

## **Chapter 5**

# **Promoter analysis in Abscission-related genes**

### **5.1 Introduction**

#### **5.1.1 Promoter**

A promoter is a defined region of DNA which regulates the spatial and temporal elements of transcription. In the process of transcription initiation, RNA polymerase must attach to the DNA in order to synthesize RNA, therefore promoters contain binding sites for RNA polymerase. Promoters also contain binding sites for binding certain transcription factors in order to regulate gene expression.

### 5.1.2 Transcription factor (TF)

A TF is a protein that binds to specific promoter of DNA, for the purpose of controlling the transcription of the DNA to mRNA (Karin, 1990; Latchman, 1997). A TF may act alone or with other proteins to activate or repress the recruitment of RNA polymerase (Roeder, 1996; Nikolov, 1997). A TF also plays a crucial role in the regulation of gene expression, which allows cells to respond rapidly to changing conditions and to attenuate protein functions. The activity of regulatory transcription factor proteins can be modulated in three ways: (1) binding of effector molecules (e.g. hormone); (2) protein-protein interaction (e.g. dimerisation) and (3) post-translational modification of the factor (e.g. phosphorylation). The position where a TF binds to a DNA sequence is called the TF binding site (TFBS), or *cis*-regulatory element. Structural features of regulatory TFs bind to specific DNA binding domains and termed motifs. Other domains may be present for other purposes e.g. to bind a small effector molecule.

### 5.1.3 Abscission-related transcription factors

The *JOINTLESS* gene has been shown to play an important role in the development of the pedicel AZ in tomato and it has been identified as a member of the MADS-box family of TFs (Mao *et al.*, 2000). In *Arabidopsis*, *AGAMOUS-LIKE15* (*AGL15*) has been shown to also be a MADS-box TF which regulates abscission through its impacts on leaf and organ patterning (Fernandez *et al.*, 2000). Plants with the mutation in *AGL15* show a delay in flowering time, maturation and abscission (Fernandez *et al.*, 2000; Harding *et al.*, 2003; Lehti-Shiu *et al.*, 2005). Gene *AtDOF4.7*

has been identified as a DOF (DNA Binding with One Finger) family member in *Arabidopsis* and constitutive expression of *AtDOF4.7* exhibits a delay in floral organ abscission accompanied with the down-regulation of *PGAZAT* (Wei *et al.*, 2010; Gonzalez-Carranza *et al.*, 2002).

#### **5.1.4 Promoter analysis**

ChIP-on-chip technology has been used to investigate protein-DNA interactions *in vivo* and using which a large number of TFs and transcription factor binding site (TFBS) have been identified (Ren *et al.*, 2000; Iyer *et al.*, 2001; Lieb *et al.*, 2001). It has been suggested that genes that share similar functional roles are often regulated by a common TF (Qiu, 2003; Werner, 2001). Previous studies have successfully identified unknown genes acting in concert using conserved motifs in promoters sequences (Werner, 2001).

In the area of abscission, whilst more and more abscission-related genes are discovered, their transcriptional regulation remains unknown. Most of the identified abscission-related genes have a specific expression in the AZ cells. It is likely that there may be certain TFs or *cis*-elements regulating this process of specific AZ expression. In order to identify putative abscission-related TFs, TFBS and common motifs, a promoter analysis of abscission-related genes was undertaken.

## 5.2 Results

### 5.2.1 Identification of the 10 bps motif

A number of genes expressed specifically in AZ of *Arabidopsis*, such as *IDA*, *IDLs*, *HAESA*, *HSL2*, *PGAZAT*, *QRT2* *et al.*, were classified by potential function and analyzed by a web-tool “GIBBS Motif Sampler” (<http://bayesweb.wadsworth.org/gibbs/gibbs.html>). A motif **AATATACATT** was found that was conserved between two abscission-related polygalacturonases (PG) genes *PGAZAT* (*At2g41850*) (Gonzalez-Carranza *et al.*, 2002) and *QUARTET2* (*QRT2*) (*At3g07970*) (Ogawa *et al.*, 2009) (Figure 5.1). The Fig 5.1 showed that the motif (the red square) is located at about 1,000 bp upstream of the translation start site of both *PGAZAT* and *QRT2*.



