

**ENLIGHTENING *Medicago truncatula* TRANSFORMATION AND  
SHADING GFP FLUORESCENCE**

A Dissertation

by

XIN ZHOU

Submitted to the Office of Graduate Studies of  
Texas A&M University  
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

August 2005

Major Subject: Biology

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Approved by:

Chair of Committee,	Timothy C. Hall
Committee Members,	Thomas McKnight
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## ABSTRACT

Enlightening *Medicago truncatula* Transformation and Shading GFP Fluorescence.

(August 2005)

Xin Zhou, B.S., University of Science and Technology of China

Chair of Advisory Committee: Dr. Timothy C. Hall

*Medicago truncatula* (*M. truncatula*) has been proposed as a model legume for molecular and genetic studies of legumes. While many genetic resources have been developed for this model legume, genetic transformation of the *M. truncatula* line A17 proved to be a problem. A reproducible transformation method is described for *M. truncatula* A17. Procedures are detailed that yielded an average regeneration frequency of 35% for recovery of transgenic shoots from cotyledonary node explants. Previously, rooting of transgenic shoots of this line has proven difficult, but media and culture procedures are described that yielded an average frequency of 39% for root induction from 419 phosphinothricin-resistant shoots. Fertile *M. truncatula* A17 plants transgenic for *35S-GFP*, *phas-GUS* or *phas-GFP* were obtained. The presence of transgenes was confirmed by expression of transgenes and by genomic DNA blots. Interestingly, although GUS and GFP driven by the *phas* promoter were very strongly and uniformly expressed in seed cotyledons of most transgenic *M. truncatula* lines, silencing of the GUS expression from the *phas* promoter was observed in several lines, indicating the

occurrence of novel epigenetic events.

The diminution of GFP fluorescence in transgenic *M. truncatula* occurs despite the presence of GFP transcript and protein. To evaluate the generality and causes of this phenomenon, fluorescence during leaf development from the same *35S-GFP* transgene was compared in *M. truncatula*, rice and *Arabidopsis*. A substantial decrease in fluorescence early in the development of *M. truncatula* and rice leaves was found to correlate with chlorophyll accumulation. Several approaches showed that chlorophyll is causally involved in the loss of GFP fluorescence. Removal of chlorophyll from leaves of transgenic *M. truncatula*, rice or *Arabidopsis* through etiolation or by extraction with ethanol yielded up to a tenfold increase in fluorescence. Direct evidence that chlorophyll is implicated in the loss of fluorescence from GFP was obtained by mixing solutions of chlorophyll and GFP. At low concentration, fluorescence loss was fourfold greater for chlorophyll b than for chlorophyll a, reflecting their relative interference with GFP excitation and emission. Thus, substantial errors in estimating promoter activity from GFP fluorescence can occur if pigment interference is not considered.

## **DEDICATION**

This dissertation is dedicated to my beloved wife Rong Zhu, my daughter Crystal Zhou,  
and my parents for their love and support.

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# CHAPTER I

## INTRODUCTION

### **The importance of the legume family**

With over 650 genera and 18,000 species, the family Leguminosae is the third largest family of higher plants and is second only to grasses in agricultural importance (Young et al. 2003). Legumes are major sources of protein and oil for humans and livestock, and also major contributors to the global nitrogen cycle due to their unique ability of symbiotic nitrogen fixation. Soybeans (*Glycine max*) account for over half of world oilseed production each year, while alfalfa (*Medicago sativa*) ranks high in acreage planted and dollar value among forage crops (Cook 1999). Aside from their agricultural importance, legumes also produce a variety of beneficial secondary compounds, many of which have been suggested to have health-promoting properties such as providing protection against human cancers (Stark and Madar 2002). Not surprisingly, legumes are among the best-studied plant families in the world.

The most interesting feature of legumes is their capability to establish atmospheric nitrogen fixing symbiotic interactions with rhizobia, and to form symbiotic root mycorrhizae with soil fungi to facilitate their uptake of phosphate, water and other nutrients from soil. Thus, legume species offer valuable research opportunities in basic and applied plant biology, especially in areas such as symbiotic nitrogen fixation,

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This dissertation follows the style and format of Planta.

mycorrhizal interaction and legume-pathogen interactions (Cook 1999). However, genetic analysis of these processes remains difficult in the major crop legumes due to features such as tetraploidy, large genome and lack of efficient methods for transformation. Since the model plant *Arabidopsis thaliana* is unable to establish either rhizobial or mycorrhizal symbiosis, the selection of a model legume is essential for many genomics applications (Thoquet et al. 2002).

### ***Medicago truncatula* as a model legume**

*Medicago truncatula* (*M. truncatula*), a close relative of an important forage legume *Medicago sativa*, has been proposed as a model legume for molecular and genetic studies of legumes. Key attributes of *M. truncatula* include its small, diploid genome (approx. 500 Mb), self-fertility, prolific seed production, and short life cycle. The existence of large number of ecotypes of *M. truncatula*, which exhibit considerable phenotypic variation for features such as growth habit, flowering time, symbiotic specificity, and disease resistance provides important resources for the genetic basis of legume functions (Bonnin et al. 1996a; Bonnin et al. 1996b).

In the past decade, researchers have been developing molecular and genetic tools for this emerging model species. Artificial hybridization method was established which resulted in a high degree of cross pollination (Pathipanawat et al. 1994). An efficient mutagenesis protocol using ethyl-methyl sulfonate (EMS) mutagenesis has yielded an ethylene-insensitive mutant as well as diverse developmental mutants of *M. truncatula*

(Penmetsa and Cook 1997; Penmetsa and Cook 2000). Bacterial artificial chromosome (BAC) library was constructed, providing basis for physical mapping and genome sequencing (Nam et al. 1999).

Rapid progress with sequencing the ~500 Mbp genome of *M. truncatula* is underway. As of January 2005, approximately 134 Mb of the genome sequence in *M. truncatula* were publicly available, with entire gene space sequence anticipated in the next few years (Young et al. 2005). The sequencing data were made available through multiple genome databases that are summarized in [www.medicago.org/genome](http://www.medicago.org/genome).

By making use of available molecular markers, cytogenetic and genetic maps for *M. truncatula* have been constructed. A molecular cytogenetic map of *M. truncatula* ( $2n = 2x = 16$ ) was constructed on the basis of fluorescence in situ hybridization (FISH) of pachytene (Kulikova et al. 2001). Using Jemalong and an Algerian natural population (DZA315) as parental homozygous lines, Thoquet *et al.* (2002) constructed a molecular genetic map that spans 1225 cM and comprises 289 markers including RAPD, AFLP and known genes. Another genetic map covering 513 cM has been established by analyzing the segregation of 288 sequence-characterized genetic markers in an F2 population (Choi et al. 2004). These results demonstrate that *M. truncatula* is amenable to diploid genetic analysis and they open the way to map-based cloning of symbiotic or other agronomically-important genes using this model plant.

cDNA libraries (Covitz et al. 1998) and EST data bases (Bell et al. 2001) of *M. truncatula* have been constructed from source tissues such as rhizobium and root and shoot tissues. In the past few years more than 190,000 *M. truncatula* ESTs have been

produced ([www.medicago.org/MtDB2/](http://www.medicago.org/MtDB2/) and [www.tigr.org/tdb/tgi/plant.shtml](http://www.tigr.org/tdb/tgi/plant.shtml)). Based on these resources, a Array-Ready Oligo Set (Qiagen) for the *M. truncatula* genome containing highly optimized, arrayable 70-mers represents 16,086 genes designed from The Institute for Genomic Research (TIGR) Gene Index Database MtGI Release 5.0 has been made available. These essential tools will greatly facilitate expression profiling which will provide us with important insight to the *M. truncatula* genome functions in symbiosis and pathogenesis.

### **The importance of transformation for functional genomics**

Rapid developments in the field of functional genomics are helping to address the functions of genes discovered by genome sequencing. To understand the function of a specific gene, its associated phenotype often provides the best insight. One way to create loss-of-function mutants is insertional mutagenesis. Transposon (Ac/Ds or En/Spm) and T-DNA tagging are two commonly used approaches to create large numbers of insertional mutants. Compared with chemical/physical mutagenesis, insertional mutagenesis enables easy recovery of the flanking sequences that facilitate cloning of the affected wild-type gene (Pereira 2000). Following mutagenesis by any procedure, confirmation that the gene-phenotype relationship is causal requires that a complementation experiment be undertaken.

Although insertional mutagenesis is a useful approach to disrupt gene function, many higher organisms fail to display an obvious knockout phenotype for many genes (Burns

et al. 1994). This may result from functional redundancy or from lethal, subtle or conditional phenotypes. For this reason, insertional sequences are engineered to become gene detector insertions to monitor or change the expression pattern of adjacent genes. Entrapment strategies (Skarnes 1990) use reporter construct to detect the expression profiles of the adjacent host gene, providing useful information about the gene function. In another approach, by incorporating strong enhancer elements in the insertion sequence, increased expression of the adjacent gene can lead to gain-of-function mutations (Walden et al. 1994).

The discovery of homology dependent gene silencing (HDGS), especially post-transcriptional gene silencing (PTGS) or RNAi, offers an attractive approach to shutdown gene function. Transformation of plants with specific silencing vectors that generate highly effective hairpin RNAs (hpRNAs) is a powerful approach for silencing homologous genes (Waterhouse et al. 1998; Wesley et al. 2001).

The above methodologies, especially T-DNA tagging and RNAi, require a robust system for genetic transformation. For this reason, large scale functional genomic analysis is only possible in model systems for which facile transformation protocols have been developed, such as *Arabidopsis* and rice (Bechtold and Pelletier 1998; Jeong et al. 2002). For soybean, corn and several other important crop plants, the lack of effective transformation procedures is a major bottleneck in the discovery of gene function.



### **Current status of *M. truncatula* transformation and functional genomics**

Given the vital importance of facile genetic transformation, it is not surprising that great efforts have been made to develop transformation protocols for *M. truncatula*.

*Agrobacterium*-mediated transformation appears to be quite routine for *M. truncatula* genotypes R108 (Hoffmann et al. 1997; Trinh et al. 1998; Scholte et al. 2002) and 2HA (Thomas et al. 1992; Chabaud et al. 2003), both of which were selected for their high regenerability through extensive tissue culture. As a result, transposon-based gene knockout research is currently underway in genotype R108 (d'Erfurth et al. 2003).

However, the single seed descendant line Jemalong A17, chosen in the U.S. for sequencing, has proven to be very recalcitrant to transformation. Although tissue-culture-based transformation methods have been published for this line (Chabaud et al. 1996; Trieu and Harrison 1996), no successful application of these two approaches has been reported.

Considerable excitement was engendered by the development of two very efficient *Agrobacterium*-mediated *in planta* transformation methods, one utilizing the inflorescence and the other seedling infiltration (Trieu et al. 2000). These approaches were of special interest as they did not require tissue culture and could potentially be used for T-DNA insertion analysis. Unfortunately, these *in planta* methods for *M. truncatula* have not been reproduced (Somers et al. 2003).

