Molecular Breeding (2005) 15: 221–231
DOI 10.1007/s11032-004-5679-9
© Springer 2005

Isolation and characterization of a novel plant promoter that directs strong constitutive expression of transgenes in plants

Kai Xiao[1,3], Celia Zhang[1,4], Maria Harrison[2] and Zeng-Yu Wang[1,*]
[1]*Forage Improvement Division, The Samuel Roberts Noble Foundation, 2510 Sam Noble Parkway, Ardmore, OK 73401, USA;* [2]*Boyce Thompson Institute for Plant Research, Cornell University, Tower Road, Ithaca, NY 14853-1801, USA;* [3]*Current address: College of Agronomy, Hebei Agricultural University, Baoding 071001, China;* [4]*Current address: Biodefence Division, Lawrence Livermore National Laboratory, Livermore, CA 94551, USA; *Author for Correspondence (e-mail: zywang@noble.org)*

Received 13 May 2004; accepted in revised form 30 October 2004

Key words: Constitutive expression, Deletion analysis, *Medicago truncatula*, Promoter, Transgenic plant

Abstract

A novel, constitutively expressed gene, designated *MtHP*, was isolated from the model legume species *Medicago truncatula*. Sequence analysis indicates that MtHP most likely belongs to the PR10 multi-gene family. The MtHP promoter was fused to a β-glucuronidase gene to characterize its expression in different plant species. Transient assay by microprojectile bombardment and hairy root transformation by *Agrobacterium rhizogenes* revealed GUS expression in leaf, stem, radicle and root in *M. truncatula*. Detailed analysis in transgenic Arabidopsis plants demonstrated that the promoter could direct transgene expression in different tissues and organs at various developmental stages; its expression pattern was similar to that of CaMV35S promoter, and the level of expression was higher than the reporter gene driven by CaMV35S promoter. Deletion analysis revealed that even a 107 bp fragment of the promoter could still lead to a moderate level of expression. The promoter was further characterized in white clover (*Trifolium repens*), a widely grown forage legume species. Strong constitutive expression was observed in transgenic white clover plants. Compared with CaMV35S promoter, the level of GUS activity in transgenic white clover was higher when the transgene was driven by MtHP promoter. Thus, the promoter provides a useful alternative to the CaMV35S promoter in plant transformation for high levels of constitutive expression.

Introduction

In order to be functionally expressed in plants, a transgene must have a promoter that is recognized by RNA polymerase in the plant cells. A promoter initiates and regulates transcription, the first and the most important step of gene expression. Isolation and characterization of promoters is therefore important for controlling the expression of foreign genes in transgenic plants. Promoters that are useful for plant transgene expression include those that are constitutive, inducible, viral, and synthetic, as recently reviewed by Potenza et al. (2004).

A constitutive promoter is able to drive gene expression in many or all tissues of a plant. The most commonly used promoter for directing strong constitutive expression has been the Cauliflower Mosaic Virus (CaMV) 35S promoter (Odell et al. 1985; Jefferson et al. 1987; Medberry et al.

1992; Potenza et al. 2004). Although a number of constitutive promoters have been isolated from plants and used for the generation of transgenic plants, there is still a great need for novel plant sequences that function as promoter elements for the high-level expression of transgenes. A wider range of effective promoters would also make it possible to introduce multiple transgenes into plant cells while still avoiding the risk of homology-dependent gene silencing.

Here we report the isolation and characterization of a constitutive promoter from the model legume species *Medicago truncatula*. The promoter is able to direct higher levels of GUS expression than that of the CaMV promoter in a range of plant species. Deletion analysis revealed that a short fragment of the promoter region is still capable of directing gene expression at levels of approximately 47% of the 'full-length' promoter.

Materials and methods

Isolation of cDNA and genomic DNA sequences from M. truncatula

The *MtHP* gene was identified during the search for a closely related, root-specific gene *Mt12a*, which was obtained by screening a *M. truncatula* cDNA library (Liu et al. 1998) using the pea RH2 (Mylona et al. 1994) cDNA sequences. *Mt12a* was used to screen a *M. truncatula* genomic library carried in Lambda Fix II (Stratagene) (Liu et al. 2003a) and a genomic clone was obtained. DNA was isolated from the genomic clone and digested by different restriction enzymes (e.g. *Bam*HI, *Hind*III, *Not*I, *Sal*I). Based on results of enzyme digestions, a restriction map was constructed. DNA of the genomic clone was double digested by restriction enzymes *Bam*HI and *Sal*I, and the digested fragments were directly subcloned into *Bam*HI digested- and *Bam*HI + *Sal*I digested-pBluescript vector. DNA was prepared from the subclones and sequenced (GenBank accession number AY751453). Unexpectedly, the sequences of the exons were different from that of the *Mt12a*. Since the exon sequences have not been characterized, we named it as *MtHP*, and continued to characterize the exon sequences and the 5′ regulatory region of the genomic clone.

Characterization of MtHP cDNA sequences

The sequence of the MtHP exons was used to search expressed sequenced tags (ESTs) in the web site of The Institute of Genomic Research (www.tigr.org). A full-length MtHP cDNA clone was obtained from the Noble Foundation EST sequencing project. The 3′ untranslated region of the cDNA clone was used as a probe to characterize the expression pattern of the gene by northern hybridization analysis. Total RNA was isolated from *M. truncatula* using TRI reagent (Molecular Research Center, Inc., Cincinnati, OH) and RNA gel blotting was carried out according to standard protocols ((Sambrook et al. 1989). Northern hybridizations were performed using the [^{32}P] dCTP-labeled MtHP cDNA as probe following the QuikHyb® Hybridization protocols (Stratagene, La Jolla, CA).

Characterization of the MtHP promoter

The MtHP promoter region was PCR amplified using primers 5′ TTTAAGCTTCTCGATCAATAGTTCAAACC 3′ (forward) and 5′ GGATCCATGGATGGTATAATGTATTAAAATGCTA 3′ (reverse). The forward primer contained a *Hind*III restriction site, and the reverse primer had a *Nco*I restriction site. The amplified promoter sequences were digested by *Hind*III and *Nco*I, and the fragment was isolated after gel electrophoresis. The isolated MtHP promoter fragment was cloned into *Hind*III and *Nco*I digested binary vector pCAMBIA3301 to replace the CaMV 35S promoter in front of the *β*-glucuronidase gene. For transient expression assays, gold particles were coated with DNA of the new MtHP-GUS vector, and a biolistic procedure described by Wang et al. (2003) was followed to bombard the leaf, stem and radicle of *M. truncatula*. For *Agrobacterium*-mediated transformation, the MtHP-GUS vector was first transferred into *A. rhizogenes* strain ARqua1 (Quandt et al. 1993), and used for hairy root transformation of *M. truncatula* following the procedure described by Boisson-Dernier et al. (2001). Because hairy root transformation can only be used to check gene expression in root tissue, the MtHP-GUS vector was transferred into *Agrobacterium tumefaciens* strain C58. Transgenic Arabisdopsis plants were generated following the

floral dip method (Clough and Bent 1998). Transgenic white clover (*Trifolium repens*) plants were obtained following the procedures described by Larkin et al. (1996). The tissue culture responsive germplasm Regen-SY (Bingham 1991) was used for alfalfa (*Medicago sativa*) transformation (Austin et al. 1995). Transgenic plants were grown at 24 °C in fluorescent light (240 μE m^{-2} s^{-1}) at a photoperiod of 16 h in the growth room.

