

**CHARACTERISING THE REGULATORY
NETWORK OF *MYB26* DURING ANTHIER
DEHISCENCE**

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ABSTRACT

Pollen development and release involves a number of important stages, which govern the success of fertilisation and thus indirectly crop yields. The secondary cell wall in the anther plays a pivotal role in anther dehiscence by offering mechanical strength required for opening and pollen release (Wilson et al. 2011).

MYB26/MALE STERILE35(MS35) is a key regulator of the secondary thickening development in anther, mutation of this gene results in a failure of anther dehiscence and functional male sterility (Steiner-Lange et al. 2003; Yang et al. 2007). However, the regulatory network of *MYB26* remains to be fully identified. To address this issue, the *MYB26* direct targets and interactive proteins were investigated.

Putative targets of *MYB26* were selected, based on their expression patterns, from previously determined expression profiles of the *ms35* mutant (Song, 2009). *PKSP*, a receptor-like cytoplasmic kinase (RLCK) was co-expressed with *MYB26*. Chromatin immunoprecipitation (ChIP)-PCR indicated that *MYB26* may bind to the first intron of *PKSP*. The *NAC SECONDARY WALL-PROMOTING FACTOR1 (NST1)* and *NST2* genes have been demonstrated as direct targets of *MYB26* in ChIP-PCR. However, electrophoretic mobility shift assay (EMSA) did not show binding and retardation, possibly due to the requirement of additional proteins to facilitate *MYB26* binding.

MYB26 interactive proteins were investigated using the yeast two-hybrid system (C. Yang, Z.A. Wilson, unpublished data) and Förster resonance energy transfer (FRET) assays. Y2H560, a CHY-type/RING-type Zinc finger protein and Y2H320/TGA9, a bZIP transcription factor family protein interacted with *MYB26* in plant cell nuclei. Y2H320/TGA9 has been shown to be functionally redundant with TGA10 in regulating anther development (Murmu et al. 2010). The *tga9tga10* double mutant produced indehiscent anthers and was male sterile due to the developmental arrest of the adaxial anther lobes and abnormal tapetum and pollen development in the abaxial anther lobes.

In summary, *MYB26* appears to regulate endothecium development and secondary thickening formation and thus anther dehiscence probably through directly regulating the subfamily VII RLCK *PKSP* and the NAC transcription factors *NST1*, *NST2* and by interacting with a CHY-type/RING-type Zinc finger protein Y2H560 and the bZIP transcription factor Y2H320/TGA9.

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ABBREVIATIONS

bp: base pair

BC: Bicellular

CaMV: Cauliflower Mosaic Virus

cDNA: complementary Deoxyribonucleic Acid

ChIP: Chromatin Immunoprecipitation

Dex: Dexamethasone

DNase: deoxyribonuclease

dNTP: Deoxynucleotide triphosphate

dUTP: Deoxyuridine triphosphate

EDTA: Ethylenediaminetetraacetic acid

EMSA: Electrophoretic Mobility Shift Assay

FRET: Förster resonance energy transfer

G: gram

HSP90: Heat Shock Protein 90

GFP: Green Fluorescent Protein

GR: glucocorticoid receptor

GST Glutathione-S-transferase

GUS: β -glucuronidase

HCl: Hydrochloric acid

HM: Homozygous

HT: Heterozygous

IPTG: Isopropyl β -D-1-thiogalactopyranoside

Kb: kilobase pair

LB: Luria Broth

min: minute

ml: millilitre

mM: milimolar

mRNA: messenger Ribonucleic Acid

MS: Murashige and Skoog Basal Medium

MS35: MALE STERILE35

NASC: Nottingham Arabidopsis Stock Centre

PBS: Phosphate buffered saline

PCR: Polymerase Chain Reaction

PMI: Pollen Mitosis I

RT-PCR: Reverse Transcriptase-Polymerase Chain Reaction

RT: Room Temperature

ST: Secondary Thickening

TAIR: The Arabidopsis Information Resource

T-DNA: Transferred-Deoxyribonucleic Acid

TF: Transcription Factor

UTR: Untranslated Regions

Wt: Wild type

Y2H: Yeast-2-Hybrid

YFP: Yellow Fluorescent Protein

CHAPTER 1 INTRODUCTION

1.1 ARABIDOPSIS ANTHER DEVELOPMENT

Arabidopsis thaliana belongs to the family *Brassicaceae*. It is a flowering dicotyledonous plant that is widely used in plant studies. Many advantages have made *Arabidopsis thaliana* the model organism for basic research in genetics and molecular biology, including fast life cycle, limited space requirement, easy to grow, numerous progeny production and relatively small, genetically tractable genome size. The whole genome sequencing of *Arabidopsis* was completed in 2000 (The Arabidopsis Genome Initiative 2000). A large number of mutant lines, as well as plenty of genomic resources, are available to facilitate the research of the cellular and molecular biology of flowering plants.

Flowers of *Arabidopsis* form into 4 concentric whorls of organs (Figure.1.1), which are sepals, petals, stamens and carpels. The third whorl consists of 6 stamens, four medial stamens and two relatively short lateral stamens. (Scott et

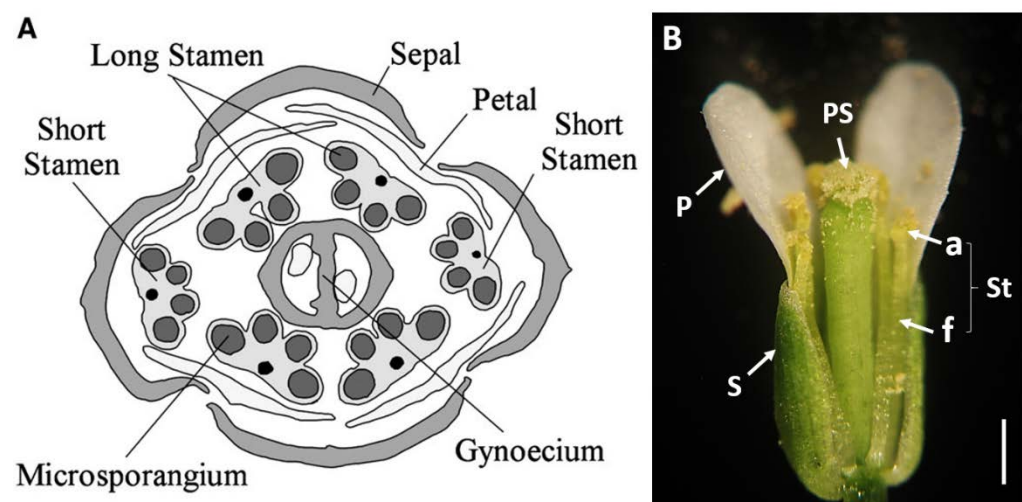


Figure 1.1 Floral structure of *Arabidopsis*.

- A. Scheme of a transverse section through an *Arabidopsis* floral bud (Scott et al., 2004).
- B. Mature flower of wild-type *Col.* with one sepal, two petals removed. S, sepals; P, petals; PS, pistil; St, stamens; a, anther; f, filament. Scale bar, 0.5mm.

al., 2004). Each of the 6 stamens consists a stalk-like filament and an upper region anther where the pollen developed (Figure 1.1). Water and nutrient are transmitted through the filament to anther and are positioned to facilitate pollen dispersal.

Anther development of the wild-type *Arabidopsis thaliana* from the emergence of stamen primordia to anther dehiscence and senescence is divided into 14 stages by Sanders et al. Stages from 1 to 8 are grouped into phase one of anther development, stages 9 to 14 belong to phase two of anther development.

At stage 1 (Figure 1.2), L1, L2 and L3 layers of floral meristem further divide and give rise to the formation of stamen primordium. From stage 2 to 4, anticlinal cell division in L1 develops into the epidermis, the surface area of the anther, while cells in L3 layer expand and differentiate into the connective and vascular tissues. Periclinal division of L2 cells lead to the formation of four anther lobes. By stage 5, anthers have four clearly defined locules, and all anther cell types are present, from outside to inside there are epidermis, endothecium, middle layer, tapetum and microspore mother cells, which undergo meiosis and turn into tetrads of microspores within each locule at stage 7. Microspores are released from tetrads at stage 8 and differentiate into pollen grains between stage 9 and 12 (Figure 1.3). During this period, the size of anther increases, and the middle layer and tapetum gradually degenerate. Secondary thickening appears in the endothecium and connective cells at stage 11. Degeneration of the septum at stage 11 and 12 generates a bilocular anther, then followed by the breakage of stomium cells and anther dehiscence with pollen release from the locules during stage 12 and 13.

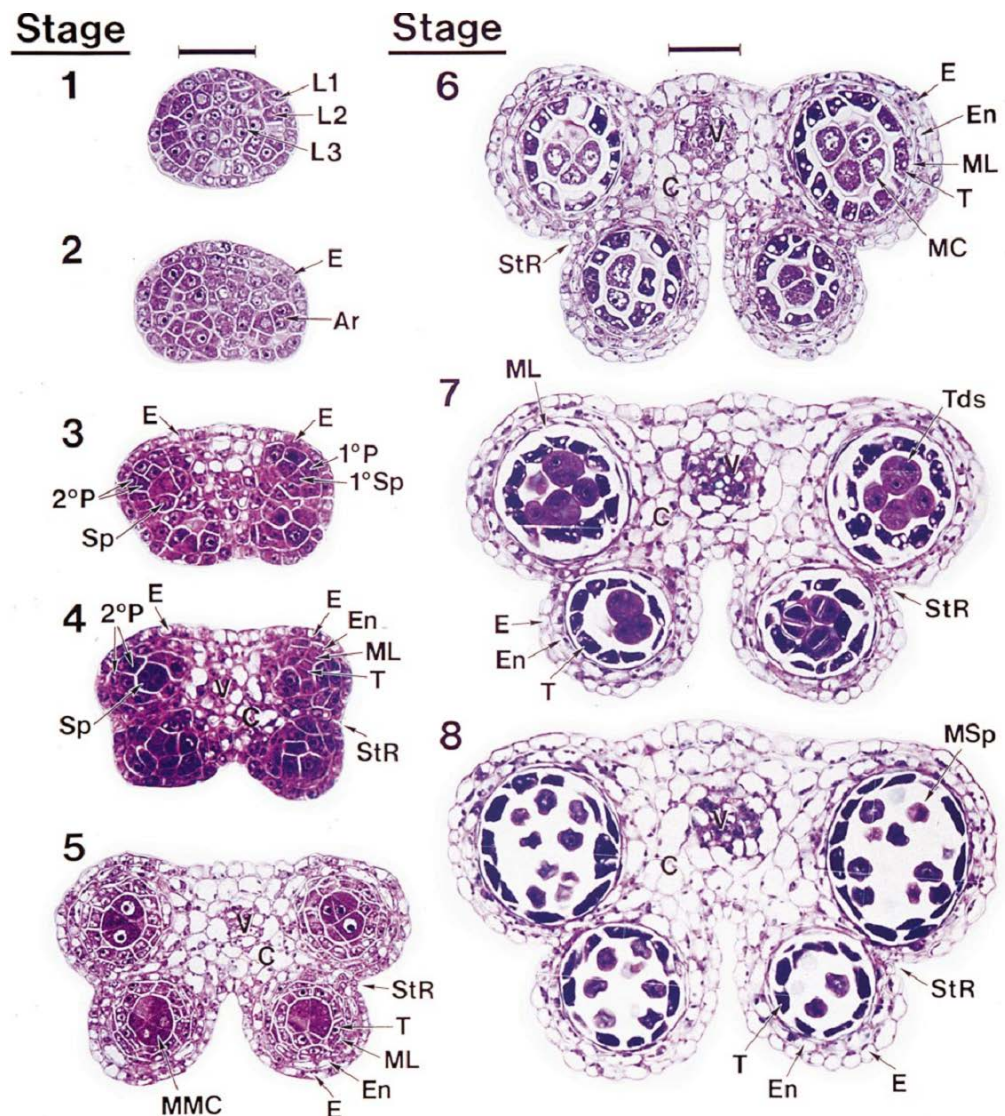


Figure 1.2 Phase one of the wild-type *Arabidopsis* anther development (Sanders, et al. 1999)

L1, L2, and L3, the three cell-layers in stamen primordia; E, epidermis; Ar, archesporial cell; 1°P, primary parietal layer; 2°P, secondary parietal cell layers; 1°Sp, primary sporogenous layer; Sp, sporogenous cells; En, endothecium; ML, middle layer; MC, meiotic cell; C, connective; MMC, microspore mother cells; StR, stomium region; T, tapetum; Tds, tetrads; V, vascular region. MSp, microspores; Bar over stage 1=25µm and this is the scale for stages 1 to 5. Bar over stage 6 = 25µm and this is the scale for stages 6 to 8.

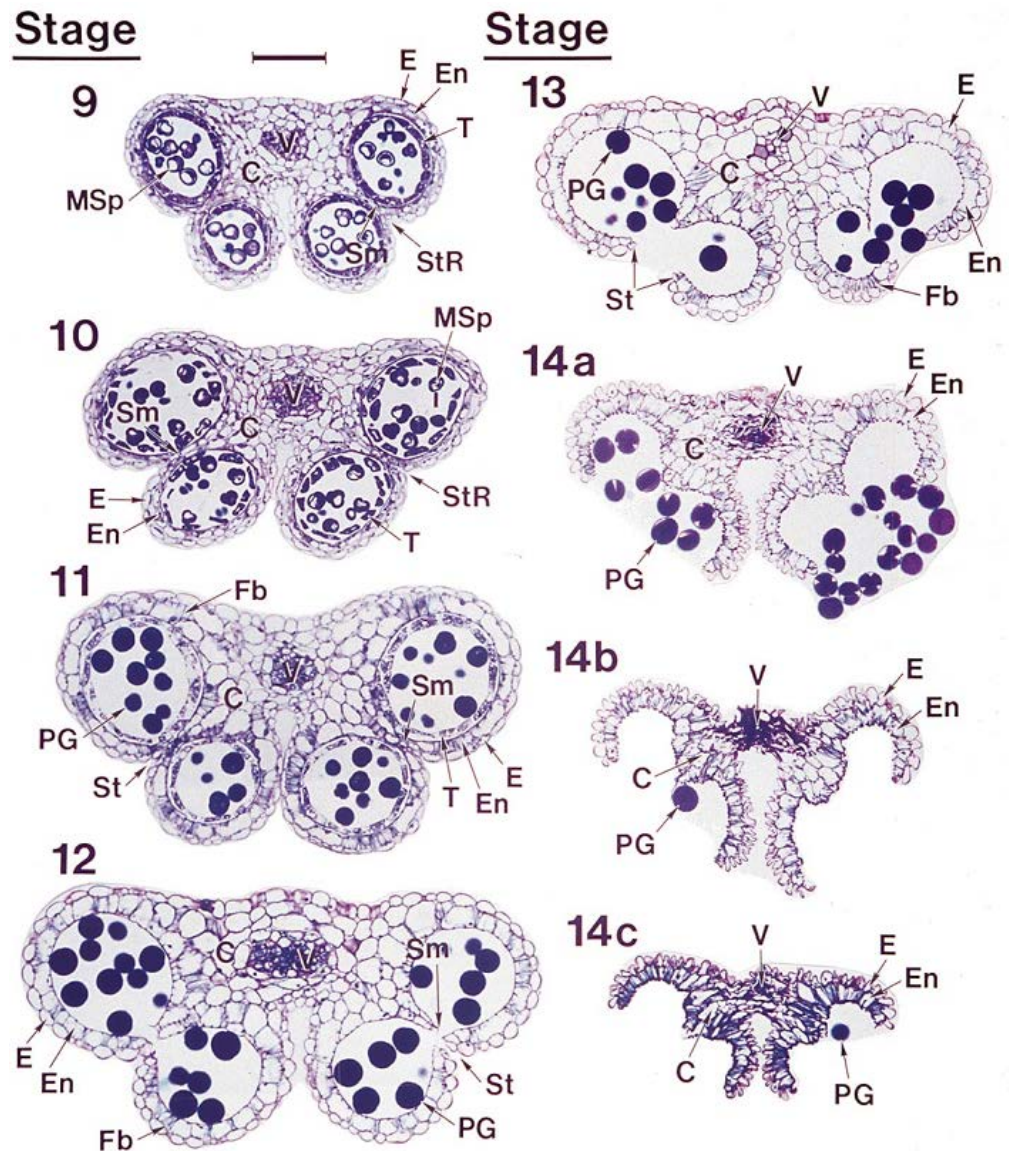


Figure 1.3 Phase two of the wild-type *Arabidopsis* anther development (Sanders, et al. 1999)

Phase two represents anther late development, dehiscence and senescence. C, connective; E, epidermis; En, endothecium; Fb, fibrous bands; MSp, microspores; PG, pollen grains; Sm, septum; St, stomium; StR, stomium region; T, tapetum; V, vascular region. Bar=50µm and applies to all stages 9–14c

The process of anther dehiscence is generally similar in many species but have subtle differences depending on anther structure (Carrizo García 2002) and types of secondary thickening in anther (García 2002). Anther dehiscence happens at the late stages of anther development after the septum degenerates and stomium ruptures to release pollen. The endothecium contributes to two steps during the anther dehiscence process (Scott et al., 2004), the secondary thickening of the

endothecium and connective cells happens at anther stage 11. Just before dehiscence, together with the lysis of the stomium cells, epidermal and endothelial cells become turgid, generating an inward force in the anther wall, and facilitate rupture of the stomium. Secondly, desiccation of the endothecium and epidermis causes differential shrinkage between the thickened and unthickened areas, causing an outward force to retract the anther wall as well as supporting the full opening of the stomium to allow pollen release (Wilson et al. 2011).

1.2 SECONDARY CELL WALL THICKENING

There are two different types of cell walls, primary and secondary cell walls in plants. Parenchyma cells, such as epidermis, pith and cortex are enclosed in a thin primary wall, which is composed of three classes of polysaccharides, cellulose, hemicellulose, and pectin (Carpita & Gibeaut 1993). The secondary cell wall is developed between the primary cell wall and the plasma membrane and is composed primarily of cellulose, hemicellulose (xylan and glucomannan) and lignin. Cell types such as fibres and tracheary elements deposit the secondary thickened walls after cessation of elongation (Demura & Fukuda 2007; Du & Groover 2010).

In stems and leaves, secondary thickening provides mechanical strength and facilitates the transport of nutrients and water. While in reproductive organs, secondary cell walls develop in the silique margin and in the anther endothecium, which are essential for seed shedding and pollen release (Wang & Dixon 2012). Secondary cell wall is also important to humans as a source of fibre, paper, pulp, timber and renewable biofuels (Pauly & Keegstra 2010).

Cellulose is the load-bearing polymer of secondary walls, in which the cellulose microfibrils cross-link with hemicellulose to form the framework of secondary cell walls. Cellulose constitutes around 40-50% of the wood component and is made of linear chains of β -1,4-linked glucosyl residues with a degree of polymerisation of 10,000 (Timell 1967). The biosynthesis of cellulose is mediated by the cellulose synthase A (CesA) of the cellulose synthase complexes located at the plasma membrane (McFarlane et al. 2014). There are ten genes encoding CesA proteins in the *Arabidopsis* genome, belonging to the glycosyltransferase family GT2 (McFarlane et al. 2014). *CesA4/IRX5*, *CesA7/IRX3* and *CesA8/IRX1* have been shown to function non-redundantly during cellulose biosynthesis of secondary cell walls (Taylor et al. 1999; Taylor et al. 2000; Taylor et al. 2003). *CesA4/IRX5*, *CesA7/IRX3* and *CesA8/IRX1* interact with each other in the cellulose synthase complex, and the normal assembly and targeting of the complex require the presence of all of them (Taylor et al. 2004; Timmers et al. 2009).

Xylan is the most abundant type of hemicellulose component in secondary cell walls of angiosperm. Xylan is a polymer with a backbone of β -1,4-linked xylosyl residues with a degree of polymerisation around 100. The xylosyl residues are often substituted with monosaccharide residues, such as α -1,2-linked glucuronic acid (GlcA), α -1,2-linked 4-*O*-methylglucuronic acid (MeGlcA), and α -1,2 and/or α -1,3 linked arabinose (Ara), the corresponding xylans are named according to these substitutions, as glucuronoxylan, arabinoxylan and glucuronoarabinoxylan (Ebringerová & Heinze 2000; Zhong & Ye 2015). Glucuronoxylan is the major form of xylan in the secondary cell walls of the dicot, while glucuronoarabinoxylan is the most abundant xylan form in the wood

of gymnosperms and grass cell walls (Zhong & Ye 2015). A number of enzymes are required for the biosynthesis of xylans, such as xylosyltransferases, glucuronyltransferases, methyltransferases, arabinosyltransferases, and acetyltransferases, they are involved in the xylan backbone elongation and substitutions. The glycosyltransferase family GT43 and GT47 play an essential role in the xylan backbone elongation. There are four GT43 genes in the *Arabidopsis* genome, including *IRX9*, *I9H/IRX9-L*, *IRX14*, and *I14H/IRX14-L*. The proteins encoded by these GT43 genes are located in the Golgi in secondary wall-forming cells such as vessels and fibres (Brown et al. 2007; Lee et al. 2010; Wu et al. 2010). Mutation of *IRX10* and *IRX10-L*, two GT47 genes, also result in a reduction of xylan xylosyltransferase activity, xylan content and secondary cell wall thickening (Brown et al. 2009; Wu et al. 2009).

Glucomannan is a minor type of hemicellulose component in dicot secondary walls, which consists of a linear chain of β -1,4-linked mannosyl residues and glucosyl residues with a ratio of 2:1 (Timell 1967). The cellulose synthase-like A (CslA) family, which belongs to the GT2 family is involved in the biosynthesis of glucomannan (Dhugga et al. 2004; Liepman et al. 2005). There are nine *CslA* genes in *Arabidopsis* genome, three of them, *CslA2*, *CslA3* and *CslA9*, are involved in the synthesis of glucomannan in secondary thickening of stems (Goubet et al. 2009).

Lignin is a complex three-dimensional polyphenolic polymer that fills the spaces between the cellulose and hemicellulose matrix and provides the compression strength to walls (Kumar et al. 2016; Zhong & Ye 2015). Lignin is derived by oxidative polymerisation of three hydroxycinnamyl alcohol precursors, p-coumaryl, coniferyl and sinapyl alcohol. These precursors polymerised into the

p-hydroxyphenyl (H), guaiacyl (G) and syringyl (S) units of lignin. The proportion of each of three type lignin units varies significantly among different plants and among different tissues within the same species. The monolignol biosynthesis is achieved through the phenylpropanoid pathway starting with phenylalanine (Figure 1.4) to produce hydroxycinnamoyl-CoA esters, which are then routed into the lignin branch pathway to generate monolignols (Vanholme et al. 2013). There are 35 monolignol biosynthetic genes identified in *Arabidopsis*. Among them, *PAL1*, *PAL2*, *C4H*, *HCT*, *C3H1*, *CSE*, *4CL1*, *4CL2*,

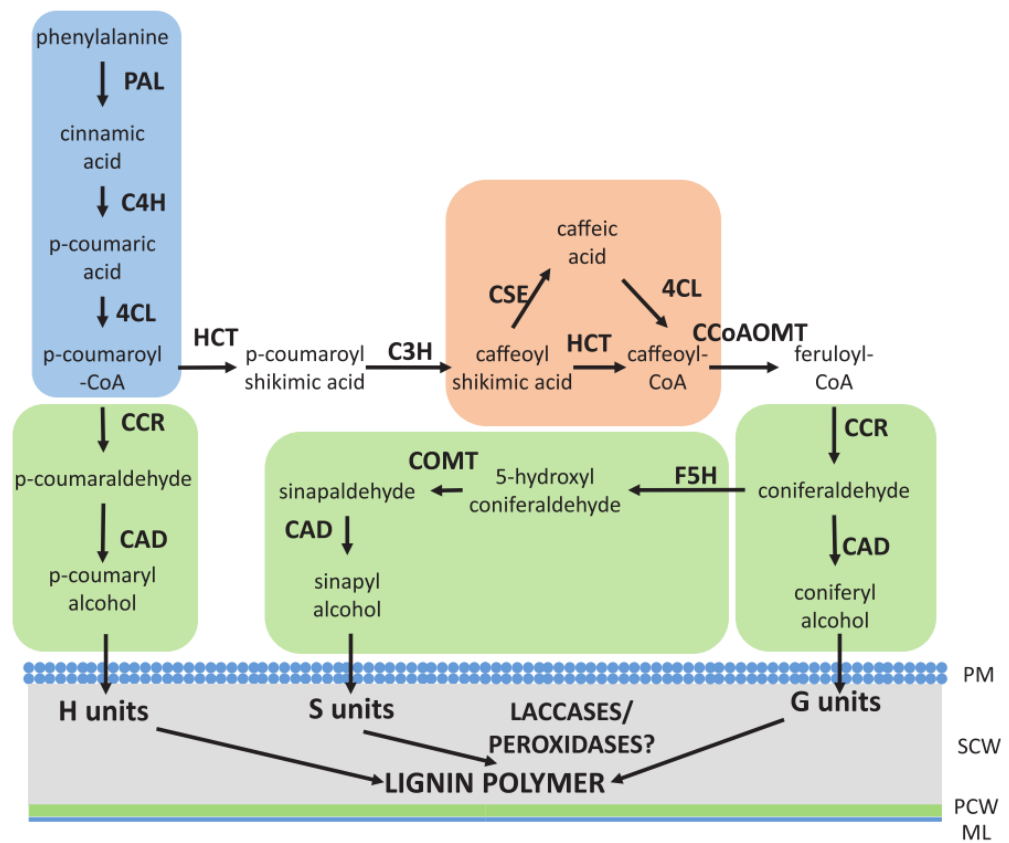


Figure 1.4 Lignin biosynthesis (Kumar et al. 2016).

A simplified monolignol biosynthetic pathway representing the most common pathways for the biosynthesis of the three major lignin units. Many additional side pathways and alternative routes are known to exist. PAL, phenylalanine ammonia-lyase; C4H, cinnamate 4-hydroxylase, 4CL, 4-coumarate:CoA ligase, HCT, p-hydroxycinnamoyl-CoA:quinic/shikimate p-hydroxycinnamoyltransferase; C3H, p-coumarate 3-hydroxylase; CSE, caffeoyl shikimate esterase; CCoAOMT, caffeoyl-CoA O-methyltransferase; CCR, cinnamoyl-CoA reductase; CAD, cinnamyl alcohol dehydrogenase; COMT, caffeic acid O-methyltransferase; F5H, ferulate 5-hydroxylase

CCoAOMT1, *CCoAOMT7*, *CCR1*, *F5H*, *COMT*, *CAD3* and *CAD6* are the core monolignol biosynthetic genes associated with the development of interfascicular fibers and lignified xylem in stems (Vanholme et al. 2013; Raes et al. 2003). Monolignols are synthesised in the cytosol before transporting to cell walls and polymerising into lignin with the help of oxidases such as laccases and peroxidases (Bonawitz & Chapple 2010). Genes encoding laccases such as *Laccase 4 (LAC4)*, *LAC7*, *LAC11*, and genes encoding peroxidases such as *AtPRX72*, *AtPRX2*, *AtPRX25* and *AtPRX71* have been shown to be involved in lignin polymerisation during secondary cell wall development (Berthet et al. 2011; Zhao et al. 2013; Herrero et al. 2013; Shigeto et al. 2013; Shigeto et al. 2014).

1.3 MYB26 ENCODES AN R2R3-MYB FAMILY PROTEIN

The MYB transcription factors represent a large group of proteins that are functionally diverse present in all eukaryotes (Dubos et al. 2010a). Transcription factor (TF) is a term used to describe proteins that have a DNA-binding domain and regulate gene's transcription through binding to the specific regulatory site of target genes. Transcription factors can function alone or form a protein complex with other proteins to act as an activator, repressor, or both, to specific genes. Based on the similarity of the DNA-binding domain, TFs have been classified into families.

1.3.1 MYB family in plants

The first MYB gene was discovered from avian myeloblastosis virus as an “oncogene” *v-MYB*, may have originated from a vertebrate gene which contains 3 members, *c-MYB*, *B-MYB*, and *A-MYB* (Klempnauer et al. 1982). The first

plant MYB gene identified was C1 in *Zea mays* (Paz-Ares et al. 1987). The MYB factors contain N-terminal conserved DNA-binding domain (MYB domain) and an activation or repression domain usually located in C-terminus.

The MYB domain consists of up to 4 imperfect repeats (R), each contains about 52 amino acids, forming 3 α -helices, and the 2nd and 3rd helices build a helix-turn-helix (HTH) structure containing a hydrophobic core. The third helices of each repeat direct contact with DNA in the major groove (Ogata et al. 1996, Jia et al. 2004). The repeats of the prototypic MYB protein c-MYB are referred to as R1, R2, and R3. Repeats from other MYB proteins are named according to their similarity to R1, R2 or R3 of c-MYB.

Based on the number of repeats in the MYB domain, MYB proteins were divided into 4 classes in the plant genome. The smallest class is the 4R-MYB proteins, which contain 4 R1/R2-like repeats, one 4R-MYB protein is encoded in several plant genomes. The second class contains five R1R2R3-MYB (3R-MYB) proteins involved in the control of cell cycle. The third class refers to MYB-related proteins, with a single or partial MYB repeats. The fourth class, R2R3-MYB proteins are the most abundant MYB factors and are specific to plants. The *Arabidopsis* genome contains 25498 genes according to the whole genome sequencing data published in 2000 (The Arabidopsis Genome Initiative 2000). There are 1600 genes (6%) predicted to encode for transcription factors and 125 of them are of the R2R3-MYB type (Riechmann et al. 2000, Stracke et al. 2001, Dubos et al. 2010).

1.3.2 R2R3-MYB proteins involved in secondary thickening and anther development

R2R3-MYB proteins have been characterised by genetic approaches and found to be involved in the control of various plant-specific processes, including primary and secondary metabolism, cell fate and identity, developmental processes and responses to biotic and abiotic stresses (Dubos et al. 2010b; Ambawat et al. 2013).

Many R2R3-MYB proteins were reported to play important roles during secondary thickening development. *AtMYB58*, *AtMYB63*, and *AtMYB85* activate lignin biosynthesis in fibres and/or vessels (Zhong et al. 2008; Zhou et al. 2009), whereas *AtMYB68* negatively regulates lignin deposition in roots (Feng et al. 2004). *AtMYB46* is a positive regulator of lignin biosynthesis in fibres and vessels and also regulates cellulose and xylan deposition (Zhong et al. 2007). *AtMYB52*, *AtMYB54* and *AtMYB69* and *AtMYB103* are positive regulators dedicated to cell wall thickening in fibre cells. *AtMYB52*, *AtMYB54*, and *AtMYB69* are proposed to regulate lignin, xylan and cellulose biosynthesis (R. Q. Zhong et al. 2008). *AtMYB61* plays a pleiotropic role, influencing lignin deposition (Newman et al. 2004), mucilage production (Penfield et al. 2001) and stomatal aperture (Liang et al. 2005), suggesting that it might act upstream of the different pathways perhaps by regulating carbon allocation.

Several R2R3-MYB genes control anther development and functionality, including *AtMYB21*, *AtMYB24*, *AtMYB57*, *AtMYB108/BOS1*, *AtMYB35/TDF1*, *AtMYB80* (formerly *AtMYB103*) and *AtMYB99* (Cheng et al. 2009; Mandaokar & Browse 2009). *AtMYB24* and *AtMYB108* are redundant, acting downstream of *AtMYB21*, to regulate jasmonate-mediated stamen maturation (Mandaokar &

Browse 2009). *AtMYB80* (formerly *AtMYB103*) regulates tapetum development, callose dissolution, exine formation and acts downstream of *AtMYB35/TDF1* (Zhang et al. 2007). *AtMYB125/DUO1* is a pollen-specific factor controlling male germ cell division and differentiation (Brownfield et al. 2009). *AtMYB33* and *AtMYB65* redundantly facilitate both anther and pollen development (Millar & Gubler 2005).

1.3.3 MYB26

The *MYB26/MALE STERILE35(MS35)* gene (AT3G13890) is located on the top arm of chromosome 3, *MYB26* encodes a transcription factor of the R2R3-MYB family, it is localised in the nucleus and regulates the endothecium development and secondary thickening (ST) in a cell-specific manner in anther (Dawson et al. 1999; Steiner-Lange et al. 2003; Yang et al. 2007).

The expression of *MYB26* is restricted to the floral tissues (Steiner-Lange et al. 2003; Yang et al. 2007). The 3.1-kb region upstream of the translation start site of *MYB26* was fused to the β -glucuronidase (GUS) reporter gene, the GUS expression was observed in anthers, filaments, style and nectaries of young post-meiotic buds through to open flowers. In anthers, the GUS expression was detected in tapetum, endothecium and connective tissues with maximum expression during pollen mitosis I (PMI) and bicellular (BC) stage (Figure 1.5, Yang et al. 2007).

The *ms35* mutant was generated by X-ray mutagenesis of the *Ler* seeds (Dawson et al., 1999), a deletion and rearrangement of a 1288 bp upstream of the translational start of *MYB26* results in a severe downregulation of the *MYB26* expression and male sterility due to lack of endothecium secondary thickening

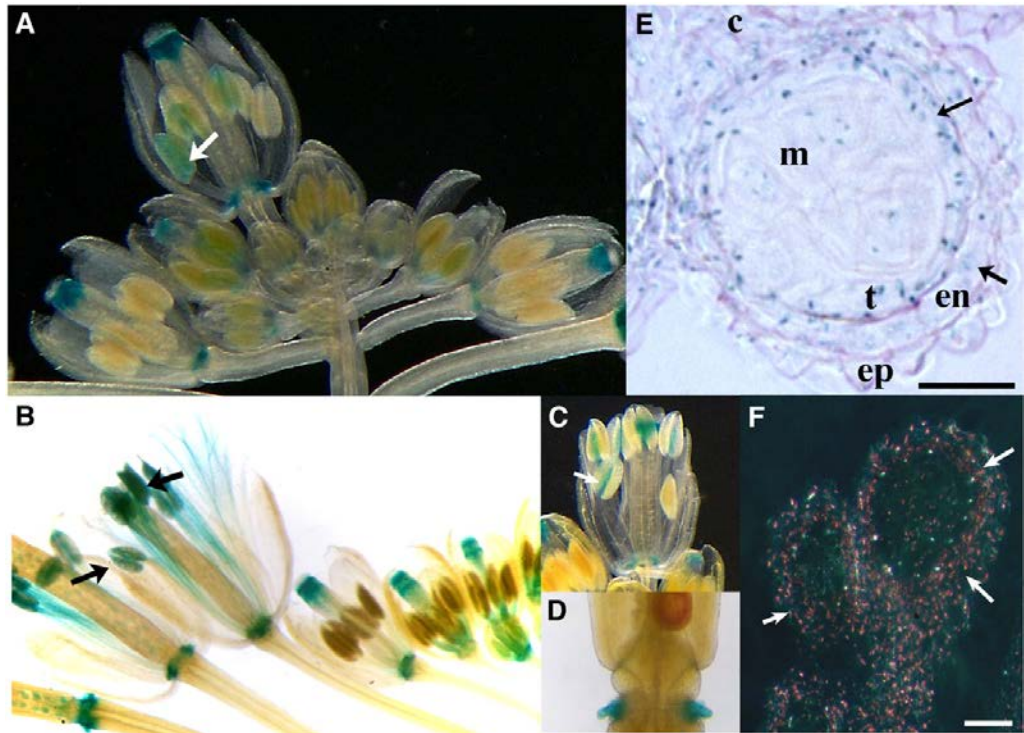


Figure 1.5 ProMYB26:GUS expression in *Arabidopsis* (Yang et al. 2007)

(A) and (B) Inflorescence showing expression of GUS in anthers, filaments, nectaries, and stylar tissues (arrows indicate expression in anther tissue). (A) is a dark-field image. (C) Close-up dark-field image of buds before opening, showing expression in postmeiotic anthers (arrow). (D) Immature silique showing GUS expression in nectaries. (E) and (F) Sections through anther at pollen mitosis I stage. GUS expression can be seen throughout the anther, particularly in the endothecium and tapetal and connective tissues (arrows). (F) is a dark-field image. c, connective tissue; en, endothecium; ep, epidermis; m, microspores; t, tapetum. Bars = 40 μ m.

in indehiscent anthers although viable pollen is produced (Figure 1.6). Two T-DNA insertion mutants, SALK_056264 and SALK_112372, are allelic to the *ms35* mutation (Figure 1.6 F), the loss-of-function (null) mutation of *MYB26* gives male sterility phenotype of failed dehiscence due to a lack of endothelial secondary thickening (Yang et al. 2007). In the *ms35* mutant, anther development appears normal until anther stage 11, when lignified secondary cell walls developed in the enlarged endothecium of the wild-type, but not in the *ms35* mutant. Degradation of the septum takes place normally in the mutant, but the breakage of stomium is hindered due to the lack of secondary thickening, the endothecium fails to expand and the lack of secondary thickening means no force

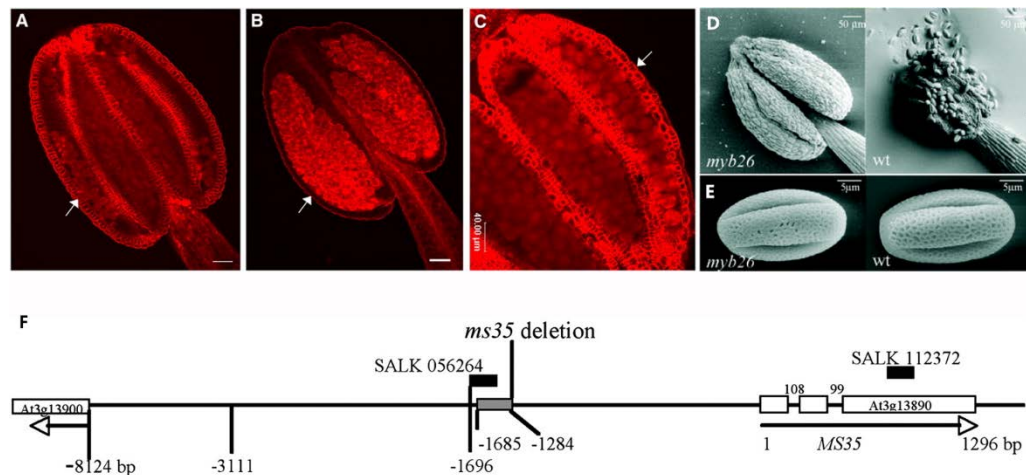


Figure 1.6 *MYB26* regulate secondary thickening and anther dehiscence.

(A-C) Lignin staining of Arabidopsis anther using acridine orange/ethidium bromide (A) *Ler* wild-type partially dehiscent anther from open flowers. ST is visible in the endothecium (arrow) (B) Anther of the *ms35* mutant, ST is absent in the endothecium (arrow) (C) Anther of the Pro35S:MYB26 overexpression lines, ectopic ST developed in the endothecium (arrow). (D-E) SEM picture of anthers of open flowers. (D) Anthers of the *ms35* mutant are closed, anthers of the wild type are fully dehiscent and have released the pollen grains. (E) Pollen of the mutant are indistinguishable from wild-type pollen. (F) Structure of the *MYB26* Gene. Arrows indicate open reading frames. (Figure is adapted from Yang et al. 2007; Steiner-Lange et al. 2003)

is generated and dehiscence fails to occur, resulting in a failure of pollen release (Dawson et al. 1999; Steiner-Lange et al. 2003; Yang et al. 2007). The pollen produced in the *ms35* mutant appears fertile and can be used to manually self-fertilise the mutant, the secondary thickening in other tissue is normally developed in the *ms35* mutant (Dawson et al. 1999).

Overexpression of *MYB26* results in ectopic secondary thickening deposition in various above ground tissues in both *Arabidopsis* and *tobacco* (Yang et al. 2007). *MYB26* regulates the expression of *NAC SECONDARY WALL THICKENING PROMOTING FACTOR1* (*NST1*) and *NST2*, which are master switches of the secondary thickening development in plants, as well as a number of biosynthetic genes associated with secondary thickening (Yang et al. 2007; Mitsuda et al.

2005). *MYB26*, therefore, is a critical regulator of the secondary thickening development in anther and is essential for anther dehiscence.

1.4 AIMS OF THE PROJECT

The aim of this project was to study the *MYB26* regulation network, by investigating *MYB26* direct targets and interactive proteins, to establish the mechanism behind anther secondary thickening development and dehiscence.

MYB26-regulated genes were investigated by analysing the previous expression profiles of the *ms35* mutant (C. Yang, Z.A.Wilson, unpublished data) (Chapter 3).

MYB26 direct targets were determined using Chromatin Immunoprecipitation (ChIP)-PCR and electrophoretic mobility shift assay (EMSA) (Chapter 4).

MYB26 interactive proteins were confirmed using a Förster resonance energy transfer (FRET) assay, and a functional study of the putative *MYB26* interactors was performed (Chapter 5).

CHAPTER 2 MATERIALS AND METHODS

2.1 PLANT MATERIALS AND GROWTH CONDITIONS

2.1.1 General growth conditions

Unless stated otherwise, the *Arabidopsis thaliana* and *Nicotiana benthamiana* seeds including wild-type and transgenic plants were obtained/generated in the Wilson lab, University of Nottingham. All T-DNA insertion mutants were obtained from the Nottingham Arabidopsis Stock Centre (NASC).

Seeds were sown onto the Levington M3 compost (The Scotts Company Ltd, UK). Plants were grown in 9cm or 13cm plastic pots at a density of 3-5 seeds per pot, or higher for screening. Transparent plastic sleeves or lids were used to avoid cross-pollination among different lines. Plants were grown in a growth room at 23/18°C (day/night) and 16hrs photoperiod at a light intensity (measured as photon intensity) of $180 \pm 20 \mu\text{mol/s/m}^2$.

2.1.2 *Arabidopsis* transformation by floral dipping

A 5ml overnight culture of *Agrobacterium* in LB medium supplied with correct antibiotics was inoculated into 95ml of same medium and grown at 28°C to OD₆₀₀=1-2. The *Agrobacterium* was pelleted down at 2200g and re-suspended into freshly made 50ml sucrose solution (5% (w/v) sucrose, 0.05% (v/v) Silwet L-77). Newly flowering *Arabidopsis* (or remove any developed siliques) were dipped into the suspension for 30 seconds at a time. To keep the plants in a humid environment, the plastic sleeves were folded down and kept away from direct light at least 24 hrs before opening the plant sleeves and moving back to normal growth condition as described in section 2.1.1.

2.1.3 Sterile germination of *Arabidopsis*

Seeds were placed in a 1.5 ml microfuge tube and washed thoroughly with 1 ml of 70% ethanol (v/v) by a vortex mixer for a few seconds. The supernatant was discarded and replaced with 1ml of fresh 70% (v/v) ethanol for 10 mins. The tube was shaken occasionally to allow seeds to fully contact with ethanol. Further steps were conducted in a laminar flow cabinet, seeds were spun down and resuspended in 0.5ml absolute ethanol for a few seconds before tipping onto a piece of sterile filter paper. After the ethanol evaporated completely, the seeds could be sown thinly on half-strength MS medium (Sigma, UK) plates with antibiotics as required.

2.2 DNA EXTRACTION

2.2.1 Sucrose method for crude DNA extraction from *Arabidopsis*

Small samples (2-3mm diameter) of young leaves (from around 3-week-old *Arabidopsis* plants) were placed directly into a PCR tube with 100µl of sucrose buffer (50mM Tris-Cl pH7.5, 300mM NaCl, 300mM Sucrose) on the ice. The tissue was then crushed with a yellow pipette tip until the supernatant turned slightly green. The samples were then put into a PCR machine and heated to 100°C for 10 mins before centrifugation at 2000-6000 x g for 5 seconds. The samples were placed on ice or stored at -20°C. 0.5µl of the supernatant was used as template in a 10 µl PCR reaction. This DNA extraction method was adapted from (Berendzen et al. 2005) and was used for the genotyping purpose.

2.2.2 Genomic DNA extraction

Plant genomic DNA was extracted using ISOLATE Plant DNA Mini Kit (Bioline, UK) according to the manufacturer's instructions. The quality and

quantity of DNA were determined using Nanodrop ND-1000 fluorospectrometer (NanoDrop Technologies, USA). DNA samples were stored at -20°C.

2.2.3 Precipitation of DNA

In order to obtain high concentration and pre-cleaned DNA, DNA samples were precipitated by adding 1/10 volume of sodium acetate (3M, pH 5.2) and 2-3 volumes of absolute ethanol or 1 volume of 100% isopropanol. The mixture was kept at -80°C for 2-3 hrs or incubated overnight at -20°C before centrifuging at 14,000g for 30 mins at room temperature. The supernatant was discarded carefully and replaced with 70% (v/v) ethanol to rinse the DNA pellet, which may, or may not, be visible at the bottom of the tube. The samples were centrifuged again for 15 mins. Supernatant was discarded and the DNA pellet was dissolved in 10-20 µl water.