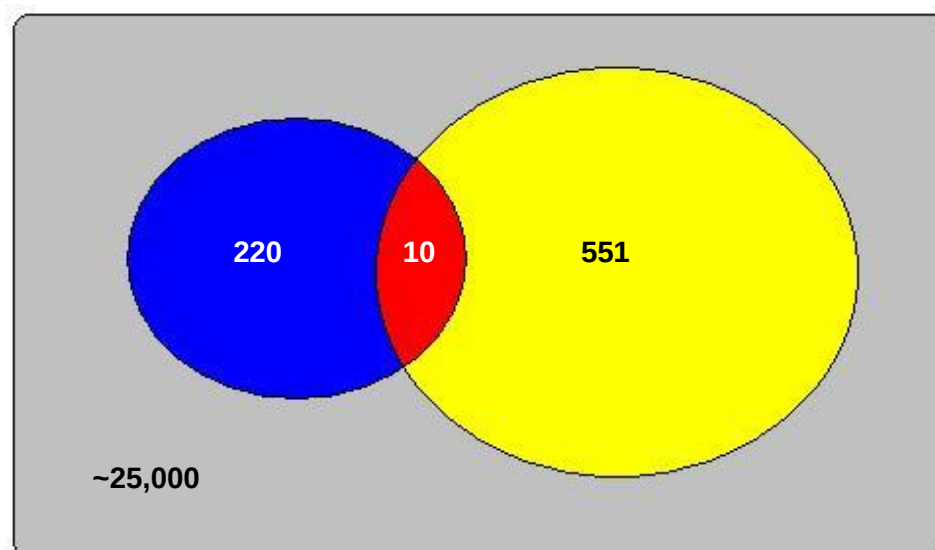
**Figure 5.1** The location of the motif **AATATACATT** in the abscission-related genes.

From this analysis the motif **AATATACATT** could be important for the following reasons.

1. In *Arabidopsis*, both of the two PGs (*PGAZAT* and *QRT2*) have been shown to play an important role in degradation of pectin in the abscission process and were expressed specifically in AZ. During previous research, a small sequence of 67 base pairs promoter area was identified by deletion

analysis and fused to a minimal promoter following GUS (Gonzalez-Carranza *et al.*, unpublished data). The 67 bp fragment was sufficient to promote GUS signal specifically in floral abscission zones of *Arabidopsis thaliana*. The motif **AATATACATT** was located in the middle of the 67 bp fragment.

2. A PetMatch search was then carried out using web-tool in TAIR (<http://www.arabidopsis.org>). The motif **AATATACATT** was used as a probe and the targets were the genes that had the same motifs at -800 to -1,200 bps up-stream of the translation start site. A total of 220 genes were screened out that contained this motif. Recently, a stamen abscission zone transcriptome profile was determined and 551 abscission-related genes were identified (Cai and Lashbrook, 2008). An analysis of these two data sets identified 10 genes that contained the **AATATACATT** motif (Figure 5.2). This is double the number predicted by random occurrence in a total of 25,000 genes in *Arabidopsis thaliana*.