To further characterize the promoter, a series of deletions of the promoter region were created by PCR using the primers D1 (5′ ACTGTACCAAA AAAAGCTTAAATAA 3′), D2 (5′ AATAGTA TATCAAGCTTTTTGTGAAC 3′), D3 (5′ ACA AATAGAAGCTTTATTTAGTCCG 3′), D4 (5′ CCCATTTTTAACTAAAGCTTTTTATT 3′), D5 (5′ AGTAAGCTTCTCCTTTAGATTGAG TC 3′) at the 5′ end and Rev (5′ GGCCATG-GATAATGTATTAAAATGCTAGGT 3′) at the 3′ end. A *Hind*III restriction site was introduced in the forward primers, and a *Nco*I restriction site was introduced in the reverse primer. The shortened promoter sequences obtained after PCR amplification were digested by *Hind*III and *Nco*I, and were cloned into *Hind*III and *Nco*I digested binary vector pCAMBIA3301 to drive the GUS gene.

Histochemical GUS staining was carried out on bombarded tissues and transformed hairy roots of *M. truncatula*, transgenic Arabidopsis plants, transgenic white clover plants and transformed alfalfa callus. The GUS expression pattern was visualized after incubating seedlings, plantlets or different tissues in 100 mM sodium phosphate, pH 7.0, 10 mM Na-EDTA, 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 0.3% (w/v) X-Gluc and 0.1% (v/v) Triton X-100 at 37 °C for 5 h (Mendel et al. 1989; Spangenberg et al. 1995). GUS enzyme activity was determined fluorometrically using the substrate 4-methylumbelliferyl β-D glucuronide (MUG) (Jefferson et al. 1987; Sessa et al. 1998). Briefly, tissues were ground in a mortar and pestle pre-cooled with liquid nitrogen, and the powder was transferred to a microcentrifuge tube containing 0.7 ml of extraction buffer [100 mM potassium phosphate (pH 7.8), 1 mM EDTA, 7 mM mercaptoethanol, 1% (v/v) Triton X-100, 10% (v/v) glycerol]. After 1 h incubation at room temperature, the tube was centrifuged at 14,000 rpm for 20 min at 4 °C. The supernatant (300 μl) was transferred into a microcentrifuge tube containing 300 μl GUS buffer (extraction buffer containing 2 mM MUG) and fully mixed. 200 μl of this mixture was transferred into an eppendorf tube containing 1 ml of GUS stop buffer (0.2 M Na_2CO_3), stored at 4 °C, and served as control. Another 200 μl of the mixture was incubated at 37 °C for 1 h and the reaction was stopped by adding 1 ml of GUS stop buffer. Fluorescence of the samples was read on a Sequoia-Turner Fluorimeter (Model 450) with emission at 455 nm and excitation at 365 nm. GUS enzyme activity was expressed as picomoles of 4-methylumbelliferone (MU) produced per minute per milligram of protein. Protein concentrations of the samples were determined using Bio-Rad Dc protein assay reagent (Bio-Rad Laboratories, Hercules, CA) with bovine serum albumin as standard. At least ten independent transgenic plants were analyzed, with triplicate samples collected for each transgenic plant. GUS activities of the transgenic plants were averaged and presented in graphs. In Arabidopsis, single insert T3 homozygous lines were used for the analysis. In white clover, primary transgenic plants were used for analysis. Significance of treatments was tested at the $p = 0.05$ level. Standard errors are provided in all figures as appropriate.

Results

Expression pattern of MtHP cDNA in M. trunctula

A root-specific gene, *Mt12a*, was obtained by screening a *Medicago truncatula* cDNA library with the pea RH2 gene (Mylona et al. 1994). During analysis of the *Mt12a* gene, we identified a family member, designated *MtHP*, that shared high sequence identity (91.5%) with *Mt12a* but showed a different expression pattern. The transcript of *Mt12a* was detected only in root (Figure 1a), while the second family member, *MtHP*, expressed not only in root, but also in leaf and stem (Figure 1b).

The open reading frame of *MtHP* is predicted to encode a protein of 158 amino acids with molecular mass of 16.81 kDa and an isoelectric point of 5.34. Alignment analysis showed that the deduced amino acid sequences share 86–87% similarity

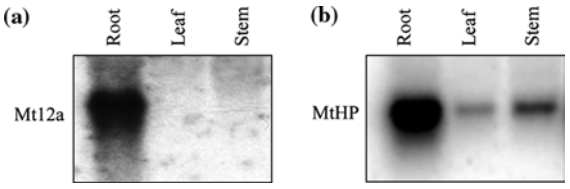

Figure 1. Northern hybridization analyses of different *M. truncatula* tissues using (a) Mt12a cDNA as a probe and (b) MtHP cDNA as a probe.

with PR10 proteins of pea and alfalfa (Breda et al. 1996; Culley et al. 1995; Fristensky et al. 1988). Thus the *MtHP* gene most likely belongs to the PR10 multi-gene family. Southern hybridization analysis revealed there are one or two copies of the *MtHP* gene in *M. truncatula* genome. A genomic clone for *MtHP* was obtained after screening a *M. truncatula* genomic library. The *MtHP* genomic clone was subcloned, sequenced and the promoter region was identified. As a strong constitutive promoter could have utility for transformation technology, further analysis of the promoter was undertaken.

