



## Analysis of the *SHP2* enhancer for the use of tissue specific activation tagging in *Arabidopsis thaliana*

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### Abstract

Activation tagging is a powerful tool to identify new mutants and to obtain information about possible biological functions of the overexpressed genes. The quadruple cauliflower mosaic virus (*CaMV*) 35S enhancer fragment is a strong enhancer, which is most commonly used for this purpose. However, the constitutive nature of this enhancer may generate lethal mutations or aberrations in different plant organs by the same overexpressed gene. A tissue-specific activation tagging approach may overcome these drawbacks and may also lead more efficiently to the desired phenotype. For this reason the *SHATTERPROOF2* (*SHP2*) promoter fragment was analysed for enhancer activity. The *SHP2* gene is involved in dehiscence zone development and expressed during silique development. The aim of the experiments described here was to identify a dehiscence zone specific enhancer that could be used for tissue-specific activation tagging. The chosen *SHP2* enhancer fragment was found to be expressed predominantly in the dehiscence zone and showed enhancer activity as well as ectopic expression activity. This activity was not influenced by its orientation towards the promoter and it was still functional at the largest tested distance of 2.0 kb. Based on these results, the *SHP2* enhancer fragment can potentially be used in a tissue-specific activation tagging approach to identify new *Arabidopsis* mutants with an altered dehiscence zone formation.

*Key words:* *Arabidopsis*, development, pod shattering, transcription factors, mutants.

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### Introduction

Activation tagging has become an upcoming tool to generate mutant plants. It is an alternative approach for gene function analysis, because loss-of-function mutations has its limitation in cases of functional gene redundancy (*Arabidopsis* Genome Initiative, 2000). Activation tagging was proposed as a novel gene isolation method in which a gene is either ectopically or constitutively overexpressed compared to normal expression levels (Walden *et al.*, 1994). Walden *et al.* (1994) designed a T-DNA based activation tagging approach to identify and isolate novel genes from tobacco, and since then it has been largely applied using either T-DNA insertion strategies (Borevitz *et al.*, 2000; Ito and Meyerowitz, 2000; Lee *et al.*, 2000; van der Graaff *et al.*, 2000; Weigel *et al.*, 2000; Huang *et al.*, 2001) or transposon based approaches (Wilson *et al.*, 1996; Marsch-Martinez *et al.*, 2002). This technology has been applied successfully to many plant species like *Arabidopsis*, rice, tomato, petunia and tobacco (Weigel *et*

*al.*, 2000; Jeong *et al.*, 2002; Zubko *et al.*, 2002; Ahad *et al.*, 2003; Mathews *et al.*, 2003).

Activation tagging is based on strong transcriptional enhancer sequences that can activate gene expression in the vicinity of the site where the enhancer was inserted into the genome. The most commonly used enhancer is a quadruple combination of the cauliflower mosaic virus (*CaMV*) 35S enhancer (Odell *et al.*, 1985; Hayashi *et al.*, 1992). These 4x35S enhancer elements have been reported to strongly enhance endogenous gene expression rather than ectopically or constitutively overexpress genes (Neff *et al.*, 1999; van der Graaff *et al.*, 2000). It has been demonstrated that it can stimulate gene expression of neighboring genes independently of its orientation, up to a distance of 3.6 kb (Weigel *et al.*, 2000) or even up to 5.0 kb (Chalfun-Junior *et al.*, 2005).

It is tempting to use this technique in a random-like approach to isolate new mutants and analyse in more detail the overexpressed genes. In some cases, the mutant displays a phenotype that can either be directly associated with the gene function of the activated gene (Zubko *et al.*, 2002) or may provide an indication of the pathway in which the gene is involved (Kardailsky *et al.*, 1999; Zhao *et al.*, 2001; Yuen *et al.*, 2003). The activation tagging method has also been used as a novel approach to isolate

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suppressor mutants of known mutant phenotypes (Neff *et al.*, 1999; van der Graaff *et al.*, 2003). However, when searching for specific mutants with a more specific phenotype, the 4x35S enhancer is not very attractive to use, as it will induce aberrations in plant tissues other than the specific one that was intended to be modified. In that case, tissue-specific enhancement could directly lead to the mutants of interest. To test this hypothesis we have characterized a tissue-specific enhancer that may be applicable to efficiently generate activation mutants with an altered pod shattering phenotype.

