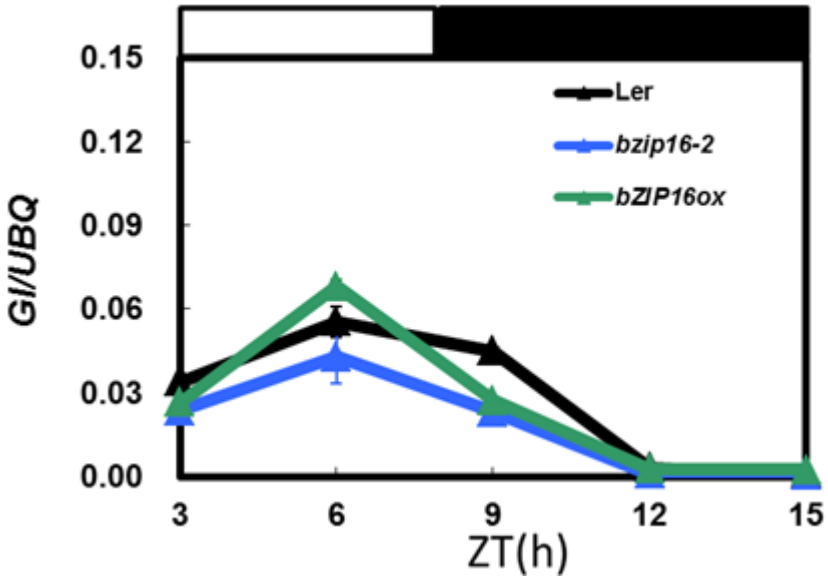

Fig.4-8 The transcript levels of *GI* in all lines were similar.

Two independent experiments were performed and showed similar results.

5. *bZIP16* directly binds to the *FLC* promoter harboring G-box motif.

To examine whether bZIP16 directly binds to the *FLC* promoter, we performed chromatin immunoprecipitation quantitative PCR (ChIP-qPCR) assay of 18-day-old *bZIP16* overexpression line (*Ler/35S-bZIP16-GFP*) with wild-type plants used as a control. In ChIP assays, cross-linked chromatin was extracted and sheared by sonication to short fragments. And we used anti-GFP to immunoprecipitate the bZIP16-GFP protein. bZIP16 was immunoprecipitated along with cross-linked DNA. According to the research of Dr. Hsieh, bZIP16 has G-box-specific binding activity (Hsieh et al., 2012). Hence, we analyzed the *FLC* promoter region and found a putative G-box motif at -255bp from *FLC* translation start site.We used specific primer pairs *pFLC* and *FLC* for amplicons of qRT-PCR to detect DNA binding enrichment of promoter region and coding sequence. *pFLC* represents a primer that spans the G-box binding site, while *FLC* represents a primer that avoids the ACGT core as a control. Besides, we used *UBC21* as an internal control.

The result demonstrated that comparing to the wild type, the percentage of input DNA of *FLC* promoter harboring G-box motif in the *bZIP16* overexpression line is 27 times higher than that in the wild type, indicating that bZIP16 directly binds to the promoter region of *FLC*.(Fig.5A, 5B).

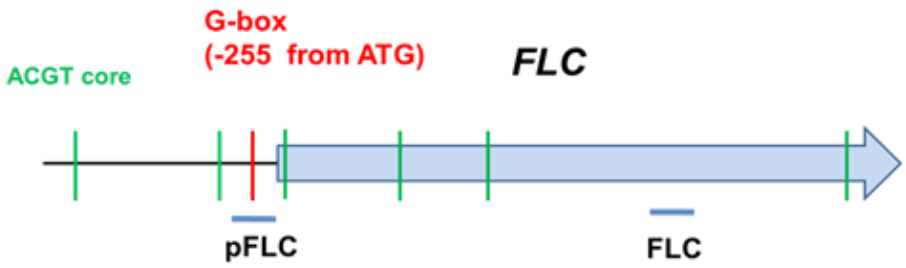

Fig.5A The structure of *FLC* promoter

The black line represents the length from 0 to-2100bp *FLC* promoter.
ACGT core is indicated with green lines.The red line denotes putative G-box motif.
The locations of PCR primers (pFLC-ABI-a-Fw&Rv, FLC-ABI-b-Fw&Rv) used for the enrichment test are indicated with blue lines

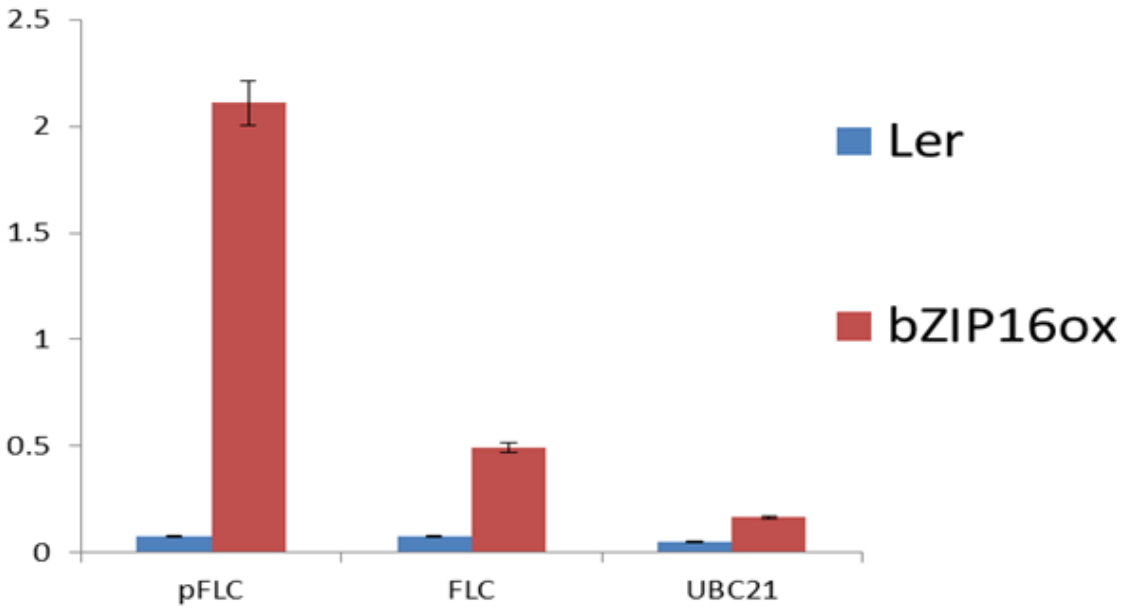

Fig.5B **bZIP16 directly binds to the *FLC* promoter harboring G-box motif**

The binding of bZIP16ox to the *FLC* promoter spanning the G-box was 27- fold enriched compared with that in wild type plants. The chromatins were extracted from 18-day-old wild-type and bZIP16ox line grown under short-day conditions (8-h light/16-h dark) at ZT12. Immunoprecipitaed DNA was quantified by qPCR with specific primer pairs for candidate fragments. Amplicons in *UBC21* were used as an internal control. Results from wild-type and Ler/*35S-bZIP16-GFP* (*bZIP16ox*) were normalized as percentage of the input DNA. Data are means±S.D.

IV. Discussion

Investigating bZIP16 protein level in different growth development stages of Arabidopsis, we found that bZIP16 protein was detected more in 4-day-old seedling under constant dark conditions than in 4-day-old seedling under constant light conditions. But bZIP16 protein was not detected in dry seeds and 1-day-old seedlings under constant light conditions. It indicated that cold treatment and light affected the expression of bZIP16 (Fig. 1-1~1-3). In addition, the accumulation of bZIP16 protein was higher in buds and flowers. Furthermore, phenotypic analysis showed that *bzip16* mutants flowered later than the wild-type plants under long-day (LD) and short-day (SD) conditions. It reveals that bZIP16 regulates flowering time.