2.3 EXPRESSION ANALYSIS

2.3.1 Total RNA extraction

Total RNA was extracted using the RNeasy Plant Mini Kit (Qiagen, UK) according to the manufacturer's instructions. In brief, less than 100 mg of fresh or frozen *Arabidopsis* tissues were ground in liquid nitrogen using plastic pestles into a fine powder and were homogenised in 450µl lysis buffer RLT with 1% (v/v) β-mercaptoethanol followed by 3 mins incubation at 56°C. The lysate was centrifuged for 2 mins at 17,000 x g, and the supernatant was mixed with 0.5 volume of absolute ethanol before loading into the RNeasy Mini Spin Columns. The columns were centrifuged at $\geq 8000 \times g$ for 15 sec and washed with 700µl of buffer RW1. To eliminate DNA contamination, 75 µl of RNase-Free DNase

solution (QIAGEN, UK) was added to the centre of column membrane and incubated at 28°C for 20 mins. The bound RNA was washed with 500 µl of buffer RPE twice before finally eluted in 20-50 µl of RNase-free water. Purified RNA was stored at -80°C until required.

2.3.2 cDNA synthesis

The concentration of RNA was measured by Nanodrop ND-1000 fluorospectrometer (NanoDrop Technologies, USA) just before cDNA synthesis. 2g of total RNA was mixed with 1µl oligo (dT) 12-18, 1µl 10 mM dNTP Mix and sterile distilled water to a total volume of 13µl. The mixture was incubated in a PCR machine at 65°C for 5 minutes then cooled on ice for at least 1 minute before adding 4µl 5X First-Strand Buffer, 1µl DTT (0.1M), 1µl RNaseOUT Recombinant RNase Inhibitor and 1µl SuperScript III Reverse Transcriptase (Invitrogen, UK). The total 20µl reaction was incubated at 50°C for 60 minutes, then the reaction was inactivated by heating to 70°C for 15 minutes. The cDNA samples was then used as template for PCR or stored at -20°C.

2.3.3 RT-PCR

The PCR reaction system was made up by mixing 0.1-0.2µl of cDNA template, 5µl REDTaq 2x Master Mix (VWR, UK), 0.5µl primer pairs (10pmol/µl), and molecular grade distilled water (SIGMA, UK) to a total volume of 10 µl. The solution was mixed gently by pipetting up and down several times. A Thermos Temperature Cycler was programmed according to the manufacturer's instructions. Depends on the melting temperature (T_m) of different primers, the annealing temperature usually ranged from 52°C to 60°C (or $T_m-3^\circ\text{C}$). The three-

step PCR programme was listed in Table 2.1. At the end of the PCR programme, the reaction product was loaded on an agarose gel for analysis.

Table 2.1 Three step PCR programme

Cycles	Step	Temperature (°C)	Duration (mm:ss)
1	Initial denaturation	95	3:00
22-35	Denaturation	95	0:30
	Annealing	52 to 60	0:30
	Elongation	72	1 min/kb
1	Final extension	72	4:00

2.3.4 Agarose gel electrophoresis

0.8-1.5% (w/v) molecular biology grade agarose (Melford, UK) in 0.5x TBE buffer (45 mM Tris-borate, 1 mM EDTA) with 0.025µg/ml ethidium bromide (Sigma, UK) was cast into gel moulds. RedTaq PCR product (loading dye included) was loaded onto the gel and electrophoresed in 0.5x TBE buffer at an electric field strength of 10 V/cm until DNA fragments were sufficiently separated. Generally, HyperLadder™ 1kb (Bioline, UK) molecular weight marker was used to evaluate the size of DNA fragment. DNA was visualised under long wave UV light, and the gel images were captured using SnapGene Software (GSL Biotech LLC).

2.3.5 Quantitative (Real-time) PCR

QPCR was adopted in this project for quantitative gene expression analysis. Maxima SYBR Green QPCR 2x Master Mix (Thermo Fisher Scientific, UK) was employed to detect and quantify gene expression. The fluorescence from SYBR Green I is detectable when bound non-specifically to double strand DNA and increased as PCR products are amplified, making it possible for the detection

of PCR product in real-time. QPCR was performed and analysed using the LightCycler® 480 System (Roche, UK).

The experimental reaction comprised 4.5µl Maxima SYBR Green QPCR 2x Master Mix, 0.2µl cDNA template, 0.2µl primer pairs (10pmol/µl) and 4µl nuclease-free water. To calculate primer efficiency, wild-type cDNA series (undiluted pool, 1:5, 1:25, 1:125; 1:625 dilution) was used as templates. Generally, for expression analysis, *ACTIN7* was chosen as a reference gene. Primers of *ACTIN7* and genes of interest were amplified in separate wells of one QPCR Light Cyclers 480 multi-well plate 384 (Roche, UK). All samples were run at least in duplicate. The plate was centrifuged at 2000g for 2 minutes before loading to the LightCycler 480 QPCR machine according to the manufacturer's instructions, following the programme as shown in Table 2.2

Table 2.2 QPCR programme

Cycles	Steps	Temperature (°C)	Duration (mm:ss)
1	Initial denaturation	95	10:00
45-50	Denaturation	95	0:30
	Annealing	52-60 (or T _m -3)	0:30
	Elongation	72	1min/kb
1	Dissociation	From annealing temperature to 95°C. Increase 1°C per cycle	0:30

2.4 MOLECULAR CLONING METHODS

2.4.1 DNA restriction digestion and ligation

The full length of *MYB26* CDS was amplified using Q5 High-fidelity DNA polymerase (NEB, UK) according to the manufacturer's instructions. Primers were designed with adapters containing recognition sites of BamHI or NotI. The PCR product was checked by electrophoresis on 1% agarose (w/v) gel and correct size DNA was recovered using QIAquick Gel Extraction Kit (Qiagen,

UK). Both the purified PCR products and plasmid pET41a(+) were digested with BamHI(HF) and NotI(HF) restriction enzyme in CutSmart™ Buffer (NEB, UK) at 37°C at least overnight. The overnight solution was run through 1% agarose (w/v) gel to check digest efficiency, DNA product of the correct size were recovered using QIAquick Gel Extraction Kit (Qiagen, UK).

DNA ligation was performed using T4 DNA ligase (NEB, UK) according to the manufacturer's instructions. Generally, around 25-50 ng of plasmid DNA was used in a ligation reaction with an insert: plasmid molar ratio of 3:1. The reaction was incubated overnight at 16°C. 5µl of the reaction was transformed into 50µl One Shot Mach1™ competent cells (Invitrogen, UK).

2.4.2 TOPO® clone and Gateway® technology

2.4.2.1 TOPO clone

The core of TOPO cloning is the enzyme DNA topoisomerase I, which functions both as a restriction enzyme and as a ligase. The topoisomerase I specifically recognises the 5'-(C/T)CCTT-3' sequence and forms a covalent bond with the phosphate group attached to the 3' thymidine and cleaves one DNA strand to unwind the DNA and then re-ligate the ends before releasing from the DNA. The TOPO® vector is Gateway®-adapted and is linearized with topoisomerase I covalently bound to the 3' phosphate on each end. This enables the vector to ligate the insertion DNA with compatible ends to produce entry clone.

pCR™8/GW/TOPO® TA Cloning Kit (Invitrogen, UK) was used to generate the Entry Clones with inserted CDS of *MYB26*, *Y2H320*, *Y2H560*, individually. According to the manufacturer's instructions, 2µl of PCR products (with 3' adenine) was mixed with 0.5µl salt solution, 0.5µl TOPO® vector and incubated

overnight at room temperature. This 3µl TOPO cloning reaction was transformed into One Shot Mach1™ competent cells (Invitrogen, UK), colony PCR and sequencing were performed to ensure the inserted DNA had the correct orientation and sequence.

2.4.2.2 Gateway Clone

Gateway cloning was performed using Gateway™ BP/LR Clonase™ II Enzyme Mix (Invitrogen, UK) according to the manufacturer's instructions using the minimum suggested reaction composition. All BP and LR reactions were incubated at 25°C overnight and the reactions were terminated by adding 1µl proteinase K and incubating for 10 mins at 37°C before transforming into DH5α competent cells.

2.4.3 *E. coli* transformation using the heat shock method

A falcon tube containing 50 µl of competent cells was thawed on ice. Around 50ng of plasmid DNA was added to the competent cells as soon as they thawed. The mixture was incubated on ice for 30 mins before moving to a pre-heated 42°C water bath for 1 minute. The cells were returned to the ice for 2 mins and 200 µl of SOC medium (2% (w/v) Tryptone, 0.5% (w/v) yeast extract, 10mM NaCl, 2.5mM KCl, 10mM MgCl₂, 20mM glucose; room temperature) was added to each tube. The cells were incubated with shaking for 1 hr at 37°C. An appropriate amount (50-100 µl) of the cell culture was spread onto the surface of LB agar plate with antibiotics required and incubated overnight at 37°C.

2.4.4 Plasmid DNA extraction and sequencing

Plasmid DNA was extracted using the GenElute™ Plasmid Miniprep Kit (Sigma, UK) according to the manufacturer's instructions with the exception that reduced

elution volume was used to obtain 100ng/μl DNA concentration for sequencing purposes.

During the cloning procedure, plasmid DNA was extracted from both entry clones and expression clones, and sequenced with primers from both ends to ensure the insertion DNA was correct. Sequencing was performed using the Sanger sequencing services provided by Source Bioscience (Nottingham). The sequencing data was analysed using SnapGene Software (GSL Biotech LLC, US).

2.4.5 *Agrobacterium* transformation by electroporation

50 μl of competent *Agrobacterium* cells (strain GV3101, Genomics facility, University of Nottingham) was thawed on ice. Around 200 ng of plasmid DNA was added and mixed gently on the ice. The *Agrobacterium* was transferred to an electroporation cuvette and loaded to the electroporation machine under the “Agr” setting before pressing the “pulse” button. An electric field at a strength of 1.25 kV/cm was then applied to the cells. The cells were immediately transferred into 1ml LB media and incubated at 28°C for 2 hrs before spreading onto LB agarose plate with antibiotics as required and incubating at 28°C for 2-3 days.

2.4.6 Storage of bacteria as glycerol stocks

Generally, 1ml overnight culture of *E. coli* or *Agrobacterium* containing sequenced construct was mixed to a final concentration of 20% (v/v) glycerol and flash frozen in liquid nitrogen. The glycerol stock was kept at -80°C.

2.5 PROTEIN METHODS

2.5.1 Induction and optimisation of expression of target proteins in *E. coli*

Freshly grown colony or glycerol stock was inoculated overnight in 5ml LB culture media containing antibiotics at 37°C with 200 rpm agitation. The overnight culture was used to inoculate a fresh culture in 1:50-1:100 dilution and grown until OD₆₀₀=0.6-0.8. A series of Isopropyl β -D-1-thiogalactopyranoside (IPTG) concentration 0.1-1mM was added before inoculation at a different temperature, such as 25°C, 28°C and 37°C. Each culture was split into two cultures, one for the induced sample and the other for pre-induced control. An aliquot of the culture (200-500 μ l) was collected over a time-course (0h, 2hrs, 4hrs, 6hrs and overnight); cells were centrifuged and the cell pellets stored at -20°C until samples of all time points were collected. The pellet cells were re-suspended in ice-cold TBS and lysed by sonication in an ice water bath (output power 320w, 6x 15 seconds pulse, 1 min rest in between). The cell debris was collected by centrifugation at 17,000g for 5 mins. 10 μ l of each supernatant (contain soluble proteins) and debris (contain insoluble proteins) were retained for analysis by protein gel electrophoresis. The target protein and solubility were checked after electrophoresis using Coomassie blue staining and Western blot.

2.5.2 Protein gel electrophoresis

Apparatus and consumables for protein 1D electrophoresis were obtained from Invitrogen, including NuPAGE[®] LDS sample buffer, NuPAGE[®] Novex 4-12% Bis-Tris Protein Gels, XCell SureLock[™] electrophoresis apparatus, etc. Bacteria culture samples were dissolved in 1X NuPAGE[®] LDS sample buffer and heated at 70°C for 10 minutes before loading to NuPAGE[®] Novex 4-12% Bis-Tris Protein Gels. Protein 1D electrophoresis was taken under 180V for 40 minutes.

Total protein was stained with Coomassie brilliant blue solution (0.1% Coomassie[®] R-250 (w/v), 40% (v/v) ethanol, 10% (v/v) acetic acid) and heated in the microwave under full power for 30 sec before gentle shaking for 15 minutes. Protein gels were then destained overnight by gently shaking in the destain solution (10% (v/v) ethanol, 7.5% (v/v) acetic acid) until the gel background became transparent. Stained protein gel was wrapped with cling film and imaged immediately using a scanner.

2.5.3 Western blot

The Western transfer was conducted using the semi-dry system (BioRad Trans-Blot SD Semi-Dry Transfer Cell). After electrophoresis, the protein gel was equilibrated in 1x NuPAGE[®] Transfer Buffer (Invitrogen, UK) for 5 minutes to remove salts. Three sheets of pre-soaked filter paper were loaded onto the anode plate, followed by pre-soaked nitrocellulose membrane, equilibrated gel and another three sheets of pre-soaked filter paper. Air bubbles were removed by rolling the stack with a blotting roller when loading each layer, any excess transfer buffer was removed from the anode plate using blue paper towel before placing the cathode plate onto the stack and putting the safety cover on.

After transferring at 12V for 1 hour, the nitrocellulose membrane was washed and blocked in 5% (w/v) non-fat milk TBST buffer and incubated overnight at 4°C. The membrane was rinsed in TBST (137mM NaCl, 20mM Tris, 0.1% (v/v) Tween-20, pH7.4) before incubating with the appropriate HRP-conjugate antibody diluted in 5% (w/v) non-fat milk TBST buffer for 2-3 hours at room temperature. The membrane was washed in TBST (five times, 5mins each time) and used for chemiluminescent detection using SuperSignal[™]West Femto Maximum Sensitivity Substrate (Thermo Fisher Scientific, UK) according to the

manufacturer's instructions except that the working solution was diluted 1:10 in water when incubating with the blot.

2.6 MICROSCOPY AND STAINING METHODS

2.6.1 DAPI staining

DAPI (4',6-diamidino-2-phenylindole) emits blue fluorescence when binding to AT regions of DNA, making it a popular nuclear and chromosome counterstain. DAPI staining was used in this project to assess the microspore and pollen developmental stage.

Arabidopsis whole inflorescences were fixed in 3:1 (v/v) ethanol: acetic acid solution for up to 24hrs. Anthers were separated under a stereomicroscope and placed in turn onto a clean slide. 20µl of 3µg/ml (w/v) DAPI working solution was added to the slide, covering 5-6 anthers from one bud. The cover slide was slightly pressed to facilitate pollen release. Since DAPI is light sensitive, samples were wrapped with aluminium foil immediately and ready to be analysed using Leica DM5000B after 5 minutes staining.

2.6.2 Alexander staining

Anthers were dissected from flowers and mature buds under a stereomicroscope and were placed onto a clean slide. 20-40 µl of Alexander staining solution (Alexander 1969) was dropped onto the sample. The cover slide was gently pressed to facilitate pollen release. The samples were observed under a light microscope after staining 30 minutes. Viable pollens are stained dark red while non-viable pollens are stained green.

2.6.3 Lignin Staining

The whole inflorescence with at least two open flowers was placed in 1X PBS (pH7.4, 137mM NaCl, 2.7mM KCl, 2mM KH₂PO₄, 10mM Na₂HPO₄.) with 2% (v/v) Tween-20 for 10 minutes at room temperature, and then rinsed with 1x PBS before staining with 0.01% (w/v) acridine orange for 1 hour. Samples were washed with 1x PBS for 5 minutes before staining in 0.005% (v/v) of 10 mg/ml ethidium bromide (Sigma, UK) for 1 hour. Finally, samples were washed twice with 1x PBS for 5 minutes and dissected under a stereomicroscope. Anthers in the open flowers and mature buds were checked under the Leica TCS SP2 Laser Scanning Confocal Microscopy under the following parameters: Ethidium bromide staining: excitation=514nm (25% laser), emission collection=580-630nm; Acridine orange staining: excitation=458nm (25% laser), emission collection=480-510nm.

CHAPTER 3 MYB26-REGULATED GENES

3.1 INTRODUCTION

Anther and pollen development is a complex biological process which is exquisitely timed and choreographed (Koltunow et al. 1990; Scott et al. 1991), Anther development could be divided into two general phases, during phase one, the morphology of anther is established, and microspore mother cells undergo meiosis, tetrads of microspores are present within the pollen sacs. During phase 2, pollen grains differentiate, the anther enlarges and is pushed upward in the flower by filament extension, endothecium secondary thickening (ST), tissue degeneration, dehiscence, and pollen grain release occurs (Goldberg et al. 1993). Many key transcription factors regulating this process have been identified. *DYSFUNCTIONAL TAPETUM1* (Zhang et al. 2006), *MALE STERILITY1* (Wilson et al. 2001), *MYB33/MYB65* (Millar & Gubler 2005), *MYB80/MYB103* (Higginson et al. 2003; Zhang et al. 2007; Zhu et al. 2010) are involved in tapetum and pollen wall development. Transcription factor MS35/MYB26 plays an important role during anther dehiscence (Steiner-Lange et al. 2003; Yang et al. 2007). The null mutant of *ms35* has a defect on endothecium ST with indehiscent anthers, while overexpression of *MYB26* results in ectopic secondary thickening. However, the regulation network of *MYB26* remains largely unknown.

The dexamethasone (Dex) inducible gene expression system was applied in this project to study genes possibly regulated by *MYB26*. Induction of transgene expression has the advantage of offering researchers more precisely temporal and spatial control of gene expression. Inducible systems are applied mainly in

tobacco, rice, *Arabidopsis*, tomato and maize. By providing the transgenic plant with appropriate chemical (such as Dexamethasone, β -estradiol etc.) or treatment (such as heat shock), inducible systems allow promoting changes in gene expression level without causing gross alterations to the whole plant development (Corrado & Karali 2009; Borghi 2010). Dexamethasone (Dex) induction system is based on the ligand-binding domain of glucocorticoid receptor (GR). GR interacts with cytosolic complexes containing heat shock protein 90 (HSP90) when there is no steroid. Transcription factors (TFs) fused with GR are inactive because they bind to HSP90 anchored in the cytoplasm thus cannot get into the nucleus. When treating with dexamethasone, a synthetic steroid hormone, GR-HSP90 interaction is disrupted and the GR-TF fusion protein is free to enter the nucleus in order to bind and activate its targets (Borghi, 2010).

With the recent advances in postgenomic technologies, a large number of genome-wide transcriptomic data sets have been generated, publically available database and community resource such as FlowerNet (Pearce et al. 2015) are valuable tools to study the regulation network of *MYB26*. A microarray analysis using the Affymetrix GeneChipTM was conducted (Jie Song, 2009) using precisely staged *Arabidopsis* stamen from both the indehiscence mutant *ms35/myb26* and the wild-type, to identify genes associated with development and dehiscence, as well as the *MYB26* regulatory targets. The above-mentioned resources can be used during bioinformatics analysis of *MYB26* putative regulating candidates.

In this chapter, *MYB26* stable overexpressing lines were generated. Increased secondary thickening was observed in *Arabidopsis* lines that were ectopically

expressing *MYB26* under the 35S promoter (P_{35S}) or ubiquitin 10 promoter (P_{UBQ10}). *MYB26* inducible overexpression expression system was used to check the response of selected genes which were down-regulated in the *ms35* mutant. The expression of the NAC transcription factors *NST1* and *NST2*, a proline-rich family protein and gibberellin 2-oxidase (*GA2ox1*) were up-regulated in a time-dependant manner after *MYB26* induction. TFs that altered expression in the *ms35* mutant during pollen mitosis I (PMI) and bicellular (BC) stage were reviewed to facilitate further study of the *MYB26* regulation network.

3.2 METHODS

3.2.1 Dexamethasone treatment

Dexamethasone (Dex) working solution was prepared using 25mM dexamethasone (Sigma, UK) stock solution and 2% (v/v) silwet L-77 (Lehle Seeds, USA) stock solution to a final concentration of 25 μ M Dex and 0.02% (v/v) silwet L-77.

4-5 weeks old *Arabidopsis* were sprayed with Dex working solution and were collected immediately as 0hr, the second treatment took place at 12hrs after the first Dex treatment. Leaf tissue was collected in time-course from 0 hrs up to 36hrs after treatment, and were flash frozen in liquid nitrogen before processing whole RNA extraction using RNeasy Mini Kit (QIAGEN, UK). Two biological replicates were collected in this experiment.

3.2.2 TFs with altered expression in the *ms35* mutant

The transcription factor (TF) database of *Arabidopsis thaliana* was obtained from PlantTFDB 3.0 (Zhang et al. 2011). A recombinant MYB26-GST random oligonucleotide binding experiment had previously determined C[A/C]GATAATT[A/C]AATC[G/A], a consensus sequence of a putative MYB26 binding motif *in vitro* (B.Taylor, 2014). Gene loci which contained this consensus sequence within the 3000bp upstream region was screened using bulk data retrieval tool on TAIR website.

Venn diagram was generated using Venny2.1 (Oliveros 2007) with TFs database, and genes contain MYB26 *in vitro* binding motif as well as the *ms35* microarray data to investigate TFs that are possibly downstream of MYB26 regulation network (Figure 3.7)

3.3 RESULTS

3.3.1 Generating *MYB26* Overexpression lines

MYB26 fully spliced CDS without stop codon was cloned into Gateway vectors pGWB5-GFP and pUBC-GFP to generate pGWB5-*MYB26*-GFP (35S::*MYB26*-GFP) and pUBC-*MYB26*-GFP (ubq10::*MYB26*-GFP) constructs. All Gateway vectors were transformed through *Agrobacterium* into wild-type Landsberg *erecta* (Ler). Transgenic plants were identified using antibiotic selection. Quantitative RT-PCR was performed to confirm the expression level of *MYB26*.

Transgenic plants containing 35S::*MYB26*-GFP constructs result in various levels of ectopic secondary thickening (ST) phenotype, many of the *MYB26* overexpression plants displayed dwarf phenotype with curled leaves, thickened and twisted stems shown in Figure 3.1 B, indicating strong ectopic ST development. Quantitative RT-PCR was performed with 4 individual lines displayed ectopic ST from severe (OEx1) to moderate phenotype alteration (OEx4). The results showed that the expression of *MYB26* was up-regulated in both buds and leaves tissue of all four representatives and with higher *MYB26* transcript level observed in plants displaying a stronger ST phenotype (Figure 3.1 D).

Generally, not like *P*_{35S} driven *MYB26* overexpression (OEx) lines, which displayed strong ST phenotype in vegetative tissue, the phenotype of *P*_{UBQ10} driven *MYB26* OEx lines were more obvious with reduced fertility to sterile phenotype, the leaves of ubq10::*MYB26*-GFP transgenic plants were slightly curled and relatively smaller compared to the wild-type, while the stems were

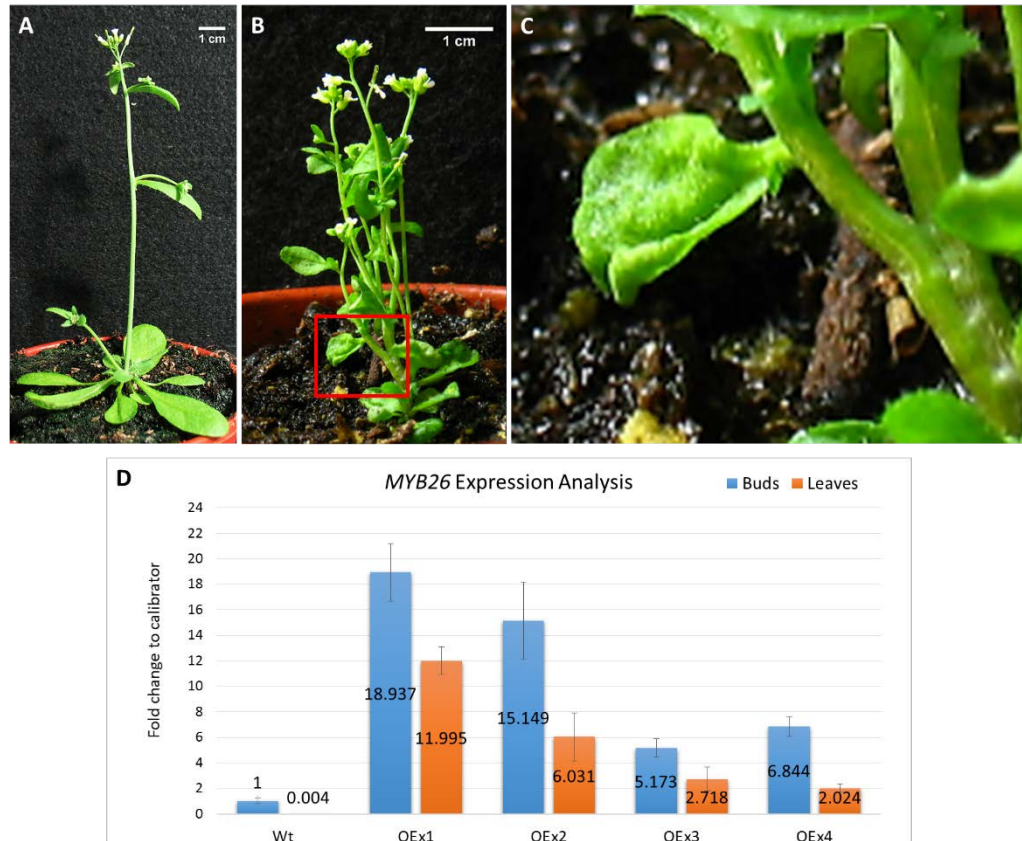


Figure 3.1 *MYB26* overexpression under the control of the 35S promoter

- A. Wild-type *Ler*. Scale bar 1cm
- B. Typical example of the 35S::MYB26-GFP transformation line displaying dwarf and curled leaves, thickened stems, indicating ectopic secondary thickening. Scale bar 1cm.
- C. Close-up of the red frame in figure B
- D. Quantitative RT-PCR of *MYB26* expression analysis in buds and leaves of the wild-type (Wt) and four representatives of the 35S::MYB26-GFP lines displaying strong (OEx1) to moderate phenotype alteration (OEx4). Data was normalised to the reference gene *Actin 7* and then compared to the *MYB26* expression in the wild-type buds. Error bar, standard error.

slim and didn't display ectopic ST (Figure 3.2 A and B). Quantitative RT-PCR of two representatives with strong (OEx1) or moderate (OEx2) ST phenotype showed that *MYB26* transcripts level was increased in both leaves and buds tissues under the driving of P_{UBQ10} and the severity of the phenotype corresponded to the level of *MYB26* expression (Figure 3.2 D).

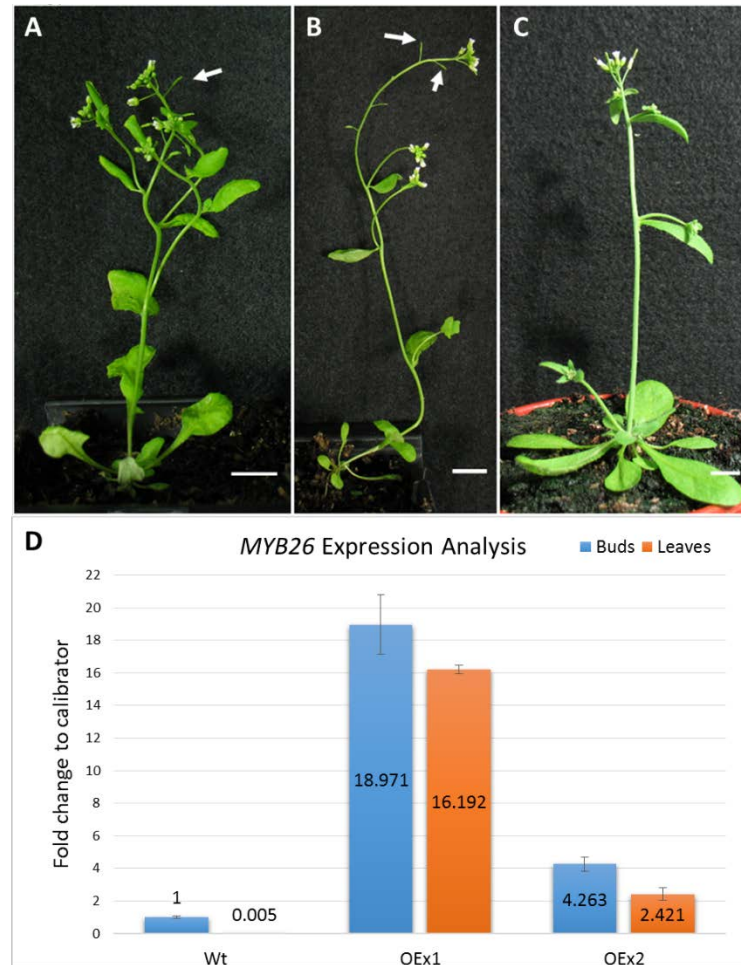


Figure 3.2 *MYB26* overexpression under the control of the ubiquitin 10 promoter

- A. Example of the *ubq10::MYB26*-GFP transformed line with curled leaves, indicating ectopic secondary thickening (ST) . Scale bar 1cm.
- B. Example of the *ubq10::MYB26*-GFP transformed line displaying moderate ST phenotype. Sterile siliques (white arrow) indicate reduced fertility. Scale bar 1cm.
- C. Wild-type *Ler*. Scale bar 1cm
- D. Quantitative RT-PCR of *MYB26* expression analysis in buds and leaves of the wild-type (Wt) and two representatives of the *ubq10::MYB26*-GFP lines displaying strong (OEx1) and moderate phenotype alteration (OEx2). Data was normalised to the reference gene *Actin 7* and then compared to the *MYB26* expression in the wild-type buds. Error bar: standard error.

Both P_{35S} and P_{UBQ10} could efficiently facilitate the overexpression of *MYB26* in both leaves and inflorescence tissues (Figure 3.1 D, and Figure 3.2 D). Although the strongest phenotype was obtained from $35S::MYB26$ -GFP plants which were severely dwarfed with ectopic ST, and sterile. Many $35S::MYB26$ -GFP

plants were still fertile although many had shorter siliques, indicating reduced fertility. However, the sterile phenotype was more commonly found in the *ubq10::MYB26-GFP* plants, although a less severe ST phenotype was displayed in the vegetative tissues compared to the *35S::MYB26-GFP* lines. In order to understand the reason behind the different fertility of the two *MYB26* OEx lines, dissected flowers of both *ubq10::MYB26-GFP* and *35S::MYB26-GFP* transgenic plants were observed under the stereomicroscope (Figure 3.3). In wild-type buds (with visible petals), anthers were dehiscent, pollen was visible on pistil as a result of pollination (Figure 3.3 I). Similarly, dehiscent anthers were observed in the *35S::MYB26-GFP* buds (Figure 3.3 F). However, in *ubq10::MYB26-GFP* lines, anthers in buds were indehiscent and turgid, filament elongation also seems hindered (Figure 3.3 C) but the extent varied between *ubq10::MYB26-GFP* open flowers (two representatives are shown in Figure 3.3 A and B), some open flowers were partially pollinated with small amount of released pollen, however anthers were not fully dehiscent to the extent seen in the wild-type and *35S::MYB26-GFP* lines, filament elongation was not fully complete and did not reach the same level as the pistils. Many other *ubq10::MYB26-GFP* flowers displayed more extreme phenotypes as shown in Figure 3.3 B, with indehiscent anthers, short filaments curled towards the pistil. In summary, the impaired anther dehiscence and reduced filament elongation appears to result in the significantly reduced fertility seen in the *ubq10::MYB26-GFP* lines.

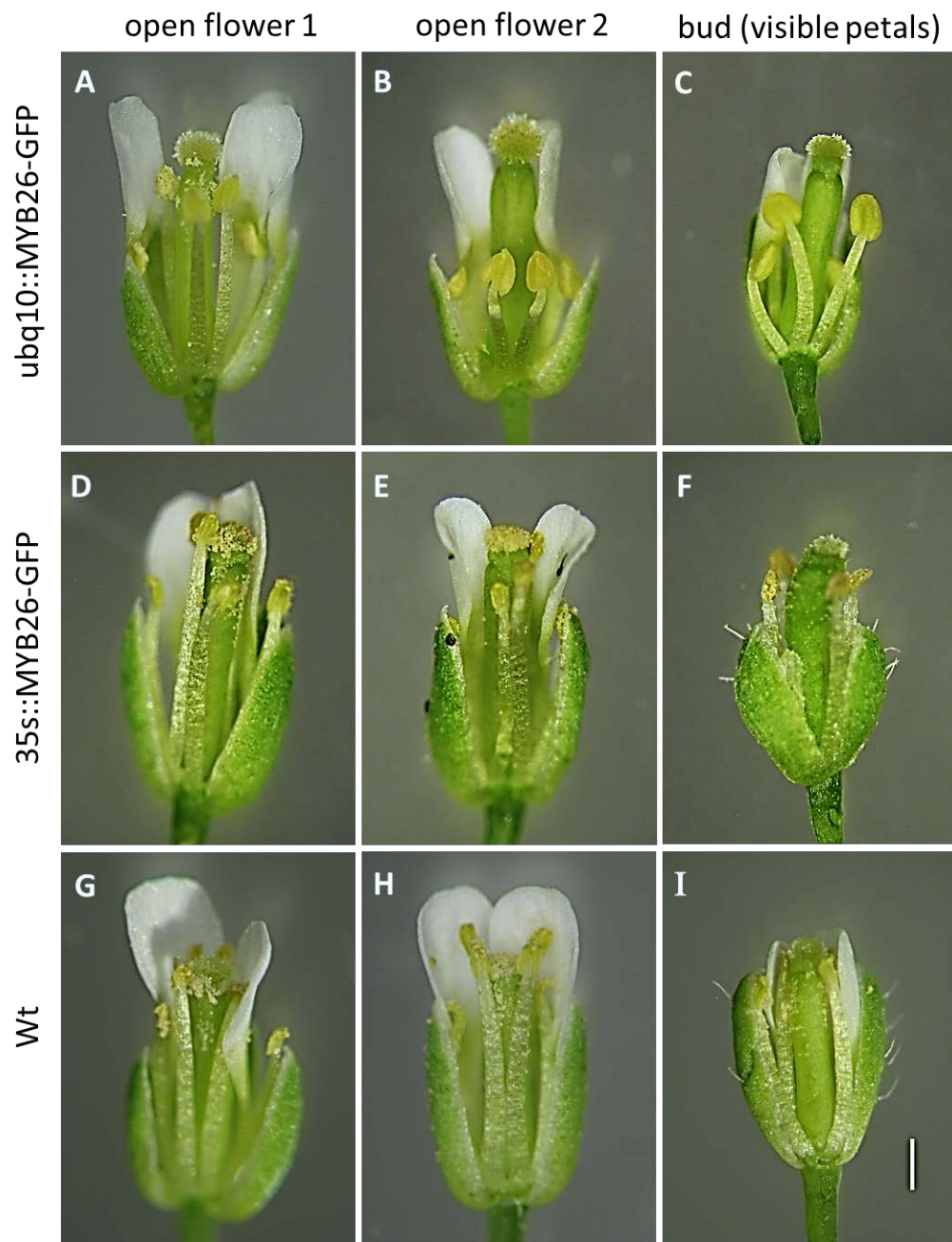


Figure 3.3 Flower phenotype of the *MYB26* overexpression lines

Examples of open flowers and bud with visible petals from each line: *MYB26* overexpression under the control of the ubiquitin 10 promoter (A-C) or cauliflower mosaic virus 35S promoter (D-F) and wild-type *Col* (G-I). Petals and sepal were partially removed. Scale bar 0.5mm.

3.3.2 Time-course gene expression analysis

The *MYB26* inducible overexpression (35S-GR::*MYB26*) line generated using the PKIGW vector (Dr C.Yang, University of Nottingham) was employed to study the downstream targets of the *MYB26*. By treating with Dexamethasone (Dex), ligand binding glucocorticoid receptor (GR)-GAL4 activation domain could be released from the cytoplasm to enter the nucleus and bind to the upstream activation sequence(UAS) which is the 35S promoter, followed by *MYB26* CDS (and GUS reporter), resulting up-regulation of *MYB26*. A time-course quantitative RT-PCR was performed to analyse the expression changes during 36hrs after Dex treatment.

The transcript level of *MYB26* was up-regulated 2hrs after Dex treatment, eventually peaking around at a 65 fold increase at 6 hrs post-induction. The *MYB26* transcript level declined afterwards but recovered after the second Dex induction taking place at 12hrs and remained relatively high until 36 hrs post-induction (Figure 3.4. D)

The *NAC SECONDARY WALL THICKENING PROMOTING FACTOR1* (*NST1*) and *NST2* function redundantly in regulating secondary wall thickening in anther walls (Mitsuda et al. 2005). The *nst1nst2* double mutant displayed anther-indehiscent phenotype due to loss of ST in anther endothecium, a similar phenotype is observed in the *ms35* mutant (Steiner-Lange et al. 2003; Yang et al. 2007). The expression of *NST1*, *NST2* peaked at bicellular (BC) stage in the wild-type but was dramatically reduced at the equivalent stage in the *ms35* stamen (Figure 3.4.B and C). In the *MYB26* inducible over-expression line (35S-GR::*MYB26*), the transcript level of *NST1* was increased after 4hrs post-

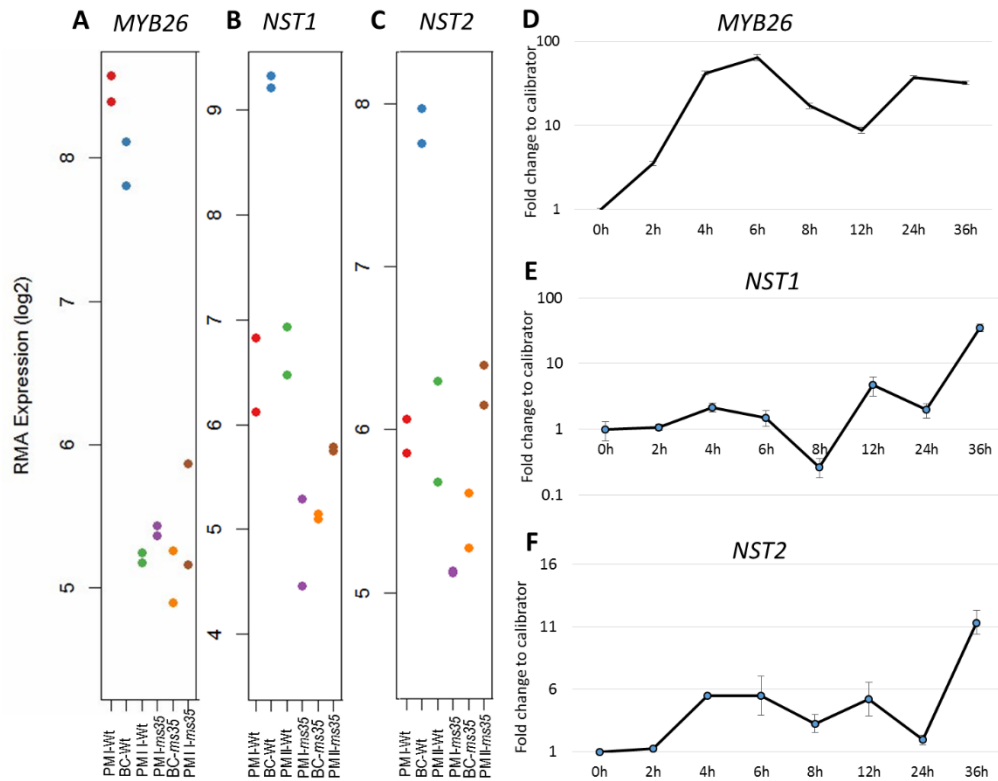


Figure 3.4 The expression of *NST1*, *NST2* was regulated by *MYB26*

A-C, Expression plots of *MYB26* (A), *NST1* (B) and *NST2* (C) in the wild-type (Wt) and the *ms35* mutant stamens at pollen mitosis I (PMI), bi-cellular (BC) and pollen mitosis II (PMII) stages. Each dot represents one replicate. Data is available from <https://www.cpib.ac.uk/anther/> (Pearce et al. 2015).

D-F, QRT-PCR of *MYB26* (D), *NST1* (E), *NST2* (F) in the *MYB26* inducible overexpression line (35S-GR::MYB26). Leaf tissue was collected over a time course after Dex treatment. Error bar, standard error.

induction, but a stable up-regulation was not observed until 12 hrs post-induction.

NST2 was up-regulated 4hrs after Dex-induction and expression remained high until 36hrs post-induction (Figure 3.4 F). The *ms35* microarray and time-course qRT-PCR results suggest that the transcript level of *NST1* and *NST2* is regulated by *MYB26*.

AT1G70990 encodes a proline-rich family protein (PRP). There is a significant down-regulation of *PRP* expression during both PMI and BC stage in the *ms35* mutant stamen (Figure 3.5 A), the transcript level of *PRP* also responded to

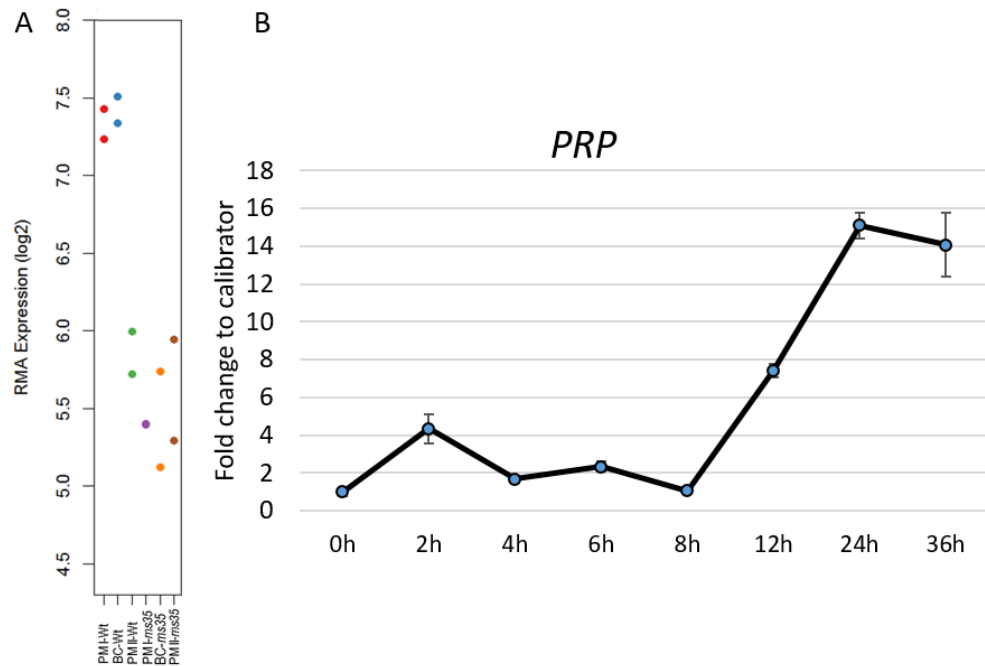


Figure 3.5 The expression of proline-rich family protein (*PRP*) was regulated by *MYB26*

- Expression plots of *PRP* in the wild-type (Wt) and the *ms35* mutant stamens at pollen mitosis I (PMI), bi-cellular (BC) and pollen mitosis II (PMII) stages. Each dot represents one replicate. Data is available from <https://www.cpib.ac.uk/anther/> (Pearce et al. 2015).
- QRT-PCR of *PRP* in the *MYB26* inducible over-expression line (35S-GR::*MYB26* lines). Leaf tissue was collected over a time course after Dex treatment. Error bar: standard error.

MYB26 induction. The up-regulation of *PRP* was first observed at 2hrs post-induction, but after 8hrs post-induction, the expression of *PRP* stably increased and peaked at 15 fold change at 24 hrs and remain up-regulated around 14 fold change at 36hrs post-induction (Figure 3.5 B). This suggests that *PRP* was regulated by *MYB26*.

GA2ox1 encodes a gibberellin 2-oxidase, which is a major member of bioactive GAs-deactivating enzymes and is responsible for gibberellin metabolism. Interestingly, the expression of *GA2ox1* was down-regulated in the *ms35* mutant stamen during all three pollen developmental stages from PMI to PMII (Figure

3.6 A). Moreover, the expression of *GA2ox1* was up-regulated in the *MYB26* inducible overexpression line at 2hrs post-induction and reached a low peak (3 fold increase) at 4hrs before decrease to the normal level at 8hrs, the expression was later up-regulated thereafter and remained high (5 fold increase) at 24hrs post-induction (Figure 3.6 B). The expression alteration of *GA2ox1* in both the *ms35* mutant and the *MYB26* inducible overexpression lines suggest that *GA2ox1* transcript level may be regulated by *MYB26*.