Precocious pod shattering is a phenomenon in which fruits lose their seeds before harvesting time, generating high losses of agronomic crops, *e.g.*, rapeseed. Engineering for shatter resistant plants allow the plants to grow until the fruits are fully ripened, resulting in an optimal product quality and increased crop yield. The closely related *MADS* box transcription factors *SHATTERPROOF1* (*SHP1*) and *SHATTERPROOF2* (*SHP2*) control seed dispersal in *Arabidopsis* by regulating the development of the dehiscence zone (Ferrandiz *et al.*, 2000; Liljegren *et al.*, 2000; Ferrandiz, 2002). In *shp1shp2* double mutant plants, the dehiscence zone is completely absent and the fruits fail to open.

Here, we describe the identification of a *SHP2* enhancer taken from its natural promoter, which contains all necessary characteristics required for a tissue-specific activation approach.

## Material and Methods

### Plant material and transformation

Constructs were introduced into *Arabidopsis thaliana* ecotype Col-0 using *Agrobacterium tumefaciens* strain GV3101 and the floral dip method (Clough and Bent, 1998). For selection of transformants, seeds were surface sterilized by vapor phase sterilization (<http://plantpath.wisc.edu/~afb/vapster.html>), and selected on medium containing kanamycin. Plants were grown in soil, under normal greenhouse conditions (22 °C, 14/10 h light/dark).

### Constructs

#### *The SHP2 enhancer fragment*

Primers were designed at -1275 bp and -55 bp from the transcription initiation site of the *SHP2* gene (forward primer *SHP2F* 5'-GTCGACAAGCTTAAGTTCTTTCTT GAAATG-3', reverse primer *SHP2R* 5'-GTCGACAA GCTTCACTTAACTGCTGCTTCAAC-3'). Both primers were extended with sequences for digestion sites to facilitate subcloning (*Hind*III and *Sal*I sites underlined). DNA of *Arabidopsis thaliana* ecotype Col-0 was used as template for PCR amplification. Using these primers a band of the expected 1220 bp was obtained, cloned (in pGEMT-Easy®, Promega) and sequenced to confirm its identity.

#### *SHP2 enhancer fused to minimal -47-35S::GUS*

The minimal -47-35S promoter *GUS* vector, *GUSXX-47* (Pasquali *et al.*, 1994) was obtained from J. Memelink, (Leiden University, Leiden, The Netherlands). The *SHP2F* primer was extended with a *Hind*III site and the reverse primer with a *Sal*I site, the fragment amplified and ligated in the *Hind*III-*Sal*I site upstream of the -47-35S promoter, which was fused to the *GUS* reporter gene. The whole fragment was subcloned in pBINPLUS (van Engelen *et al.*, 1995) using *Hind*III-*Kpn*I, resulting in vector pGD751.

#### *SHP2 fragment fused to the pFBP1::GUS*

Both *SHP2* primers were extended with both *Hind*III and *Sal*I sites to facilitate the cloning of the vectors described next. Several constructs were generated to test the enhancer activity of the *SHP2* fragment. For that reason the *SHP2* fragment (as a *Hind*III-*Hind*III fragment) was ligated upstream of the *FBP1* promoter present in vector pFBP12E (Angenent *et al.*, 1993). This resulted in a sense fusion of the *SHP2* enhancer to the 'short' 220 bp *pFBP1::GUS* (pGD418).