There are at least four flowering pathways in Arabidopsis, namely photoperiod, autonomous, vernalization, and gibberellins dependent pathways (Mouradov et al., 2002). The photoperiod pathway refers to regulation of flowering in response to day length and quality of light perceived. The autonomous pathway refers to endogenous regulators that are independent of the photoperiod and gibberellin pathways (Koornneef M,et al.,1991). The vernalization pathway refers to the acceleration of flowering on exposure to a long period of cold. *FLC* delays flowering by blocking the transcription of genes in the photoperiodic flowering pathway. Vernalization inhibits transcription of *FLC*. *FLC* inhibits flowering by directly repressing the key promoters of flowering *FT*, *SOC1*, and *FD* (Michaels, 2009). The *FT*, *LFY* and *SOC1* genes act as central floral integrators (Blazquez et al., 1998; Nilsson et al., 1998; Samach et al., 2000). The gibberellin pathway refers to the requirement of gibberellic acids for normal flowering patterns. *SOC1* integrates the GA-mediated flowering-time signal with these environmental cues (Moon et al., 2003). The *FT* protein and mRNA appear to be the

flowering stimulus that moves fromleaves into the shoot apical meristem region, where it evokes the transitionfrom vegetative to reproductive meristem identity (Corbesier et al., 2007).

During the early stages of Arabidopsis seedling development, bZIP16 represses *RGL2* to promote seed germination and hypocotyl elongation. Through the transcriptomic comparison of 43 flowering-related genes in wild-type plants and *bzip16* mutants and analyzing the expressions of the selected genes *CO, CI, FT, SOC1, FLC, RGA* and *RGL2* in wild-type plants, *bzip16* mutants and *bZIP16* overexpression line by qRT-PCR, my studies revealed that bZIP16 does not repress *RGL2* in adult plants. It indicates that bZIP16 may function differently in different developmental stages. Besides, bZIP16 does not affect the genes involved in GA pathway, photoperiodic pathway, vernalization pathway and autonomous pathway. Therefore, bZIP16 likely functions independently of the GA and the photoperiodic pathway.

A major role of the genes in the autonomous pathway is to repress *FLC* expression. Arabidopsis plants with mutations in autonomous-pathway genes (*fca, fld, fpa, fve, fy* and *ld*) have increased levels of *FLC* expression (Michaels and Amasino, 1999). Unlike the photoperiod pathway late-flowering mutants only in LD conditions, the autonomous pathway mutants are delayed in flowering under under SD and LD conditions but still respond to photoperiod by flowering earlier in long days than in short days (Mouradov et al., 2002). bZIP16 represses *FLC* and promotes the expressions of *SOC1* and *FT* to regulate the flowering (Fig. C). In addition, the *bzip16* mutants show late-flowering under LD and SD conditions. Moreover, the transcriptomic study and qRT-PCR analyses demonstrated that bZIP16 does not affect the genes involved in GA pathway, photoperiodic pathway, vernalization pathway and autonomous way. Maybe bZIP16 participates in the autonomus pathway. But, to date we know that the

autonomous-pathway genes repress the expression of *FLC* through chromatin-remodeling complexes and small RNAs. And the vernalization-pathway genes repress the expression of *FLC* through histone modifications. Our chromatin immunoprecipitation assay showed that bZIP16 directly binds to the *FLC* promoter harboring G-box motif. bZIP16 is the first one transcription factor among the upstream genes of *FLC* that represses *FLC* by binding to the *FLC* promoter directly in *vivo.* Hence, bZIP16 maybe belong to a new unknown pathway of its own and function in parallel of the vernalization and autonomous pathways.

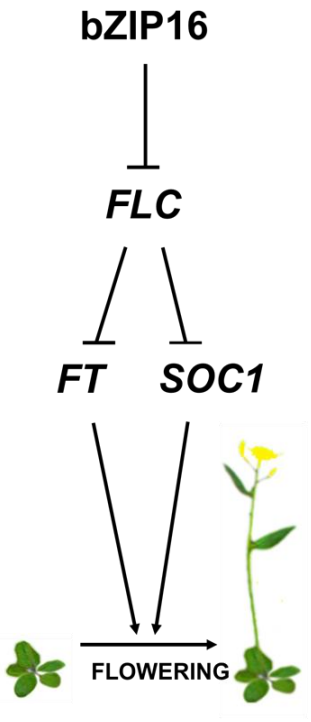

Fig.C Proposed model for the role of *bZIP16* in the regulation of Arabidopsis flowering time. Arrow indicates promotion and blunt arrow indicates repression.

V. Conclusions

Our studies showed the high bZIP16 protein accumulation in buds and flowers. Through the observation of the phenotypes, *bzip16* mutants show late-flowering phenotypes under both short-day and long-day conditions. According to the transcriptomic comparison and qRT-PCR, bZIP16 does not affect those genes in photoperiodic pathway, GA pathway, autonomus and vernalization pathway. Our chromatin immunoprecipitation assay showed that bZIP16 directly binds to the *FLC* promoter harboring G-box motif. It demonstrates that bZIP16 regulates flowering time by binding to the *FLC* promoter to repress its expression, which in turn de-repressing the expressions of *FT* and *SOC1* to promote flowering. Our findings provide strong evidence that bZIP16 not only promotes seed germination and hypocotyl elongation during the early stages of Arabidopsis seedling development but also plays a positive role in floral induction. We report a novel physiological function of bZIP16 *in planta.* Furthermore, our study reveals bZIP16 as a new floral regulator in repressing the floral integrator *FLC* via binding to the *FLC* promoter directly to control flowering time.

The most exciting part of doing research is that we are not always able to predict the results. However, concentrating on investigation alway brings us unexpected surprises. At the beginning, we hypothesized that bZIP16 might promote flowering by repressing *RGL2.* Although the results are not what we expected, we are lucky to find bZIP16 as a new floral regulator in controlling flowering time.