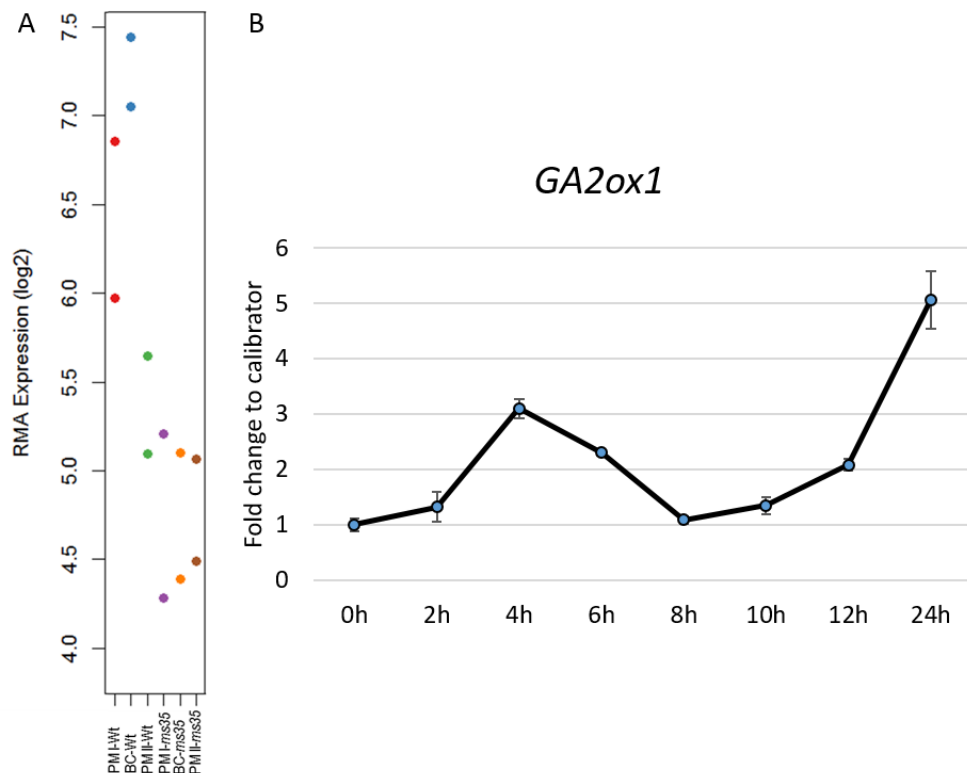


Figure 3.6 The expression of *GA2ox1* was regulated by *MYB26*

- A. Expression plots of *GA2ox1* in the wild-type (Wt) and the *ms35* mutant stamens at pollen mitosis I (PMI), bi-cellular (BC) and pollen mitosis II (PMII) stages. Each dot represents one replicate. Data is available from <https://www.cpib.ac.uk/anther/> (Pearce et al. 2015).
- B. QRT-PCR of *GA2ox1* in the *MYB26* inducible overexpression line (35S-GR::MYB26). Leaf tissue was collected over a time course after Dex treatment. Error bar, standard error.

3.3.3 Transcription factors with altered expression in the *ms35* mutant.

Secondary cell walls provide mechanical support, facilitate critical biological processes such as nutrient and water transport, plant organ movement, silique shattering, anther dehiscence (Hussey et al. 2013). Genes participated in secondary thickening (ST) development are under strict transcriptional control, when the considerable diversity of transcription factors (TFs) are involved. The most predominant families regulate ST appear to be the NAC and R2R3-type MYB families (Dubos et al. 2010a; Hussey et al. 2013; Zhong & Ye 2014). *NST1* and *NST2* have been reported to be master switches of ST synthesis and among top tier regulators in the ST regulation network (Hussey et al. 2013).

The expression of *NST1* and *NST2* was down-regulated in the *ms35* mutant, while up-regulated responding to *MYB26* induction (Figure 3.4) and in the *MYB26* overexpression line (Yang et al. 2007), suggesting that *MYB26* may possibly regulate ST through *NST1*, *NST2*. Moreover, *MYB26* may be one of the top tier regulators during ST development. Thus in order to study the regulation network of *MYB26*, gene expression comparisons were conducted to highlight the TFs that altered expression in the staged anthers of the *ms35* mutant, especially during pollen mitosis I (PMI) and Bicellular (BC) stage when *MYB26* is highly expressed in the wild-type. The aim of this analysis is to find TFs that may be putative downstream targets of the *MYB26* regulation network involving ST development in anther. The transcription factor database of *Arabidopsis thaliana* was obtained from PlantTFDB 3.0 (Zhang et al. 2011). A recombinant MYB26-GST random oligonucleotide binding experiment determined a consensus sequence of the MYB26 putative binding motif *in vitro* (Taylor, 2014).

Gene loci which contained the MYB26 *in vitro* binding motif C[A/C]GATAATT[A/C]AATC[G/A] within the 3000bp upstream of ATG transcription start site were screened using bulk data retrieval tool on TAIR website. Four out of 13 TFs which show altered expression in the *ms35* mutant during both PMI and BC stages, contained the MYB26 *in vitro* binding motif 13 (Figure 3.7). According to the Venn diagram results (Figure 3.7), Thirteen TFs that are possible downstream of the MYB26 regulation network are listed in Table 3.1 with expression fold change compared to the wild-type.

Among the 13 transcription factors, the expression of *AGAMOUS-LIKE 87* (*AGL87*), a MADS-box family protein; *At3g53310*, a AP2/B3-like

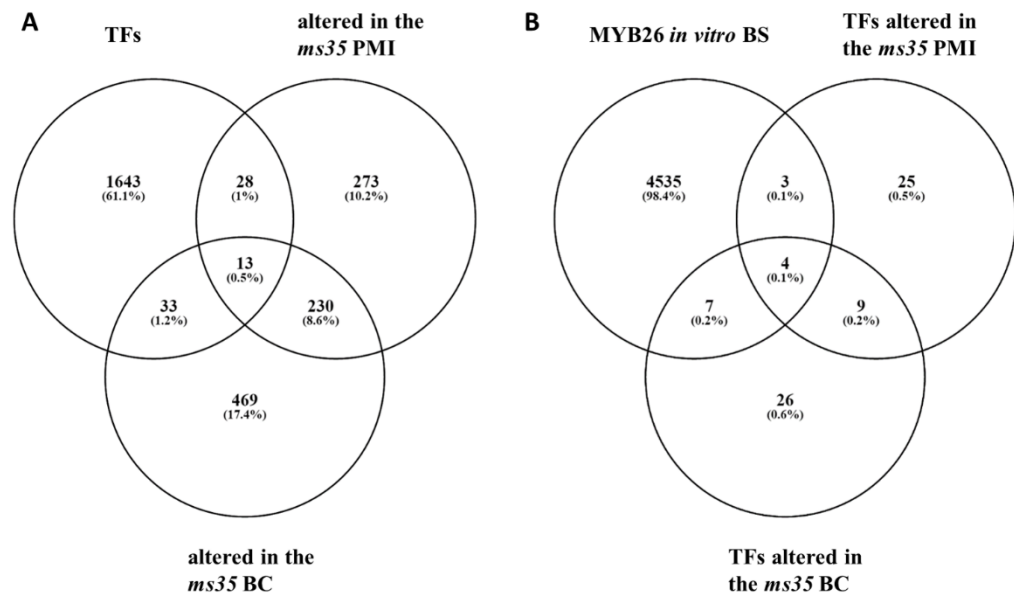


Figure 3.7 Venn diagrams of the TFs that altered expression in the *ms35* mutant

- Venn diagram¹ of the transcription factors² (TFs) and genes with altered expression in the *ms35* at pollen mitosis I (PMI) stage, bicellular stage (BC).
- Venn diagram of TFs that altered expression in PMI or BC stage in the *ms35* and contain the MYB26 *in vitro* binding site (BS) (Taylor, 2014) within 3000bp upstream of the translation start site.

¹ Venn diagram was generated using Venny2.1 (Oliveros 2007)

² The transcription factor database of *Arabidopsis thaliana* was obtained from PlantTFDB 3.0 (Zhang et al. 2011).

Table 3.1 Transcription factors with altered expression in the *ms35* in PMI and BC stage.

Common elements	AGI code	Other name	<i>ms35</i> PMI	<i>ms35</i> BC
Binding motif PMI BC	AT1G19510	RL5	3.168 ↓	2.679 ↓
	AT1G22590	AGL87	2.712* ↑	2.122* ↑
	AT1G66810	C3H14	2.089 ↓	21.273*** ↓
	AT2G38090	NA	2.013 ↓	3.258* ↓
PMI BC	AT2G46770	NST1	3.118 ↓	17.976*** ↓
	AT3G53310	NA	11.388*** ↑	4.63 ↑
	AT5G43410	ERF96	6.108*** ↑	2.912 ↑
	AT5G10140	FLC	8.048* ↓	8.686* ↓
	AT1G62990	KNAT7	2.069* ↓	8.712* ↓
	AT1G73410	MYB54	3.323 ↑	3.838 ↓
	AT2G16720	MYB7	2.031 ↓	3.271 ↓
	AT3G13890	MYB26	9.014*** ↓	8.177*** ↓
	AT4G09460	MYB6	2.066 ↓	2.784*** ↓

↓, down-regulated; ↑, up-regulated; Statistical significance: *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$; Binding motif, *MYB26 in vitro* binding motif (Taylor, 2014); PMI, pollen mitosis I; BC, bicellular stage. This table is a detail illustration of Figure 3.7 B. Values represent the fold change of genes expression in the *ms35* compared to the wild-type.

transcriptional factor family protein; and *ERF96*, a member of the ERF(ethylene response factor) family protein, were up-regulated during both PMI and BC stages in the *ms35* mutant, while the rest of TFs were down-regulated except *MYB54* (Table 3.1). Available functional studies revealed that some of these TFs are involved in secondary thickening development, including *C3H14* (Chai et al. 2015), NAC transcription factor *NST1* (Mitsuda et al. 2005), *KNOTTED-LIKE HOMEODOMAIN OF ARABIDOPSIS THALIANA 7*, *KNAT7* (Li et al. 2011; Li et al. 2012), *MYB54* (Zhong & Ye 2012; R. Q. Zhong et al. 2008); phenylpropanoid metabolism and/or Phytohormone response such as *AtMYB7* (Fornalé et al. 2014; Kim et al. 2015), *ERF96* (Wang et al. 2015; Catinot et al. 2015); Anthocyanin biosynthesis pathway such as transcriptional repressor *AtMYB6* (Rowan et al. 2009); Flower development such as *FLOWERING LOCUS C (FLC)*, a repressor of floral transition (Michaels & Amasino 1999; Simpson & Dean 2002; Rouse et al. 2002).

3.4 DISCUSSION

3.4.1 35S promoter or Ubiquitin 10 promoter

Promoters play a key role in the regulation of gene expression, and the selection of these plays a significant role on level and specificity of expression. In order to generate *Arabidopsis* lines that stably overexpress *MYB26*, constructs containing the full-length *MYB26* CDS without stop codon with GFP tag were prepared under the control of the ubiquitin 10 promoter (P_{UBQ10}) or Cauliflower Mosaic Virus 35S promoter (P_{35S}), and transformed into the wild-type *Ler*.

The transcript level of *MYB26* varied in different tissues of a single plant with higher expression detected in floral tissues than in leaves, levels also varied between different transgenic lines driving by the same promoter. Presumably as a consequence of position effects of the insertions. No obvious differences were observed between P_{UBQ10} or P_{35S} when overexpressing *MYB26* in terms of the transcript level; the highest transcript level of *MYB26* obtained in floral tissue with either P_{UBQ10} or P_{35S} could reach around 18.9 fold change compared to wild-type in our system. However, different phenotypes were observed when using different promoters/constructs. Transgenic plants containing the 35S::*MYB26*-GFP construct displayed ectopic secondary thickening (ST) in vegetative tissues with small curled leaves and thickened stems, and most of the transgenic plants were fertile, although fertility was often reduced with shorter siliques compared to the wild-type. Whilst most transgenic plants containing the UBQ10::*MYB26*-GFP construct had moderate ST deposition indicated by small slightly curled leaves and thin stems, however, most transgenic plants were sterile probably due to delayed anther dehiscence and incompatible filament elongation (Figure 3.2

and Figure 3.3). Whether the severity of the different phenotypes was due to different vectors (pGWB5 equipped with P_{35S} and pUBC-DEST-GFP equipped with P_{UBQ10}) or by different promoters, is not known.

Many studies have been conducted to evaluate different constitutive promoters, however it seems difficult to simply answer which promoter is better without considering the plant species, transient/stable expression system and different genes. Grefen et al. 2010 compared P_{35S} and P_{UBQ10} both in tobacco and in *Arabidopsis* after transient transformations, they found the expression of *SYP121* under P_{35S} gave around 20 fold greater signal than the same construct under P_{UBQ10} in tobacco, however, the difference in fluorescence signals was much smaller when expressed in *Arabidopsis*.(Grefen et al. 2010). While Behera et al claimed a superior performance of the P_{UBQ10} than P_{35S} when expressing the Ca^{2+} indicator *Yellow Cameleon 3.6* (YC3.6) with regard to expression pattern, levels and expression stabilities in both *Arabidopsis thaliana* and *O. sativa* (Behera et al. 2015). In a transient expression system, P_{UBQ10} was reported to be temporally more stable than P_{35S} in tobacco, *Arabidopsis* and *O.sativa*, and was less prone to gene silencing over time (Grefen et al. 2010; Behera et al. 2015). Moreover, the expression patterns may vary between different promoters, P_{UBQ10} -driven *SYP121*-GFP shows fluorescence polarised to the base of trichoblast cells and to the tips of root hairs rather than evenly distributed on the cell membrane of the equivalent tissue in the P_{SYP121} -driven control (Grefen et al. 2010). A comparison of 5 promoters, including CaMV-35S, CaMV-35S_{omega}, *Arabidopsis* ubiquitin UBQ1, barley leaf thionin BTH6 and soybean heat shock promoter Gmhsp17.3 in stably transformed *Arabidopsis thaliana* showed that the P_{BTH6} was almost inactive in majority of the lines and clearly different in organ

specificity compared to the others, slight differences could be seen between the other four promoters, P_{UBQ1} -driven seedlings show a stronger reporter signal in the hypocotyl than the rest, indicating different promoters may focus on particular tissues when driving gene transcription (Holtorf et al. 1995). Tissue-specific patterns of different promoters may be a possible reason why different phenotype was observed in P_{35S} -driven and P_{UBQ10} -driven transformed *Arabidopsis*.

3.4.2 TFs in *MYB26* regulation network

Genes participating in secondary thickening (ST) development are under strict transcriptional control, with a considerable diversity of transcription factors (TFs) involved in this pathway. Some TFs listed in Table 3.1 have already been reported to participate in various biological processes.

AtC3H14 (At1g66810) and its homologue, *AtC3H15* are plant-specific CCCH-type zinc-finger proteins that belong to the 68-member CCCH family in *Arabidopsis thaliana* (Wang et al. 2008), *C3H14* and *C3H15* have overlapping roles in the regulation of secondary wall formation and anther development (Chai et al. 2015; Kim et al. 2014). Although the *c3h14c3h15* double mutants have reductions in stem secondary wall thickening and defects in anther and pollen development, the secondary wall thickening in anther dehiscence zones was not affected. *C3H14* was reported to be one of the direct targets of *MYB46*, which is a key regulator of secondary wall formation (Kim et al. 2012). *MYB46* was highly expressed at BC stage in wild-type anther, but dramatically down-regulated in the *ms35* mutant at equivalent stage, thus the down-regulation of

C3H14 could possibly due to the significant down-regulation of *MYB46* in the *ms35* mutant during BC stage.

The *NAC SECONDARY WALL THICKENING PROMOTING FACTOR1 (NST1)* and *NST2* function redundantly in regulating secondary wall thickening in anther walls (Mitsuda et al. 2005). The double mutant *nst1nst2* displayed anther-indehiscent phenotype due to loss of ST in the anther endothecium, a similar phenotype was observed in the *ms35* mutant. The expression of *NST1* and *NST2* was down-regulated in the *ms35* mutant and was up-regulated in responding to *MYB26* induction (Figure 3.4), indicating that *NST1* and *NST2* were regulated by *MYB26*. To investigate whether *NST1* and *NST2* are direct targets of *MYB26*, further experiment such as ChIP-PCR and EMSA were performed and illustrated in Chapter 4, Section 4.3.5.

ETHYLENE-RESPONSIVE ELEMENT BINDING FACTORS 96 (ERF96) was reported to regulate plant defence response by binding to GCC element of jasmonate acid (JA) and ethylene (ET) responsive defence genes (Catinot et al. 2015). *ERF96* is also a positive regulator of abscisic acid (ABA) response in *Arabidopsis* (Wang et al. 2015). *ERF96* expressed relatively high in flowers and seeds. The *ERF96* overexpression plants had smaller rosette size, delayed in flowering time, and reduced stomatal aperture in the presence of ABA. There hasn't been any report showing whether *ERF96* is involved in ST synthesis or anther development, however, genes belonging to JA signalling, redox stage of plant cells and glutathione transferase, as well as cell wall development were numerously altered in *ERF96* over-expression lines (Catinot et al. 2015).

FLOWERING LOCUS C (FLC) encodes an MADS-box transcription factor, is well known as a repressor of floral transition and contributes to temperature compensation of the circadian clock (Sheldon et al. 1999; Michaels & Amasino 1999; Simpson & Dean 2002). *FLC* was also reported to be involved in regulating stomatal opening. Closed-stomata phenotype was obtained in *FRI-Col* plants with increased *FLC* expression and decreased expression of *FT*, *TSF* and *SOC1* in guard cells (Kimura et al. 2015). *FLC* was also reported to have cross-talk with pleiotropic phytohormones such as gibberellic acid (GA) and abscisic acid (ABA). *FLC* was shown to be regulated by DELLA proteins, which are master transcriptional regulators of the GA pathway (Davière et al. 2008). Exogenous melatonin treatment stabilised the DELLA proteins and delayed floral transition through *FLC* in *Arabidopsis* (Shi et al. 2016). *ABSCISIC ACID-INSENSITIVE 4 (ABI4)*, a key component in the ABA signalling pathway, negatively regulates floral transition by directly promoting *FLC* transcription (Shu et al. 2016). Interestingly, the transcript level of *FLC* was significantly reduced in the *ms35* anther during both PMI and BC stages (Table 3.1), there has no direct link between *FLC* and ST synthesis or anther development been reported, but the involvement of phytohormones could possibly explain the link between *FLC* and *MYB26* during anther development. In the meantime, whether the *ms35* mutant has altered flowering time needs to be further confirmed.

It is worth noticing that *KNOTTED ARABIDOPSIS THALIANA7 (KNAT7)*, which is a transcriptional repressor that negatively regulate SCW formation (Li et al. 2012), was significantly down-regulated in the *ms35* mutant. The expression of *KNAT7* is co-expressed with SCW biosynthetic enzymes (Ehlting et al. 2005; Brown et al. 2005) and is reported to be one of the direct targets of

both *SND1/VND6* (Zhong et al. 2008) and *MYB46* (Ko et al. 2009). *KNAT7* function as a potent transcriptional repressor, the repression ability is enhanced by physical interaction with OVATE FAMILY PROTEIN4 (OFP4) (Li et al. 2011) and BLH6 (Liu et al. 2014). *KNAT7* also physically interacts with MYB75, which regulates plant secondary cell wall biosynthesis (Bhargava et al. 2010). *KNAT7* was proposed to act in a negative feedback loop in the SCW synthesis network, working antagonistically to NAC family and MYB family positive regulators (Li et al. 2012). The down-regulation of *MYB26* in the *ms35* mutant may possibly be one of the input signals that allow *KNAT7* to fine tune metabolic commitment during secondary thickening biosynthetic processes.

MYB54 encodes a putative R2R3-MYB family protein and is involved in regulating ST development (Zhong et al. 2008). Dominant repression of *MYB54* could reduce the secondary wall thickening in fibre cells, also, *MYB54* is able to induce secondary wall biosynthetic genes. *MYB54* is regulated by the ST master switch *SECONDARY WALL-ASSOCIATED NAC DOMAIN PROTEIN1 (SND1)*, which is a functional homolog of *NST1/2*, *VND6/7* (Zhong et al. 2008). Moreover, *MYB54* is a direct target and activated by *MYB46* and *MYB83* through the secondary wall MYB-responsive element (SMRE) (Zhong & Ye 2012). In the *ms35* mutant, *MYB54* was upregulated during PMI stage but down-regulated during BC stage, both changes were not significant (Table 3.1).

MYB7 were moderately down-regulated in the *ms35* mutant during both PMI and BC stage. *MYB7* belongs to the subgroup 4, which clusters the only R2R3-MYB factors acting as transcriptional repressors of different branches of phenylpropanoid metabolism (Fornalé et al. 2014), and the phenylpropanoid metabolic pathway generates a wide variety of compounds that are essential for

biosynthesis of lignin, flavonoids and the *Brassicaceae*-specific sinapate esters (Dixon et al. 2002, Milkowski and Strack, 2010, Vogt 2010). Also, the expression of *AtMYB7* is partially controlled by *AtMYB4* and induced by salt stress (Fornalé et al. 2014).

CHAPTER 4 DIRECT TARGETS OF MYB26

4.1 INTRODUCTION

Secondary walls in wood and fibres constitute the most abundant plant biomass widely used in our daily life. Secondary thickening (ST) biosynthesis is a complex biological process which requires a complex regulatory mechanism to ensure ST biosynthetic production in various cell types, such as tracheary elements and fibers in xylem, sclereids in some fruits, seed coats and leaves, endothecium in anthers, valve margin fibers in seed pods, trichomes, and extraxylary fibers (Zhong & Ye 2014). Genomic and molecular studies have identified a number of transcription factors (TFs) that act as master switches during ST synthesis (Wang & Dixon 2012; Hussey et al. 2013; R. Q. Zhong et al. 2008), by investigating the targets of ST key players will lead to a better understanding of the transcriptional control network for secondary wall biosynthesis.

The *MYB26* gene encodes an R2R3-type MYB transcription factor. Many R2R3-MYB proteins have been characterized by genetic approaches and found to be involved in the control of plant-specific processes including primary and secondary metabolism, cell fate and identity, developmental processes and responses to biotic and abiotic stresses (Stracke et al. 2001; Dubos et al. 2010a). The transcription factor MYB26/MS35 plays an important role during anther dehiscence (Steiner-Lange et al. 2003; Yang et al. 2007). The null mutant of *ms35* displays a sterile phenotype due to anther indehiscence, although the pollen of *ms35* is fertile. The secondary wall thickenings are absent in the endothecium layer of *ms35* anthers, this is needed to provide the mechanical force necessary

for the rupture and dehiscence of stomium to facilitate pollen release. *MYB26* has been shown to be a master switch regulating secondary thickening in anther development (Caiyun Yang 2007). However, the regulation network of *MYB26* remains largely unknown.

Several methods can be applied to study protein-DNA binding activities, such as the classic *in vitro* method electrophoretic mobility shift assay (EMSA) (Fried & Crothers 1981; Garner & Revzin 1981), and *in vivo* method chromatin immunoprecipitation (ChIP) (Gilmour & Lis 1985; Solomon et al. 1988; Jackson 1978) combined with PCR or high throughputs methods such as Microarray Chip and next generation sequencing (Ho et al. 2011; Kaufmann et al. 2010a). The ChIP assay captures protein-DNA complexes in living cells and therefore gives valuable insights of protein-DNA binding *in vivo*, while EMSA examines purified protein's capability of binding to given DNA or RNA sequence *in vitro*. In this thesis, ChIP-PCR and EMSA were adopted to study *MYB26* direct binding targets *in vivo* and *in vitro* respectively.

Microarray-based global gene expression profiling has been performed using staged *Arabidopsis* stamen from the *ms35* mutant and the wild-type to identify genes associated with anther development and dehiscence (Song, 2009). The *ms35* mutant microarray data, gene expression plots and FlowerNet, a correlation network generated with publicly available *Arabidopsis* gene expression data (Pearce et al. 2015) are the main resources that were used to screen *MYB26* regulating targets. The upstream region of selected *MYB26* putative targets with the cis-regulatory element such as conserved non-coding sequence (CNS) among crucifer (Reineke et al. 2011), MYB binding site

(Davuluri et al. 2003), MYB26 *in vitro* binding motif (Taylor, 2014), were tested in ChIP-PCR.

The *NAC SECONDARY WALL THICKENING PROMOTING FACOTR1 (NST1)* and *NST2* function redundantly in regulating secondary wall thickening in anther walls (Mitsuda et al. 2005). The chimeric repression of *NST1* or *NST2*, and the double mutant *nst1nst2* displayed an anther-indehiscent phenotype due to loss of ST in the anther endothecium, a similar phenotype was observed in the *ms35* mutant. The expression of *NST1* and *NST2* is down-regulated in *ms35* mutant and up-regulated in *MYB26* overexpression lines (Chapter 3 figure 3.4; Yang et al. 2007), indicating that *MYB26* may regulate secondary thickening synthesis through regulating *NST1* and *NST2*. The promoter regions of *NST1* and *NST2* were enriched in ChIP-PCR (J.Song, B.Taylor, Z.A.Wilson, unpublished data), and further analysed using both ChIP-PCR and EMSA in this thesis.

4.2 METHODS

4.2.1 Chromatin isolation and DNA sonication

Chromatin Immunoprecipitation protocol was adapted from Professor D. Zhang's Lab (Shanghai Jiao Tong University), with modification from (Kaufmann et al. 2010 , Bowler et al., 2004 ,Haring et al., 2007). All steps were carried out at 4°C, or on ice unless stated otherwise.

5g of fresh buds tissue for each sample was collected on ice within one hour. Weighed sample were submerged in 25ml freshly prepared MC buffer (10mM sodium phosphate pH7, 50mM NaCl and 0.1M sucrose, 5mM β -Mercaptoethanol, 0.1mM PMSF, 0.1% (v/v) protease inhibitors) with 1% (V/V) formaldehyde under vacuum infiltration for 15 minutes on ice. After that, 1.57ml of 2M glycine was mixed into each tube and vacuum infiltrated for 5 minutes on ice to quench the cross-linking reaction. Tissue was washed 3 times with plenty of water then dried on paper towels.

Cross-linked tissue was ground in liquid nitrogen to a fine, dry powder. Then suspended in 30ml ice-cold extraction buffer 1 (0.4M Sucrose, 10mM Tris-HCl, pH 8.0, 5mM β -Mercaptoethanol, 0.1mM PMSF, 0.1% (v/v) protease inhibitors) and Incubated for 30 minutes on ice with manual gentle shaking every 5 minutes. The solution was filtered through 4 layers of Miracloth into a new ice-cold 50ml tube, then centrifuged at 2880g at 4°C for 20 minutes. The supernatant was disposed and the pellet was re-suspended in 1 ml ice-cold extraction buffer 2 (0.25M sucrose, 10mM Tris-HCL pH8.0, 10mM MgCl₂, 1% Triton X-100, 5mM β -Mercaptoethanol, 0.1mM PMSF, 0.1% protease inhibitors). The solution was transferred to a 1.5ml microcentrifuge tube. Centrifuged at 12,000g for 10

minutes at 4°C. The pellet was re-suspended in 300µl ice-cold extraction buffer 3 (1.7M sucrose, 10mM Tris-HCl pH8.0, 2mM MgCl₂, 0.15% Triton X-100, 5mM β-Mercaptoethanol, 0.1mM PMSF, 0.1% (v/v) protease inhibitors). 500 µl of ice-cold extraction buffer 3 was added to a new 1.5ml microcentrifuge tube. The re-suspended pellet was overlaid to this layer and centrifuged for 1 hour at 16000g at 4°C. And the pellet was re-suspended in 320µl ice-cold Nuclei lysis buffer (50mM Tris-HCl pH8.0, 10mM EDTA, 1% SDS, 0.1mM PMSF, 0.1% protease inhibitors), incubated on ice for 20 minutes, 10µl aliquot was taken from this step as “unsheared” chromatin.

Sonication was performed in an ice water bath with Diagenode's Bioruptor® device under high power setting, 30 sec ON, 1min OFF, 10-20 cycles to shear DNA into manageable size fragments. The amount of times of sonication was based on the average fragment size required (400-800bp for ChIP-QPCR). The sonicated solution was spun 5 minutes at 16000g at 4 °C to pellet the insoluble fraction. The clear supernatant was transferred to an Eppendorf® DNA LoBind tube and was kept on ice. 10µl aliquot was taken from the chromatin solution. Ethanol Purified DNA from un-sheared and sonicated aliquots was analysed by agarose gel electrophoresis to check sonication efficiency.

4.2.2 Chromatin immunoprecipitation

300µl of the aforementioned sonicated supernatant was diluted in 1660µl of ice-cold ChIP dilution buffer (16.7mM Tris-HCl pH8.0, 1.2mM EDTA, 1.1% Triton X-100, 167mM NaCl, 0.1mM PMSF, 0.1% protease inhibitors). 55 µl aliquot was taken from the chromatin solution as the “input sample”, 100µl TE buffer,

6.5µl 5M NaCl and 8µl 20% SDS was added to the “input sample” then stored at -20°C.

550µl of chromatin solution was transferred to new 1.5ml Eppendorf® DNA LoBind tubes. To one tube 1µl anti-HA antibody was added as a non-specific antibody control. To the other two tubes 3 or 5 µl anti-GFP antibody (Ab290, Abcam, UK) was added. Each tube was topped up with 430µl ChIP dilution buffer and was left at 4°C overnight with gentle agitation (Rotator at 12 rpm).

20µl of protein A Agarose beads (Dynabeads® Protein A, Invitrogen, UK) was added to each sample and then agitated at 4°C for 1 hour. A magnetic bar was used to gather the beads and the content was washed with the following buffers: 1x Low Salt buffer (20mM Tris-HCl pH8.0, 2mM EDTA, 1% (v/v) Triton X-100, 0.1% SDS, 150mM NaCl), 1x High Salt buffer (20mM Tris-HCl pH8.0, 2mM EDTA, 1% Triton X-100, 0.1% SDS, 500mM NaCl), 1x LiCl buffer (10mM Tris-HCl pH8.0, 1mM EDTA, 1% NP-40, 250mM LiCl, 1% sodium deoxycholate) and 2x with TE buffer (10mM Tris-HCl pH8.0, 1mM EDTA). 1ml of each buffer was used to wash the beads for 10 minutes at 4°C with gentle agitation. After that, 50µl Elution buffer was added to the pelleted beads to elute the immune complexes followed by 15 minutes incubation at 65°C with gentle agitation. The supernatant was then transferred into a new DNA Low bind tube and the elution step was repeated. Formaldehyde cross-linking was reversed by adding 4µl 5M NaCl to each tube and incubating at 65°C overnight.

DNA isolation was conducted by adding 1.2µl of 20mg/ml Proteinase K (Invitrogen, UK), 2µl 0.5M EDTA and 4µl 1M Tris-HCl pH6.5, incubating at 45°C for 4 hours. DNA was purified using QIAquick PCR Purification Kit

(QIAGEN, UK) and finally eluted in 12-20µl nuclease free water. DNA quantity and quality were tested using the NanoDrop ND-1000 fluorospectrometer (NanoDrop Technologies, USA) before using as template in QPCR analysis to determine the enrichment of candidate promoter sequences.

4.2.3 EMSA

DIG labelled probes (NST1_P2a and NST2_P8) were obtained by PCR with DIG labelling Mix (Roche, UK) according to the manufacture's instruction, probe sensitivity detection was performed on 10-fold serial dilutions up to 10^{-5} diluted labelled probes, undiluted probes was used in EMSA experiment.

EMSA was performed with the LightShift EMSA Kit (Thermo Fisher Scientific, UK), the positive control provided in the kit was performed along with the test system to evaluate the results. DNA and protein binding reactions were listed in Table 4.1 and Table 4.2 and was performed according to the manufacture's instruction. In brief, the reactions were mixed and incubated at room temperature for 20 mins. 4µl of 6x loading buffer (glycerol only) was mixed into the protein/DNA solution before loading onto the 6% DNA Retardation Gels (Thermo Fisher Scientific, UK). The gel was then run with pre-cooled buffer for 2hrs at 100V until the dye had migrated to the end of the gel. Protein/DNA was further transferred to a nylon membrane by electrophoresis. The nylon membrane was then dried at room temperature and binding was detected using either anti-Digoxigenin-AP, Fab fragments (Roche, UK) for DIG labelled probes or SuperSignal West Pico Chemiluminescent Substrate (Thermo Fisher Scientific, UK) for 3' biotin labelled positive control probes.

Table 4.1 EMSA binding reactions for EBNA system (positive control)

Component	Final amount	Positive Control reactions		
		#1	#2	#3
Ultrapure water	--	12 µl	11 µl	9 µl
10x binding buffer	1X	2 µl	2 µl	2 µl
50% (v/v) glycerol	2.5%	1 µl	1 µl	1 µl
100mM MgCl ₂	5mM	1 µl	1 µl	1 µl
1µg/µl Poly(dI.dC)	50ng/ µl	1 µl	1 µl	1 µl
1% (V/V) NP-40	0.05%	1 µl	1 µl	1 µl
Unlabelled EBNA DNA	4 pmol	--	--	2 µl
EBNA Extract	1 Unit	--	1 µl	1 µl
Biotin-EBNA control DNA	20 fmol	2 µl	2 µl	2 µl
Total Volume	--	20 µl	20 µl	20 µl

Table 4.2 EMSA binding reactions for the test systems

Component	Final amount	Positive Control reactions		
		#1	#2	#3
Ultrapure water	--	12 µl	2 µl	--
10x binding buffer	1X	2 µl	2 µl	2 µl
1µg/µl Poly(dI.dC)	50 ng/ µl	1 µl	1 µl	1 µl
50% (v/v) glycerol	2.5%	1 µl	1 µl	1 µl
100mM MgCl ₂	5mM	1 µl	1 µl	1 µl
200mM EDTA	1 mM	0.1 µl	0.1 µl	0.1 µl
1% (V/V) NP-40	0.05%	1 µl	1 µl	1 µl
Unlabelled DNA probes	4 pmol	--	--	2 µl
MYB26-GST Extract	System-dependent	--	10 µl	10 µl
DIG-labelled DNA probes	System-dependent	2 µl	2 µl	2 µl
Total Volume	--	20 µl	20 µl	20 µl

4.3 RESULTS

4.3.1 MYB26 putative regulatory targets.

Microarray-based global gene expression profiling was previously performed using GeneChip™ *Arabidopsis* ATH1 Genome Array, using staged *Arabidopsis* stamen from the *ms35* mutant and the wild-type to identify genes associated with anther development and dehiscence (J.Song, Z.A.Wilson, unpublished data). Besides the *ms35* microarray data, the gene expression plots and FlowerNet, a correlation network generated with publicly available *Arabidopsis* gene expression data (Pearce et al. 2015) and Laser Capture Microscopy (LCM) sectioning data from wild-type anther tissues (Gema Vizcay-Barrena, unpublished data) were the main resources used to identify MYB26 putative regulatory targets.

MYB26 putative target genes were selected from the above-mentioned datasets according to the following criteria, (1) gene expression was significantly altered in the *ms35* mutant (Figure 4.1), (2) the expression stage was similar to MYB26 (Figure 4.2) in the wild-type, (3) highly expressed in endothecium layer where the secondary thickening developed (Figure 4.3). Moreover, a recombinant MYB26-GST random oligonucleotide binding experiment determined a consensus sequence C[A/C]GATAATT[A/C]AATC[G/A] of a putative MYB26 binding motif *in vitro* (Taylor, 2014). Gene loci which contain this consensus sequence within the 3000bp upstream region were screened using the bulk data retrieval tool of TAIR (<https://www.arabidopsis.org/tools/bulk/index.jsp>). Together with the aforementioned 3 requirements, ten MYB26 putative target genes were selected (Table 4.3) and analysed using ChIP-PCR.

Table 4.3 List of ten putative MYB26 targets.

Name	AGI code	Brief Gene Description	Cluster	Cluster member/Co-expression
GBA2	AT1G33700	Beta-glucosidase, GBA2 type family protein; INVOLVED IN: glucosylceramide catabolic process, sphingolipid metabolic process;	1904	AT3G15870: 0.902 AT1G78440: ATGA2OX1 0.89
DUF662	AT2G27740	Family of unknown function.	781	AT1G78440: ATGA2OX1 0.905 AT5G16490: RIC4 0.9 AT3G03910: GDH3 0.9 AT2G43840: UGT74F1 0.881
PKSP	AT2G39110	Protein kinase superfamily protein; FUNCTIONS IN: protein kinase activity, kinase activity, ATP binding	1865	AT3G13890: MYB26 0.909 AT3G15870: 0.904
GH3.9	AT2G47750	Encodes GH3.9, a member of the GH3 family auxin-responsive genes. gh3.9-1 mutants had greater primary root length, increased sensitivity to indole-3-acetic acid (IAA)-mediated root growth inhibition, but no obvious effects on apical dominance or leaf morphology.	3568	AT2G18420: 0.886
2OG	AT3G13610	2-oxoglutarate and Fe(II)-dependent oxygenase superfamily protein	NA	
ADS3	AT3G15870	Fatty acid desaturase family protein; FUNCTIONS IN: oxidoreductase activity, oxidoreductase activity, acting on paired donors	1904	AT2G39110: 0.904 AT1G33700: 0.902
	AT4G30170	Peroxidase family protein; FUNCTIONS IN: peroxidase activity	NA	
	AT4G39320	microtubule-associated protein-related; LOCATED IN: plasma membrane	NA	
RIC4	AT5G16490	encodes a member of a novel protein family that contains contain a CRIB (for Cdc42 /Rac-interactive binding) motif required for their specific interaction with GTP-bound Rop1 (plant-specific Rho GTPase). It interacts with Rop1 and is involved in pollen tube growth and function, and exocytosis in the pollen tube tip.	781	AT5G44030: CESA4 0.913 AT2G27740: DUF662 0.9 AT1G09610: DUF 579 0.891 AT2G46770: NST1 0.887 AT1G78440: ATGA2OX1 0.886
IRX15-L	AT5G67210	Encode a DUF579 (domain of unknown function 579) containing protein essential for normal xylan synthesis and deposition in the secondary cell wall.	209	AT3G16920: CTL2 0.94 AT3G62020: GLP10 0.929 AT3G18660: PGSI1 0.919 AT5G44030: CESA4 0.911 AT3G21240: 4CL2 0.911 AT2G38080: IRX12 0.904 AT1G24030: 0.903 AT4G27435: DUF1218 0.901 AT5G60020: LAC17 0.885

Brief gene description is obtained from the TAIR website <https://www.arabidopsis.org/>
Cluster information is available from FlowerNet, <https://www.cpib.ac.uk/anther/> (Pearce et al. 2015), values = correlation.

In *Arabidopsis* anthers, *MYB26* expression occurs early during endothelial development, with maximal expression during pollen mitosis I (PMI) and bicellular (BC) stages. In the *ms35* mutant, the expression of *MYB26* is significantly reduced. The 10 candidates of *MYB26* putative regulatory targets have a similar expression pattern, with maximal expression during PMI stage (*PKSP*, *GH3.9*, *2OG*) or BC stage (*GBA2*, *DUF662*, *ADS3*, *At4g30170*, *At4g39320*, *RIC4*, and *IRX15-L*). These genes were all significantly down-regulated in the *ms35* mutant at PMI and BC stages (Figure 4.1).

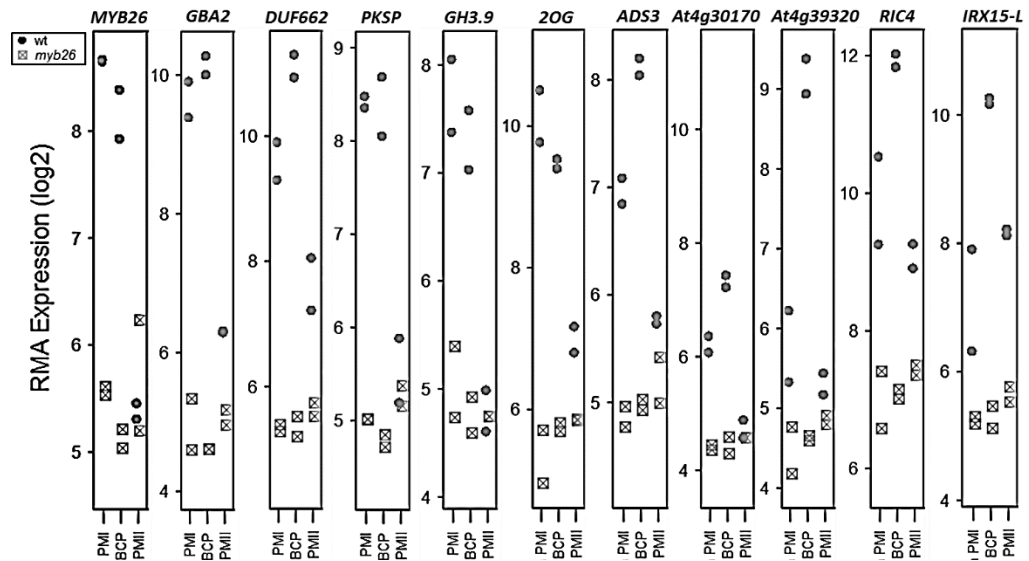
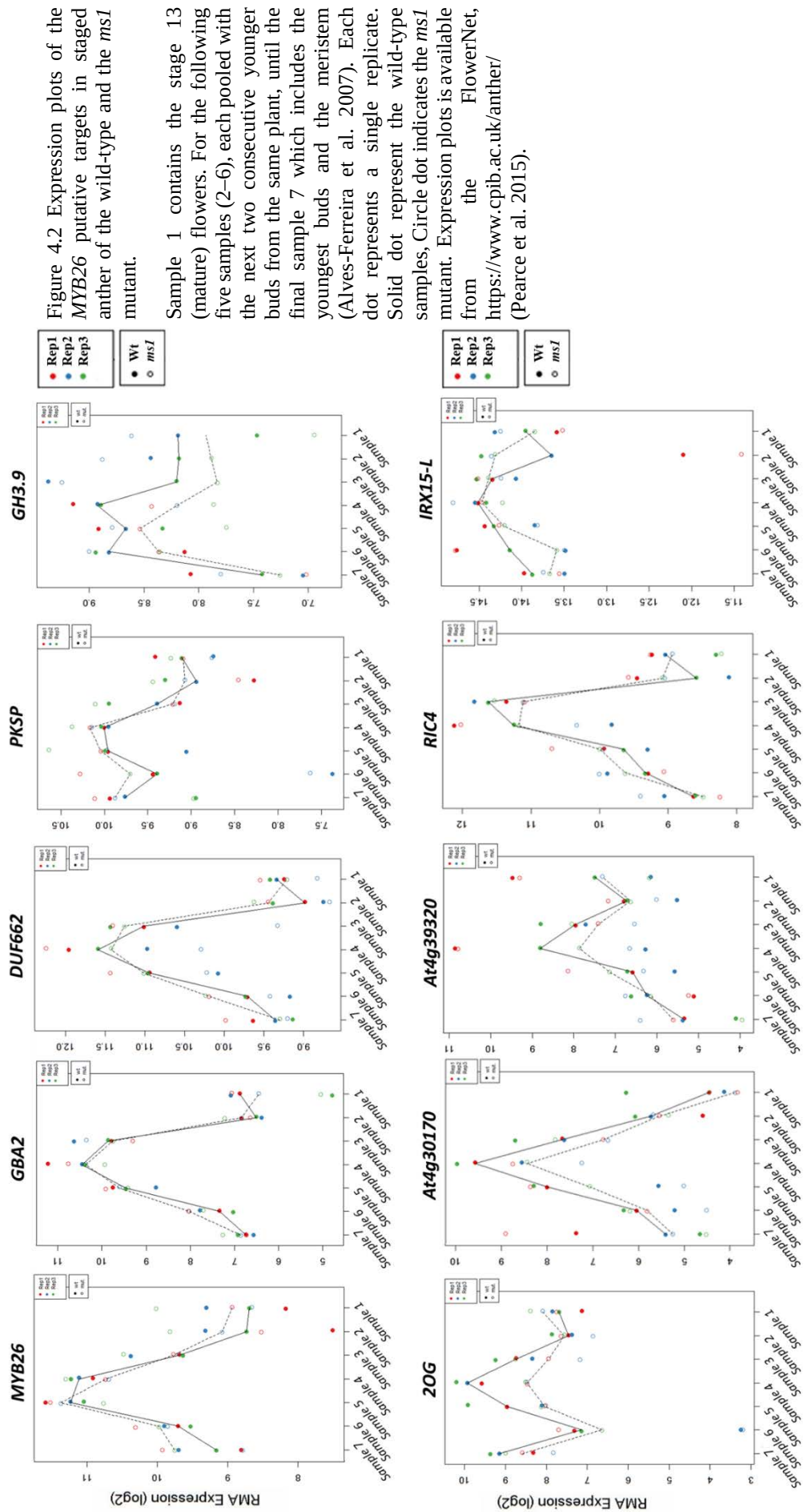
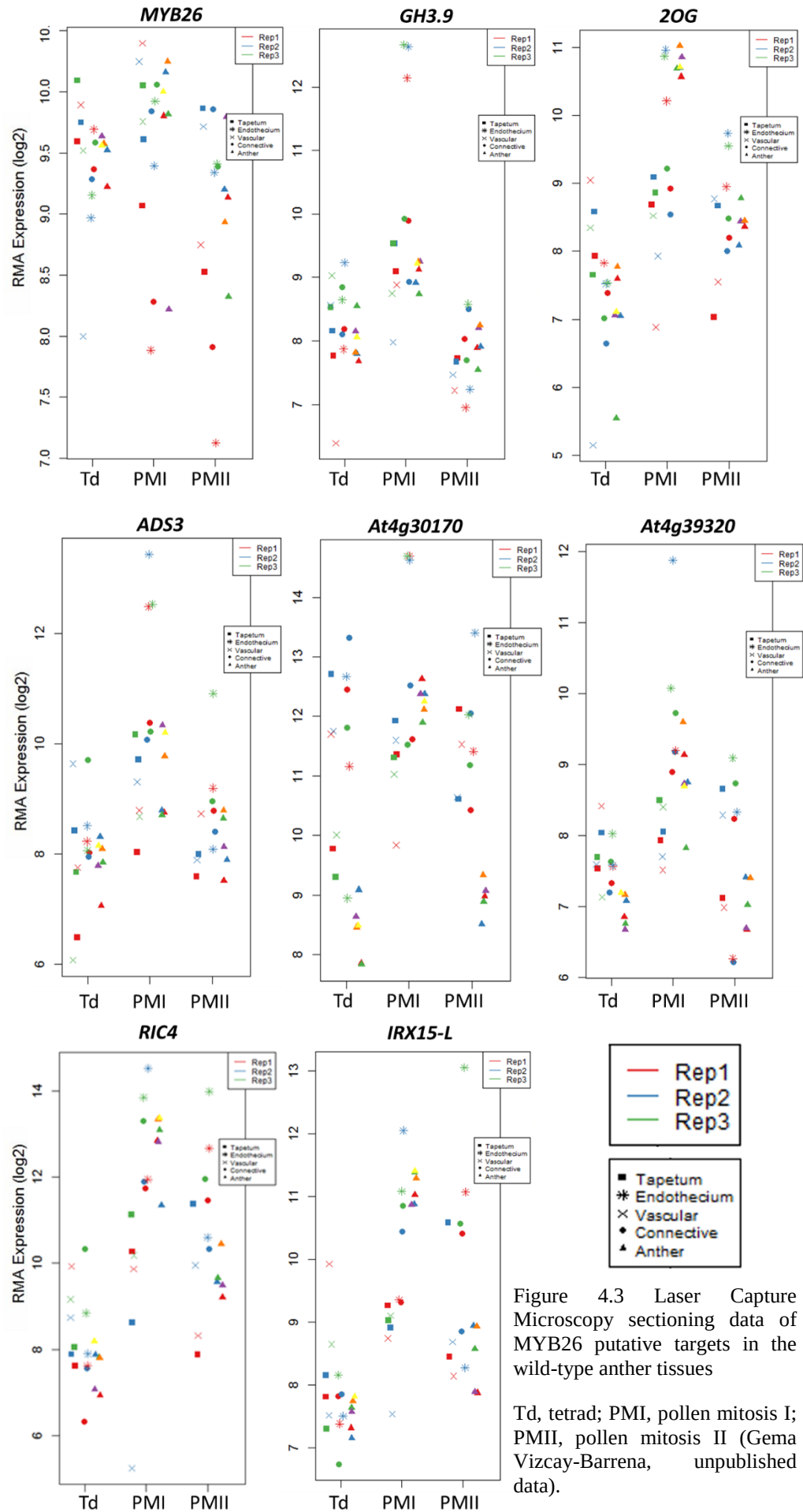


Figure 4.1 Expression plots of *MYB26* putative regulatory candidates in the *ms35* mutant and wild-type

Expression plots showed the expression changes of *MYB26* and its putative targets in the anthers of the wild-type and the *ms35* mutant at three developmental stages, pollen mitosis I (PMI), bicellular (BCP), and pollen mitosis II (PMII). The microarray data was normalised using RMA normalisation, each dot represents a single replicate (Jie, 2009). Expression plots is available from the FlowerNet, <https://www.cpib.ac.uk/anther/> (Pearce et al. 2015).

