Investigation of the regulatory network involving *AGAMOUS-LIKE 24* in floral transition of *Arabidopsis*

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List of Abbreviations

Chemicals and reagents

dGTP	deoxyguanosine triphosphate
dNTP	deoxynucleoside triphosphate
EDTA	ethylene-diamine-tetra-acetate
Gly	glycine
HCl	hydrochloric acid
$K_3Fe(CN)_6$	potassium ferricyanide
$K_4Fe(CN)_6$	potassium ferrocyanide
KPO_4	potassium phosphate
LB	Luria bertani
LiCl	lithium chloride
$MgCl_2$	magnesium chloride
NaCl	Sodium chloride
Na_2HPO_4	disodium Phosphate
NaH_2PO_4	sodium Phosphate (dibasic)
$NaPO_4$	sodium phosphate
PBS	phosphate buffered saline
PMSF	phenylmethylsulfonylfluoride
SDS	sodium dodecylsulphate
Tris	Tris (hydroxymethyl)-aminomethane

Units and measurements

bp	base pairs
g	gram(s)
h	hour(s)
kb	kilo base-pairs
kDa	kilo Dalton(s)
M	Molar
min	minute(s)
ml	mililitre(s)
mM	Milimolar
ng	nanogram(s)
OD_{600nm}	absorbance at wavelength 600 nm
rpm	revolution per minute
sec	second(s)
U	unit(s)
v/v	volume per volume
w/v	weight per volume
°C	Degree Celsius
µg	microgram(s)
µl	microlitre(s)
µM	Micromolar

Others

BLAST	Basic Local Alignment Search Tool
DNA	deoxyribonucleic acid
et al.	et alter (and others)
GA	gibberellin
i.e.	that is
LD	long day
mRNA	messenger ribonucleic acid
PCR	polymerase chain reaction
RT-PCR	Reverse Transcription Polymerase Chain Reaction
SAM	short apical meristem
SDS-PAGE	SDS Polyacrylamide Gel Electrophoresis
SD	short day
TAE buffer	tris acetate electrophoresis buffer

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Summary

Flowering is one of the most essential processes in the life cycle of a flowering plant. It requires a transition from vegetative to reproductive growth, which is regulated by four genetic pathways, including gibberellin (GA), photoperiod, venalization and autonomous promotion pathways. Photoperiod and venalization pathways respond to environmental signals, while GA and autonomous pathways sense endogenous signals. They activate the expression of a group of flowering time integrators to promote the transformation of the vegetative shoot apical meristem (SAM) into the inflorescence SAM, which has the capacity to generate floral meristems on its flankings.

We have known that a MADS-domain containing transcription factor, *AGAMOUS-LIKE 24 (AGL24),* plays an important role in integrating flowering time signals in *Arabidopsis*. *AGL24* is a dosage-dependent promoter of flowering in *Arabidopsis*, because loss-of-function *agl24* mutants show late flowering and overexpression of *AGL24* transgenic plants show early flowering. Loss of *AGL24* function can suppress the premature flowering phenotype of overexpression of *SOC1* and overexpression of *AGL24* can partially rescue the late flowering phenotype of *soc1*. Thus, *AGL24* acts partly downstream of *SOC1*. In addition, *AGL24* affects the transcriptional induction of a floral meristem identity gene, *LEAFY (LFY)* because *LFY* expression is delayed and reduced in *agl24* mutants during floral transition, whilst over-expression of *LFY* is able to compensate the late flowering phenotype of *agl24*. Therefore, AGL24 functions upstream of *LFY*. Although the above results suggest that *AGL24* is an essential flowering time integrator, it is hitherto unknown what are the direct regulators upstream or downstream of *AGL24*.

In this study, chromatin immunoprecipitation (ChIP) was applied to identify the downstream target genes of *AGL24*. Firstly, *35S::AGL24-12HA* tagging transgenic lines were generated and showed the similar phenotypes as *35S::AGL24*, indicating that *35S::AGL24-12HA* fusion protein is biologically functional. Secondly, by using these tagging lines and the specific HA antibody, it was purified that the *in vivo* complex containing the AGL24-12HA fusion protein and associated DNAs. Lastly, several putative target genes were identified by cloning and sequencing isolated DNA fragments in ChIP experiments.

To study the molecular basis of the regulation of *AGL24*, the *AGL24* promoter was isolated and this regulatory region was investigated by promoter deletion analysis.

To facilitate our further studies of *AGL24* target genes, an *in situ* hybridization system was established to examine the spatial and temporal expression of a specific gene. By using this system, the altered expression of two putative target genes of *AGL24* has successfully detected. Through these studies, we gained sights into the mechanism of *AGL24* function in the control of floral transition in *Arabidopsis*.

1 Literature review

1.1 Introduction

Flowering is one of the most essential phases in the life cycle of a flowering plant. It requires a transition from vegetative to reproductive growth, which is controlled by a group of flowering time genes in response to environmental and endogenous developmental signals. The complexity of this transition is reflected by an intricate network of several flowering promotion pathways (Mouradov *et al*., 2002).

At least four genetic pathways have been suggested to control flowering time in plants. Vernalization and photoperiod pathways capture environmental signals, while autonomous and GA pathways respond endogenous developmental signals. The genes immediately downstream of these four pathways are flowering integrators that integrate environmental and endogenous cues regulating floral transition and the subsequent flower development. *LFY, FLOWERING LOCUS T (FT)* and *SOC1* are three critical flowering time integrators. The expression of *FT* and *SOC1* are regulated positively by the photoperiod pathway, but negatively by *FLOWEIRNG LOCUS C* (*FLC),* which is an important regulator integrating the signals from the vernalization and autonomous pathways. *SOC1* can be up-regulated by the GA pathway as well (Komeda, 2004). *LFY* is the most critical regulator located very downstream of flowering time pathways, which controls the switch form flowering time control to flower development (Blazquez *et al*., 1997).

Arabidopsis is an excellent model system to study the control of flowering time, because it responds to typical environmental conditions that affect flowering in a wide

range of plant species (Mouradov *et al*., 2002). *Arabidopsis* also offers several additional advantages for the research in the regulation of flowering, including the rapid life cycle, prolific seed production, efficient transformation systems and established genetic molecular tools for studying *Arabidopsis* genes. Genetic and molecular studies in *Arabidopsis* have shown that a lot of MADS-box genes are required for plant reproduction development (Pařenicová *et al*., 2003). *AGL24* is one of the MADS-domain containing transcription factors, which plays an important role in the regulation of flowering time (Yu *et al*., 2002; Michaels *et al*, 2003). As a dosage-dependent mediator of the flowering signals, the levels of *AGL24* expression determine the flowering time in *Arabidopsis*. Although it has been suggested that *AGL24* acts downstream of *SOC1* and upstream of *LFY* (Yu *et al*, 2002), there is no evidence for the direct relationships between these genes. Some other intermediators may function in the gaps. To investigate the regulatory components downstream and upstream of *AGL24*, we planned to use ChIP to identify *AGL24* target genes and to use promoter deletion analysis to dissect cis-elements responsible for the regulation of *AGL24*.

1.2 Flowering Time

The general body plan of plants is established during embryogenesis, when the undifferentiated meristematic regions of root and shoot are set aside. Much of plant development occurs postembryonically, through the reiterative production of organ primordia at the SAM. In flowering plants, the SAM initially gives rise to vegetative organs such as leaves, but at a specific point the SAM makes the transition to reproductive development and then produces flowers (Levy & Dean, 1998).The induction of flowering in plants is the most important part from the standpoints of reproductive strategy and allocation of limited resources. Monocarpic plants, such as *Arabidopsis*, perform the flowering only once in their lifecycle, and the reproductive success depends entirely on this one opportunity (Komeda, 2004).

During floral transition, the change in the developmental fate of primordia initiated at the SAM is controlled by environmental and endogenous signals (Bernier, 1988; McDaniel *et al*., 1992). Unlike many developmental transitions in animals, the SAM of plants is not irreversibly "committed" to reproductive development once flowering commences. In some species and genotypes under certain environmental conditions, flowers can be transformed back into leafy shoots in a phenomenon known as the inflorescence reversion (Battey & Lyndon, 1990; Pouteau *et al*., 1997). This observation implies that the genes and processes involved in the transition to flowering are required for both the initiation and maintenance of flower development.

The endogenous signals for floral transition in many species can accumulate in vegetative tissues. These internal cues include plant size or number of vegetative nodes.

The vegetative SAM is thought to first pass through a "juvenile" phase in which it is incompetent to respond to internal or external signals that would be able to trigger flowering in an "adult" meristem (Levy & Dean, 1998). The acquisition of reproductive competence is then marked by changes in the morphology or physiology of vegetative structures. For example, the alteration of leaf morphology may indicate the competence for floral transition in a process known as vegetative phase change (Poethig, 1990; Lawson & Poethig, 1995). It is likely that some of the important genes in controlling the transition from vegetative to reproductive development are also involved in vegetative phase change.

In some species, the timing of flowering is primarily influenced by environmental factors, which serve to communicate the timing and/or growth conditions favorable for sexual reproduction and seed maturation. These factors include photoperiod (i.e., day length), light quality (spectral composition), light quantity (photon flux density), vernalization (exposure to a long period of cold), nutrient and water availability. However, flowering can also be induced by stresses such as nutrient deficiency, drought, and overcrowding. These kinds of response enable plants to produce seeds, which will facilitate plants to survive the stress and pass the genetic information to the next generation (Levy & Dean, 1998).

1.3 Genetic Pathways in the Control of Flowering Time

Over the years, four genetic pathways in the control of flowering time have been well investigated in the model plant species *Arabidopsis*. They are vernalization, photoperiod, autonomous and gibberllin pathways (Fig. 1).

1.3.1 Autonomous Pathway

Genes in autonomous pathway can promote flowering by sensing endogenous developmental signals. These genes include *FCA, LUMINIDEPENDENS (LD), FVE, FPA, and FY*. Loss-of-function mutants of these genes show late flowering in both long days (LD) and short days (SD) (Koornneef *et al*., 1991). The *LD* gene encodes a protein carrying nuclear localization signal, homologous to mammalian transcription domain and plant DNA-binding homeo domain (Lee *et al*., 1994). The *FCA* gene encodes a RNA-binding protein, containing WW-protein interaction domain (Macknight *et al*., 1997). It is homologous to *SEX-LETHAL (SX-1)* and *EMBRYONIC LETHAL ABNORMAL VISUAL SYSTEM (ELAV)* genes of *Drosophila* (Macknight *et al*., 1997). Both *LD* and *FCA* promote flowering by repressing a MADS-box transcription factor, *FLC,* which is a major repressor converging on both autonomous and venalization pathways.

FY is an RNA 3′ end-processing factor that interacts with *FCA* to regulate flowering time (Simpson *et al*., 2003). *FCA* expression is autoregulated through the selection of different polyadenylation sites within the *FCA* pre-mRNA, and the *FCA/FY*

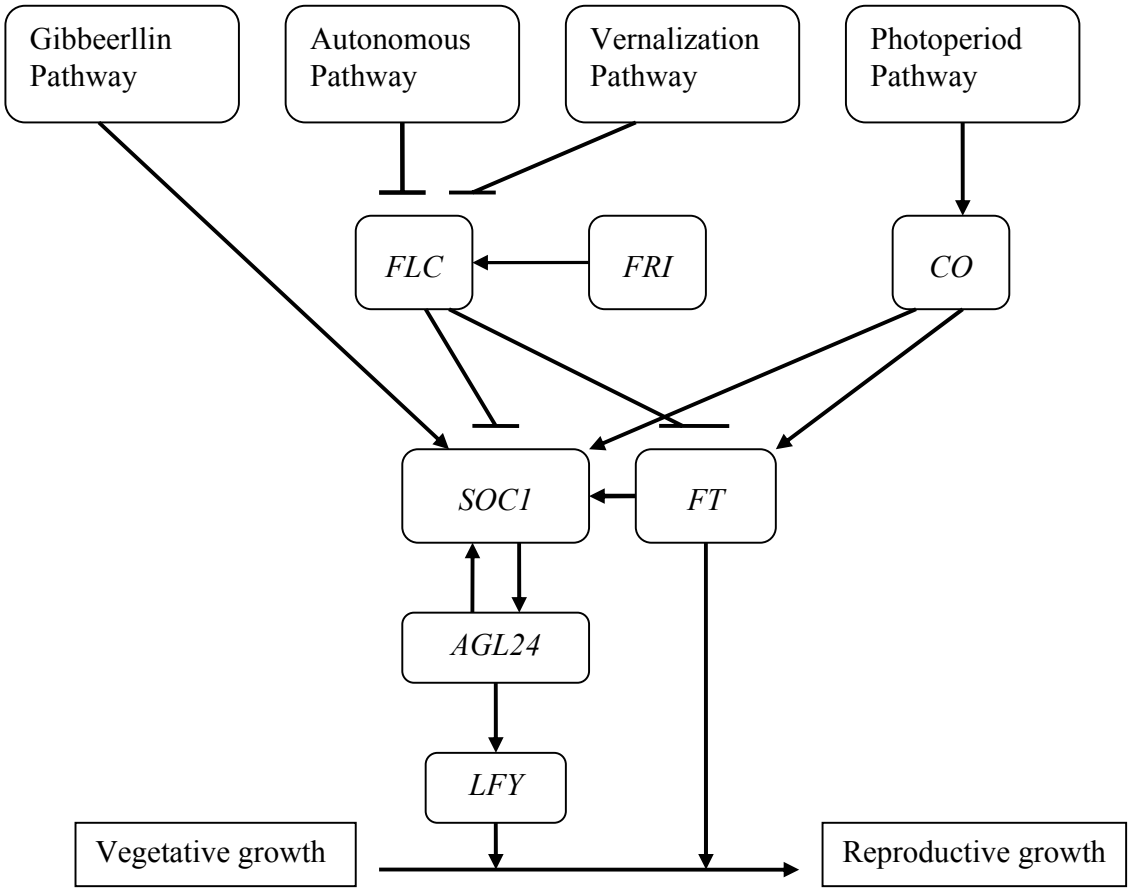

Fig. 1 Four distinct genetic pathways regulate flowering time in *Arabidopsis*. *FT*, *SOC1*, *AGL24* and *LFY* serve as flowering time integrators acting downstream of four genetic pathways in the control of flowering time. Arrows indicate promotion while T bars represent repression.

interaction is required for efficient selection of the promoter-proximal polyadenylation site. Their interaction is also required for the downregulation of *FLC*. *FPA* encodes a protein which contains three RNA recognition motifs in N-terminal region. It is expressed strongly in developing tissues, similar to the expression of *FCA* and *LD* (Schomburg *et al*., 2001). Mutation of *FPA* result in extremely delayed flowering (Schomburg *et al*., 2001). All the above genes in the autonomous pathway can repress *FLC* and thus promote flowering (Komeda, 2004). These redundant genes in the same pathway ensure the developmental tuning of flowering when a plant develops to a certain age or size (Komeda, 2004).

1.3.2 Vernalization Pathway

The genes in this pathway can sense the alteration of temperature and promote flowering by repressing *FLC* activity. There are two genes *VERNALIZATION 1* (*VRN1*) and *VERNALIZATION 2* (*VRN2*) isolated in this pathway (Chandler *et al*., 1996). The function of *VRN1* is unknown, while *VRN2* is a polycomb group protein that is essential for the stable repression of *FLC* by modulating its chromatin (Sheldon *et al*., 2000; Gendall *et al*., 2001). Autonomous and vernalization pathways are conveged on the *FLC* gene, which functions in repressing a group of downstream flowering time integrators, including *SOC1* and *FT*. By repression of *FLC*, both pathways can eventually promote the expression of other downstream flowering time integrators, leading to the initiation of floral meristem development. *FRIGIDA (FRI)* is a positive regulator of *FLC*. Some ecotypes of *Arabidopsis*, such as Columbia, Lansberg *erecta*, and Wassilewskija (WS),

have mutations in the *FRI* gene, which causes the downregulation of *FLC* and early flowering (Johanson *et al*., 2000)

1.3.3 Photoperiod Pathway

The genes in this pathway can sense the length and quality of light and promote flowering by upregulating *SOC 1* and *FT* through a critical regulator *CONSTANT (CO)*. The *CO* gene encodes a protein that has homology to the Zinc-finger domain protein family of transcriptional factors (Putterill *et al*., 1995). The *co* mutants are late flowering only under LDs (Koornneef *et al*., 1991). The quantity of the *CO* message was proportional to the earliness of flowering in transgenic plants overexpressing *CO* (Samach *et al*., 2000). These results indicate that *CO* is a major regulator in the photoperiod pathway. The transgenic plants overexpressing the *CO* gene to some extent rescued the late flowering demonstrated by the mutants in the autonomous pathway (Komeda, 2004). Thus, it is clear that the autonomous pathway in *Arabidopsis* is partially redundant to the photoperiod pathway (Komeda, 2004).

PHYTOCHROME A (PHYA), *PHYTOCHROME A (PHYB)*, *CRYPTOCHROME 1 (CRY1)*, and *CRYPTOCHROME 2 (CRY2)* are several important regulators upstream of *CO* in the photoperiod pathway (Levy & Dean, 1998). Red light is absorbed by the phytochrome proteins encoded by *PHYA* and *PHYB* in *Arabidopsis* (Briggs *et al*., 2001; Parks & Quail, 1993; Reed *et al*., 1993), while blue light are absorbed by cryptochrome proteins, encoded by *CRY1* and *CRY2* (Ahmad & Cashmore, 1993; Lin *et al*., 1998). The *PHYA* expression is abundant and labile while the *PHYB* expression is low abundant but

more stable (Parks & Quail, 1993). Moreover *PHYA* responds to far-red light but *PHYB* percepts red light (Parks & Quail, 1993). Under red light, *PHYB* functions in repressing *CO* function (Putterill *et al*., 1995), while under blue light, *CRY2* inhibits *PHYB* and induces flowering (Lin *et al*., 1998; Mockler *et al*., 1999). Another cryptochome gene *CRY1* cooperatively functions with the *CRY2* gene to repress the function of the *PHYB* (Mockler *et al*., 1999). The genes *LATE ELONGATED HYPOCOTYL (LHY)*, *CIRCADIAN CLOCK ASSOCIATED 1 (CCA1)*, *EARLY FLOWERING 3 (ELF3)*, *EARLY IN SHORT DAY 4 (ESD 4)* and *TIMING OF CAB EXPRESSION 1 (TOC1)* function to process the physical signals in the photoperiod pathway (Doyle *et al*., 2002; Hicks *et al*., 2001; Reeves *et al*., 2002). The processed signals are transmitted to the *GI* gene encoding a membrane protein with a membrane-spanning region (Fowler *et al*., 1999; Park *et al*., 1999), which further activates the *CO* gene to promote flowering (Suarez-Lopez *et al*., 2001).

1.3.4 Gibberellin Pathway

GAs are one important class of plant hormones affecting many plant developmental programs from seed germination, stem elongation, flowering time and flower development. In *Arabidopsis*, GA primarily affect flowering time in SD conditions. The GA-deficient *ga1-3* mutants of *Arabidopsis thaliana* never flower under SD conditions (Wilson *et al*., 1992). Blazquez *et al*. (1998) found that the *ga1-3* mutants contained very low levels of expression of the floral meristem identity gene *LFY* in SDs, while overexpression of *LFY* could rescue the non-flowering phenotype of *ga1-3* under SDs. Thus, GA signaling pathway regulates flowering time by activation of several

downstream integrators including *LFY*. This process is mediated mainly by two GA signaling DELLA proteins: REPRESSOR OF GA1-3 (RGA) and GA INSENSITIVE (GAI), because the non-flowering phenotype of *gal-3* in SDs can be rescued by *rga* and *gai* loss-of-function mutants (Dill & Sun, 2001).