The *Sal*I-*Sal*I fragment was introduced in the *Sal*I digested pFBP12E vector, resulting in a sense fusion of the *SHP2* enhancer to the 'long' 1040 bp *FBP1* promoter fused to the *GUS* reporter gene (pGD393). For the other constructs, the *Sal*I-*Sal*I fragment of the cloned PCR *SHP2* enhancer fragment was first subcloned in pBluescript SK<sup>+</sup> vector (Promega) in both orientations (pARC012 and pARC013). The 'long' *pFBP1::GUS* fragment was also subcloned as an *Eco*RI-*Eco*RI from pFBP12E into pBluescript SK<sup>+</sup> vector in both orientations (pARC014 and pARC015). A *Cla*I-*Xho*I fragment containing the sense or antisense *SHP2* enhancer was then inserted adjacent to the *pFBP1::GUS* fragment in both vectors pARC014 and pARC015. This resulted in an antisense fusion of the *SHP2* enhancer upstream of the 'long' *pFBP1::GUS* fragment and in vectors where the *SHP2* enhancer was downstream of this fragment in either sense or antisense orientation. The total inserts of these vectors were subcloned in the binary vector pBINPLUS® by using *Xba*I and *Kpn*I. All vectors were checked in detail by restriction sites and fragment length analysis.

### GUS activity

Histochemical localization of GUS activity was performed as described by Jefferson *et al.* (1987).

## Results

### Tissue specificity of the *SHP2* enhancer

It has been shown previously that the *SHP2* gene is expressed in the dehiscence zone of *Arabidopsis* siliques (Savidge *et al.*, 1995; Liljegren *et al.*, 1998; Liljegren *et al.*, 2000). To test whether a putative *SHP2* enhancer fragment

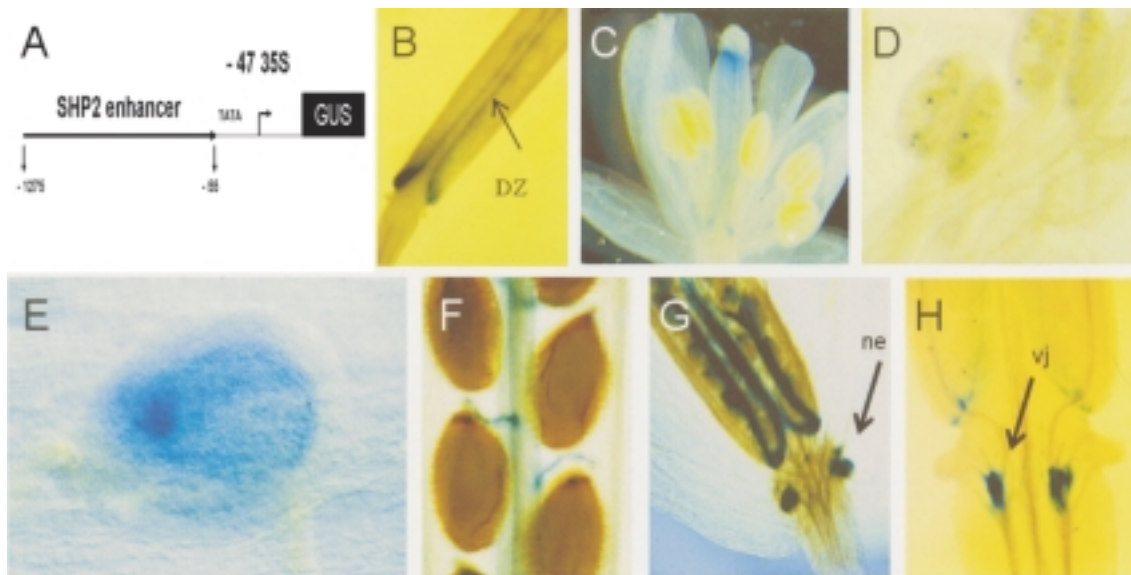
contains the *cis*-acting elements and all its tissue specific regulating sequences, a 1220 bp upstream sequence was tested for its promoter/enhancer activity. This 1220 bp fragment was chosen from -1275 bp to -55 bp upstream of the transcription initiation site, which was also 8 bp upstream of a putative TATA box. This 1220 bp *SHP2* promoter fragment contains a CarG-box like sequence (Savidge *et al.*, 1995), which is the putative *AGAMOUS* binding site and which is very likely to be important for its regulation. This promoter fragment (from now on referred to as the *SHP2* enhancer) was fused to a minimal -47-35S promoter, which was fused to the *GUS* reporter gene (*GUSXX-47*) (Pasquali *et al.*, 1994). This construct will allow the analysis of its transcriptional enhancer activity and tissue specificity. This binary vector (Figure 1A) and a control construct, which carries only the empty vector (*GUSXX-47*), were introduced into *Arabidopsis* plants ecotype Col-0. In Table 1, the *GUS* expression patterns of 24 analyzed T1 plants are summarized. No *GUS* expression was observed in plants containing only the empty vector. In most of the *GUS* positive plants, *GUS* staining was observed in the dehiscence zone (Figure 1B). This expression in the dehiscence zone was seen at earlier stages during flower development (Figure 1C). Besides *GUS* expression in the dehiscence zone, some plants also displayed *GUS* expression in other tissues, for instance in pollen grains (Figure 1D), ovules (Figure 1E), funiculus (Figure 1F), nectaries (Figure 1G) and the vascular junction at the receptacle (Figure 1H). Similar to the results obtained here, *SHP1* and *SHP2* genes were previously described to be expressed in tissues like septum, ovules and funiculus (Ma *et al.*, 1991), in addition to the dehiscence zone (Savidge *et al.*, 1995; Liljegren *et al.*,