Comparison of the expression levels of GUS driven by MtHP and CaMV35S promoters

The binary vector pCAMBIA3301 carries a β-glucuronidase gene (*gusA)* driven by CaMV35S promoter (Figure 2). A new MtHP-GUS construct (Figure 2) was created by replacing the CaMV35S promoter of pCAMBIA3301 with 1548 bp of the MtHP promoter. For ease of understanding and illustration, here we refer pCAMBIA3301 as 35S-GUS (Figure 2). Transient expression assays by particle bombardment revealed GUS expression in radicle, stem and leaf tissues of *M. truncatula* (data not shown). The results indicate that MtHP promoter could direct gene expression in above ground tissues in *M. truncatula*. The *M. truncatula* roots transformed by *A. rhizogenes* showed blue color after staining with GUS solution (data not shown), confirming that the promoter could also direct GUS expression in root and root hairs.

To further characterize the expression pattern of the reporter gene driven by the MtHP promoter, transgenic Arabidopsis plants were generated using the MtHP-GUS and 35S-GUS constructs. More than 30 independent transgenic lines were evaluated for each construct. GUS expression levels varied with the transgenic lines, a result that is consistent with those of other studies and can be attributed to positional effects (Peach and Velten 1991; Grec et al. 2003). Non-typical expression pattern (blue staining only in root or only in shoot) was observed for both 35S-GUS and MtHP-GUS plants, although these were rare case events ($< 10\%$). Exceptionally high levels of GUS (71,000 pmoles/min/mg protein) were detected in 2 out of 30 MtHP-GUS plants; their GUS activity was six times higher than that of the highest expressing 35S-GUS plants. However, these were also rare events ($< 10\%$) and were not included in the final analysis. Ten typical GUS positive plants of each gene construct were used in the final analysis, as described by Grec et al. (2003) in other promoter studies.

Staining of the transgenic Arabidopsis plants revealed strong GUS expression at different developmental stages when MtHP promoter was used to drive the *gusA* gene (Figure 3). Although both 35S promoter and MtHP promoter could lead to GUS staining in 3-day, 1-week, 2-week and 3-week old plants (Figure 3a), levels of GUS expression were consistently higher in MtHP-GUS transgenic

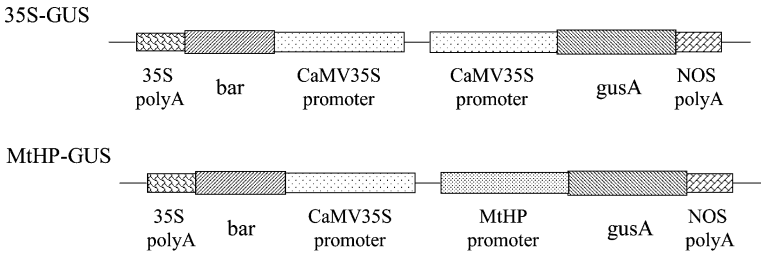

Figure 2. Schematic illustrations of the CaMV35S-GUS and MtHP-GUS binary vectors used for *Agrobacterium*-mediated transformation.

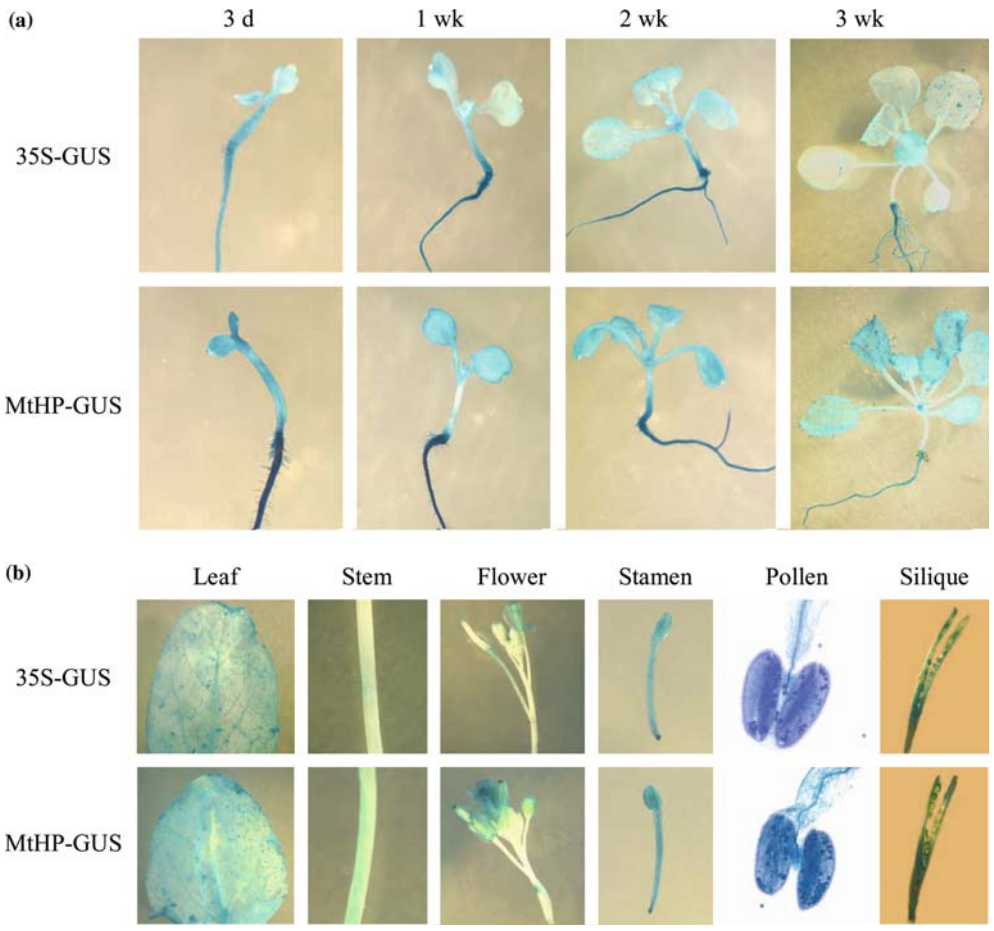

Figure 3. GUS staining of transgenic Arabidopsis carrying 35S-GUS and MtHP-GUS gene constructs. (a) GUS staining of Arabidopsis plants at different developmental stages. (b) GUS staining of different tissues and organs of transgenic Arabidopsis.

plants than that of 35S-GUS plants (Figure 4a). In mature transgenic Arabidopsis plants, GUS expression was analyzed in different organs: leaf, stem, flower, stamen, pollen and siliques (Figure 3b). Again, GUS expression level in different organs was higher in MtHP-GUS transgenic plants than those of 35S-GUS plants (Figure 4b).

To test the function of the promoter in widely grown forage legume species, MtHP-GUS and CaMV35S-GUS were transferred into the genome of white clover and alfalfa through *A. tumefaciens*-mediated transformation. Transgenic white clover plants showed dark blue staining in leaf, petiole, root and flower (Figure 5). Compared with 35S-GUS, a higher level of GUS activity was detected in transgenic white clover plants and transgenic alfalfa calli when MtHP-GUS was introduced into the plant genome (Figure 6).