1.4 Integration of Flowering Time Control Pathway

All of the above four genetic pathways eventually activate the expression of a group of downstream flowering time integrators to promote the transition of the vegetative SAM into inflorescence SAM, which can further generate floral meristems on its flankings. These key flowering time integrators include *FT, SOC1,* and *LFY* (Moon *et al*., 2005).

FT is a promoter of flowering that acts downstream of various regulatory pathways. The *FT* gene encodes a small and mobile protein that is expressed in the vasculature of cotyledons and leaves. In *Arabidopsis,* the day-length response depends on the induction of *FT*, which interacts with a bZIP transcription factor *FD* to activate downstream genes including a floral meristem identity gene *APETALA1 (AP1)* (Wigge *et al*., 2005; Abe *et al*., 2005). It has been suggested that *FT* is a primary target of *CO* in the photoperiod pathway (Samach *et al*., 2000), and a target of *FLC* in both vernalization and autonomous pathways (Hepworth *et al*., 2002)

SOC1, also called *AGAMOUS-LIKE 20*, is another important flowering time integrator. It encodes a MADS-box transcription factor, whose expression is dramatically increased in the SAM during floral transition (Lee *et al*., 2000; Borner *et al*., 2000).

Vernalization can increase *SOC1* expression via reduction of *FLC* levels (Lee *et al.,* 2000), and GA treatment can also upregulate its expression. These results suggest that *SOC1* acts downstream of the vernalization and GA pathways (Borner *et al.*, 2000). In addition, *SOC1* has been identified as a direct target of *CO* in the photoperiod pathway (Hepworth *et al.*, 2002; Samach *et al*., 2000).

LFY is a key regulator that integrates the signals from several genetic pathways in the control of flowering time and subsequently specifies floral meristem identity (Weigel *et al*., 1992; Blazquez *et al*., 1997). High levels of *LFY* mRNA are detectable in young floral primordia during floral transition. The *lfy* mutants have an extended vegetative phase and the flowers are often incompletely converted to vegetative shoots (Komeda, 2004). *LFY* specifies floral meristem identity and regulates floral organ formation mainly through promoting the expression of *AP1* and other floral organ identity genes (Wagner *et al*., 1999; William *et al*., 2004).

1.5 *AGL24*

AGL24 is another important MADS-box transcription factor that acts as a flowering time integrator (Yu *et al*., 2002; Michaels *et al.,* 2003). Loss or reduction of *AGL24* function causes late flowering (Fig. 2A) and there is a strong correlation between the severity of phenotype and the expression levels of *AGL24*. Overexpression of *AGL24*

Fig. 2 Phenotype of *AGL24* mutant plants. (A) A loss-of-function *agl24* mutant shows late flowering phenotype; (B) A transgenic plant with overexpression *AGL24* plant shows early flowering phenotype; (C) A wild type plant

shows early flowering under both LDs and SDs (Yu *et al*., 2002; Fig 2B and 2C). Thus, *AGL24* is a dosage-dependent promoter of flowering in *Arabidopsis* (Yu *et al*., 2002).

Over-expression of *AGL24* could partially rescue the late flowering phenotype of *soc1,* while loss of *AGL24* function could suppress the premature flowering phenotype of overexpression of *SOC1* (Yu *et al*., 2002). These results indicate that *AGL24* acts partly downstream of *SOC1*. On the other hand, over-expression of *SOC1* causes an increase of *AGL24* expression (Michaels *et al*., 2003). Therefore, it seems that *AGL24* and *SOC1* are able to positively regulate each other and the actual relationship needs to be further investigated. Mutants with loss- or gain-of-function of *SOC1* and *AGL24* are still responsive to the difference of photoperiod, despite the fact that both *SOC1* and *AGL24* act downstream of *CO*. Therefore, the gene cascade from *CO* to *FT* might contribute a larger part to the photoperiod pathway than the gene cascade from *CO* to *SOC1* (Yu *et al.*, 2002). Furthermore, *AGL24* affects the transcriptional induction of *LFY* because *LFY* expression profile is delayed and reduced in *agl24* mutant during floral transition, whilst over-expression of *LFY* is able to compensate the late flowering phenotype of *agl24* (Yu *et al.*, 2002). However, whether or not *LFY* is a direct target of *AGL24* is unknown. In *in vitro* assays, *AGL24* can be specifically bound to and phosphorylated by a meristemic receptor-like kinase (MRLK). The MRLK signaling promotes translocation of *AGL24* from the cytoplasm to the nucleus (Fujita *et al.*, 2003), which may be important for the *AGL24* function as a transcription factor.

It is noteworthy that *AGL24* can promote both flowering and the inflorescence identity, because overexpression of *AGL24* not only shows early flowering, but also converts floral meristems into inflorescence meristems (Yu *et al*., 2004). Thus, repression

of *AGL24* is a crucial step for flower development. Floral meristem identity gene *LFY* and *AP1* promote establishment and maintenance of floral identity in newly formed floral primordia (Parcy *et al*., 2002; Irish & Sussex, 1990; Weigel *et al*., 1992) by repressing *AGL24*. Without this repression, ectopic expression of *AGL24* would revert floral meristems into inflorescence meristems (Yu *et al*., 2004). This explains the phenotype of floral reversion (from floral to shoot meristem) in *ap1* and *lfy* loss-of-function mutants. Loss of *AGL24* function can significantly reduce the inflorescence characteristics in the flowers of *ap1-1*and *lfy-6* mutants (Yu *et al*., 2004), further suggesting that *AGL24* activity has to be repressed during flower development.

1.6 The MADS-box Protein Family

A lot of key regulators in the control of flowering time including *FLC*, *SOC1* and *AGL24* belong to the MADS-box gene family, which encode transcription factors that are found in a wide range of eukaryotic kingdoms. In flowering plants, MADS proteins are involved in many important developmental programs ranging from embrogenesis, vegetative growth, flowering time control, flower and fruit development. MADS is the acronym of *MINICHROMOSOMEMAINTENANCE 1* (*MCM1*) gene in yeast, *AGAMOUS* (*AG*) in *Arabidopsis*, *DEFICIENS* (*DEF*) in *Antirrhinum* and *SERUM RESPONSE FACTOR* (*SRF*) in human (Riechmann & Meyerowitz, 1997). They are collectively called as MADS-box proteins because they share a highly conserved MADS domain, a DNA binding domain that binds to a CC-$(A/T)_6$-GG (CArG box) motif in the regulatory regions of their target genes (Hayes *et al*., 1988; Riechmann *et al*., 1996).

The biological function of MADS-box proteins, the interaction among MADS-box proteins and other cofactors, and the evolutionary significance of these proteins have been extensively investigated (Shore & Sharrocks, 1995; Riechmann & Meyerowitz, 1997; Jack 2001; Ng and Yanofsky, 2001; Saedler *et al.*, 2001; Becker & Theißen, 2003; De Bodt *et al,* 2003; Messenguy & Dubois, 2003**;** Pařenicová *et al*., 2003; Zhang *et al.*, 2004). The MADS-box genes family in *Arabidopsis* can be divided into five major classes termed MIKC, Mα, Mβ, Mγ and Mδ based on the phylogenetic relationship of the conserved MADS-box domains (Table 1; Pařenicová *et al*., 2003). MIKC-type genes have a MIKC domain structure, including a MADS-box (M), an intervening (I), a keratin-like (K), and a C-terminal (C) domain (Hasebe & Banks, 1997; Ma *et al*., 1991; Münster *et al*., 1997; Theißen *et al*., 1996). The MADS-box domain is usually located at the N-terminus of a MADS-box protein, which is responsible for DNA-binding (Shore and Sharrocks, 1995). This is the most conserved domain of MIKC domains. The I domain is a less conserved domain located between MADS and K domains, which may contribute to the specificity of dimerization of MADS-box protein (Pařenicová *et al*., 2003). The K domain is characterized by a coiled-coil structure, which facilitates protein –protein interactions (Davies *et al*., 1996; Fan *et al*., 1997). The C domain is the least conserved domain located at the C-terminus of a MADS-box protein. It has shown different functions in different MIKC-type MADS-box genes. For example, it may contribute to the formation of multimeric MADS-box protein complexes (Egea-Cortines *et al*., 1999; Honma & Goto, 2001). The genomic distribution of the MIKC genes suggests that these genes already existed at the time of the polyploidization of the *Arabidopsis* genome (75 million years ago). MIKC-type genes play essential and important roles in plant

development, because almost all the MADS-box genes identified so far to have important regulatory functions belong to this type of MADS-box genes (Pařenicová *et al.* 2003). Furthermore, MIKC genes have long sequences containing five to eight exons, while Mα, Mβ, and Mγ groups have no introns or a single intron (Pařenicová *et al.* 2003; Table 1). The protein sequence analysis suggests C-terminal part of the MADS-box domain in the Mα, Mβ, and Mγ groups are more divergent (Pařenicová *et al.* 2003). The origin of the Mδ group is unique. This group is small and contains genes that have the structural complexity comparable to the MIKC genes (5 to 10 exons). However, they do not contain the K domain, and their functions are unknown.

Interactions between MADS proteins or between MADS proteins and proteins of other classes to form homo/heterocomplexes appear to be a common theme in the MADS proteins family (Shore & Sharrocks, 1995). These protein-protein interactions are essential in the formation of specific transcriptional regulatory complexes to determine some key developmental programs, such as the formation of floral organs. However, this kind of protein interaction has not been found among the MADS-box genes involved in the control of flowering time.

Table 1. Differences between subfamilies of MADS-box genes (Pařenicová *et al.* 2003).

Subfamily	Intron and exons distribution	Genome distribution	Duplication
MIKC	Multiple introns, 5 to 10 exons	Across all five chromsomes	Duplication occurs between two differnent chromosomes
Mα	No intron or single intron	Mainly on chromosome I and V	Internal chromosome duplication
Mβ	No intron or single intron	Mainly on chromosome I and V	Internal chromosome duplication
Mγ	No intron or single intron	Mainly on chromosome I and V	Internal chromosome duplication
Mδ	Multiple introns, no K domain	Mainly on chromosome I and V	Internal chromosome duplication

1.7 Methods Used for the Investigation of AGL24-related Regulatory Network

Although *AGL24* has been suggested as a novel integrator of flowering pathways, acting downstream of *SOC1* and upstream of *LFY*, genes interacting directly with *AGL24* are thus far unclear. To address this question, we applied the following three methods in this study.

1.7.1 Promoter Studies

GUS (β-glucuronidase) is a widely used reporter gene in plants (Jefferson *et al*., 1987). It encodes a stable enzyme which hydolyses a wide range of β-glucuronides and has low endogenous activity in plants. Its enzyme activity can be assayed easily by using X-Gluc (5-bromo-4-chloro-3-indolyl β-D-glucuronide) as a substrate (Jefferson & Wilson, 1991). The reaction first produces an unstable intermediate, which would then undergo oxidative dimerization to form the intensive blue ClBr-indigo. ClBr-indigo immediately precipitates upon formation, thus allowing precise localization of GUS activity. Therefore, by monitoring GUS localization, the fusion of *AGL24* regulatory regions with GUS reporter gene can be used for detection of the spatial and temporal regulation of *AGL24* expression.

1.7.2 Chromatin Immunoprecipitation (ChIP)

Protein-DNA interactions mediate transcription, DNA replication and repair, which are central problems to the biology of all organisms. The ability to provide direct

information that a given protein is associated "in time and space" with specific genomic regions is a key determinant of the merits of the various techniques (Orlando, 2000). In particular, the method of ChIP appears to have significant advantages over other approaches, because it allows the analysis of the interaction of transcriptional factors with DNAs in living cells, thereby providing an *in vivo* picture of the native chromatin structure and its bound factors at a specific developmental stage (Sandoval *et al.*, 2004). In ChIP technique, biological materials are firstly fixed, where the targeted protein and DNA is corsslinked into a chromatin complex. The crosslinked chromatin is then isolated and sonicated to produce small fragments. Subsequently, the antibody that recognizes the target protein is used to precipitate the chromatin complex. Finally, the crosslink between the protein and DNA is reversed, and the released DNA fragments are purified for further analysis. Although the whole ChIP process appears to be simple, a lot of parameters should be adjusted to achieve different levels of accuracy to determine the interaction of a protein and its associated DNA sequences. With appropriate controls, ChIP can serve as a valuable tool for studying nuclear events of protein and DNA interactions (Spencer *et al.*, 2003). In this study, *AGL24* was fused with haemagglutinin (HA) tag, because of the unavailability of AGL24 antibody. Then ChIP was applied to identify the downstream targets of *AGL24* by using the transgenic lines containing the AGL24-HA fusion proteins.

1.7.3 *In Situ* Hybridization

In situ hybridization techniques allow specific nucleic acid sequences to be detected in morphologically preserved chromosomes, cells or tissue sections. In combination with immunocytochemistry, *in situ* hybridization can relate microscopic

topological information to gene activity at the DNA, mRNA, and protein levels. The technique was originally developed by Pardue and Gall (1969) and (independently) by John *et al*. (1969).

With the completion of many genome projects, a large number of gene sequences are readily available for comprehensive functional analysis. Gene expression analysis is a key procedure in determining the function of genes and the activity of promoters. Most of gene expression studies involve the hybridization of a specific nucleic acid probe to either genomic DNA (Southern blotting) or mRNA (Northern blotting), which has been isolated from homogenized tissues, separated by electrophoretic methods, and transferred to a stable matrix such as nitrocellulose or more recently, nylon. Whilst these techniques can provide a great deal of information regarding gene expression, they are limited by the crude spatial discrimination of the technique. Comparatively, *in situ* hybridization allows the precise cellular or subcellular localization of genes. Furthemore, some mRNA species present at low levels are often expressed in only a few cells, the sensitivity of *in situ* hybridization may exceed that of Northern hybridization where the target mRNAs are inevitably diluted by the relatively large-scale homogenization of tissue.

In this study, it was investigated that the expression patterns of several *AGL24* target genes in wild-type plants and *agl24* loss-of-function mutants by *in situ* hybridization, which facilitates the precise localization of these genes in the SAM. If these genes were regulated by *AGL24,* it would be expected that their expression patterns are different with or without *AGL24*.

2 Material and Methods

2.1 Plant Material

Seeds of *Arabidopsis thaliana* ecotype Columbia (wild type, transgenic plants and mutants) were geminated at 22 °C under 16 h light/8 h dark condition. T1, T2 and T3 transgenic plants containing transgenes with the pGreen backone were screened with 0.3% Basta after producing two rosette leaves under the same growth condition. Seedlings at different developmental stages were collected for analysis of GUS reporter genes. For ChIP and *in situ* hybridization, inflorescence and vegetative SAMs were collected for fixation.

2.2 Vector Construction

2.2.1 Genomic DNA Extraction

Leaves were ground in 200 µl extraction buffer consisting of 0.2 M Tris-HCl pH 9.0, 0.4 M LiCl, 25mM EDTA, 1% SDS in an eppendorf tube. After centrifugation at 13,000 rpm for 5 min, the supernatant was transferred to a new tube containing 150 µl isopropanol and mixed well. After centrifugation at 13000 rmp for 10 min, the supernatant was poured off and the pellet was rinsed with 70% ethanol, dried by vaccum concentrator, and resuspended in 100 µl TE buffer (10 mM Tris pH 8.0, 1 mM EDTA). The extracted genomic DNA was stored at -20 ºC.

2.2.2 Promoter reporter constructs

For promoter deletion analysis, four vectors have constructed containing four different fragments of *AGL24* regulatory regions fused with the GUS reporter gene (Fig. 3). Four *AGL24* genomic fragments were amplified from the extracted genomic DNA by polymerase chain reaction (PCR) using primers shown in Table 1. A 50 µl PCR reaction system consisted of 2 µl of DNA template, 1 µl of 10 mM dNTPs, 1 ul of 10 mM forward primer, 1 µl of 10mM reverse primer and 1 µl of Advantage^{TM} 2 polymerase in 1X PCR buffer (clontech). PCR was performed by 94 ºC for 2 min of initial denaturation, which was followed by 35 cycles of 94 ºC for 1 min, 58 ºC (P2 and P3) or 56.6 ºC (P4 and P5) (Table 2) for 1 min, elongation at 68 ºC for 2.5 min for P2, 3.6 min for P3, 4.8 min for P4 and 3.6 min for P5, and a final extension at 68 ºC for 10 min. After PCR amplification, the resulting PCR products were separated on agarose gels and the expected bands were cut and purified by PCR purification Kit (Roche, USA). The purified products were digested at the designed restriction enzyme cutting sites, which were corresponding to the sites in the cloning vectors.

Genomic fragments of P2, P3, P4 and P5 were cloned into pGreen-HY107 (Fig. 4A), which was derived from the pGreen 0229 vector (Hellens *et al*., 2000) by deleting the 35S promoter and inserting the GUS reporter gene. For the purpose of ligation, the cloning vectors were firstly digested by the corresponding restriction exzymes and then dephosphorylated by alkaline phosphatase (CIP, New England Biolab, USA). The vectors were further purified by the PCR purification column (Roche, Germany). The treated inserts and vectors were ligated in a 10 µl reaction system consisting of 1 µl T4 DNA ligase and 1 µl 10X ligation buffer (New England Biolab, USA). After overnight ligation

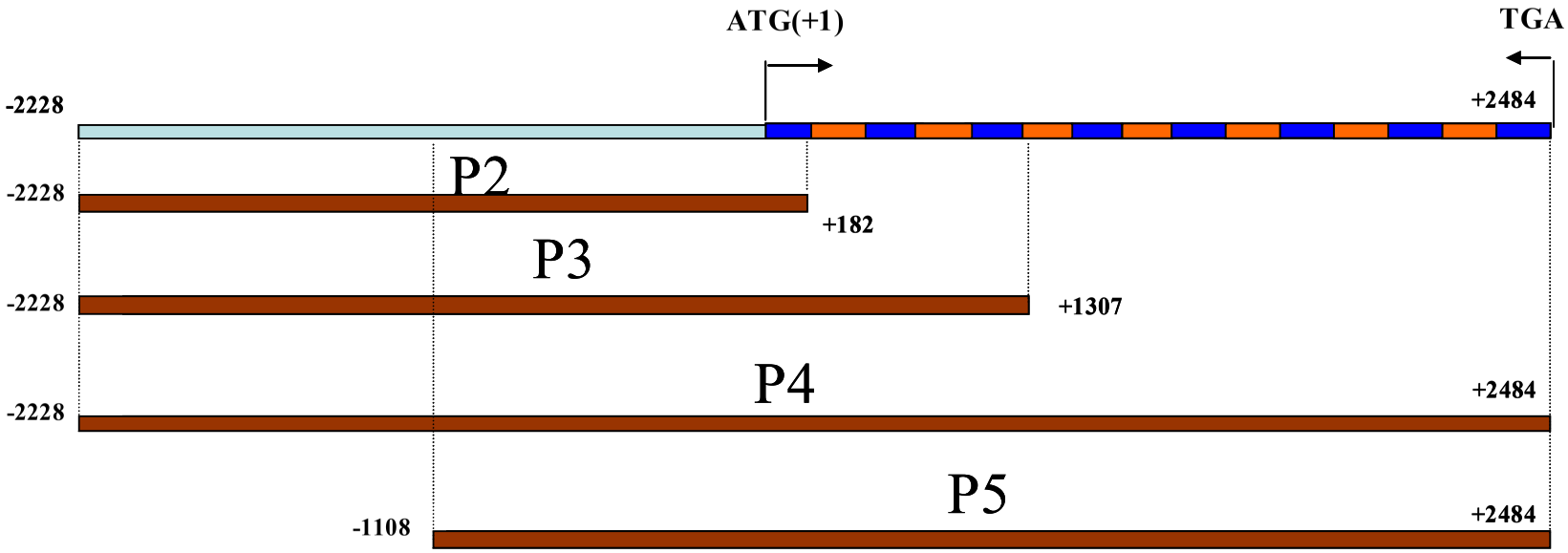

Fig. 3 Diagram showing the fragments of AGL24 genomic sequence used for promoter analysis. The fragments of P2, P3, P4 and P5 for promoter analysis were amplified from the genomic DNA and fused to GUS reporter gene. The genomic sequence of *AGL24* including 2228 bp of 5' upstream region (light blue box), exons (dark blue boxes) and introns (orange boxes). The numbers represent the position relative to the transcription start site (+1).