Besides *ms35* mutant microarray data, at least another two independent microarray datasets were used to evaluate the expression pattern of *MYB26* putative targets. Alves-Ferreira et al. (2007) used developmentally staged samples of the wild-type and the male sterile 1 (*ms1*) mutant to study the effect of *MS1* on global gene expression. In their experiment, sample 1 contains stage 13 flowers, with each following stages pooling with two younger buds from the same plant, until the final sample 7 which includes the youngest buds and the meristem. The expression plots from the two-colour microarrays were publically available <https://www.cpib.ac.uk/anther/>, and the wild-type expression plots (Figure 4.2) were used in conjunction with the wild-type anther Laser Capture Microscopy (LCM) sectioning data (Figure 4.3) to temporally and spatially evaluate the expression pattern of *MYB26* putative targets. A line was drawn





through the median values of these samples, for both the wt and the *ms1*. This was used to see how the expression pattern changed across the developmental time course.

The Laser Capture Microscopy (LCM) sectioning data from the wild-type anther tissues, including tapetum, vascular, endothecium, connective tissues and the whole anther at three developmental stages, was performed at Nottingham using three-colour microarrays (Gema Vizcay-Barrena, unpublished data). The expression of *MYB26* was observed throughout the *Arabidopsis* anther tissue, especially endothecium, tapetum and connective tissues (Yang et al. 2007), and the *ms35* mutant has a defect in endothelial secondary thickening development. Among the selected *MYB26* putative targets, gene *GH3.9*, *2OG*, *ADS3*, *At4g30170*, *At4g39320*, *RIC4*, *IRX15-L* were highly expressed in endothecium at PMI stage (Figure 4.3).

4.3.2 Optimisation of ChIP experiment

Chromatin Immunoprecipitation was conducted to study the binding activity between *MYB26* and its putative targets (Table 4.3) *in vivo*.

The materials used for ChIP experiments was *MYB26* inducible expression lines in the *ms35* background, the sterility of *ms35* was recovered only after Dexamethasone (Dex) treatment when the *MYB26*-GR-YFP could enter the nucleus to bind and activate its targets. The pG0229-*MYB26* line (prom*MYB26*:*MYB26*-GR-YFP, *ms35* background) was generated by Dr. C. Yang (University of Nottingham). YFP signal was observed under the confocal microscope in anthers of Dex-treated material but absent in mock (water) treated anthers (Figure 4.4 A and B). 5g of Dex/Mock-treated whole inflorescences was

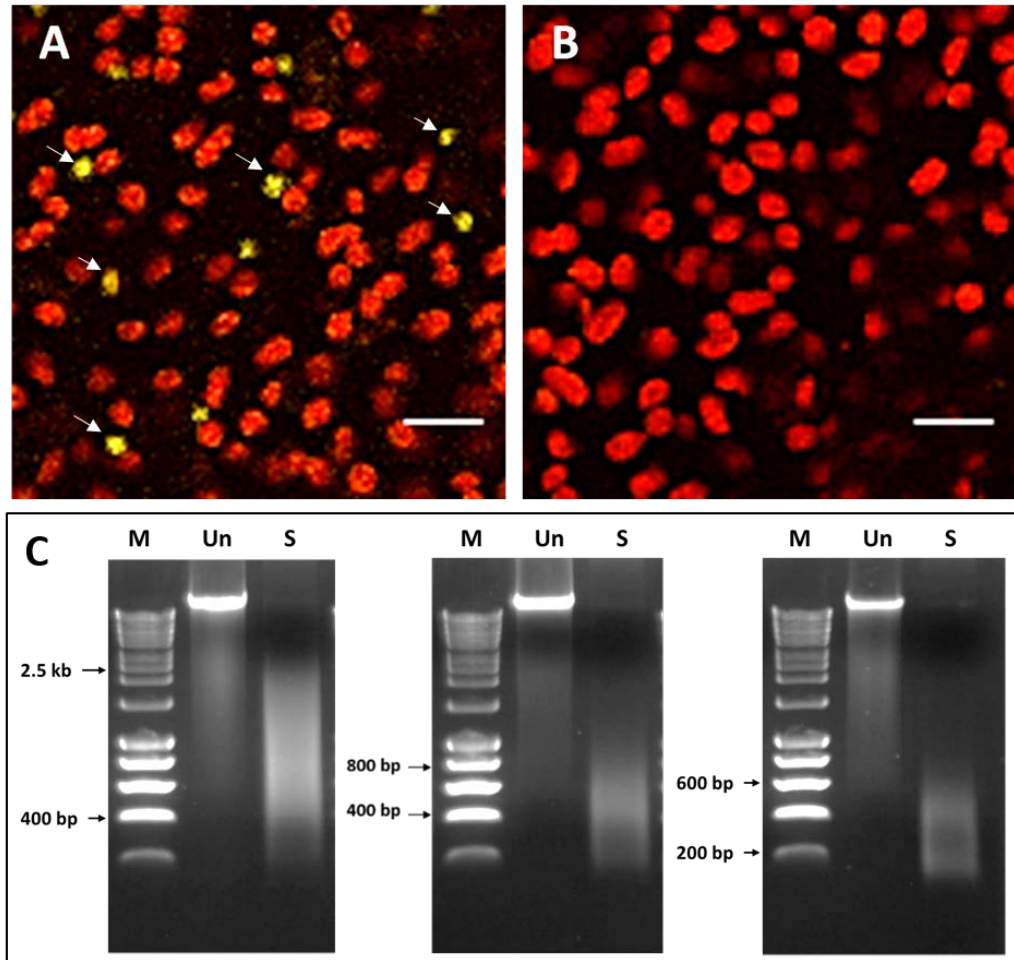


Figure 4.4 ChIP material and sonication efficiency.

A-B, YFP signal (arrow) was observed in the nucleus after Dex treatment (A) but not in the mock treated anther (B) of the promMYB26::MYB26-GR-YFP transgenic lines, scale bar 50 μ m.

C, Sonication efficiency. Three examples of sonication were performed under the same condition using Bioruptor sonicator. M, hyperlayer I; Un, unsonicated sample; S, sonicated sample.

collected and cross-linked with 1% formaldehyde for 15 minutes at room temperature. Nuclear protein was extracted as described in section 4.2.1, and sonication was processed with Bioruptor sonicator under with the following parameters: high power setting, 30 sec ON, 1 min OFF, 15-20 cycles. Sonication efficiency varies between batches of materials, even under same cell lysis/sonicating conditions. Three examples with different sonication results, with fragmented DNA size from 400-2500bp, 400-800bp, and 200-400 bp are

shown in Figure 4.4 C. The results indicate that even under the same sonicating parameters, the sonication efficiency may vary between different batch of materials and how the material was processed, thus checking sonication efficiency every time is necessary to obtain proper DNA fragment size.

4.3.3 Investigating MYB26 binding targets by ChIP

YFP-tagged MYB26-DNA complex was precipitated with commercial anti-GFP (ab290 ChIP grade) antibody (Abcam, UK), and reversed cross-linked to recover binding DNA, the purified DNA was used as template in PCR/QPCR to determine the enrichment of selected upstream regions from the 10 candidates (Table 4.3).

Due to the low amount of DNA that could be purified from the ChIP experiment, it was not possible to analyse all promoter regions of the 10 MYB26 putative targets, thus 13 pairs of primers were designed to analyse regions that contain conserved non-coding sequences, MYB binding site, or MYB26 *in vitro* binding motif (Figure 4.5 and Figure 4.6). According to the ChIP-PCR results (Figure 4.7), the *PKSP* intron region was enriched in both IP1 and IP2, immunoprecipitation (IP) with anti-GFP (ab290, ChIP-grade) antibody at 3‰ and 5‰ (v/v) concentration, but not enriched in the non-specific antibody control (IgG) or any IP sample in mock treated material control, indicating that MYB26 could possibly bind to the 1st intron region of *PKSP*, which encodes a protein kinase superfamily protein. *RIC4-p1* region also had enrichment only in anti-GFP IP sample of Dex-treated group, but the signal was relatively weak. *ADS3* prom and *IRX15-L p1* also seemed enriched in anti-GFP IP samples only in Dex-treated material, however, a background could be observed in the non-specific antibody (IgG) control, making their binding results not as convincing

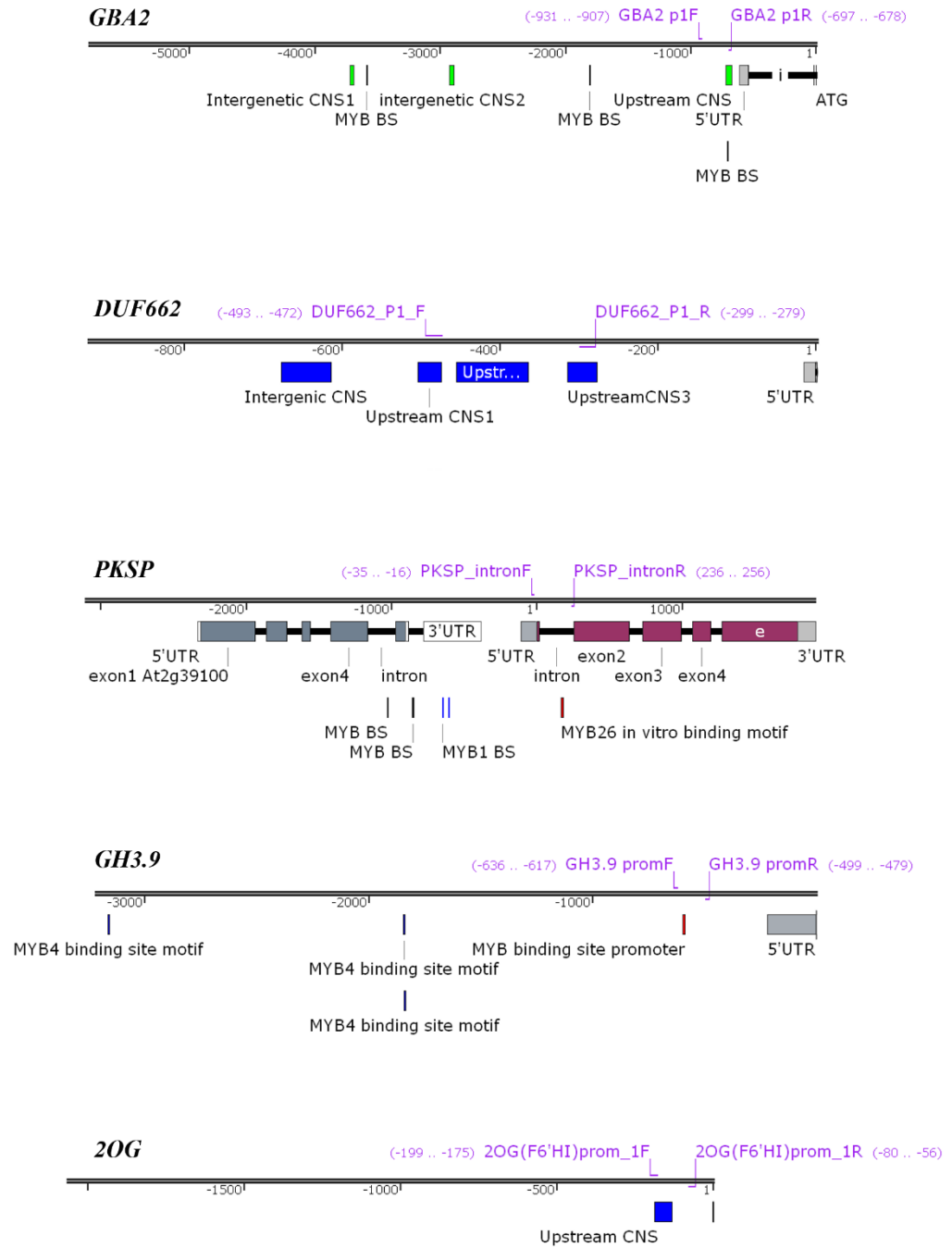


Figure 4.5 ChIP-PCR primers design (part I)

Maps of MYB26 putative targets upstream region, showing ChIP primers and the cis-regulatory elements such as conserved non-coding sequence (CNS) among crucifer (Reineke et al. 2011), MYB protein binding site (Davuluri et al. 2003) and MYB26 *in vitro* binding motif (Taylor, 2014). The translation start site of each gene was numbered as base 1.

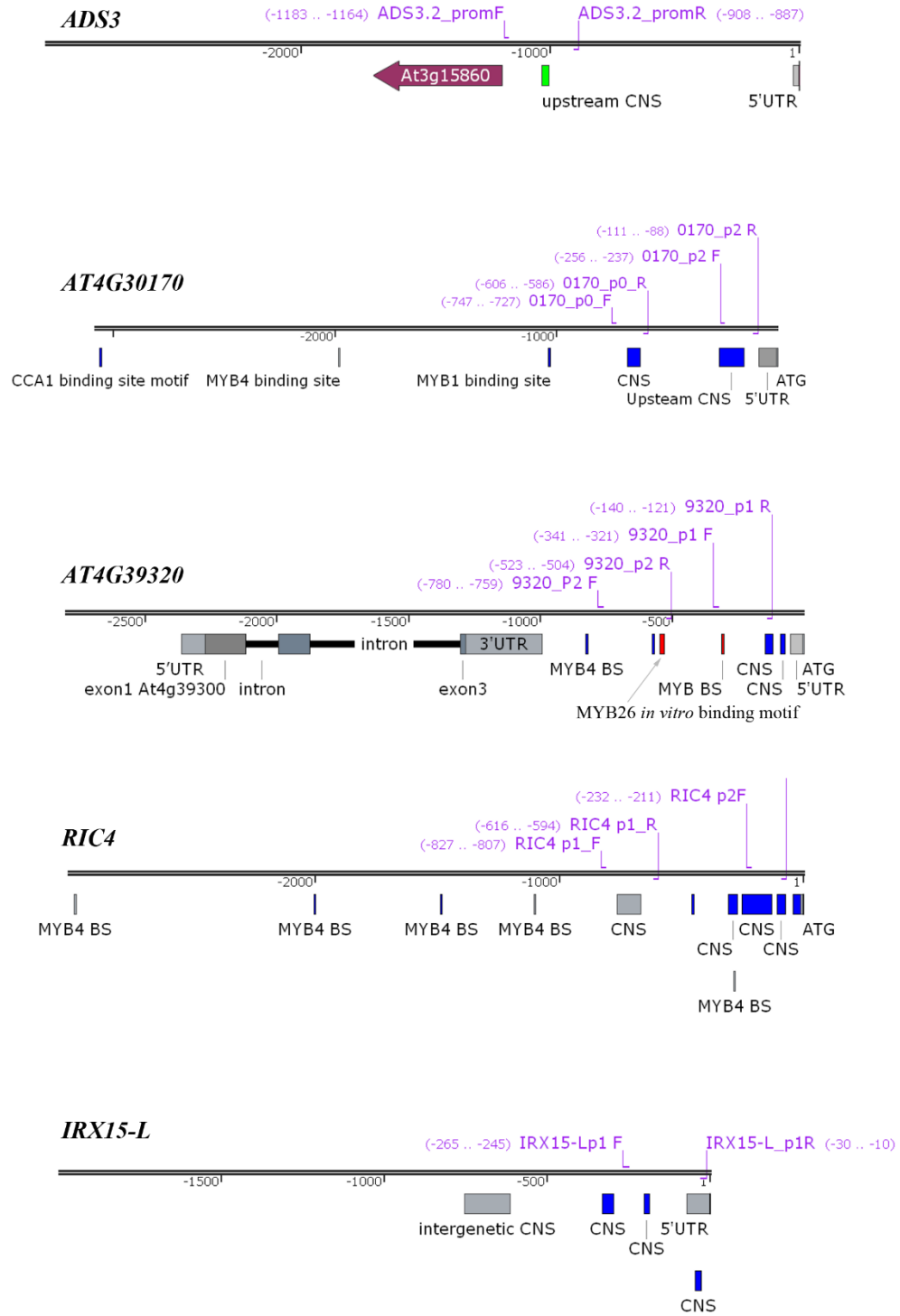


Figure 4.6 ChIP-PCR primers design (part II)

Maps of MYB26 putative targets upstream region, showing ChIP primers and the cis-regulatory elements such as conserved non-coding sequence (CNS) among crucifer (Reineke et al. 2011), MYB protein binding site (Davuluri et al. 2003) and MYB26 *in vitro* binding motif (Taylor, 2014). The translation start site of each gene was numbered as base 1.

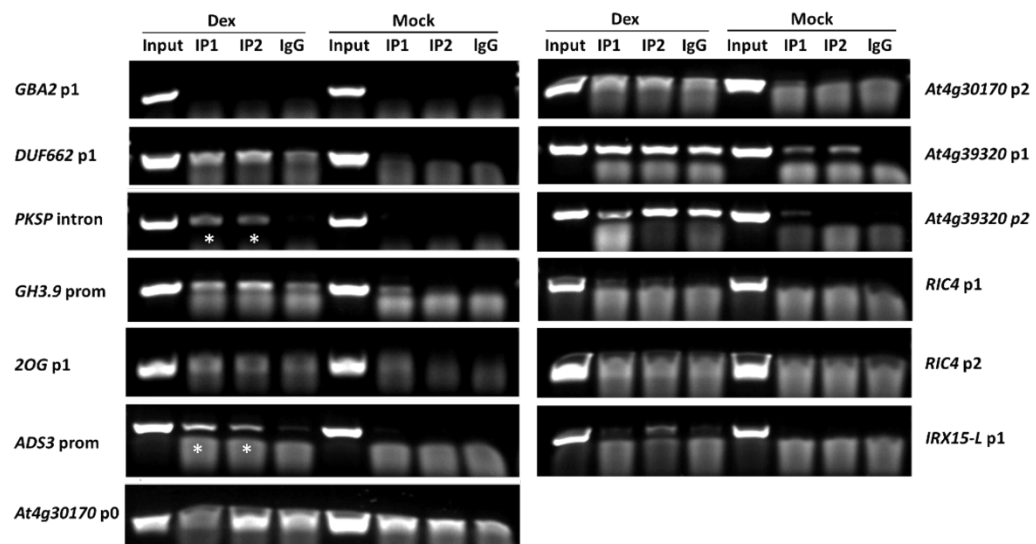


Figure 4.7 ChIP-PCR of MYB26 putative targets

Thirteen pairs of primers were analysed in ChIP-PCR, *PKSP* intron and *ADS3* prom showed enrichment in MYB26 ChIP-PCR (star). Input, input genomic DNA control; IP1, immunoprecipitation using 3% (v/v) anti-body; IP2, immunoprecipitation using 5% (v/v) anti-body; IgG, nonspecific antibody control. Dex, Dex induced material, Mock, mock treated material.

as *PKSP*. *GBA2* p1 region didn't enrich in either Dex or Mock-treated IP samples, indicating that there was no binding activity taking place between MYB26 and *GBA2* p1. Many other selected regions, such as *DUF662* p1, *GH3.9* prom, *2OG* p1, *At4g30170* p0 p2; *At4g39320* p1-2, *RIC4*-p2, were enriched in the negative control as well, indicating their enrichment were false positive, may due to the background of immunoprecipitation.

Independent ChIP-qPCR was conducted to further confirm the binding activity between MYB26 and *PKSP* intron, *ADS3* prom, *RIC4* p1 and *IRX15-L*. *GBA2* p1 was also analysed in ChIP-qPCR as a negative control to evaluate ChIP-qPCR results (Figure 4.8). *RIC4* p1 and *IRX15-L*, however, didn't amplify in the independent ChIP-qPCR (no Cp value was produced in anti-GFP IP samples). The fold enrichment of *PKSP* intron was 7.45 compared to the nonspecific antibody (IgG) IP control. The fold enrichment of *ADS3* prom was around 1.98

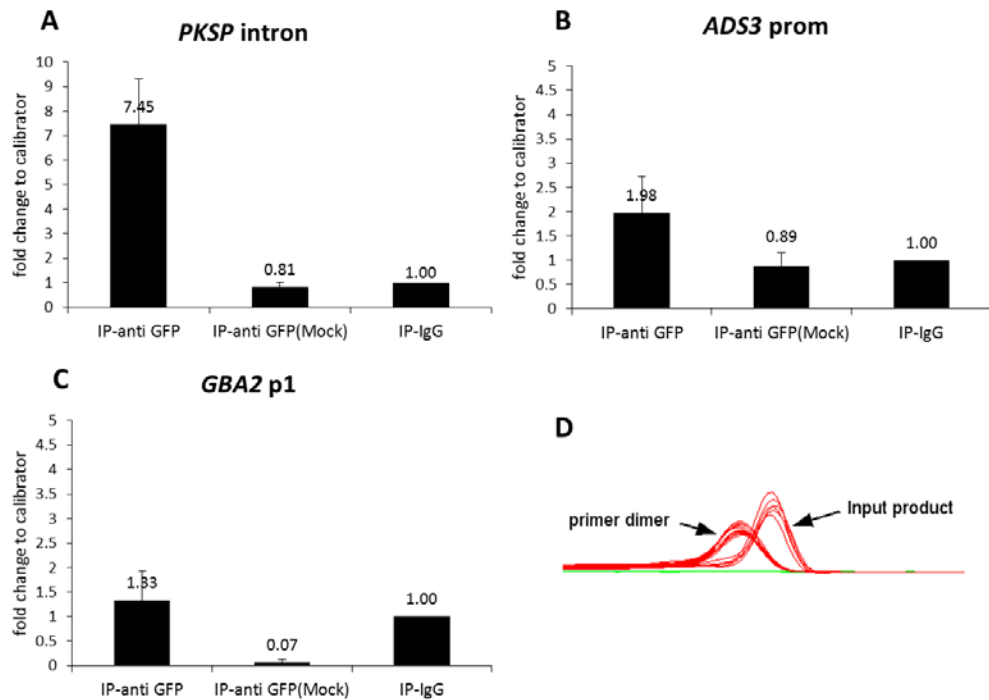


Figure 4.8 ChIP-qPCR of *PKSP* intron, *ADS3* prom, and *GBA2* p1

An independent ChIP-qPCR was performed to analyse the fold enrichment of *PKSP* intron (A), *ADS3* prom (B) and *GBA2* p1 (C). *GBA2* p1 was selected as a negative control to evaluate the ChIP results. (D) Melting peak analysis of *GBA2_p1*.

compared to IgG IP control. According to melting peak analysis, *GBA2* p1 didn't amplify in any IP samples, consistent with the ChIP-PCR results (Figure 4.7). The independent ChIP-qPCR data indicates that *PKSP* could possibly be an MYB26 binding target.

4.3.4 Expression and knock-out analysis of *PKSP*

PKSP belongs to protein kinase superfamily, involved in protein amino acid phosphorylation. No functional analysing study of *PKSP* has been reported. *PKSP* is co-expressed with *MYB26* according to FlowerNet (Pearce et al. 2015). The expression of *PKSP* was dramatically down-regulated in the *ms35* mutant during PMI and BC stages. In the *MYB26* overexpression line, *PKSP* was moderately up-regulated in both leaves and buds tissue (Figure 4.9).

Protein amino acid sequence blast results indicate that PKSP has relatively high sequence similarity to three protein kinase superfamily proteins, which are PCRK1 (At3g09830) , PCRK2 (AT5G03320), and At2g28940 (Figure 4.10 A)

The expression stage of *PKSP*, *PCRK1* and *PCRK2* was further analysed using qPCR with dissected anthers pooling into 5 developmental stages from mature tricellular pollen stage to younger than pollen mitosis I stage (Figure 4.10 C and D). QRT-PCR results showed that the expression of *PKSP* synchronized with *MYB26*, with expression peaked during pollen mitosis I stage and decreased afterwards. The expression level of *PCRK1* and *PCRK2* were much lower compared to *MYB26* and *PKSP*, *PCRK2* may express higher at PMI and before, while *PCKR1* may express at pollen mitosis II anther stage (Figure 4.10.B)

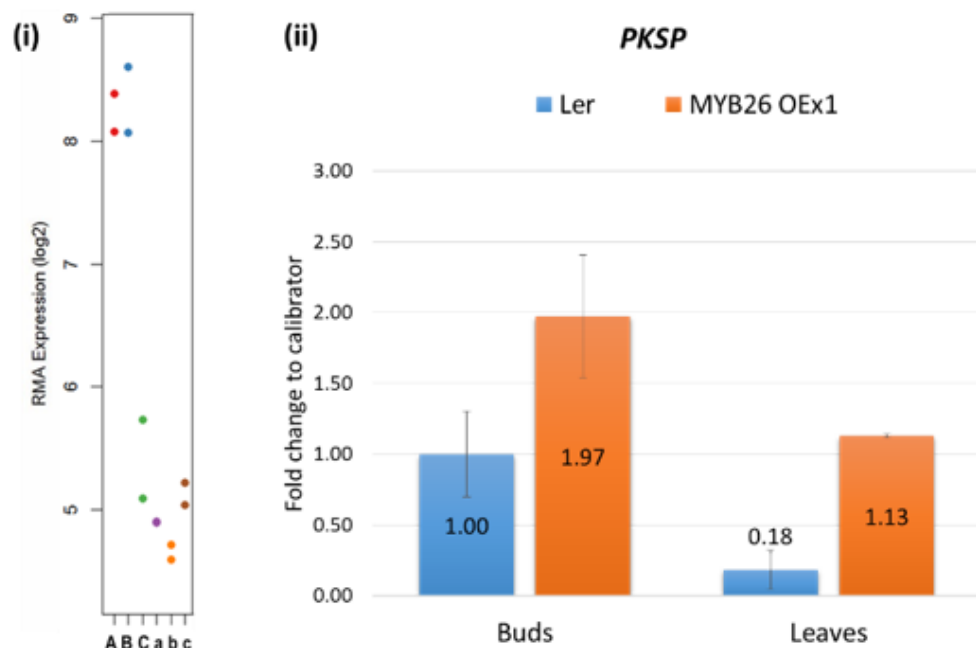


Figure 4.9 *PKSP* expression was regulated by *MYB26*

- i. The expression of *PKSP* in the wild-type (A-C) and the *ms35* mutant (a-c) anthers. A/a, pollen mitosis I; B/b, pollen mitosis II; C/c, bi-cellular stage (Jie song, 2009). The microarray data was renormalised using RMA normalisation. Each dot represents a single replicate. The expression plots is available from FlowrNet (Pearce et al. 2015)
- ii. QRT-PCR of the *PKSP* expression in buds and leaves of the wild-type and the *MYB26* overexpression lines 1(Chapter 3 section 3.3.1).

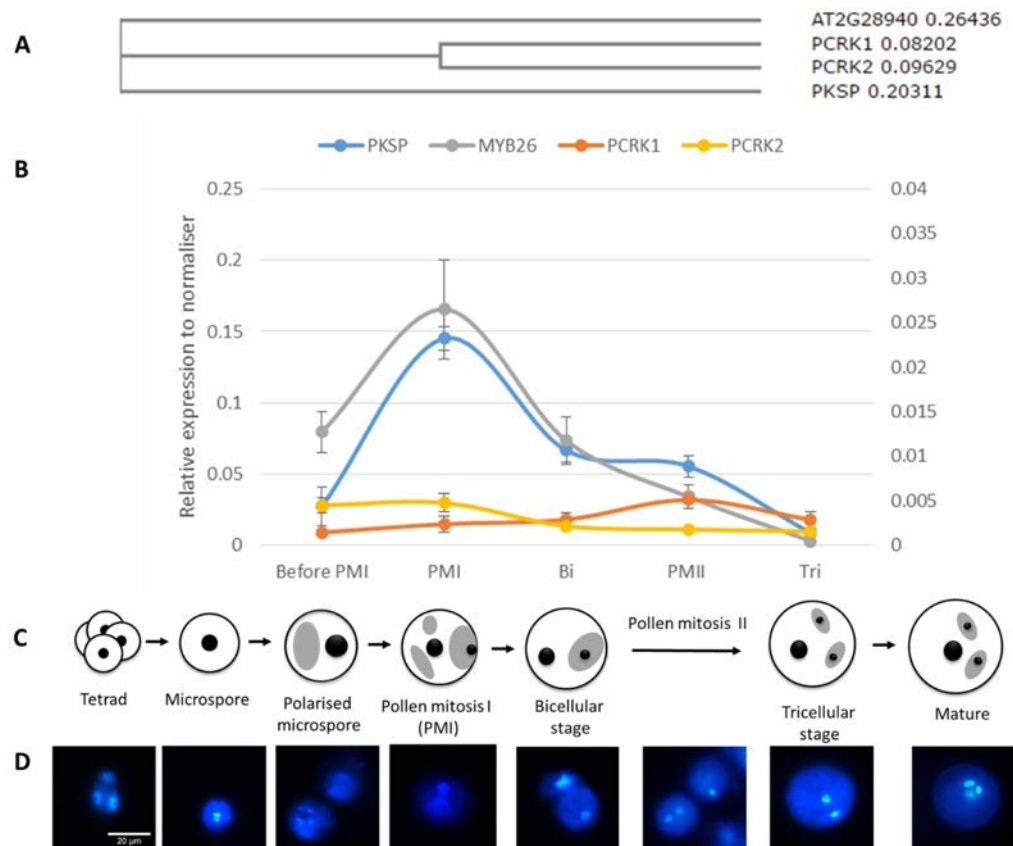


Figure 4.10 Expression analysis of the protein kinase family protein

- Phylogenetic tree of the four closely related protein kinase family proteins. This is an unrooted neighbour-joining tree without distance corrections, produced with T-coffee multi sequence alignment server (<http://tcoffee.crg.cat/>)
- Expression of *PKSP*, *MYB26*, *PKRK1* and *PKRK2* in dissected anthers of five developmental stages from young to mature. Primary axis (left) applied to the relative expression level of *PKSP* and *MYB26*, while *PKRK1* and *PKRK2* was indicated by the secondary axis (right). Before PMI, before pollen mitosis I; PMI, pollen mitosis I; Bi, bi-cellular stage, PMII, pollen mitosis II; Tri, tri-cellular stage. Data was normalised to reference gene *Actin7*.
- Diagram of pollen development from tetrad stage to mature pollen
- Example of DAPI staining at equivalent pollen stages in figure C, scale bar 20 μ m

In order to study the possible function of *PKSP* during the biological process, single T-DNA insertion mutant SAIL_416_A03 was ordered from NASC, genotyping results indicated that SAIL_416_A03 is a homozygous line (Figure 4.11. A), and the expression of *PKSP* was knocked-out in both leaves and buds tissue of the homozygous plants (Figure 4.11 B). However, no significant fertility alteration was observed in *pksp* mutant compared to wildtype (Figure 4.11 C and D). *PKSP* were expressed higher in roots compared to other tissues

in *Arabidopsis* (e-FP browser, <http://bar.utoronto.ca/efp/cgi-bin/efpWeb.cgi>), a possible defect on lateral root development seems observed in several *pksp* mutant seedlings grown on ½ MS medium (Figure 4.11 E), however, more biological replicates are required to confirm if there was any significant alteration on root phenotype.

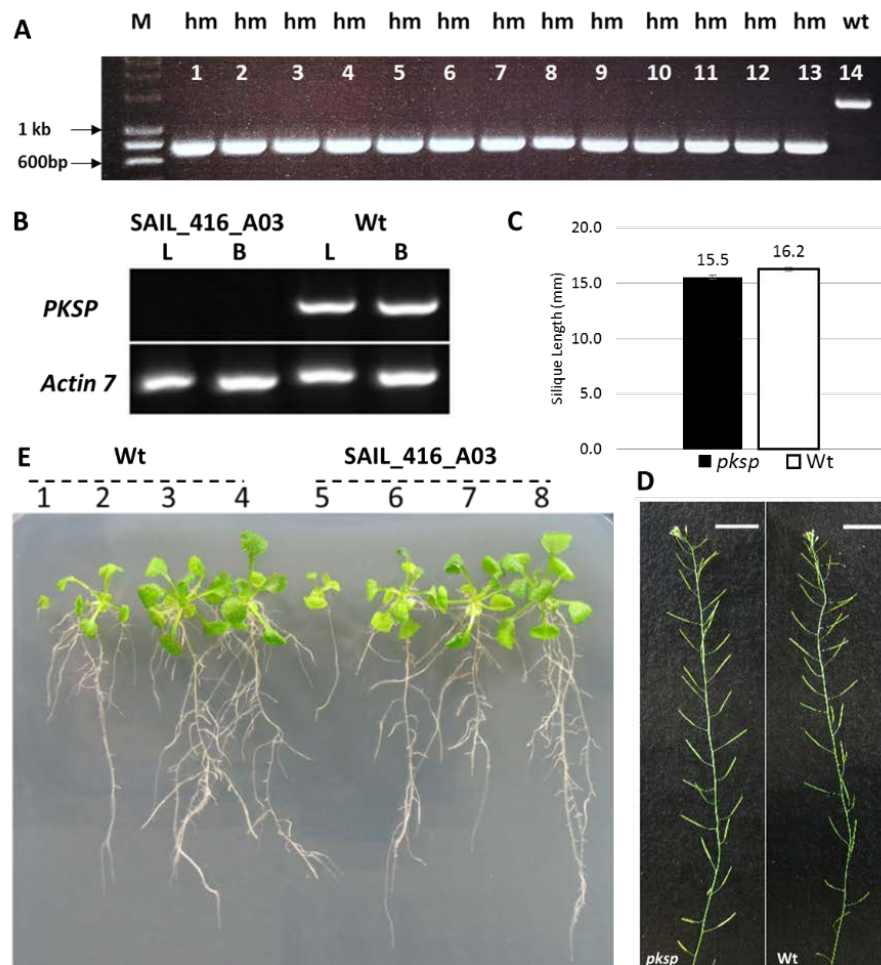


Figure 4.11 T-DNA insertion mutant analysis of *PKSP*

- Genotyping of the *PKSP* T-DNA insertion mutant SAIL_416_A03. M, HyperLadder™ 1kb; hm, homozygous; wt, wild-type. PCR was performed with SAIL_416_A03 LP/RP, LB3 primer mix.
- PKSP* expression Analysis. *PKSP* expressed both in leaves (L) and Buds (B) in the wild-type (Wt) but not in the SAIL_416_A03 mutant.
- Silique length of the *pksp* mutant (SAIL_416_A03) and the wild-type. Silique length was measured from the 4th to the 20th siliques of the main stem. Error bar, standard error from at least two biological replicates.
- Main stem of the *pksp* mutant and the wild-type *Col*. Scale bar, 3 cm
- Root phenotype of the *pksp* mutant. 24-days-old wild-type *Col* (1-4) and the *pksp* mutant (SAIL_416_A03, lane 5-8) were grown on ½ MS media.

The T-DNA insertion mutant of *PCRK1*, SALK_057158, and *PCRK2*, SAIL_129_D02 were ordered to generate *pksp pcrk1 pcrk2* triple mutant to analyse possible redundant functions within these protein kinase superfamily proteins, however according to genotyping and RT-PCR expression analysis, both SALK_057158 and SAIL_129_D02 were not successfully knocked out but showed induced the expression of gene of interest (Figure 4.12). Considering the above T-DNA mutant analysis of *PKSP*, *PCRK1* and *PCRK2*, creating *PKSP*

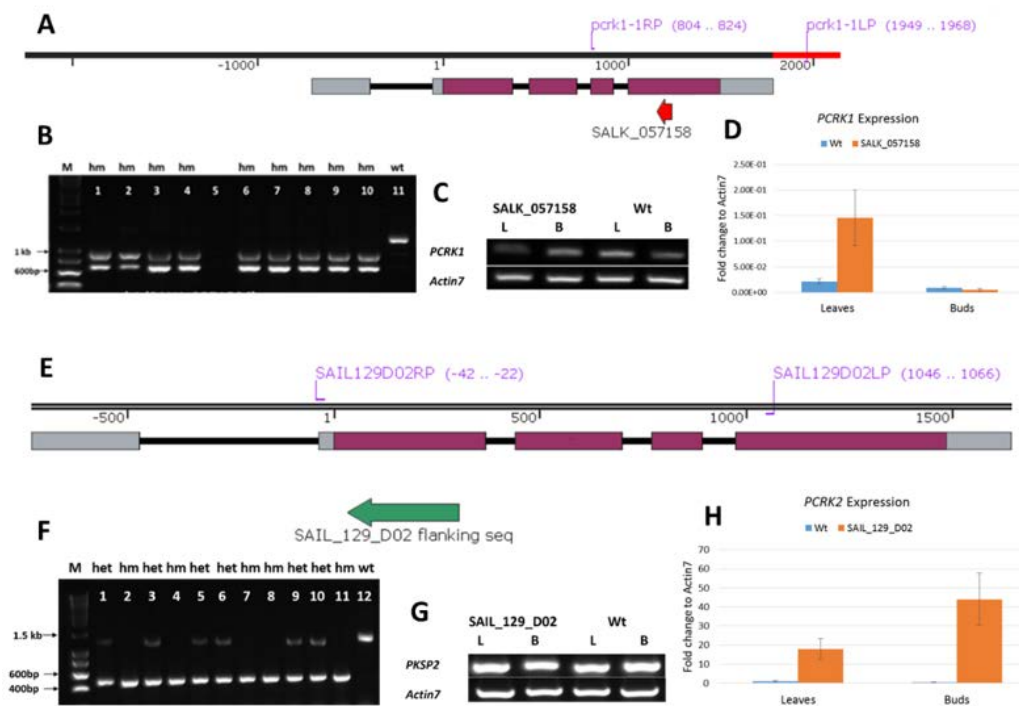


Figure 4.12 T-DNA insertion mutant of *PCRK1* and *PCRK2*

- Map of gene *PCRK1*, showing flanking sequence of the T-DNA insertion mutant SALK_057158 (Red arrow, sequencing data is available from T-DNA express website) and primers used for genotyping. Plum box, exon; solid black line, intron; grey box, untranslated region (UTR).
- Genotyping of the SALK_057158 using primers indicated in figure A and LBb1. M, hyperladder I marker; Wt, wild-type *Col*; hm, homozygous.
- RT-PCR of *PCRK1* in SALK_057158 and wild-type (Wt). L, leaves; B, buds; reference gene *Actin7*.
- QRT-PCR of *PCRK1* in leaves and buds of the SALK_057158 line and Wt.
- Map of gene *PCRK2*, showing the flanking sequence of the T-DNA insertion mutant SAIL_129_D02 (Green arrow, sequencing data is available from T-DNA express)
- Genotyping of the SAIL_129_D02 line,
- RT-PCR of *PCRK2* in SAIL_129_D02 and the wild-type.
- QRT-PCR of *PCRK2* in SAIL_129_D02 and the wild-type.

dominant repression line in future work may be more efficient to analyse *PKSP* biological function.