1998; Liljegren *et al.*, 2000). In some plants, no *GUS* staining was observed. Although no molecular analyses were performed on them, it is very likely that these plants were not transgenic or were not expressing the *GUS* gene properly (Table 1). Based on these results, it can be concluded that the 1220 bp *SHP2* enhancer still contains the dehiscence specific regulatory sequences of the *SHP2* promoter.

### Activity of the *SHP2* enhancer

Enhancers are defined as *cis*-acting DNA sequences that can increase the transcription level of genes by the binding of specific transcription factors. Full promoter activity also requires the DNA region containing the TATA box directly upstream of the transcription start site (Lewin, 2000). Enhancers usually can function in either orientation and separated from a minimal promoter domain. To obtain an enhancer for efficient tissue-specific activation tagging, these features are essential. To test all these features of the *SHP2* enhancer, several constructs were generated (Figure 2).

Because enhancers are not able to act alone (Lewin, 2000), a minimal or natural promoter is necessary. Therefore, the *FLORAL BINDING PROTEIN1* (*FBP1*) promoter was chosen because this petunia promoter regulates expression in whorl 2 and 3 of the flower (petals and stamens respectively) (Angenent *et al.*, 1993). In petunia, two different lengths of the *FBP1* promoter fragment were analysed, a 220 bp ‘short’ promoter and a 1040 bp ‘long’ *FBP1* promoter. Both promoter fragments showed the same specificity and levels of expression in petunia (Angenent *et al.*, 1993). Analysis of both promoter fragments in *Arabidopsis* showed the same results as for petunia (unpublished re-

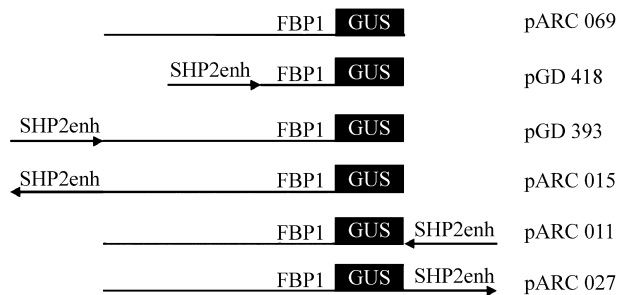


**Figure 1** - *GUS* expression pattern in *Arabidopsis* plants containing the construct *SHP2* fused to the minimal 35S promoter (-47-35S:::*GUS*). (A) Schematic representation of the construct (which is not drawn to scale). (B) *Arabidopsis* silique showing *GUS* expression in the dehiscence zone (DZ, arrow). (C) A young flower with *GUS* expression in the DZ. *GUS* expression is also present in (D) pollen grains, (E) ovules, (F) funiculus and (G) nectary (ne), and (H) vascular junction (vj) in the receptacle.