Table 2. List of primers used to amplify various genomic fragments used for promoter analysis.

construct	Primers		Restriction enzyme used
P2	Forward	5'-CGCGTCGACTCGTTCCTTATAGCGGTGGAT-3'	Sal I
	Reverse	5'-GCTCTAGACTTGAGCTGGAGAACTCGAA-3'	Xba I
P3	Forward	5'-CGCGTCGACTCGTTCCTTATAGCGGTGGAT-3'	Sal I
	Reverse	5'-GCTCTAGACGTAGCTGCTTGGTTTTGTC-3'	Xba I
P4	Forward	5'-AACTGCAGTCGTTCCTTATAGCGGTGGAT-3'	Pst I
	Reverse	5'-GGACTAGTTTCCCAAGATGGAAGCCTAACCAAC-3'	Spe I
P5	Forward	5'-AACTGCAGGATGACGGTGGGAGACGAGTGATC-3'	Pst I
	Reverse	5'-GGACTAGTTTCCCAAGATGGAAGCCTAACCAAC-3'	Spe I

The underlined nucleotides represent the restriction enzyme cutting sites.

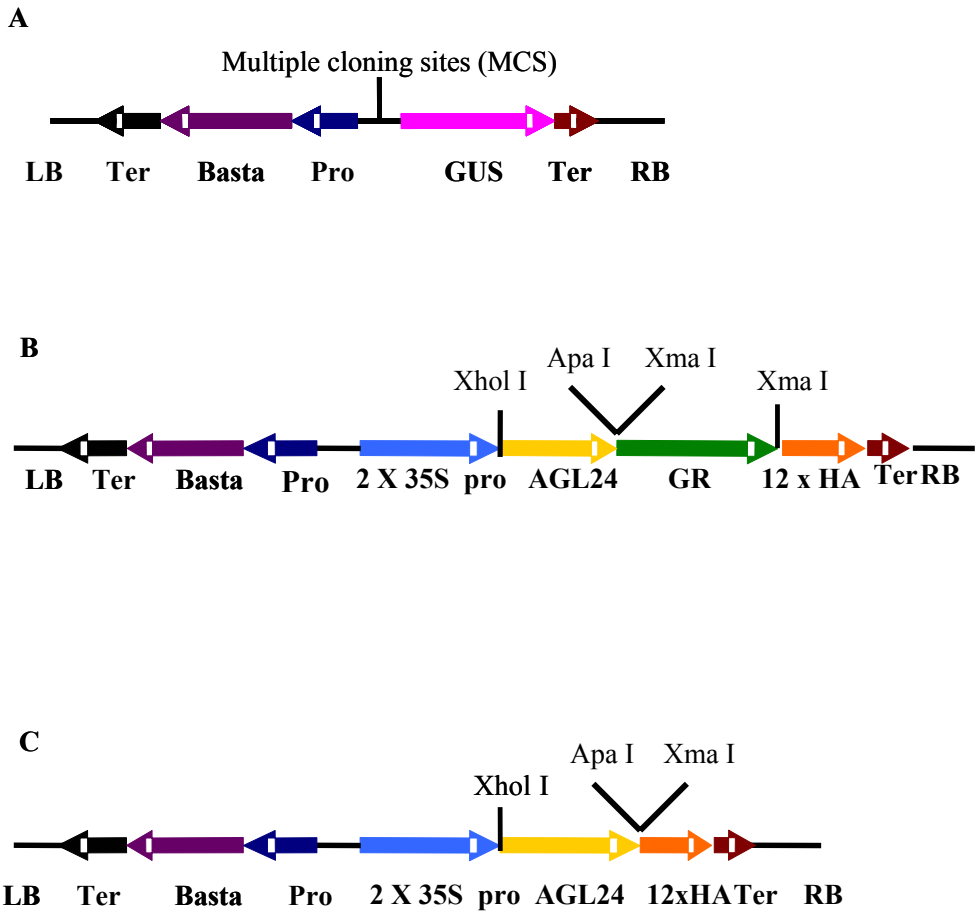

Fig. 4 pGreen vectors used for cloning. (A) pGreen HY107 vector; (B) pGreen 35S-AGL24-GR-12HA; (C) pGReen 35S-AGL24-12HA.

at 16 ºC, 2 µl of ligation product was transformed into E. coli competent cells, which were prepared according to the published protocol (Ausubel *et al*., 1995). A tube containing 50 µl of frozen competent cells was thawed on ice. Two microliters of ligation reaction was mixed with the competent cells and incubated on ice for 30 min. The tube was subsequently incubated at 42 ºC for 90 seconds and placed on ice for 2 min. LB broth was then mixed with competent cells at 37 ºC for 1 hour. Cultured competent cells were precipitated and spreaded on LB agar plates supplied with 50 µg ml^{-1} kanamycin. After incubation at 37 ºC for 16 hours, colonies containing the successfully ligated vectors were selected and cultured overnight at 37 ºC. The plasmids extracted from these bacteria cultures were further confirmed by sequencing.

DNA sequencing was performed by the dideoxy chain-termination method (Sanger *et al*., 1977) utilizing ABI BigDye^{TM} Terminator Cycle Sequencing Kit (Applied Biosystem, USA). About 300 ng of purified plasmid was mixed with 3.2 pmols of forward or reverse primer. The sequencing PCR was performed with 25 cycle of denaturation at 96 °C for 10 sec, annealing at 50 °C for 5 sec, and extension at 60 °C for 3 min. PCR products were then precipitated with 80 µl of 75 % isopropanol, pelleted by centrifugation, washed with 500 µl of 70 % ethanol, and finally air-dried. The DNA pellet was sequenced by the DNA Sequencing Laboratory in the Department of Biological Sciences, National University of Singapore.

2.2.3 Construction of pGreen 35S-AGL24-12HA

To identify the target genes of *AGL24*, ChIP was performed to isolate the protein complex containing the AGL24 protein and promoter sequences of its target genes. Since AGL24 antibody was not available, we firstly produced the AGL24-12HA tag, which can be precipitated by commercially available HA antibodies. The pGreen 35S-AGL24-12HA vector containing the AGL24-12HA tag was derived from pGreen 35S-GR-12HA (a gift from Dr. Toshiro Ito, Temasek Life Science Laboratory, Singapore).

AGL24 cDNA fragments were amplified from the cDNA template (Yu *et al*., 2002) by PCR using forward primer: 5'-CCGCTCGAGGTAGTGAAGGAGAGATCTGG-3' and reverse primer: 5'-ATGGGCCCTTCCCAAGATGGAAGCCCAA-3'. 50 µl PCR reaction system consisted of 2 µl of DNA template, 1 µl of 10 mM dNTPs, 1 ul of 10 mM forward primer, 1 µl of 10mM reverse primer and 1 µl of Advantage^{TM} 2 in 1X PCR buffer (Clontech, USA). PCR was performed by 94 ºC for 2 min of initial denaturation, which was followed by 35 cycles of 94 ºC for 1 min, 60 ºC for 1 min, elongation at 68 ºC for 1 min, and a final extension at 68 ºC for 10 min. After PCR amplification, the resulting PCR products were separated on agarose gels and the expected bands were cut and purified by Gel Extraction Kit (Qiange, USA). The purified products were digested by restriction enzymes *Xho*I and *Apa*I, which were corresponding to the coloning sites in the vector pGreen 35S-GR-12HA.

pGreen 35S-GR-12HA vector was digested by restriction enzymes *Xho*I and *Apa*I and ligated with *AGL24* fragment prepared as described above. This resulted in the generation of pGReen 35S-AGL24-12HA (Fig. 4B)

To produce pGreen 35S-AGL24-12HA (Fig. 4), the pGReen 35S-AGL24-12HA was digested by *Xma*I to delete GR fragment, and self-ligated.

2.3 Plant transformation

The constructed vectors were introduced into *Arabidopsis* via *Agrobacterium*-mediated transformation. Fristly, these constructs were transformed into *Agrobacterium* strain GV3101 by electroporation. The competent GV3101 bacteria mixed with constructs were electroporated in 1mm Gene Pulser® cuvettes (Bio-Rad, USA). The bacteria after electroporation were cultured in 1 ml of LB broth for 4 hours at 28 °C. The bacteria were precipitated and plated on the LB agar medium supplemented with 25 μg/ml gentamycin, 10 μg/ml tetracycline and 50 μg/ml kanamycin. The plates were incubated for 48 hours at 28 °C. The colonies for different constructs were screened by PCR verification using one gene-specific primer and one primer located on the vector. The confirmed colonies with transgene constructs were selected for subsequent plant transformation.

Plant transformation was performed by floral dipping method (Clough & Bent, 1998). The selected *Agrobacteria* strains containing transgenes were cultured in a large scale LB broth containing 25 μg/ml gentamycin, 10 μg/ml tetracycline, and 50 μg/ml kanamycin at 28 °C until OD_{600nm} of the culture reached 0.8. *Agrobacteria* were then precipitated at 4000 rpm for 10 min and resuspended thoroughly in a same volume of 5%

sucrose with 0.015% Silwet L-77 (OSi Specialties, USA). Inflorescences of wild-type plants containing different stages of floral buds were dipped into the *Agrobacterium* suspension for 5 seconds. The inoculated plants were then covered in black plastic bags overnight, which kept humidity and thus promoted transformation efficiency. The inoculated plants were grown under normal growth conditions after removing the covers on the next day.

2.4 Detection of GUS Reporter Gene

The GUS reporter gene was detected according to the pulished protocol (Rodrigues-Pousada *et al*., 1993).

2.4.1 Fixation

The transgenic seedlings carrying various promoter constructs were collected and immersed in 90% ice-cold acetone immediately and incubated on ice for 15-20 min.

2.4.2 Staining

After fixation, actone was removed and fixed tissues were washed three times with the rinse solution consisting of 50mM $NaPO_4$ pH 7.2, 0.5mM $K_3Fe(CN)_6$, 0.5mM $K_4Fe(CN)_6$. The tissues were then incubated in staining solution consisting of 50mM $NaPO_4$ (pH 7.2), 0.5mM $K_3Fe(CN)_6$, 0.5mM $K_4Fe(CN)_6$) and 2mM X-Gluc for overnight at 37ºC .

2.4.3 Dehydration

To remove chlorophyll, tissues were dehydrated through ethanol series solution of 15%, 30%, 50%, 70%, 85%, 95% and 100%.

2.4.4 Observation Under Microscope

All tissues were observed under dissecting microscope (Nikon SM21500, Japan). Tissues were lain on slides with some ethanol. All pictures were captured by the digital camera (DXM1200F, Japan) for microscope.

2.5 ChIP

ChIP assays were carried out as the published protocol with minor modification (Ito *et al*., 1997; Wang *et al*., 2002).

2.5.1 Nuclear fixation

Collected shoot apical meristems were immediately soaked in MC buffer (10 mM KPO_4, pH 7.0, 50 mM NaCl, and 0.1 M sucrose) with 1% formaldehyde that was freshly added. The materials were then infiltrated under vacuum pressure for 90 min at 4 ºC. When vacuum pressure was released, the formaldehyde was quenched by incubating it with 0.15 M glycine for 15 min at 4 ºC. This was followed by washing the fixed tissues with fresh MC buffer three times at 4 ºC for 20 min. After these treatments, the fixed materials were either stored at -80 ºC for future use or applied immediately into following procedures.

2.5.2 Homogenization and sonication

About 1.0 g of fixed materials were ground with mortar and pestle using liquid nitrogen. The ground powder was quickly transferred into a beaker and mixed thoroughly with M1 buffer (10 mM KPO_4, pH 7.0, 0.1 M NaCl, 10 mM beta-mercaptoethanol, 1M 2-methyl-2,4-pentanediol, and 1mM phenylmethylsulfonylfluoride (PMSF)). This slurry was centrifuged at 15300 rpm for 10 min at 4 ºC. After removing the supernatant, the green pellet was resuspended into M2 buffer (10 mM KPO_4, pH 7.0, 0.1 M NaCl, 10 mM beta-mercaptoethanol, 1 M 2-methyl 2, 4-pentanediol, 10 mM $MgCl_2$, and 0.5% Triton X-100), and subsequently centrifuged at 15300 rpm for 10 min at 4 ºC. The supernatant was discarded and the pellet was thoroughly washed with M2 buffer again for other three times. The pellet was resuspended with M3 buffer (10 mM KPO_4, pH 7.0, 0.1M NaCl, and 10 mM beta-mercaptoethanol), and centrifuged at 15300 rpm for 10 min at 4 ºC. The supernatant was discarded and the pellet was subjected to the sonication step.

For sonication, the crude nuclear extract was resuspended in 0.5 ml sonication buffer (10 mM KPO_4, pH 7.0, 0.1 mM NaCl, 0.5% sarkosyl, and 10 mM EDTA), and mixed with 1/3 volume of 106 μm glass beads (Sigma, USA), which were prepared by washing them twice with 1 M HCl for 30 min, 0.1 M HCl for 30 min, and distilled water for 30 min, and then rinsing them twice with the sonication buffer and 5 μl 100 mM PMSF. After resuspension, the nuclear extract with glass beads was then sheared with a probe sonicator (Materials Inc., VC 130 PB Sonicator®). The output power was controlled at about 104 W with each pulse for 15 sec for 12 times. Between two continuous pulses, the chromatin solution was cooled down for 1 min on ice. After

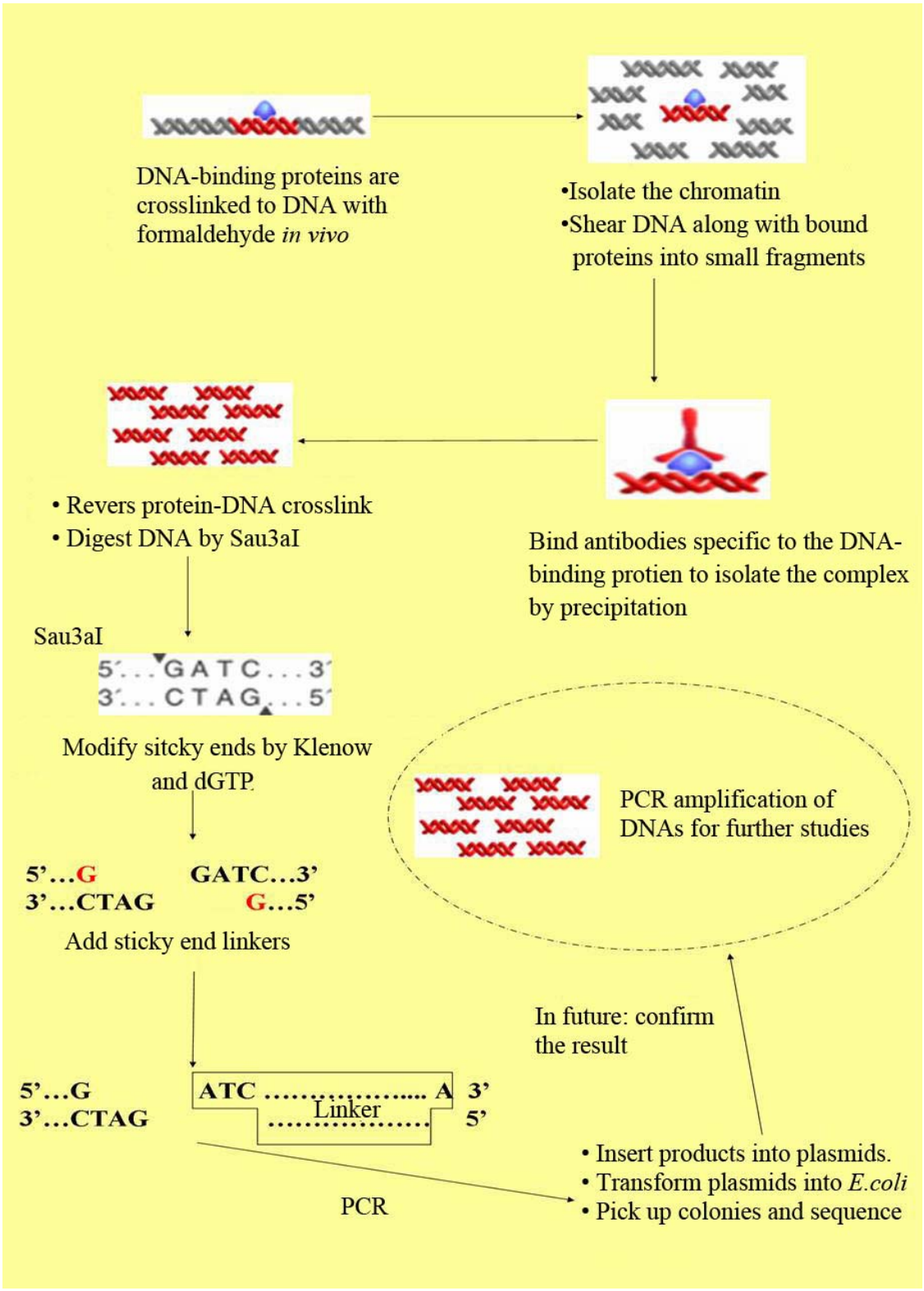

Fig. 5 Flowchart of ChIP work.

sonication, the solution was centrifuged at 14000 rpm for 5 min at 4 ºC and the supernatant was transferred into a new eppendorff tube. About 1/10 of the sonicated sample was saved, from which the DNA recovered was designated as “input DNA”. The remaining sample was subjected to further immunoprecipitation.

2.5.3 Immunoprecipitation

Before incubating the sheared chromatin with the immunoprecipitation column, 30 μl of anti-HA antibody conjugated with agarose beads was washed 5 times with the sonication buffer. After the chromatin solution was incubated with the antibody for 2 h at 4 ºC on a shaker, the mixture was centrifuged at 3500 rpm for 1 min. The supernatant was removed as the “post-bind sample”, while the remaining anti-HA antibody beads were washed 4 times with the sonication buffer with each time for 5 min. Washing by high salt solution was then carried out by using the sonication buffer with 500 mM NaCl for 5 min, which was followed by four rounds of washing with the sonication buffer. The last step of washing was conducted in a new eppendorff tube. To elute, the anti-HA antibody beads were incubated with 300 μl of elution buffer (50 mM Tris, pH 8.0, 1% SDS, and 10 mM EDTA) at 65 °C for 30 min on a shaker. The beads were precipitated at 3500 rpm at room temperature for 1 min and the supernatant was collected. Additional 100 μl of elution buffer was incubated with beads at 65 °C for 5 min to elute more chromatin complex. From a total volume of 400 μl eluted solution, 20 μl was saved for Western blot analysis, and the remaining was used for the DNA recovery step.