4.3.5 MYB26 bind *NST1* and *NST2* *in vivo* but not *in vitro*.

NAC SECONDARY WALL THICKENING PROMOTING FACTOR1 (*NST1*) and *NST2* are key regulators of secondary thickening (ST), the *nst1nst2* double mutant has indehiscent anthers due to lack of ST in endothecium layer (Mitsuda et al. 2005). The expression of *NST1* and *NST2* was down-regulated in the *ms35* mutant and was up-regulated in the *MYB26* overexpression lines (Chapter 3 section 3.3.1, Yang et al. 2007), indicating that *MYB26* possibly regulates *NST1* and *NST2*. Thus, *NST1* and *NST2* promoters were tested using ChIP-PCR to determine whether *MYB26* directly regulates *NST1* and *NST2*. Primers were designed to cover 3000 bp upstream region of both *NST1* and *NST2* (Song, 2009), among them *NST1_P2a* (-2409bp to -2260bp from *NST1* ATG), *NST2_P8* (-440bp to -235bp from *NST2* ATG) showed enrichment in ChIP-PCR (Song, 2009; Taylor, 2014). In this thesis, *NST1_P2a* and *NST2_P8* were tested in *MYB26* ChIP using anti-GFP antibody (Ab290 ChIP grade, Abcam, UK) and anti-*MYB26* antibody (Roderick, 2008, raised by Invitrogen, Appendix) (Figure 4.13). Conserved non-coding sequence(CNS) among crucifer and reported binding site were annotated on the map of *NST1* and *NST2* upstream region using UCSC genome browser (Haudry et al. 2013) and AGRIS (Davuluri et al. 2003; Yilmaz et al. 2011). *NST1_P2a* covered a secondary wall MYB-responsive element (SMRE), which is reported as the binding site of the ST master switches MYB46 and MYB83 as well as their poplar orthologues PtMYB2/3/20/21 during secondary wall biosynthesis activation (Zhong & Ye 2012). *NST1_P2a* was significantly enriched in ChIP-qPCR with a fold change of 5.17 (anti-GFP

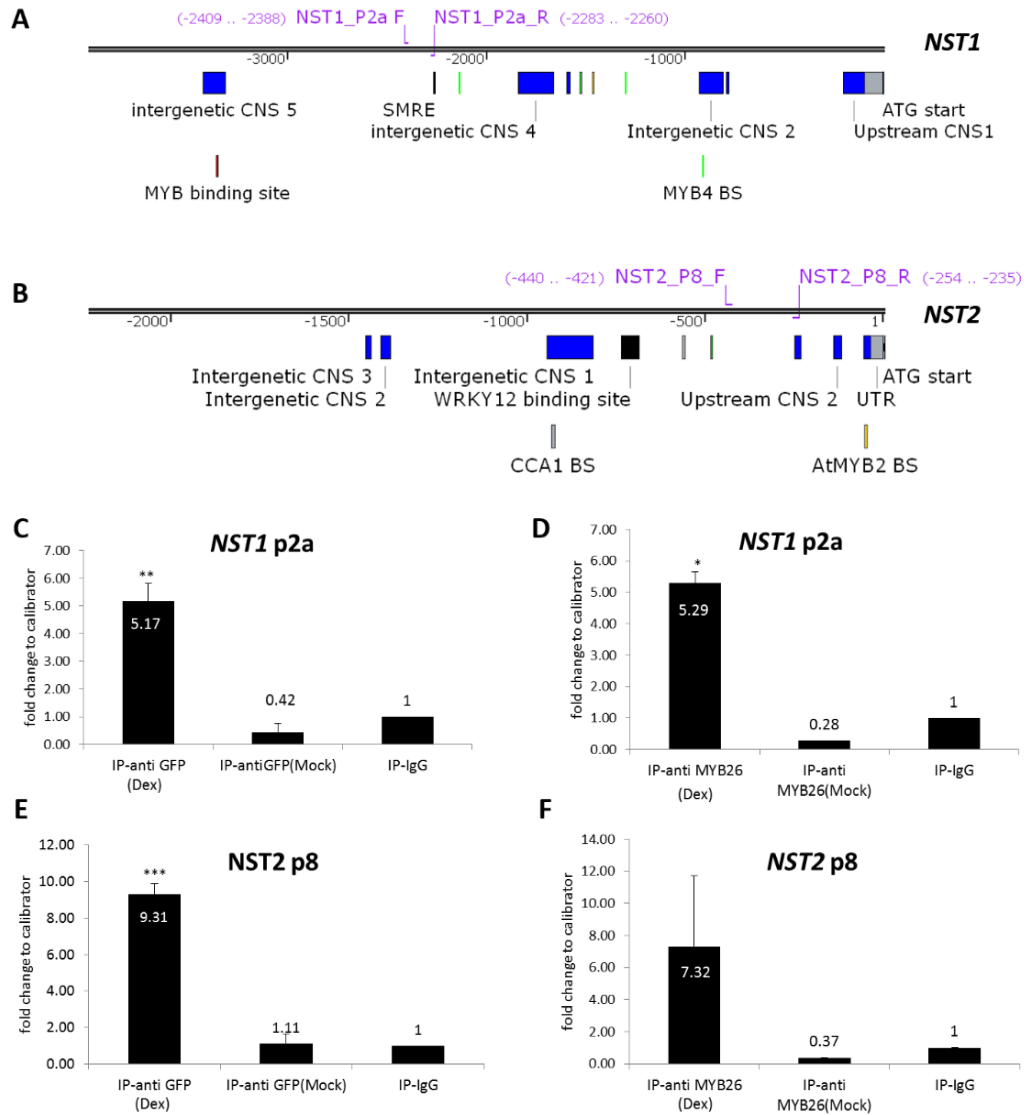


Figure 4.13 MYB26 binds to *NST1* and *NST2* promoter in ChIP

- Map of the *NST1* upstream region. Primers shown on map indicate the region tested in ChIP-PCR and EMSA. Conserved non-coding sequence (CNS) and published binding sites were highlighted on the map.
- Map of *NST2* upstream region
- QPCR showed enrichment of *NST1* p2a in ChIP using the anti-GFP antibody. Immunoprecipitation (IP) was conducted using Dex-treated material and Mock-treated material. Nonspecific antibody (IgG) was included as a negative control.
- Enrichment of *NST1* p2a in ChIP-QPCR with anti-MYB26 antibody
- Enrichment of *NST2* p8 in ChIP-qPCR with anti-GFP antibody.
- Enrichment of *NST2* p8 in ChIP-qPCR with anti-MYB26 antibody.

antibody) and 5.29 (anti-MYB26 antibody) compared to the non-specific antibody control (Figure 4.13 C and D). *NST2_P8* region covers a CNS and is close to several reported binding sites such as WRKY12 binding site. WRKY12 is a negative regulator of ST formation in pith, WRKY12 was reported to be a

direct negative regulator binds to *NST2* in EMSA and in planta (Wang et al. 2010). The fold enrichment of *NST2_P8* in ChIP-qPCR was 9.31 (anti-GFP antibody) and 7.32 (anti-MYB26 antibody) compared to non-specific antibody control. ChIP-qPCR results indicate that MYB26 possibly regulates the expression of *NST1* and *NST2* by binding to *NST1_P2a* and *NST2_P8* respectively during secondary thickening biosynthesis.

Besides ChIP experiment, electromobility shift assay (EMSA) was conducted to further investigate the binding activity between MYB26 and *NST1/2* *in vitro*. MYB26 fully spliced CDS was cloned into pET41 (a+) plasmid and transformed into *E.coli* expression strain BL21 Star (DE3), MYB26 with N-terminal GST tag recombinant protein was ectopically expressed under Isopropyl β -D-1-thiogalactopyranoside (IPTG) induction. Although most of the recombinant protein GST-MYB26 (67.6KDa) was not soluble and remained as inclusion bodies, by optimising culture conditions, increased amount of soluble GST-MYB26 was obtained with 0.5mM or 1mM IPTG inducement and cultured at 28°C for 6 hours (Figure 4.14 A). Soluble MYB26-GST recombinant protein was then purified from large-scale cultures after 0.5mM IPTG induction for 6 hrs at 28°C. Purification was conducted using Pierce™ Glutathione Magnetic Beads (ThermoScience, UK) according to manufacturer's instruction except that the cell lysis input concentration was optimised. Because most recombinant proteins remained in inclusion bodies while relatively low amount of GST-MYB26 was soluble, thus a gradient concentration, 50/250, 100/200, 200/100 (V/V) cell lysis supernatant/binding buffer was used during purification, followed by Western blot detection using anti-MYB26 antibody (Figure 4.14 B). With the purified GST-MYB26, EMSA was performed with binding buffer

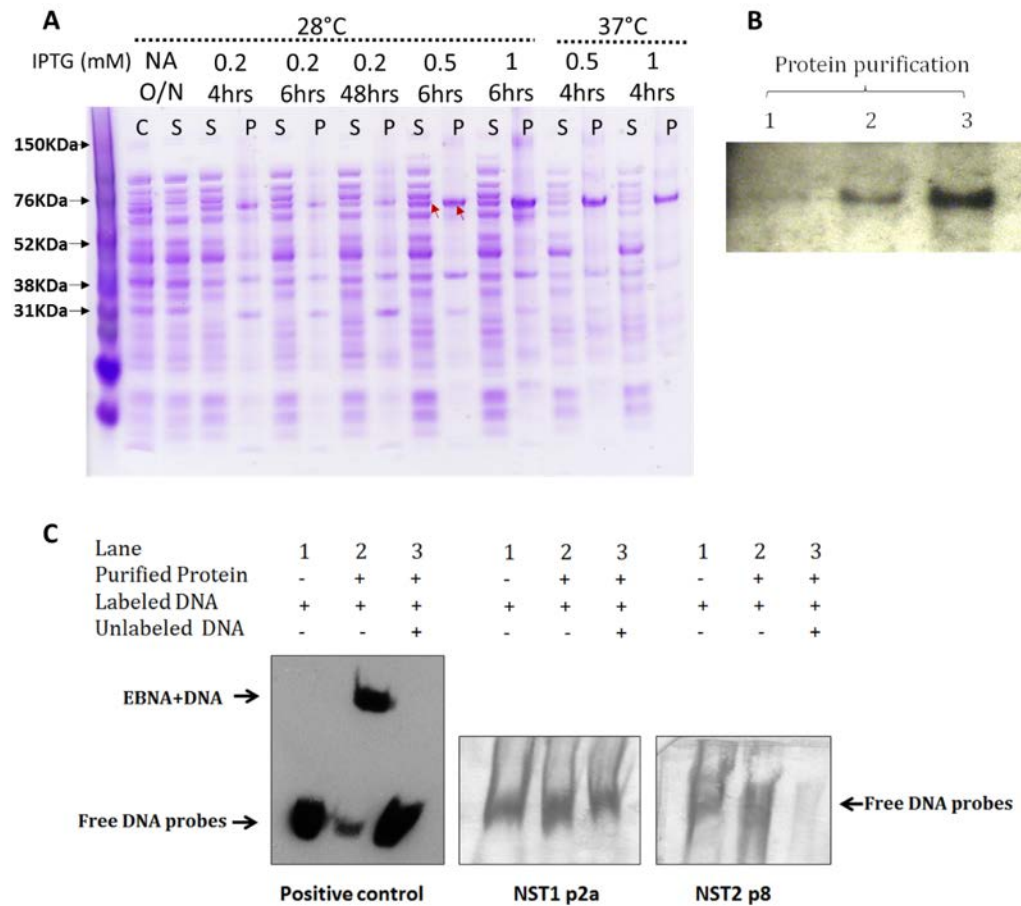


Figure 4.14 MYB26 did not bind to NST1_p2a or NST2_p8 in EMSA.

- A. Optimisation of MYB26-GST expression in *E.coli*, expression strain BL21* contain pET41-MYB26-GST were incubated at either 28 °C or 37°C, induced with 0.2-1mM IPTG and incubated from 4hrs to 48hrs. Red arrow indicates examples of MYB26-GST (67.6kDa) in both soluble condition in cell lysis supernatant (S) or in inclusion body of the cell lysis pellet (p). Leaking expression was found from the whole cell lysis (C) without IPTG induction.
- B. Western-blot (WB) of the purified MYB26-GST protein using anti-MYB26 antibody., Increased input of the cell lysis were used in protein purification system. From 1-3: 50 µl /300 µl, 100 µl /300 µl, 200 µl /300 µl cell lysis/purification system.
- C. EMSA tested the binding activity between MYB26 and NST1_p2a, NST2_p8 *in vitro*. Positive control was provided by the LightShift™ Chemiluminescent EMSA Kit (Thermo, UK). The biotin labeled positive control (included in kit) and the DIG labeled NST1_p2a, NST2_p8 were performed under the same binding condition.

provided in the LightShift™ Chemiluminescent EMSA Kit (Thermo Fisher Scientist, UK). The same binding condition was used between GST-MYB26 and DIG-labeled/unlabelled NST1_P2a, NST2_P8 as well as a positive control (EBNA+ 3' biotin labelled/unlabeled DNA) provided in the EMSA kit and performed according to the manufacturer's instruction. In positive control, a

heavier, slower moving band (lane 2) was detected, while the chemiluminescence disappeared when 20x the amount of unlabelled competitor DNA was introduced. For NST1_P2a and NST2_P8, only free DNA probes were detected in all three lanes, indicating that MYB26 didn't bind either NST1_P2a or NST2_P8 *in vitro* under the binding condition used.

In summary NST1_P2a (-2409bp to -2260bp from *NST1* ATG), NST2_P8 (-440bp to -235bp from *NST2* ATG), were enriched in MYB26 ChIP experiment but did not show binding to MYB26 under the condition tested in EMSA.

4.4 DISCUSSION

4.4.1 MYB26 putative targets selection

Ten putative targets of MYB26 were selected based on the *ms35* microarray data, and their temporal and spatial expression pattern according to expression plots, FlowerNet (Pearce et al. 2015), and Laser Capture Microscopy (LCM) sectioning data from wild-type anther tissues (G. Vizcay-Barrena, Z.A.Wilson, unpublished data). The expression of these candidates all dramatically changed in the *ms35* mutant. In wildtype, these candidates were highly expressed in anthers during PMI to BC pollen stages, many of them showed enriched expression in the anther endothecium layer, indicating a possible expression overlap between these candidates and *MYB26* according to the previously described bioinformatics analysis.

Primers were designed to test upstream regions of the 10 selected MYB26 putative targets, one or two regions were selected for each gene that contained cis-regulatory elements and were closer to the translation start site to test in ChIP-qPCR. Those which did not enrich in ChIP indicate that the tested regions may not bind by MYB26 *in vivo*. Considering the fact that not all promoter regions were covered in ChIP-qPCR, there is still possibility that genuine MYB26 binding target may locate in the untested regions among these candidates. It is worth giving attention to these candidates (Table 4.3) for further research due to promising expression pattern. Also, ChIP combined with the next generation sequencing (Kaufmann et al. 2010c) in future work will give a more straightforward clue to uncover MYB26 direct binding targets during secondary thickening development in anther.

4.4.2 Receptor-like cytoplasmic kinases (RLCKs)

A protein kinase superfamily protein *PKSP* (At2g39110) showed co-expression with *MYB26*, the expression of *PKSP* was down-regulated in the *ms35* mutant and was up-regulated in the *MYB26* overexpression lines (Figure 4.9 and Figure 4.10), the first intron of *PKSP* was enriched in ChIP-qPCR experiment, indicating that *MYB26* may directly regulate *PKSP*.

The eukaryotic protein kinases are enzymes that catalyse the reversible transfer of the γ -phosphate from ATP to amino acid side chains of proteins. Eukaryotic protein kinases have been typically divided into those that phosphorylate serine and/or threonine, and those that phosphorylate tyrosine (Stone & Walker 1995). The phosphorylation status of a protein can profoundly affect its activity and interaction with other proteins. An estimated 1 to 2% of functional eukaryotic genes encode protein kinases (Lehti-Shiu & Shiu 2012), over 1000 protein kinases has been identified in *Arabidopsis* (The Arabidopsis Genome Initiative 2000), indicating importance in many aspects of cellular regulation in both animals and plants.

The catalytic domains of eukaryotic protein kinases contain 250 to 300 amino acids with alternating regions of high and low conservation. Based on the sequence alignment of catalytic domains, the protein kinase superfamily has been divided into several groups. *PKSP* contain a predicted Serine/threonine-protein kinase-like domain and has been classified into the receptor-like cytoplasmic kinase (RLCK) VII according to public available databases such as Arabidopsis Protein Phosphorylation Site Database (PhosPhAt) and plant protein phosphorylation database (P3DB)(Yao et al. 2014; Heazlewood et al. 2008). RLCKs belong to the superfamily of receptor-like kinases (RLKs) which

are transmembrane proteins with an extracellular receptor domain for signal perception and an intracellular kinase domain for signal transduction (Shiu & Bleecker 2001). RLCKs show homology to RLKs on the kinase domain but lack the transmembrane domain (Figure 4.15).

RLCKs, such as members of subfamily VII of RLCKs, have been reported to play roles in stress responses, signalling, and plant immunity, (Shiu & Bleecker 2001), are overrepresented among genes induced by biotic stress (Lehti-Shiu et al. 2009). OsRLCK185 was reported to be an essential immediate downstream signalling partner of OsCERK1 in mediating chitin- and peptidoglycan-induced plant immunity (Yamaguchi et al. 2013). The RLCK subfamily VII member, *BOTRYTIS-INDUCED KINASE1 (BIK1)*, is an essential component in microbe-associated molecular patterns (MAMP) signal transduction. BIK1 is rapidly phosphorylated by BAK1, a leucine-rich repeat-receptor-like kinase, upon flagellin perception. BIK1 also directly phosphorylates BAK1 and FLS2 to recognise bacterial flagellin and initiates immune signalling in *Arabidopsis* (Lu

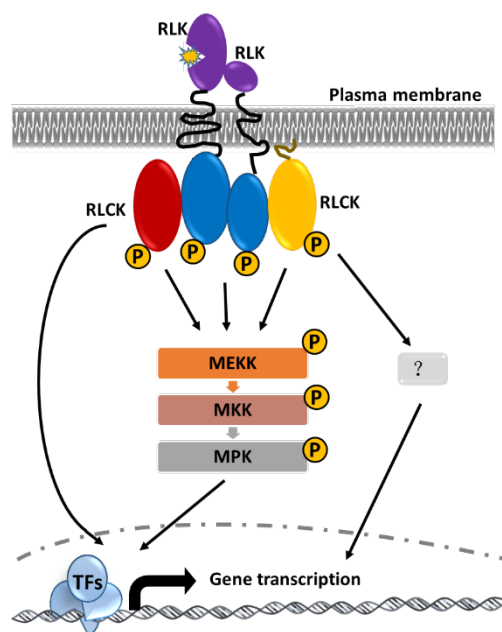


Figure 4.15 A model of the receptor-like cytoplasmic kinase (RLCK) functions. Redrawn from (Lin et al. 2013)

RLCK/RLK complexes could activate mitogen-activated protein kinase (MAPK) cascade (MEKK-MKK-MPK) and/or MAPK-independent signalling to regulate gene expression via transcription factors (TFs) or other physiological responses.

et al. 2010). Functional characterisation of four rice RLCKs, *OsRLCK57*, *OsRLCK107*, *OsRLCK118*, and *OsRLCK176*, showed that they negatively regulate brassinolide signalling, while positively regulating immune responses. Enlarged leaf angles and hypersensitiveness to brassinolide treatment were observed in transgenic lines that silenced each of 4 *OsRLCKs*. Transgenic plants silenced for *OsRLCK57* had fewer tillers and reduced panicle secondary branching, and lines silenced for *OsRLCK107* and *OsRLCK118* produce fewer seeds (Zhou et al. 2016). The receptor-like cytoplasmic kinase *PCRK1* was reported to be important for defence against the pathogen *Pseudomonas syringae* pv. *maculicola* ES4326. The pathogen defence ability and callose deposition were reduced in *pcrk1* mutant lines, but double mutant of *pcrk1* and two phylogenetically closely related genes *PCRK2* (At5g03320) or At5g03320 didn't significantly reduce the immunity against *Pma* ES4326 compared to the single mutant of *pcrk1* (Sreekanta et al. 2015). However, Kong et al suggest that the *pcrk1pcrk2* double mutant, but neither of the single mutants, exhibits reduced accumulation of salicylic acid (SA), a signalling molecule in plant defence, and enhanced disease susceptibility to bacterial pathogens, indicating a redundant function between *PCRK1* and *PCRK2* (Kong et al. 2016).

RLCKs also regulate plant growth and development as well as involved in hormone signalling. Expression profile of rice *OsRLCKs* showed that among the total 339 *OsRLCKs*, 98 *OsRLCKs* expressed differentially during reproductive developmental stages, include stages of panicle and seed development; 82 *OsRLCKs* differentially expressed under any one of the cold, salt, and dehydration abiotic stress conditions. An overlap of 36 *OsRLCKs* both altered expression during reproductive developmental stages and abiotic stress

conditions (Vij et al. 2008). BSK1 and CDG1 belong to RLCK subfamily VII and XII, respectively (Shiu et al. 2004), which function in parallel to positively regulate Brassinosteroids (BR) signalling (Kim & Wang 2010; Kim et al. 2011). *Arabidopsis thaliana* *BOTRYTIS-INDUCED KINASE1* (*BIK1*) regulates immune responses to pathogens, and also function as a component of the ripening hormone ethylene (ET) signalling. Altered expression of ET-related genes was found in *bik1* mutant (Laluk et al. 2011). Cast away (CST), a subfamily VII RLCK, acts as a spatial and temporal inhibitor of organ separation (Burr et al. 2011). M locus protein kinase (MPLK), another subfamily VII RLCK, is essential for self-incompatibility responses in *Brassica* (Murase et al. 2004)

As exemplified by the previous studies, PLCKs are involved in distinct physiological responses from immunity, hormone-mediated growth, organ abscission to self-incompatibility, however, remarkable similarity can be found in the framework of their signal transduction cascades (Figure 4.15). Since secondary thickening is a dramatic complex biological process, requiring precise transcriptional regulation of many TFs and biosynthetic genes, it could be hypothesized that the MYB26 putative binding target *PKSP*, subfamily VII RLCK, may function in a signal transduction pathway to regulate gene expression probably via TFs involved in secondary thickening development.

4.4.3 MYB26 possibly regulate ST through *NST1* and *NST2*

The *NAC SECONDARY WALL THICKENING PROMOTING FACTOR1* (*NST1*) and *NST2* function redundantly regulating secondary thickening (ST) development (Mitsuda et al. 2005; Nelson et al. 2012). The expression of *NST1* was detected in various tissues in which ST developed, while the *NST2* expression was stronger in anthers. Overexpression of *NST1* or *NST2* induced

ectopic ST in various aboveground tissues. The *nst1nst2* double mutant produced indehiscent anthers due to lack of ST development in the anther endothecium layer, similar to the phenotype observed in the *ms35* mutant. Moreover, the expression of *NST1* and *NST2* were up-regulated in *MYB26* inducible expression line (Chapter 3, Figure 3.4). It was hypothesised that *MYB26* possibly regulate *NST1/2* during ST development; ChIP-qPCR (Figure 4.11) suggests that *NST1_P2a* (-2409bp to -2260bp from *NST1* ATG), *NST2_P8* (-440bp to -235bp from *NST2* ATG) could contain *MYB26* binding sites, however, this was variable with enrichment observed in some biological replicates but not in others. The lack of a positive control is a difficult issue when evaluating *MYB26* ChIP experiments. EMSA results suggest that *MYB26* did not bind *NST1_p2a* and *NST2_p8* *in vitro* (Figure 4.12), however this could be due to the binding conditions not favouring *MYB26* binding activity or possibly *MYB26* requiring additional partners to form a protein complex, which is common among transcription factors, to fulfil its function. Based on the abovementioned research and the fact that *MYB26* affect the expression of *NST1/2*, it is possible that *MYB26* regulate secondary thickening through *NST1/2*.

CHAPTER 5 MYB26 INTERACTING PROTEINS

5.1 INTRODUCTION

In eukaryotes, gene transcription frequently is mediated by multi-protein complexes in a combinatorial fashion, rather than via single protein as often seen in prokaryotes. The protein complex assembly involves the combinatorial action of transcription factors that bind conserved DNA elements in precise spatial orientation and on the basis of both protein-DNA and protein-protein interaction (Wolberger 1999). Interacting with specific partners and DNA binding often lead to various adaptation in protein conformation and function (Lefstin & Yamamoto 1998). Transcription factors can sometimes bind multiple partner proteins in a similar manner or use a different set of interaction surfaces. This combinatorial and flexible mechanism integrates different signalling pathways, providing a complex network to meet the higher regulatory demands of a higher organism (Reményi et al. 2004).

The MYB and basic helix-loop-helix (bHLH) families provide excellent examples of combinatorial plant gene regulation. The *Arabidopsis* genome contains more than 150 bHLH proteins, making bHLH family the second largest class of transcription factors in plants. About 30% of bHLH proteins have been functionally characterized to varying degrees (Pires & Dolan 2010; Toledo-Ortiz 2003). The bHLH domain is highly conserved and consists of around 60 amino acids forming two functionally distinct regions, the N-terminus basic (primary basic amino acid) region and α -helix-loop-helix region. Proteins containing the bHLH motif often form homo- or heterodimers with other bHLH proteins, which are required for DNA recognition and contribute to DNA-binding specificity

(Grove et al. 2009). The cooperation between MYB and bHLH proteins have been found in a variety of biological process such as flavonoid biosynthesis, trichome and root hair development and vacuolar acidification, cold stress tolerance, abscisic acid signalling, phytochrome A signalling (Feller et al. 2011). In-depth analysis of protein-protein interaction specificities of MYB protein in *Arabidopsis* uncovered that [DE]L_{x2}[RK]_{x3}L_{x6}L_{x3}R is conserved in several R2R3-MYB (Chapter 1 Introduction, section 1.3 MYB family and MYB26) subgroups, and contribute to the biological specificity and stability of the MYB-bHLH complex. The interaction motif has been identified in all anthocyanin-accumulating plants studied. This motif has been successfully used to predict new MYB/bHLH interactions in *Arabidopsis* (Zimmermann et al. 2004; Lin-Wang et al. 2010). MYB26 does not contain the bHLH interaction motif, however, based on the above-mentioned studies, it is tempting to suggest that many R2R3-MYBs could possibly function in a combinatorial fashion.

There are various methods to study protein-protein interactions. The most common methods for characterising plant interactomes can be divided into two main approaches, untargeted systematic approach and targeted pair-wise analysis (Braun et al. 2013). The unbiased systematic approach includes heterologous expression in yeast, such as yeast two-hybrid (Y2H) assays, split-ubiquitin (sUbq) assay; protein complex purification from plants, such as affinity purification combined with ultrasensitive mass spectrometry (AP-MS) and protein arrays. The high-throughput systematic approach is powerful to gain insight into the systems organization of proteomes, however, targeted pair-wise analysis is necessary to validate, characterise and confirm protein interactions. Protein-fragment complementation assays (PCA) such as bimolecular

fluorescence complementation (BiFC); co-immunoprecipitation; resonance energy transfer methods such as Förster resonance energy transfer (FRET), bioluminescence resonance energy transfer (BRET); and nucleic acid programmable protein array (NAPPA) are widely used methods to validate plant protein interactomes.

In this project Yeast two-hybrid (Y2H) assay and FRET analysis were conducted to validate putative MYB26 interacting proteins; AP-MS was applied to systematically analyse MYB26 interactomes *in vivo* (Figure 5.1). A Y2H stamen library screen was performed using full-length MYB26 as the bait (Caiyun Yang and Zoe A Wilson, unpublished data), and five positive Y2H proteins were further validated using FRET analysis; these results indicate that two proteins, Y2H320 and Y2H560 could possibly interact with MYB26.

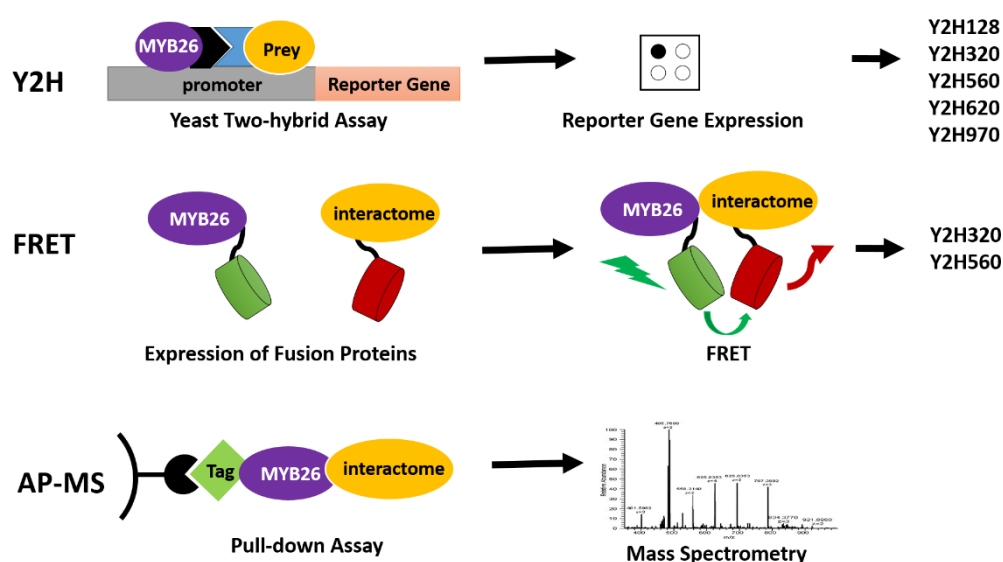


Figure 5.1 Schematic of methods used to study the MYB26 interacting proteins

Y2H, yeast two-hybrid assay. FRET, Förster resonance energy transfer. AP-MS, affinity purification followed by mass spectrometry. Five MYB26 interacting proteins were identified from the Y2H library screen using full-length MYB26 as the bait (C Yang and Z. A. Wilson, unpublished data). Five Y2H proteins were further tested in the FRET assay, results indicated that Y2H320 and Y2H560 interact with MYB26 in plant cell. AP-MS will be conducted for this project, material generation and preliminary protein purification results are documented in this thesis.

Y2H320 (bZIP21/TGA9, At1g08320) belongs to the TGACG (TGA) motif-binding proteins, which is a distinct subclade in the basic leucine-zipper (bZIP) transcription factor superfamily. This subclade contains 10 members, TGA1-TGA7, PERIANTHIA (PAN), TGA9 and TGA10 (bZIP65, At5g06839) (Jakoby et al. 2002). TGA9 and TGA10 were reported to function redundantly in anther development. The *tga9tga10* double mutant has defects in male gametogenesis, discordant adaxial and abaxial anther lobe development, abnormal lignification in anther middle layer, and lack of septum and stomium degeneration in indehiscent anthers. *TGA9* and *TGA10* are expressed throughout early anther primordia but resolve to the middle and tapetum layers during meiosis of pollen mother cells according to in-situ hybridisation data. TGA9 and TGA10 directly interact with ROXY proteins in yeast and in plant cell nuclei, suggesting that activation of TGA9 and TGA10 may relay changes in intracellular redox status (Murmu et al. 2010).

Y2H560 (At5g25560) is a member of CHY-type/CTCHY-type/RING-type Zinc finger protein, which encodes a protein containing both a CHY zinc finger and a C3H2C3-type RING domain. Y2H560 shares a high level of protein sequence similarity with CHY ZINC-FINGER PROTEIN 1 (CHYR1), which is reported to function positively in ABA response and drought tolerance. Loss-of-function mutation of *CHYR1* and *OsRZFP34*, a *CHYR1* homolog in rice, decreased relative stomata aperture under unstressed conditions, overexpression of *OsRZFP34* in rice and *Arabidopsis* increased relative stomatal opening (Hsu et al. 2014). Further study revealed that CHYR1 is a ubiquitin E3 ligase, which interacts with SNF1-RELATED PROTEIN KINASE2 (SnRK2) kinases and could be phosphorylated by SnRK2.6 (Ding et al. 2015). *CHYR1* was also

reported to be a sugar-responsive gene (Kunz et al. 2014), the expression of *CHYR1* correlate with glucose in roots, with sucrose in shoots of *Arabidopsis* seedlings, and repressed by hexokinase 1 (Kunz et al. 2015).

In this thesis, the interaction between MYB26 and two Y2H proteins, Y2H320 (TGA9) and Y2H560, were further validated by FRET assay. Expression overlap between MYB26 and the two Y2H proteins according to quantitative RT-PCR supports the hypothesis that they could interact with MYB26 during anther development. In the *tga9tga10* double mutant, lignin deposition seems altered in endothecium layer and anther development is abnormal. Overexpression of *Y2H320* affects fertility, possibly due to late anther dehiscence and filament elongation. *Y2H560* single mutant did not show apparent phenotype compared to wild-type. Preliminary results indicate that *Y2H560* over-expression (T1 generation) may have a minor effect on fertility.

5.2 METHODS

5.2.1 Cloning and transient expression constructs.

Vectors and *E.coli* strain Dh5 α were provided by the Genomic Facility, Plant and Crop Sciences, University of Nottingham. Full-length CDS of *MYB26* and *SAG101* without stop codon were cloned into Gateway compatible GFP destination vector pUBC-DEST-GFP to create pUBC-MYB26-GFP and pUBC-SAG101-GFP. Full-length CDS of *Y2H320*, *Y2H560*, *EDS1* without stop codon were individually cloned into Gateway compatible RFP destination vectors to create pUBN-Y2H320-RFP, pUBC-560-RFP, and pUBC-EDS1-RFP. Plasmid DNA was prepared from an *E.coli* overnight culture (100ng/ μ l DNA) and was used for sequencing.

5.2.2 *Agrobacterium*-mediated transient expression in tobacco leaves

200 ng of plasmid DNA were used for *Agrobacterium* (GV3101, provided by genomic facility, University of Nottingham) transformation. *Agrobacterium* carrying clones of interest were cultured overnight (28°C, 200 rpm) in LB medium contain appropriate antibiotics. An aliquot from the overnight culture was diluted 1:10 into fresh medium to start a second culture for 6 hours (28°C, 200rpm) to give final OD around 1-2. The bacteria were spun down at 2200g for 5 mins and re-suspended in infiltration buffer (50 mM MES pH 5.6, 2 mM Na₃PO₄, 0.5% glucose, 100 μ M Acetosyringone) to OD=0.2-0.4. The bacteria suspension of each clone were mixed with P19 (gene silencing suppressor protein) clones and gently agitate for 1hr at room temperature in the dark before infiltrating into tobacco leaves with a syringe. Plants were incubated under the

normal growing condition (see chapter 2, section 2.1 plant materials and growth conditions) for 2-3 days before conducting FRET assay.

5.2.3 Förster Resonance Energy Transfer (FRET) Assay

Agrobacterium contain either donor clones or acceptor clones were injected next to each other and slightly touching, allowing FRET samples and donor only control as well as acceptor only control to be observed in one specimen under the confocal microscope. Co-expression of the two constructs and subcellular localisation of the two proteins were confirmed by sequential scanning under Leica SP5 confocal microscope. A positive control, SAG101-GFP, and EDS1-RFP (Zhu et al. 2011) were selected to evaluate the FRET result. A random selection of donor (SAG101-GFP) and acceptor (Y2H320-RFP) was used as negative control.

FRET assay was conducted according to the manufacturer's instruction (FRET Sensitized Emission method, Leica TCS SP5). Measurements were taken by detection of the fluorescent signals of the donor and FRET as well as the acceptor in a line by line sequential scan acquisition. Beam path settings was: Donor = GFP excitation 488nm (30% laser), Emission 500-525nm; Acceptor = RFP excitation 561 nm (30% laser), Emission 570-630 nm. After analysis of the donor, FRET, and acceptor the fluorescence intensity was adjusted, and the PMT gain, zoom factor etc. were modified to ensure signal in all channels was not saturated. After setting the experimental conditions, the donor only control and acceptor only control were defined for calibration. FRET samples were then taken within one specimen. When the specimen was changed the calibration routine etc. was repeated and started over as a new experiment. Throughout the entire experiment and calibration routine, all measuring parameters such as gain,

emission detection window, excitation intensities, zoom, scan speed, format, pinhole etc. remained constant.

FRET efficiency was automatically calculated using the application wizard (FRET Sensitized Emission method, Leica), according to the manufacturer's instruction. In brief, calibration factors β , α , γ , δ were generated using Donor only reference ($\beta = \frac{FRET\ Channel}{Donor\ Channel}$) and Acceptor only reference ($\alpha = \frac{Donor\ Channel}{Acceptor\ Channel}$, $\gamma = \frac{FRET\ Channel}{Acceptor\ Channel}$, $\delta = \frac{Donor\ Channel}{FRET\ Channel}$) to correct the donor/acceptor cross-talk. A, B, C correspond to the intensities of the 3 signals (donor, FRET, acceptor). FRET efficiency = $\frac{B-A\times\beta-C\times(\gamma-\alpha\times\beta)}{C\times(1-\beta\times\delta)}$. (van Rheenen, Langeslag, & Jalink, 2004; Confocal application letter, Leica, 2006)

5.2.4 Tissue fixation and embedding

Whole inflorescences were fixed in fresh fixative (4% paraformaldehyde, 1XPBS pH 7.2, 0.1% Triton X-100, 0.1% Tween 20.) for 30 mins with vacuum infiltration followed by overnight fixation in the cold room under gentle rotation. Fixed tissues were washed with PBS twice, 30min each time, in the fume hood at room temperature before dehydration using an ethanol series (30%, 50%, 70%, 90% (v/v) 1 hr each and 100% two times at least 1 hr each).

The resin was prepared according to the manufacturer Technovit® 7100 (Kulzer Histo-Technik, Germany). Tissue was pre-infiltrated using a mixture of ethanol/resin (solution A) as following ratios: 2:1, 1:1 and 1:2 for one hour each solution, then infiltrate using 100% resin (solution A) overnight in the fridge. Embedding was prepared by mixing 375µl of resin solution A and 25 µl Hardener II for each inflorescence sample. The solution was aliquoted into a single gelatine capsule, the inflorescence tissue was transferred into the capsule

as far down the tube (flower pointing downwards and as straight as possible). When it has solidified the capsule lid was added and left to fully harden on the bench overnight.

The capsule was subsequently melted in warm water and the resin secured (inflorescence pointing up) using a little superglue to the top of TAAB Technovit Histobloc (T395) moulds. A small amount of TAAB Cold Curing Resin liquid was mixed and poured into the moulds to further secure the samples and left to harden overnight in the fume hood.

5.2.5 Tissue Sectioning

Before section, the mould body was secured in the microtome's vice and the holding block was orientated so the inflorescence sample was facing straight in front. When the blade was placed into the vice and tightened, the angle of the blade was set to 20° and the cutting width to 10µm. Samples were sectioned either manually or using the automatic drive. Sections were collected from the blade and flicked into a beaker of water to flatten before fishing out onto a coverslip. The coverslip was labelled on the reverse and left side up to dry at 25 °C on a warm block. The samples were flooded gently with 0.02% (w/v) Toluidine blue solution for 20 seconds, rinsed gently with water, dried and preserved with DPX mounting medium (Thermo Fisher Scientific, UK) on a microscope slide and left overnight in the fume hood to set. The slide was ready to observe under the light microscope.

5.3 RESULTS

5.3.1 Investigate MYB26 interacting proteins by FRET Analysis

Agrobacterium GV3101 containing donor or acceptor constructs were injected into 4-week-old *Nicotiana benthamiana* leaves together with clones expressing P19 (suppressor of gene silencing protein). FRET experiment was designed using the green fluorescent protein (GFP) as donor FP and the red fluorescent protein (RFP) as acceptor FP. MYB26-GFP was tested in FRET assays with Y2H320-RFP and Y2H560-RFP respectively. Because the crosstalk between fluorescent proteins is an important issue for the FRET sensitized emission method, controls and correction calculations are crucial for result evaluation. SAG101 and EDS1 are common regulators of plant immunity against many pathogens. SAG101 interacts with EDS1 and form a ternary complex with PAD4 *in planta* (Zhu et al. 2011). In this thesis, SAG101-GFP and EDS1-RFP protein pair was selected as FRET positive control. A random selection of donor (SAG101-GFP) and acceptor (Y2H320-RFP) was used as negative control (Table 5.1).

Table 5.1 FRET Assay Experiment Design

Protein pair (AGI)	Donor	Acceptor	Note
MYB26 (AT3G13890) Y2H320 (AT1G08320)	pUBC-MYB26-GFP	pUBN-Y2H320-RFP	
MYB26 (AT3G13890) Y2H560 (AT5G25560)	pUBC-MYB26-GFP	pUBC-Y2H560-RFP	
SAG101 (AT5G14930) EDS1 (AT3G48090)	pUBC-SAG101-GFP	pUBC-EDS1-RFP	Positive Control
SAG101 (AT5G14930) Y2H320 (AT1G08320)	pUBC-SAG101-GFP	pUBN-Y2H320-RFP	Negative Control

Donor MYB26-GFP, SAG101-GFP and acceptor Y2H320-RFP were nucleus localised (Figure 5.2 A, Figure 5.4 A), acceptor Y2H560-RFP were located in the nucleus and cell membrane, but higher RFP signal was observed in the nucleus (Figure 5.3). Acceptor EDS1-RFP showed preferential localisation on the membrane only, with a very low incidence of both nucleus and membrane location observed. However when co-expressed with SAG101-GFP, EDS1-RFP only located in the nucleus (Figure 5.4 A).

Y2H320 (TGA9, AT1G08320) is a member of the bZIP transcription factor family. Transient expression in *Nicotiana benthamiana* indicated that Y2H320 co-localised with MYB26 in the nucleus (Figure 5.2 A). In one typical FRET experiment (Figure 5.2 B ii), after excitation of the donor GFP, an emission wave of GFP was detected by the acceptor RFP due to protein-protein interaction (distance <10 μ m), resulting in a strong fluorescence detected in the acceptor RFP's emission channel. The energy transfer value, after corrections of excitation and emission cross-talk, was calculated by the FRET sensitized emission wizard (Leica SP5). FRET between MYB26-GFP and Y2H320-RFP was measured in 10 biological replicates, FRET efficiency was calculated using the application wizard (FRET Sensitized Emission method, Leica), according to the manufacturer's instruction (Methods 5.2.3), giving an average FRET efficiency around 0.357 (Figure 5.2 C).

Y2H560 (At5g25560) is a member of CHY-type/CTCHY-type/RING-type Zinc finger protein, which encodes a protein containing both a CHY zinc finger and a C3H2C3-type RING domain. Transient expression in *Nicotiana benthamiana* indicated that Y2H560 co-localised with MYB26 in the nucleus (Figure 5.3 A). FRET between MYB26-GFP and Y2H560-RFP was measured from 10

biological replicates, giving an average FRET efficiency of around 0.261 (Figure 5.3 C).

When transiently expressed in tobacco, SAG101 and EDS1 co-localised in the nucleus (Figure 5.4 A 1), EDS1 located both in membrane and nucleus, but preferentially located in the nucleus with SAG101 (Figure 5.4 A 2). FRET efficiency between SAG101-GFP and EDS1-RFP was measured from 12 biological replicates, giving an average of 0.293 FRET efficiency (Figure 5.4 C), indicated a physical interaction between SAG101 and EDS1. For the negative control, average FRET efficiency between SAG101-GFP and Y2H320-RFP is 0.034, measured from 10 biological replicates (Figure 5.5 C). A summary of FRET experiment (Figure 5.6) indicated that, compared to positive and negative controls, Y2H320 and Y2H560 physically interacted with MYB26 in plant cells.

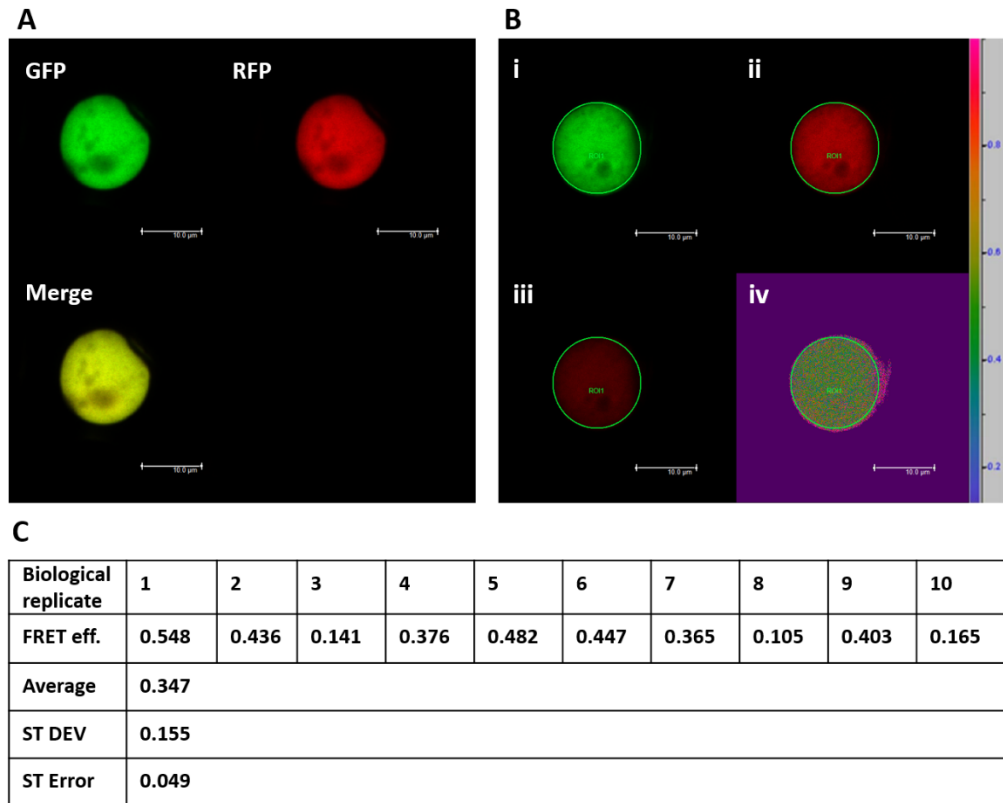


Figure 5.2 FRET between MYB26 and Y2H320

- A. Co-localisation of MYB26-GFP and Y2H320-RFP in the *Nicotiana benthamiana* nucleus. Scale bar 10 µm.
- B. One example of FRET between MYB26-GFP and Y2H320-RFP.
 - i. Donor (GFP) emission under donor excitation;
 - ii. Acceptor (RFP) emission under donor (GFP) excitation;
 - iii. Acceptor (RFP) emission under acceptor excitation;
 - iv. FRET efficiency of the region of interest (ROI1), calculated by Leica SP5 FRET SE (sensitized emission) wizard. Heat map indicates FRET efficiency. Scale bar 10 µm.
- C. A table summary of the FRET efficiency from 10 biological replicates. ST DEV, standard deviation. ST Error, standard error.