Deletion analysis of the MtHP promoter

To characterize the promoter in further detail, a series of 5′-deletions of the promoter region were created. The MtHP promoter sequence and corresponding deletion segments are illustrated in Figure 7. GUS expression was detected in transgenic Arabidopsis plants carrying shortened MtHP-GUS gene constructs with varying lengths of promoter regions (Figure 8). As shown in Figures 8 and 9, reducing the promoter sequence to 1118 bp (D5), 827 bp (D4), 523 bp (D3) had little effect on GUS activity. A significant reduction in GUS activity was observed when the promoter was reduced to 252 bp (D2) and 107 bp (D1). To our surprise, even the shortest promoter segment (107 bp, D1) was still able to direct GUS expression in transgenic plants (Figures 8 and 9),

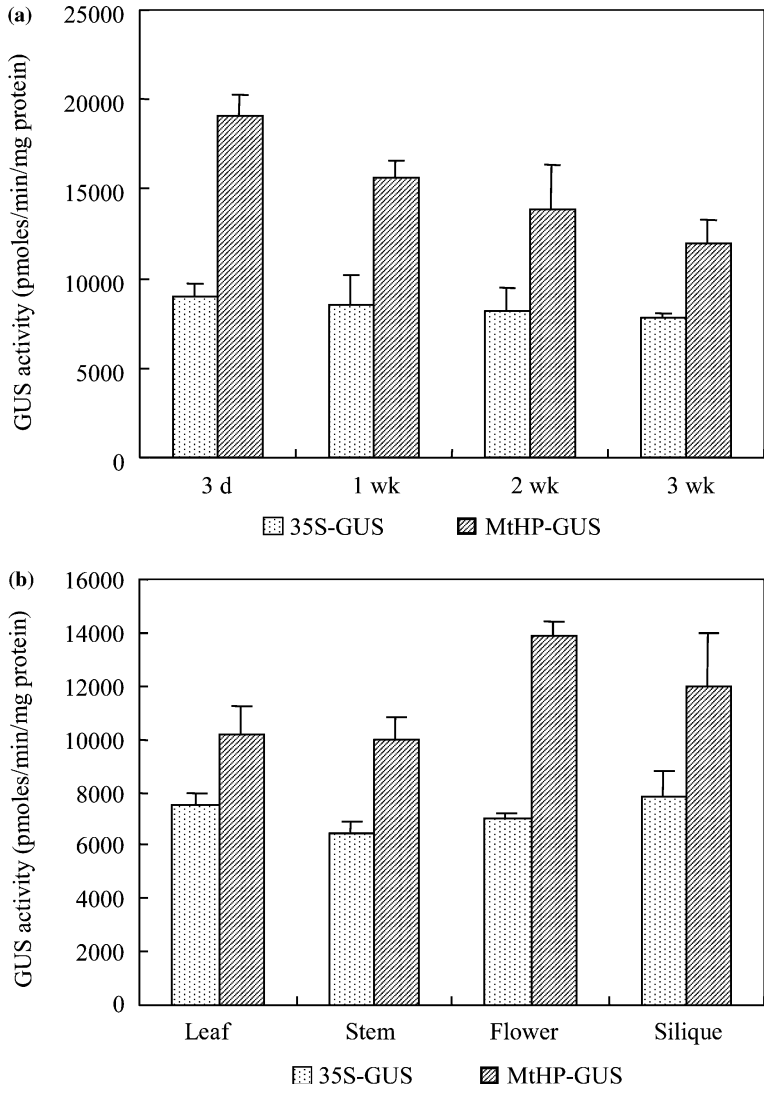

Figure 4. GUS activity of transgenic Arabidopsis plants carrying 35S-GUS and MtHP-GUS gene constructs. (a) GUS activity of 3-day, 1-week, 2-week and 3-week old plants. (b) GUS activity of different tissues and organs of 8-week old transgenic plants. Error bars represent standard error.

although the level of expression was about 47% of the 'full-length' MtHP promoter.

Discussion

Although the deduced amino acid sequences of MtHP showed high similarity to a PR10 protein of alfalfa, their gene expression patterns were different. Expression of the PR10 gene in alfalfa was detected only in roots and was not detectable in hypocotyls, stems or leaves (Breda et al. 1996), while the *MtHP* gene was expressed in different tissues in *M. truncatula*. It is known that the PR10 proteins belong to a multi-gene family and are often responsive to biotic and abiotic stresses (Truesdell and Dickman 1997; Robert et al. 2001; Hwang et al. 2003; Liu et al. 2003b; Hashimoto et al. 2004;). Despite their proposed roles in plant defense response, the exact biological functions of the PR10 protein family are still unknown (Liu and Ekramoddoullah 2003). Some PR10 proteins are involved in plant development and display constitutive expression patterns that are unrelated

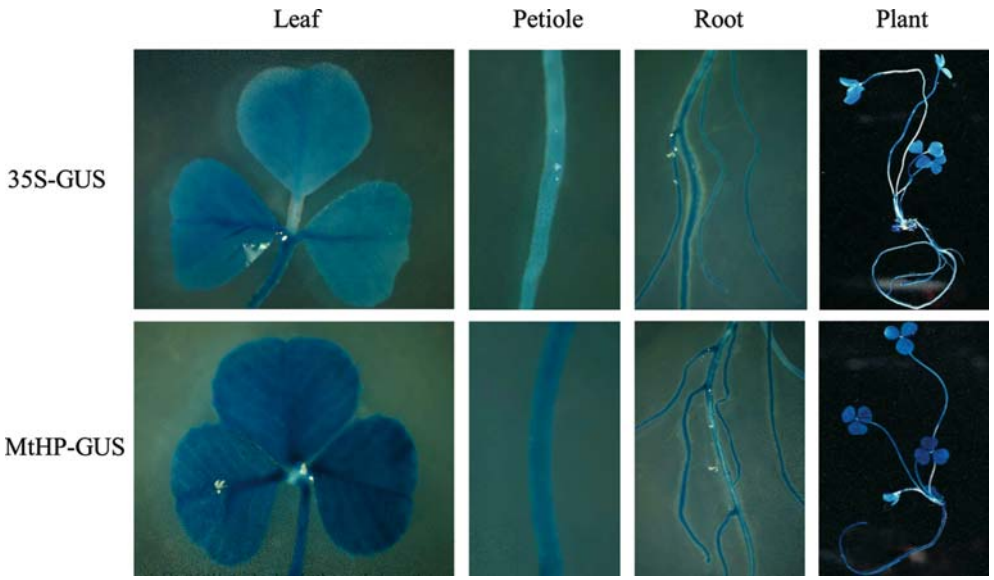

Figure 5. GUS staining of transgenic white clover plants carrying 35S-GUS and MtHP-GUS gene constructs.