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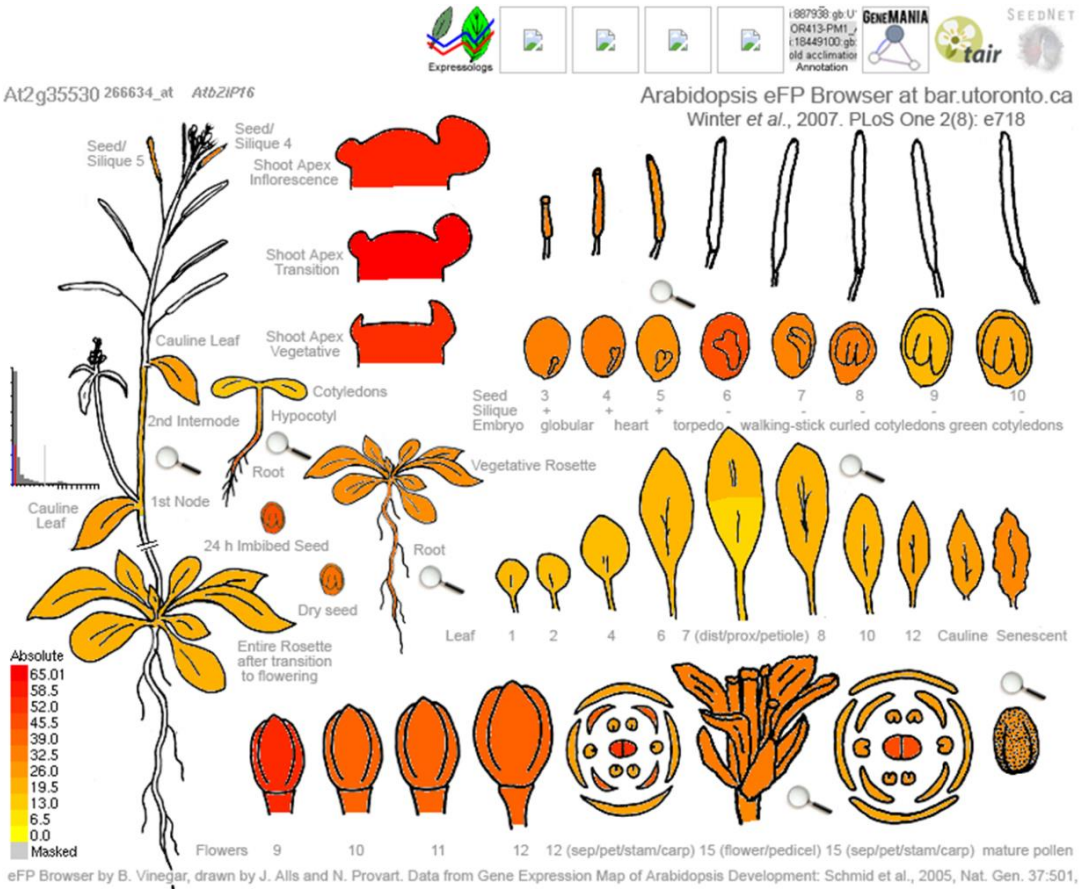
VII. Appendices

Appendix 1

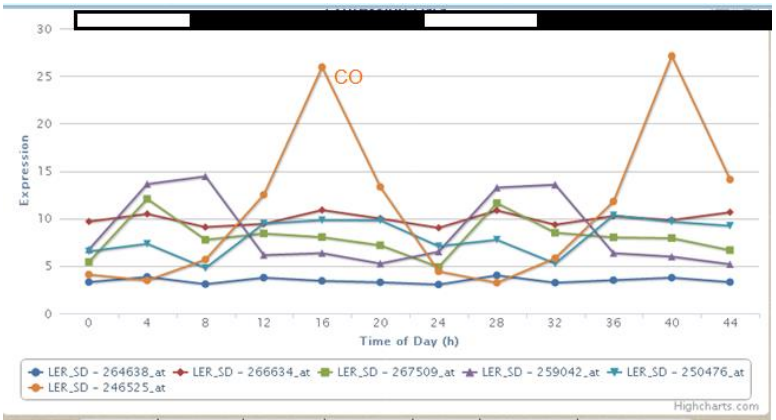
The distribution of bZIP16 transcript in different growth development stages of Arabidopsis. The expression patterns retrieved from Arabidopsis eFP Browser. http://bbc.botany.utoronto.ca/efp/cgi-bin/efpWeb.cgi?primaryGene=AT2G35530&modeInput=Absolute



The developmental map shows that the bZIP16 mRNA accumulates more in seeds, buds, shoot apexes and flowers.

Appendix 2.

Data about the expression level of *FT*, *bZIP16*, *SOC1*, *RGL2*, *FLC* and *CO* transcripts retrieved from http://diurnal.mocklerlab.org (Mockler Lab, U.S.A)



	Probe	Locus	Genome	Condition	Phase	Correlation	
FT	264638_at	AT1G65480	A. thaliana	LER_SD	4	0.692813	View Orthologs
bZIP16	266634_at	AT2G35530	A. thaliana	LER_SD	21	0.549075	View Orthologs
SOC1	267509_at	AT2G45660	A. thaliana	LER_SD	5	0.858959	View Orthologs
RGL2	259042_at	AT3G03450	A. thaliana	LER_SD	6	0.990479	View Orthologs
FLC	250476_at	AT5G10140	A. thaliana	LER_SD	16	0.895611	View Orthologs
CO	246525_at	AT5G15840	A. thaliana	LER_SD	17	0.934655	View Orthologs

	Probe	Locus	Genome	Condition	Phase	Correlation	
bZIP16	266634_at	AT2G35530	A. thaliana	LER_SD	21	0.549075	View Orthologs
SOC1	267509_at	AT2G45660	A. thaliana	LER_SD	5	0.858959	View Orthologs
RGL2	259042_at	AT3G03450	A. thaliana	LER_SD	6	0.990479	View Orthologs
FLC	250476_at	AT5G10140	A. thaliana	LER_SD	16	0.895611	View Orthologs

According to web-based tool, DIURNAL, we found that time points of the high expressions of the mentioned genes above is between 4 to 16 hour after light on. Therefore, we collected the samples of each line at ZT 3, 6, 9, 12 and 15hr after light on, respectively

Appendix 3. Observation of flowering phenotype

Plants and mutants grown under long-day conditions (LD)

Experiment 1

Rosette Leaves	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**AVE**	**STEDV**
Ler	4	4	4	4	4	4	4	4	4	4	4	4	4.00	0.00
bzip16-2	5	5	5	5	5	5	5	5	5	5	5	5	5.00	0.00
bzip16-2/35S-bZIP16-GFP	4	5	5	5	5	5	5	5	5	4	5	5	4.83	0.39
Ler/35S-bZIP16-GFP#1	4	4	4	4	4	4	4	4	4	4	4	4	4.00	0.00
Ler/35S-bZIP16-GFP#6	4	4	4	4	4	4	4	4	4	4	4	4	4.00	0.00

Cauline Leaves	1	2	3	4	5	6	7	8	9	10	11	12	AVE	STEDV
Ler	2	2	2	2	2	2	2	2	2	2	2	2	2.00	0.00
bzip16-2	3	2	2	2	3	2	2	2	2	2	3	2	2.25	0.45
bzip16-2/35S-bZIP16-GFP	2	2	2	2	2	2	2	2	2	2	2	3	2.08	0.29
Ler/35S-bZIP16-GFP#1	2	3	2	2	2	2	2	3	2	2	2	2	2.17	0.39
Ler/35S-bZIP16-GFP#6	2	2	2	2	2	2	2	2	2	2	2	2	2.00	0.00

Genotype	Rosette Leaves	STEDV	Cauline Leaves	STEDV	T-test
Ler	4.00	0.00	2.00	0.00	
bzip16-2	5.00	0.00	2.25	0.45	1.6922E-40
bzip16-2/35S-bZIP16-GFP	4.83	0.39	2.08	0.29	1.33374E-05
Ler/35S-bZIP16-GFP#1	4.00	0.00	2.17	0.39	0.338800696
Ler/35S-bZIP16-GFP#6	4.00	0.00	2.00	0.00	0.338800696

Appendix 4. Observation of flowering phenotype

Plants and mutants grown under long-day conditions (LD)

Experiment 2

Rosette Leaves	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**AVE**	**STEDV**
Ler	4	5	5	4	4	5	4	5	4	5	5	4	4.50	0.52
bzip16-2	6	6	6	6	5	5	5	5	5	5	6	6	5.50	0.52
Ler/35S-bZIP16-GFP#6	4	4	5	4	4	5	4	4	4	4	4	4	4.17	0.39