To bypass the difficulty in transformation of line A17, *Agrobacterium rhizogenes*-mediated RNAi analysis has been used to study genes involved in root biology (Limpens et al. 2004). However, to understand gene function throughout the plant, *Agrobacterium tumefaciens*-mediated transformation is still required. Therefore, the major objective of this research was to develop an efficient and reproducible *Agrobacterium*-mediated transformation procedure for *M. truncatula* Jemalong A17. The approach was originally based on that described by Trieu and Harrison (1996), but involved multiple modifications. A plant-optimized green fluorescent protein (GFP) coding sequence, *mGFP5er* (Haseloff et al. 1997), driven by the *cauliflower mosaic virus* (*CaMV*) 35S promoter (Odell et al. 1985) was included in the T-DNA region of the transformation vector to enable early detection and localization of transformation events as well as to assist in evaluating the efficacy of experimental modifications.

### **The advantage of GFP as a reporter in plant research**

The ability to visualize, track and quantify gene products in living tissues is essential towards an understanding of the function of genes and the biological processes in which they are involved. The advent of fluorescent proteins, such as green fluorescent protein (GFP) from the jellyfish *Aequorea victoria* (Chalfie et al. 1994) and its variants, has endowed researchers with powerful tools to monitor gene expression. The lack of requirement for an exogenous substrate, cofactors or for histochemical fixation makes GFP a particularly valuable reporter for real time documentation of gene expression and

promoter activity in living tissue. Shortly after its emergence, GFP was rapidly adopted for use as a reporter in cells of a wide variety of organisms, including bacteria (Chalfie et al. 1994), yeast (Niedenthal et al. 1996), *Drosophila* (Yeh et al. 1995), zebra fish (Amsterdam et al. 1995), human (Rizzuto et al. 1995), and plants (Baulcombe et al. 1995; Haseloff and Amos 1995; Sheen et al. 1995).

Wild-type GFP has significant deficiencies, such as low fluorescence yield, slow fluorophore formation and maturation and photo bleaching at the major excitation peak (395 nm) (Cubitt et al. 1995). Extensive modifications were made to perfect its use as a universal marker and reporter. The exchange of amino acid serine 65 to cysteine (S65C) or threonine (S65T) increased the intensity of green fluorescence dramatically, thereby significantly raising the detection level for GFP (Reichel et al. 1996). Optimization of codon usage for S65T yielded sGFP(S65T) which accumulates in maize leaf cells to a level 20-fold higher than that of the native GFP (Chiu et al. 1996). Further, elimination of a cryptic intron together with mutation and site-directed mutagenesis produced a thermostable folding mutant mGFP5 that can be efficiently excited using either long-wavelength ultraviolet or blue light (Siemering et al. 1996). An endoplasmic reticulum (ER) target sequence was later fused to this version of GFP to avoid the putative toxicity of GFP in the cytoplasm (Haseloff et al. 1997).

Various forms of GFP are now widely used as reporter proteins in many organisms; in plants they are attractive alternatives to  $\beta$ -glucuronidase (GUS) (Jefferson et al. 1987) for assessment of promoter function (Harper et al. 1999; Sunilkumar et al. 2002), optimization of plant transformation (Stewart 2001), analysis of gene silencing (Ruiz et

al. 1998), and definition of spatial (Johnson et al. 2005), developmental (Marton et al. 2005) and quantitative (Tang and Newton 2004) properties of promoters.

### **The observed GFP fluorescence decreases with leaf age in plants**

The *35S-GFP* construct proved to be of value as a screenable marker during our development of a transformation method. However, we were surprised to discover that a dramatic decrease in fluorescence from GFP occurred shortly after leaf emergence in transgenic *M. truncatula* (Zhou et al. 2004). An initial assumption was that the decrease reflected gene silencing. However, experimental evidence was obtained showing that the loss of GFP fluorescence was not accompanied by the absence of GFP transcript or protein, making silencing an unlikely explanation. In a search for reports of similar observations, we found that a decrease in observed GFP fluorescence from a similar *35S-GFP* construct with leaf maturation has been reported in transgenic tobacco (Harper and Stewart 2000) and oilseed rape (Halfhill et al. 2003). While the latter article attributed the decrease in GFP fluorescence to changes in soluble protein content, we found that our observations do not support this explanation. Since GFP is playing an increasingly important role in plant research and the success of GFP as an expression marker hinges on a close correlation between the detected intensity of GFP fluorescence and GFP protein content in the plant tissue, it is certainly of great importance to find out the cause of the diminution of GFP fluorescence in mature leaves of *M. truncatula* and also investigate the generality of this observation in other model plant systems.

## CHAPTER II

### *IN PLANTA* TRANSFORMATION OF *M. truncatula*

#### **Introduction**

The generation of genetically homogeneous plants carrying the same transformation event in all cells has typically presented two separate hurdles: transformation of plant cells and regeneration of intact, reproductively competent plants from those transformed cells (Birch 1997; Hansen and Wright 1999). The latter stage usually requires refined tissue-culture procedure and participation of experts. It is also unfortunate that plant regeneration from single transformed cells often produces mutations ranging from single base changes or small rearrangements to the loss of entire chromosomes. In addition, significant epigenetic changes (for example, in DNA methylation) can also occur (Phillips et al. 1994). For these reasons, researchers have been pursuing transformation procedures that avoid tissue-culture. The real breakthrough was made on the model plant *Arabidopsis* when Bechtold et al. reported successful transformation by vacuum infiltration (Bechtold and Pelletier 1998). *Arabidopsis* plants at early stages of flowering are vacuum infiltrated with a suspension of *Agrobacterium*, grown to set seeds, and stably transformed T1 seedlings can be selected. This method has been reproduced in the hands of researchers all over the world and greatly enhanced the role of *Arabidopsis* as the model plant.

The publication of two *in planta* transformation procedure for the model legume *M. truncatula* A17 by Maria Harrison's lab in 2000 generated considerable excitement among *M. truncatula* community (Trieu et al. 2000). The first flower infiltration approach is similar to the "vacuum infiltration" method developed for transformation of *Arabidopsis*. The second seedling infiltration approach is a novel method that involves infiltration of young seedlings with *Agrobacterium*. The reported transformation frequency ranges from 4.7 to 76% for the flower infiltration method, and from 2.9 to 27.6% for the seedling infiltration method. With the expectation that these results could be reproduced, we decided to evaluate the possibility of transforming *M. truncatula* with reporter genes driven by *phaseolin* promoter and studying gene regulation of the major seed storage protein gene of *Phaseolus vulgaris* in a legume background.

## **Materials and methods**

### *Agrobacterium* strains and binary vectors

*Agrobacterium* strain GV3101 (Koncz and Schell 1986) and EHA105 (Hood et al. 1993) carrying either pCB302-phasGUS or pCB302-phasGFP (Fig. 2.1) were used for transformation.

The following experiments were performed essentially as described by Trieu *et al.* (Trieu et al. 2000) with minor changes.

### Sterilization and vernalization of *M. truncatula* seeds

*M. truncatula* seeds were soaked in concentrated sulphuric acid for approximately 10 min, rinsed three times with sterile distilled water and then sterilized in 30% chlorox, 0.1% Tween 20 for 5 min with gentle agitation. The seeds were then washed three times in sterile distilled water and spread on 0.8% water agar (Sigma) in petri plates (10×100 mm), approximately 25 seeds per plate. The plates were wrapped with parafilm and aluminum foil. The seeds were then vernalized by incubating at 4°C for 2 weeks.

### Preparation of *Agrobacterium* for flower infiltration

A liquid culture of *Agrobacterium* was grown at 28°C in YEP medium (Bacto-peptone 10 g, yeast extract 10 g, NaCl 5 g) containing the appropriate antibiotics for the strain, to an O.D.600 of 1.6-1.8. The cells were pelleted by centrifugation at 5000 g for 5 min at room temperature, and resuspended in flower infiltration media (0.5×MS salts, 1×Gamborg's vitamins, 0.04 mM BAP, 0.02% Silwet77 (OSI specialities, Inc., Danbury, CT, USA), pH 5.7.

### Transformation of *M. truncatula* by vacuum infiltration of flowering plants

The experiments were performed essentially as described by Trieu et al. (2000) with minor changes. Vernalized seedlings were grown until the plants had small flower buds and a few opened flowers. The plants were watered heavily on the day before infiltration. To infiltrate the plants, the pots were inverted and the above-ground portion of the plant submerged in a container filled with a suspension of *Agrobacterium* in flower infiltration medium. Usually the soil was held in the pot by the roots, but if the soil appeared loose the pot was packed with cotton wool to ensure that the soil did not fall out. The pot and tray were placed in a vacuum chamber and a vacuum drawn to 25 inches Hg and held for 3 min. The vacuum was released very rapidly and the procedure repeated once. The pots were removed from the *Agrobacterium* and placed on their sides in a tray. The tray and pots were transferred to a growth chamber set at 18°C, 95% humidity, 16/8 h photoperiod. The plants were incubated in the chamber for a week. After 2-3 days the pots were placed in an upright position again. The pots were not watered during this time. After a week, the infiltration process was repeated and the plants returned to the growth chamber for a week. In some cases the plants required water during the second week and this was applied carefully to the bottom of the pots. After the second week the plants were returned to normal growing conditions and allowed to set seed. The transformants were selected in the subsequent generation.



### Transformation of *M. truncatula* by vacuum infiltration of seedlings

Vernalized seedlings were removed from the cold room and transferred to petri plates (10×100 mm) containing approximately 10 ml vacuum infiltration medium suspension. The medium coats, but does not completely submerge, the seedlings. The petri plates were placed in a vacuum chamber and a vacuum drawn to 25 inches Hg. This was held for 1 min and then released rapidly. The vacuum step was repeated once more and then the seedlings blotted on sterile filter paper (3MM, Whatman) and spread onto seedling co-cultivation medium, approximately 20 seedlings per petri plate (10×100 mm).

Seedling co-cultivation medium is based on M<sub>2</sub>C medium (Trieu and Harrison 1996) and contains 1×PDM salts and vitamins (Chabaud et al., 1996), sucrose 10 g l<sup>-1</sup>, acetosyringone 100 mM, agar-agar 0.75% (Sigma), pH 5.8. The plates were incubated in a growth chamber at 20°C, 16 h days for 2 days. Following incubation the seedlings were rinsed briefly in distilled water and planted into pots containing RediEarth. The pots were covered with a plastic dome and placed in the growth conditions. The dome was removed after 1 week. The seeds were collected from these plants for further selection.

### Selecting *M. truncatula* transformants with PPT

Seeds collected from the infiltrated plants were treated with acid as described, but were not surface-sterilized. They were placed on damp filter paper in petri plates and

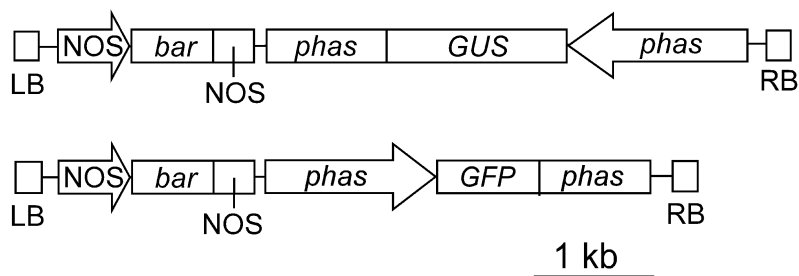
incubated at 4°C for 3-4 days to help germination. They were then planted in RediEarth (Scott) in large trays, grown to the first trifoliolate stage and sprayed thoroughly with Ignite (AgrEvo, Wilmington, Delaware, USA) diluted to contain PPT at 80 mg l<sup>-1</sup>. The seedlings were initially covered with a plastic dome that was removed after 3-4 days. Two weeks later the seedlings were sprayed with 560 mg l<sup>-1</sup> PPT.