2.5.4 DNA recovery

The eluted chromatin complex was first incubated with 1 μl of RNase A (1 mg/ml) at 37 °C for 30 min, and then treated with 0.5 mg/ml Proteinase K at 37 °C for at least 3 hours. After incubation, Proteinase K was added again and the solution was incubated at 65 °C for 6 hours to reverse the formaldehyde crosslink. The released DNAs were subsequently extracted by phenol:chloroform and precipitated with standard protocols. Briefly, an approximately equal volume of extraction solution A (50% v/v Tris saturated phenol, 48% v/v chloroform, and 2% v/v isoamyl alcohol) was mixed with the chromatin solution. After centrifugation, the aqueous layer was collected and then mixed with the same volume of extraction solution B (96% v/v chloroform and 4% v/v isoamyl alcohol). After centrifugation, the aqueous layer was transferred into a new eppendorf tube and precipitated overnight at -20 °C by adding 1 μl glycogen (20 mg/ml), 1/10 volume of 3 M sodium acetate, and 2.5 volumes of absolute ethanol. DNAs were eventually precipitated at 15300 rpm at 4 °C for 20 min and washed with 70% ethanol twice. After the DNA pellets were completely dried, they were dissolved in 50 μl water. The recovered DNAs were further modified with the designed linkers to allow their amplification via PCR.

2.5.5 Linker Modification and PCR Amplification

To facilitate the amplification of precipitated DNAs from ChIP, we modified the recovered DNAs by sticky end linker. Thirteen microliters of co-precipitated DNAs were incubated with 2 units of restriction enzyme Sau3AI (New England Biolab, USA) at 37

°C for 2 h, and inactivated at 65 °C for 20 min. After cooling, 1 μl of 10 mM dGTP and 0.5 μl Klenow Enzyme (Roche, Germany) were added to perform single G fill-in reaction at 37 °C for 10 min. The resulting products were then purified with High Pure PCR Product Purification Kit (Roche, Germany) following the manufacturer's instruction. After purification, DNAs were incubated in a T4 DNA ligase mixture with the pre-made linkers overnight at 16 °C. The linkers were annealed by heating two oligonucleotides: oligonucleotide 1 (5'-ATCGAGATATTAGAATTCTACTCA-3') and oligonucleotide 2 (5'-GAGTAGAATTCTAATATCTC-3') to 95 °C and allowing them to cool down gradually to 16 °C over 2 hours.

After linker modification, the DNAs in ligation mixture were purified by High Pure PCR Product Purification Kit again to remove excessive linkers. To monitor self ligation of linkers, a mock ligation mixture without co-precipitated DNAs was also simultaneously prepared as a negative control.

For PCR amplification, two microliters of modified DNAs were added into a buffered PCR reaction system containing 0.2 mM dNTP, 1 unit of KlenTaq LA polymerase (Sigma, USA), and 0.4 mM linker primer (5'-GAGTAGAATTCTAATATCTCGATC-3'). PCR was performed by denaturation at 94 °C for 3 min, followed by 40 cycles of denaturation at 94 °C for 30 sec, annealing at 58 °C for 30 sec, and extension at 68 °C for 1.5 min, and final extension at 68 °C for 5 min. About 4 μl of PCR products were analyzed on 1% Tris acetated electrophoresis (TAE) agarose gel, and the rest was used for cloning and subsequent sequence analysis.

2.5.6 Western Blot Analysis

To monitor the process of ChIP, the samples collected from several critical steps were subject to Western Blot analysis. For Western blot analysis, 20 μl of protein samples were mixed with 4.5 μl of 6×SDS-PAGE loading buffer (300 mM Tris-HCl, pH 6.8, 12% SDS, 0.6% bromophenol blue, and 60% glycerol) and 2.7 μl of 1 M beta-mercaptoethanol in boiling water for 5 min. The denatured protein samples were then separated on 12.5% (w/v) denaturing polyacrylamide gels and blotted onto immun-Blot^{TM} PVDF membrane (Bio-Rad). The membrane was blocked with 5% non-fat dry milk that was dissolved in PBS buffer (130 mM NaCl, 7 mM Na_2HPO_4, and 3 mM NaH_2PO_4) for 1 hour, and incubated with 1:10000 (v/v) anti-HA alkaline phosphatase conjugate antibody (Sigma, USA) (diluted with PBS buffer supplemented with 0.05% Tween 20) at room temperature for 1 hour. The membrane was subsequently washed three times with PBS buffer containing 0.05% Tween 20 for 15min each. Finally, the membrane was treated with CDP-Star (Roche, Germany) and exposed to CL-X Posure X-ray film (Pierce, USA).

2.5.7 Analysis on Co-precipitated DNA

2.5.7.1 Cloning

PCR products obtained in section 2.5.5 were purified with High Pure PCR Product Purification Kit (Roche, Germany). To clone these purified PCR products into pGEM®-T Easy Vector (Promega, USA), 20 ng DNA was ligated overnight at 16 °C with 50 ng vector in the reaction mix containing 1×T4 ligase buffer and 3 unit of T4 ligase.

The recombinant plasmids were then transformed into competent cells of *E.coli* DH5α as described in 2.2.2.

After cell transformation, the bacteria were plated on the agar medium supplemented with 100 μg/ml ampicillin and 20 μl of 2% X-gal. The agar plates were incubated overnight at 37 °C, and the white colonies were selected for further verification.

2.5.7.2 Verification of clonies using PCR

For verification of colonies containing successfully ligated vectors, each selected colony was suspended in 5 μl LB broth. Two microliters of bacterial suspension were added to a buffered PCR reaction mix containing 0.2 mM dNTP, 1 unit of Dynazyme^{TM} Thermostable DNA Polymerase, 0.2 mM T7 (5'-GTAATACGACTCACTATAGGGC-3') and SP6 (5'-TATTTAGGTGACACTATAG-3') primers. PCR was performed by denaturation at 94 °C for 5 min, 30 cycles of denaturation at 94 °C for 30 sec, annealing at 52.5 °C for 30 sec, and extension at 72 °C for 1 min, and final extension at 72 °C for 5 min. PCR products were separated on a 1% TAE agarose gel by electrophoresis. Colonies with PCR products that had distinguishably different sizes were selected for DNA sequencing, while those with PCR products at the same size were subject to a pre-screening process as described below.

For pre-screening of colonies, 8 μl of PCR products were digested by 0.1 unit of RsaI (New England Biolab, USA) at 37 °C for 20 min. The digested products were separated on a 1% TAE agarose gel. Colonies showing different digestion patterns were

selected for DNA sequencing, while for colonies with a same digestion patterns, only one was selected for further sequencing.

2.5.7.3 Sequence analysis

Plasmids from the selected colonies were purified with Wizard® Plus SV Minipreps DNA Purification System (Promega, USA) according to the manufacturer's instruction. The plasmid sequence was determinded by BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, USA) as described in 2.2.2. The identity of the DNA inserts was identified by comparing the sequence with the published databases in the BLAST program at the web site of National Centre for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov).

2.6 Non-radioactive RNA-RNA *In Situ* Hybridization

Non-radiocactive *in situ* hybridization was performed as described previously (Yu *et al*., 2004).

2.6.1. Synthesis of DIG Labeling mRNA Probe

Both DNA and RNA probes can be used for *in situ* hybridization, but RNA probes show high sensitivity and give strong signals. Thus, we chose RNA probes for all *in situ* hybridization experiments. We cloned a gene of interest into the pGEM®-T Easy Vector (Promega, USA) that contains both SP6 and T7 promoters on the flanks of the

insertion site. To get run off transcripts with either SP6 or T7 polymerase, the cloned plasmids were digested into linearized plasmids by a restriction enzyme, which was able to generate a 3' overhang. An excess amount of enzyme was usually used to ensure a complete digestion. The digestion plasmids were purified by phenol/chloroform to get rid of any RNase contamination, and then resuspended in DEPC-treated H_2O to reach the concentration of 0.5μg/μl.

The RNA probes were generated from the digested plasmids by a digoxigenin RNA labeling kit (Roche, Germany). The 20 μl labeling reaction system contained 1μg template cDNA, 4 μl 5xtranscription buffer, 2 μl 10×DIG labeling Mix, 1 μl RNase inhibitor, 2 μl RNA polymerase. The reaction was incubated at 37°C for 2 hours and added with 2 μl of RNase-free DNase (Roche, Germany) for additional 30 min to remove the DNA template. A small portion of the resulting products could be verified on a 1% TAE agarose gel.

The labeled RNA probes should be partially hydrolyzed to about 150 bp by alkaline treatment to improve permeability to tissue sections. We calculated the reaction time for alkaline treatment with the following formula: Time=(Li-Lf)/0.11*Li*Lf (Li=initial length of probe in kb; Lf=desired final length of probe in kb).

For alkaline treatment, the volume of the transcription reaction was increased to 100 μl by adding DEPC H_2O. The reaction was then incubated with 2×CO_3 buffer (80mM $NaHCO_3$ and 120mM Na_2CO_3) at 60°C for the calculated time, neutralized by 10 μl of 10% acetic acid, and precipitated with 1/10 volume of 3M NaAc (pH 5.2), 2.5 volumes of ethanol, and 2μl of 10mg/ml tRNA. The pellet was rinsed with 70% ethanol,

and resuspended in 80 µl of 50% formamide. The final concentration of probes used in the hybridization solution was 0.5ng/µl/kb.

2.6.2 Fixation of *In Situ* Materials

The fixative for *in situ* materials was freshly prepared by dissolving 4% (w/v) paraformaldehyde in 1xPBS (0.13 M NaCl, 7 mM Na_2HPO_4, and 3 mM NaH_2PO_4, pH 7.0). Firstly, the required amount of 1xPBS was adjusted to pH 11.0 with NaOH. Secondly, PBS solution was heated to 60-70°C in waterbath, and mixed with paraformaldehyde by vigorously shaking. After paraformaldehyde was completely dissolved, the fixative solution was placed on ice until it was cooled to 4°C. Lastly, the solution was adjusted to pH 7.0 by H_2SO_4, and placed on ice for immediate fixation of plant materials.

SAMs of *agl24* mutants and wild type plants were collected and immediately immersed into ice-cold fixative. Vacuum was applied to samples until the fixative started to bubble. After the fixed materials were kept in vacuum for 15 min, the vacuum was released slowly. The above vacuum operation was repeated until tissues began to sink. The fixative was then replaced and the plant samples were incubated with new fixative by shaking overnight at 4°C.

2.6.3 Dehydration, Embedding and Section

After the fixed, materials were washed twice by 1XPBS at 4°C for 30 min each. They were dehydrated and washed by 30%, 40%, 50%, 60%, 70%, and 85% ethanol for 1

hour each at 4°C. In the step of dehydration by 95% ethanol at 4°C, eosin was added to provide tissue staining, which was important for locating samples in the wax. The samples were further dehydrated by 100% ethanol and eosin for 4 times. For histoclear infiltration, the samples were immersed in 25% histoclear and 75% ethanol for 60 min, and were continuously exchanged by 50% histoclear and 50% ethanol for 60 min, 75% histoclear and 25% ethanol for 60 min, and 100% histoclear for 60 min twice. For paraffin infiltration, 1/4 volume paraplast chips were added, and the samples were incubated at room temperature overnight. In the next day, samples were placed at 42°C, until paraplast chips melt completely. We added additional 1/4 volume of chips, until chips completely melted. Samples were then moved to 58°C for several hours. Wax/histoclear was replaced with freshly melted wax overnight at 58°C. On the following three days, wax was changed twice per day. For paraffin embedding, tissues and wax were poured together into a weighing boat. The plant samples were orientated by a syringe needle, which had been warmed up in a flame. The weighing boat was placed in cold water to speed up solidification of the paraffin. The solidified blocks could be stored at 4 °C.

We used are ProbeOn Plus slides (Fisher Biotechnology, USA). They are pre-cleaned and charged with a white frosting on them that allows to sandwich them together in the hybridization/detection steps. During sectioning, continual ribbons were floated on water to get rid off wrinkles. After attaching the ribbons on the surface of slides, the slides were incubated overnight in slide-warmer at 42 ºC. Sectioned tissue could be stored at 4°C for several weeks.

2.6.4. *In Situ* Section and Pretreatment

For deparaffinization, slides were soaked twice in 100% histoclear for 10 min. The slides were rehydrated with 100%, 95%, 90%, 80%, 60%, 30% ethanol and DEPC-treated water for 1-2 min in each step. The slides were then incubated in 2X SSC for 15-20 min. After treated with proteinase K (1μg/ml) in 100 mM Tris pH 8.0, and 50 mM EDTA for 30 min at 37°C, the slides were soaked in 2 mg/ml glycine in PBS solution for 2 min. After washed twice in PBS for 2 min, the slides were teated with 4% (w/v) paraformaldehyde in PBS solution (pH 7) for 10 min, and washed twice with PBS solution for 5 min. The slides were further treated with 0.1 M triethanolamine and acetic anhydride for 10 min. The slides were subsequently washed twice by PBS solution for 5 min, and dehydrated through 30%, 60%, 80%, 90%, 95%, and 100% ethanol for 30 sec in each step. The slides were stored in a container with a little ethanol at the bottom for up to several hours at 4°C before theywere used for *in situ* hybridization.

2.6.5 Hybridization

For *in situ* hybridization, the slides were incubated with the hybridization buffer that contains 0.5ng/μl/kb of RNA probes at 55°C overnight. Every 800μl of hybridization solution contains 100 μl 10X in situ salts, 400 μl deionized formamide, 200 μl 50% dextran sulfate, 20 μl 50X Denhardts solution (warm to 50^{O}C before pipetting), 10 μl tRNA (10 mg/ml), and 70 μl DEPC-treated water. For each pair of slides, 60 μl RNA probes in 50% formamide were heated to 80°C for 2 min, chilled on ice, spun down and mixed with 240 μl of hybridization solution. The probes were then applied on the slides

with one of the following methods. One technique was to apply 150µl probes to each slide, spreading them out over the entire slide with the side of a pipette tip so that all of the tissues were exposed to probes. Then two slides were slowly sandwiched together. Second technique was to put all of the probes in the middle of one slide and slowly massage the other slide on top until the two slides were merged together. Hybridized slides were elevated above wet paper towels in a plastic container that was sealed tightly, and incubated at 50-55°C overnight.

2.6.6 Wash and Detection

Hybridized slides were washed via a series of solutions to reduce background signals. Before washing, 0.2X SSC and NTE (0.5 M NaCl, 10 mM Tris pH 8.0, 1mM EDTA) solutions were pre-warmed to 55°C and 37°C respectively. Hybridized slides were dipped and rinsed in 0.2X SSC and then washed twice fresh in 0.2X SSC solution for 60 min with gentle agitation. The slides were treated with RNase A (20 µg/ml RNase A in NTE) for 30 min at 37°C with gentle agitation, and washed twice in NTE for 5 min at 37°C. The slides were further washed in 0.2X SSC for 60 min with gentle agitation, and incubated in PBS for 5 min at room temperature. The slide s were blocked with 1% Boehringer block (Roche, Germany) in 100 mM Tris pH 7.5, 150 mM NaCl for 45 min on rocker platform at room temperature. After blocking, the slides were incubated in 1.0% Bovine Serum Albumin (BSA) in 100 mM Tris pH 7.5, 150 mM NaCl, 0.3% Triton X-100 for 45 min on rocker platform. Anti-dig antibody (Roche, Germany) was diluted in a ratio of 1:100-500 in 2 ml BSA/Tris/NaCl/Triton solution prepared as in the last step a plastic weighing dish. The slides were sandwiched together and dipped into the antibody solution, which allowed capillary action to pull up solution throughout the slides. After

the slides with antibody were elevated above wet paper towels in a plastic container for 2 hours, the antibody solution was drained on Kimwipes and the slides were separated. The slides were washed 4 times in BSA/Tris/NaCl/Triton solution for 15 min each at room temperature on rocker platform, and further washed twice in 100 mM Tris pH 9.5, 100 mM NaCl, 50 mM MgCl2 for 10 min each to ensure all of detergent was washed off.

To prepare substrate solution to detect in situ signals, 10% (w/v) polyvinyl alcohol (PVA, either 40 kD or 70-100 kD, Sigma, USA) was dissolved in Tris pH 9.5/NaCl/$MgCl_2$ solution to make a Tris-NaCl-PVA stock solution. To detect 30-50 slides, 200 µl NBT/BCIP stock solution (Roche, Germany) was mixed vigorously with 10 ml of Tris-NaCl-PVA stock solution. Around 300 µl solution was applied to each pair of slides, which were sandwiched and kept overnight in a wet plastic container in darkness. In the next day, the slides were separated and rinsed in tap water to stop reaction. They were dehydrated through 70% and 100% ethanol for less than 10 sec in each step, because color products were soluble in ethanol. The slides were air-dried and mounted with 50% glycerol.

3 Results and Discussion

3.1 Investigation of the Regulatory Region of *AGL24*

3.1.1 GUS Constructs

To study the regulatory region of *AGL24*, different *AGL24* promoter::β-glucuronidase (GUS) gene fusion constructs were generated. Genomic sequence of *AGL24* is around 4.5 kb, which is composed of 8 exons, 7 introns, and 2.2 kb of 5' upstream sequence (Fig. 6). Sequence analysis of *AGL24* genomic region revealed the presence of some *cis* elements thought to be the binding sites of certain important regulatory proteins in plant development. As shown in Figure 6, several putative CArG box motifs have at least a nine out of ten match with the core consensus binding sites of MADS-box proteins (Dolan & Fields, 1991, Treisman, 1992). Several putative DNA-binding core sequences (CCANTGK) of the *LFY* gene are also present in the *AGL24* gnomic sequence (Matinspector analysis result). We also found that *AGL24* genomic sequence had *ABA INSENSITIVE PROTIEN 4 (ABI4)*, *TCP CLASS 1 TRANSCRIPTION FACTOR (ATTCP20)* and *CAACTC REGULATORY ELEMENTS, GA-INDUCIBLE (CARE)* binding sites. Moreover, there were 4 *CA-RICH ELEMENT (CARICH)* transcription binding sites and 8 *CIRCADIAN CLOCK ASSOCIATED 1 (CCA1)* binding sites.