Cauline Leaves	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**AVE**	**STEDV**
Ler	**2**	**2**	**1**	**1**	**2**	**1**	**1**	**2**	**2**	**2**	**1**	**2**	1.58	0.51
bzip16-2	**1**	**1**	**1**	**2**	**2**	**1**	**2**	**2**	**2**	**2**	**2**	**2**	1.67	0.49
Ler/35S-bZIP16-GFP#6	**2**	**2**	**2**	**2**	**2**	**2**	**1**	**2**	**2**	**2**	**2**	**2**	1.92	0.29

Genotype	Rosette Leaves	STEDV	Cauline Leaves	STEDV	T-test
Ler	4.50	0.52	1.58	0.51	
bzip16-2	5.50	0.52	1.67	0.49	0.00011
Ler/35S-bZIP16-GFP#6	4.17	0.39	1.92	0.29	0.09124

Appendix 5. Observation of flowering phenotype

Plants and mutants grown under short-day (SD)conditions

Experiment 3

Rosette Leaves	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**AVE**	**STEDV**
Ler	23	24	22	23	25	24	25	27	24	X	22	20	23.55	1.86
bzip16-2	30	35	32	30	33	28	X	32	31	33	33	28	31.36	2.20
bZIP16ox	23	22	25	22	X	21	21	23	22	23	21	24	22.45	1.29

Cauline Leaves	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**AVE**	**STEDV**
Ler	7	7	4	5	6	7	6	8	7	X	4	5	6.00	1.34
bzip16-2	7	8	9	8	8	8	X	10	8	9	8	6	8.09	1.04
bZIP16ox	7	5	7	6	X	8	7	5	5	6	5	7	6.18	1.08

Genotype	Rosette Leaves	STEDV	Cauline Leaves	STEDV	T-test	leaf range
Ler	23.55	1.86	6.00	1.34		20~25
bzip16-2	31.36	2.20	8.09	1.04	2.33E-08	28~33
bZIP16ox	22.45	1.29	6.18	1.08	0.128284334	21~25

Appendix 6. Observation of flowering phenotype

Plants and mutants grown under short-day (SD)conditions

Experiment 4

Rosette Leaves	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**AVE**	**STEDV**
Ler	19	15	22	23	25	24	26	22	29	26	25	22	23.17	3.64
bzip16-2	22	20	23	25	29	26	33	32	39	26	32		27.91	5.66
bZIP16ox	18	18	18	23	16		23	24		21	16		19.67	3.12

Genotype	Rosette Leaves	STEDV	Cauline Leaves	STEDV	T-test	leaf range	
Ler	23.17	3.64	8.00	1.28		15~29	
bzip16-2	27.91	5.66	9.00	1.34	3.03E-02	20~39	**
bZIP16ox	19.67	3.12	5.60	2.50	2.90E-02	16~24	*

Appendix 7. The data of the biological repeat 1 by qRT –PCR

RGA

		R1		R2		R3	
	hrs	AVE (UBC-0.5)	AVE (RGA-0.5)	AVE (UBC-0.5)	AVE (RGA-0.5)	AVE (UBC-0.5)	AVE (RGA-0.5)
Ler	3	20.476	24.426	20.616	24.480	20.487	24.587
	6	20.814	26.007	20.692	26.195	20.705	26.153
	9	20.024	25.946	20.045	25.894	20.012	25.834
	12	20.775	25.048	20.818	25.019	20.690	25.022
	15	20.626	25.228	20.664	25.301	20.718	25.213
bzip16-2	3	20.434	24.803	20.417	24.746	20.416	24.739
	6	20.731	26.455	20.732	26.471	20.738	26.489
	9	20.135	25.868	20.139	25.990	20.127	26.064
	12	20.606	24.986	20.638	25.093	20.596	25.104
	15	20.462	25.709	20.416	25.791	20.424	25.775
bZIP16/bzip16-2	3	20.693	25.645	20.638	25.110	20.618	25.310
	6	20.684	26.497	20.685	26.485	20.643	26.549
	9	19.515	24.152	19.498	23.932	19.609	23.875
	12	20.748	24.969	20.722	24.929	20.957	24.946
	15	20.497	24.918	20.545	24.914	20.670	24.902
bZIP16ox	3	20.290	23.478	20.232	23.274	20.297	23.477
	6	20.085	24.382	20.094	24.397	20.132	24.269
	9	19.809	25.787	19.969	25.757	19.944	26.120
	12	20.650	24.709	20.695	24.759	20.641	24.855
	15	20.226	24.425	20.188	24.884	20.220	24.694

c	R1-2	R1-3	R1-1	R1-2	R1-3	(R1+R2)/3	STEDV/√n
ΔCT(RGA-UBQ-ct)	ΔCT(RGA-UBQ-ct)	ΔCT(RGA-UBQ-ct)	$2^{-\Delta CT}$	$2^{-\Delta CT}$	$2^{-\Delta CT}$	$2^{-\Delta CT}$	SEM
3.950	3.864	4.100	0.0647	0.0687	0.0583	0.0639	0.0030
5.193	5.503	5.448	0.0273	0.0221	0.0229	0.0241	0.0016
5.922	5.849	5.822	0.0165	0.0173	0.0177	0.0172	0.0004
4.273	4.201	4.332	0.0517	0.0544	0.0497	0.0519	0.0014
4.602	4.637	4.495	0.0412	0.0402	0.0443	0.0419	0.0013
4.369	4.329	4.323	0.0484	0.0498	0.0500	0.0494	0.0005
5.724	5.739	5.751	0.0189	0.0187	0.0186	0.0187	0.0001

5.733	5.851	5.937	0.0188	0.0173	0.0163	0.0175	0.0007
4.380	4.455	4.508	0.0480	0.0456	0.0439	0.0459	0.0012
5.247	5.375	5.351	0.0263	0.0241	0.0245	0.0250	0.0007
4.952	4.472	4.692	0.0323	0.0451	0.0387	0.0387	0.0037
5.813	5.800	5.906	0.0178	0.0179	0.0167	0.0175	0.0004
4.637	4.434	4.266	0.0402	0.0463	0.0520	0.0461	0.0034
4.221	4.207	3.989	0.0536	0.0541	0.0630	0.0569	0.0030
4.421	4.369	4.232	0.0467	0.0484	0.0532	0.0494	0.0020
3.188	3.042	3.180	0.1097	0.1214	0.1103	0.1138	0.0038
4.297	4.303	4.137	0.0509	0.0507	0.0568	0.0528	0.0020
5.978	5.788	6.176	0.0159	0.0181	0.0138	0.0159	0.0012
4.059	4.064	4.214	0.0600	0.0598	0.0539	0.0579	0.0020
4.199	4.696	4.474	0.0544	0.0386	0.0450	0.0460	0.0046

RGL2

		R1		R2		R3	
	hrs	AVE (UBC-0.5)	AVE (RGL2-0.5)	AVE (UBC-0.5)	AVE (RGL2-0.5)	AVE (UBC-0.5)	AVE (RGL2-0.5)
Ler	3	20.476	26.714	20.616	26.926	20.487	26.886
	6	20.814	28.726	20.692	28.596	20.705	28.912
	9	20.024	31.028	20.045	31.438	20.012	30.923
	12	20.775	29.552	20.818	29.283	20.690	29.575
	15	20.626	28.956	20.664	28.893	20.718	28.911
bzip16-2	3	20.434	26.682	20.417	26.816	20.416	27.017
	6	20.731	29.177	20.732	29.054	20.738	29.202
	9	20.135	31.820	20.139	31.556	20.127	31.795
	12	20.606	29.347	20.638	29.817	20.596	29.580
	15	20.462	29.183	20.416	29.429	20.424	29.524
bZIP16/bzip16-2	3	20.693	28.094	20.638	27.947	20.618	27.891
	6	20.684	28.749	20.685	28.843	20.643	28.719
	9	19.515	30.754	19.498	31.194	19.609	30.955
	12	20.748	29.683	20.722	30.711	20.957	30.231
	15	20.497	27.752	20.545	29.407	20.670	29.206
bZIP16ox	3	20.290	26.621	20.232	26.656	20.297	26.708
	6	20.085	27.963	20.094	27.960	20.132	27.982
	9	19.809	30.291	19.969	30.709	19.944	30.213
	12	20.650	29.383	20.695	29.392	20.641	29.509
	15	20.226	29.169	20.188	28.822	20.220	28.783