## Results

Extensive attempts have been made to transform *M. truncatula* with reporter genes driven by the phas promoter. The reporter constructs used in these experiments were pCB302-phasGUS and pCB302-phasGFP which both carry the bar (phosphinothricin resistance) gene as a selectable marker (Fig. 2.1). Both constructs have been confirmed to be functional by bombardment or transforming into *Arabidopsis*. The *Agrobacterium* strains used in the transformation experiments were GV3101 (Koncz and Schell 1986) and EHA105 (Hood et al. 1993), both of which were reported to be used successfully for transformation (Trieu et al. 2000).

After initial attempts that strictly followed the published protocol failed to yield any transformants, multiple adjustments were made to the original protocol. Three different co-cultivation temperatures, 18°C, 20°C and 25°C were tested since co-cultivation temperature affects transformation rate (Baron et al. 2001). Modifications to the infiltration were also made, including different vacuum strengths and the concentration

of the detergent Silwet 77. Despite these changes, I have been unable to identify any PPT resistant seedlings (Table 2.1).



**Fig. 2.1** T-DNA constructs used for *in planta* transformation of *M. truncatula*.

**Table 2.1** Summary of *in planta* transformation.

Infiltration methods	Agro strains	Constructs	Selectable marker	Plants treated	Total T1 seedlings sprayed	PPT resistant seedlings
seedling	GV3101	<i>phas-GUS</i>	<i>nos-bar</i>	160	>1000	0
infiltration	EHA105	<i>phas-GFP</i>				
flower	GV3101	<i>phas-GUS</i>	<i>nos-bar</i>	126	>1000	0
infiltration	EHA105					

## Discussion

Failure to repeat the initial success reported by the Harrison laboratory (Trieu et al. 2000), appears to be experienced by all who have attempted to recapitulate their procedures. However, we have analyzed progeny of the transformant derived from the *in planta* transformation approach (kindly provided by Dr. Harrison) and found strong GUS expression in all tissues, including mature leaves. Genomic blotting confirmed the presence of an integrated *35S-GUS* insert, although an identical pattern was obtained from all seed available to us. Nevertheless, these results support the original work and indicate that the *in vivo* approaches are feasible.

**CHAPTER III**

***AGROBACTERIUM*-MEDIATED TRANSFORMATION OF**

***M. truncatula* A17 AND FUNCTIONAL ANALYSIS**

**OF GUS AND GFP EXPRESSION**

**Introduction**

With over 650 genera and 18,000 species, the family *Leguminosae* is the third largest family of higher plants and is second only to grasses in agricultural importance (Young et al. 2003). Legumes are major sources of protein and oil for humans and livestock. Some legumes have beneficial medicinal qualities, others can be highly allergenic. While *Arabidopsis* is proving to be an excellent organism for gene discovery and functional genomics, it does not have the ability to establish symbiotic interactions with rhizobia and mycorrhizae exhibited by legumes such as *Lotus japonicus* and *Medicago truncatula*. The lack of symbiosis in *Arabidopsis* justifies the development of a legume model. In Europe, *Lotus japonicus* has been established as a valuable legume system for gene discovery. Effective systems for *Agrobacterium*-mediated transformation have been established (Handberg and Stougaard 1992; Stiller et al. 1997), enabling the use of

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transposon (Thykjaer et al. 1995) and T-DNA (Martirani et al. 1999) tagging as tools to identify novel plant genes.

*M. truncatula* is also emerging as a model legume system for future gene discovery. Attractive features are its small, diploid genome, self-fertility and short generation time. Rapid progress with sequencing its ~500 Mbp genome and in comparative genomics is underway (Lamblin et al. 2003). However, a facile system for molecular transformation is of great importance for functional genomics. Tissue-culture-based transformation methods have been reported for four genotypes of *M. truncatula*: R108-1 (Hoffmann et al. 1997; Trinh et al. 1998; Scholte et al. 2002), Jemalong 2HA (Thomas et al. 1992; Chabaud et al. 2003), Jemalong J5 (Kamaté et al. 2000) and Jemalong A17 (Chabaud et al. 1996; Trieu and Harrison 1996). Among these, Jemalong A17 is of special interest because the genome of this single-seed descent line was selected for sequencing. Considerable excitement was engendered by the development of two very efficient *in planta* transformation methods, one utilizing the inflorescence and the other seedling infiltration by a medium containing *Agrobacterium* (Trieu et al. 2000). These approaches were of special interest as they did not require tissue culture and could potentially be used for T-DNA insertion analysis.

Unfortunately, the *in planta* methods have not been reproduced (Somers et al. 2003) and difficulty has been experienced with *M. truncatula* A17 in effectively establishing previously-described tissue culture-based transformation systems (Chabaud et al. 1996; Trieu and Harrison 1996). Therefore, great need exists for a reproducible and convenient transformation method for this line. Here, we describe in detail an effective method for

transformation and regeneration of *M. truncatula* A17 based on shoot organogenesis from cotyledonary node. It has thus far been successful in producing transgenic shoots from each of eight completely independent experiments, five of which resulted in stable fertile transformants that express both *GUS* and *GFP* transgenes through the second ( $T_1$ ), and in the four lines tested, the  $T_2$  generation. Recent improvements to the regeneration protocol have yielded rooting rates approaching 50%.

Functional analysis is described for transgenic plants bearing two reporter gene constructs. In one, the *GFP* reporter is under control of the CaMV 35S promoter, that is often regarded as constitutive (Benfey et al. 1989; Battraw and Hall 1990). In the other, the *GUS* reporter is driven by the *phas* promoter, which is under stringent spatial regulation (van der Geest et al. 1995; Li et al. 2001). Genomic blot analysis showed that both single copy and multiple copy plants were recovered.

## **Materials and methods**

### Plant material

*Medicago truncatula* cv. Jemalong (line A17) was used for all experiments. Individual wild-type plants were grown in Redi-Earth (Scotts) in 4" pots under greenhouse conditions of 14 h day length (23°C day, 20°C night). Plants were watered daily and once weekly with Miracle-Gro® nutrient at the concentration recommended by the

manufacturer. Primary transgenic plants were grown under similar conditions in a growth chamber.

#### Preparation of the *Agrobacterium* suspension medium

pCB302-*phas-GUS* (Fig. 3.1a) was electrophoretically transformed into *Agrobacterium tumefaciens* strain EHA105 or AGL1; the bacterial cells were selected on 100 mg l<sup>-1</sup> kanamycin and a single resistant colony was streaked on solid YEP medium (10 g l<sup>-1</sup> Bacto-peptone, 10 g l<sup>-1</sup> yeast extract, 5 g l<sup>-1</sup> NaCl) containing 100 mg l<sup>-1</sup> kanamycin and incubated at 28°C for 2 days. After restriction digestion confirmation of the presence of the desired gene construct, liquid YEP medium (20 ml) containing 100 mg l<sup>-1</sup> kanamycin was inoculated with a single colony of *Agrobacterium* and cultured overnight at 28°C, with shaking at 250 r.p.m., to an OD<sub>600</sub> of 1.0 to 2.0. The cells were then pelleted by centrifugation at 4000 x g for 10 min and resuspended to a final OD<sub>600</sub> of 0.8 in inoculation medium, pH 5.5, consisting of SH salts and vitamins (Sigma), 20 g l<sup>-1</sup> sucrose, 3 mg l<sup>-1</sup> BAP, 0.1 mg l<sup>-1</sup> NAA, 100 µM acetosyringone, 400 mg l<sup>-1</sup> L-cysteine (Sigma), 1 mM DTT (Sigma) and 3 mM MES.



## Preparation of cotyledonary explants and infection with *Agrobacterium*

Procedures for permeabilization and surface-sterilization of *M. truncatula* seeds were modified from Trieu *et al.* (2000) and the preparation of cotyledonary node explants was altered from Trieu & Harrison (1996). Mature seeds were exposed to conc. H<sub>2</sub>SO<sub>4</sub> for 8 min, rinsed three times with sterile distilled water, then sterilized in 33% Chlorox® (2% NaOCl) for 10 min with gentle agitation. The seeds were thoroughly rinsed, immersed in sterile distilled water (~2 h) and arranged on 25x100 mm Petri dishes (15 per dish) containing a germination medium of SH salts vitamins, 20 g l<sup>-1</sup> sucrose, 1 mg l<sup>-1</sup> BAP in 0.8% agar (Sigma), pH 5.8. After incubation for 4 days at 25°C under fluorescent lights (16/8 h light/dark), the radicle and most of the hypocotyl were removed from the emerging seedlings by transection 1-2 mm below the cotyledonary node. The remaining tissues were bisected so that each explant had 1 cotyledon and half of the embryonic axis.

The explants were inoculated by immersion in the *Agrobacterium* inoculation medium described above, with gentle shaking for 30 min, then briefly blotted on sterile filter paper to remove excess *Agrobacteria*. They were then placed adaxial side face-up on co-cultivation medium (inoculation medium solidified with 0.8% agar) and incubated at 25°C for 5 days with a 16/8 h (light/dark) photoperiod.

## Plant regeneration and selection

Co-cultivated explants were washed twice by shaking in sterile distilled water at 100 r.p.m. (10 min each time), blotted dry on sterile filter paper and placed on 25x100 mm Petri dishes (~10 explants per dish) containing a regeneration medium consisting of SH salts and vitamins, 20 g l<sup>-1</sup> sucrose, 3 mg l<sup>-1</sup> BAP, 0.1 mg l<sup>-1</sup> NAA, 10 mg l<sup>-1</sup> AgNO<sub>3</sub>, 3 mM MES, 100 mg l<sup>-1</sup> Claforan® (cefotaxime sodium: Hoechst-Roussel, Somerville, NJ), 500 mg l<sup>-1</sup> Timentin® (ticarcillin disodium and clauvulanate potassium: GlaxoSmithKline, Research Triangle Park, NC) in 0.8% agar, pH 5.8. The explants were incubated for 15 days under the same conditions as those used for co-cultivation and then transferred to selection medium (regeneration medium supplemented with 1.6 mg l<sup>-1</sup> PPT from Duchefa Biochemie, Netherlands) and transferred to fresh medium every other week. Untransformed shoots started to die after one week and were removed at the time of transfer.

## Induction of adventitious roots

Well-developed resistant shoots (~1.5 cm) were separated from explants with a surgical blade and laid with the cut end exposed on the surface of a plant development and rooting medium consisting of SH salts and vitamins, 10 g l<sup>-1</sup> sucrose, 0.5 mg l<sup>-1</sup> IBA (Sigma), 100 mg l<sup>-1</sup> Claforan, 300 mg l<sup>-1</sup> Timentin and 0.25% Phytigel (Sigma), pH 5.8, in Phytatrays (Sigma), with 3 to 4 shoots per Phytatray. If no roots were induced within

one month, the ends of the shoots were cut with a surgical blade and transferred to fresh medium. The plantlets with well-developed roots were transplanted into soil and cultured (25°C, 16/8 h photoperiod) in a growth chamber, initially with high humidity by covering with plastic bags. The plants usually started to flower after one month.