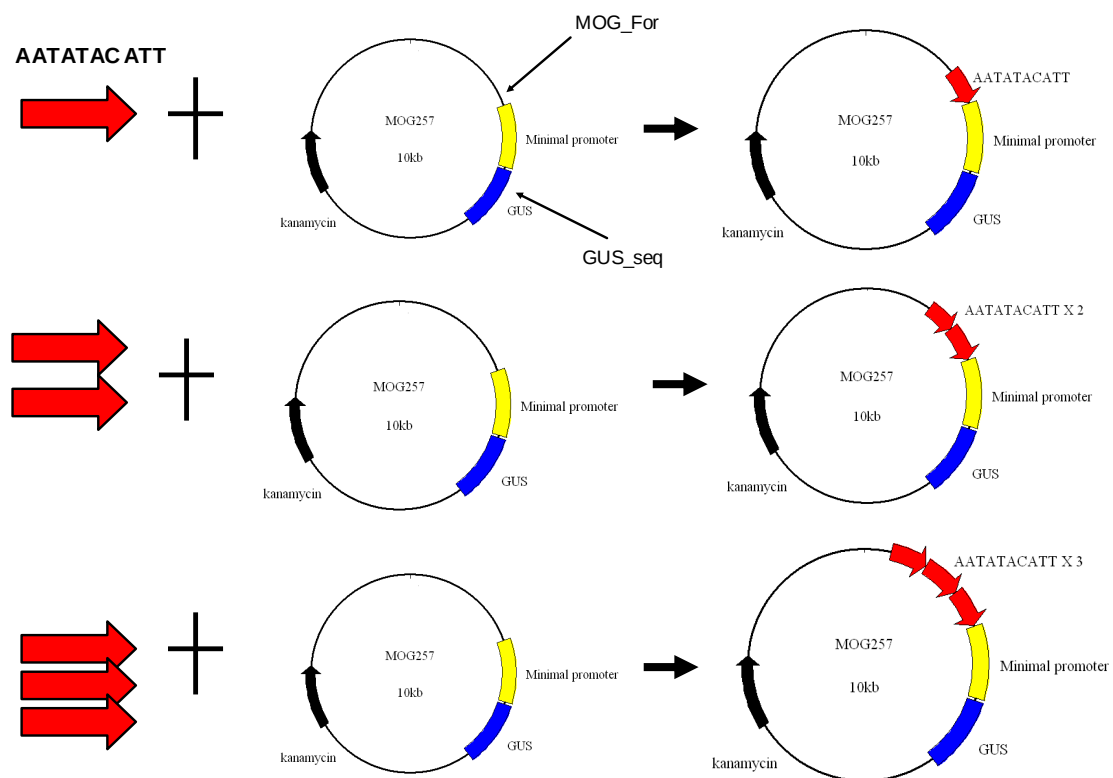


- Genes that were found by using PetMatch tool in TAIR.
- Genes that were generated by micro-array data from the stamen abscission zone transcripts.
- Genes that are shared between the two.
- Total amount of *Arabidopsis* genes.

**Figure 5.2** The picture shows the genes that are shared between the micro-array data and the PetMatch BLAST result.

### 5.2.2 Analysis of the function of the motif AATATACATT.

The motif **AATATACATT** could be a binding site for a TF that regulates the expression of abscission-related genes. To explore this hypothesis we fused this motif with a minimal promoter following the GUS reporter gene. The vector MOG257 was applied. The information of MOG257 is described in chapter 2. Three constructs were generated with the motif **AATATACATT** repeated one, two and three times in three different insertions (Figure 5.3). The resulting constructs were named *RP1:GUS*, *RP2:GUS* and *RP3:GUS*.