A lot of studies have shown that a regulatory region is not always located in the upstream promoter region of a gene. For example, the second intron sequences of *AGAMOUS (AG)*, a critical regulator of floral reproductive organs, are sufficient to confer the same expression as *AG*, indicating the *AG* expression is regulated via the *cis*-

cgttccttatagcggtggattccgactgtttcaccgcgagtttggttaag -2178
P2, P3 and P4 forward primer -2228

tctactgatcgccgatcggtctcgtcttttgtgtgtctggtggtgaggt -2128
ggttcacgttttaccatttgccgtcgttatcgtgaagcttcttcatgaga -2078
cggagggttctgtgtttttgtgaattatgatttcttgttcttatatgggc -2028
CArG box

ctatttttaagacatcaatatggcccaaatttcgaacttgttatgagttt -1978
CArG box

aaggaaataagtagtaagtactataaatgatggttcgatctcggaggaga -1928
aaaaaaaaaacattgtttacgaggaagcaaaatgtgagttgatataaagg -1878
gtacaacacataatttatttttggaagtcaaaactttgaggattaagctg -1828
acaacgaaggttagtgaagactttcgggatcgagcaatcgggagatatac -1778
atgagcctagagggctgacaagatgaccaagcattccaaatgaaaggctt -1728
aagatttttctttttctaaactcaagtaagaaacacaagatatatgaaag -1678
ggtaacaagggtcaacaacaagtctaagcttttaaacgtgttagatgat -1628
tcttcttgaacactattacaattactgtttagtttcacatttatatgacc -1578
ttgggagtcttctagctcgtcccaaatatattttcaacatattactataa -1528
gatcctaaagaccaataacattgatctacaccaaaaactctcactttctg -1478
CCAAT-box

attttgcactcgctttttttcctcccataaacaaaaccaaaggcttacaa -1428
tactaaatctgtctcacattcttagtgcttatttgttttagtcataaaga -1378
acttaatcttatacagattgaagtcttaaagtcatctatattacttttca -1328
catgtatcattatgagatggtacgtttcccacgaatttatcagtttagt -1278
ttaattttcagttgtactttgggagaaaaaatttacaagatacttgtcgg -1228
ccatgatatcaccctagagttaccggagtccggtgatatatcatttctaa -1178
CArG box

ttagggttaaaacttaaaagggtataaatggctgatcaaacccaaaaata -1128
CArG box

aaagataatgatgacggtgggagacgagtgatcttatcaggtgtcgcatc -1078
P5 forward primer -1108

tagcatatataggtgaaagactataaaaaagacatgaaatatttaataga -1028
CArG box

cacaacttttgtaataaaccaaaaccaaaaaggtagatgaactgatgaac -978
agcatcttctaattacgaataaaaaaagtaaccaaactttctttccatta -928
gaattggtacgtagttccttgtgtattgtgatttctttcattttccaatt -878
CArG box