#### Genomic DNA blot analysis of transgenic plants

Total genomic DNA from transgenic plants was extracted using cetyltrimethylammonium bromide (CTAB) (Woodhead et al. 1998), digested with *Hind*III, loaded (10 µg per lane) onto an agarose gel (1%) and, after electrophoretic separation, blotted onto Hybond-N+ membrane (Amersham). The *Eco*RV-*Xba*I fragment of the T-DNA construct containing the *GUS* coding sequence was used as a *GUS* probe (Fig. 3.1a). A DNA fragment PCR amplified from the binary vector pCB302-*phas-GUS* with the primer pair 5'-CACTGGAGTTGTCCCAATTCTTG-3' and 5'-GTCTGGTAAAAGGACAGGGC-3' was used as a *GFP* probe (Fig. 3.1a). Hybridization was carried out in ULTRAhyb ultrasensitive hybridization solution (Ambion) for 14 h at 42°C. The hybridization results were recorded using a Fuji BAS 2000 phosphorimager system.

### PCR analysis of *GUS* transgenic plant

Total genomic DNA was used as template for PCR analysis with the primer pair 5'-GGTGGGAAAGCGCGTTACAAG-3' and 5'-GTTTACGCGTTGCTTCCGCCA-3' and 30 cycles of amplification (94°C for 1 min, 60°C for 1 min and 72°C for 2 min).

### Detection of GFP expression in transgenic plants

The expression profile for intact transgenic plants was observed using a LT9700 Little Luma excitation light (Lighttools, Encinitas, CA) with 470 nm excitation and 500 nm emission wavelengths. Precise localization of GFP expression in transgenic plants was undertaken using a Zeiss Stemi SV11 microscope with a Zeiss AttoArc 2 light source. Excitation and emission wavelengths were 470 nm and 500 nm, respectively. Expression patterns were recorded using a Zeiss AxioCam HRc.

### Histochemical localization of GUS activity

Embryos and leaves from transgenic plants were stained for GUS activity as described (Jefferson et al. 1987). Tissue samples were immersed in the GUS staining solution, vacuum infiltrated for 5 min to facilitate penetration, and then incubated at 37°C in the dark. The stained samples were then rinsed with distilled water and immersed in 95% ethanol to remove chlorophyll.

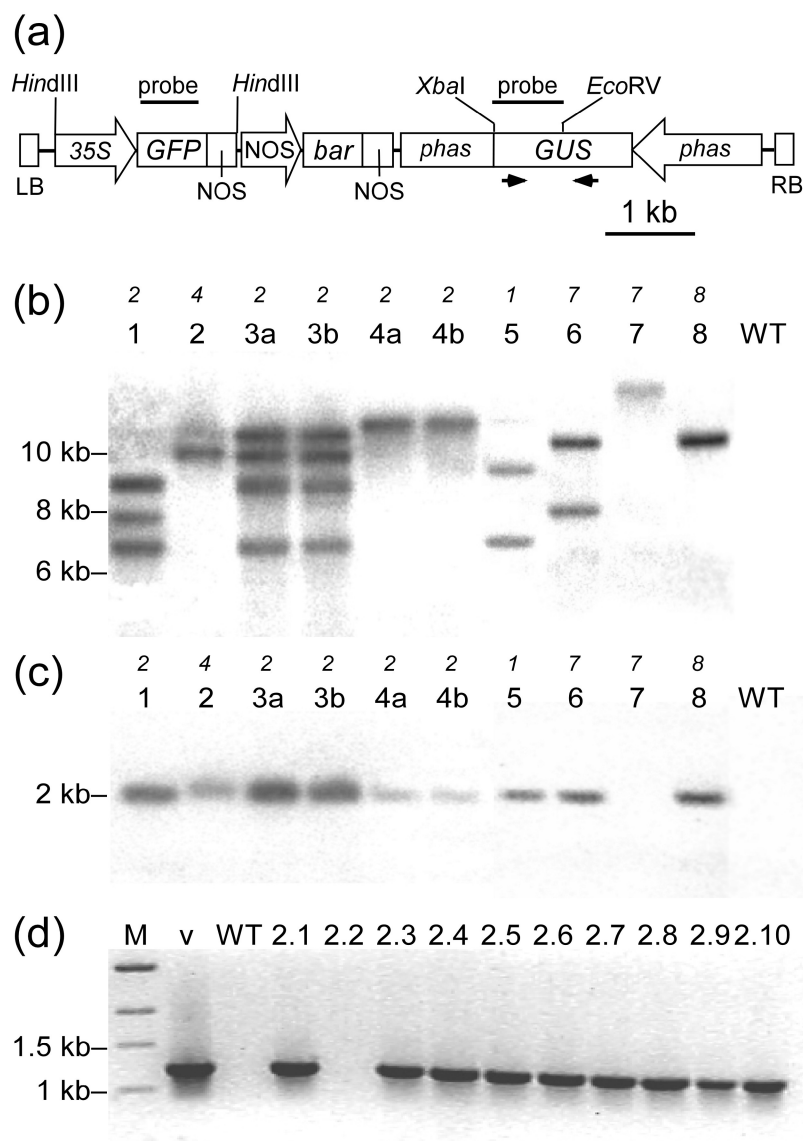
### Detection of *GFP* transcript using RT-PCR

Total RNA was extracted from young and mature leaves of *M. truncatula* plants transgenic for *35S-GFP* using TRIzol reagent (Invitrogen) and treated with DNaseI (Invitrogen, 1 unit/1  $\mu$ l) at 25°C for 15 min to remove DNA and then heated at 65°C for 10 min in the presence of 2.5 mM EDTA to inactivate DNase. RT-PCR reactions were performed using a OneStep-RT-PCR kit (Qiagen) according to the manufacturer's recommendations. Reverse transcription was performed at 50°C for 30 min, followed by PCR activation at 90°C for 15 min and then 25 cycles of PCR amplification (94°C for 1 min, 52°C for 1 min and 72°C for 1 min) using primer pair 5'-CACTGGAGTTGTCCCAATTCTTG-3' and 5'-GTCTGGTAAAAGGACAGGGC-3' for *GFP* transgene and primer pair 5'-CTCTCCGCCACCGATTCATC-3' and 5'-CCATCTCTCTCACTCACTGCATAC-3' for *M. truncatula EF-1 $\alpha$* .

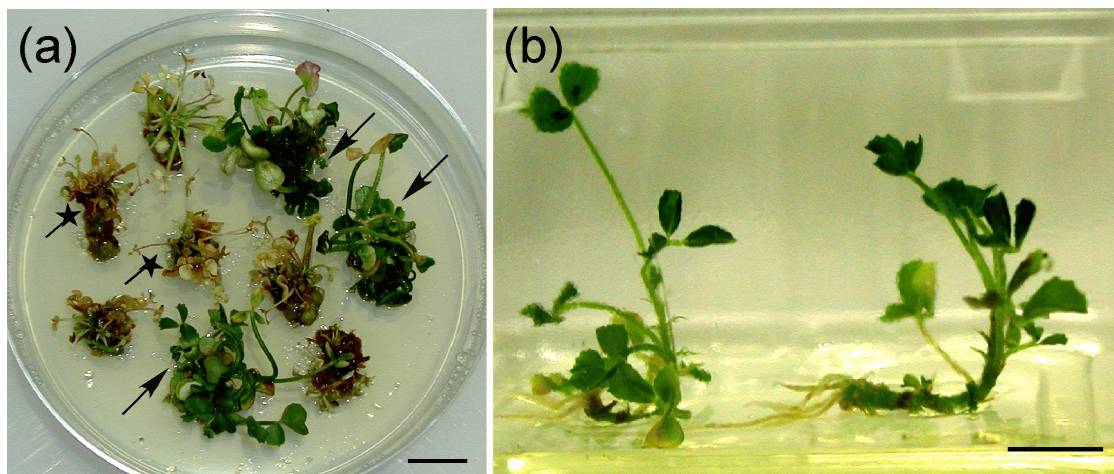
## Results

### *Agrobacterium*-mediated transformation of *M. truncatula*

Eight independent transformation experiments were performed using CaMV 35S-*GFP* and *phas-GUS* constructs (Fig. 3.1a) inserted into binary vector pCB302 (Xiang et al. 1999) in *Agrobacterium* strain EHA105 (Hood et al. 1993) for experiments 1 to 3, and in strain AGL1 (Lazo et al. 1991) for experiments 4 to 8 (Table 3.1). In these experiments, 100 to 150 explants were inoculated with *Agrobacterium* suspension. Phosphinothricin (PPT)-resistant shoots were induced in 3% to 15% of the explants, with an average of 6% of the explants treated. More than one resistant shoot was often induced from a single explant; the ratios of resistant shoots to inoculated explants ranged from 3% to 174%, with an average of 35%. The clear distinction between transgenic, PPT-resistant explants with green leaves and non-transformed with browning leaves on selection medium is shown in Fig. 3.2a. The difficulty in inducing roots in the initial attempts using Trieu & Harrison's protocol (1996) is evident from the data shown for experiments 3, 5 and 6 (Table 3.1) where no rooted plants were recovered. However, using the improved rooting procedure (see experimental procedures for details), considerably higher proportions of the regenerating resistant shoots form roots, as seen for experiments 1 (27%) and 8 (45%). Efficient induction of roots was very dependent on both the composition of the medium and the positioning of the cut shoot, as shown in Fig. 3.2b.



**Fig. 3.1** Transgene construct and insertion patterns. (a) Diagram of the T-DNA region of pCB302-*phas-GUS*. The locations of the *GFP* and *GUS* probes are indicated by *lines* and primer positions by *arrows*. The *HindIII* sites used for the genomic blots are indicated, as are the *XbaI* and *EcoRV* sites used to obtain the *GUS* probe. (b) Genomic blot of leaf DNA from 10  $T_0$  plants probed for *GUS*. Plants 3a, 3b are siblings, as are 4a, 4b. WT, wild-type *M. truncatula* A17. The *italic* numerals above the lanes correspond to the experiment numbers in Table 1; the **bold** Roman numerals correspond to the plant line numbers. (c) The blot was stripped and re-probed for *GFP*. (d) PCR confirmation of the *GUS* insert in 10  $T_1$  progeny of line 2 (lanes 2.1-2.10). v, binary vector pCB302-*phas-GUS*. M, 1 kb marker ladder.



**Fig. 3.2.** Regeneration of transgenic plants. (a) Selection of regenerating *M. truncatula* A17 shoots on medium containing  $1.6 \text{ mg l}^{-1}$  PPT. Green, resistant shoots are indicated by *arrows*. (b) Optimal positioning of cut shoots for root induction; note that roots emerge from the upper edge of the cut surface. Both panels are for experiment 8 in Table 3.1. Scale bars denote 1 cm in both (a) and (b).

In total, on the basis of GFP expression, more than 30 transgenic plants have been obtained from five independent experiments. To confirm the integration of T-DNA into the genomes of these plants, genomic DNA was extracted from ten of the  $T_0$  plants derived from five separate experiments (experiments 1, 2, 4, 7 and 8) and subjected to hybridization analysis using the *GUS* and *GFP* probes indicated in Fig. 3.1a. The results showed that, except for line 7, the  $T_0$  plants regenerated from PPT-resistant shoots carry at least one copy of each reporter (Fig. 3.1b,c). Of the ten lines tested, eight were shown to be independent. Lines 3a, 3b and 4a, 4b were siblings, reflecting the recovery of multiple PPT-resistant shoots from a single transformation event. The hybridization fragments for *GUS* are all larger than 6 kb, as expected from the 5.8 kb distance from the *HindIII* site between the *nos* terminator and *nos* promoter and the right T-DNA border



(Fig. 3.1a). The 2.0 kb *GFP* hybridization fragments reflect the distance between the two *HindIII* sites flanking the *35S-GFP* gene. The hybridization patterns are consistent with the presence of one copy of the transgene insert in lines 4 and 8, two copies in lines 5 and 6, three copies in line 1 and four copies in line 3. Line 2 appears to have one intact copy and a second, partial copy.