R1-1	R1-2	R1-3	R1-1	R1-2	R1-3	(R1+R2)/3	STEDV/√n
ΔCT(RGL2 -UBQ-ct)	ΔCT(RGL2 -UBQ-ct)	ΔCT(RGL2 -UBQ-ct)	$2^{-\Delta CT}$	$2^{-\Delta CT}$	$2^{-\Delta CT}$	$2^{-\Delta CT}$	SEM
6.238	6.310	6.399	0.0132	0.0126	0.0118	0.0126	0.0004
7.912	7.904	8.207	0.0042	0.0042	0.0034	0.0039	0.0003
11.004	11.393	10.911	0.0005	0.0004	0.0005	0.0005	0.0000
8.777	8.465	8.885	0.0023	0.0028	0.0021	0.0024	0.0002
8.330	8.229	8.193	0.0031	0.0033	0.0034	0.0033	0.0001
6.248	6.399	6.601	0.0132	0.0118	0.0103	0.0118	0.0008
8.446	8.322	8.464	0.0029	0.0031	0.0028	0.0029	0.0001
11.685	11.417	11.668	0.0003	0.0004	0.0003	0.0003	0.0000
8.741	9.179	8.984	0.0023	0.0017	0.0020	0.0020	0.0002
8.721	9.013	9.100	0.0024	0.0019	0.0018	0.0020	0.0002
7.401	7.309	7.273	0.0059	0.0063	0.0065	0.0062	0.0002
8.065	8.158	8.076	0.0037	0.0035	0.0037	0.0036	0.0001
11.239	11.696	11.346	0.0004	0.0003	0.0004	0.0004	0.0000
8.935	9.989	9.274	0.0020	0.0010	0.0016	0.0015	0.0003
7.255	8.862	8.536	0.0065	0.0021	0.0027	0.0038	0.0014
6.331	6.424	6.411	0.0124	0.0116	0.0118	0.0119	0.0002
7.878	7.866	7.850	0.0043	0.0043	0.0043	0.0043	0.0000
10.482	10.740	10.269	0.0007	0.0006	0.0008	0.0007	0.0001
8.733	8.697	8.868	0.0024	0.0024	0.0021	0.0023	0.0001
8.943	8.634	8.563	0.0020	0.0025	0.0026	0.0024	0.0002

FLC

		R1		R2		R3	
	hrs	AVE (UBC-0.5)	AVE (FLC-0.5)	AVE (UBC-0.5)	AVE (FLC-0.5)	AVE (UBC-0.5)	AVE (FLC-0.5)
Ler	3	20.476	29.331	20.616	29.540	20.487	29.334
	6	20.814	29.496	20.692	29.466	20.705	30.084
	9	20.024	29.664	20.045	29.767	20.012	29.839
	12	20.775	29.317	20.818	29.244	20.690	28.999
	15	20.626	28.979	20.664	29.368	20.718	28.939
bzip16-2	3	20.434	26.966	20.417	26.787	20.416	26.878
	6	20.731	26.984	20.732	27.030	20.738	26.997
	9	20.135	26.725	20.139	26.705	20.127	26.674

	12	20.606	26.505	20.638	26.441	20.596	26.420
	15	20.462	26.647	20.416	26.546	20.424	26.422
bZIP16/bzip16-2	3	20.693	27.606	20.638	27.683	20.618	27.612
	6	20.684	27.807	20.685	27.860	20.643	27.321
	9	19.515	26.832	19.498	26.930	19.609	27.119
	12	20.748	26.929	20.722	27.053	20.957	27.198
	15	20.497	26.860	20.545	26.926	20.670	26.958
bZIP16ox	3	20.290	29.605	20.232	29.246	20.297	29.107
	6	20.085	29.282	20.094	29.320	20.132	29.157
	9	19.809	29.889	19.969	29.554	19.944	29.595
	12	20.650	29.962	20.695	29.908	20.641	29.692
	15	20.226	29.556	20.188	29.707	20.220	29.358

R1-1	R1-2	R1-3	R1-1	R1-2	R1-3	(R1+R2)/3	STEDV/√n
ΔCT(FLC -UBQ-ct)	ΔCT(FLC -UBQ-ct)	ΔCT(FLC -UBQ-ct)	$2^{-\Delta CT}$	$2^{-\Delta CT}$	$2^{-\Delta CT}$	$2^{-\Delta CT}$	SEM
8.855	8.924	8.847	0.0022	0.0021	0.0022	0.0021	0.0000
8.682	8.774	9.379	0.0024	0.0023	0.0015	0.0021	0.0003
9.640	9.722	9.827	0.0013	0.0012	0.0011	0.0012	0.0000
8.542	8.426	8.309	0.0027	0.0029	0.0032	0.0029	0.0001
8.353	8.704	8.221	0.0031	0.0024	0.0034	0.0029	0.0003
6.532	6.370	6.462	0.0108	0.0121	0.0113	0.0114	0.0004
6.253	6.298	6.259	0.0131	0.0127	0.0131	0.0130	0.0001
6.590	6.566	6.547	0.0104	0.0106	0.0107	0.0105	0.0001
5.899	5.803	5.824	0.0168	0.0179	0.0177	0.0174	0.0003
6.185	6.130	5.998	0.0137	0.0143	0.0156	0.0146	0.0006
6.913	7.045	6.994	0.0083	0.0076	0.0078	0.0079	0.0002
7.123	7.175	6.678	0.0072	0.0069	0.0098	0.0080	0.0009
7.317	7.432	7.510	0.0063	0.0058	0.0055	0.0058	0.0002
6.181	6.331	6.241	0.0138	0.0124	0.0132	0.0131	0.0004
6.363	6.381	6.288	0.0121	0.0120	0.0128	0.0123	0.0002
9.315	9.014	8.810	0.0016	0.0019	0.0022	0.0019	0.0002
9.197	9.226	9.025	0.0017	0.0017	0.0019	0.0018	0.0001
10.080	9.585	9.651	0.0009	0.0013	0.0012	0.0012	0.0001
9.312	9.213	9.051	0.0016	0.0017	0.0019	0.0017	0.0001
9.330	9.519	9.138	0.0016	0.0014	0.0018	0.0016	0.0001