**Table 1** - GUS expression of *SHP2* enhancer-*GUS* plants (pARC751).

| Number of plants | L* | S* | P* | O* | N* | F* | C* | DZ* young | DZ* mature |
|------------------|----|----|----|----|----|----|----|-----------|------------|
| 5 (21%)          | -  | -  | -  | -  | -  | -  | -  | -         | -          |
| 10 (42%)         | -  | -  | -  | -  | -  | -  | -  | -         | -          |
| 3 (12.5%)        | -  | -  | -  | -  | -  | -  | -  | -         | -          |
| 3 (12.5%)        | -  | -  | -  | -  | -  | -  | -  | -         | -          |
| 1 (4%)           | -  | -  | -  | -  | -  | -  | -  | -         | -          |
| 1 (4%)           | -  | -  | -  | -  | -  | -  | -  | -         | -          |
| 1 (4%)           | -  | -  | -  | -  | -  | -  | -  | -         | -          |

\* L (Leaf); S (Sepals); P (Pollen); O (Ovules); N (Nectary); F (Funiculus); C (Carpels); DZ (dehiscence zone).



**Figure 2** - Schematic representation of the constructs in which the *SHP2* enhancer was fused to the *pFBPI::GUS* promoter-gene construct in different orientations. pARC069: the *pFBPI::GUS* construct used as control. pGD418: *SHP2* in sense orientation upstream of the short version of the *pFBPI* promoter. pGD393: the *SHP2* enhancer fragment is located upstream of the long *pFBPI::GUS* version. pARC015: the *SHP2* is also located upstream of the long *pFBPI::GUS* construct, however in antisense orientation. pARC011: the *SHP2* is placed downstream of the *GUS* gene in sense orientation and *SHP2* is placed in antisense orientation in the same long version of the *FBPI* promoter, in pARC027. This schematic representation is not drawn to scale.

sults). Therefore, as control, a binary vector harbouring the 'long' *FBPI* promoter sequence fused to *GUS* was introduced into *Arabidopsis* plants. Several transgenic plants were obtained with different levels of GUS expression in petals (often very weak) and filaments (stronger) (Table 2 and Figure 3A, B). This result indicates that this promoter acts as expected, with the same specificity as in petunia (Angenent *et al.*, 1993), and can be used to test the *SHP2* enhancer. Figure 2 summarises the vectors that were used to test the *SHP2* enhancer activity based on its ectopic expression in combination with the *FBPI* promoter. In the first two tester constructs, the *SHP2* enhancer was fused in sense orientation, with both the 'short' and the 'long' versions of the *FBPI* promoter resulting in pGD418 and pGD393 respectively (Figure 2). These constructs were used to test whether the *SHP2* enhancer fragment could ectopically activate GUS expression in combination with the *FBPI* promoter and, moreover, whether the enhancer still keeps its tissue-specific activity when combined with a natural promoter. By using the 'short' and 'long' version of the *FBPI* promoter, the influence of the distance towards

the promoter was analysed. The other three constructs should identify whether the *SHP2* enhancer acts independently of its orientation and position related to the coding sequence. The results of the GUS staining of the transgenic *Arabidopsis* plants expressing these constructs are summarised in Table 2.