The inheritance of *GUS* in the T<sub>1</sub> generation of line 2 was confirmed by PCR amplification. A fragment with the predicted length of 1.2 kb was amplified from nine out of ten T<sub>1</sub> plants (Fig. 3.1d). For lines 1, 2, 3 and 4, the presence of transgenes in T<sub>1</sub> and T<sub>2</sub> progeny was confirmed by the expression of GFP and GUS.

**Table 3.1.** Efficacy of transgenic rooted shoot recovery from cotyledonary node explants

Expt.	Explants			Resistant shoots			
	Inoculated	Resistant <sup>1</sup>	Resistant (%)	Regenerated <sup>2</sup>	Per inoculated explant (%)	Rooted	Rooted (%)
1	140	11	8	41	29	11	27
2	150	23	15	261	174	103	39
3	150	5	3	12	8	0	0
4	150	9	6	53	35	21	40
5	150	4	3	15	10	0	0
6	100	3	3	3	3	0	0
7	140	6	4	24	17	10	42
8	100	7	7	40	7	18	45

<sup>1</sup>Denotes the number of inoculated explants from which shoots resistant to 1.6 mg l<sup>-1</sup> PPT were recovered.

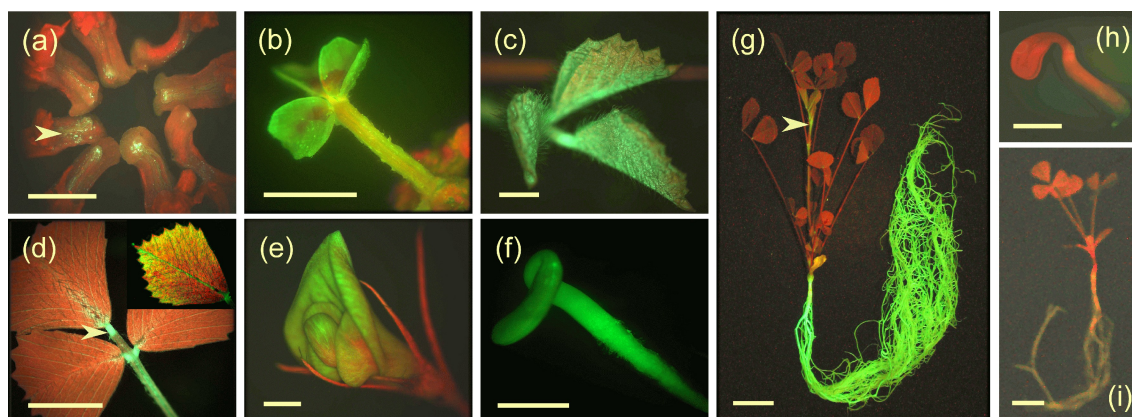
<sup>2</sup>Denotes the number of PPT-resistant shoots recovered from the resistant explants.

### Diminution of 35S-driven GFP expression in mature tissues

Expression of GFP has proven a useful visual screenable marker for plant transformation (Haseloff et al. 1997). To assess its value in *Agrobacterium*-mediated transformation of *M. truncatula* A17, a 35S-GFP fusion construct (Fig. 3.1a) was used. Strong green fluorescence was detected at some cut surfaces of inoculated explants 2 days after co-cultivation and also in newly regenerating shoots from the cotyledonary node regions of the explants (Fig. 3.3a,b), permitting early identification of transformed tissues. Strong expression was also seen in young (~ 1 day old) leaves of transgenic lines 1 (Fig. 3.3c) and 2; weaker expression was evident for lines 3 to 6 and 8. However, GFP expression diminished in all lines as the leaves aged, as seen for the 5-day-old trifoliolate leaf of line 1 in Fig. 3.3d (although fluorescence at the leaf margin sometimes remained, as shown in the inset). Interestingly, as the shoots elongated, newly emerging leaves showed high GFP expression; again, this diminished as the leaves aged. The possibility that decreased expression resulted from gene silencing was evaluated by analysis of RNA transcript level using RT-PCR. Similar levels of transcript were apparent in extracts of young and old leaves (Fig. 3.4), indicating that, as reported by Halfhill *et al.* (2003) for aging leaves of *Brassica napus*, silencing was not the primary cause of decreased GFP expression.

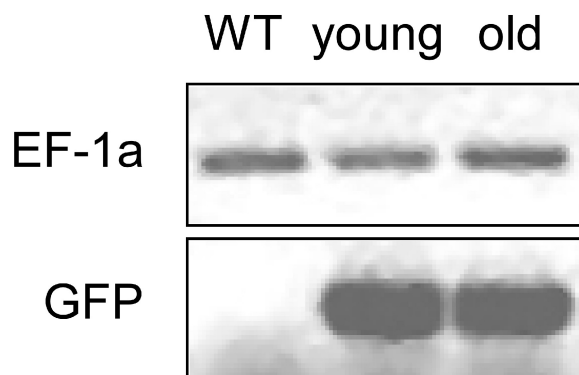
Petals showed high levels of expression, but expression in the calyx was low (Fig. 3.3e). Evaluation of GFP expression in seed tissues was difficult because high levels of fluorescence were present in untransformed plants (not shown). The eight lines examined in detail were fertile although line 1 produced only a few pods. The

germination rate for line 1 (~30%) was also lower than that of the other transgenic lines, which was similar to wild type.



**Fig. 3.3** GFP expression in transgenic *M. truncatula* A17. (a) Transient GFP expression (*arrowhead*) in explants 2 days after cocultivation. (b) Transgenic shoot 1 month after initiating regeneration. (c) Emerging trifoliolate leaf after transfer of plant to soil. (d) Diminished GFP fluorescence in the mature trifoliolate leaf, except at the margin of some leaves (inset); bright green fluorescence remains at the petiole nodes (*arrowhead*). (e) Strong fluorescence in the corolla but weak fluorescence in the calyx. (f) Ubiquitous fluorescence in germinating (2 day)  $T_1$  seedling. (g) Entire  $T_1$  plant (~20 day) showing strong fluorescence in roots and nodal meristem (*arrowhead*), but no or low expression in mature leaves or stem. (h) A 2-day wild-type seedling. (i) A 10-day wild-type seedling. ( Scale bars denote: 5 mm in (a), (b), (d), (f) and (h); 1 mm in (c) and (e); 1 cm in (g) and (i).

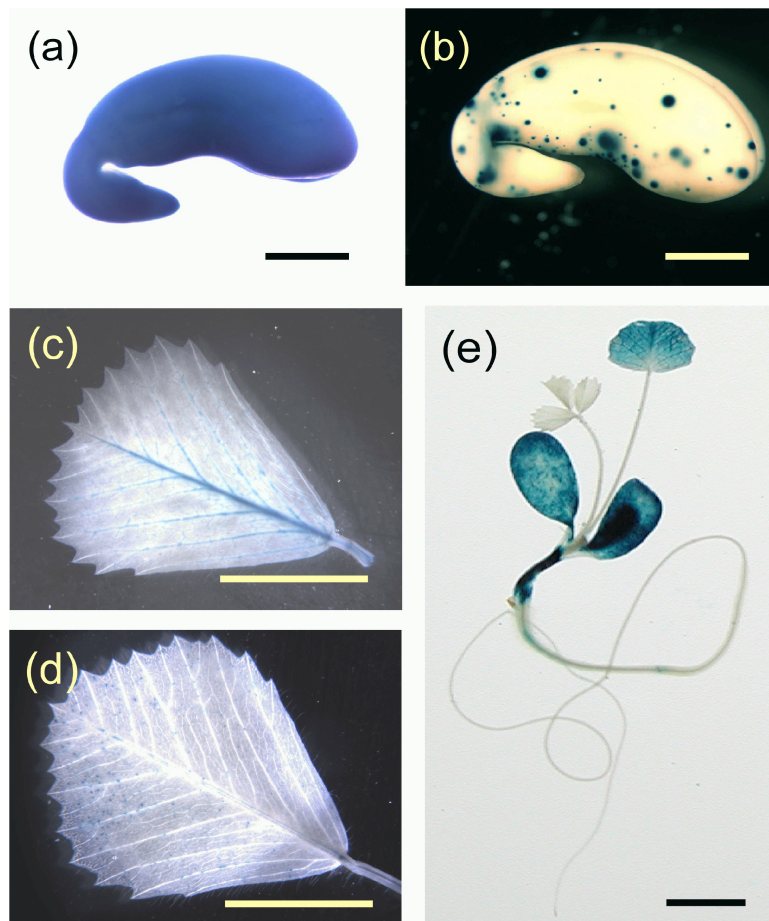
GFP expression was evident in all tissues of the emerging  $T_1$  progeny seedling of line 1 shown in Fig. 3.3f. Seedlings of other lines had lower overall levels of expression than did line 1, but, in each case, the radicle region showed higher fluorescence than did the other regions. An entire 20-day-old plant of line 1 is shown in Fig. 3.3g; bright green fluorescence was seen throughout the root tissues but, in the shoot, GFP expression was limited to nodes and young leaves.



**Fig. 3.4** RT-PCR analysis of *GFP* transcription in young and old leaves of transgenic *M. truncatula* A17. Bottom panel shows *GFP* transcript levels in young and old leaf tissues of transgenic plants normalized relative to that of the internal control, *EF-1 $\alpha$*  (top panel). WT, wild-type.

Diminished spatial-specificity of GUS expression driven by the *phaseolin* promoter in transgenic *M. truncatula*

Histochemical staining was performed to detect GUS expression driven by the *phas* promoter in transgenic plants. For lines 1, 2 and 4 (Fig. 3.1b), blue staining was evident for embryos within a few minutes of incubation, and overnight staining resulted in intense blue staining throughout the embryo, indicating a high level of GUS expression (Fig. 3.5a). For line 3, discrete, dark blue spots were evident throughout the embryo (Fig. 3.5b), suggesting the occurrence of epigenetic silencing in this multicopy line.



**Fig. 3.5.** GUS expression in transgenic *M. truncatula* A17. (a) Mature embryo exhibiting strong GUS expression in all tissues. (b) Mosaic GUS expression pattern seen in line 3. (c) GUS expression pattern typical of leaf veins in all lines except lines 3a and 3b, for which a mosaic GUS expression pattern was seen (d). (e) Entire T<sub>1</sub> plant of line 1 showing GUS expression in the cotyledons, the plumular leaf, and the hypocotyl and adjacent root region. Scale bars denote: 1 mm in (a) and (b); 5 mm in (c), (d) and (e).

Although expression from the *phas* promoter is known to be strictly confined to embryogenesis and microsporogenesis in beans, transgenic tobacco (Sengupta-Gopalan et al. 1985; van der Geest et al. 1995) and *Arabidopsis* (Chandrasekharan et al. 2003), histochemical staining revealed GUS expression in the leaf veins of all the transformants (Fig. 3.5c); as for the embryo, line 3 exhibited spotty expression in the leaves (Fig. 3.5d).