SOC1

		R1		R2		R3	
	hrs	AVE (UBC-0.5)	AVE (SOC1-0.5)	AVE (UBC-0.5)	AVE (SOC1-0.5)	AVE (UBC-0.5)	AVE (SOC1-0.5)
Ler	3	20.476	24.555	20.616	24.796	20.487	24.777
	6	20.814	24.308	20.692	24.291	20.705	24.417
	9	20.024	24.105	20.045	23.955	20.012	24.320
	12	20.775	25.231	20.818	25.242	20.690	25.235
	15	20.626	25.582	20.664	25.781	20.718	25.632
bzip16-2	3	20.434	24.870	20.417	25.181	20.416	25.197
	6	20.731	24.987	20.732	24.796	20.738	24.847
	9	20.135	25.261	20.139	25.232	20.127	25.140
	12	20.606	25.896	20.638	25.929	20.596	25.704
	15	20.462	26.395	20.416	26.380	20.424	26.311
bZIP16/bzip16-2	3	20.693	25.205	20.638	25.465	20.618	25.448
	6	20.684	24.230	20.685	24.419	20.643	24.272
	9	19.515	24.603	19.498	24.631	19.609	24.685
	12	20.748	25.659	20.722	25.684	20.957	25.547
	15	20.497	25.973	20.545	25.982	20.670	25.997
bZIP16ox	3	20.290	23.887	20.232	24.001	20.297	24.034
	6	20.085	23.187	20.094	23.200	20.132	23.274
	9	19.809	24.022	19.969	24.267	19.944	24.227
	12	20.650	24.472	20.695	24.576	20.641	24.578
	15	20.226	24.846	20.188	24.908	20.220	24.938

R1-1	R1-2	R1-3	R1-1	R1-2	R1-3	(R1+R2)/3	STEDV/√n
ΔCT(SOC1 -UBQ-ct)	ΔCT(SOC1 -UBQ-ct)	ΔCT(SOC1 -UBQ-ct)	$2^{-\Delta CT}$	$2^{-\Delta CT}$	$2^{-\Delta CT}$	$2^{-\Delta CT}$	SEM
4.079	4.180	4.290	0.0592	0.0552	0.0511	0.0552	0.0023
3.494	3.599	3.712	0.0888	0.0825	0.0763	0.0825	0.0036
4.081	3.910	4.308	0.0591	0.0665	0.0505	0.0587	0.0046
4.456	4.424	4.545	0.0456	0.0466	0.0428	0.0450	0.0011
4.956	5.117	4.914	0.0322	0.0288	0.0332	0.0314	0.0013
4.436	4.764	4.781	0.0462	0.0368	0.0364	0.0398	0.0032
4.256	4.064	4.109	0.0523	0.0598	0.0580	0.0567	0.0022
5.126	5.093	5.013	0.0286	0.0293	0.0310	0.0296	0.0007

5.290	5.291	5.108	0.0256	0.0255	0.0290	0.0267	0.0011
5.933	5.964	5.887	0.0164	0.0160	0.0169	0.0164	0.0003
4.512	4.827	4.830	0.0438	0.0352	0.0352	0.0381	0.0029
3.546	3.734	3.629	0.0856	0.0752	0.0808	0.0805	0.0030
5.088	5.133	5.076	0.0294	0.0285	0.0296	0.0292	0.0003
4.911	4.962	4.590	0.0332	0.0321	0.0415	0.0356	0.0030
5.476	5.437	5.327	0.0225	0.0231	0.0249	0.0235	0.0007
3.597	3.769	3.737	0.0826	0.0734	0.0750	0.0770	0.0029
3.102	3.106	3.142	0.1165	0.1161	0.1133	0.1153	0.0010
4.213	4.298	4.283	0.0539	0.0508	0.0514	0.0520	0.0010
3.822	3.881	3.937	0.0707	0.0679	0.0653	0.0680	0.0016
4.620	4.720	4.718	0.0407	0.0379	0.0380	0.0389	0.0009

CO

		R1		R2		R3	
	hrs	AVE (UBC-0.5)	AVE (CO-0.5)	AVE (UBC-0.5)	AVE (CO-0.5)	AVE (UBC-0.5)	AVE (CO-0.5)
Ler	3	20.476	33.192	20.616	33.112	20.487	33.207
	6	20.814	33.324	20.692	32.819	20.705	32.420
	9	20.024	28.475	20.045	28.293	20.012	28.628
	12	20.775	27.932	20.818	27.970	20.690	27.738
	15	20.626	27.815	20.664	27.813	20.718	27.851
bzip16-2	3	20.434	33.113	20.417	32.429	20.416	34.016
	6	20.731	32.953	20.732	32.204	20.738	32.967
	9	20.135	27.578	20.139	27.975	20.127	28.073
	12	20.606	26.832	20.638	27.430	20.596	27.839
	15	20.462	28.237	20.416	27.735	20.424	28.002
bZIP16 /bzip16-2	3	20.693	33.947	20.638	34.094	20.618	33.609
	6	20.684	32.890	20.685	32.969	20.643	32.355
	9	19.515	27.865	19.498	27.969	19.609	28.060
	12	20.748	27.696	20.722	28.037	20.957	27.975
	15	20.497	28.070	20.545	28.215	20.670	28.370
bZIP16ox	3	20.290	33.612	20.232	33.709	20.297	33.787
	6	20.085	31.320	20.094	31.511	20.132	32.018
	9	19.809	27.446	19.969	28.001	19.944	28.471
	12	20.650	27.075	20.695	27.421	20.641	27.743
	15	20.226	28.035	20.188	27.859	20.220	27.950

R1-1	R1-2	R1-3	R1-1	R1-2	R1-3	(R1+R2)/3	STEDV/√n
ΔCT(CO-UBQ-ct)	ΔCT(CO-UBQ-ct)	ΔCT(CO-UBQ-ct)	$2^{-\Delta CT}$	$2^{-\Delta CT}$	$2^{-\Delta CT}$	$2^{-\Delta CT}$	SEM
12.716	12.496	12.720	0.0001	0.0002	0.0001	0.0002	0.0000
12.510	12.127	11.715	0.0002	0.0002	0.0003	0.0002	0.0000
8.451	8.248	8.616	0.0029	0.0033	0.0025	0.0029	0.0002
7.157	7.152	7.048	0.0070	0.0070	0.0076	0.0072	0.0002
7.189	7.149	7.133	0.0069	0.0070	0.0071	0.0070	0.0001
12.679	12.012	13.600	0.0002	0.0002	0.0001	0.0002	0.0000
12.222	11.472	12.229	0.0002	0.0004	0.0002	0.0003	0.0000
7.443 6.226	7.836 6.792	7.946 7.243	0.0057 0.0134	0.0044 0.0090	0.0041 0.0066	0.0047 0.0097	0.0005 0.0020
7.775	7.319	7.578	0.0046	0.0063	0.0052	0.0054	0.0005
13.254	13.456	12.991	0.0001	0.0001	0.0001	0.0001	0.0000
12.206	12.284	11.712	0.0002	0.0002	0.0003	0.0002	0.0000
8.350	8.471	8.451	0.0031	0.0028	0.0029	0.0029	0.0001
6.948	7.315	7.018	0.0081	0.0063	0.0077	0.0074	0.0006
7.573	7.670	7.700	0.0053	0.0049	0.0048	0.0050	0.0001
13.322	13.477	13.490	0.0001	0.0001	0.0001	0.0001	0.0000
11.235	11.417	11.886	0.0004	0.0004	0.0003	0.0003	0.0000
7.637	8.032	8.527	0.0050	0.0038	0.0027	0.0039	0.0007
6.425	6.726	7.102	0.0116	0.0094	0.0073	0.0095	0.0013
7.809	7.671	7.730	0.0045	0.0049	0.0047	0.0047	0.0001