In general, all constructs that contained the *SHP2* enhancer fused in either way to the *pFBPI::GUS*, showed the *FBPI*-driven expression in whorls 2 and 3 (Figure 3C) and, also ectopic GUS expression in the dehiscence zone (Figure 3D). In whorls 2 and 3, the GUS expression driven by the *FBPI* promoter was similar to or in some lines slightly enhanced compared to the expression observed in plants that contained the control construct with only the 'long' *pFBPI::GUS* construct (pARC069). The *SHP2* enhancer region drove ectopic expression to the dehiscence zone, however it did not seem to promote up-regulation of the *FBPI* promoter activity. As expected, this ectopic expression driven by the *SHP2* enhancer was mostly seen in the dehiscence zone tissue. However in some plants, GUS staining in pollen grains was observed as well (data not shown).

The distance of the *SHP2* enhancer relative to the promoter sequence did not seem to influence the frequency of plants showing ectopic expression in the dehiscence zone. When the *SHP2* enhancer was situated downstream of the *GUS* gene (pARC011 and pARC027) (Figure 2), the enhancer was still able to ectopically activate the expression with the same frequency as observed for the upstream sense position of the *SHP2* enhancer (Table 2).

The only construct that did not result in ectopic expression in dehiscence zone tissue was construct pARC015 (Table 2), which contains the *SHP2* enhancer positioned upstream, in an anti-sense orientation, linked to the 'long' *pFBPI::GUS* fragment. Out of fourteen T1 plants analysed, 10 showed expression in whorls 2 and 3 only.

## Discussion

Tissue specific activation tagging is a novel approach to generate mutants for a specific trait of interest. Requirements for such a strategy are that: i) the used enhancer frag-

**Table 2** - GUS expression observed in tissues of transgenic *Arabidopsis* plants containing different constructs (see Figure 2).

| Name    | Construct  | N. of plants | FPB1 expression |    |    | DZ*   |     | N* | O* | V* |
|---------|--|--------------|-----------------|----|----|-------|-----|----|----|----|
|         |  |              | P*              | A* | F* | Young | Old |    |    |    |
| pARC069 | Long <i>FBP1::GUS</i>                              | 7            | +               | +  | +  | -     | -   | -  | -  | -  |
|         |  | 6            | -               | -  | -  | -     | -   | -  | -  | -  |
| pGD418  | <i>SHP2</i> enh sense<br>Short <i>FBP1::GUS</i>    | 2            | +               | +  | +  | -     | -   | -  | -  | -  |
|         |  | 2            | +               | +  | +  | +     | +   | -  | -  | -  |
|         |  | 1            | +               | +  | +  | -     | -   | -  | +  | -  |
|         |  | 1            | +               | +  | +  | +     | +   | +  | +  | -  |
|         |  | 5            | -               | -  | -  | -     | -   | -  | -  | -  |
| pGD393  | <i>SHP2</i> enh sense<br>Long <i>FBP1::GUS</i>     | 16           | +               | +  | +  | -     | -   | -  | -  | -  |
|         |  | 13           | +               | +  | +  | +     | +   | -  | -  | -  |
|         |  | 9            | -               | -  | -  | +     | +   | -  | -  | -  |
|         |  | 5            | -               | -  | -  | -     | -   | -  | -  | -  |
| pARC015 | <i>SHP2</i> enh antisense<br>Long <i>FBP1::GUS</i> | 10           | +               | +  | +  | -     | -   | -  | -  | -  |
|         |  | 4            | -               | -  | -  | -     | -   | -  | -  | -  |
| pARC011 | Long <i>FBP1::GUS</i><br><i>SHP2</i> enh sense     | 2            | +               | +  | +  | -     | -   | -  | -  | -  |
|         |  | 13           | +               | +  | +  | +     | +   | -  | -  | -  |
|         |  | 4            | +               | +  | +  | +     | +   | -  | -  | +  |
|         |  | 2            | +               | +  | +  | -     | -   | -  | -  | -  |
|         |  | 7            | -               | -  | -  | -     | -   | -  | -  | -  |
| pARC027 | Long <i>FBP1::GUS</i><br><i>SHP2</i> enh antisense | 1            | +               | +  | +  | -     | -   | -  | -  | -  |
|         |  | 3            | +               | +  | +  | +     | +   | -  | -  | -  |
|         |  | 1            | -               | -  | -  | +     | +   | -  | -  | -  |
|         |  | 1            | -               | -  | -  | -     | -   | -  | -  | -  |

\* DZ (dehiscence zone) P (petals); A (anthers); F (filaments); N (nectary); O (ovule); V (vascular bundle).