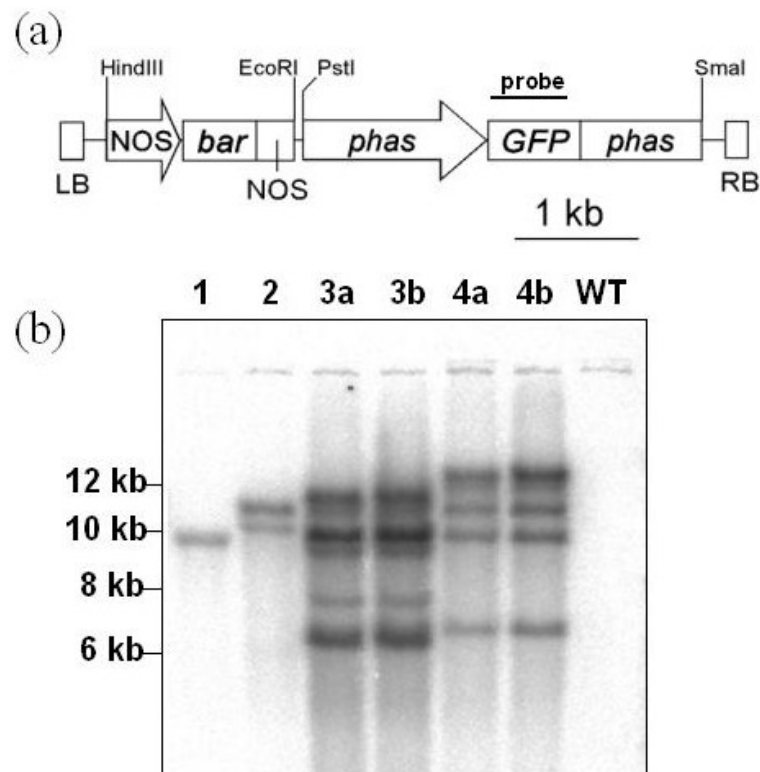
GUS expression was assessed histochemically for 7-day seedlings of lines 1, 2 and 4; a representative seedling is shown in Fig. 3.4e. GUS has a half-life of approximately 50 h (Jefferson *et al.*, 1987) and residual expression was seen for tobacco seedlings 5 days after germination (Frisch *et al.* 1995). Similarly, residual GUS expression was detected (Fig. 3.5e) for 7-day *M. truncatula* seedlings of lines 1, 2 and 4. The hypocotyl and cotyledons stained strongly, moderate staining was observed for the plumular leaf and faint staining was seen in the upper parts of developing roots. No staining was observed for the cotyledonary node, the first trifoliate leaf, nor for most of the root.

#### Transformation of *M. truncatula* with *phas-GFP*

Twelve *M. truncatula* plants from four independent transformation experiments using a pCB302-*phas-GFP* construct (Fig. 3.6a) have been obtained following the established transformation procedure. Genomic blot were performed using the coding sequence of *GFP* as probe (Fig. 3.6a) to confirm the integration of the T-DNA into the genome of these plants (Fig. 3.6b). The results showed that all six lines tested carry at least one copy of the *GFP* reporter gene. Of the six lines tested, four were shown to be independent, while line 3a, 3b and 4a, 4b were siblings.

The expression pattern of GFP driven by the *phas* promoter is determined in the six transgenic lines. For lines 1, 2 and 4, strong GFP fluorescence can be detected in embryonic tissues of transgenic seed even with 100 ms of exposure (Fig. 3.7a), while no GFP fluorescence was observed in vegetative tissues. For the single-insertion line (line

1), an expected 3 to 1 segregation ratio in T1 seeds was observed (Fig. 3.7a). Removal of seed coat reveals more intense fluorescence in embryonic tissues (Fig. 3.7b). On germination GFP is quickly degraded in cotyledons, while the emerging plumular leaf showed no GFP fluorescence (Fig. 3.7c, d).

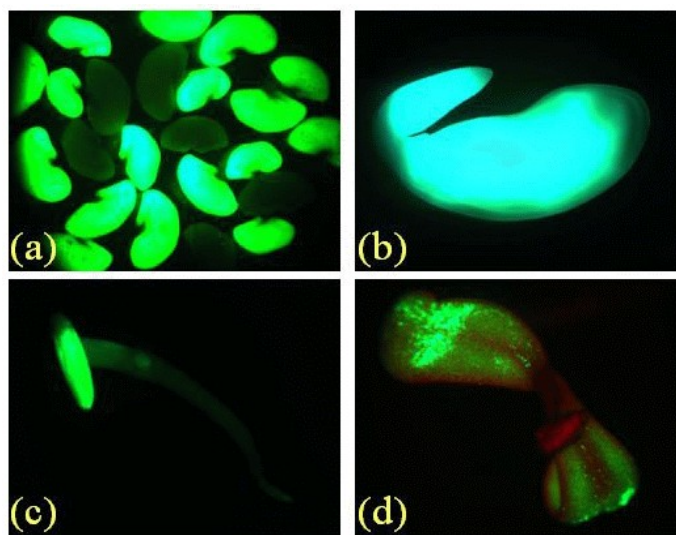


**Fig. 3.6** Transgene construct and insertion patterns. (a) Diagram of the T-DNA region of pCB302-*phas-GFP*. The locations of the *GFP* probe is indicated by lines. The *HindIII* sites used for the genomic blots are indicated. (b) Genomic blot of leaf DNA from 6 T<sub>0</sub> plants probed for *GFP*.

Aberrant expression from the *phas* promoter was also observed for the *phas-GFP* construct even though the *phas* promoter in this construct is not close to the T-DNA border. The transgenic lines 3a and 3b showed substantially lower level of GFP

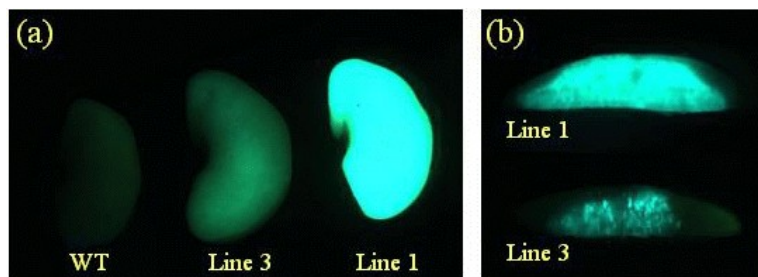
expression in the T1 seeds (Fig. 3.8a). The comparison between the cross section of the seeds of line 1 and line 3 showed that GFP fluorescence was not only detectable in the center region of the cotyledon, but also less intense in the embryo of line 3 compared with that of line 1 (Fig. 3.8 a, b).

The successful recovery of transgenic *M. truncatula* without the aid of a screenable visible *35S-GFP* marker verifies the effectiveness of the current transformation method. The occurrence of weak expression from *phas-GFP* in the seeds of line 3 corroborated our findings for aberrant expression of GUS from the *phas* promoter.



**Fig. 3.7.** GFP expression driven by *phas* promoter in transgenic *M. truncatula*. (a) T1 seeds of line 1. (b) An excised T1 embryo of line 1. (c) A 1-d-old seedling of line 1. (d) A 3-d-old seedling of line 1. The emission wavelength was 500 nm. The exposure times were 100 ms for (a) and (b), and 1 sec for (c) and (d).





**Fig. 3.8** Aberrant expression of *phas-GFP* in line 3. (a) A comparison of seed fluorescence among wild-type (WT), line 3 and line 1. (b) Cross section of cotyledons of lines 1(top) and line 3(bottom). The emission wavelength was 525 nm. The exposure times were 300 ms for (a), 100 ms for line 1 in (b) and 2 sec for line 3 in (b).

## Discussion

### Optimization of transformation, selection and regeneration conditions

The direct induction of shoots from cotyledonary nodes has been used successfully for regeneration of transgenic plants following *Agrobacterium*-mediated infection for several legume species (Somers et al. 2003). Although an early report exists of *M. truncatula* A17 transformation by this approach (Trieu and Harrison 1996), we are not aware of any subsequent articles that have utilized this system. While the work reported here is based on this earlier work, many small but important modifications were made to obtain reproducible, high frequencies for recovery of transgenic plants.

Compared with other tissue culture-based transformation methods, the cotyledonary-node approach is relatively fast and does not involve the induction of embryogenic calli and somatic embryos, which can be difficult. On the other hand, common problems with this approach are the low rates of transformation and regeneration, and recovery of

transgenic plants can be less than 1% (Zhang et al. 1999; Donaldson and Simmonds 2000). In this work, up to 15% inoculated explants gave rise to PPT-resistant shoots and up to 45% PPT-resistant shoots were rooted (Table 3.1). Excluding experiments 3, 5 and 6, for which no rooted transgenic shoots were obtained, the average recovery of rooted shoots was 39% from a total of 419 explants. Use of the conditions detailed in the Experimental Procedures section should greatly facilitate research in gene discovery and gene function in the model plant *M. truncatula*.

In our initial attempts to establish *M. truncatula* transformation, *Agrobacterium* strain LBA4404 was used, as in the original protocol (Trieu and Harrison 1996), but without success. *Agrobacterium* strains EHA105 (Hood et al., 1993) and AGL1 (Lazo et al., 1991) were then tested because of their reported virulence; both proved to be effective in transforming *M. truncatula*.

An important component of the transformation procedure is inclusion of DTT and L-cysteine in both the inoculation and the co-cultivation media. The presence of these compounds in inoculation and co-cultivation media substantially reduces tissue browning resulting from the release of phenolics (McCown et al. 1968) and greatly improved transformation efficiency in soybean (Olhoft et al. 2001; Olhoft et al. 2003). The inclusion of these compounds improved transient GFP expression immediately after co-cultivation and yielded a higher percentage of explants from which PPT-resistant shoots were regenerated.

For successful transformation of *M. truncatula*, the selection stringency must be carefully determined, and should be coupled with the strength of the promoter used to

drive the resistance gene to get effective selection of transformants, i.e. if a weak promoter is used, a lower concentration of selection agent should be employed. The presence of  $1.6 \text{ mg l}^{-1}$  PPT in the selection medium was effective in killing untransformed shoots within two weeks while enabling the transgenic shoots to survive and develop. To avoid untransformed shoots from escaping, selection pressure was maintained for two months.

In summary, the parameters we found to be of major importance to successful transformation of *M. truncatula* A17 include the usage of hypervirulent *Agrobacterium* strains for inoculation, the incorporation of L-cysteine and DTT in inoculation and co-cultivation media to suppress tissue browning, and careful determination of selection stringency.

#### Induction of adventitious roots from transgenic shoots

Rooting of the regenerating shoots has been a bottleneck for transformation of *M. truncatula* A17. In the present work, a large array of modifications were tested towards improving rooting efficiency. Changes from the conditions used by Trieu and Harrison (1996) found to be effective were: supplementation of full strength Schenk and Hildebrandt salts and vitamins (Schenk and Hildebrandt 1972) with  $0.5 \text{ mg l}^{-1}$  indole-3-butyric acid (IBA) instead of  $0.2 \text{ mg l}^{-1}$  and reduction of the sucrose concentration from  $20 \text{ g l}^{-1}$  to  $10 \text{ g l}^{-1}$ ; the use of 0.25% Phytigel as the gelling agent instead of 0.8% agar, and the exclusion of the PPT selection agent. Various combinations of IAA and NAA

were tested, but they were much less effective than IBA. A procedure found to be of vital importance is that the shoots must be laid on the surface of the medium with freshly cut ends exposed in the air, as illustrated in Fig. 3.2b. For shoots that did not root within one month, the ends were cut again and the shoot transferred to fresh medium. Recent experiments revealed that a pH of 6.5 is more effective for root induction than is pH 5.8, yielding rooting frequencies approaching 50%. Even shoots that had failed to root after a year of culture responded favorably to this procedure and the numbers of rooted shoots, and hence their percentage of resistant shoots (Table 3.1) is increasing.