FT

		R1		R2		R3	
	hrs	AVE UBC-0.5)	AVE (FT-0.1)	AVE (UBC-0.5)	AVE (FT-0.1)	AVE (UBC-0.5)	AVE (FT-0.1)
Ler	3	20.476	34.546	20.616	37.570	20.487	35.532
	6	20.814	33.247	20.692	33.591	20.705	33.173
	9	20.024	33.251	20.045	33.571	20.012	33.748
	12	20.775	35.863	20.818	33.929	20.690	33.864
	15	20.626	35.170	20.664	35.569	20.718	35.375
bzip16-2	3	20.434	37.477	20.417	35.020	20.416	37.477
	6	20.731	35.151	20.732	36.531	20.738	36.154
	9	20.135	34.843	20.139	34.785	20.127	34.785
	12	20.606	35.228	20.638	34.712	20.596	36.474
	15	20.462	38.113	20.416	37.417	20.424	37.387

bZIP16/bzip16-2	3	20.693	33.933	20.638	35.990	20.618	36.160
	6	20.684	35.488	20.685	36.463	20.643	35.278
	9	19.515	34.631	19.498	33.772	19.609	34.636
	12	20.748	34.357	20.722	35.466	20.957	35.243
	15	20.497	36.883	20.545	36.588	20.670	36.552
bZIP16ox	3	20.290	35.342	20.232	35.197	20.297	35.288
	6	20.085	33.573	20.094	32.734	20.132	33.333
	9	19.809	34.132	19.969	35.468	19.944	35.178
	12	20.650	33.183	20.695	33.621	20.641	33.818
	15	20.226	33.256	20.188	33.587	20.220	34.682

R1-1	R1-2	R1-3	R1-1	R1-2	R1-3	(R1+R2)/3	STEDV/√n
ΔCT(FT-UBQ-ct)	ΔCT(FT-UBQ-ct)	ΔCT(FT-UBQ-ct)	$2^{-\Delta CT}$	$2^{-\Delta CT}$	$2^{-\Delta CT}$	$2^{-\Delta CT}$	SEM
14.070	16.954	15.045	0.0001	0.0000	0.0000	0.0000	0.0000
12.433	12.899	12.468	0.0002	0.0001	0.0002	0.0002	0.0000
13.227	13.526	13.736	0.0001	0.0001	0.0001	0.0001	0.0000
15.088	13.111	13.174	0.0000	0.0001	0.0001	0.0001	0.0000
14.544	14.905	14.657	0.0000	0.0000	0.0000	0.0000	0.0000
17.043	14.603	17.061	0.0000	0.0000	0.0000	0.0000	0.0000
14.420	15.799	15.416	0.0000	0.0000	0.0000	0.0000	0.0000
14.708	14.646	14.658	0.0000	0.0000	0.0000	0.0000	0.0000
14.622	14.074	15.878	0.0000	0.0001	0.0000	0.0000	0.0000
17.651	17.001	16.963	0.0000	0.0000	0.0000	0.0000	0.0000
13.240	15.352	15.542	0.0001	0.0000	0.0000	0.0000	0.0000
14.804	15.778	14.635	0.0000	0.0000	0.0000	0.0000	0.0000
15.116	14.274	15.027	0.0000	0.0001	0.0000	0.0000	0.0000
13.609	14.744	14.286	0.0001	0.0000	0.0001	0.0001	0.0000
16.386	16.043	15.882	0.0000	0.0000	0.0000	0.0000	0.0000
15.052	14.965	14.991	0.0000	0.0000	0.0000	0.0000	0.0000
13.488	12.640	13.201	0.0001	0.0002	0.0001	0.0001	0.0000
14.323	15.499	15.234	0.0000	0.0000	0.0000	0.0000	0.0000
12.533	12.926	13.177	0.0002	0.0001	0.0001	0.0001	0.0000
13.030	13.399	14.462	0.0001	0.0001	0.0000	0.0001	0.0000

LFY

		R1		R2		R3	
	hrs	AVE (UBC-0.5)	AVE (LFY-0.2)	AVE (UBC-0.5)	AVE (LFY-0.2)	AVE (UBC-0.5)	AVE (LFY-0.2)
Ler	3	20.476	33.429	20.616	Undetermined	20.487	33.470
	6	20.814	Undetermined	20.692	35.165	20.705	Undetermined
	9	20.024	39.815	20.045	Undetermined	20.012	Undetermined
	12	20.775	33.010	20.818	33.241	20.690	33.294
	15	20.626	Undetermined	20.664	Undetermined	20.718	34.592
bzip16-2	3	20.434	34.581	20.417	35.164	20.416	34.127
	6	20.731	35.103	20.732	35.077	20.738	Undetermined
	9	20.135	34.967	20.139	Undetermined	20.127	Undetermined
	12	20.606	35.334	20.638	33.503	20.596	34.140
	15	20.462	Undetermined	20.416	33.817	20.424	33.729
bZIP16/bzip16-2	3	20.693	34.199	20.638	Undetermined	20.618	35.157
	6	20.684	35.040	20.685	35.412	20.643	34.972
	9	19.515	35.930	19.498	35.361	19.609	33.646
	12	20.748	34.202	20.722	35.133	20.957	35.040
	15	20.497	34.508	20.545	35.108	20.670	33.451
bZIP16ox	3	20.290	35.986	20.232	34.839	20.297	Undetermined
	6	20.085	33.362	20.094	Undetermined	20.132	35.085
	9	19.809	35.120	19.969	Undetermined	19.944	Undetermined
	12	20.650	35.095	20.695	35.266	20.641	35.123
	15	20.226	34.975	20.188	35.232	20.220	34.362

R1-1	R1-2	R1-3	R1-1	R1-2	R1-3	(R1+R2)/3	STEDV/√n
ΔCT(LFY-UBQ-ct)	ΔCT(LFY-UBQ-ct)	ΔCT(LFY-UBQ-ct)	$2^{-\Delta CT}$	$2^{-\Delta CT}$	$2^{-\Delta CT}$	$2^{-\Delta CT}$	SEM
12.953	#VALUE!	12.983	0.0001	#VALUE!	0.0001	#VALUE!	#VALUE!
#VALUE!	14.473	#VALUE!	#VALUE!	0.0000	#VALUE!	#VALUE!	#VALUE!
19.791	#VALUE!	#VALUE!	0.0000	#VALUE!	#VALUE!	#VALUE!	#VALUE!
12.235	12.423	12.604	0.0002	0.0002	0.0002	0.0002	0.0000
#VALUE!	#VALUE!	13.874	#VALUE!	#VALUE!	0.0001	#VALUE!	#VALUE!
#REF!	14.164	14.748	#REF!	0.0001	0.0000	#REF!	#REF!
#REF!	14.371	14.339	#REF!	0.0000	0.0000	#REF!	#REF!
#REF!	14.828	#VALUE!	#REF!	0.0000	#VALUE!	#REF!	#REF!