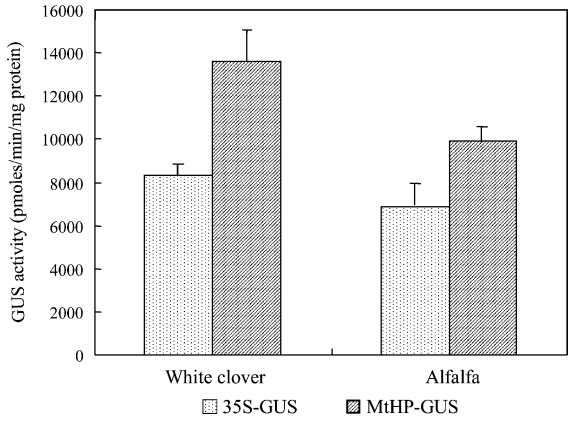

Figure 6. GUS activity of transgenic white clover plants and transformed alfalfa calluses carrying 35S-GUS and MtHP-GUS gene constructs. Leaf and petiole of transgenic white clover plants 4 wk after regeneration were harvested and used for fluorometric assay. Error bars represent standard error.

to a pathogenic response (Liu and Ekramoddoullah 2003). The MtHP may belong to this category.

Certain promoters can direct gene expression at higher levels than others. Promoters that drive high, constitutive expression have become a valuable tool in the genetic engineering of plants (Potenza et al. 2004). In monocot species, certain constitutive promoters of plant origin, such as rice actin promoter (McElroy et al. 1990) and maize ubiquitin promoter (Christensen et al. 1992), have been isolated and are often used for transformation of grasses (Zhang et al. 1991; McElroy et al. 1995; Christensen and Quail 1996; Chen et al. 2003, 2004; Wang et al. 2004). In dicot plants, although a number of endogenous constitutive promoters have been isolated (An et al. 1996; Plesse et al. 2001; Potenza et al. 2004), they are not yet widely used or tested in other species, particularly in legumes. The most commonly used promoter for constitutive expression is still the CaMV35S promoter. Due to viral origin of the CaMV promoter, it has been shown that CaMV infection can destabilize a commercially important genetic trait in oilseed rape by silencing the bar transgene, altering the plant phenotype from herbicide resistance to susceptibility (Al-Kaff et al. 2000). Overuse of CaMV35S promoter may increase the chances of transcriptional inactivation (Matzke and Matzke 1995; Stam et al. 1997). Numerous transgenic plant systems have exhibited the phenomenon of homology-dependent gene silencing, which can involve interactions between closely linked repetitive elements on one DNA molecule or homologous sequences on separate DNA molecules, at either allelic or non-allelic positions (Park et al. 1996; Jakowitsch et al. 1999). It is not uncommon to find in the literature that CaMV 35S promoter

```
      CTCGATCAAT AGTTCAAACC AAAGAAAACA AAAATGAATT CAAGAATTAG CCAAAGCCCA AACACATATA GGGATTAAGC   -1469
      CACCAATTAT GATAAAAAAA AAAAAAGAAG ACTTTTTTGC ATTCAGTCAT CGATAGAAAA TGAGCTTAAT GTTATCTAAA   -1389
      AATTGAAGAG CTGAGCTTGT TGTATTATTA AATATCTTGT CGTTTCTTTC TTTCTGTATA ACCTACGTGC AAGCAAACCA   -1309
      AATCATCAAC ATAAATGAGC GGCGGGATTT TGAAACACCT TCTAAACAAC TTTTTTTCTA AATAAAACTT AATGAATGTT   -1229
      TCCTTTTTTG AATCTTTAAC AAAAACACTT AGAACACTTG TTAATAGTAA AAAATGAAGA ATATAATTTG TATTTTATTA   -1149
D5    AAATTTACTA AAAATAGTAT GATCCTCCTT TAGATTGAGT CCATATAAGA TGTCCCGTAA GTTTAAATAT AAAAATCATG   -1069
      GTTTGGCTCT CTCTATCGGG AAACCAAATA TGTAAAATTG ATTTTGCCAT ATTTGTCCGA TGCTTATTGA AATTGATTTT   -989
      TATCTCTATA ATTATATAAC TTGAAGTTAG AACTTAGAAC TGCAGCTTTT AATTTTTAAT ATGATTTTTA TACTCAAATT   -909
D4    TAGCGTTCAA GGCACGCGGA CATGAATATA TTTAAATATA ACTAATTCAC ATTTAACCCA TTTTTAACTA AATCATTTTA   -829
      TTTGAAAAAA AATTTGTTGG CAAAACTAAA CATGCACTAA ACCAAGTTGT TTGGCCTCCG GTGGAAAGGA GCTCTTTCCA   -749
      AGGATGTATC CGGAAAATAC CGGACTCGAT TCACAGAGGG AACAACGCTT GACCAATTTG CATGAGCCAG ATTAGTCACC   -669
      AACCTTTGGT GGGTCGAAAA CTGGTGCAAA AGCCAAAAAA TCCATTAAAA AAACTAAACA CGCACTAAAT ATTTCCAACT   -589
D3    AAAGTATTAG TATTATTTAA AAAAAAAACA AAAACTATAT TGTAAAACAA ATAGTAGTAT TATTTAGTCC GGATTCTAGA   -509
      ATGGCGTCTT GAGACTGAAT TGTTTAATAT TAAACAAATA AAATAAAAAA TGAGAGACTG CAACATAATT TCCTCTTCTT   -429
      TTTTGGGTTT GCATTTGATC GTGTAAAAGA GAAATCTAGA TACAAGAATA CGCAGATTTT GAATATATTG CCATAGCTTT   -349
      TGTGGTTATT TGAATAGTAT ATCAGGAATT TTGAAATTTG ACTGGTCTTA GAGGAGATAA AAGAAGAAAA AAAAAATAGT   -269
D2    ATATCAAGAA TTTTGTGAAC CCAAATAATT TTTTTCTTTA AAATTCATAT CTCAATGTGA ATAATAAAAT GGTTCCAACA   -189
D1    ACCTGATATT AGTTAGTCAT CATCTTTATC TAATTAGCAA TCTAAAGTCC AAACAATACT GTACCAAAAA AAGTCTAAAT   -109
      AATACAACTT CAGTTCCTAT AAATACTAGC CATAACTACA TTCATAAACC ACACATTACG ACCATTATTT GTTATTTCTT   -29
Rev   ACCTAGCATT TTAATACATT ATACCATCAT GGGTGTGTTC AATTTTGAGG ATGAAACCAC CTCTAATGTA GCTCCTGCTA    52
```

Figure 7. MtHP full-length promoter sequences and deletions. Deleted promoter segments are shown by arrows.