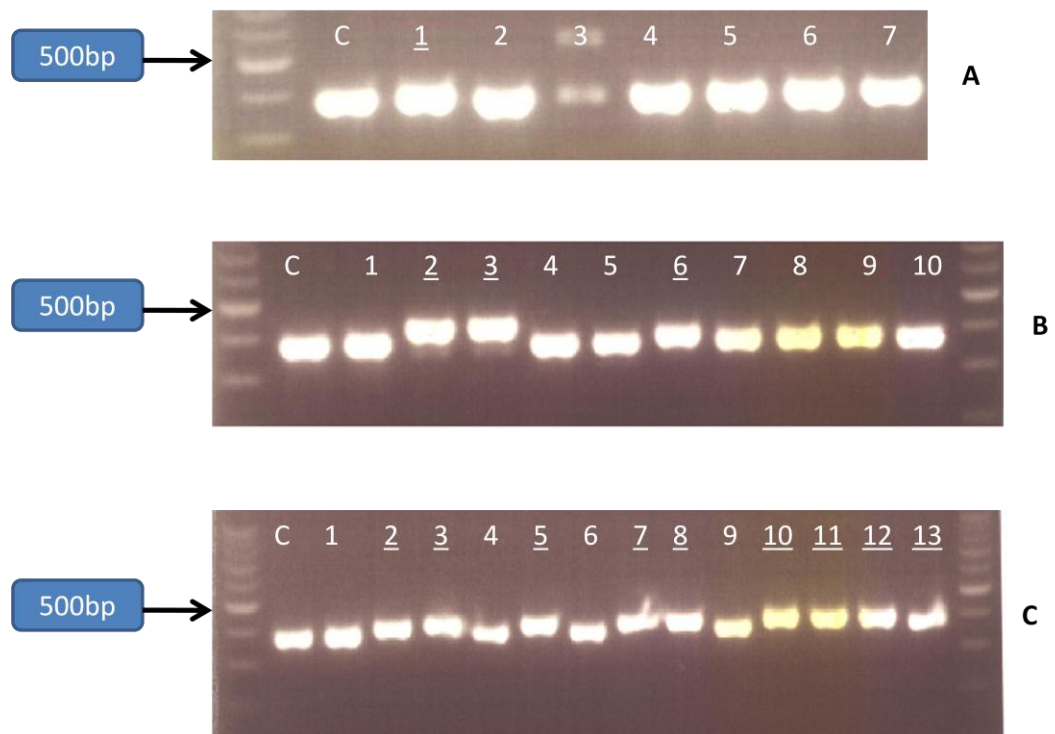
**Figure 5.3** The 10 bp motif **AATATACATT** was transformed into MOG257 vector. The positions of primers MOG\_Forward (MOG\_For) & GUS\_sequence (GUS\_Seq) are shown in the picture. The diagrams are not drawn to scale.

The insertions were designed with the repeated motifs, restriction enzyme digestion sites HindIII and XhoI, and protection base pairs. Primers MotifRepeat1, 2 and 3 were designed to achieve this objective. The information of the primers is shown in section 2.1.3. The primers were annealed together to get the double stranded DNA fragments following the procedure described in section 2.2.14.

After the annealing, both the inserts and the vector were digested by restriction enzymes HindIII and XhoI as described in section 2.2.15. In case the inserts fused together, the inserts were dephosphorylated

following the protocol described in section 2.2.16. The inserts and vector were purified before a ligation reaction was carried out (section 2.2.9).

A PCR was then performed to confirm the constructs using the primers MOG\_Forward and GUS\_sequence (Figure 5.4). The confirmed constructs were then sent for sequencing before they were transformed into *Agrobacterium tumefaciens* C58 following the protocol in section 2.2.11. The Kanamycin & Rifampicin resistant colonies were confirmed by PCR using primers MOG\_Forward and GUS\_sequence (Figure 5.5) before they were transformed into *Arabidopsis thaliana* by the “Floral Dip method” which has been described in section 2.2.12.

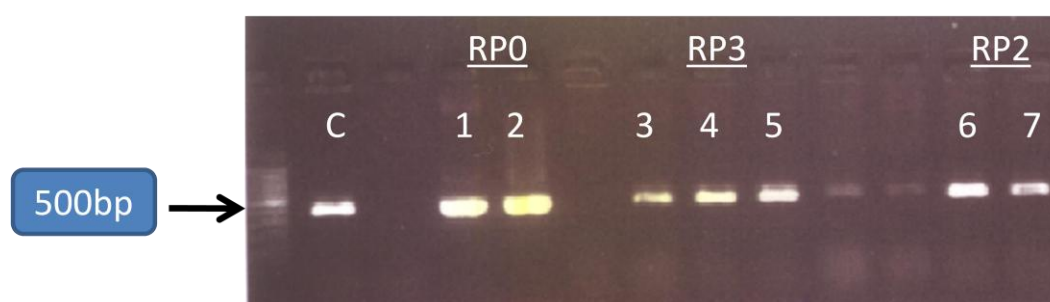


**Figure 5.4** PCR for selection of the colonies that contain the RP1:GUS (**A**), RP2:GUS (**B**) and RP3:GUS (**C**) using primers MOGForward and GUSSequece. **C**: Positive control using MOG257 vector. The Arabic numbers showed the colony numbers. Numbers underlined showed the successful constructs.