atgtttttttatttatcatgttacatttttgatagtgggtaacttttgt -828
atcattttatttgacctagccatatataaatctattaacttatacggagt -778
agtatttcacgtcatttatttttatttgtttttagatgggaagttattc -728
aaaactagactaaaacagtaaaactaggaaacccgctactgaataaagtt -678
acaattccacattattccatgacagactaattgaattagaaggttaggta -628

```
aattattaaatcataactgtagcagtctcttcgtctggcagctcagtcag      -578
acaaaacacaaagtgtgtttatgtgttatttttaatgattatagtttggg      -528
aaaaagacataatcaaaagggatacaaaacatatggcccattgataagta      -478
                                      LFY binding site

tagatcactgtttagctaaaaaaagcagactctttttccaatcttgaac      -428
                                           CCAAT-box

acaaacacagtcaccatctctctctctttctctctcactcacacatta      -378
gggagtaaacagctaccagaaaaaccttttttatcttctcacaaatttaa      -328
taaagtgggtgctgagattgaataacgtaatccaagatcctccaactcac      -278
agaaaggtaaaagctgtgaatctgtgttctttcttcttaagcaaagtgtt      -228
tgatgaattcatctagtcctgtccattcttttgcttctcatggtttatgg      -178
atctgatctctctttctctctctctctagccattagggtttcctaagaat      -128
attatataaactctctttagctaacaccgttccaattggtttctttcttt       -78
                                 CCAAT-box

gttcttggtctaaaatctaaatggtgttatgggtataggcagattcaaga       -28
                                     TATA box

acagtagtgaaggagagatctggtaaaATGGCGAGAGAGAAGATAAGGAT       +23
AAAGAAGATTGATAACATAACAGCGAGACAAGTTACTTTCTCAAAGAGAA       +73
GAAGAGGAATCTTCAAGAAAGCCGATGAACTTTCAGTTCTTTGCGATGCT      +123
GATGTTGCTCTCATCATCTTCTCTGCCACCGGAAAGCTCTTCGAGTTCTC      +173
CAGCTCAAGgtatattctatcttttgttagtagttgtcttatttttttc       +223
P2 reverse primer +182

aatccatgtttgtgtttttgagaatatggttggataaatatattaagata      +273
tgtatttaaatgagatttttatttctcgtttactctctaaagttaatta       +323
tcagtaggctcggagatctcatgtacggcataatttgatgacctaaatta      +373
ttatactttaaagtataggattgatgttttattacttttatgtataacac      +423
atcatgtatttaattccgtttaacataatatgggtttttaacgtgtaatt      +473
                               CArG box

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taagagagcagaatattatagtgtgatttttgttaattaggaaagcatat      +573
gtatatatggatacatagtacttaccacaattagaatgaatttcttttcc      +623
ctttttttcatttgactttgtgtattacaaaagtctttgacactgtcactt      +673
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gcattaccataattgggaaacgagtcatctgtctgtatcgtgatggctac      +773
       CArG box

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ggattgtttgttgaaacgtgtagAATGAGAGACATATTGGGAAGGTATAG      +923
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TCCGGgtattttcgatatcacttactctttttttttttgtggattttaa      +1023
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```

tagctgtaagtctaaatatagaaacattgagtggcatataatcattaatc +1173
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ctgttgactttgcatctagcagCTTGAGAATTGTAACCTCTCCAGACTAA +1273
GTAAGGAAGTCGAAGACAAAACCAAGCAGCTACGgtatggctccattgat +1323
P3 reverse primer +1307 LFY binding site

atgttatgcagataaacctattttcatataggctatagctgtaagagatc +1373
atctatttcatgtgtgtggtttttttttttatgtttttttcaatgatgtgt +1423
gcatgctatttttaggttttagaatctatttcatggaaattgaagatatt +1473
tcatttcacgtgtaagttcgtcaagttgtggcgtgtgtcttggaaattga +1523
tgttttgtttgtagattttaagagctacttctaaaatttacaagagtttt +1573
gtaattttcaattatggcccattattctcattaattcattaaaaaaatta +1623
CArG box

tatacattactatctatatctagcataggtagtttttttttttcttttttct +1673
ttggtagacctactgaacaaatatctgatatatcactgactggataaata +1723
tctatagagatatttttgatagaaatgagtgttaatttaacgtaaaacag +1773
GAAACTGAGAGGAGAGGATCTTGATGGATTGAACTTAGAAGAGTTGCAGC +1823
GGCTGGAGAAACTACTTGAATCCGGACTTAGCCGTGTGTCTGAAAAGAAG +1873
gtttactactatacataaactaatagcatgcatattttccttaacgtggc +1923
atataaataataagctgtacatatataaaagtttgactttgttgttgtta +1973
ttggtaaatagGGCGAGTGTGTGATGAGCCAAATTTTCTCACTTGAGAAA +2023
CGGgttagtagttagtacatacaattcgtataactaatggatcataagcc +2073
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TGGATGAGAATAAGAGACTGAGGGATAAAgtacggctctaaacccttata +2173
gatatcatggaataaccttAatctatttttttatgtataagaaaatatga +2223
tgagggaacgtatattatatatcggcagCTAGAGACGTTGGAAAGGGCAA +2273
AACTGACGACGCTTAAAGAGGCTTTGGAGACAGAGTCGGTGACCACAAAT +2323
GTGTCAAGCTACGACAGTGGAACTCCCCTTGAGGATGACTCCGACACTTC +2373
CCTGAAGCTTGGgtataatttgtttaactgaacatatttcaaacttttg +2423
ttgacattttgtatgtggatgtttactaactgtttgttggttagGCTTCC +2473
ATCTTGGGAA**TGA** +2487
P4 and P5 reverse primer +2484

Fig. 6 Genomic sequence of *AGL24*. Black letters indicate upstream sequence. Orange captial letters present exons and blue letters present introns. The transcription start site and stop codon are bold. The CCAAT-box and TATA box are underlined. The putative MADS-box genes and *LFY* binding sites are indicated.

elements in the second intron (Deholos & Sieburth, 2000). Therefore, for the investigation of *AGL24* regulatory region, we included its upstream putative promoter region, exons, and introns in the promoter GUS constructs.

3.1.2 Transgenic Plants

As shown in Figure 7, the phenotypes of transgenic plants containing different promoter constructs usually showed no apparent differences from wild-type plants, indicating that the insertion of transgenes have not affected normal plant development and these transformants could be used for monitoring promoter functions. After transforming *Arabidopsis* wild-type plants with different *AGL24* promoter constructs, we identified different number of transgenic plants bearing these constructs at the T1 generation (Table 3). After they were segregated at the T2 generation, we identified several transgenic plants with only single-insertion of the transgene for each construct, based on their segregation ratio. Since the insertion locus in the chromosome would sometimes affect the GUS expression in the transgene, the selection of single-insertion transgenic lines could reduce this possibility to the minimal level.

3.1.3 GUS staining results

As a promoter of flowering, *AGL24* mRNA is present in all of the tissues with the strongest expression in stems (Yu *et al*., 2002). During floral transition, the *AGL24* expression gradually increases in the inflorescence SAM. However, once floral meristems are generated on the flanks of the inflorescence meristem, *AGL24*

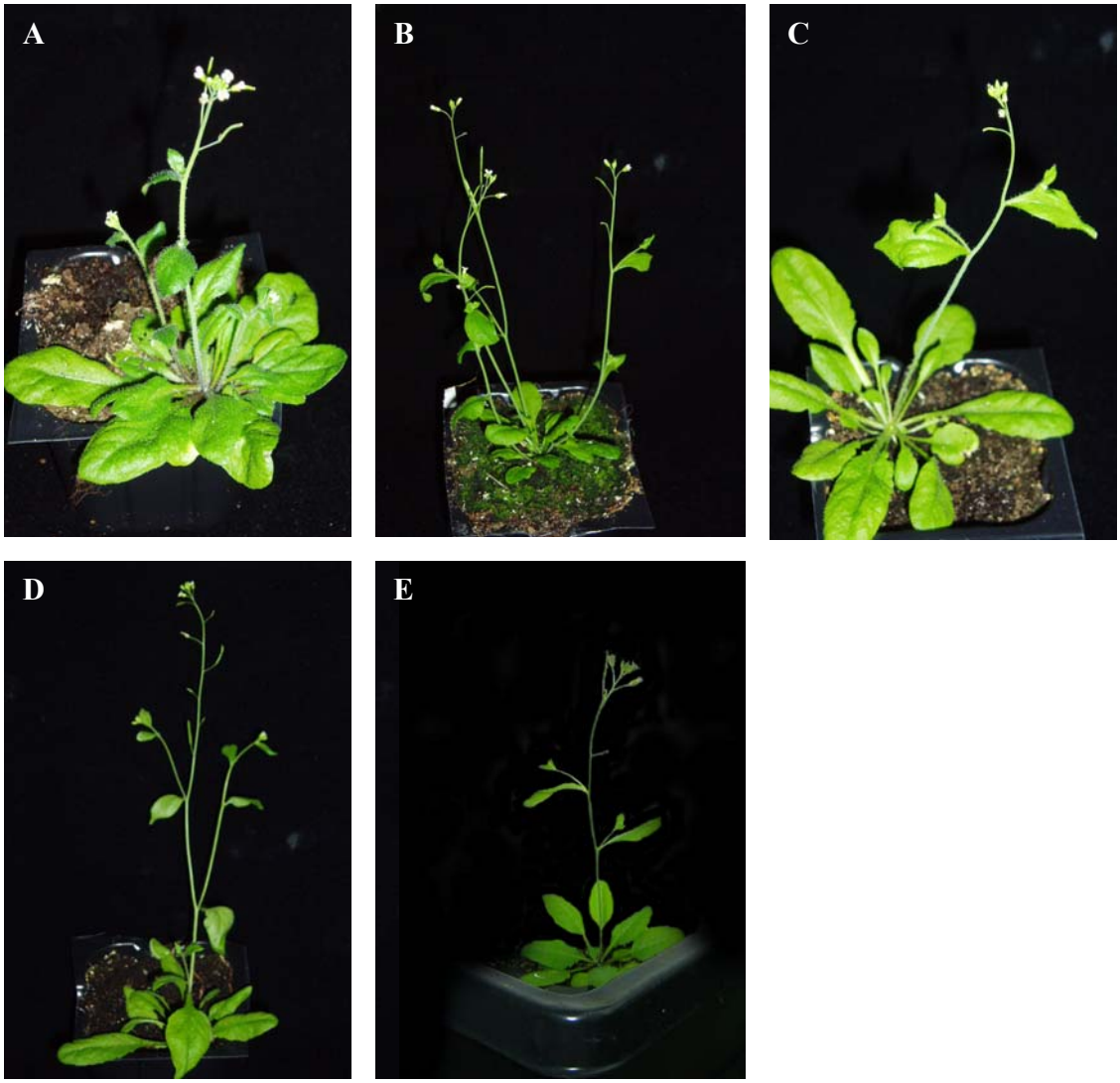


Fig. 7 Transgenic plant containing different AGL24 promoter:: GUS constructs. (A) a AGL24-P2 transformant; (B) a AGL24-P3 transformant; (C) a AGL24-P4 transformant; (D) a AGL24-P5 transformant; (E) a wild-type plant

Table 3 Isolation of transgenic plants containing different promoter constructs

Construct	No. of T1 transformants identified	No. of single-insertion transformants used for further studies
P2	42	15
P3	39	14
P4	28	14
P5	35	13

expression is dramatically reduced in floral meristems (Yu *et al*., 2004). With these expression patterns as a reference, we compared the GUS expression in transgenic plants containing different *AGL24* promoter constructs. GUS staining patterns was examined in at least 10 lines of independent transgenic plants for each promoter construct. Although slightly varied intensity of staining was observed in the lines carrying the same construct, the patterns of GUS staining were usually consistent.

In AGL24-P4 plants, the GUS reporter gene was driven by a 4.7 kb sequence including 2 kb upstream promoter and 2.7 kb intragenic sequence of *AGL24* (Fig. 3). GUS expression was firstly detected at the SAM and young leaf primordia of AGL24-P4 seedlings 8 days after germination (Fig. 8A). The GUS expression gradually increased in the same regions from 8 to 16 days after germination (Fig. 8 B-E). The GUS expression reached the highest level on 16 days after germination (Fig. 8E). It was obvious that GUS expression in AGL24-P4 plants were only limited in the SAM and the coupled young leaf primordialm, but not in adult leaves. These GUS expression patterns were consistent with the *AGL24* exprssion during floral transition of *Arabidopsis*. When AGL24-P4 plants stared to bolt 18 days after germination, GUS expression was dramatically reduced in floral buds (Fig. 9A). The staining was only obvious at the apical region of the inflorescence and coupled cauline leaves (Fig. 9A). During inflorescence stem elongation from 20 to 25 days after germination, GUS expression was mainly confined in the provascular strands of the inflorescence stem and in anthers of mature flowers (Fig. 9B-D). In general, the GUS expression pattern in AGL24-P4 plants completely mimicked the endogenous *AGL24* expression, suggesting that the P4 fragment contains all the *cis*-elements required for the regulation of *AGL24* expression in wild-type plants.

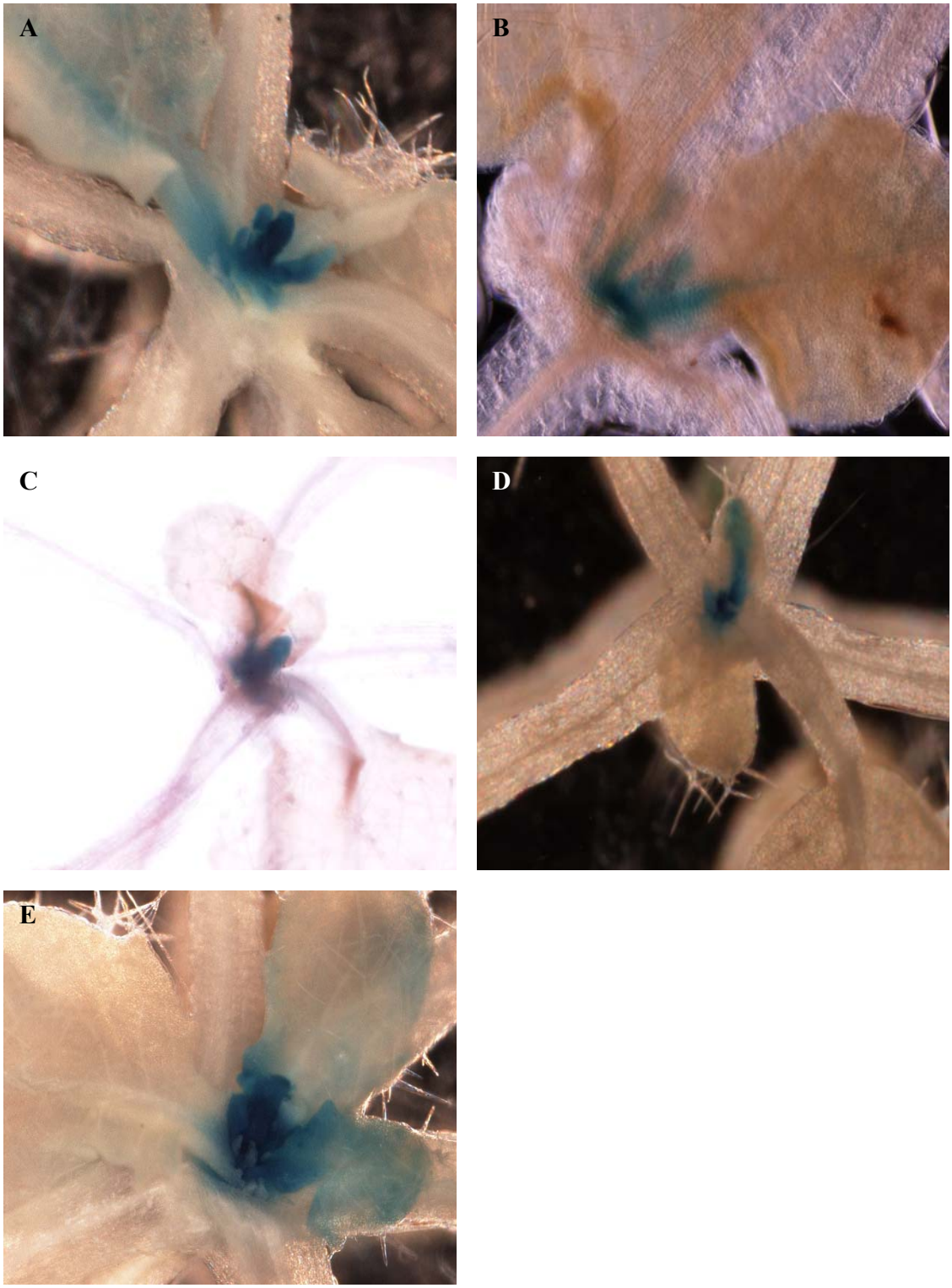


Fig. 8 GUS expression in AGL24-P4 plants. (A) 8 days after germination; (B) 10 days after germination; (C) 12 days after germination; (D) 14 days after germination; (E) 16 days after germination.

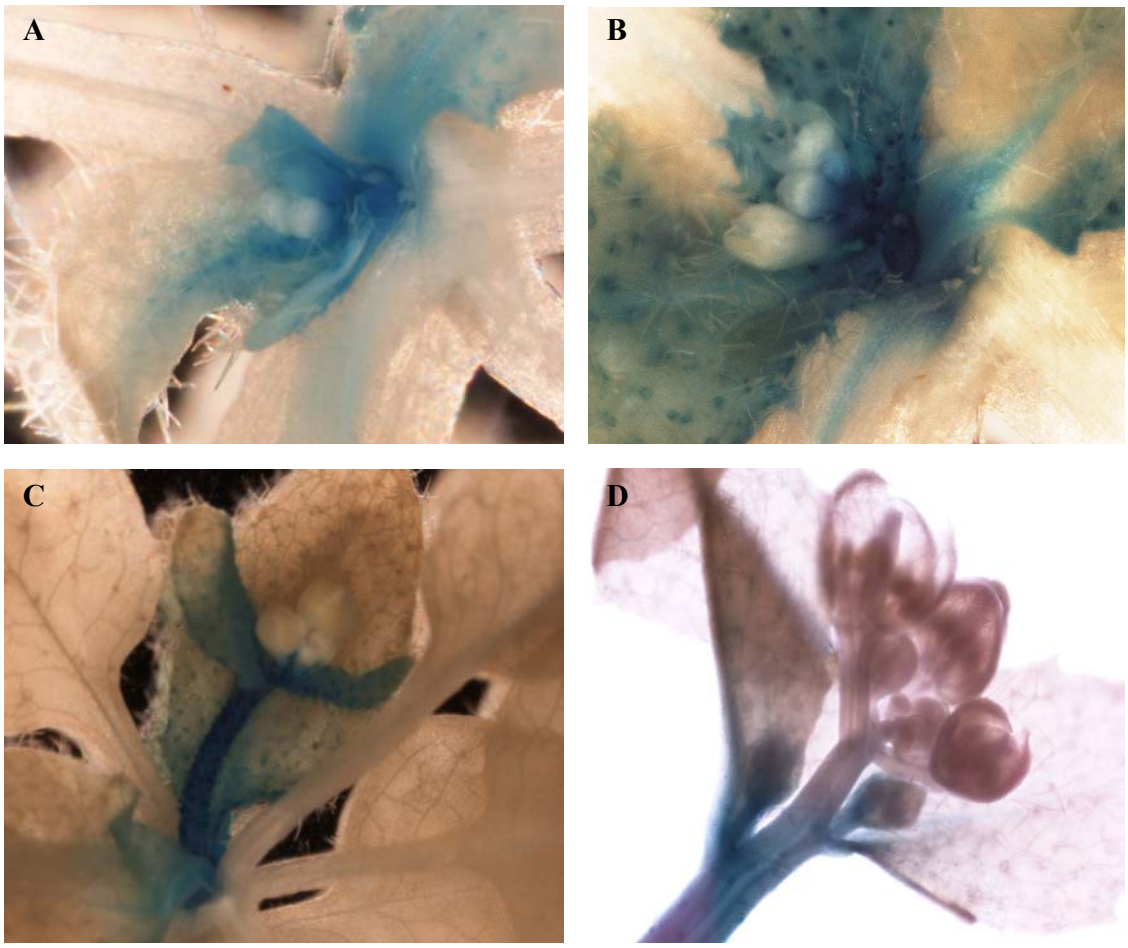


Fig. 9 GUS expression in AGL24-P4 plants. (A) 18 days after germination; (B) 20 days after germination; (C) 22 days after germination; (D) 25 days after germination.

In AGL24-P5 plants, the GUS reporter gene was driven by a 3.6 kb *AGL24* genomic fragment including 1 kb upstream promoter and 2.6 kb intragenic sequence (Fig. 3). The only different between AGL24-P4 and AGL24-P5 was that AGL24-P5 lacked the upstream promoter region from -2228 to -1109. In transgenic plants harboring the AGL24-P5 construct, GUS expression was barely detectable in the seedlings from 8 to 12 days after germination (Fig. 10A-C). On 14 days after germination, we could detect the GUS expression at the SAM and young leaf primordia (Fig. 10D). This expression was further enhanced in the seedling 16 days after germination (Fig. 10E) The GUS expression patterns in AGL24-P5 plants at later developmental stages (Fig. 11A-D) were similar to those in AGL24-P4 plants. The different GUS expression between AGL24-P4 and AGL24-P5 transformants implies that the promoter region from-2228 to -1109 may be responsible for the initial regulation of *AGL24* in the control of flowering time.

In AGL24-P2 construct, the GUS gene was fused with 2 kb upstream promoter and the first exon of AGL24 (Fig. 3). In transgenic plants with the AGL24-P2 constructs, GUS expression was early detected in the seedling 8 days after germination (Fig. 12A). However, its expression was neither at the SAM nor at the young leaf primordia. The irregular distribution of GUS expression at this stage and the total loss of its expression in the seedlings at later stages (Fig. 12B-E) strongly suggest that the *AGL24* 5' upstream promoter sequence is not sufficient to provide the normal regulation of *AGL24*.

Compared with the AGL24-P2 construct, the AGL24-P3 construct contained additional two introns and two exons after the first exon (Fig.3). The GUS expression in AGL24-P3 transformants was in great contrast to that in AGL24-P2 transformants. In AGL24-P3 transformants, Gus expression was early detected in the seedling 8 days after

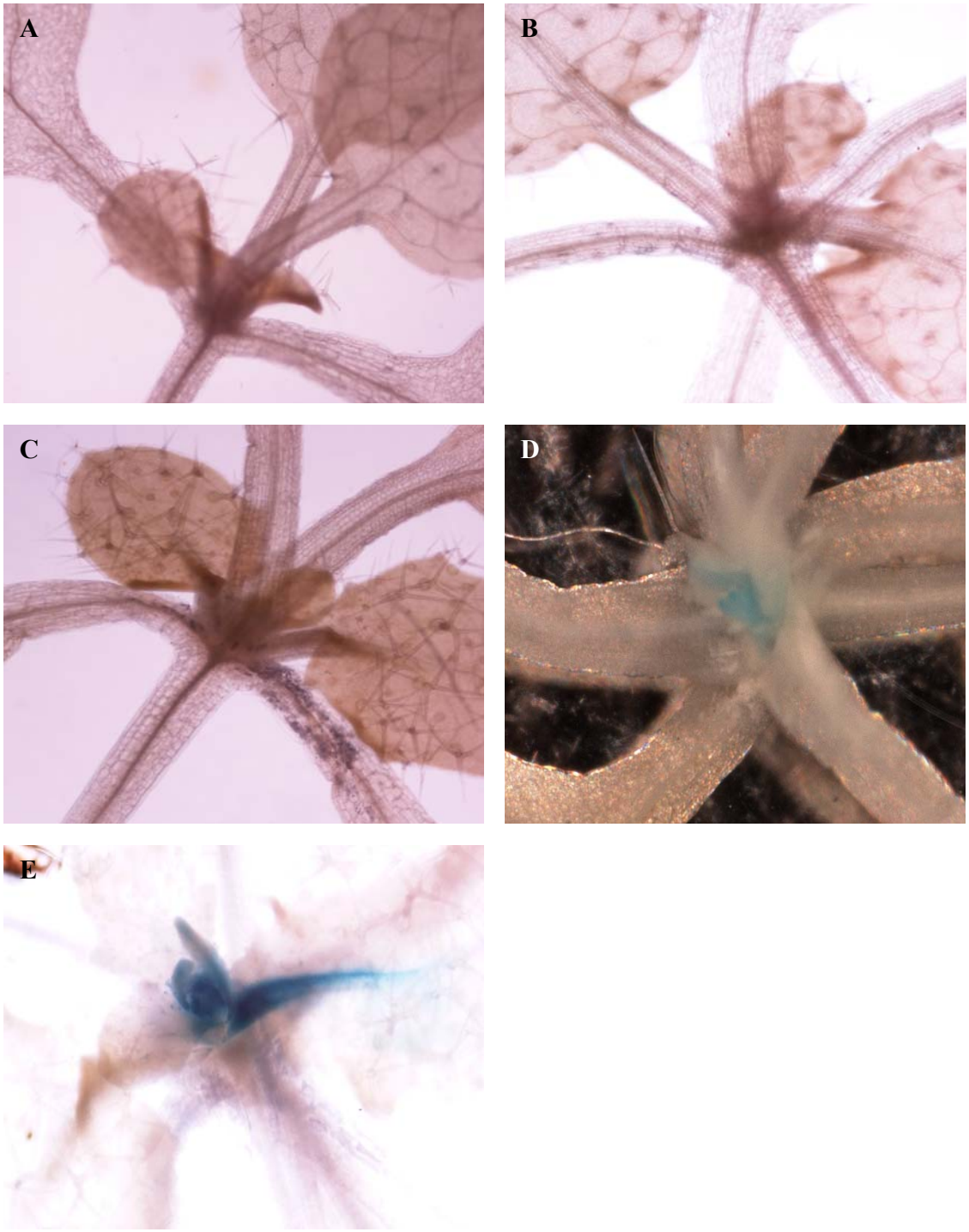


Fig. 10 GUS expression in AGL24-P5 plants. (A) 8 days after germination; (B) 10 days after germination; (C) 12 days after germination; (D) 14 days after germination; (E) 16 days after germination.

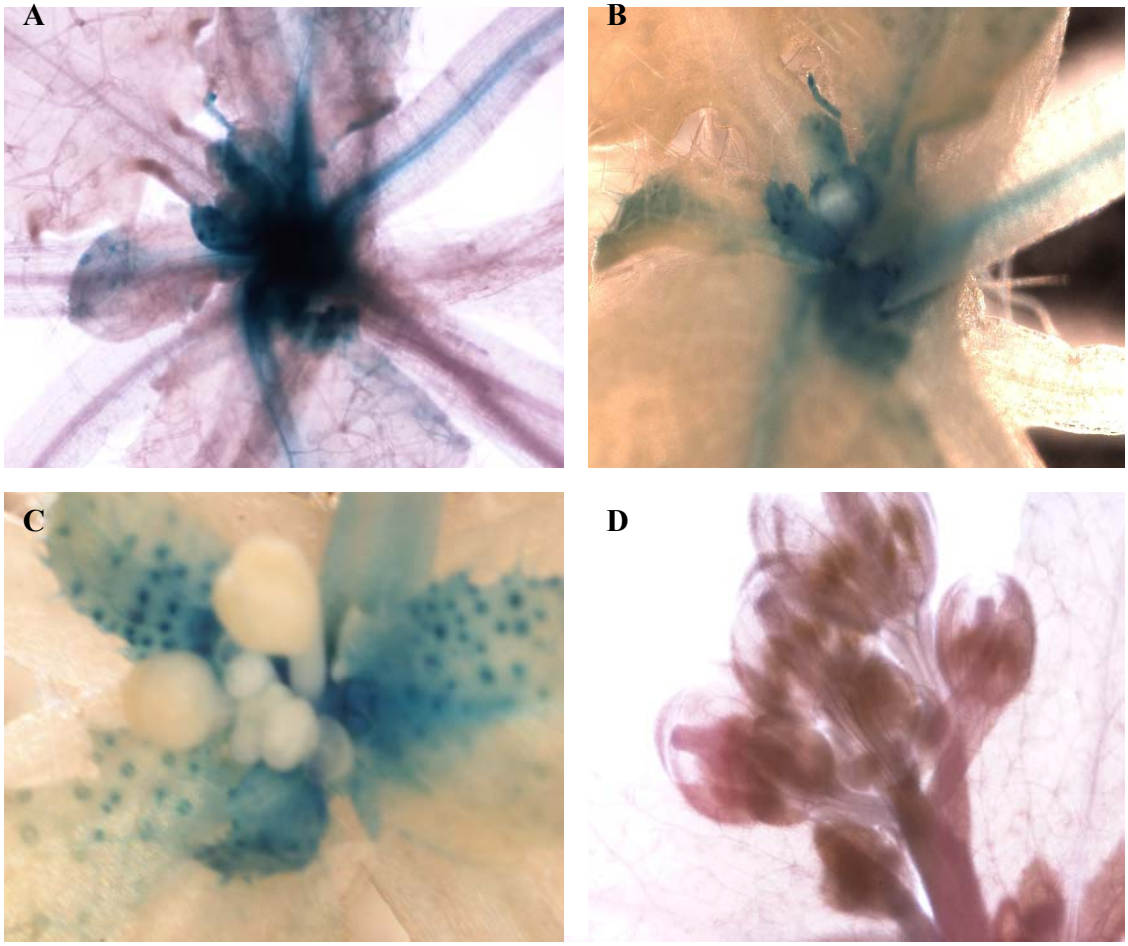


Fig.11 GUS expression in AGL24-P5 plants. (A) 18 days after germination; (B) 20 days after germination; (C) 22 days after germination; (D) 25 days after germination.

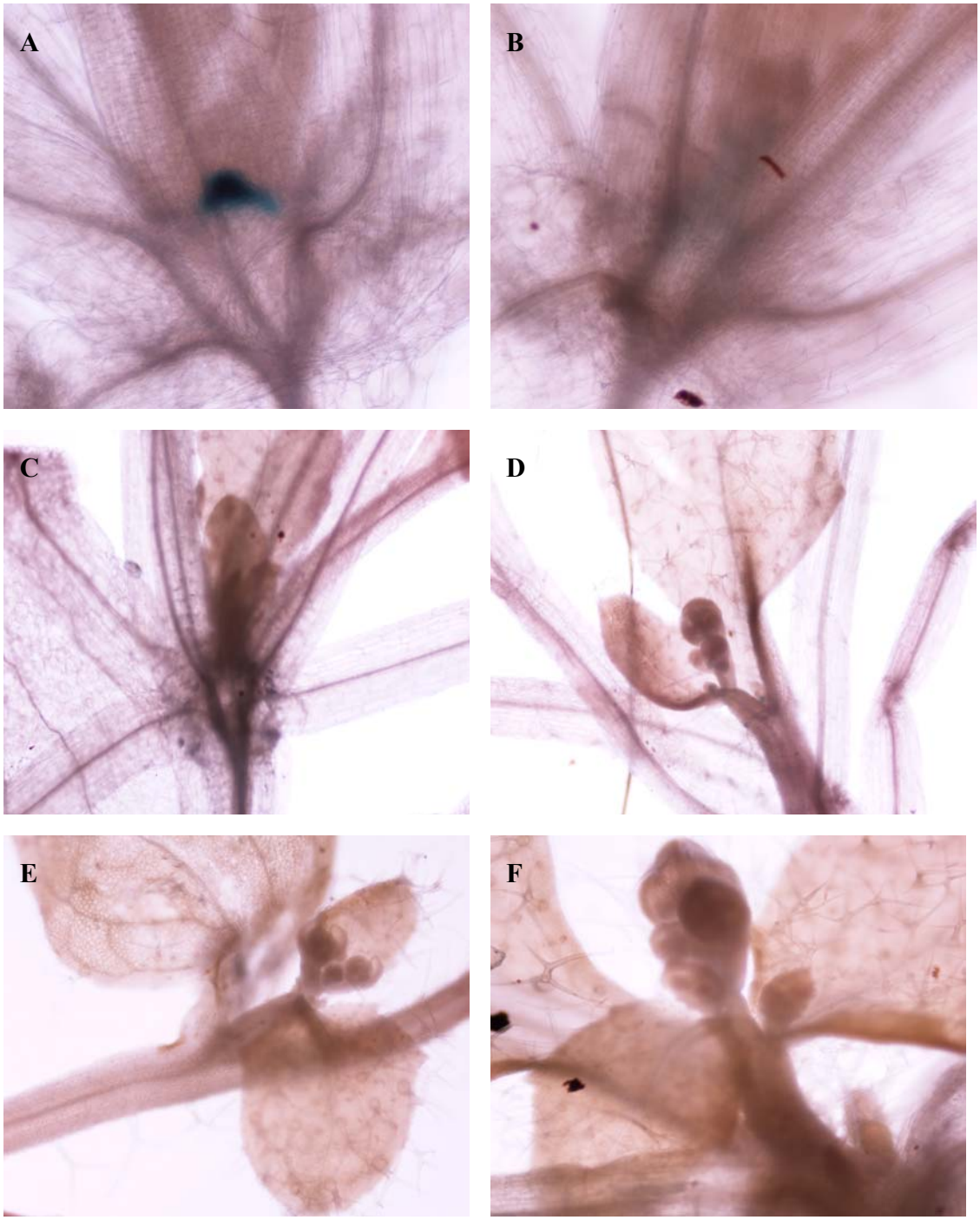


Fig. 12 GUS expression in AGL24-P2 plants. (A) 8 days after germination; (B) 10 days after germination; (C) 12 days after germination; (D) 16 days after germination; (E) 18 days after germination; (F) 20 days after germination.