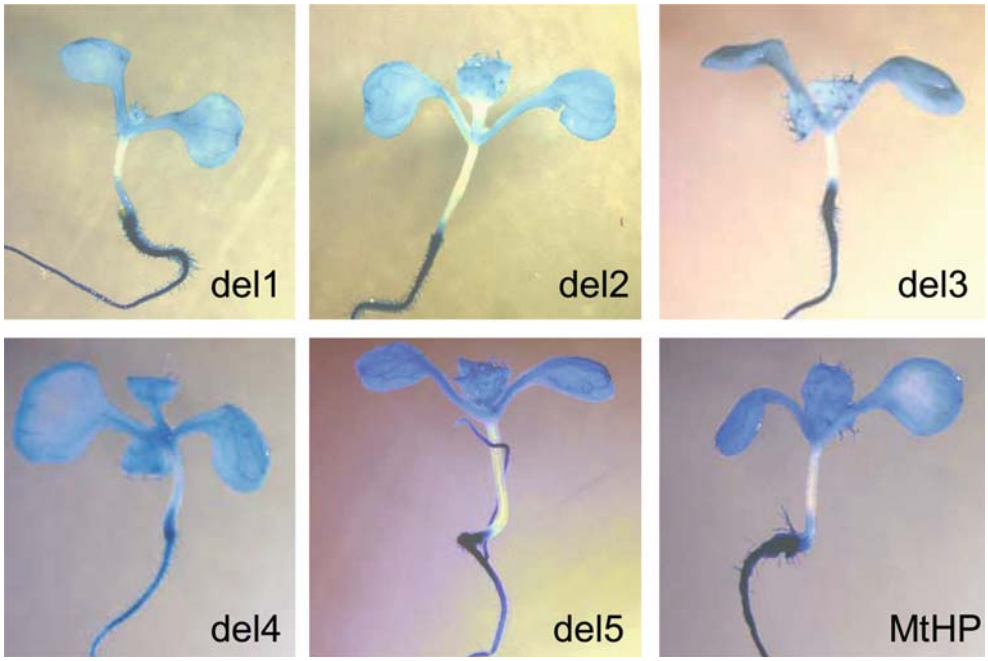

Figure 8. GUS staining of 12-day old transgenic Arabidopsis carrying deleted MtHP-GUS gene constructs with varying lengths of promoter regions.

is used to drive two or more chimeric genes in the same transformation vector. In this case, gene silencing caused by inactivation of CaMV35S promoter has been observed (Matzke and Matzke 1995; Park et al. 1996; McCabe et al. 1999). In other cases, such as to introduce another gene into transgenic plants, if the resident transgene contains the CaMV35S promoter, the introduction of additional copies of the 35S enhancer may result in methylation and silencing of unlinked homologous copies (Mette et al. 2000; Dong and Von Arnim 2003). Thus, a novel alternative promoter to CaMV35S will be very useful for directing strong constitutive expression in plants.

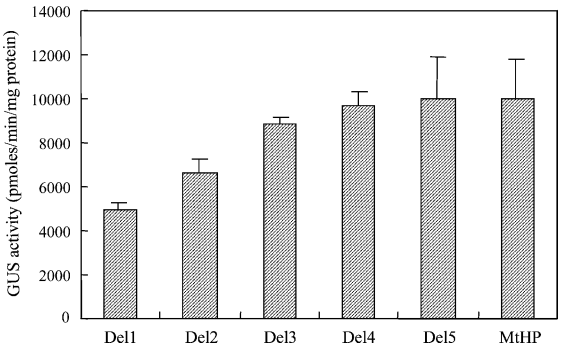

Figure 9. GUS activity of transgenic Arabidopsis carrying deleted MtHP-GUS gene constructs with varying lengths of promoter regions. Shoots of 12-day old plants were harvested and used for fluorometric assay. Error bars represent standard error.

Medicago truncatula is a forage legume that has been developed as a model legume (Barker et al. 1990; May 2004). We isolated a novel promoter (MtHP) from *M. truncatula* and fused it to a GUS reporter to test its expression in different species. Transient expression analysis by particle bombardment and hairy root transformation of *M. truncatula* revealed the potential usefulness of MtHP as a constitutive promoter. Detailed analysis in Arabidopsis demonstrated that the promoter could lead to GUS expression at various developmental stages and in different tissues and organs. In comparison with CaMV35S promoter, higher levels of expression were consistently observed with the MtHP promoter. Because pCAMBIA vectors are widely used, and the only difference between the two vectors in our study is the promoter in front of the *GUS* gene, the comparison of the strengths of MtHP and CaMV35S promoters is straightforward and practical. Even though gene silencing might have occurred in the 35S-GUS construct due to the presence of two 35S promoters, we tried to address this potential problem by screening a large number of transgenic plants for each gene construct and by analyzing only the plants with typical expression pattern. In order to test the influence of gene silencing, future research is needed in which a promoter other than the CaMV35S is used to drive the selectable marker gene in the 35S-GUS construct. It is also interesting to note that a very short fragment of the promoter (107 bp) could still lead to a moderate level of expression approximately 47% of the 'full-length' promoter. Previous promoter deletion analyses have shown that expression levels decrease drastically, or are minimal when the promoter regions are less than 200 bp (McElroy et al. 1990; Santamaria et al. 2001; Lauvergeat et al. 2002; Grec et al. 2003; Kim et al. 2003; Wang and Oard 2003). Thus it may be useful to conduct further studies of the elements involved in the short MtHP regulatory sequences.

The MtHP promoter was further tested in the most widely grown forage legumes, white clover and alfalfa. The gene expression patterns were similar to that of the CaMV35S promoter but the levels of gene expression were considerably higher. Therefore, the MtHP promoter described in this paper provides a practical choice to direct high level and constitutive expression of transgenes.

Acknowledgments

The authors thank Jeremey Bell and Yaxin Ge for their valuable help and Carol Resz for editing the manuscript. The work was supported by the Samuel Roberts Noble Foundation.

References

Al-Kaff N.S., Kreike M.M., Covey S.N., Pitcher R., Page A.M. and Dale P.J. 2000. Plants rendered herbicide-susceptible by cauliflower mosaic virus-elicited suppression of a 35S promoter-regulated transgene. Nat. Biotechnol. 18: 995–999.