**Figure 5.5** PCR analysis of plasmids from the colonies transformed into *Agrobacterium tumefaciens* C58. 0: MOG257 vector without any insert as a control. 1, 2 & 3 are the MOG257 vectors with the motif **AATATACATT** repeating for 1, 2 and 3 times respectively. A clear 10bp difference among 2, 3 and 0 indicates the successful construct. Lane 1 showed an unsuccessful construct.

A total of 2, 2 and 3 independent primary transformants for *RP0:GUS*, *RP2:GUS* and *RP3:GUS* respectively were obtained from Kanamycin-resistant screening. A genomic PCR was then performed to confirm the presence of the GUS gene using primers MOG\_Foward and GUSSequence (Figure 5.6).



**Figure 5.6** PCR analysis of the primary transformants using primers MOG\_Foward and GUS\_Sequence. C: Positive control using MOG257 vector. RP0: Primary transformants containing MOG257 vector. RP3: Primary transformants containing MOG257 vector with motif **AATATACATT** repeating for 3 times. RP2: Primary transformants containing MOG257 vector with motif **AATATACATT** repeated for 2 times.

In order to confirm the GUS expression of the T1 transformants, GUS assay was then carried out using flower tissue of *RP2:GUS* and *RP3:GUS* using *RP0:GUS* as negative control and *G2:GUS* as positive control.

However the results showed that no GUS accumulation was detected at any time during floral organ shedding.

## 5.3 Discussion and future work

### 5.3.1 Identification of potential abscission-related motifs

Specific TF genes including binding sites in the promoter region regulate gene expression. It has been suggested that genes that share similar functional contexts are often driven by common transcription factors combinations (Qiu, 2003; Werner, 2001). Analysis of these transcription factor combinations could also be used to identify new candidate genes that are involved in the same biological processes (Werner, 2001; Moss, 2007).

The motif **AATATACATT** has been identified as a potential DNA binding site and we have reasons (chapter 5.3.1) to believe that it may play an important role in regulating expression of the genes *PGAZAT* and *QRT2* specifically in the AZ. To test this hypothesis, this motif coupled to a minimal promoter was fused to reporter gene *GUS*. However the result showed that two or three copies of this motif were not sufficient to promote *GUS* expression.

One possible explanation for this is that the AZ specific expression requires not only the motif **AATATACATT** but also a combination of TF binding sites (TFBS) in the promoter.

The motif combinations have been shown to be essential in directing gene expression (Krivan and Wasserman 2001; Aerts *et al.*, 2003). In

eukaryotes, TFs often work together (transcription factor binding sites complex) in a combinatorial fashion to regulate a cell to respond to environmental and developmental signals (Zhu *et al.*, 2005). There is evidence that the spacing between TF binding sites in a complex can regulate similar biological events that these are often highly conserved (Moss *et al.*, 2007). In the case of abscission-related TFs, a previous study has identified a 67 bps DNA fragment, together with minimal promoter, to be sufficient to promote GUS expression in AZ (Gonzalez-Carranza *et al.*, unpublished data). The 67 bps fragment, which includes motif **AATATACATT**, could be a TFBS module. If motif **AATATACATT** is a TFBS, it might need other motifs in the complex to act together and correct motif spacing might be needed. The other motif(s) are likely to be located within the 67 bps.

In order to test if **AATATACATT** is an important motif in the transcription of abscission-related genes, the motif could be deleted in the 67 bps fragment, which will be used to promote GUS expression with a minimal promoter. The loss of GUS signal in AZ may lead to a phenotype that could test the function of motif **AATATACATT**. At the same time, further identification of new potential abscission-related motifs should be carried out at the benefit of various online databases and techniques such as ChIP-on-chip technology.