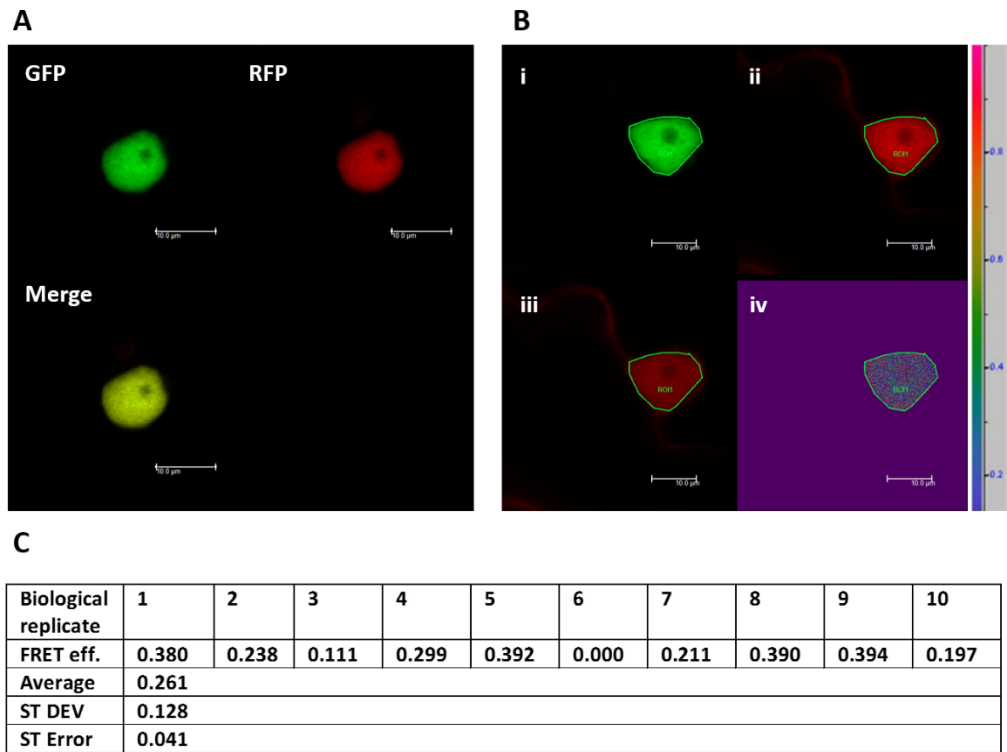


Figure 5.3 FRET between MYB26 and Y2H560

- A. Co-localisation of MYB26-GFP and Y2H560-RFP in the *Nicotiana benthamiana* nucleus. Scale bar 10 µm.
- B. One example of FRET between MYB26-GFP and Y2H560-RFP.
- Donor (GFP) emission under donor excitation;
 - Acceptor (RFP) emission under donor (GFP) excitation;
 - Acceptor (RFP) emission under acceptor excitation;
 - FRET efficiency of the region of interest (ROI1), calculated by Leica SP5 FRET SE (sensitized emission) wizard. Heat map indicates FRET efficiency. Scale bar 10 µm.
- C. A table summary of the FRET efficiency from 10 biological replicates. ST DEV, standard deviation. ST Error, standard error.

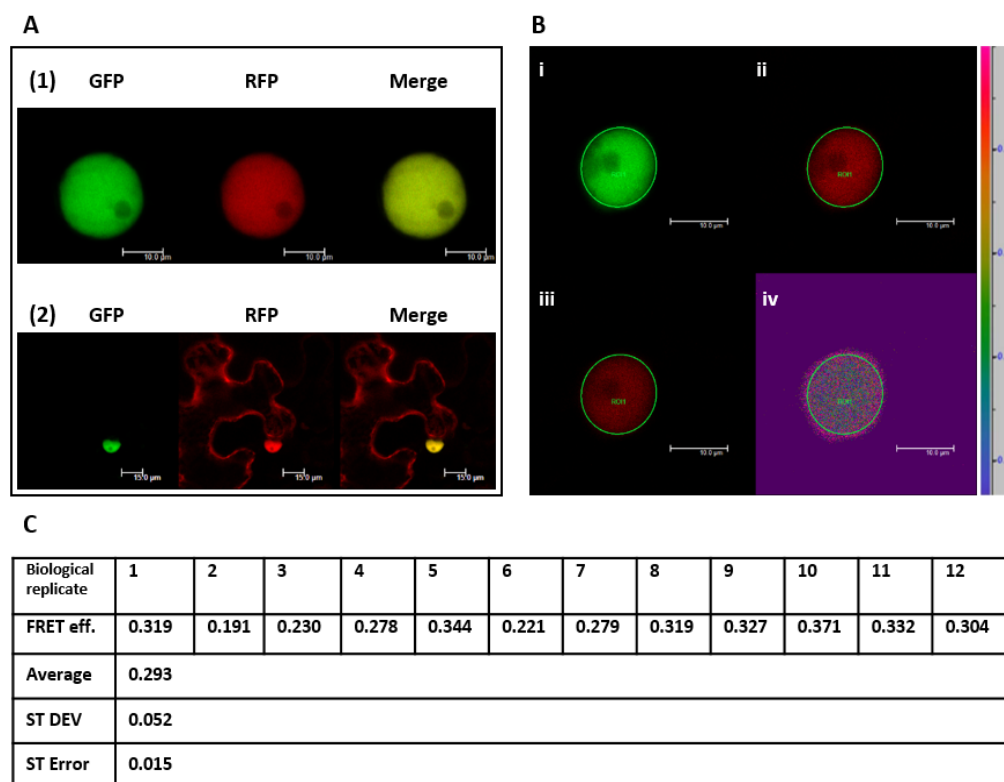


Figure 5.4 FRET between positive control SAG101 and EDS1

SAG101 interacts with EDS1 and PAD4 to form a protein complex which is preferentially nucleus localised and regulates plant immunity against many pathogens (Zhu et al. 2011)

- A. Co-localisation of SAG101-GFP and EDS1-RFP in *Nicotiana benthamiana*.
- SAG101 and EDS1 co-localised in nucleus. Scale bar 10 µm.
 - Different location of EDS1. With absence of SAG101, EDS1 preferred locating on membrane. While in the neighbour cell, EDS1 localised in nucleus with SAG101. Scale bar 15 µm.
- B. One example of FRET between SAG101-GFP and EDS1-RFP.
- Donor (GFP) emission under donor excitation;
 - Acceptor (RFP) emission under donor (GFP) excitation;
 - Acceptor (RFP) emission under acceptor excitation;
 - FRET efficiency of region of interest (ROI1), calculated by Leica SP5 FRET SE (sensitized emission) wizard. Heat map indicates FRET efficiency. Scale bar 10 µm.
- C. Table summary of FRET efficiency from 12 biological replicates. ST DEV, standard deviation. ST Error, standard error.

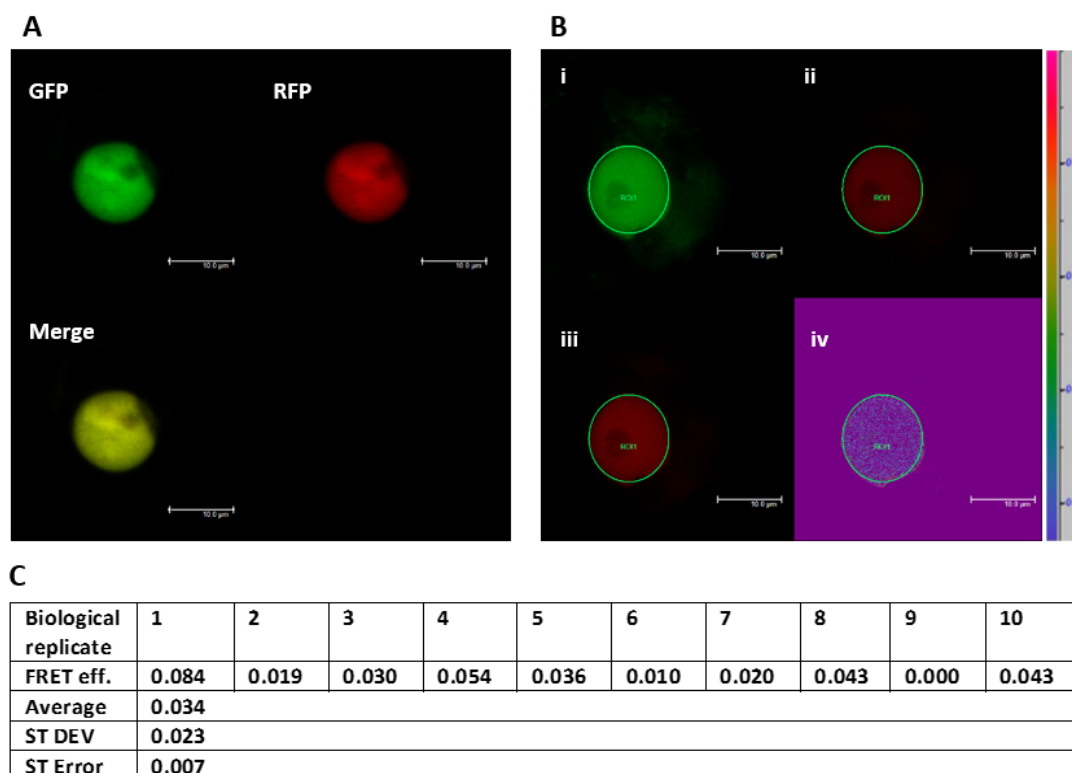


Figure 5.5 FRET between the negative control SAG101-GFP and Y2H320-RFP.

- A. Co-localisation of SAG101-GFP and Y2H320-RFP in the *Nicotiana benthamiana* nucleus. Scale bar 10 µm
- B. One example of FRET between SAG101-GFP and Y2H320-RFP.
 - i. Donor (GFP) emission under donor excitation;
 - ii. Acceptor (RFP) emission under donor (GFP) excitation;
 - iii. Acceptor (RFP) emission under acceptor excitation;
 - iv. FRET efficiency of the region of interest (ROI1), calculated by Leica SP5 FRET SE (sensitized emission) wizard. Heat map indicates FRET efficiency. Scale bar 10 µm.
- C. A table summary of FRET efficiency from 10 biological replicates. ST DEV, standard deviation. ST Error, standard error.

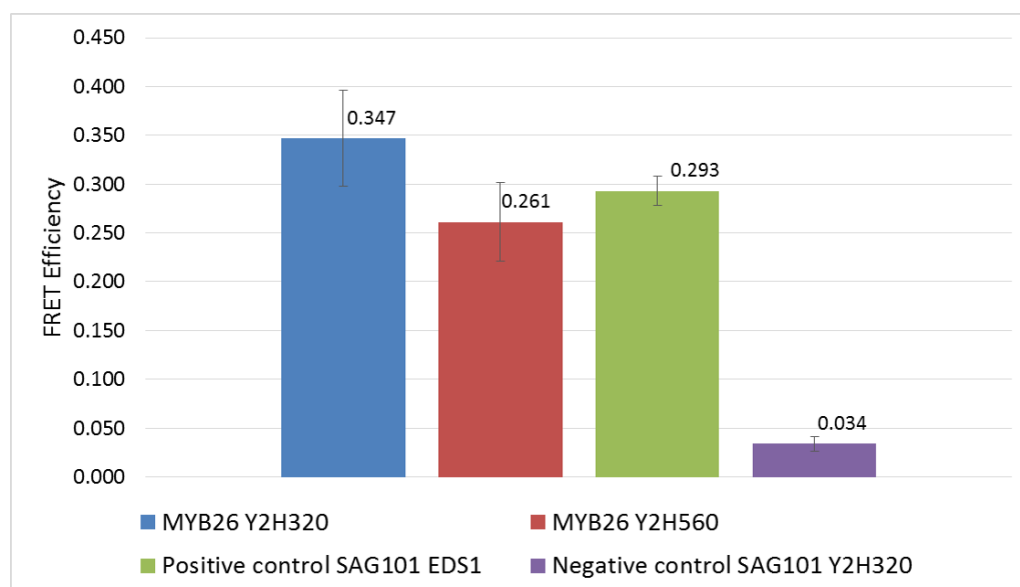


Figure 5.6 FRET Efficiency summary

FRET assay was conducted between MYB26 and putative interacting proteins Y2H320 and Y2H560 respectively. SAG101-GFP and EDS1 (At3g8090)-RFP were reported to form a protein complex (Zhu et al. 2011) and were selected as a positive control. A random selection of donor and acceptor can be used as a negative control. In this experiment SAG101-GFP and Y2H320-RFP were used as negative control. The average FRET efficiency was calculated with at least 10 biological replicates from each pair of proteins. Error bar = standard error.

5.3.2 Expression profile of MYB26 putative interacting proteins

- *Expression pattern of TGA9 and TGA10*

The expression pattern of *TGA9* and *TGA10* was investigated by quantitative RT-PCR using cDNA isolated from dissected anthers grouped into five anther developmental stages. Anthers of wild-type *Ler.* were dissected using a stereomicroscope from mature bud to the central younger bud in the whole inflorescence. Freshly dissected anthers were stained with 3 mM 4',6' diamino-2-phenylindole (DAPI) solution, and incubated for several minutes before observation using a Leica fluorescent microscope. According to the DAPI staining (Figure 5.7 C), anthers were collected from young to mature into 5 groups: before pollen mitosis I (PMI), PMI, Bicellular (Bi), PMII, and Tricellular (Tri) stage.

The expression of *MYB26* peaked at PMI stage (Figure 5.7 A), consistent with the reported *MYB26* expression stage (Yang et al. 2007). *TGA9* and *TGA10* were expressed throughout early anther primordia but resolved to the middle and tapetum layers during meiosis of pollen mother cells (Murmu et al. 2010, in-situ hybridisation data). *TGA10* transcript level in the qRT-PCR result did not appear to agree with the published in-situ hybridisation data. While higher transcript level of *TGA9* was observed in early anther stages (before PMI), consistent with Murmu et al's report, however *TGA9* transcript level was lower at PMI stage; it

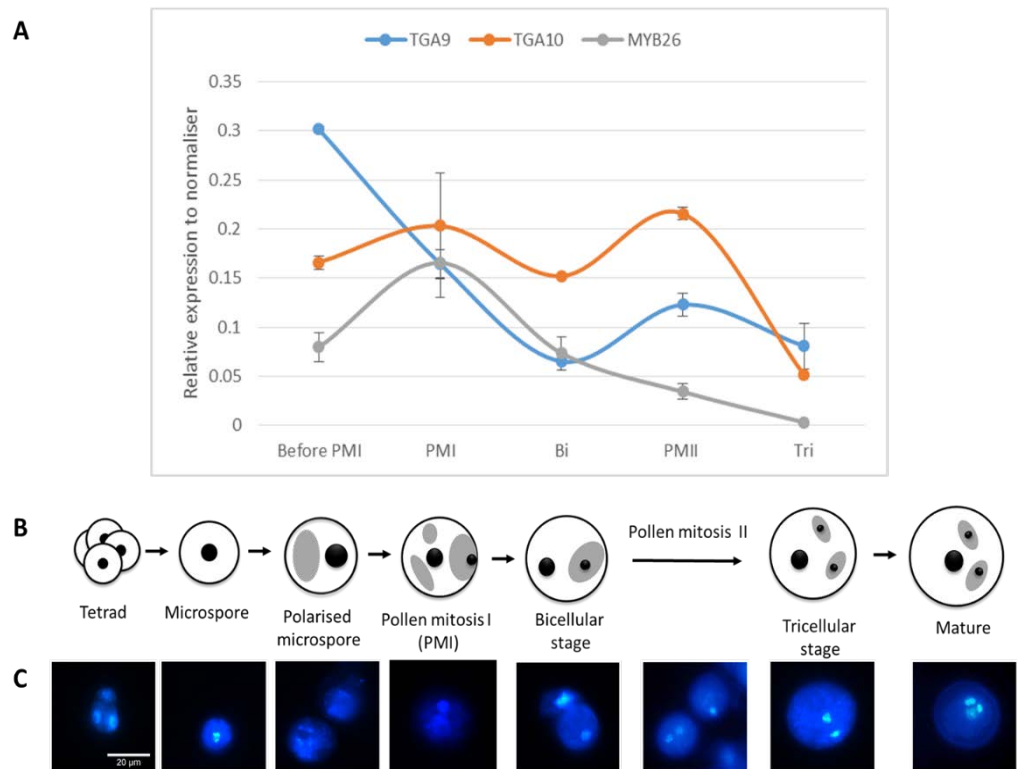


Figure 5.7 Expression profile of *MYB26*, *TGA9* and *TGA10* in dissect staged anthers of wild-type.

- Expression of *TGA9*, *TGA10* and *MYB26* in dissected anthers of five developmental stages from young to mature. Before PMI, before pollen mitosis I; PMI, pollen mitosis I; Bi, bi-cellular stage, PMII, pollen mitosis II; Tri, tri-cellular stage. Data normalised to reference gene *Actin7*.
- Diagram of pollen development from tetrad stage to mature pollen
- DAPI staining of pollen at equivalent stages in figure B, scale bar 20 μ m

therefore seems possible that there is an overlap of the expression stage between *MYB26* and *TGA9* as well as *TGA10*.

In order to study whether the expression of *TGA9* and *TGA10* was affected by altered expression of *MYB26*, the *ms35* mutant microarray dataset was analysed (Figure 5.8 A) and QRT-PCR conducted with *MYB26* overexpression tissues (Figure 5.8.B). In wild-type, *MYB26* was expressed highly at pollen mitosis I (PMI) and bi-cellular (BC) stage; it was significantly down-regulated in *ms35*

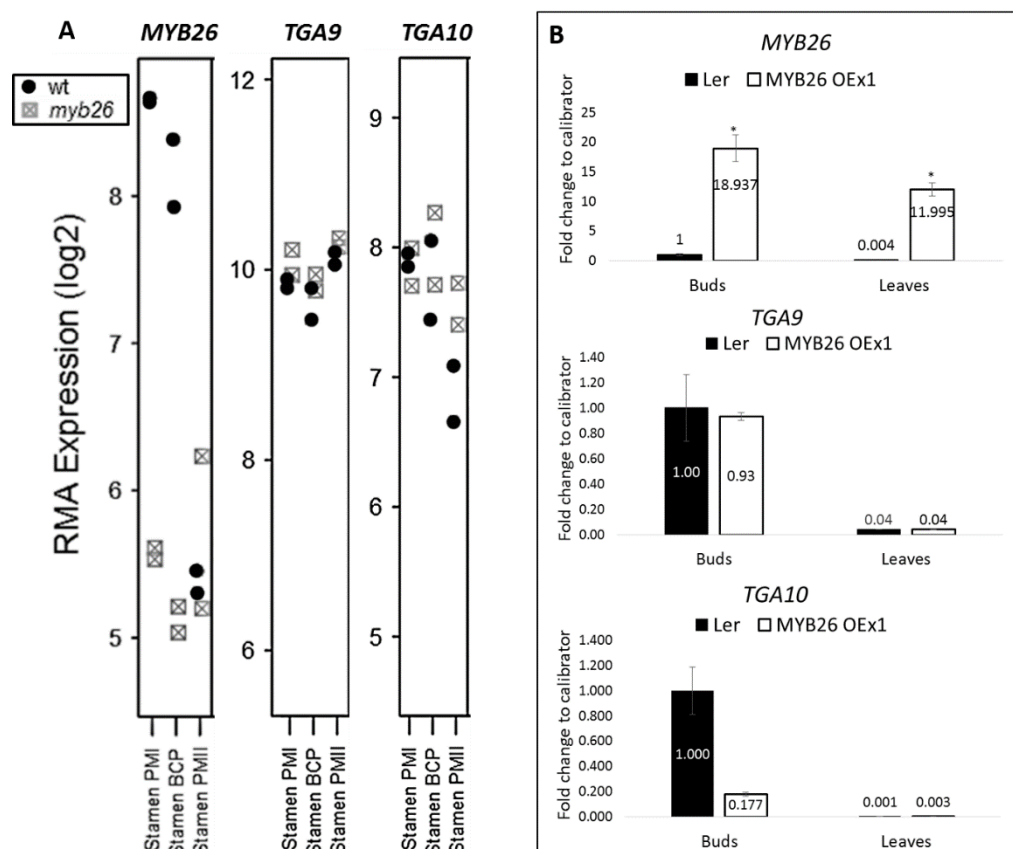


Figure 5.8 *TGA9* and *TGA10* expression in the *ms35* mutant and the *MYB26* overexpression line.

- The expression of *MYB26*, *TGA9* and *TGA10* in anthers at three developmental stages in the wild-type (wt) and the *ms35/myb26* mutant (Song, 2009). PMI, pollen mitosis I; BCP, bi-cellular pollen stage; PMII, pollen mitosis II. The data was re-normalised using RMA normalisation. Each dot represents a single replicate. The expression plots is available from the FlowerNet (Pearce et al. 2015).
- The expression of *MYB26*, *TGA9* and *TGA10* in the *MYB26* over-expression (OEx) line 1 and the wild-type *Ler*. Data was normalised to reference gene *Actin7*, the expression level in the wild-type *Ler* buds was set as 1.

mutant at the relevant stages. The expression of *TGA9* remained constant in *ms35* mutant during three anther developmental stages (PMI, BC, PMII); no significant alteration of *TGA9* expression was observed in the *MYB26* overexpression line. The expression of *TGA10* at PMII stage appeared slightly up-regulated in the *ms35* mutant and down-regulated in *MYB26* overexpression lines, although the difference was not statistically significant. These results indicated that the expression of *TGA9* is not affected by *MYB26*, but *MYB26* may have a negative effect on *TGA10*, more biological replicates, and further experiments are needed to confirm this hypothesis.

- *Expression pattern of Y2H560 and CHYR1*

Alignments show that the amino acid sequence of Y2H560 and CHYR1 share a significant similarity, may indicate a close functional, structural, evolutionary relationships (Figure 5.9 A). The expression level of *Y2H560* and *CHYR1* is higher than *MYB26* when normalised to reference gene *Actin 7*. *Y2H560* seems stably expressed through all pollen developmental stages, while the expression of *CHYR1* peaked during pollen mitosis stage, and overlapped with *MYB26* at PMI (Figure 5.9 B). In order to study whether the expression of *Y2H560* and *CHYR1* was affected by altered expression of *MYB26*, the *myb26* mutant microarray data was analysed (Figure 5.10 A) and qRT-PCR was conducted using *MYB26* overexpression lines (Figure 5.10.B). There was no significant change of *CHYR1*'s expression in both *myb26* mutant and *MYB26* overexpression line. *Y2H560* seems slightly down-regulated in the bud and leaf tissues in *MYB26* overexpression line, but this alteration was not statistically significant.

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Y2H560 1 MGDVYFNHFAQQLQMSDQEKGEISRHSHPHSINEESESSTLERVAAESLTNKLDRGLME
CHYR1 1 -----MD-----MGFHENEQNQEFANLMEICSGH

Y2H560 61 YGCPHYRRRCIRAPCCNEIFGCHHCHYEAKNNINVDQKORHDI PRHVEQVICLLCGTE
CHYR1 25 YGCSHYRRRCIRAPCCDEIFDCRHCHNEAKDSLHLEQHRHELPRHEVSKVICSLCETE

Y2H560 121 QEVGCHCIHCGVCMGKYFCVKVCLYDDDSKKQYHCDGCGICRIGGRENFFHCYKCGCCY
CHYR1 85 QDVQCNCSNCGVCMGKYFCSKCFEDDELSSKKQYHCDGCGICRTGGRENFFHCRCRCY

Y2H560 181 SILLKNGHPCVEGAMHHDCPTCFERLFESRNDVTVPFGHTTHQKLEEMRDHYQYACPL
CHYR1 145 SKIMEDKHQCVGAMHHNCPVCFEYLFDSFRDITVLRGHTMHLECKDMGLHNRYTCPV

Y2H560 241 CSKSVCDMSKIVWEKFDMEIAATPMEEFYCNRMVNSLQRLWEESRGTVFCGGSEMPKLOLI
CHYR1 205 CSKSLCDMSNLWKKLDBEVAAMPKMYENKMVWILCND-----CGSNTNVRFHLLI