#### Intriguing expression patterns of *35S-GFP* and *phas-reporter* in *M. truncatula*

The progressive loss of GFP expression in aerial parts as the plant aged was dramatic and occurred in each of the four transgenic plants studied. Consequently, transgene copy number does not appear to be a major factor. The possibility that decreased expression resulted from gene silencing was evaluated by analysis of RNA transcript level using RT-PCR, but the similar GFP transcript levels in young (1-2 days) and old leaves (>7 days) (Fig. 3.5) detracts from this notion. In detailed studies of aging leaves of *Brassica napus*, Halfhill *et al.* (2003) found that the decrease in GFP expression was positively correlated with soluble protein content. However, this does not satisfactorily explain the rapid disappearance of GFP seen as the *M. truncatula* leaves age. Possibilities to be examined in the future include the synthesis of a quenching agent in the maturing leaves or the accumulation of a degrading agent such as a protease. The present analysis of

transcript levels was not exhaustive and it also remains formally possible that gene silencing is associated with the observed loss of GFP expression. This possibility is supported by the finding from one of the first transformants we obtained that T<sub>1</sub> seedlings cultured in the presence of 20 mg l<sup>-1</sup> 5-azacytidine exhibited green fluorescence whereas those grown in the absence of 5-azacytidine showed no fluorescence (data not shown).

The expression of *35S-GFP* gene fusion constructs in transgenic *M. truncatula* has previously been reported for genotypes R-108-1 and Jemalong J5 (Kamaté *et al.*, 2000) and 2HA (Chabaud *et al.*, 2003). GFP expression was evident in transgenic calli, somatic embryos, flowers, roots and germinating seedlings (Chabaud *et al.*, 2003; Kamaté *et al.*, 2000). However, no GFP expression in leaf tissue is shown in any of these reports, suggesting that, as found in our experiments, loss of expression may have occurred. Indeed, Kamaté *et al.* (2000) comment that GFP expression in embryogenic calli became weaker or ceased at embryo initiation.

From the limited number of independent transformants studied in any detail thus far, it is not possible to determine if the diminished expression observed in mature leaf tissues reflects aberrant activity of the *35S* promoter, or is a commonly occurring feature of GFP in aging tissues that is especially apparent in *M. truncatula*. Both possibilities exist. While the *35S* promoter was originally thought to be constitutive in transgenic plants (Battraw & Hall, 1990; Benfey *et al.*, 1989), more recent studies suggest that it is subject to spatial and other fluctuations in expression (Li *et al.* 1999) and that it may be especially vulnerable to rearrangement (Kohli *et al.* 1999; Kumpatla and Hall 1999).

Certainly, the difference in GFP expression above and below the cotyledonary node evident in Fig. 3.3g (and seen in three additional lines) is dramatic. Further resolution of the situation will require evaluation of expression of GFP from alternative promoters and, conversely, to examine expression of GUS from the *35S* promoter.

Evidence that aberrant transgene expression in *M. truncatula* is not confined to the *35S* promoter is provided by the patchy pattern of GUS expression from the *phas* promoter in T<sub>1</sub> embryos of lines 3a and 3b. The non-uniform expression suggests silencing, a phenomenon not previously encountered for the *phas* promoter. This may reflect the fact that the transgene construct is present in relatively high copy number in lines 3a and 3b (Fig. 3.1b) since intense, uniform staining, and hence GUS expression, was seen for all other lines tested (1, 2 and 4). Lack of the usual spatial stringency of expression from the *phas* promoter in transgenic *M. truncatula* was also evidenced by the weak expression of GUS in mature leaf veins (Fig. 3.5c,d). While this may reflect the genomic environment of the transgene, the observation of expression in several independent lines detracts from this explanation. These findings are especially interesting since it might be assumed that the *phas* promoter would behave more, rather than less, faithfully in a legume background than in evolutionarily distant species such as tobacco and *Arabidopsis*. As evidenced in Table 3.1, many additional transformants will be available, and their analysis will permit a more statistically sound evaluation of transgene expression patterns in *M. truncatula*.

## CHAPTER IV

### THE DARK SIDE OF GFP

#### Introduction

The ability to visualize, track and quantify gene products in living tissues is essential towards understanding of the function of genes and the biological processes in which they are involved. The advent of fluorescent proteins, such as green fluorescent protein (GFP) from the jellyfish *Aequorea victoria* (Chalfie et al. 1994) and its variants, has endowed researchers with powerful tools to monitor gene expression (Harper et al. 1999; Sunilkumar et al. 2002) and gene silencing (Ruiz et al. 1998), and to define spatial (Johnson et al. 2005), developmental (Marton et al. 2005) and quantitative (Tang and Newton 2004) properties of promoters. The lack of requirement for exogenous substrate, cofactors or for histochemical fixation makes GFP a particularly valuable reporter for plant cells, which are not as permeable as animal cells (Hanson and Köhler 2001). Several modifications of the native GFP have improved its thermotolerance and fluorescence, especially in plants (Siemering et al. 1996; Haseloff et al. 1997). For these reasons, GFP and its variants are widely used as attractive alternatives to  $\beta$ -

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glucuronidase (GUS) (Jefferson et al. 1987) for assessment of promoter function and in optimizing plant transformation (Stewart 2001).

The success of GFP to macroscopically monitor the existence and level of transgene expression in intact tissues or organs in promoter or coding region fusions relies upon the existence of a close correlation between the detected intensity of GFP fluorescence and GFP protein content in the plant tissue. Therefore, in studies characterizing transgene expression in the model legume *M. truncatula*, a surprising discovery was that a dramatic decrease in fluorescence (using either a 500 nm long-pass filter or a 525 nm band-pass filter) from GFP occurred shortly after leaf emergence. In contrast, no striking change in fluorescence with age was detected for nonphotosynthetic organs such as roots and petals (Zhou et al. 2004). Similar results were obtained using either 500 nm long-pass filter or 525 nm band-pass filters. Another surprise was the presence of similar levels of GFP transcript for young and old leaves, making it unlikely that gene silencing was the primary cause.

A decrease in observed GFP fluorescence with leaf maturation from a similar 35S-*mGFP5er* construct has been reported in transgenic tobacco (Harper and Stewart 2000) and oilseed rape (Halfhill et al. 2003). Whereas the latter article attributed the decrease in GFP fluorescence to changes in soluble protein content, we now provide several lines of evidence that chlorophyll is a major culprit in the loss of detectable GFP fluorescence as leaves mature. In addition, we show that a decrease in the proportion of GFP in the total protein plays a role. The novel finding of the interference of chlorophyll with GFP



fluorescence reveals a major complication in the application of GFP to plant research that deserves careful evaluation.

## **Materials and methods**

### Plant materials

Transformation of *35S-mGFP5er* into *M. truncatula* using the binary vector pCB302-phas-GUS in *Agrobacterium tumefaciens* strain EHA105 was performed as described (Zhou et al. 2004). *Arabidopsis* (*Arabidopsis thaliana*) plants also transformed (Bechtold and Pelletier 1998) with *35S-mGFP5er* were kindly provided by Tao Wang and rice (*Oryza sativa*) plants transformed (Dong et al. 1996) with *35S-mGFP5er* were generously donated by Yiming Jiang.

### Fluorescence imaging and quantification

For qualitative detection of whole plants, an LT-9700 Little Luma excitation light (Lighttools, CA) was used together with a 470/40 nm filter and 500 nm (long-pass) emission filter and images were taken with an Olympus Camedia C-3040ZOOM digital camera (Olympus, Japan).

Green fluorescence in leaves of plants transgenic for *35S-mGFP5er* was recorded with a Zeiss SV11 stereomicroscope (Carl Zeiss, Germany) coupled to an AxioCam HRc digital camera (Carl Zeiss), using 470 nm excitation and 500 or 525 nm emission filters. Quantification of fluorescence, as the average signal intensity in a selected area, was performed on digitized pictures captured in the presence of a 525 nm emission filter using Zeiss AxioVision software (Carl Zeiss).

Leaf segments were vacuum infiltrated with water and mounted on a glass slide under a coverslip. Hand-cut cross-sections were mounted in the same manner. Specimens were viewed using a Zeiss Axiophot (Carl Zeiss) microscope equipped with a 63x/1.4 Plan Apochromat oil immersion objective and a GFP fluorescence filter set (excitation 470/20 nm, emission 525/25 nm). Image stacks were acquired at a 0.5  $\mu\text{m}$  step between optical sections, using a Coolsnap cf CCD camera (Photometrics, Tucson, AZ). Contrast and brightness of the stacks were then adjusted with ImageJ (<http://rsb.info.nih.gov/ij>) and projected (Brightest Point method) for extended depth of field. Differential interference contrast images of the same field of view were also captured. Assembly and annotation of figures were performed using Canvas 9 (ACD Systems, Saanichton, BC, Canada).

#### Protein extraction

Approximately 300 mg of leaf tissue was ground into fine powder in liquid nitrogen using a mortar and pestle, and transferred into an Eppendorf tube. Total leaf protein was

then extracted with TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0) for 20 min and centrifuged at  $1.2 \times 10^4$  g for 20 min. The supernatant was collected and the total protein concentration was determined (Bradford 1976).

### GFP fluorometry

The fluorescence of plant samples (100  $\mu$ g protein) was determined (Bio-Rad VersaFluor fluorometer) relative to a standard curve for a GFP (Clontech) concentration range of 100 ng ml<sup>-1</sup> to 1  $\mu$ g ml<sup>-1</sup> using 480/20 nm excitation and 510/10 nm emission filters.

To determine the effect of chlorophyll on GFP fluorescence, 1 mg of HPLC-purified chlorophyll a or b (Sigma) was dissolved in 2 ml methanol containing 20 mg ml<sup>-1</sup> Na cholate (Griffiths 1978), dried in a Speed Vac Concentrator (Savant), and resolublized in 2 ml TE buffer. Various amounts of chlorophyll solution were then mixed with 1  $\mu$ g of standard EGFP (Clontech) in a total volume of 1 ml TE buffer containing 20 mg ml<sup>-1</sup> Na cholate. The relative fluorescence of the samples was determined with a Bio-Rad VersaFluor fluorometer calibrated to 0 RFU (relative fluorescence units) with a blank of TE buffer containing 20 mg ml<sup>-1</sup> Na cholate and 1,000 RFU with 1  $\mu$ g of EGFP in TE buffer (1 ml) containing 20 mg ml<sup>-1</sup> Na cholate.

## Western blot analysis

*M. truncatula* leaf extract (5 µg protein), or rice leaf extract (20 µg protein), from various leaf positions, was separated on an SDS-polyacrylamide (5% stacking and 10% resolving) gel, then electrophoretically transferred to Immun-Blot PVDF membrane (Bio-Rad) using a Mini Trans-Blot Electrophoretic Transfer Cell (Bio-Rad). The membrane was incubated with a primary rabbit anti-GFP polyclonal antibody (BD Biosciences-Clontech) at 1:2,000 dilution for 5 h at room temperature, and then incubated with a secondary goat-anti-rabbit antibody conjugated with alkaline phosphatase (Novagen) at 1:10,000 dilution for 1 h at room temperature. Hybridization signals were visualized by exposing the membrane to 1 ml CDP-Star Substrate (Novagen) and then recorded with a UVP BioImaging system (UVP Inc, Upland, CA).