#REF!	14.696	12.907	#REF!	0.0000	0.0001	#REF!	#REF!
#REF!	#VALUE!	13.393	#REF!	#VALUE!	0.0001	#REF!	#REF!
13.506	#VALUE!	14.539	0.0001	#VALUE!	0.0000	#VALUE!	#VALUE!
14.356	14.727	14.329	0.0000	0.0000	0.0000	0.0000	0.0000
16.415	15.863	14.037	0.0000	0.0000	0.0001	0.0000	0.0000
13.454	14.411	14.083	0.0001	0.0000	0.0001	0.0001	0.0000
14.011	14.563	12.781	0.0001	0.0000	0.0001	0.0001	0.0000
15.696	14.607	#VALUE!	0.0000	0.0000	#VALUE!	#VALUE!	#VALUE!
13.277	#VALUE!	14.953	0.0001	#VALUE!	0.0000	#VALUE!	#VALUE!
15.311	#VALUE!	#VALUE!	0.0000	#VALUE!	#VALUE!	#VALUE!	#VALUE!
14.445	14.571	14.482	0.0000	0.0000	0.0000	0.0000	0.0000
14.749	15.044	14.142	0.0000	0.0000	0.0001	0.0000	0.0000

bZIP16

		R1		R2		R3	
	hrs	AVE (UBC-0.5)	AVE (*bzip16*-0.5)	AVE (UBC-0.5)	AVE (*bzip16*-0.5)	AVE (UBC-0.5)	AVE (*bzip16*-0.5)
Ler	3	20.476	25.985	20.616	25.998	20.487	26.311
	6	20.814	26.803	20.692	26.94	20.705	26.896
	9	20.024	26.511	20.045	26.648	20.012	26.591
	12	20.775	26.564	20.818	26.661	20.690	26.499
	15	20.626	26.576	20.664	26.85	20.718	26.453
bzip16-2	3	20.434	31.779	20.417	32.431	20.416	32.329
	6	20.731	32.437	20.732	32.086	20.738	32.566
	9	20.135	31.859	20.139	32.493	20.127	32.119
	12	20.606	30.863	20.638	31.187	20.596	31.242
	15	20.462	31.319	20.416	31.431	20.424	31.348
bZIP16/bzip16-2	3	20.693	24.787	20.638	24.91	20.618	25.171
	6	20.684	25.014	20.685	25.193	20.643	25.225
	9	19.515	24.77	19.498	24.727	19.609	24.93
	12	20.748	24.964	20.722	25.079	20.957	24.935
	15	20.497	24.942	20.545	24.921	20.670	25.083
bZIP16ox	3	20.290	24.267	20.232	24.463	20.297	24.563
	6	20.085	23.565	20.094	23.839	20.132	23.945
	9	19.809	22.966	19.969	23.072	19.944	23.113
	12	20.650	22.16	20.695	22.384	20.641	22.39
	15	20.226	21.645	20.188	21.59	20.220	21.641

R1-1	R1-2	R1-3	R1-1	R1-2	R1-3	(R1+R2)/3	STEDV/√n
ΔCT(*bzip16*-UBQ-ct)	ΔCT(*bzip16*-UBQ-ct)	ΔCT(*bzip16*-UBQ-ct)	$2^{-\Delta CT}$	$2^{-\Delta CT}$	$2^{-\Delta CT}$	$2^{-\Delta CT}$	SEM
5.509	5.382	5.824	0.0220	0.0240	0.0177	0.0212	0.0019
5.989	6.248	6.191	0.0157	0.0132	0.0137	0.0142	0.0008
6.487	6.603	6.579	0.0111	0.0103	0.0105	0.0106	0.0003
5.789	5.843	5.809	0.0181	0.0174	0.0178	0.0178	0.0002
5.950	6.186	5.735	0.0162	0.0137	0.0188	0.0162	0.0015
11.345	12.014	11.913	0.0004	0.0002	0.0003	0.0003	0.0000
11.706	11.354	11.828	0.0003	0.0004	0.0003	0.0003	0.0000
11.724	12.354	11.992	0.0003	0.0002	0.0002	0.0002	0.0000
10.257	10.549	10.646	0.0008	0.0007	0.0006	0.0007	0.0001
10.857	11.015	10.924	0.0005	0.0005	0.0005	0.0005	0.0000
4.094	4.272	4.553	0.0586	0.0518	0.0426	0.0510	0.0046
4.330	4.508	4.582	0.0497	0.0439	0.0418	0.0451	0.0024
5.255	5.229	5.321	0.0262	0.0267	0.0250	0.0260	0.0005
4.216	4.357	3.978	0.0538	0.0488	0.0635	0.0554	0.0043
4.445	4.376	4.413	0.0459	0.0482	0.0469	0.0470	0.0007
3.977	4.231	4.266	0.0635	0.0533	0.0520	0.0562	0.0036
3.480	3.745	3.813	0.0896	0.0746	0.0711	0.0785	0.0057
3.157	3.103	3.169	0.1121	0.1164	0.1112	0.1132	0.0016
1.510	1.689	1.749	0.3511	0.3101	0.2975	0.3196	0.0162
1.419	1.402	1.421	0.3740	0.3784	0.3735	0.3753	0.0016

Appendix 8. The data of chromatin immunoprecipitation q-PCR assay

		Input	IP	ΔCT(IP-input)	$2^{-\Delta CT}$	Ave $2^{-\Delta CT}$	STEDV	Template ratio	%
Ler	pFLC	24.973	31.992	7.01900100708007	0.0077	0.0070	0.0012	10	0.077102801
Ler	pFLC	24.943	32.400	7.45700073242187	0.0057			10	
Ler	pFLC	24.932	31.958	7.02600097656250	0.0077			10	
Ler	FLC	23.593	30.580	6.98699951171875	0.0079	0.0106	0.0028	10	0.078832186
Ler	FLC	23.716	30.273	6.55700111389160	0.0106			10	
Ler	FLC	23.965	30.182	6.21699905395507	0.0134			10	
Ler	UBC21	25.150	32.730	7.57999992370605	0.0052	0.0051	0.0001	10	0.052262798
Ler	UBC21	24.961	32.619	7.65799903869628	0.0050			10	
Ler	UBC21	24.978	32.596	7.61800003051757	0.0051			10	
bZIP16ox	pFLC	24.883	27.129	2.24600028991699	0.2108	0.2054	0.0053	10	2.108077352
bZIP16ox	pFLC	24.902	27.187	2.28499984741210	0.2052			10	
bZIP16ox	pFLC	24.826	27.147	2.32099914550781	0.2001			10	
bZIP16ox	FLC	23.696	28.040	4.34400177001953	0.0492	0.0507	0.0066	10	0.492408075
bZIP16ox	FLC	23.834	27.943	4.10900115966796	0.0580			10	
bZIP16ox	FLC	23.547	28.022	4.47499847412109	0.0450			10	
bZIP16ox	UBC21	25.063	30.977	5.91399955749511	0.0166	0.0173	0.0007	10	0.165847427
bZIP16ox	UBC21	25.107	30.947	5.84000015258789	0.0175			10	
bZIP16ox	UBC21	24.989	30.791	5.80200004577636	0.0179			10	

		Input	IP	ΔCT(IP-input)	2-ΔCT	Ave 2-ΔCT	STEDV	Template ratio	%	% STEDV	fold
Ler	pFLC	24.949	32.117	7.16733423868815	0.0070	0.0070	0.0012	10	0.077102801	0.000230996	
Ler	FLC	23.758	30.941	6.58699989318847	0.0106	0.0106	0.0028	10	0.078832186	0.000556002	
Ler	UBC21	25.030	32.648	7.61866633097331	0.0051	0.0050	0.0001	10	0.052262798	2.75062E-05	
bZIP16ox	pFLC	24.870	27.154	2.28399976094564	0.2053	0.2053	0.0053	10	2.108077352	0.001068391	27.34113
bZIP16ox	FLC	23.692	28.002	4.30933380126953	0.0507	0.0507	0.0066	10	0.492408075	0.001323541	6.386384
bZIP16ox	UBC21	25.053	30.905	5.85199991861979	0.0173	0.0173	0.0006	10	0.165847427	0.000135926	2.150991

評語

探討 bZIP16 對開花的影響，創新度很好，深度也夠，是一件頗為完整的作品，學生對研究主題瞭解透徹，對問題的對答，有令人激賞的表現。