An Y.Q., McDowell J.M., Huang S., McKinney E.C., Chambliss S. and Meagher R.B. 1996. Strong, constitutive expression of the Arabidopsis ACT2/ACT8 actin subclass in vegetative tissues. Plant J. 10: 107–121.

Austin S., Bingham E.T., Mathews D.E., Shahan M.N., Will J., Burgess R.R., Cassells A.C. and Jones P.W. 1995. Production and field performance of transgenic alfalfa (*Medicago sativa* L.) expressing alpha-amylase and manganese-dependent lignin peroxidase. Euphytica 85: 381–393.

Barker D.G., Bianchi S., Blondon F., Dattée Y., Duc G., Essad S., Flament P., Gallusci P., Génier G., Guy P., Muel X., Tourneur J., Dénarié J. and Huguet T. 1990. *Medicago truncatula*, a model plant for studying the molecular genetics of the *Rhizobium*-legume symbiosis. Plant Mol. Biol. Rep. 8: 40–49.

Bingham E.T. 1991. Registration of alfalfa hybrid Regen-SY germplasm for tissue culture and transformation research. Crop Sci. 31: 1098.

Boisson-Dernier A., Chabaud M., Garcia F., Becard G., Rosenberg C. and Barker D.G. 2001. *Agrobacterium rhizogenes*-transformed roots of *Medicago truncatula* for the study of nitrogen-fixing and endomycorrhizal symbiotic associations. Mol. Plant Microbe Interact. 14: 695–700.

Breda C., Sallaud C., El-Turk J., Buffard D., Kozak I.d., Esnault R. and Kondorosi A. 1996. Defense reaction in *Medicago sativa*: a gene encoding a class 10 PR protein is expressed in vascular bundles. Mol. Plant Microbe Interact. 9: 713–719.

Chen L., Auh C., Dowling P., Bell J., Chen F., Hopkins A., Dixon R.A. and Wang Z.-Y. 2003. Improved forage digestibility of tall fescue (*Festuca arundinacea*) by transgenic down-regulation of cinnamyl alcohol dehydrogenase. Plant Biotechnol. J. 1: 437–449.

Chen L., Auh C., Dowling P., Bell J., Lehmann D. and Wang Z.-Y. 2004. Transgenic down-regulation of caffeic acid *O*-methyltransferase (COMT) led to improved digestibility in tall fescue (*Festuca arundinacea*). Funct. Plant Biol. 31: 235–245.

Christensen A.H. and Quail P.H. 1996. Ubiquitin promoter-based vectors for high-level expression of selectable and/or screenable marker genes in monocotyledonous plants. Transgenic Res. 5: 213–218.

Christensen A.H., Sharrock R.A. and Quail P.H. 1992. Maize polyubiquitin genes: Structure, thermal perturbation of expression and transcript splicing, and promoter activity following transfer to protoplasts by electroporation. Plant Mol. Biol. 18: 675–689.

Clough S.J. and Bent A.F. 1998. Floral dip: a simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*. Plant J. 16: 735–743.

Culley D.E., Brown S., Angela Parsons M., Hadwiger L.A. and Brian F. 1995. Cloning and sequencing of disease-resistance response gene DRR49a from *Pisum sativum* (Accession No. U31669). Plant Physiol. 109: 722.

Dong Y. and Von Arnim A.G. 2003. Novel plant activation-tagging vectors designed to minimize 35S enhancer-mediated gene silencing. Plant Mol. Biol. Rep. 21: 349–358.

Fristensky B., Horovitz D. and Hadwiger L.A. 1988. cDNA sequences for pea disease resistance response genes. Plant Mol. Biol. 11: 713–715.

Grec S., Vanham D., De Ribaucourt J.C., Purnelle B. and Boutry M. 2003. Identification of regulatory sequence elements within the transcription promoter region of *NpABC1*, a gene encoding a plant ABC transporter induced by diterpenes. Plant J. 35: 237–250.

Hashimoto M., Kisseleva L., Sawa S., Furukawa T., Komatsu S. and Koshiba T. 2004. A novel rice PR10 protein, RSOsPR10, specifically induced in roots by biotic and abiotic stresses, possibly via the jasmonic acid signaling pathway. Plant Cell Physiol. 45: 550–559.

Hwang H., Kim H., Yu H., Oh M., Lee I. and Kim S. 2003. Gene encoding pathogenesis-related 10 protein of *Lithospermum erythrorhizon* is responsive to exogenous stimuli related to the plant defense system. Plant Sci. 165: 1297–1302.

Jakowitsch J., Papp I., Moscone E.A., Winden J.v.d., Matzke M. and Matzke A.J.M. 1999. Molecular and cytogenetic characterization of a transgene locus that induces silencing and methylation of homologous promoters in *trans*. Plant J. 17: 131–140.

Jefferson R.A., Kavanagh T.A. and Bevan M.W. 1987. GUS fusion: ß-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. EMBO J. 6: 3901–3907.

Kim K.Y., Kwon S.Y., Lee H.S., Hur Y., Bang J.W. and Kwak S.S. 2003. A novel oxidative stress-inducible peroxidase promoter from sweetpotato: molecular cloning and characterization in transgenic tobacco plants and cultured cells. Plant Mol. Biol. 51: 831–838.

Larkin P.J., Gibson J.M., Mathesius U., Weinman J.J., Gartner E., Hall E., Tanner G.J., Rolfe B.G. and Djordjevic M.A. 1996. Transgenic white clover: Studies with the auxin-responsive promoter, GH3, in root gravitropism and lateral root development. Transgenic Res. 5: 325–335.

Lauvergeat V., Rech P., Jauneau A., Guez C., Coutos T.P. and Grima P.J. 2002. The vascular expression pattern directed by the *Eucalyptus gunnii* cinnamyl alcohol dehydrogenase *Eg-CAD2* promoter is conserved among woody and herbaceous plant species. Plant Mol. Biol. 50: 497–509.

Liu H., Trieu A.T., Blaylock L.A. and Harrison M.J. 1998. Cloning and characterization of two phosphate transporters from *Medicago truncatula* roots: regulation in response to phosphate and to colonization by arbuscular mycorrhizal (AM) fungi. Mol. Plant Microbe Interact. 11: 14–22.

Liu J., Blaylock L.A., Endre G., Cho J., Town C.D., VandenBosch K.A. and Harrison M.J. 2003a. Transcript profiling coupled with spatial expression analyses reveals genes involved in distinct developmental stages of an arbuscular mycorrhizal symbiosis. Plant Cell 15: 2106–2123.