germination in a similar pattern as that in AGL24-P4 transformants (Fig. 13A). Before 16 days after germination, the GUS expression in AGL24-P3 was almost similar to the patterns in AGL24-P4 transformants (Fig. 13B). However, on 16 days after germination, the GUS expression pattern was not consistent in different lines of AGL24-P3 transgenic plants. Some lines showed very strong GUS expression throughout the SAM and the coupled young leaf primodia (Fig. 13C), while some lines showed weak GUS expression (Fig. 13D). On 20 days after germination, GUS expression was usually high in the inflorescence stalk and the coupled young flowers (Fig. 13E). These results suggest that the region from +182 to +1307 containing exons 1 and 2 as well as introns 1 and 2 is necessary to confer the initial and enhanced *AGL24* expression in a correct spatial and temporal pattern during floral transition, while the region emcompassing the intragenic region after exons 3 of *AGL24* gene may be capable of maintaining *AGL24* expression during floral transition and regulating *AGL24* in floral meristem development.

Comparison of GUS expression pattern in AGL24-P2, -P3, -P4 and –P5 provided important insights for the regulation of *AGL24* expression (Fig. 14). First, the regulatory region required for conferring the full spectrum of *AGL24* expression was in the sequence from -2228 to +2484 as demonstrated by GUS expression in AGL24-P4 trangenic plants (Fig. 8A-E). Second, the different GUS expression pattern revealed in AGL24-P4 and AGL24-P5 transformants indicates that the region between -2228 and -1108 contains basic *cis*-elements required for the initial expression of *AGL24* during floral transition. Deletion of this region caused the significant delay of *AGL24* expression in the SAM and the coupled leaf primordia, as shown by GUS expression in AGL24-P5 transformants (Fig. 10A-E). Third, the region from +182 to +1307 seems crucial for providing normal

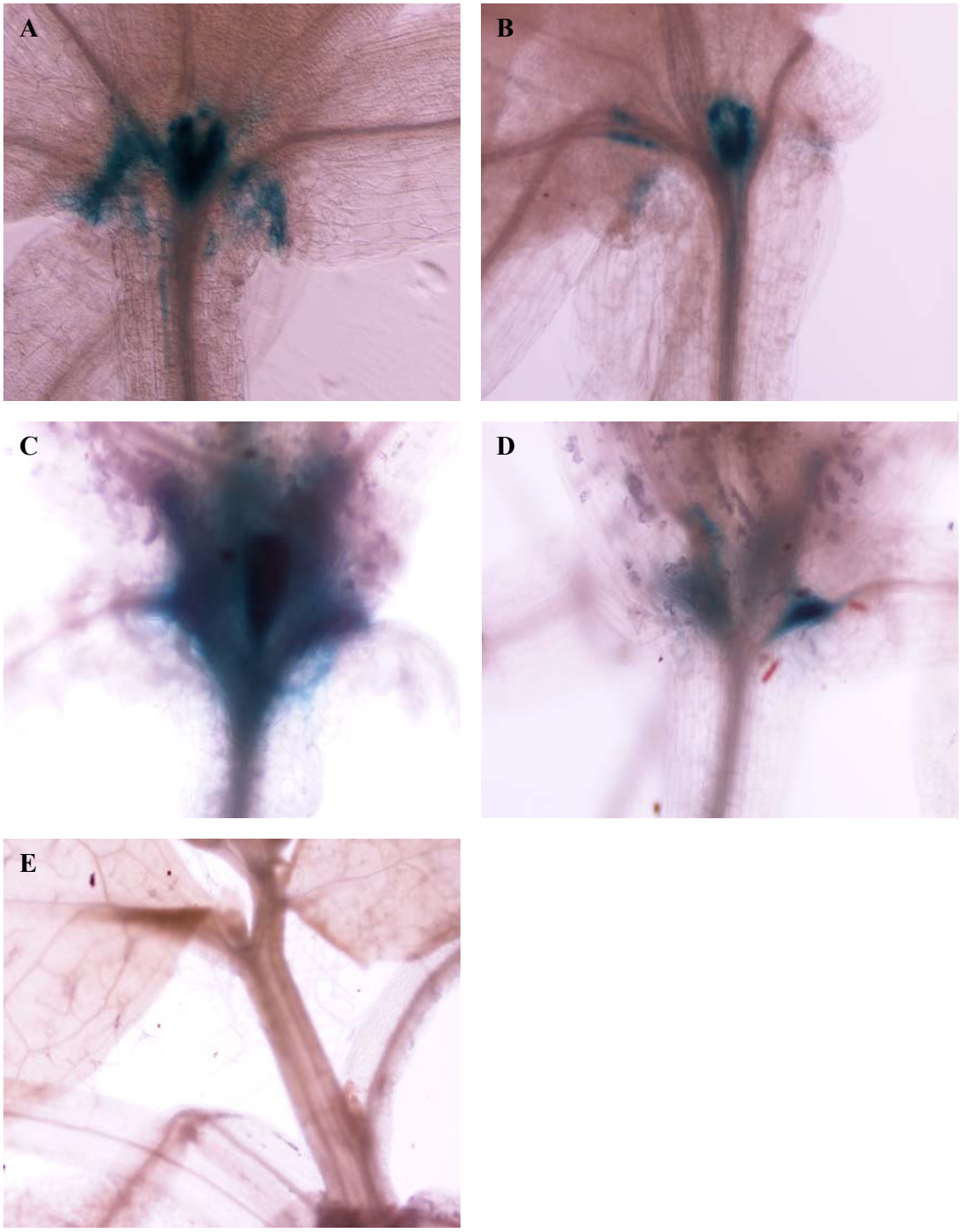


Fig. 13 GUS expression in AGL24-P3 plants. (A) 8 days after germination; (B) 10 days after germination; (C) 16 days after germination (strong expression); (D) 16 days after germination (weak expression); (E) 20 days after germination.

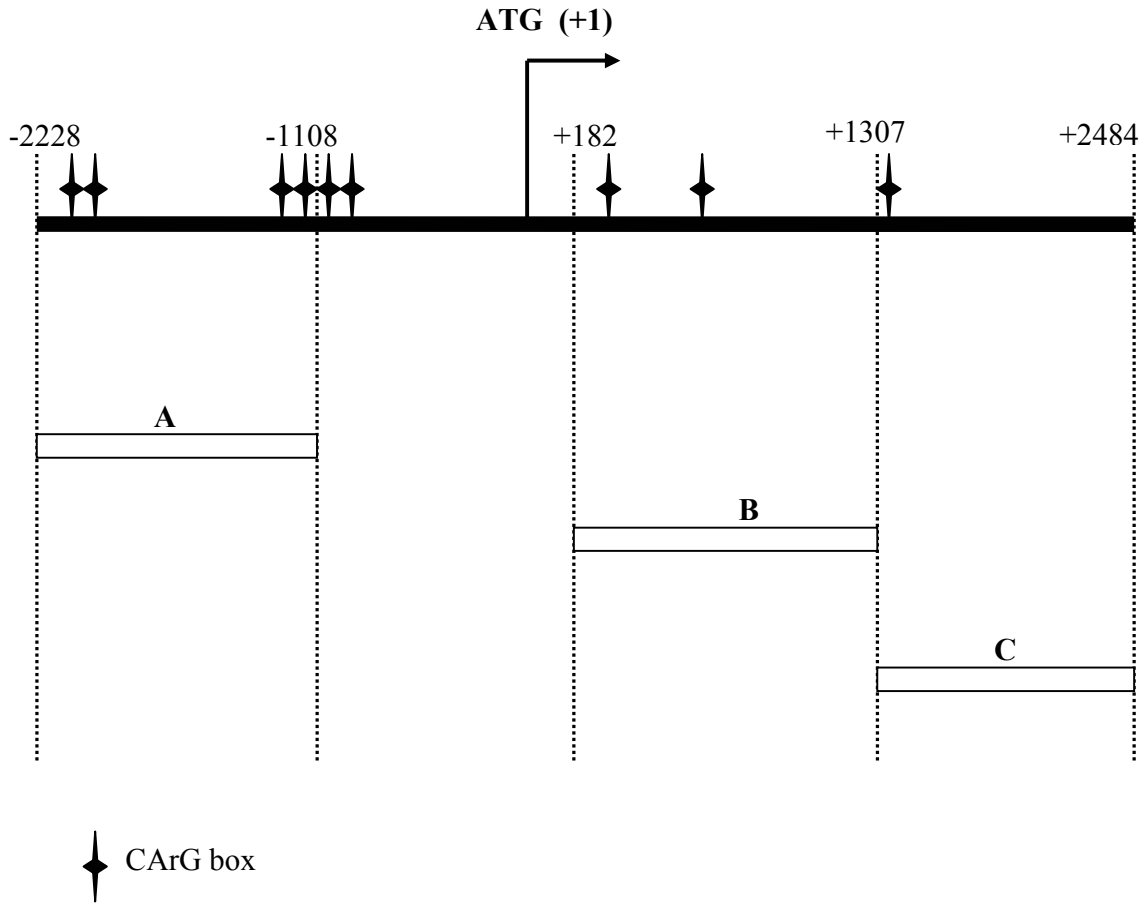


Fig. 14 Schematic diagram summarizing the required regulatory elements for the normal *AGL24* expression. The full-length *AGL24* genomic sequence is shown on the top with the labeled sequence position corresponding to the end of each promoter fragment relative to the transcription start site (+1). The critical regulatory regions required for the normal expression of *AGL24* are represented by horizontal boxes. Region A is required for the initial expression of *AGL24* during floral transition. Region B required for normal spatial and temporal expression of *AGL24* during floral transition, while region C is required for maintaining *AGL24* expression during floral transition and repressing *AGL24* expression in floral meristems.

spatial and temporal expression of *AGL24* during *Arabidopsis* development, because deletion of this fragment could totally abolish the *AGL24* expression as shown by GUS expression in AGL24-P2 transfomants (Fig. 12A-F). Last, the intragenic region of *AGL24* gene after exon 3 may be responsible for the maintenance of *AGL24* expression during floral transtion and for the repression of *AGL24* in floral meristems, as suggested by the distinct GUS expression patterns in AGL24-P3 and AGL24-P4 transgenic plants.

It is noteworthy that from GUS expression analyses, the *AGL24* intragenic regions, especially exons 1 and 2 and introns 1 and 2, play an important role in regulating AGL24 expression in a normal spatial and temporal pattern. This result is consistent with the regulatory pattern of *AG* gene in *Arabidopsis*, which also encodes a MADS-box transcription factor and is responsible for the development of floral meristems and floral reproductive organs (Mizukami & Ma, 1997). *AG* RNA accumulates in the centre of floral meristems and later in the stamen and carpel primordia of young flowers (Mizukami & Ma, 1997; Weigel & Meyerowitz, 1993). Thus, the *AG* expression needs to be tightly regulated at different developmental stages.

It has been reported that the transgenic plants containing the GUS fusion construct including ~6 kb upstream and ~3.8 kb intragenic sequence of *AG* showed a staining pattern similar to the endogenous expression pattern of *AG* in wild type plants (Sieburth & Meyerowitz, 1997; Bowman *et al*., 1991; Drews *et al*., 1991). Loss of *AG* intragenic sequence resulted in loss of negative control of *AG*, thus producing spatial distribution of *AG* floral in both floral and vegetative tissues, and caused loss of positive regulation of *AG*, thus delaying activation of *AG* in floral meristems (Sieburth & Meyerowitz, 1997). Further deletion analyses of the intagenic sequence of *AG* gene found that a specific

region in second intron sequences (1653 bp of DNA from the 3' end of the second intron) of *AG* was sufficient to confer a normal *AG* expression pattern (Deyholos & Sieburth, 2000)

Investigation of *AG* and *AGL24* regulatory region suggests that gene regulation may involve the genomic sequences that are not conventionally located at the upstream promoter regions. Further dissection of *AGL24* regulatory regions may provide more insights into the regulatory mechanism of *AGL24* in the control of flowering.

3.2 Identification of *AGL24* Target Genes by ChIP

3.2.1 Production of Functional Trasngeneic Tagging Lines

To proceed in isolation of putative *AGL24* targets by ChIP, firstly AGL24-12HA tagging transgenic lines were produced because of unavailability of *AGL24* antibody. Fusion of a small tag sequence that has an available antibody with a protein of interest allows monitoring or purifying the protein of interest much more easily. However, sometimes the original structure of the target protein might be disrupted by the fused tag, and hence its biological function could be severely affected. Therefore, it is essential to test if the fusion protein still maintains its biological function in an *in vivo* context. In this study, a preliminary checkup has been conducted to test if AGL24-12HA fusion protein keeps the basic function as AGL24. Transgenic plants of *35S::AGL24-12HA* were created, and their phenotypes were compared with those of *35S::AGL24* and wild-type plants under same conditions. For each genotype, the flowering time was calculated based on the number of rosette leaves when a plant began to bolt. Compared with *35S::AGL24* transgenic plants, the stature of *35S::AGL24-12HA* transgenic plants was smaller, while on average, both of them produced 5-6 rosette leaves before bolting (Fig. 15). On the other hand, wild-type plants produced 8-9 rosette leaves before bolting (Fig. 15). Therefore, over-expression of *AGL24-12HA* caused the same early flowering phenotype as over-expression of *AGL24*, indicating that AGL24-12HA fusion protein was biologically functional as endogenous AGL24 in terms of flowering time regulation.

WT

35S:: AGL24

35S:: AGL24-12HA

Fig. 15 Over-expression of AGL24-12HA fusion protein was able to induce early flowering as overexpression of *AGL24*. Two transgenic (*35S::AGL24-12HA* and *35S::AGL24*) and a wild-type (WT) plants were sowed and grown under same conditions.

Besides the early flowering phenotype that was observed in both 3*5S::AGL24-12HA* and *35S::AGL24* transgenic plants, they showed somehow different phenotypes in flower development. In *35S::AGL24,* the reversion from floral meristems to inflorescence meristems was usually observed, while *35S::AGL24-12HA* transgenic plants produced flowers similar to wild-type plants (Fig. 16). This suggests that over-expression of *AGL24* and *AGL24-12HA* has different impact on flower organ development. The presence of AGL24 activity in floral meristems severely interferes the function of floral homeotic genes, and thus repression of *AGL24* expression is a crucial step in promoting flower development (Yu *et al.,* 2004). It is possible that due to the HA tagging sequence, the fused AGL24 protein could not interfere with other factors that play critical roles in regulating floral organ formation during flower development.

Phenotypic variation was also observed among different *35S::AGL24-12HA* transgenic lines. Figure 16D showed four *35S::AGL24-12HA* transgenic lines that were grown under same conditions. One out of these four lines was significant larger than the others. We found that AGL24-12HA expression level was significantly lower in the plants with a large stature, as Western blot analysis was only able to detect AGL24-12HA fusion protein in 4 mg of plant leaves collected from small transgenic plants but not from large ones. Therefore, in addition to its influence on flowering time, the expression level of AGL24-12HA also affected plant size as well, which was similar to the effect of overexpression of other flowering time genes.

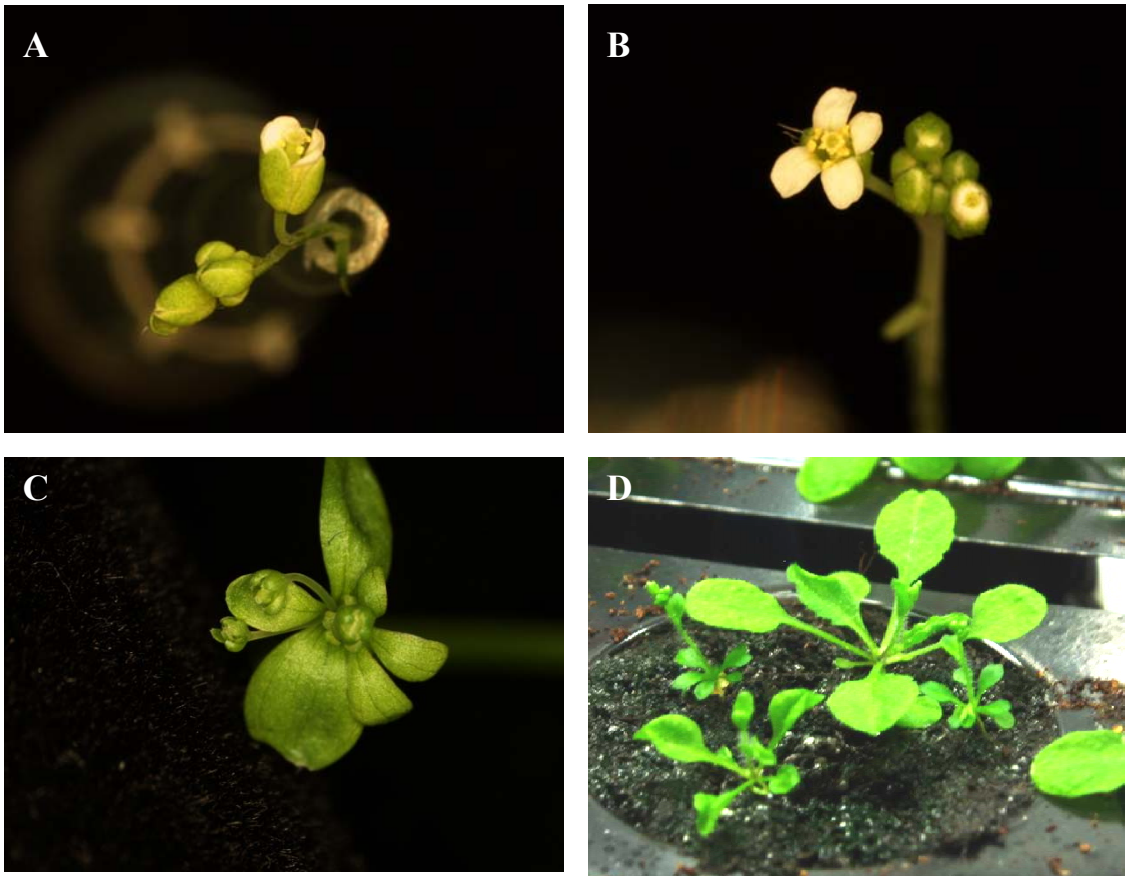


Fig. 16 Flower phenotypes in 35S::AGL24-12HA transgenic plants. Flowers of *35S::AGL24-12HA* (A) show the similar phenotype as those of wild-type plants (B), while flower of *35S::AGL24* (C) demonstrate floral reversion in that floral meristems are converted inflorescence meristem. (D) 35S::AGL24-12HA plants show different stature.

3.2.2 Sonication

The sonication step determines the extent of fragmentation of chromosomes, and an efficient sonication step in ChIP should result in sheared DNAs with an average length of 1000 bp. It was suggested that the extent of sonication mainly depends on pulse time, pulse strength, and sample volume. An average fragment size of 1 kb could be reached when the output power was increased to around 65 W. Under this condition, the number of pulses could be increased in proportion to the increased sample volume. For example, if 1 ml sample was subjected to sonication, 14 pulses were required to achieve the same shearing effect as that of 6 pulses for 0.5 ml sample. In addition, tip immersion depth would greatly affect sonication efficiency as well (Orlando *et al.* 1997). Thus, the tip immersion depth should be adjusted in a way that samples neither circulated around the container nor produced too much foam.

3.2.3 Western Blot

Immunoprecipitation, washing, and elution steps in ChIP were performed as described in "Material and Methods". To monitor the ChIP process, Western blot analyses were performed for the samples collected from several critical steps. The AGL24-12HA fusion protein was detected in crude extract of *35S::AGL24-12HA* inflorescence tissues (Fig. 17). After immunoprecipitation, the fusion protein was detected in the beads conjugated with anti-HA antibody, but not in the supernatant (post-

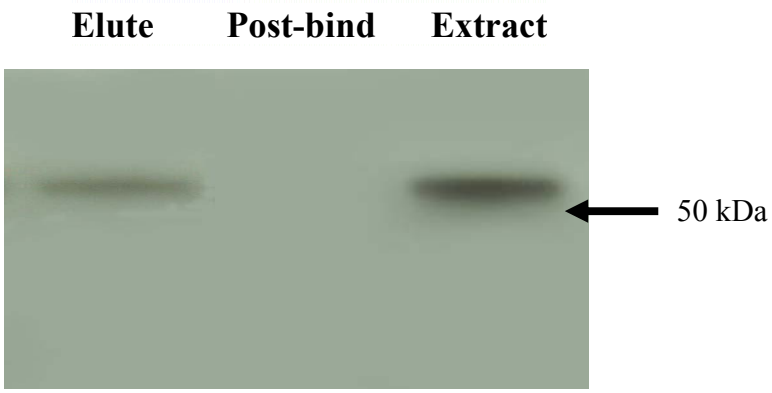


Fig. 17 AGL24-12HA fusion protein was purified. Elute: AGL24-12HA fusion protein could be eluted from beads by SDS elution buffer; Post-bind: AGL24-12HA was absent in the solution that has been incubated with beads, confirming that AGL24-12HA has bound with beads; Extract: crude extract of *35S::AGL24-12HA* inflorescence tissues.

bind solution), indicating that beads containing the HA antibody could specifically bind with AGL24 -12HA fusion protein. Further, the fusion protein was also detectable in the elution buffer, suggesting that the whole process of ChIP was able to isolate the chromatin complex associated with AGL24-12HA fusion protein.

In Western blot analyses, we found that the molecular size of AGL24-HA protein was at 50 kDa, which was larger than the theoretical value of 39.7 kDa (Figure 17), as predicted by the Biology WorkBench Program from San Diego Supercomputer Center (SDSC) (http://workbench.sdsc.edu). This discrepancy between experimental and theoretical data was acceptable, since the difference of the magnitude between the theoretical mass and the mass estimated by SDS/PAGE was commonly found (Bisht *et al.,* 2004). In fact, analyses of *in vitro* synthesized AGL24 in SDS/PAGE gel also showed a larger mass at around 35 kDa than its theoretical size at 25.1 kDa (Fujita *et al.,* 2003).