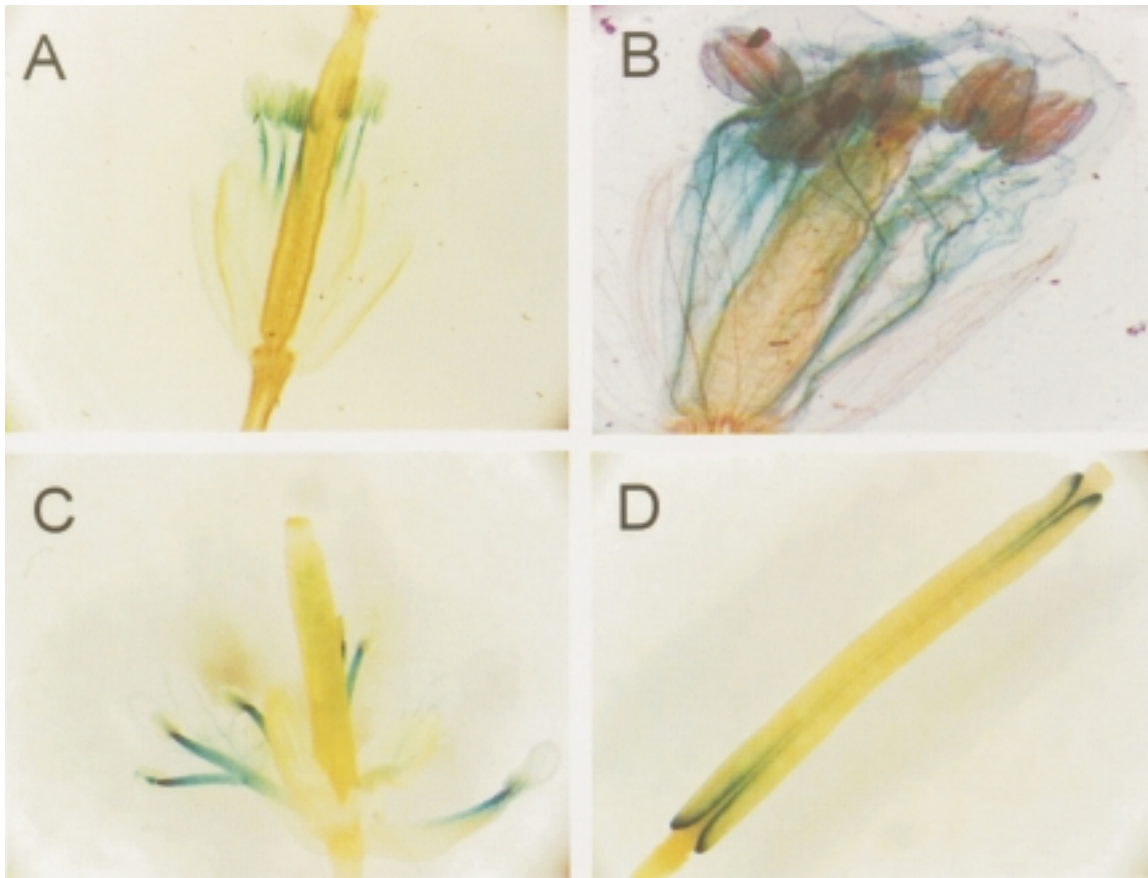
ment shows tissue specificity, avoiding unwanted side effects in other tissues ii) the activity of the enhancer should still be functional at least at several kbs from the natural promoter or gene, increasing the efficiency that genes will be activated, and iii) the insertion in the genome should be carried out by a system that minimizes the chances of complex insertion integrations (Nacry *et al.*, 1998), which can cause silencing of the introduced enhancer (Chalfun-Junior *et al.*, 2003).