Y2H560 301 QHSPNQRLKFLK-----LICYQMQLRAFHLLQ---
CHYR1 256 AHKSSCGSYNTRQTQRGSDSHSCSSG---MPQVVGSTG

```

B

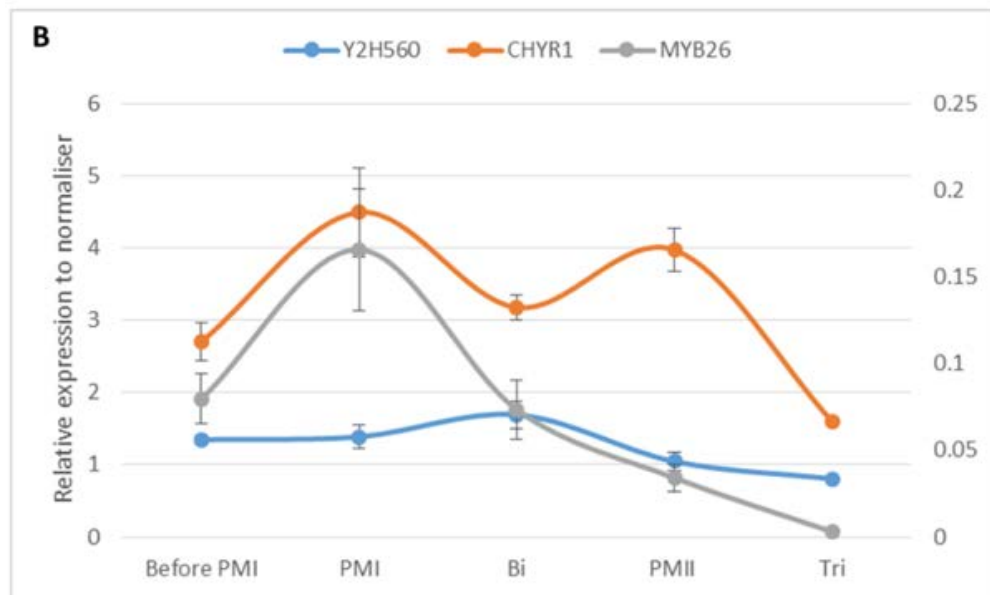


Figure 5.9 Expression pattern of *Y2H560* and *CHYR1*.

- Alignment of *Y2H560* and *CHYR1*. Amino acid sequences were aligned using Clustal Omega and presented in BoxShade format.
- Expression of *Y2H560*, *CHYR1* and *MYB26* in dissect anthers at five developmental stages from young to mature. The expression level of *MYB26* was indicated in secondary axis (right axis), *Y2H560* and *CHYR1* used primary axis (left axis). Before PMI, before pollen mitosis I; PMI, pollen mitosis I; Bi, bi-cellular stage, PMII, pollen mitosis II; Tri, tri-cellular stage. Data was normalised to reference gene *Actin7*. Details of Pollen developmental stages was shown in figure 5.6 (B) and (C).

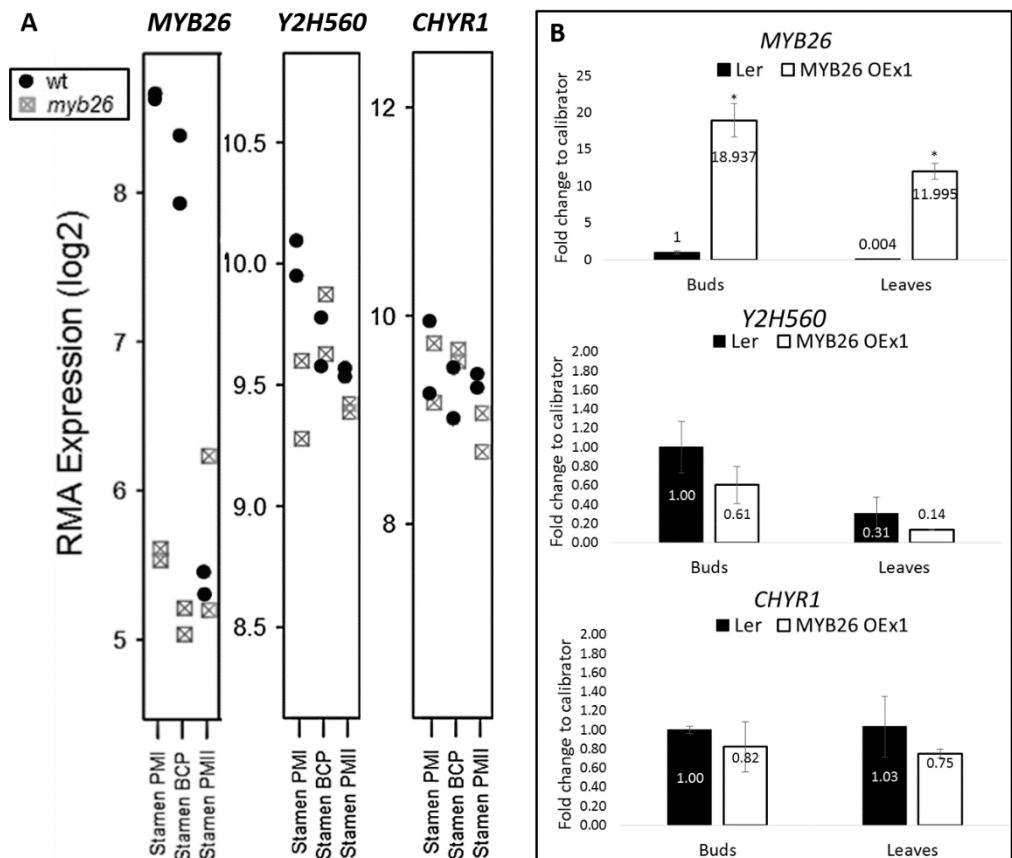


Figure 5.10 Expression of *Y2H560* and *CHYR1* in the *ms35* mutant and the *MYB26* OEx line 1

- A. The expression of *MYB26*, *Y2H560* and *CHYR1* in anthers of the wild-type (wt) and the *ms35/myb26* mutant at three developmental stages (Song, 2009). In the wild-type, *MYB26* expressed highly during pollen mitosis I (PMI) and bi-cellular (BC) stage and was significantly down-regulated in the *ms35/myb26* mutant at equivalent stages. The data was re-normalised using RMA normalisation. Each dot represents a single replicate. The expression plots is available from the FlowerNet (Pearce et al. 2015).
- B. The expression of *MYB26*, *Y2H560* and *CHYR1* in the *MYB26* over-expression (OEx) line 1 and the wild-type *Ler*. Data was normalised to reference gene *Actin7*, the expression level in wild-type *Ler* buds was set as 1.

5.3.3 Y2H320 (TGA9) is required for normal anther development.

- The *tga9tga10* double mutant displayed sterile phenotype.

Single T-DNA insertion mutants of *TGA9* (SALK_057609) and *TGA10* (SALK_124227) were obtained from Nottingham Arabidopsis Stock Centre (NASC). The genotyping result indicated that both lines were segregating (Figure 5.11 B and E), RNA of the homozygous line was extracted using the RNeasy mini kit (QIAGEN,UK), The results of RT-PCR indicated that *TGA9*

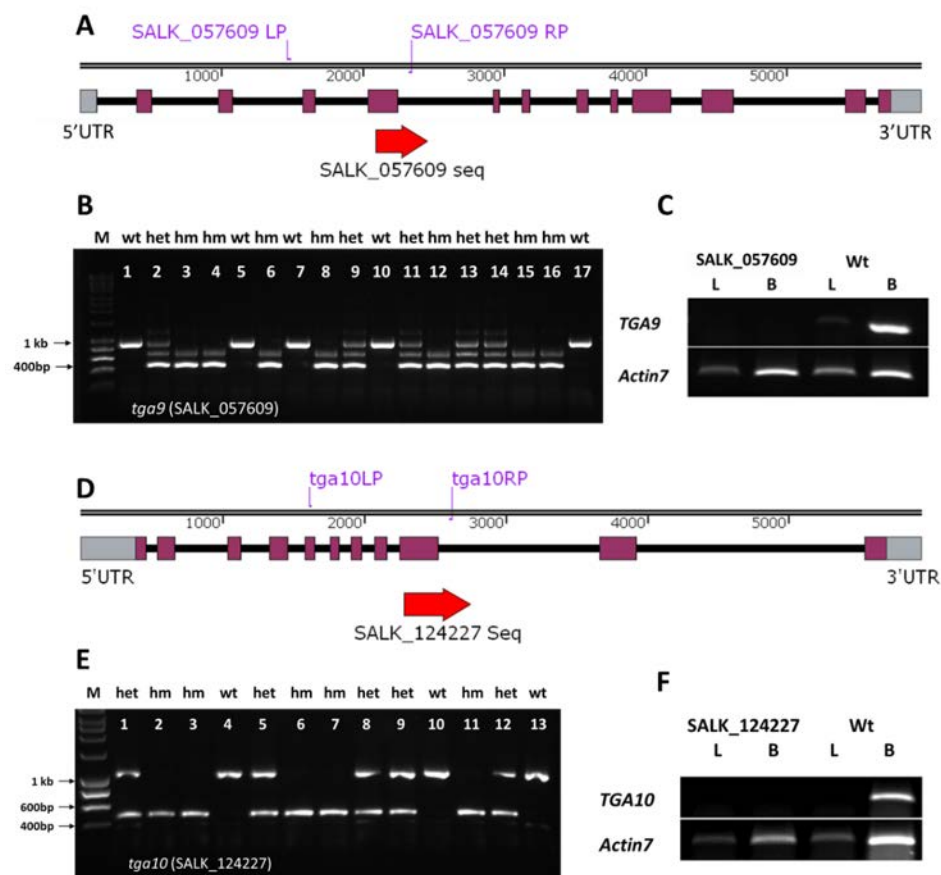


Figure 5.11 T-DNA insertion mutants of *TGA9* and *TGA10*

- Map of *TGA9*, showing flanking sequence of the T-DNA insertion mutant SALK_057609 (Red arrow, sequencing data is available from T-DNA express) and primers used for genotyping. Plum box, exon; solid black line, intron; grey box, untranslated region (UTR).
- Genotyping of SALK_057609, using primers indicated in figure A and LBb1. M, hyper ladder I marker; Wt, wild-type *Col.*; het, heterozygous; hm, homozygous.
- Expression of *TGA9* in the SALK_057609 homozygous line and the wild-type (Wt). L, leaves; B, buds; reference gene *Actin7*.
- Map of gene *TGA10*.
- Genotyping example of SALK_124227.
- Expression of *TGA10* in the SALK_124227 homozygous line and the wild-type (Wt).

and *TGA10* were mainly expressed in bud tissue and were silenced in the T-DNA insertion mutant respectively (Figure 5.11 C and F)

The *tga9* or *tga10* single mutant did not show obvious phenotype alterations compared to wild-type. Thus, homozygous lines used for RNA extraction (Figure 5.11. C and F) were crossed; the *tga9tga10* double mutant was screened in the F₂ generation and further confirmed by RT-PCR. Interestingly, RT-PCR indicated that the expression of *TGA10* seems turned on in *tga9tga10* double mutant (Figure 5.12.A), qRT-PCR confirmed that *TGA10* was knock-down in the double mutant (Figure 5.12 B).

The *tga9tga10* double mutant displayed sterile phenotype (Figure 5.12 E). The silique length in *tga9tga10* was significantly reduced to 4mm compared to 14mm in wild-type *Col.* (Figure 5.12.C). Indehiscent anthers were observed in *tga9tag10* open flowers (Figure 5.12 G). In order to investigate pollen viability in the mutant, Alexander staining was conducted; anthers of the double mutant and wild-type were stained overnight. Some *tga9tga10* mutant pollen remained green indicating inviable pollen (Figure 5.12 H), while others were partially stained red (Figure 5.12 I). Pollen in wild-type *Col.* was stained dark red, indicating normal pollen viability (Figure 5.12 J). These results suggest that sterility in *tga9tga10* was due to reduced pollen viability and anther indehiscence, constant with Murmu et al (2010).

In order to investigate whether secondary thickening was affected in the *tga9tga10* double mutant, lignin staining with acridine orange/ethidium bromide was conducted in open flowers of wild-type (Figure 5.13 A-C) and the *tga9tga10* mutant (Figure 5.13 D-F). Acridine orange stains both live and dead cells, while

ethidium bromide stains lignified cells. In *tga9tga10*, closed stomium region indicated indehiscent anthers (Figure 5.13 D), secondary thickening was developed but seems not as intensive as in wild-type. The transverse sections from stage 10 to stage 12 highlighted the endothecium development in wild-type (Figure 5.13 G-I) and *tga9tga10* (Figure 5.13 J-L). In wild-type, endothecium

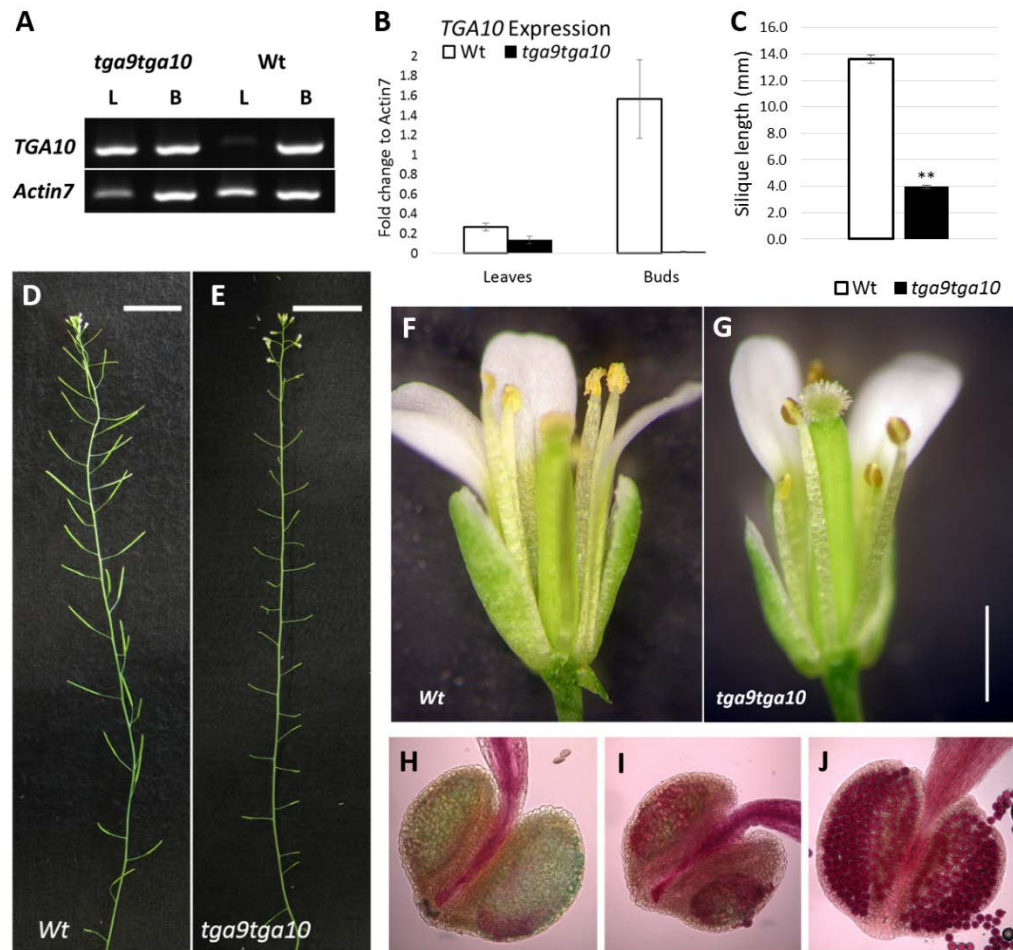


Figure 5.12 The *tga9tga10* mutant is sterile with indehiscent anthers

- RT-PCR of *TGA10* transcripts in the *tga9tga10* mutant and the wild-type. L, leaves; B, buds; reference gene *Actin7*.
- QRT-PCR of *TGA10* in the *tga9tga10* mutant. Data was normalised to the reference gene *Actin7*.
- Silique length of the *tga9tga10* mutant and the wild-type. Silique length was measured from the 4th to the 15th siliques from the main stem of three biological replicates. Asterisks indicate significant differences from the wild-type value (** $P < 0.01$).
- D-E Main stem of the wild-type *Col.* (D) and the *tga9tga10* mutant (E). Scale bar 3 cm.
- F-G. Dissected flower of the wild-type (F) and the *tga9tga10* mutant (G). Petals and sepals were partially removed. Scale bar 1 mm.
- H-J. Anther Alexander staining of the *tga9tga10* mutant (H-I) and the wild-type (J).

expanded from stage 10, secondary thickening deposited at stage 11. In *tga9tga10* the endothecium developed in a similar pattern as in wild-type, but it appeared less turgid than in the wild-type, indicating possible limited expansion in the *tga9tga10* mutant. Also, the tapetum in *tag9tga10* was abnormal with enlarged vacuoles at stage 10, when the tapetum degradation initiated in the wild-type, and remained swollen at stage 11.

Transverse sections were conducted with whole inflorescences of the *tga9tga10* mutant and the wild-type to investigate anther development at the cellular level

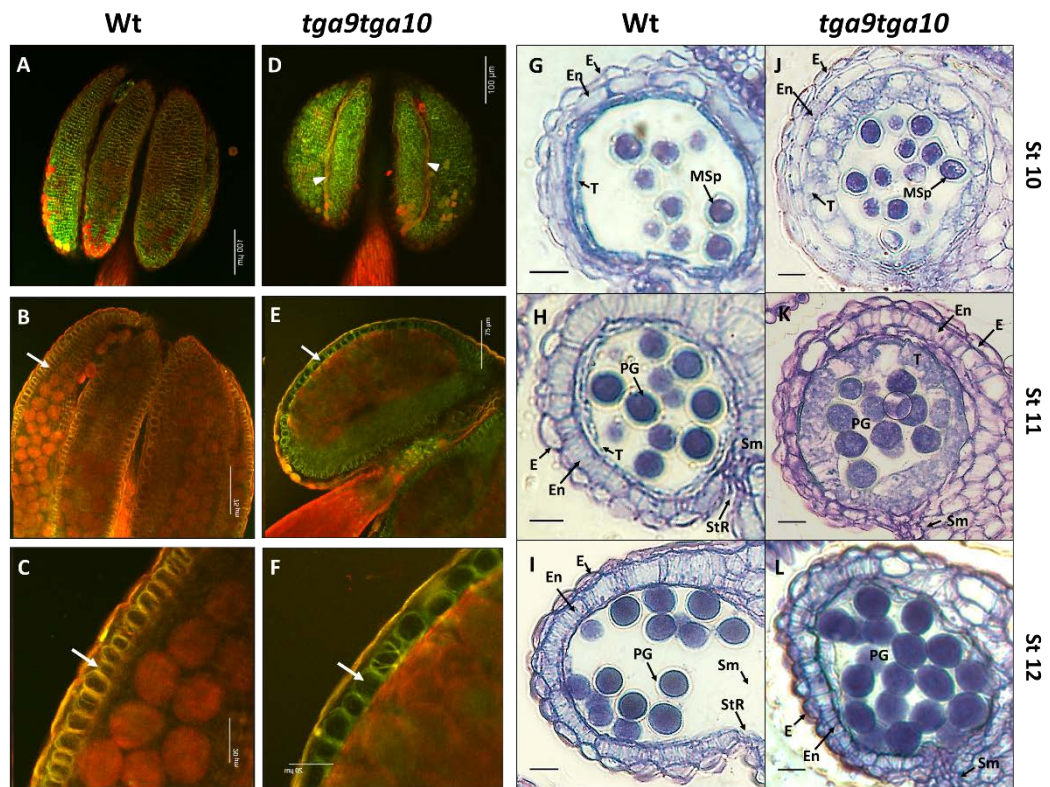


Figure 5.13 Secondary thickening development in *tga9tga10* mutant and wild-type

A-F. Lignin staining of wild-type (Wt, A-C) and *tga9tga10* (D-F) anthers from open flowers. Anthers were stained with acridine orange/ethidium bromide and visualized by confocal microscopy. Endothecium secondary thickening was developed (arrow). Stomium region seems closed in *tga9tga10* (arrowheads). Scale bar: A and D 100 μm, B and E 75 μm, C and F 20 μm.

G-L. Transverse sections of anthers from stage 10 to stage 12 in wild-type (Wt, G-I) and *tga9tga10*. (J-L) The sections were stained with toluidine blue and photographed by bright-field microscopy. E, epidermis; En, endothecium; MSp, microspore; PG, pollen grain; Sm, septum; StR, stomium region; T, tapetum. Scale bar 20 μm.

from the emergence of the stamen primordia to anther dehiscence (Figure 5.14, Figure 5.15). Anther stages were defined using Sanders' staging (Goldberg et al. 1993). Abnormal anther development was observed from anther stage 5 in the *tga9tga10* mutant. In wild-type, 1°parietal and 1°sporogenous cell differentiated into the endothecium, middle layer, tapetum and microspore mother cells in all four lobes. In the *tga9tga10* mutant, sporogenous cell differentiation was various and disorganised in the two adaxial lobes, most of them resulted in developmental arrest from stage 5, but some had differentiated tissues observed in either one or both of the adaxial lobes but the development was disorganised and uncoordinated with the abaxial lobes and was arrested in the later stage (Figure 5.14, Figure 5.15). The abaxial lobes developed in a similar pattern as in wild-type but the tapetum began to expand and had enlarged vacuoles from anther stage 6. At anther stage 10, tapetum degradation was initiated in the wild-type, but not in *tga9tga10*. Until anther stage 12, debris of tapetum could still be seen in the abaxial lobes in *tga9tga10*. Secondary thickening deposited in *tga9tga10* at anther stage 11, appeared to be the same as in the wild-type, however, the endothecium expansion did not appear as turgid as in the wild-type. In the wild-type at anther stage 12, the septum degenerated and the stomium region ruptured to facilitate pollen release in wild-type. However, both septum and stomium did not breakdown in the *tga9tga10* mutant, resulting in indehiscent anthers.

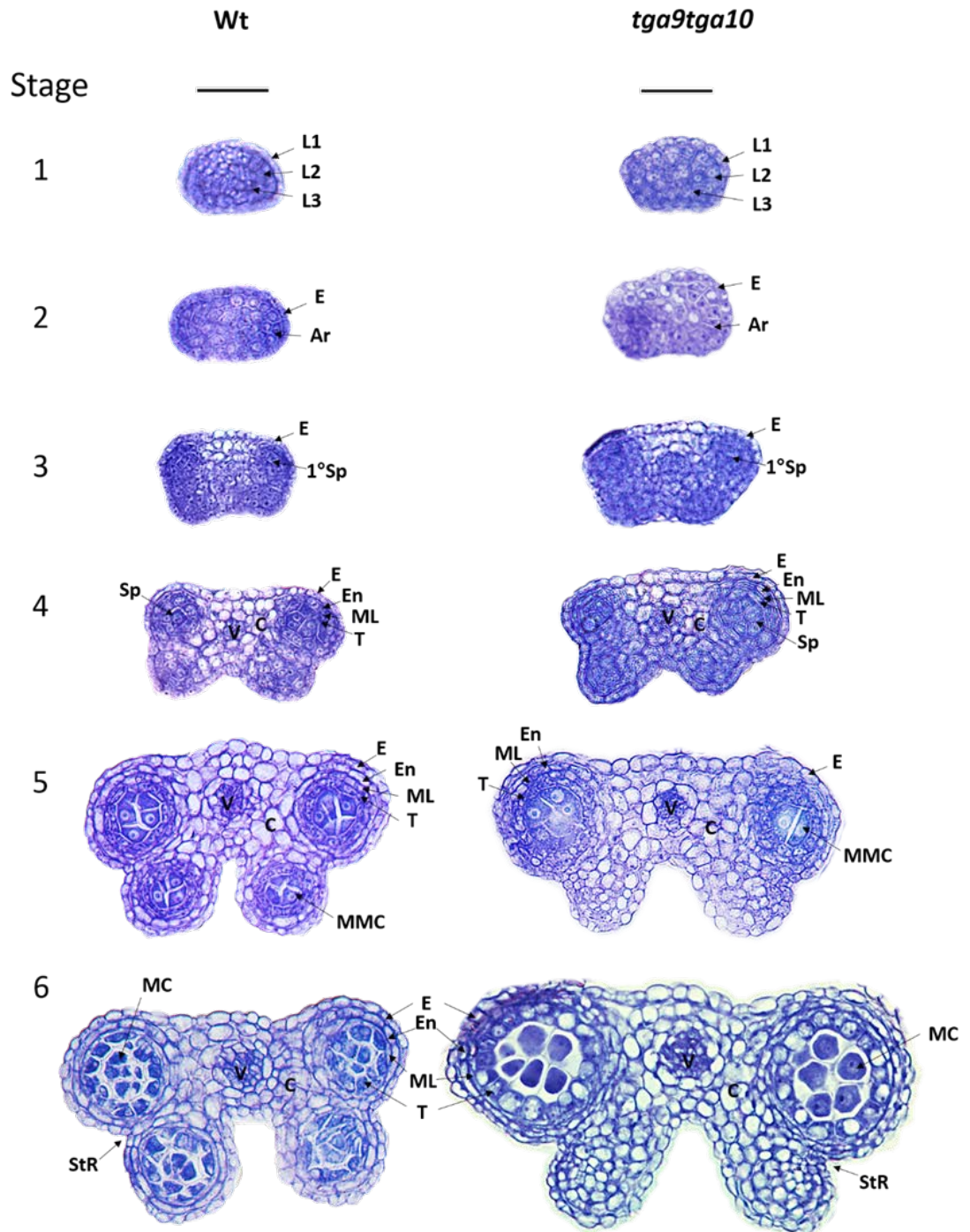


Figure 5.14 Anther development of the *tga9tga10* mutant and the wild-type. Transverse sections of anthers from stage 1 to 6.

Inflorescences were fixed and embedded in resin (Technovit 7100), transverse sectioned and stained with toluidine blue; anthers were photographed by bright-field microscopy. Sections were selected from at least two biological replicates from both the wild-type and the *tga9tga10* mutant. Ar, archesporial cell; C, connective; E, epidermis; En, endothecium; L1, L2, and L3, the three cell-layers in stamen primordia; MC, meiotic cell; ML, middle layer; MMC, microspore mother cells; 1°Sp, primary sporogenous layer; Sp, sporogenous cells; StR, stomium region; T, tapetum; V, vascular region. Scale bar 50 μ m

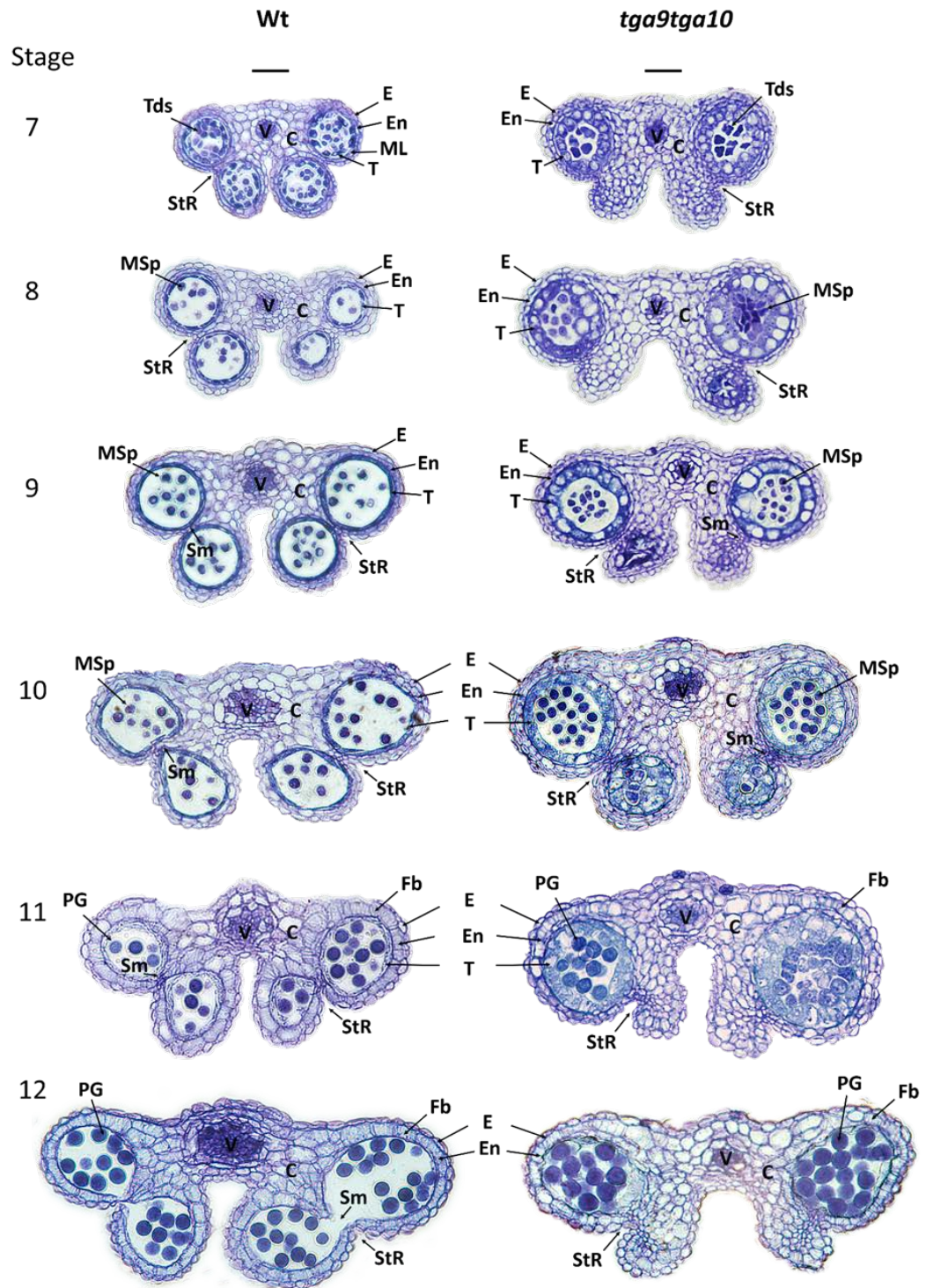


Figure 5.15 Anther development of the *tga9tga10* mutant and the wild-type. Transverse sections of anthers from stage 7 to stage 12.

Inflorescences were fixed and embedded in resin (Technovit 7100 embedding kit) and sliced as described in materials and method. The transverse sections were stained with toluidine blue and anthers were photographed by bright-field microscopy. Section pictures were edited from at least two biological replicates from both the wild-type and the *tga9tga10* mutant.; C, connective; E, epidermis; En, endothecium; Fb, fibrous bands; ML, middle layer; MSp, microspore; PG, pollen grains; Sm, septum; StR, stomium region; T, tapetum; Tds, tetrads; V, vascular region. Scale bar 50 μ m

- *Overexpression of Y2H320 (TGA9) may affect fertility*

Y2H320 (TGA9) overexpression lines were generated by transforming *Ler.* with the pUBN-Y2H320-RFP construct. More than 10 transgenic plants (T1 generation) survived Basta selection; these transgenics displayed either normal fertility or reduced silique length to various degrees. Four representatives of the transgenic plants are shown in Figure 5.16 A, representing silique length from short (OEx1, OEx3), medium (OEx2), and normal (OEx4). Silique length of OEx1, 2 and 4 was measured from the 4th to the 15th silique of the main stem of each individual line (Figure 5.16 B). Quantitative RT-PCR confirmed that the expression of *TGA9* in OEx1 and OEx2 was increased. Since tapetum development was abnormal in *tga9tga10* mutant, we investigated whether overexpression of *TGA9* would affect the transcript level of *MS1*, which is required for normal tapetum development. qRT-PCR indicated that the expression of *TGA10*, *MYB26*, and *MS1* was not significantly affected by over-expressed *TGA9* (Figure 5.16 C).

Dissected flowers revealed a possible delayed anther dehiscence phenotype in *TGA9* OEx 3, which displayed a reduced silique length. In wild-type buds with visible petals (just before opening), anthers were already dehiscent (Figure 5.16 F) and the pistil was covered with released pollen. In *TGA9* OEx3 buds just before opening, anthers dehiscence condition varied from indehiscent anthers supported by the filament, which hadn't been fully elongated (Figure 5.16 D) to partially dehiscent anthers (Figure 5.16 E). In fully open flowers, anthers from *TGA9* OEx3 were dehiscent to a similar level as in wild-type, but pollen release and pollination was reduced, as indicated by clean pistil in *TGA9* OEx3 (Figure 5.16 G and H).

Lignin staining was conducted to check the endothecium secondary thickening (ST) development in open flowers (Figure 5.16 J-L). In *MYB26* overexpression line (Figure 5.16 L), anther secondary thickening was increased and epidermal cells displayed ectopic lignification, however, no obvious alteration of ST was observed in *TGA9* OEx 3 using this method. Further study is required to investigate cellular level changes in stable *TGA9* overexpression lines.

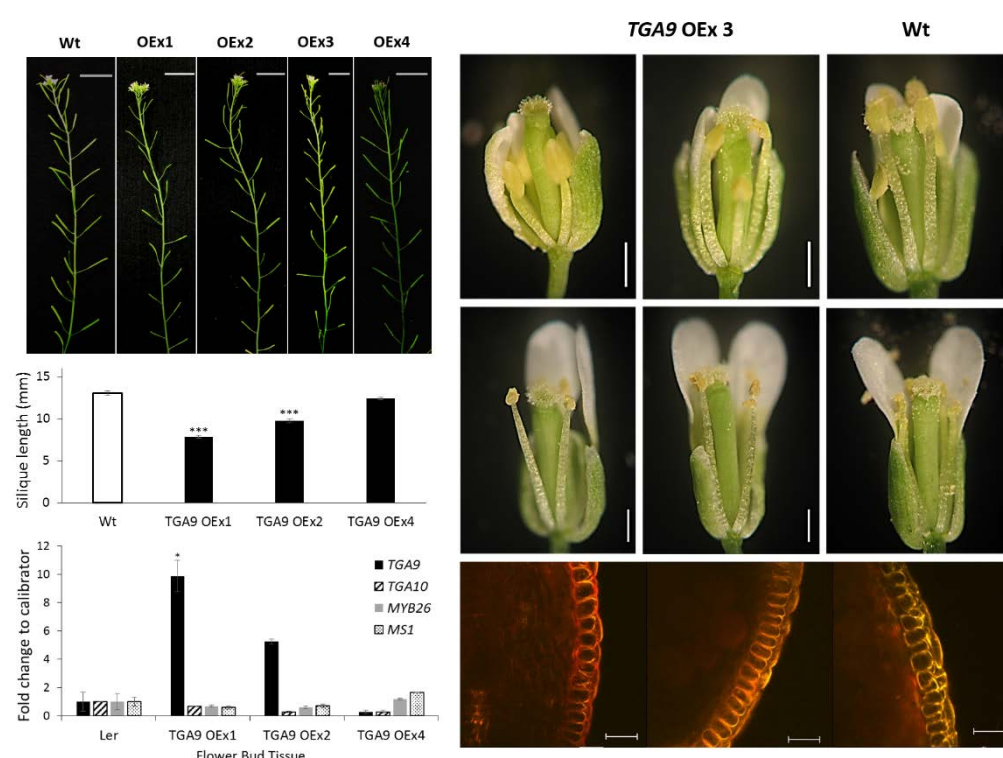


Figure 5.16 Overexpression (OEx) of *TGA9* may affect fertility

- A. Examples of *TGA9*-OEx transgenic plants showing reduced fertility. The silique length may correlate with the *TGA9* expression level. OEx1-4, pUBN-*TGA9*-RFP transformed lines (T1 generation) survived from Basta selection. Wt, wild-type. Scale bar, 2cm.
- B. Silique length of OEx1, 2 and 4 displayed severe to moderate reduction of fertility. Silique length was measured from the 4th-15th siliques from the main stem.
- C. QRT-PCR showed the expression of *TGA9*, *TGA10*, *MYB26* and *MS1* in bud tissue of the *TGA9* OEx1, 2, 4. Asterisks indicate significant differences from the wild-type value (* $P \leq 0.05$. *** $P \leq 0.001$).
- D-I. Dissected Flowers of the *TGA9* OEx3 (D, E, G, H) and the wild-type (F, I). Petal and sepal were partially removed. D-F from bud with visible petal. G-I were open flowers. Scale bar 0.5 cm
- J-L. Anther lignin staining of the wild-type (J), *TGA9* OEx 3 (K), *MYB26* overexpression line (ubq10::*MYB26*-GFP). Arrows indicate secondary thickening in endothecium layer. Arrow heads indicate ectopic secondary thickening in epidermal. Scale bar 20

5.3.4 Y2H560 may affect *Arabidopsis* fertility

Single T-DNA insertion mutant of *Y2H560* (GK-944F09) and *CHYR1* (SALK_045606C) were obtained from Nottingham Arabidopsis Stock Centre (NASC). The genotyping results indicated that GK-944F09 was segregating and SALK_045606C was homozygous (Figure 5.17 B and E). RT-PCR indicated that *Y2H560* and *CHYR1* were expressed in both buds and leaves tissue of wild type, and that expression was knocked out in the T-DNA insertion mutants respectively (Figure 5.17). However the single mutants did not show obvious

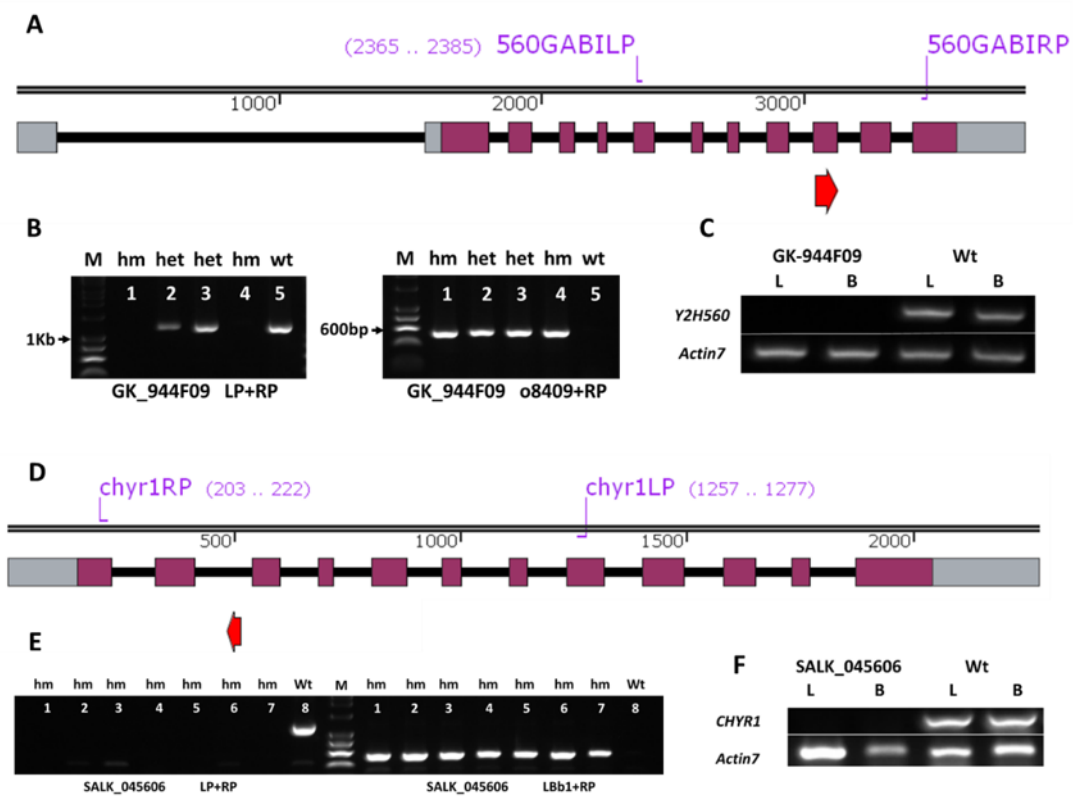


Figure 5.17 T-DNA insertion mutants of *Y2H560* and *CHYR1*

- Map of *Y2H560*, showing flanking sequence of the T-DNA insertion mutant GK-944F09 (Red arrow, sequencing data is available from T-DNA express) and primers used for genotyping. Plum box, exon; solid black line, intron; grey box, untranslated region (UTR).
- Genotyping of the GK-944F09, using primers indicated in figure A and o8409. M, hyper ladder I marker; Wt, wild-type *Col.*; het, heterozygous; hm, homozygous.
- Expression of *Y2H560* in the GK-944F09 homozygous line and the wild-type (Wt). L, leaves; B, buds; reference gene *Actin7*.
- Map of gene *CHYR1*.
- Genotyping of SALK_045606.
- Expression of *CHYR1* in the SALK_045606 homozygous line and the wild-type (Wt).

phenotype alterations as compared to wild-type. Thus functional study of *Y2H560* may require generating the *y2h560chyr1* double mutant or dominant repression of *Y2H560*.

Full-length CDS of *Y2H560* was cloned into the pUBC vector under the control of ubiquitin 10 promoter with a C-terminal red fluorescent protein (RFP) tag. This construct was transferred into wild-type *Ler*. Three transgenic plants (T1 generation) survived from Basta selection (Figure 5.18). Silique length was measured from the 4th to the 15th silique of the main stem of the individual lines.

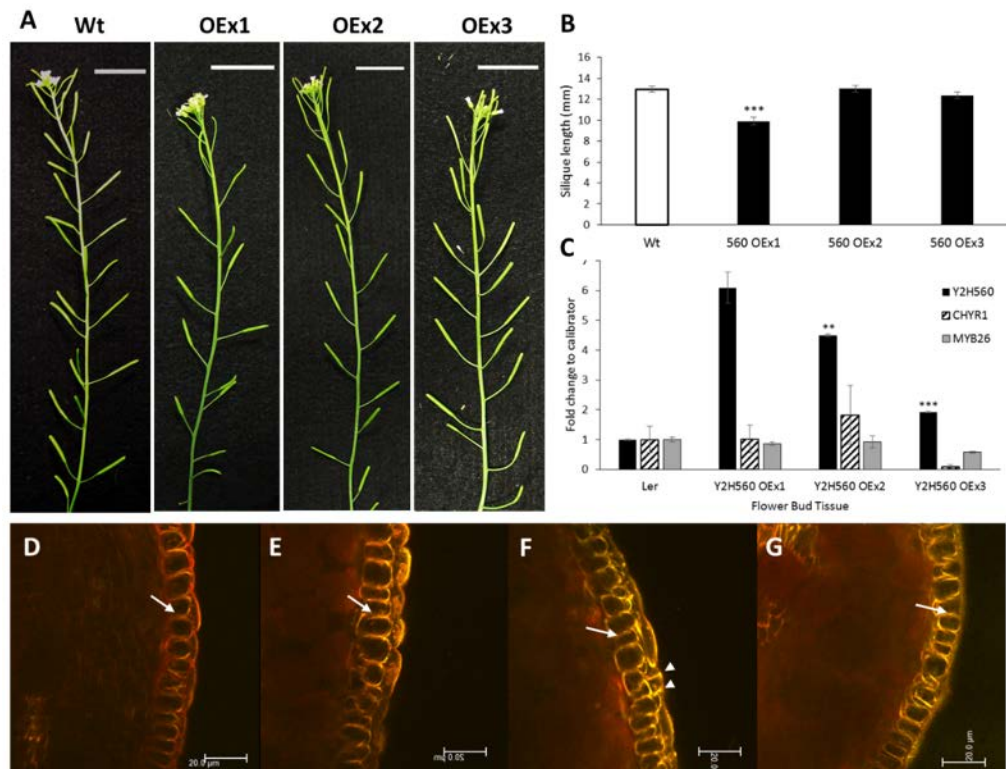


Figure 5.18 Phenotypic analysis of the *Y2H560* overexpression line

- Main stem of the *Y2H560* overexpression (OEx) lines. Three pUBC-*Y2H560*-RFP transformed lines are from T1 generation. Scale bar 2 cm.
- Silique length summary. Silique length was measured from the 4th to the 15th siliques from the main stem of *Y2H560* OEx1-3 (T1 generation).
- QRT-PCR showed expression changes of *Y2H560*, *CHYR1* and *MYB26* in the bud tissues of the *Y2H560* OEx lines.
Error bar, standard error. Asterisks indicate significant differences from the wild-type (** $P \leq 0.01$, *** $P \leq 0.001$)
- D-G. Anther lignin staining of the wild-type (D), *Y2H560* OEx1 (E), *MYB26* OEx lines ubq10::MYB26-GFP (F) and 35S::MYB26-GFP (G). Arrows indicate secondary thickening (ST) in anther endothecium layer. Arrow heads indicate ectopic ST in epidermal layer. Scale bar 20 μ m.

Y2H560 OEx1 seemed to display reduced silique length compared to wild-type (Figure 5.18). The QRT-PCR results confirmed that *Y2H560* was significantly up-regulated in all three transgenic plants, while the expression of *CHYR1* and *MYB26* did not significantly change (Figure 5.18 C). Lignin staining with acridine orange/ethidium bromide showed no convincing changes in *Y2H560* OEx1 (Figure 5.18 E). However, as compared to the wild-type, increased secondary thickening was displayed in the *MYB26* overexpression lines, *ubq10::MYB26-GFP* and *prom35S::MYB26-GFP* (Figure 5.18 F)

5.3.5 Affinity purification of MYB26 from *Arabidopsis*

Full-length *MYB26* CDS was cloned into the pUBC vector (ubiquitin 10 promoter) and pGWB5 promoter (CaMV 35S promoter) with a C-terminal GFP tag. This construct was transformed into wild-type *Ler*. Expression of *MYB26* was checked using qRT-PCR (Chapter 3, Figure 3.1 D and Figure 3.2 D) in transgenic plants.

GFP signal could be observed in anthers of both *ubq10::MYB26-GFP* and *35S::MYB26-GFP* transformed plants, however, no GFP signal was observed from leaf tissue, probably due to multiple-layer structure of leaf cell and high chlorophyll background. The leaves of *35S::MYB26-GFP* were strongly curled (Figure 5.19 A), indicating that ectopic secondary thickening (ST) was induced by *MYB26*, presumably through the same mechanism in anther during ST development. Thus leaf materials of *35S::MYB26-GFP* and *Ler*. were used for affinity purification followed by Mass spectrometry (AP-MS) analysis. In *ubq10::MYB26-GFP* lines, secondary thickening was not very obvious in leaf

tissues, but most plants displayed a sterile phenotype due to increased anther thickening. Thus buds of *ubq10::MYB26-GFP* and *Ler.* were therefore used for AP-MS.

Nuclear proteins were isolated from 5 g of buds, run on 4%-12% Bis-Tris protein gels and stained with Ponceau Red solution. Proteins were successfully transferred from gels to nitrocellulose membrane (Figure 5.19 E) and Western

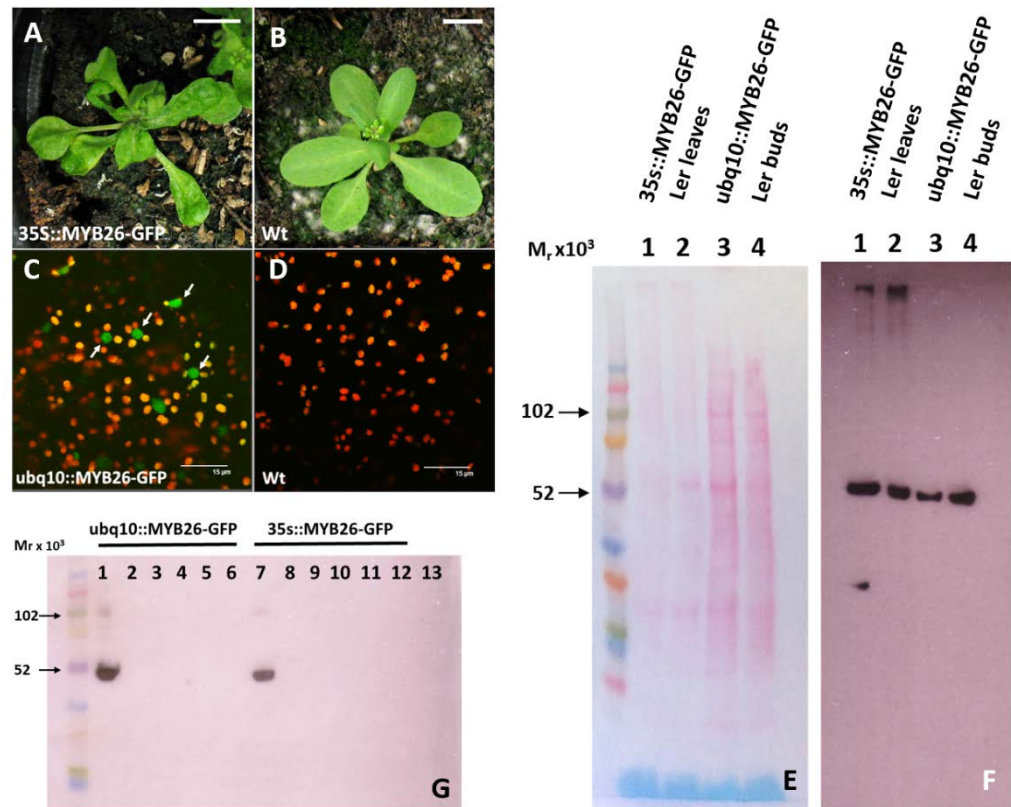


Figure 5.19 Affinity purification of MYB26 from *Arabidopsis*.

A-B. Leaf phenotype of the *35S::MYB26-GFP* line (A) and the wild-type *Ler.* (B). Scale bar 1cm.

C-D. GFP signal in the anthers of *ubq10::MYB26-GFP* line (C) and *Ler.* (D). Scale bar 15 μ m.

E. Ponceau Red staining of nucleus protein extracted from 5g of the following tissues: 1 *35S::MYB26-GFP* leaves; 2 *Ler.* leaves; 3 *ubq10::MYB26-GFP* buds; 4 *Ler.* buds.

F. Western-blot of proteins in figure E with anti-GFP-HRP antibody (GeneTex, US) 52 KDa band was detected in all protein extraction. Size of MYB26-GFP is around 68.58 KDa.

G. Affinity purification with GFP-trap (ChromoTech, Germany) from nucleus lysis of *ubq10::MYB26-GFP* line (1-6) and *35S::MYB26-GFP* line (7-12). Aliquots were saved from every step of the purification, and detected with western blot using anti-GFP-HRP antibody. Samples were loaded in the following order, nucleus lysis (1 and 7), nucleus lysis supernatant (Input, 2 and 8); unbound (3 and 9); 1st wash (4 and 10), 2nd wash (5 and 11); bound elution (6 and 12). 2x LDS sample buffer control (13).

blotted using anti-GFP-HRP (GeneTex, Germany). A band of approximately 52 KDa was detected in all tissues, including *Ler.* leaves and buds control (Figure 5.19 F), this indicated that it could be nonspecific recognition by the anti-GFP antibody. However, this 52 KDa protein was found in all biological replicates in four independent experiments. Thus, the GFP-Trap protein purification was continued via nuclear lysis of *ubq10::MYB26-GFP* and *35S::MYB26-GFP*, using anti-GFP VHH coupled to agarose beads for immunoprecipitation of MYB26-GFP (68.58 KDa). Aliquots were kept from every step during the protein purification. Western blots showed that only nuclear lysis contained the 52 KDa protein. From the input (nucleus lysis supernatant) to final elution product, no protein was detected by the GFP antibody, indicating that the 52 KDa protein is not soluble, and MYB26-GFP was not successfully extracted from MYB26 over-expression tissues (Figure 5.19 G).

5.4 DISCUSSION

5.4.1 FRET Assay evaluation.

Five proteins have been identified as interacting with MYB26 by Yeast two-hybrid screening of a stamen specific yeast 2 hybrid library (C. Yang and Z. A. Wilson, unpublished data). Except Y2H128, which was membrane localised, the other four Y2H320, Y2H560, Y2H620 and Y2H970 were co-localised with MYB26 in the nucleus (J. Song and Z. A. Wilson, unpublished data). Due to the high false positive drawback of Y2H system, a FRET assay was conducted using the tobacco transient expression system. Results suggest that Y2H320 and Y2H560 were possibly interacting with MYB26 in plant cells (Figure 5.2, Figure 5.3.)

In the FRET assay, spectral overlap between fluorescent proteins (FP) need to be considered when selecting Donor/Acceptor FP pairs. GFP and RFP have a spectral overlap, which means that RFP could be excited using GFP excitation. Proper control and calibration are crucial for FRET evaluation. SAG101 and EDS1 were previously reported forming a protein complex (Zhu et al. 2011) and were therefore included in this FRET assay as a positive control. EDS1 located in both nucleus and cell membranes, however in the tobacco transient expression system, EDS1 with C-terminal tagged RFP showed preferential localisation to the membrane when expressed alone, making it very time-consuming to find an acceptor only control for FRET calibration purpose. Thus, we suggest that EDS1 may not be a convenient positive control when using the FRET-sensitized emission method.

The FRET assay is extremely sensitive to small changes in distance. Many factors could influence the efficiency of FRET, such as donor/acceptor spectral overlap, distance, and orientation. Y2H320 with C-terminal tagged YFP and MYB26 with C-terminal tagged CFP were not FRET positive (J.Song and Z.A Wilson, unpublished data), however, high FRET efficiency was observed between Y2H320 with N-terminal tagged RFP and MYB26 with C-terminal tagged GFP. Thus the interaction between MYB26 and the FRET negative Y2H proteins such as Y2H970 and Y2H620 may need further experiment to confirm.

Intensive studies have shown that Y2H620 (TCP14), a class I TCP family protein with the conserved bHLH motif, are involved in the diverse biological process and hormone responses in plants. TCP14 functions redundantly with TCP15 in promoting cell division in young proliferating tissue, affecting internode length and leaf shape in *Arabidopsis*. *promTCP::TCP14::SRDX* transgenic plants displayed a general alteration of floral organ development (Kieffer et al. 2011). TCP14 has been reported to interact with variable partners in different biological process, such as DOF6, SPINDLY, DELLA in seed, leaf and flowers, shoot apex respectively, and appears closely related to phytohormone such as ABA, cytokinin, gibberellin and auxin responses (Lucero et al. 2015; Steiner, Yanai, et al. 2012; Daviere et al. 2014; Steiner, Efroni, et al. 2012; Rueda-Romero et al. 2012; Kieffer et al. 2011). TCP24, a class II TCP family protein, is a target of miR319 and negatively regulates secondary thickening (ST) in anther and roots, but may function in an MYB26-independent manner (H. Wang et al. 2015).

5.4.2 Functional study Y2H320 and Y2H560

Y2H320 (TGA9) was reported to function redundantly with TGA10 in regulating anther development, abnormal lignification was found in the middle

layer of the *tga9tga10* mutant (Murmu et al. 2010). In this thesis ST did not appear altered however the endothecium of *tga9tga10* appeared less turgid than wild-type during ST development (Figure 5.13). Overexpression of *TGA9* in *Ler.* background appeared to reduced fertility in the T1 generation plants due to a possible delayed anther dehiscence, however, more stable transgenic plants from T2 afterwards generation are required for convincing phenotypic analysis. Also, lignin staining and transverse sections maybe not the best way to evaluate ST changes in *tga9tga10* mutant, thus, we suggest that cell wall composition analysis using Mass spectrometry may give more details on cell wall polysaccharides composition in the mutant. In the Y2H system and FRET assay, Y2H320 interact with MYB26, however, it remains unclear whether they are functionally linked. Analysing the *MYB26* promoter activity using the GUS reporter system, and MYB26 protein activity with MYB26-GFP in the *tga9tga10* mutant may give a clue whether *MYB26* transcripts or protein level are affected by Y2H320.

Y2H560 (At5g25560) is a member of CHY-type/CTCHY-type/RING-type Zinc finger protein. There hasn't been any report on the function of Y2H560. However, *CHYR1*, which shares a high level of protein sequence similarity with Y2H560, has been shown to be involved in ABA response and drought tolerance by positively regulating stomata opening (Hsu et al. 2014). *CHYR1* also responded to sugar such as glucose and sucrose levels and was repressed by hexokinase 1 in *Arabidopsis* (Kunz et al. 2014, Kunz et al. 2015). Water status is changing during late anther developmental stages to facilitate anther dehiscence and pollen release. It would be interesting to conduct a functional study on *y2h560chyr1* or *y2h560* dominant repression lines. Similarly, more

stable *Y2H560* overexpression lines are required to conduct further phenotypic analysis.

5.4.3 Protein pull-down assay

MYB26 protein purification from *MYB26* overexpression tissue was documented in this thesis. A strong secondary thickening phenotype was displayed from *MYB26* overexpression lines indicating an ectopic accumulation of MYB26 protein. However, western blot analysis failed to detect MYB26-GFP in nuclear protein preparations, probably due to insufficient nuclear lysis. Lysis buffer contains strong detergent such as SDS, which may break unstable protein-protein interactions, however, insufficient lysis may result in failure of protein extraction.

There are several types of protein-protein interactions, some proteins form a stable structural protein complex, while many others may contact transiently, such as in a cascade of a signalling pathway. Weak interactions can be identified by covalently crosslinking the proteins during Co-IP or pull-downs. Besides optimising cell lysis condition, crosslinking combined with strong detergent lysis is worth trying for MYB26 AP-MS study.

CHAPTER 6 GENERAL DISCUSSION

Pollen development and release in flowering plants is critical for reproduction and selective breeding. Hybrids typically show superior growth rates, performance and yield, termed “hybrid vigour” or “heterosis”, compared with the inbred lines (Lippman & Zamir 2007), however there is a need for fertility control to produce hybrids. Male-sterile lines are favoured in hybrid seed production to avoid the time consuming and labour intensive emasculation process (Wilson & Zhang 2009). Male sterility results not only from defects during pollen development, but also can be due to the failure of anther dehiscence and pollen release. Viable pollen produced in indehiscent anthers has the advantage that it can potentially be used for rescue of fertility, making the male fertility controllable during hybrid generation and thus has potential commercial value.

MYB26/MALE STERILE35(MS35) encodes a transcription factor (TF) of the R2R3-MYB family, regulating the secondary thickening development in the endothecium of anther and is required for anther dehiscence (Steiner-Lange et al. 2003; Yang et al. 2007). The *ms35/myb26* mutant is male sterile due to a lack of endothecium secondary thickening, resulting in indehiscent anthers although viable pollen is produced. *MYB26* regulates the expression of the NAC Transcription factor *NST1* and *NST2* as well as a number of biosynthetic genes associated with secondary thickening (Yang et al. 2007). However, the regulation network of *MYB26* remains largely unknown. To address this issue, the direct regulating targets and interacting proteins of *MYB26* during anther dehiscence were investigated in this project.

6.1 MYB26 PUTATIVE BINDING TARGETS

6.1.1 MYB26 binds to a receptor-like cytoplasmic kinase *PKSP*

Coexpression analysis was used to identify possible direct regulatory targets for MYB26. *PKSP* (At2g39110) encodes for a protein kinase superfamily protein, was found to be co-expressed with *MYB26*, and the expression of *PKSP* was down-regulated in the *ms35/myb26* mutant and up-regulated in the *MYB26* overexpression lines, indicating that *PKSP* was regulated by *MYB26* (Figure 4.7 and Figure 4.8). Chromatin immunoprecipitation (ChIP)-PCR was performed to study MYB26 binding targets *in vivo*, a region within the first intron of *PKSP* was enriched in ChIP sample, indicating that MYB26 may directly bind to the first intron of *PKSP*.

Protein kinase superfamily is important in many aspects of cellular regulation in both animals and plants. An estimated 1 to 2% of functional eukaryotic genes encode protein kinases (Lehti-Shiu & Shiu 2012), over 1000 protein kinases have been identified in *Arabidopsis* (The Arabidopsis Genome Initiative 2000). Eukaryotic protein kinases have been typically divided into those that phosphorylate serine and/or threonine, and those that phosphorylate tyrosine on side chains of proteins (Stone & Walker 1995). The phosphorylation status of a protein can profoundly affect its activity and interaction with other proteins.