3.2.4 Linker Ligation

After ChIP, usually there is very little amount of DNAs that could be recovered (Orlando, 2002). To facilitate the following examination of DNA sequences, co-precipitated DNA had to be amplified by PCR. Thus, adaptors (linkers) were required to be ligated with the co-precipitated DNAs to provide known sequences for primer binding during the subsequent PCR step. To prevent linker self ligation, an additional step was added after linker modification, but before PCR amplification, to remove the extra adaptors by the High Pure PCR Product Purification Kit (Roche, Germany), because the columns in the kit were only able to purify DNA fragments with sizes more than 100 bp.

3.2.5 Sequence analysis

The co-precipitated DNAs modified with linkers were amplified by PCR and cloned into pGEM-T Easy vectors for sequencing. In total, there were 246 different sequences identified by sticky end ligation. Among them, 211 sequences were at unique genomic sites and the remaining 35 sequences were located at repetitive chromosome regions including 27 centromeric repeats and 8 ribosomal repeats. Some other sequences not from the nuclear source, such as chloroplast DNA and mitochondria DNA as well as the DNAs that could not be identified through BLAST, were considered as "contaminants".

It was expected that the ChIP-enriched sequences would be observed at a high frequency. However, every one of the identified 211 unique loci appeared only once. This might be caused by the problem in cloning the targets of a transcription factor directly from immunoprecipitated chromatin, where the overall abundance of non-specifically co-precipitated DNAs were dominant. Although the specific DNA sequences for a targeted protein could be typically 10 to 30 fold more abundant than those representing random DNA loci, the absolute amount of non-specifically precipitated fragments still overwhelmd in the precipitated DNAs. Hence, large-scale sequencing work has to be performed to enlarge the sequence library to identify the enriched sequences. Alternatively, there are other methods such as Southern blot and genomic microarray analyses that are available to find out candidate targets. However, these alternative ways are only well established in yeast, and their applications in *Arabidopsis* need to be further investigated (Burski & Frenkel, 2004).

Table 4. Candidate genes isolated by ChIP can be used for further functional studies.

Gene Locus	CArG motif* in co-precipitaed DNA	Gene Locus	CArG motif* in co-precipitaed DNA
AT5G45780	CCTTATAAGA, ACAATTATGG	**AT2G35230**	CCATATATGC
AT5G54570	CGTAAATTGG	**AT2G47010**	CCTAAATAGC
AT5G57620	CCATAATTGC	**AT2G47980**	CCTAAAAAGT
AT5G66430	CTAAATATGG	**AT3G10980**	TCAAATTTGG
AT5G66770	TCATTTTTGG, CTTTAATTGG	**AT3G17850**	CCTAATTTCG
AT4G02110	CCATATAAAG	**AT1G30810**	CGATATTAGG, CGATATTAGG
AT4G25500	CATTTAATGG	**AT1G43670**	CCATATATTG
AT4G26700	CCTAAATAGC	**AT1G45230**	CCATTATTGA, CAAAAAATGG
AT4G30470	TCTTTTTAGG	**AT1G52980**	CCATATTAGG**
AT4G38710	CCAAAATTGT	**AT1G71380**	CCTTATATGA
AT5G05620	CCTAAATAGG**	**AT1G73100**	CCTAAAAAGC
AT5G06090	CCAAAAAAGG**	**AT1G76510**	CCTTATATGT
AT5G16120	CGTTTTTAGG	**AT1G76970**	CATAATTTGG
AT5G17160	CCAAAAAAAG	**AT2G07729**	CCTTTTTTTGG,
AT5G22950	CGAATAATGG, CCTTTAAATG	**AT2G07735**	CCTTATTTGA
AT5G23610	CCATTAAAGC	**AT2G07787**	CCTATTAAGT
AT5G28463	CCAAAATAAG	**AT2G32580**	CCTAATAAGT
AT3G32897	CCTAATAAGT	**AT1G07570**	CCATTAATGC, ACATTTTTGG
AT3G41979	TCTTAATTGG	**AT1G09050**	CCATTATTAG, CTATTATTGG,
AT3G50560	CCTTTTTTGT	**AT1G11670**	CCTAAATAGC, GCATTAATGG
AT3G54300	ACAAAAAAGG, GCATATATGG, TCAATATAGG	**AT1G16330**	ACATTTTTGG, ACTTTTAAGG
AT3G57800	CCAATAATCG, CCAATTTTGG**	**AT1G24260**	GCTAAATAGG

* Maximally one nuleotide mismatch is allowed

** Perfect CArG box

The CArG motif with the CC-$(A/T)_6$-GG pattern that is a potential binding site of MADS-box genes was also searched in these sequences, since AGL24 is a typical MADS-box protein. Recent studies on AGL15, another MADS-box protein, revealed that in addition to the consensus CC-$(A/T)_6$-GG sequence, AGL15 was also able to bind with a relaxed form of CArG motif known as C-$(A/T)_8$-G (Tang and Perry, 2003). Therefore, in our study, the sequences with one base mismatch of CArG motif were also counted. In total, we identified 44 sequences with at least one CArG motif (Table 4). Four of them (AT1g52980, At3g57800, AT5g05620, and AT5g06090) were good candidate genes for further enrichment test because they all contained the prefect CArG motif in their genomic sequences.

3.3 Nonradioactive RNA-RNA *In situ* Hybridization

In parallel with the ChIP experiment, we also tried to establish an in situ hybridization system, which could be potentially used for detection of genes isolated from ChIP experiments.

3.3.1 Putative *AGL24* Target Genes

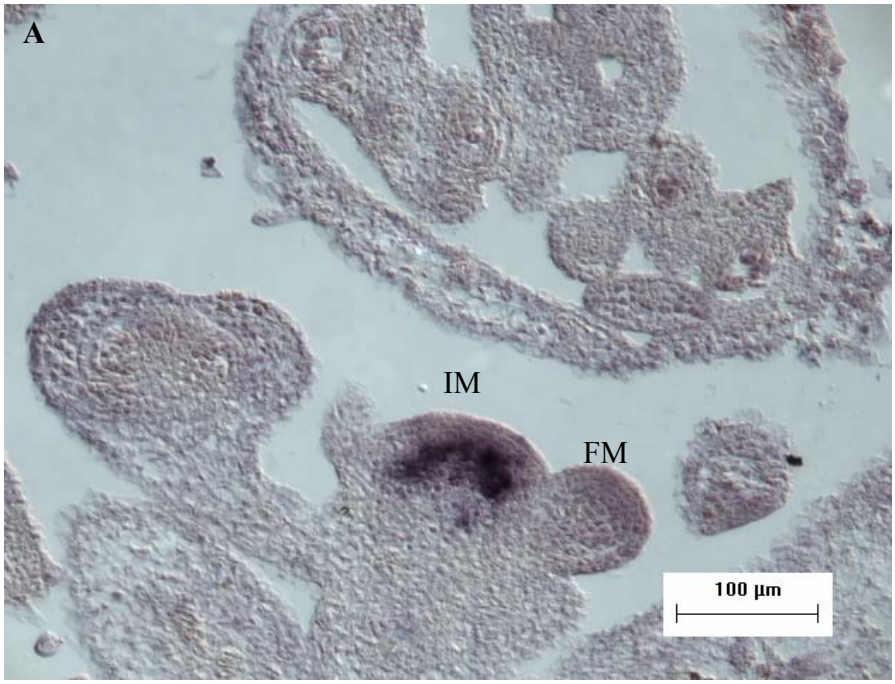
TERMINAL FLOWER 1 (TFL1) is a pivotal gene that affects the growth phases of *Arabidopsis* by regulating the phase transition in the short apical meristem (Shannon & Meeks-Wagner, 1991; Schultz & Haughn, 1991; Alvarez *et al*., 1992; Ray *et al*., 1996). The TFL1 protein has similarity to animal phosphatidylethanolamine-binding proteins, which can associtate with membrane protein complexes. Its exact function in celluar process is unknown (Bradley *et al*., 1997). In *Arabidopsis*, the *tfl1* loss-of-function mutants showed early flowering and converted inflorescence meristems into floral meristems shortly after bolting, while overexpression of *TFL1* caused late flowering and prolonged life cycle (Ohshima *et al*., 1997; Ratcliffe *et al*., 1998). It has been found that *TFL1* could delay the upregulation of *LFY* and *AP1* expression in the inflorescence apical meristem (Ratcliffe *et al*., 1998), and prevent the floral meristem from responding to the activity of *LFY* or *AP1* (Ratcliffe *et al*., 1999). Since *AGL24* acts as a promoter of flowering and it functions upstream of LFY, *AGL24* may act antagonistic to *TFL1* function. Therefore, we checked *TFL1* expression in the background of *agl24* loss-of-function mutants and wild-type plants.

SHORT VEGETATIVE PHASE (SVP), is another MADS-box transcription factor, involved in the control of flowering time. Loss-of-function of *SVP* caused significant early flowering (Hartman *et al*., 2000), while overexpression of *SVP* resulted in late flowering (Zhou *et al*., unpublished data). These results suggest that *SVP* is an important repressor of flowering. Genetic crossings showed that *SVP* could suppress the late flowering phenotype of *agl24,* and *35S::SVP* could also suppress the early flowering phenotype of *35S::AGL24* (Zhou *et al*., unpublished data), indicating that *SVP* is epistatic to *AGL24*. Thus, *SVP* might be a downstream regulator of *AGL24*. In this study, we used *SVP* as a marker gene to compare the different expression of *SVP* corresponding to *AGL24* activity.

3.3.2 *In Situ* Hybridization Results

In situ hybridization showed that in wild-type plants, *TFL1* was strongly expressed in the centrol zone of the SAM, but not in the apical two layers of cells (Fig. 18A). Also, *TFL1* expression was not detected in the floral meritems at different developmental stages. These expression patterns were consistent with previous reports (Kardailsky *et al*., 1999; Ratcliffe *et al*., 1999), suggesting that *TFL1* is responsible for the phase transition in the SAM.

On the other hand, *TFL1* expression in *agl24* was expanded into the whole zone of the SAM (Fig. 18B), which was in great contrast to the confined expression of *TFL1* in the central region of the SAM in wild type plants. This result implies that *AGL24* activity



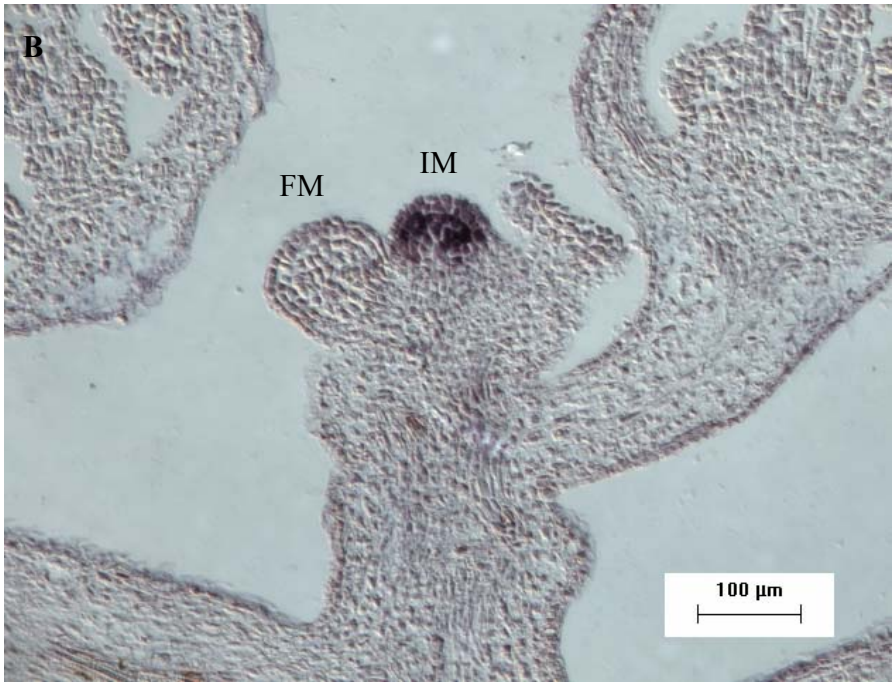


Fig. 18 *TFL1* expression pattern in wild type plants (A) and in *agl24* mutants (B)
IM: inflorescence meristem; FM: floral meristem

may be responsible for restricting *TFL1* expression in the central zone of the SAM, where a small group of cell essential for meristem maintenance is present.

It has been reported that *TFL1* expression in the shoot apex accounted for indeterminate growth and flowering time control (Bradley *et al*., 1997). Despite the strong phenotypes observed in *tfl1* and *35S::TFL1*, the exact function of *TFL1* is so far unknown. The results shown in our study suggest that *AGL24* may serve as an upstream regulator of *TFL1* to regulate flowering time in *Arabidopsis*. Some studies have demonstrated that *TFL1* could antagonize the activity of *LFY* and *AP1* to mediate the formation of floral meristems (Ratcliffe *et al*., 1998; Ratcliffe *et al*., 1999). Thus, a mormal floral transition leading to the generation of floral meristems may be achieved by repression of *TFL1* in the peripheral zone of the SAM by *AGL24*.

The interesting phenomenon was that *TFL1* expression in the central zone of the SAM was not regulated by *AGL24*, since *TFL1* expression was always there regardless of *AGL24* expression. This observation strongly suggests that *AGL24* can only limit *TFL1* expression in the peripheral zone, but not in the central zone of the SAM. Some other factors should be responsible for the stable expression of *TFL1* in the centre of the SAM.

In wild-type plants, *SVP* was strongly expressed in the central zone of the SAM and the coupled young leaf primodia during the vegetative stage, while its expression disappeared in the inflorescence meristem during floral transition (Hartman *et al*., 2000; Fig 19A). This expression pattern was consistent with *SVP* function as a repressor of flowering.

In *agl24* mutant plants, *SVP* expression remained the same in the SAM dring the vegetative stage as in wild-type plants (data not shown). However, *SVP* was still

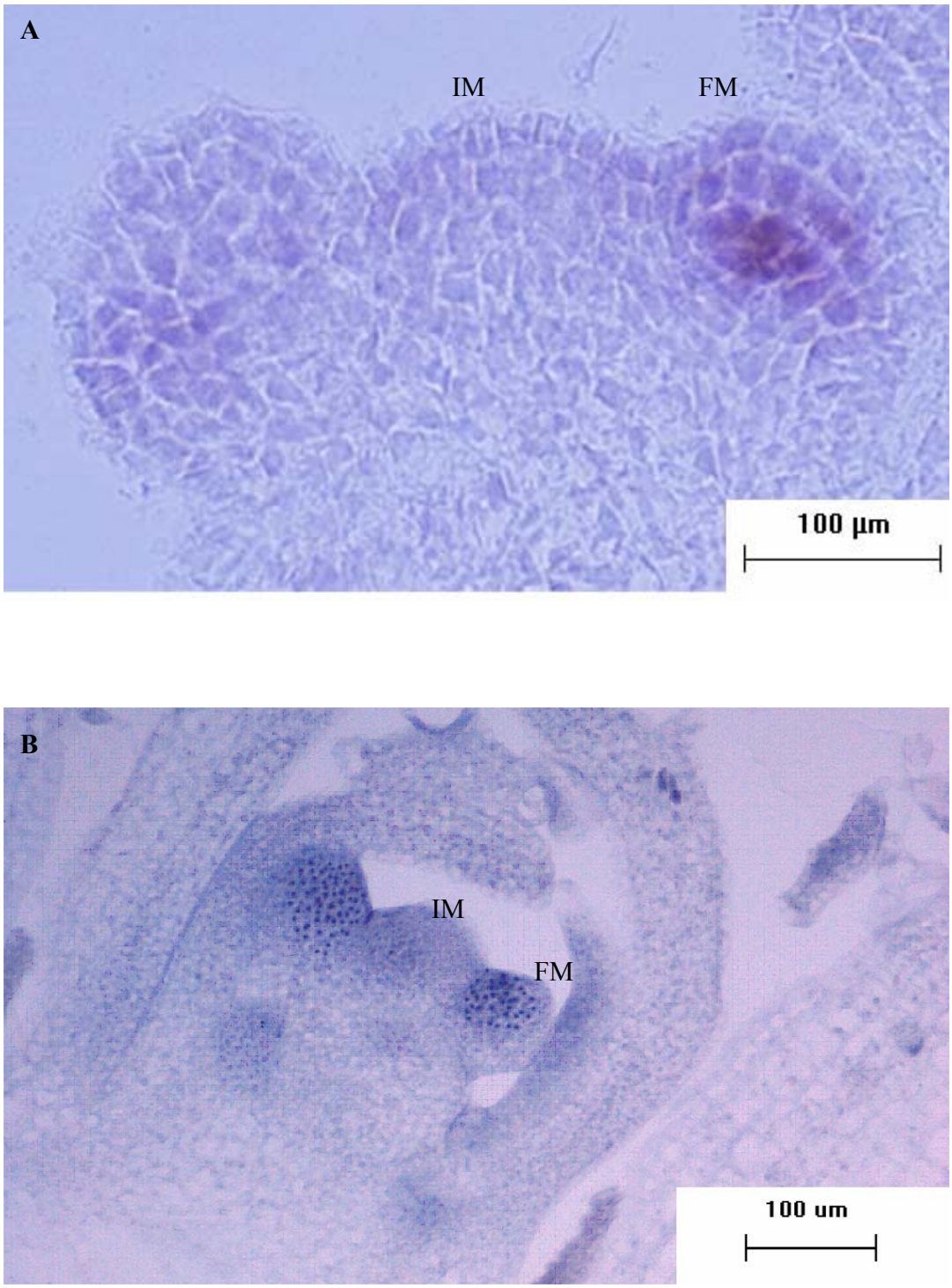


Fig. 19 *SVP* expression pattern in wild type plants (A) and in *agl24* mutants (B).
IM: inflorescence meristem; FM: floral meristem

expressed in the inflorescence meristem of *agl24* mutants (Fig 19B). This result implies that *AGL24* activity may repress *SVP* expression in the SAM during floral transition.

Conclusion

AGL24, a MADS-box DNA binding transcription factor, is a promoter of flowering. It acts downstream of *SOC1* and upstream of *LFY* (Yu *et al*., 2002). Although the linear hierarchy from *SOC1* to *LFY* via *AGL24* is well documented, it is still unknown what are direct regulators or targets of *AGL24*.

In this study, we have applied several molecular methods to analyze the regulation of *AGL24* and its target genes. First, by utilizing GUS reporter gene, we dissected the regulatory regions of *AGL24*. Several concrete regions required for the regulation of *AGL24* at different developmental stages were identified. Further identification of *cis*-elements in these regions can help to identify the upstream regulators of *AGL24*. Second, we isolated a group of putative target genes of *AGL24* by ChIP. The genomic sequences of these target genes contained the consensus binding site of MADS-box transcript factors including *AGL24*. Further molecular and genetic studies on these target genes will reveal whether they are directly regulated by *AGL24* or not. Last, we established an *in situ* hybridization system to detect genes expression in the background of *agl24* loss-of-function mutants. We successfully detected the alteration of gene expression of two marker genes, *TFL1* and *SVP*, in the inflorescence meristem of wild-type and *agl24* plants. The established *in situ* system can be applied for detection of other *AGL24* target genes.

Take together, this study has provided important insights into the regulatory network involving *AGL24* in floral transition of *Arabidopsis*. The results derived form this study pave ways on the eventual elucidation of *AGL24* function in flowering process.

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