Liu J., Ekramoddoullah A.K.M. and Yu X. 2003b. Differential expression of multiple PR10 proteins in western white pine following wounding, fungal infection and cold-hardening. Physiol. Plant. 119: 544–553.

Liu J.J. and Ekramoddoullah A.K.M. 2003. Root-specific expression of a western white pine PR10 gene is mediated by different promoter regions in transgenic tobacco. Plant Mol. Biol. 52: 103–120.

Matzke M.A. and Matzke A.J.M. 1995. How and why do plants inactivate homologous (trans)genes? Plant Physiol. 107: 679–685.

May G.D. 2004. From model to crops: integrated *Medicago* genomics for alfalfa improvement. In: Hopkins A., Wang Z.Y., Mian R., Sledge M. and Barker R.E. (eds), Molecular Breeding of Forage and Turf. Kluwer Academic Publishers, Dordrecht, pp. 325–332.

McCabe M.S., Schepers F., Arend A.v.d., Mohapatra U., Laat A.M.M.d., Power J.B. and Davey M.R. 1999. Increased stable inheritance of herbicide resistance in transgenic lettuce carrying a *petE* promoter- *bar* gene compared with a CaMV 35S-*bar* gene. Theor. Appl. Genet. 99: 587–592.

McElroy D., Chamberlain D.A., Moon E. and Wilson K.J. 1995. Development of gusA reporter gene constructs for cereal transformation: availability of plant transformation vectors from the CAMBIA Molecular Genetic Resource Service. Mol. Breed. 1: 27–37.

McElroy D., Zhang W., Cao J. and Wu R. 1990. Isolation of an efficient actin promoter for use in rice transformation. Plant Cell 2: 163–172.

Medberry S.L., Lockhart B.E.L. and Olszewski N.E. 1992. The commelina yellow mottle virus promoter is a strong promoter in vascular and reproductive tissues. Plant Cell 4: 185–192.

Mendel R.R., Mueller B., Schulze J., Kolesnikov V. and Zelenin A. 1989. Delivery of foreign genes to intact barley cells by high-velocity microprojectiles. Theor. Appl. Genet. 78: 31–34.

Mette M.F., Aufsatz W., vanderWinden J., Matzke M.A. and Matzke A.J.M. 2000. Transcriptional silencing and promoter

methylation triggered by double-stranded RNA. EMBO J. 19: 5194–5201.

Mylona P., Moerman M., Yang W.C., Gloudemans T., Van D.K.J., Van K.A., Bisseling T. and Franssen H.J. 1994. The root epidermis-specific pea gene RH2 is homologous to a pathogenesis-related gene. Plant Mol. Biol. 26: 39–50.

Odell J.T., Nagy F. and Chua N.H. 1985. Identification of DNA sequences required for activity of the cauliflower mosaic virus 35S promoter. Nature 313: 810–812.

Park Y.D., Papp I., Moscone E.A., Iglesias V.A., Vaucheret H., Matzke A.J.M. and Matzke M.A. 1996. Gene silencing mediated by promoter homology occurs at the level of transcription and results in meiotically heritable alterations in methylation and gene activity. Plant J. 9: 183–194.

Peach C. and Velten J. 1991. Transgene expression variability (position effect) of CAT and GUS reporter genes driven by linked divergent T-DNA promoters. Plant Mol. Biol. 17: 49–60.

Plesse B., Criqui M.C., Durr A., Parmentier Y., Fleck J. and Genschik P. 2001. Effects of the polyubiquitin gene Ubi.U4 leader intron and first ubiquitin monomer on reporter gene expression in Nicotiana tabacum. Plant Mol. Biol. 45: 655–667.

Potenza C., Aleman L. and Sengupta-Gopalan C. 2004. Targeting transgene expression in research, agricultural, and environmental applications: promoters used in plant transformation. In Vitro Cell. Dev. Biol. Plant 40: 1–22.

Quandt H.J., Pühler A. and Broer I. 1993. Transgenic root nodules of *Vicia hirsuta*: a fast and efficient system for the study of gene expression in indeterminate-type nodules. Mol. Plant Microbe Interact. 6: 699–706.

Robert N., Ferran J., Breda C., Coutos-Thévenot P., Boulay M., Buffard D. and Esnault R. 2001. Molecular characterization of the incompatible interaction of *Vitis vinifera* leaves with *Pseudomonas syringae* pv. *pisi*: expression of genes coding for stilbene synthase and class 10 PR protein. Eur. J. Plant Pathol. 107: 249–261.

Sambrook J., Fritsch E.F. and Maniatis T. 1989. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, New York.

Santamaria M., Thomson C.J., Read N.D. and Loake G.J. 2001. The promoter of a basic PR1-like gene, *AtPRB1*, from Arabidopsis establishes an organ-specific expression pattern and responsiveness to ethylene and methyl jasmonate. Plant Mol. Biol. 47: 641–652.

Sessa G., Borello U., Morelli G. and Ruberti I. 1998. A transient assay for rapid functional analysis of transcription factors in *Arabidopsis*. Plant Mol. Biol. Rep. 16: 191–197.

Spangenberg G., Wang Z.-Y., Wu X.L., Nagel J., Iglesias V.A. and Potrykus I. 1995. Transgenic tall fescue (*Festuca arundinacea*) and red fescue (*F. rubra*) plants from microprojectile bombardment of embryogenic suspension cells. J. Plant Physiol. 145: 693–701.

Stam M., Mol J.N.M. and Kooter J.M. 1997. The silence of genes in transgenic plants. Ann. Bot. 79: 3–12.

Truesdell G.M. and Dickman M.B. 1997. Isolation of pathogen/stress-inducible cDNAs from alfalfa by mRNA differential display. Plant Mol. Biol. 33: 737–743.

Wang J. and Oard J.H. 2003. Rice ubiquitin promoters: Deletion analysis and potential usefulness in plant transformation systems. Plant Cell Rep. 22: 129–134.

Wang Z.-Y., Bell J., Ge Y.X. and Lehmann D. 2003. Inheritance of transgenes in transgenic tall fescue (*Festuca arundinacea*Schreb.). In Vitro Cell. Dev. Biol. Plant 39: 277–282.

Wang Z.-Y., Bell J. and Lehmann D. 2004. Transgenic Russian wildrye (*Psathyrostachys juncea*) plants obtained by biolistic transformation of embryogenic suspension cells. Plant Cell Rep. 22: 903–909.

Zhang W., McElroy D. and Wu R. 1991. Analysis of rice Act1 5′ region activity in transgenic rice plants. Plant Cell 3: 1155–1165.