PKSP contains a predicted serine/threonine-protein kinase-like domain and has been classified into the receptor-like cytoplasmic kinase (RLCK) subgroup VII based on the sequence alignment of the catalytic domains. RLCKs belong to the superfamily of receptor-like kinases (RLKs) which are transmembrane proteins with an extracellular receptor domain for signal perception and an intracellular

kinase domain for signal transduction (Shiu & Bleecker 2001). RLCKs show homology to RLKs in kinase domain but lack the transmembrane domain (Figure 4.13). By activating the mitogen-activated protein kinase (MAPK) cascade, or the MAPK independent signalling, RLK/RLCK can regulate gene expression via transcription factor(s) or other physiological responses (Lin et al. 2013).

RLCKs has been reported to play roles in stress responses, signalling, and plant immunity. Particularly members of RLCKs subfamily VII, are overrepresented among genes induced by biotic stress (Lehti-Shiu et al. 2009). PKSP has a high protein sequence similarity with another receptor-like cytoplasmic kinase, PCRK1 (Figure 4.8), which was reported to be important for defence against the pathogen *Pseudomonas syringae* pv. *maculicola* ES4326. The pathogen defence ability and callose deposition were reduced in the *pcrk1* mutant, but the double mutant of *PCRK1* with two phylogenetically closely related genes *PCRK2* (At5g03320) and At5g03320 didn't significantly reduce the immunity against *Pma* ES4326 compared to the single mutant of *pcrk1* (Sreekanta et al. 2015). However, Kong et al (2016) suggested that the *pcrk1pcrk2* double mutant, but neither of the single mutants, exhibited reduced accumulation of salicylic acid (SA), and enhanced disease susceptibility to bacterial pathogens, indicating a redundant function between *PCRK1* and *PCRK2* (Kong et al. 2016).

RLCKs also regulate plant growth and development as well as involved in hormone signalling. Expression profiles of rice OsRLCKs show that among the total 339 OsRLCKs, 98 OsRLCKs were expressed differentially during reproductive developmental stages, including stages of panicle and seed development; 82 OsRLCKs were differentially expressed under any one of the

cold, salt, and dehydration abiotic stress conditions. An overlap of 36 OsRLCKs showed both altered expression during reproductive developmental stages and abiotic stress conditions (Vij et al. 2008). *BSK1* and *CDG1* belong to RLCK subfamily VII and XII, respectively (Shiu et al. 2004), which function in parallel to positively regulate Brassinosteroids (BR) signalling (Kim & Wang 2010; Kim et al. 2011). *Arabidopsis thaliana* *BOTRYTIS-INDUCED KINASE1* (*BIK1*) regulates immune responses to pathogens, and also functions as a component of the ripening hormone ethylene (ET) signalling. Altered expression of ET-related genes was found in the *bik1* mutant (Laluk et al. 2011). *CAST AWAY* (*CST*), a subfamily VII RLCK acts as a spatial and temporal inhibitor of organ separation (Burr et al. 2011). *M LOCUS PROTEIN KINASE* (*MPLK*), another subfamily VII RLCK, is essential for self-incompatibility responses in *Brassica* (Murase et al. 2004).

The *PKSP* single T-DNA insertion mutant did not display morphological alteration compared to the wild-type (Figure 4.9); this may be due to functional redundancy with other phylogenetically-related RLCKs, such as *PCRK1*, *PCRK2* and *At2g28940* (Figure 4.8). However, functional study of *PKSP* by generating double or triple mutant of *PKSP* with its putative redundant RLCKs is time-consuming and challenging due to lack of suitable T-DNA insertion mutants (Figure 4.10). Therefore there is a need in future work, to generate *PKSP* dominant repression lines and overexpression lines to study the biological function of *PKSP*.

Although RLCKs are involved in distinct physiological responses, remarkable similarity can be found in the framework of signal transduction cascades (Figure 4.13). RLCK/RLK complexes can activate mitogen-activated protein kinase

(MAPK) cascade and/or MAPK-independent signalling to regulate gene expression via transcription factors (TFs) or other physiological responses (Lin et al. 2013). Since secondary thickening is a complex biological process, requiring precise transcriptional regulation of many TFs and biosynthetic genes, it could be hypothesised that the MYB26 putative binding target *PKSP*, a subfamily VII reception-like cytoplasmic kinase (RLCK), may function in a signal transduction way to regulate gene expression probably via TFs during secondary thickening development.

6.1.2 MYB26 regulates ST through *NST1* and *NST2*

The *NAC SECONDARY WALL THICKENING PROMOTING FACTOR1* (*NST1*) and *NST2* function redundantly regulating secondary thickening (ST) development (Mitsuda et al. 2005; Nelson et al. 2012). The expression of *NST1* was detected in various tissues in which ST developed, while the *NST2* expression was stronger in anther. Overexpression of *NST1* or *NST2* induced ectopic ST in various aboveground tissues. The *nst1nst2* double mutant produced indehiscent anthers due to lack of ST development in anther endothecium layer, similar to the phenotype observed in the *ms35* mutant.

The expression of *NST1* and *NST2* was affected by ectopic expression of *MYB26* (Figure 3.4) and it was hypothesised that *MYB26* possibly regulate *NST1/2* directly during ST development. Chromatin Immunoprecipitation (ChIP)-QPCR suggested enrichment of a region *NST1_P2a* (-2409bp to -2260bp from *NST1* ATG) and *NST2_P8* (-440bp to -235bp from *NST2* ATG) (Figure 4.11), however, the enrichment was only observed in around half of all biological replicates tested. Nevertheless this ChIP enrichment was seen with two separate antibodies, a MYB26 peptide antibody and anti-GFP in the CaMV35S::*MYB26*-GFP line.

Lacking a positive control makes the evaluation of MYB26 ChIP experiment very difficult. Electrophoretic mobility shift assay (EMSA) results suggested that MYB26 didn't bind *NST1_p2a* and *NST2_p8* *in vitro* (Figure 4.12), however this could be due to the binding conditions not being suitable for MYB26 binding activity, or that MYB26 may require additional partners to form a protein complex, which is commonly found among transcription factors (Berg et al. 2010), to fulfil its function.

6.2 MYB26 INTERACTING PROTEINS

Five proteins have been previously shown in the Yeast two-hybrid system to interact with MYB26 (C. Yang, Z.A. Wilson unpublished data). Except for Y2H128, which is membrane localised, the other four, Y2H320, Y2H560, Y2H620 and Y2H970, are co-localised with MYB26 in the nucleus (J. Song, PhD Thesis), Förster resonance energy transfer (FRET) assays were conducted using the tobacco transient expression system, the results further confirm that Y2H320 and Y2H560 interact with MYB26 in plant cells (Figures 5.2 and 5.3).

The FRET assay is extremely sensitive to small changes in distance. Many factors could influence FRET efficiency, such as donor/acceptor spectral overlap, distance, and orientation. However, Y2H320 with C-terminal tagged YFP and MYB26 with C-terminal tagged CFP, did not display FRET interactions in onion cells (Song 2009), however, high FRET efficiency was observed between Y2H320 with N-terminal tagged RFP and MYB26 with the C-terminal tagged GFP.

Intensive studies have shown that *Y2H620 (TCP14)*, encodes for a class I TCP family protein with a conserved bHLH motif; it is involved in diverse biological

process and hormone responses in plants. *TCP14* functions redundantly with *TCP15* in promoting cell division in young proliferating tissue, affecting internode length and leaf shape in *Arabidopsis*, with promTCP:TCP14:SRDX transgenic plants displaying a general alteration of floral organ development (Kieffer et al. 2011). TCP14 has been shown to interact with variable partners in different biological process, such as DOF6, SPINDLY, DELLA in seed, leaf and flowers, shoot apex respectively, and has crosstalk with phytohormones such as ABA, cytokinin, gibberellin and auxin (Lucero et al. 2015; Steiner, Yanai, et al. 2012; Daviere et al. 2014; Steiner, Efroni, et al. 2012; Rueda-Romero et al. 2012; Kieffer et al. 2011). MYB26 with C-terminal tagged CFP and Y2H620 with C-terminal tagged YFP didn't show FRET phenomena in onion epidermal cells (Song, 2009). Thus optimisation of the donor/acceptor fluorescent protein tag is required to further confirm whether the previous FRET negative candidates Y2H620 and Y2H970 interact with MYB26 in plant cell

6.2.1 Y2H320/TGA9 and Y2H560 expression overlap with MYB26

MYB26 is highly expressed during pollen mitosis I (PMI) and bicellular (BC) stage, the *MYB26* promoter activity, as indicated by GUS reporter expression, was observed throughout the anther at stage 10, particularly in the endothecium, tapetal and connective tissues (Yang et al. 2007). *TGA9* promoter activity was previously observed using a GUS reporter from anther stage 4 and peaked at stage 5-6, then gradually declined during stage 7 to 11 (Murmu et al. 2010). In situ hybridisation showed that the *TGA9* transcripts accumulated in the lateral and adaxial portion of the anther primordia during stage 2 and 3, and the expression was throughout sporogenic tissue and surrounding cells in both abaxial and adaxial lobes during stage 4 and 5. At stage 6, the expression was

restricted to tapetum and middle layers before gradually fading with degeneration of these cell layers until stage 11. The expression level of *TGA10* was lower than *TGA9*, but in a similar pattern (Murmu et al. 2010). According to the above study, it is possible that *MYB26* and *Y2H320/TGA9* have a temporal and special expression overlap, which is the prerequisite for functional interaction between *MYB26* and *Y2H320/TGA9* in developing anthers. Quantitative RT-PCR was performed with staged anthers, the transcript level of *Y2H320/TGA9* was higher in anthers before pollen mitosis I (PMI) and decreased afterwards but was still detectable during PMII. These data showed that *Y2H320/TGA9* and *MYB26* are coexpressed during PMI stage of anther development (Figure 5.7 A). The transcript level of *TGA10* was co-expressed with *MYB26* during PMI stage and had a second expression peak during PMII stage (Figure 5.7 A), however, the *TGA10* expression stages observed with QRT-PCR was much later than suggested by Murmu et al (2010). Nevertheless, since *TGA9* and *TGA10* function redundantly and both interact with ROXY proteins to regulate anther development, it would be interesting to see whether *TGA10* could interact with *MYB26* in future work.

Y2H560 (At5g25560) interacted with *MYB26* in yeast two-hybrid system (C. Yang, Z.A. Wilson unpublished data) and FRET assay (Figure 5.3). *Y2H560* is a member of CHY-type/CTCHY-type/RING-type Zinc finger protein. Quantitative RT-PCR showed that the transcript level of *Y2H560* is higher than *MYB26*, and expressed throughout the whole anther development with a small peak at bicellular (BC) stage (Figure 5.9), suggesting that there is an expression overlap between *Y2H560* and *MYB26*. The transcript level of *CHY ZINC-FINGER AND RING PROTEIN 1* (*CHYR1*), which is phylogenetically closely

related to *Y2H560*, is also co-expressed with *MYB26* during PMI stage and had a second expression peak during PMII stage (Figure 5.9). There has not been any previous reports on the function of *Y2H560*, and no obvious phenotypes were observed in the single T-DNA insertion mutant of *Y2H560* (GK-944F09). *CHYR1* is involved in the ABA response and drought tolerance, by positively regulating stomata opening. The loss of function mutation of *CHYR1* results in a reduction of stomatal aperture under non-stress condition in rice and *Arabidopsis* (Hsu et al. 2014). *CHYR1* also responds to sugar such as glucose, sucrose level and repressed by *Hexokinase 1* in *Arabidopsis* (Kunz et al. 2014, Kunz et al. 2015). It is known that water status is changing during late anther developmental stages to facilitate anther dehiscence and pollen release (Wilson, 2011), it could be hypothesised that *Y2H560* and *CHYR1*, which are phylogenetically close related to each other, may play a role during anther dehiscence by regulating stomata aperture and thus affecting water status in anther. In future work, generating an *y2h560chyr1* double mutant, or *y2h560* dominant repression lines, could be performed to investigate the function of *Y2H560*. Transgenic plants overexpressing *Y2H560* were generated and a slight reduction of the silique length was observed in one of these transgenic plants (Figure 5.17), however, these are preliminary results from the T1 generation, more stable *Y2H560* overexpression lines are required to perform the phenotypic analysis.

It remains unclear whether *Y2H320* and *Y2H560* are functionally linked with *MYB26* in developing anthers and if so in what manner they are acting. Analysing *MYB26* promoter activity using the GUS reporter system (ref), and investigating *MYB26* protein activity using the GFP reporter in the *tga9tga10*

mutant and *y2h560* mutant may give a clue as to whether Y2H320 and Y2H560 are required for normal MYB26 transcripts and protein expression and activity to regulate the secondary thickening development.

6.2.2 Functional study of Y2H320 (TGA9)

Y2H320 (TGA9) interacted with MYB26 in the Yeast two-hybrid system (C. Yang, Z.A. Wilson unpublished data) and FRET assay (Figure 5.2). *TGA9* has been reported to function redundantly with *TGA10* in regulating anther development (Murmu et al. 2010). They observed that the development of the adaxial and abaxial anther lobes in the *tga9tga10* mutant was differentially affected. The adaxial lobe development was blocked at an early stage, while in the abaxial lobe, the pollen was inviable and the anthers failed to dehisce and had abnormal tapetum and middle layer lignification (Murmu et al. 2010).

In this project, the *tga9tga10* mutant was generated with single T-DNA insertion mutants of *TGA9* (SALK_057609) and *TGA10* (SALK_124227) as described in Murmu et al. (2010). Consistent with their results, the *tga9tga10* mutant was sterile due to abnormal anther development. To study the morphological alterations of the *tga9tga10* mutant anther, light microscopy was conducted. In the wild-type, the cell development of anther lobe was not synchronised until anther stage 5, with the adaxial lobes smaller than the abaxial lobes. The parietal cells and sporogenous cells were defined in all four anther locules and differentiated into microspore mother cells surrounded by tapetum, middle layer, endothecium and epidermis (Figure 5.14, Figure 5.15, and Sanders et al. 1999). However in the *tga9tga10* mutant, abnormal anther development was clearly observed from stage 5, with reliable microspore mother cells produced only in the abaxial lobes, while the cell differentiation. Development in the adaxial lobes

was variable, most of them resulted in developmental arrest from stage 5, but some had differentiated tissues observed in either one or both of the adaxial lobes but the development was disorganised and uncoordinated with the abaxial lobes and was arrested in the later stage (Figure 5.14, Figure 5.15). In the wild-type, coordinated with the pollen development was a general increase in anther size, degeneration of several cell layers including the middle layer, tapetum, and septum. The endothecium underwent expansion and lignification between stage 10 and 11 to facilitate the rupture of stomium region followed by pollen release (Figure 5.14, Figure 5.15 and Sanders et al. 1999). In the *tga9tga10* mutant, cell differentiation in the abaxial lobes followed a pattern similar to the wild-type, however, the tapetum became expanded and the abnormal vacuoles were retained after stage 6, rather than initiating programmed cell death and the release of microspores that was seen in the later stages in the wild-type. The development of microspores in the *tag9tag10* mutant was mostly impaired. Although pollen was produced in the abaxial lobes of the *tga9tga10* mutant, the septum and stomium were not functional, resulting in indehiscent anthers (Figure 5.14, Figure 5.15). Pollen from the *tga9tga10* mutant could not be manually released since they were clumped together and there was much less pollen than wild type; Alexander staining also indicated that pollen viability was reduced in the *tga9tga10* mutant (Figure 5.12).

Nevertheless endothecium lignification took place in the abaxial anther lobes of the *tga9tga10* mutant at anther stage 11, the same stage as in the wild-type, although the endothecium didn't fully expand to the same extent as in the wild-type. The lignification in adaxial lobes was visible in connective tissue but less intensive as in the wild-type. Lignin staining using acridine orange and ethidium

bromide indicated that the secondary thickening (ST) was developed in the *tga9tga10* mutant. However, both cross-sections and lignin staining methods couldn't provide details on any changes in the cell wall composition. Future work using Mass Spectrometry to further investigate the cell wall polysaccharides composition in the *tga9tga10* mutant would be of interest.

The fertility of *TGA9* overexpression (OEx) lines (T1) in *Ler* background was reduced due to a possible delayed anther dehiscence (Figure 5.16 D and E), and the fertility of *TGA9* OEx lines indicated by silique length was negatively correlated with the expression level of *TGA9* (Figure 5.16 A-C). These are preliminary results from the T1 generation of the transformed plants, more stable lines from T2 afterwards are required for convincing the phenotypic alteration.

6.3 FUTURE WORK

Secondary thickening in the endothecium is critical for anther dehiscence, which is a multistage process involving cellular differentiation, programmed cell death, changes in the structure and water status (Wilson et al. 2011). The regulation network of *MYB26* during endothecium development and secondary thickening in *Arabidopsis* anther was investigated in this project by studying *MYB26* regulating targets and interacting proteins.

Numerous transcription factors (TFs) are required for the complex transcriptional regulation of secondary cell wall (SCW) biosynthesis, (Hussey et al. 2013). TFs that show altered expression in the *ms35/myb26* mutant, especially during the pollen mitosis I (PMI) and bicellular (BC) stage when *MYB26* is highly expressed in the wild-type, may play an important role in the regulation network of *MYB26* during secondary thickening development in anther. Besides

MYB26, twelve TFs showed altered expression with at least a two-fold change in the *ms35* mutant compared to the wild-type during both PMI and BC stage (Figure 3.7, Table 3.1), four of them contain the *MYB26 in vitro* binding site (Taylor, PhD thesis, 2014). Some of these TFs have been reported to participate in various biological processes. *AtC3H14* is involved in post-transcriptional regulation and required for cell elongation, secondary cell wall development in stem and pollen development (Kim et al. 2014; Chai et al. 2015). NAC Transcription factor *NST1*, a master switch of secondary thickening, functions redundantly with *NST2* in regulating anther dehiscence (Mitsuda et al. 2005). *ETHYLENE-RESPONSIVE ELEMENT BINDING FACTORS 96 (ERF96)* is a regulator of the ethylene (ET), jasmonate (JA) and abscisic acid (ABA) responses in *Arabidopsis* (Catinot et al., 2015; Wang et al, 2015), genes associated with JA signalling, redox stage of plant cells and glutathione transferase, cell wall development were numerously altered in the *ERF96* over-expression lines (Catinot et al. 2015). The floral transition repressor *FLOWERING LOCUS C (FLC)* also involves in regulating stomatal opening and associated with pleiotropic phytohormones such as gibberellic acid (GA) and abscisic acid (ABA) (Rouse et al. 2002; Bastow et al. 2004; Shu et al. 2016; Shi et al. 2016; Kimura et al. 2015; Michaels & Amasino 1999; Simpson & Dean 2002). *KNOTTED ARABIDOPSIS THALIANA7 (KNAT7)*, a transcriptional repressor that negatively regulates SCW formation (Li et al. 2012). MYB7, an R2R3-MYB factor acting as transcriptional repressors of different branches of phenylpropanoid metabolism (Fornalé et al. 2014). Not all the TFs listed in table 3.1 have been functional studied, and in future work, these TFs are promising

candidates and should be given attention when study MYB26 regulating targets during secondary cell wall development in anther.

Besides transcription factors, another ten genes were selected from the *ms35* mutant microarray data according to the temporal and special expression pattern (Table 4.3). These genes were tested by chromatin immunoprecipitation (ChIP)-PCR, and the results indicated that a receptor-like cytoplasmic kinase (RLCK) *PKSP* could possibly be a direct binding target of *MYB26 in vivo*. However due to limited amount of ChIP DNA, up to two ChIP probes were designed for each gene, with consideration of cis-regulatory elements and distance to the translation start site. Genes that didn't enrich in ChIP-PCR could only indicate that the tested regions did not bind by MYB26 *in vivo*. Further study is required on genes listed in Table 4.3. In future work the global map of MYB26 binding sites in *Arabidopsis* using ChIP combined with next generation sequencing will give a more straightforward clue to uncover MYB26 binding targets during secondary thickening development in anther.

In order to investigate MYB26 interacting proteins in *Arabidopsis*, MYB26 protein pull-down was performed using tissues overexpressed *MYB26* and developed ectopic secondary thickening (Chapter 5, Section 5.3.5). Optimisation of the cell lysis buffer is required to successfully extract target protein without disturbing protein-protein interactions. There are several types of protein-protein interactions, some proteins form a stable structural protein complex, while many others may contact transiently, such as in a cascade of the signalling pathway. Weak interactions can be identified by covalently crosslinking the proteins during co-IP or pull-down. Thus, crosslinking combined with strong detergent lysis is another option to pull down MYB26 from *Arabidopsis* tissue before

continuing with mass spectrometry to identify MYB26 interaction partners during secondary cell wall development in *Arabidopsis* anther.

6.4 CONCLUSION

Pollen development and release involves a number of important stages, which govern the success of fertilisation and thus indirectly crop yields. The secondary cell wall in the anther plays a pivotal role in anther dehiscence by offering mechanical strength required for opening and pollen release (Wilson et al. 2011). *MYB26* appears to regulate the endothecium development, particularly cell expansion and secondary cell wall thickening and thus anther dehiscence. In this thesis, the MYB26 direct targets and interacting proteins were investigated.

MYB26 acts upstream of the lignin biosynthesis pathway and regulates secondary thickening development probably through directly regulating the subfamily VII RLCK *PKSP* and the NAC transcription factor *NST1*, *NST2*. *MYB26* interacts with CHY-type/RING-type Zinc finger protein Y2H560 and the bZIP transcription factor Y2H320/TGA9 in tobacco transient expression system, suggesting that additional interacting proteins may be required for the function of MYB26 during secondary thickening and anther development.

In future work, chromatin Immunoprecipitation (ChIP) followed by next generation sequencing will be needed to construct a global map of the MYB26 binding sites across the *Arabidopsis* genome. In parallel, the combination of MYB26 protein pull down from *Arabidopsis* with Mass spectrometry will help to generate a predictive model of the MYB26 regulation network during secondary thickening development in anther.

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APPENDIX

CHEMICALS AND REAGENTS

ChIP Buffers

MC buffer

Component	Concentration
Sodium phosphate (pH 7.0)	10 mM
NaCl	50 mM
Sucrose	0.1 M
β -Mercaptoethanol	5 mM
PMSP	0.1 mM

Extraction buffer 1

Component	Concentration
Sucrose	0.4 M
Tris-HCl, pH 8.0	10 mM
β -Mercaptoethanol	5 mM
PMSF	0.1 mM

Extraction buffer 2

Component	Concentration
Sucrose	0.25 M
Tris-HCl, pH 8.0	10 mM
MgCl ₂	10 mM
Triton X-100	1% (v/v)
β -Mercaptoethanol	5 mM
PMSF	0.1 mM

Extraction buffer 3

Component	Concentration
Sucrose	1.7 M
Tris-HCl, pH 8.0	10 mM
MgCl ₂	2 mM
Triton X-100	0.15% (v/v)
β-Mercaptoethanol	5 mM
PMSF	0.1 mM

Nuclei lysis buffer

Component	Concentration
Tris-HCl, pH 8.0	50 mM
EDTA	10 mM
SDS	1% (w/v)
PMSF	0.1 mM

ChIP dilution buffer

Component	Concentration
Tris-HCl, pH 8.0	16.7 mM
EDTA	1.2 mM
Triton X-100	1.1% (v/v)
NaCl	167 mM
PMSF	0.1 mM

Low salt wash buffer

Component	Concentration
Tris-HCl, pH 8.0	20 mM
EDTA	2 mM
Triton X-100	1% (v/v)
NaCl	150 mM
SDS	0.1% (w/v)

High salt wash buffer

Component	Concentration
Tris-HCl, pH 8.0	20 mM
EDTA	2 mM
Triton X-100	1% (v/v)
NaCl	500 mM
SDS	0.1% (w/v)

LiCl wash buffer

Component	Concentration
Tris-HCl, pH 8.0	10 mM
EDTA	1 mM
NP-40	1% (v/v)
LiCl	250 mM
Sodium deoxychlorate	1% (w/v)

TE buffer

Component	Concentration
Tris-HCl, pH 8.0	10 mM
EDTA	1 mM

ChIP Elution buffer

Component	Concentration
SDS	1% (w/v)
NaHCO ₃	0.1 M

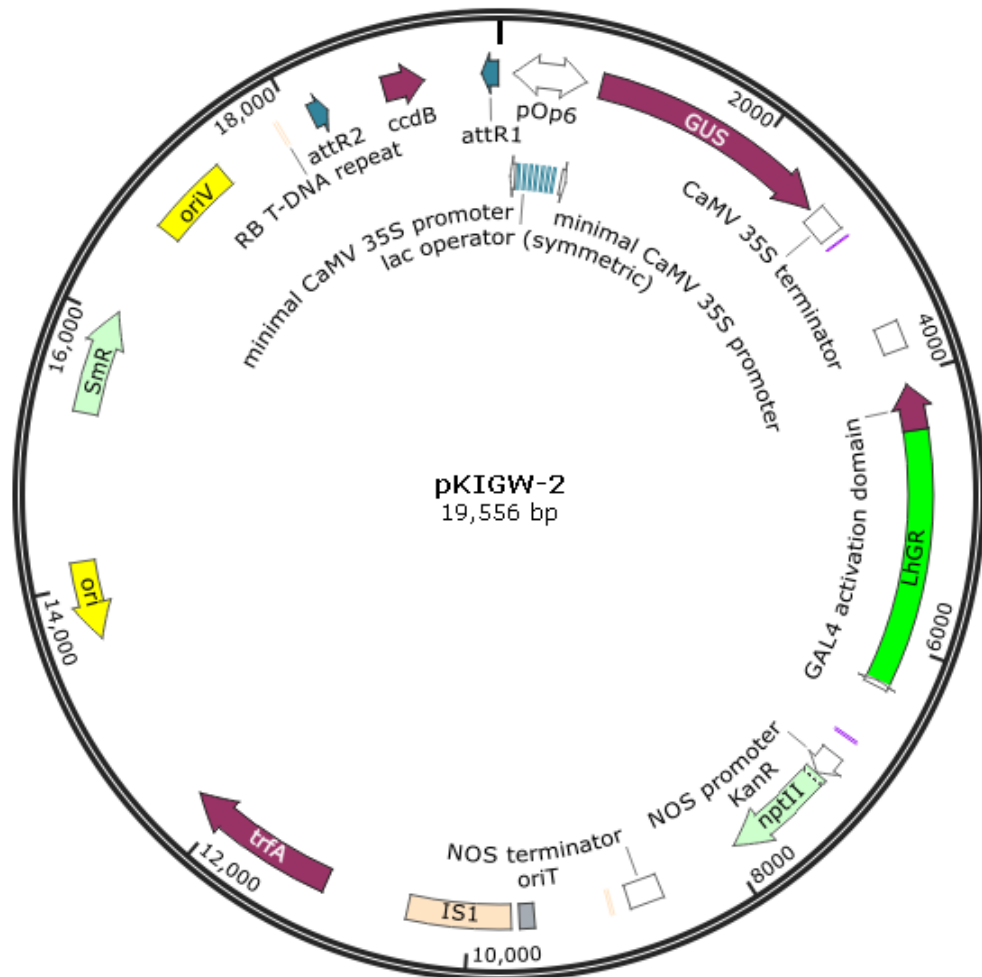
Anti-MYB26 Antibody

The anti-MYB26 antibody used in chromatin immunoprecipitation (ChIP) assays (Figure 4.13 D and F) was produced by Invitrogen and was tested using western blot (Roderick 2008).

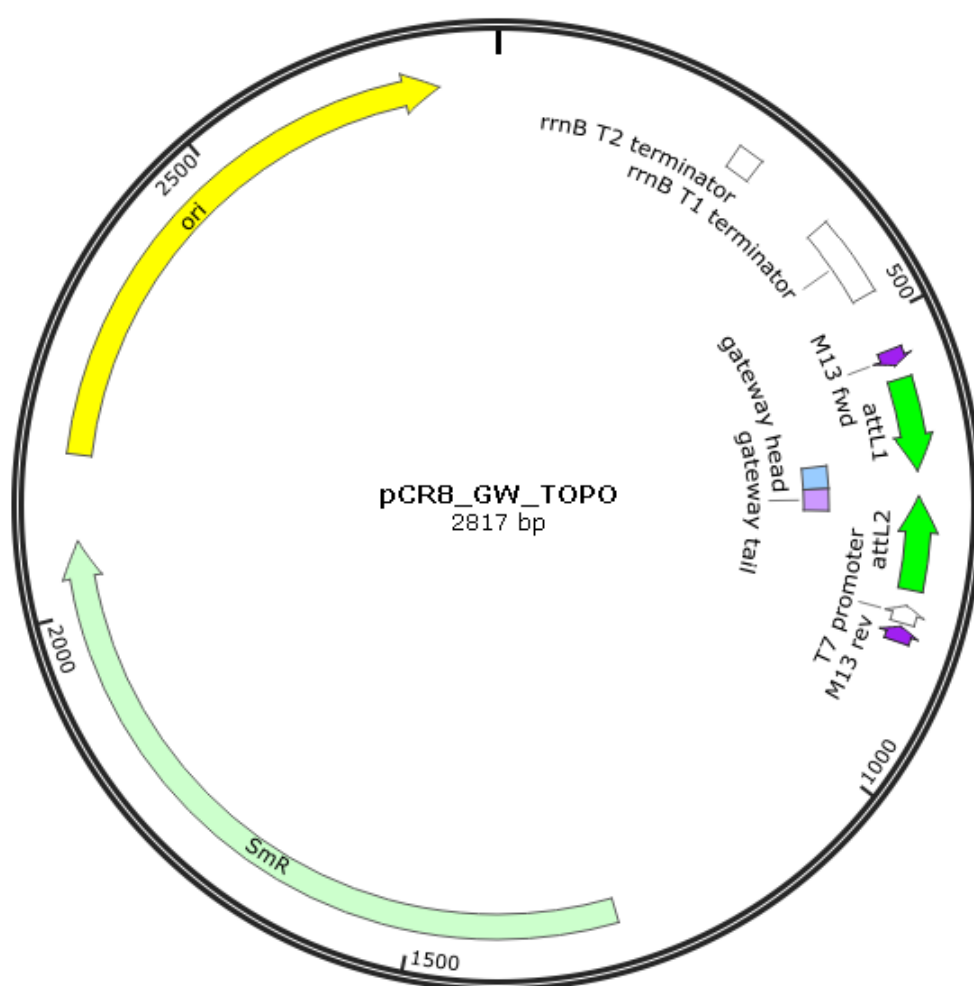
Excluding the conserved MYB domain, two peptides with the lowest similarity to other *Arabidopsis* protein were identified (Invitrogen), they are MYB26-2 (CFQETLQDYDDANK, Invitrogen reference Q9LVL0, #260~272) and MYB26-3 (CGFNPTTVDDSSR, Invitrogen reference Q9LVL0, #144~156). MYB26-2 and MYB26-3 peptides were injected into two specific pathogen free New Zealand rabbits followed by additional three booster injections at 2 weeks, 6 weeks and 8 weeks post immunisation (pi). The antiserum was collected by Invitrogen at 10 weeks pi. Enzyme-linked immunosorbent assay (ELISA) was performed to determine the reactivity of the antibody to the target peptides. The anti-MYB26 antibody was further tested using western blot and immunolocalisation by Roderick (Roderick, 2008; Song, 2009).

VECTORS

pKIGW



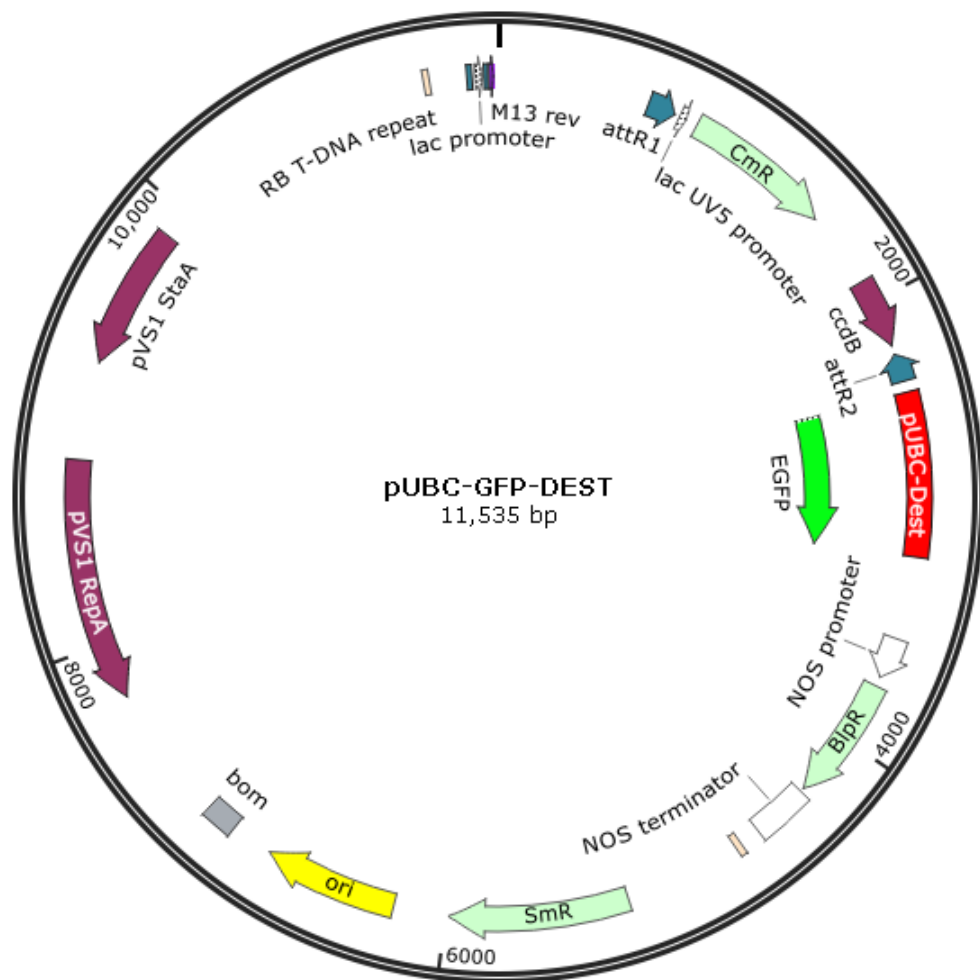
pCRTM8/GW/TOPO Vector (InvitrogenTM)



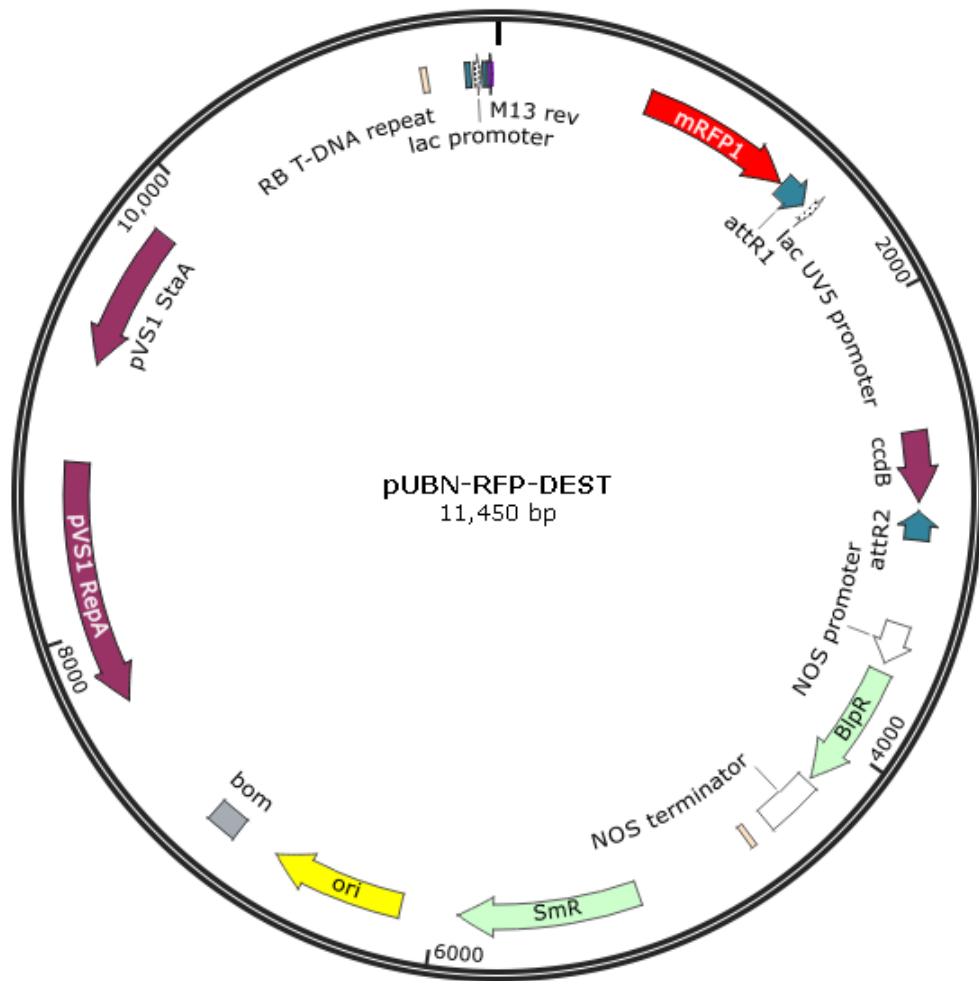
Gateway®pDONR™221 Vector (Invitrogen™)



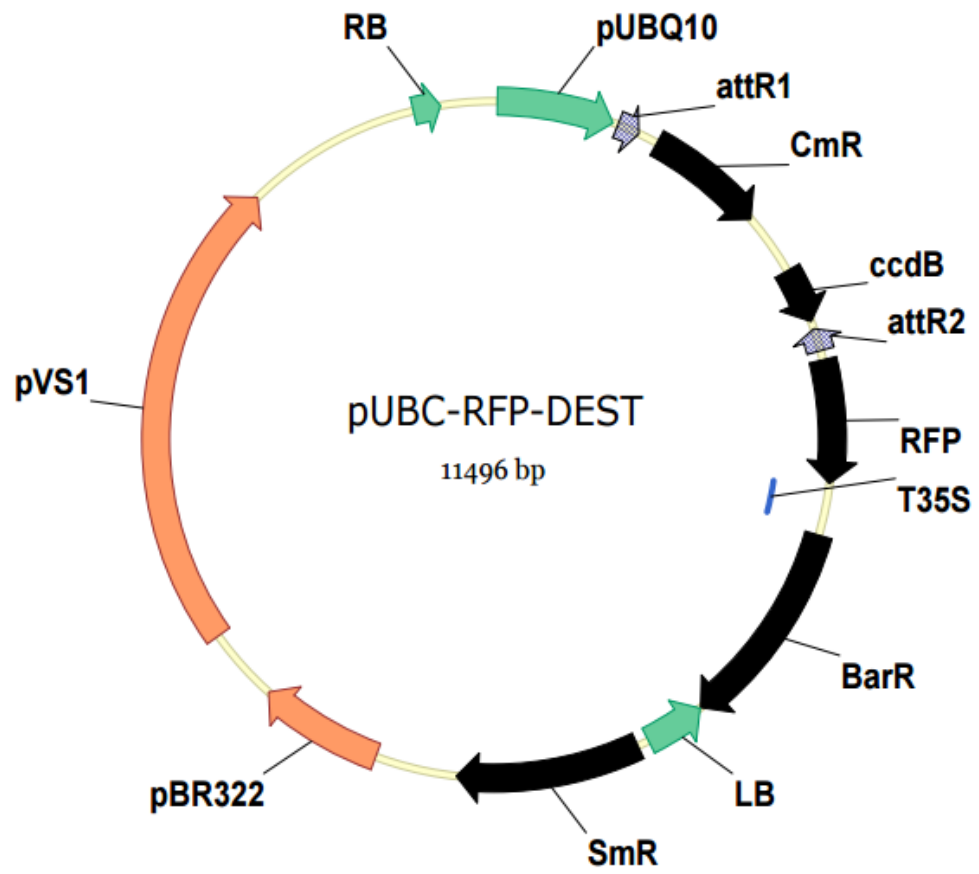
PUBC-GFP-DEST (Grefen et al. 2010)



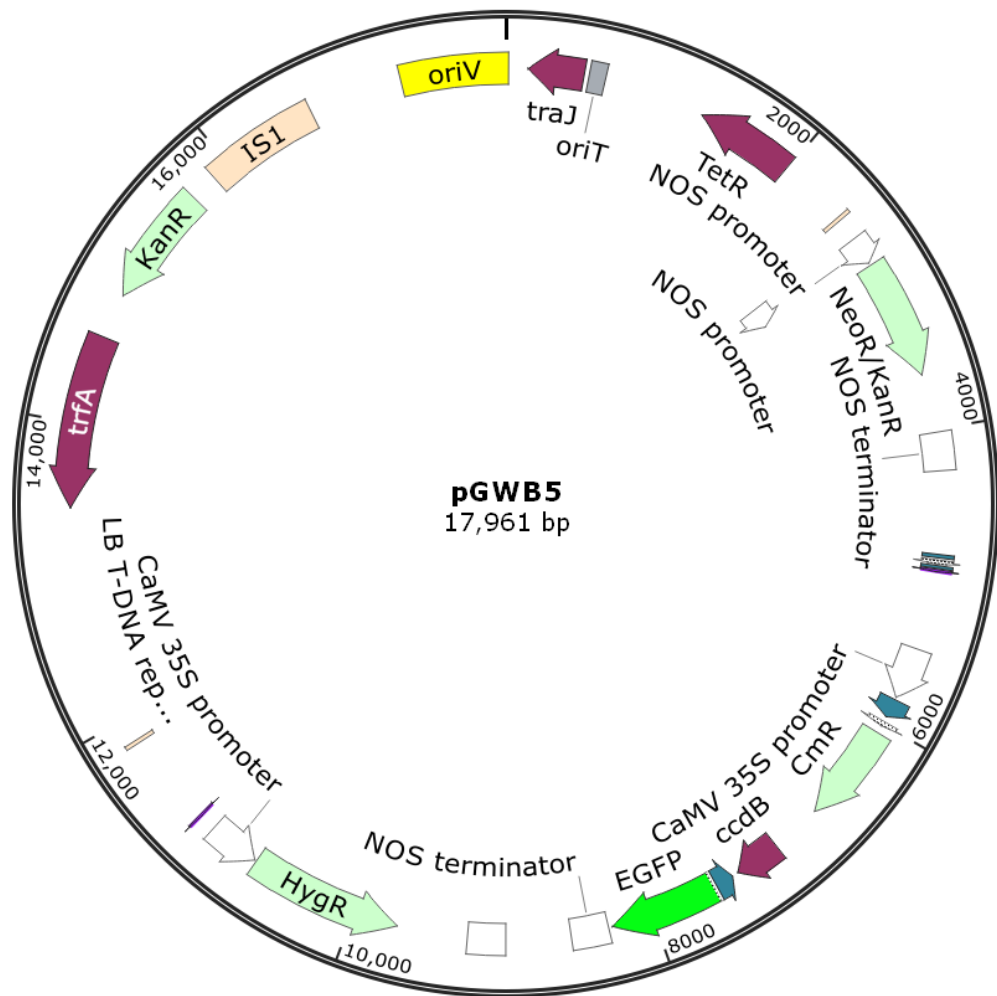
pUBN-RFP-DEST (Grefen et al. 2010)



pUBC-RFP-DEST (Grefen et al. 2010)



pGWB5(Nakagawa et al. 2007)



PRIMERS

ChIP probes		
Name	Sequence	AGI Code
NST1_P2a F	CTATTGATTCGGGTTGACATGA	At2g46770
NST1_P2a R	AGGTTTGATTTCAGAATCTTCATT	At2g46770
NST2_P8 F	AAAACCAAAGGCCGAAGAAG	At3g61910
NST2_P8 R	GGCATCGTAATAATTTGTGCTAA	At3g61910
9110_intronF	ATGAAGACCCCCATCACAGC	At2g39110
9110_intronR	TCCAAACCAGGTCCACATTCC	At2g39110
F6'HIprom1_R	GGGATTTTGATTGTGTTGTGAAGAT	At3g13610
At1g33700prom1_F	GAGATTTTGCTACAGAATCCTTGCT	At1g33700
At1g33700prom1_R	CATGCGGGCCACAAAATAGC	At1g33700
DUF662_Prom1_F	GCCTTCAACTTCACACCTAACA	At2g27740
DUF662_Prom1_R	CCAAGTGGGGCTAAAAGGTCT	At2g27740
DUF662_prom2_F	GTTTAATCAGGGATAGTGTGGTTGAC	At2g27740
DUF662_prom2_R	CATTGTTCTCAGATCCTTTTTCTC	At2g27740
DUF662_prom0_F	CCGTAAGTTTCAGCCAGAGTACA	At2g27740
DUF662_prom0_R	TGTTAGGTGTGAAGTTGAAGGC	At2g27740
GH3.9_promF	AGCTAGTGGTGGACTTTCGC	At2g47750
GH3.9_promR	TCAGGGAAGCAATCACAACCA	At2g47750
9320_p1_F	ACATGACTCAGCCTTCCGTTA	At4g39320
9320_p1_R	GTGCCCCTCCATCTCGTTAC	At4g39320
9320_p2_F	TGAGCATGGAAGGGAGTTTAGC	At4g39320
9320_p2_R	AAGCTAAAGGCTGGCGAGAT	At4g39320
0170_p0_F	TCCGATCACATTCCACGTTGT	At4g30170
0170_p0_R	TGTCTTTGACAACTCAGTGGC	At4g30170
0170_p1_F	TGCGATTAAAGCCAGACGAG	At4g30170
0170_p1_R	GAGGCTAAGGTGAGGCATCG	At4g30170
0170_p2_F	GATGCCTCACCTTAGCCTCC	At4g30170
0170_p2_R	AGGTATGGTACAATTTGGGCTAGT	At4g30170
ADS3.2_promF	GTGTGTGTTCTCGCTCGTTC	At3g15870
ADS3.2_promR	GACTGCTCAAATAAACAGGCCC	At3g15870
0950_promF	CGTAAATGGCCACATGAAAC	At4g10950
0950_promR	GCCTACCTACCTCGGATCAAA	At4g10950
IRX15-L_p1F	GTCGCCTTCAGAAATCTCCCA	At5g67210
IRX15-L_p1R	TCGCTCTGTTTTGGTCAGAGA	At5g67210
IRX15-L_p2F	CCGTAAAAACCAGTCAACAGCAT	At5g67210
IRX15-L_p2R	TGGGAGATTTCTGAAGGCGA	At5g67210
RIC4_p1F	TGGCCTTGCCATCATAACCAT	At5g16490
RIC4_p1R	AACTCTACTTCTGTGGACTGTGT	At5g16490
RIC4_p2F	ACCGCAACTTCATATACACACG	At5g16490
RIC4_p2R	CGATGAAAATGACAACCCACCA	At5g16490

Genotyping primers		
Name	Sequence	AGI Code
LBb1	GCGTGGACCGCTTGCTGCAACT	
LB3	TAGCATCTGAATTTTCATAACCAATCTCGA TACAC	
O8409	ATATTGACCATCATACTCATTGC	
pcrk1(2)LP	TCCATAACTTGCCAAACCAAC	At3g09830
pcrk1(2)RP	GGGTTACGGAAGTGAACCTCC	At3g09830
pcrk1(1)LP	TTTTCTCCATTCCACAAGC	At3g09830
pcrk1(1)RP	ATATTCGCGGATTTCAAGTCC	At3g09830
SAIL129D02LP	ATGAAAACCTCCGTATCCCCAC	At5g03320
SAIL129D02RP	ATATCAGTGGAGGAACCTCGG	At5g03320
tag10LP	CTCATCGGACCATGACATACC	At5g06839
tag10RP	AATTATTGTGTGGGATGTGGC	At5g06839
SALK_057609L P	TCAAAAACACATATACATGCATGCACA	At1g08320
SALK_057609R P	TCATGATGACTCCAAGTACGTACCATGC	At1g08320
SALK_140489.L P	TATCACGCAAATCATCTGCAG	At2g39110
SALK_140489.R P	TCTTGGAGTGGATCAGACCAC	At2g39110
chyr1LP	ACACATTGGTGTTCCTCC	At5g22920
chyr1RP	AATGGAGATTGGATCTGGGC	At5g22920
560GABILP	TGTATCCACTGTGGAGTGTGC	At5g25560
560GABIRP	GCCACCACATGGTACTGTACC	At5g25560
560SALKLP	CCTCCAAGCACTTCTGATGAATT	At5g25560
560SALKRP	CCCAGTTGACAAGACCTCTATC	At5g25560

Expression analysis		
Name	Sequence	AGI Code
MYB26 F	TGACCCCATTTGATGTTCCCAACC	At3g13890
MYB26 R	GGTAACGCTGGCCATTGGAGA	At3g13890
At1g08320_288F	AGATGGATCGTCCAGTTTCG	At1g08320
At1g08320_Jie_491 R	TCGCTGCTCTCTCCTCCTGAC	At1g08320
MS1_b_F	GAGAAGGCACTTGAGTCGGTCC	At5g22260
MS1_b_R	TCTCGGTAAAGGCTGGTTAGGT	At5g22260
Actin7_F	TGGCCGATGGTGAGGATATT	At5g09810
Actin7_R	AACGGCCTGAATGGCAACAT	At5g09810
TGA10F	GCTGCAACAGTCCACACAAG	At5g06839
TGA10R	TAAAGAGGTCAAGCTGGCGG	At5g06839
At1g78440_F	TGGTGACTCTCTCCAGGTGAT	At1g78440
At1g78440_R	CCTCGTACAACCTCTCGTCC	At1g78440
At1g10770_F	ATCTTTGTGCCGACCTCTGG	At1g10770
At1g10770_R	CAGACACAGCCGCAGTTAGA	At1g10770
NST1_432F	GCCGTAGAATTGGGATGAGA	At2g46770
NST2_1392R	GAAGCTCCTCCGACGGGACT	At2g46770
NST2_F	CCAAGTCAACAACCTGCCACG	At3g61910
NST2_R	GTCGTTCTCGCGAAATCTGC	At3g61910
PKSP2_653F	AAAGAGGGTTGCAGGGTCAT	At5g03320
PKSP2_1081R	CTGGAGCTGCGTATCCCATT	At5g03320
PCRK1_321F	GGTGTCTGTCAGTGGACAAA	At3g09830
PCRK1_752R	TATGCCCCTGCAACCCTCTT	At3g09830
At2g39110_F	TAGAAGAGGTCTACAGGGGCA	At2g39110
At2g39110_R	CGTGAGGTGTCCTGTTTGGA	At2g39110