Here we show that the 1220 bp *SHP2* promoter fragment taken from the upstream sequence of the *SHP2* gene contains most of the *cis*-regulatory sequences. It has been previously reported that the *SHP2* gene is expressed in the dehiscence zone of the *Arabidopsis* silique, in addition to other tissues like septum, ovules and funiculus (Ma *et al.*, 1991; Savidge *et al.*, 1995; Liljegren *et al.*, 1998; Liljegren *et al.*, 2000). The *SHP2* enhancer sequence chosen in our study is mainly expressed in the dehiscence zone, although GUS expression was also observed in ovules, funiculi and nectaries. The expression occasionally observed in a few pollen grains, nectaries and the vascular junction at the receptacle were not described previously. We did not test GUS expression driven by the complete endogenous *SHP2*

promoter in this same setup, which makes a comparison between published *in situ* hybridisation data and our data obtained with the enhancer studies more difficult. In conclusion, we demonstrated that it is possible to confer specific and ectopic expression of a particular gene by using an upstream enhancer sequence combined with minimal promoter elements.

The second prerequisite for an efficient activation tagging approach is that the enhancer activity should reach as far as possible from the minimal promoter elements (*e.g.*, the TATA box). Based on the results obtained with the constructs in which the *SHP2* enhancer was fused to the *FBP1* promoter (in combination with the *GUS* reporter gene), we detected ectopic expression driven by the *SHP2* enhancer in most of the combinations. In some of the plants, the GUS staining in the dehiscence zone was very strong and comparable with *-47-35S::GUS* transgenics. This was even the case when the enhancer was inserted downstream of the *GUS* gene, which resulted in GUS expression levels comparable with those for the upstream sense positions.

It still remains unclear why the construct pARC015 revealed no ectopic activation. This improper function may be specific for the *FBP1* promoter and it is unlikely that this



**Figure 3** - GUS expression pattern in *Arabidopsis* plants containing the *SHP2* enhancer inserted in the *pFBP1::GUS* construct (pGD418). (A-B) *pFBP1::GUS* (pARC069) flower, showing the *FBP1*-driven expression in whorls 2 and 3 (petals and stamens, respectively), (A) in stamens and (B) in petals. (C) Flower of a line containing pGD418 construct, showing GUS expression in petals (vasculature) and anther filaments, and weak expression in the ovules. (D) Silique of a pGD418 line. Besides the *FBP1*-driven expression in petals and filaments, ectopic GUS expression is observed in the dehiscence zone.

is a general feature for an enhancer inserted in antisense orientation upstream of a native promoter. Our results demonstrate that the enhancer activity acts over a distance of at least 2.0 kb, making it interesting to explore it in a tissue-specific activation tagging strategy. When an even stronger activity would be required, it may be possible to create a tagging construct with a repeated *SHP2* enhancer, similar to experiments performed with the *35S* enhancer (Weigel *et al.*, 2000; Marsch-Martinez *et al.*, 2002). And this brings us to the third requirement for an efficient tagging strategy, *i.e.* avoidance of silencing, which could be induced by the insertion of repeated DNA sequences (Chalfun-Junior *et al.*, 2003).

Based on the findings described by Marsch-Martinez *et al.* (2002) and our own observations on the methylation of T-DNA based activation tagging using the quadruple *35S* enhancer (Chalfun-Junior *et al.*, 2003), we propose a transposon-based tagging strategy as being the most promising. Hopefully this strategy, in which the described *SHP2* enhancer could be used as tissue-specific enhancer, will lead to a better understanding of pod dehiscence and to

novel approaches to control the shattering behaviour of crops.

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