## Chlorophyll content determination

Relative chlorophyll concentrations in leaves were determined using a SPAD-502 Chlorophyll Meter (Minolta).

## Results

GFP fluorescence driven by the *CaMV 35S* promoter diminishes in maturing transgenic *M. truncatula* leaves

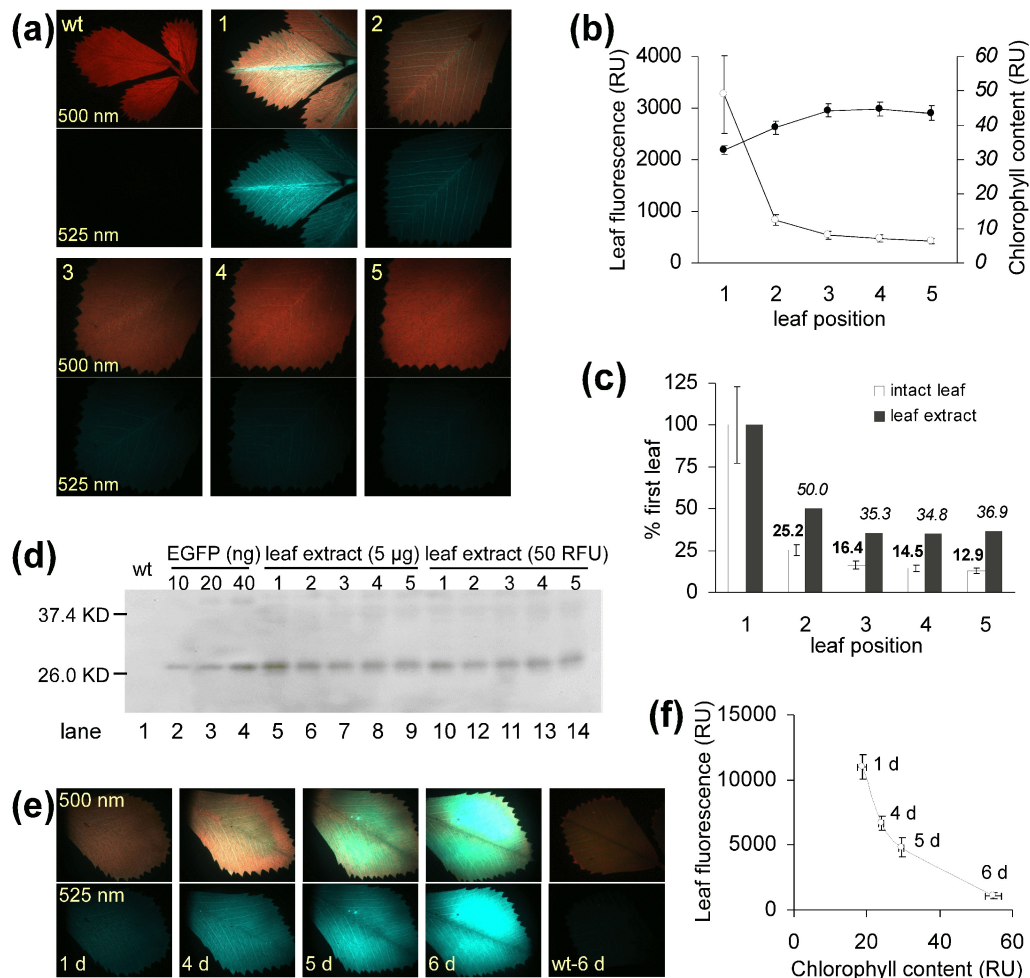
*M. truncatula* plants transgenic for the *mGFP5er* reporter driven by the putatively constitutive *CaMV 35S* promoter display an intriguing temporal-spatial pattern of fluorescence (Zhou et al. 2004). Using an excitation wavelength of 470 nm together with 500 nm or 525 nm emission filters, that reduce or eliminate red fluorescence from chlorophyll, strong GFP fluorescence can be observed in young seedlings, the root system, petals and meristematic tissue and emerging leaves. However, as young *M. truncatula* leaves develop, a progressive loss of GFP fluorescence in aerial parts takes place, eventually resulting in the apparent absence of green fluorescence in mature leaves. Only red fluorescence from chlorophyll or a blank background is observed when 500 nm or 525 nm emission filters are used, respectively (Fig. 4.1a). To follow changes in fluorescence with leaf maturation, *M. truncatula* leaves were numbered according to their relative positions on the shoot, with the emerging leaf closest to the shoot tip being labeled as number 1. To quantitate the intensity of green fluorescence in leaves, digital images were taken (525 nm emission filter) using a Zeiss SV11 microscope fitted with an AxioCam Hrc digital camera. The average intensity of green light in a selected area was measured using AxioVision software (Carl Zeiss, Germany). A dramatic decrease in green fluorescence per unit area was evident during leaf maturation, the most rapid loss

being during early development (from leaf 1 to leaf 3); leaves 5 and older had only approximately one eighth (12.9/100) of the fluorescence level of the first leaf (Fig. 4.1b,c).

Decrease in GFP fluorescence during leaf maturation greatly exceeds the loss of GFP as a proportion of total *M. truncatula* leaf protein

Leaves of various stages of maturity from *M. truncatula* plants transformed with *35S-mGFP5er* were ground in liquid nitrogen and extracted with 1 ml of TE buffer (see Materials and Methods). Total protein content was determined (Bradford 1976) and the fluorescence of 100 µg protein determined relative to a standard curve for GFP (Clonetech) using 480 nm excitation and 510 nm emission filters. A substantial decrease in relative GFP fluorescence was evident commensurate with leaf age (position) as shown in Fig. 4.1(c).

The decrease in GFP content with leaf maturation was further substantiated by immuno-hybridization analysis using antibodies to GFP (Fig. 4.1d). As shown in Fig. 4.1(d), using equal amounts (5 µg) of *M. truncatula* leaf protein, the youngest leaf (leaf 1) yielded a stronger signal than did older leaves, whereas loading samples on the basis of equal RFUs yielded similar western signal intensities. These data show that, in *M. truncatula*, the relative concentration of GFP in total protein decreases with leaf maturity, contributing to the loss of fluorescence with leaf age. However, the ratio for



**Fig. 4.1** Loss of fluorescence in maturing *M. truncatula* leaves reflects increased chlorophyll and decreased GFP concentrations. **(a)** Comparison of GFP fluorescence from non-transgenic (wt) with that from young to mature leaves (leaf positions 1 to 5) of *M. truncatula* transgenic for *35S-mGFP5er*. Images were recorded using 500 nm or 525 nm emission filters (top and bottom panels, respectively). The wild-type leaf is equivalent to a position 1 leaf. **(b)** Fluorescence (*open circle*) decreased and chlorophyll content (*closed circle*) increased with leaf maturity. Error bars indicate SD. **(c)** Comparison of relative GFP fluorescence for intact leaves and leaf extracts with leaf age (position). **(d)** Western blot analysis of GFP in *M. truncatula* transformed with *35S-mGFP5er*. Extracts of leaves from positions 1 to 5 were loaded on a basis of equal total protein (5  $\mu$ g; lanes 5 to 9) or fluorescence (50 relative fluorescence units: lanes 10 to 14). EGFP standards are shown in lanes 2 to 4 and a wild-type leaf extract (5  $\mu$ g protein) was used as control (lane 1). **(e)** Leaves covered for 1, 4, 5, and 6 days were recorded using either 500 emission filter (top panels) or 525 nm emission filter (bottom panels). Wild-type leaves covered for 6 days were included as controls (wt). **(f)** The relative fluorescence (*open circle*) was plotted against chlorophyll content for leaves shown in panel (e). All pictures were exposed for 5 seconds. RU denotes relative units.

loss of GFP fluorescence in intact leaves ( $100/12.9 = 7.8$ ) was almost three-fold greater than that in leaf extracts ( $100/36.9 = 2.7$ ), unveiling the presence of a major interfering factor (or factors) in *M. truncatula* leaves that are not present in leaf extracts (Fig. 4.1c).

Inverse correlation between chlorophyll level and fluorescence intensity observed *in vivo* implies the involvement of chlorophyll in the disappearance of GFP fluorescence

Since the apparent loss of GFP fluorescence is most evident in leaves, a likely candidate is interference by chlorophyll. That chlorophyll absorbs strongly at the excitation wavelength of GFP protein, and the absence of chlorophyll in aqueous leaf extracts (for which there is little loss of fluorescence with leaf age, adjusted for GFP content; Fig. 4.1c,d), provide clues that chlorophyll is a likely culprit in the disappearance of GFP fluorescence.

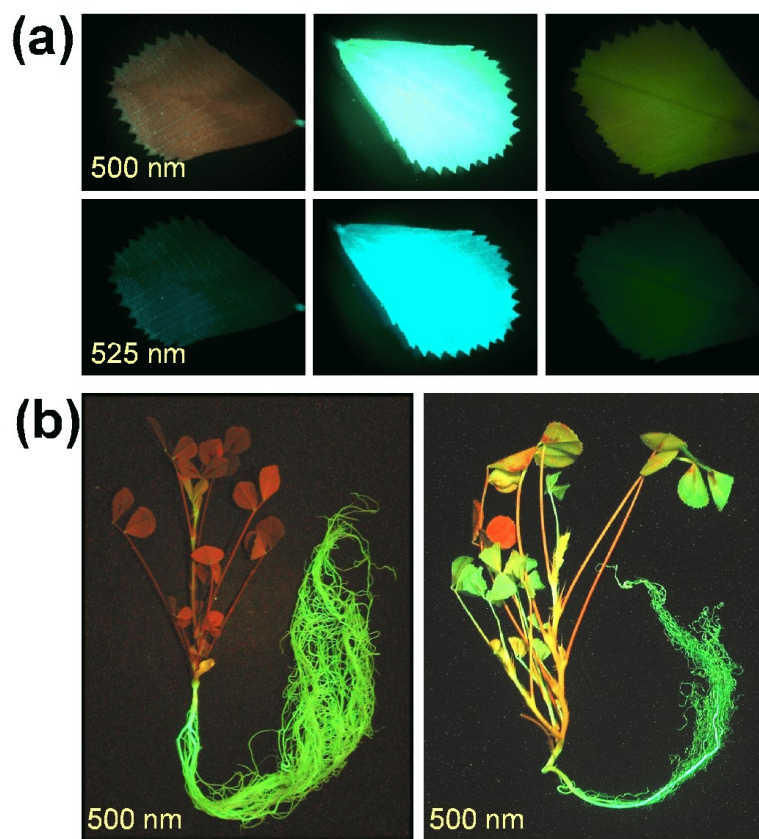
A SPAD 502 chlorophyll meter (Minolta) was used to measure the relative chlorophyll levels in *M. truncatula* leaves of various developmental stages (positions). As leaves aged, GFP fluorescence decreased with increasing chlorophyll content (Fig. 4.1b), the changes being most dramatic at early stages of leaf development (between leaves 1 and 3).



Removal of chlorophyll recovers strong fluorescence in both young and mature *M. truncatula* leaves

Another approach used to study the effect of pigment concentration on fluorescence was to etiolate mature *M. truncatula* leaflets by wrapping them in aluminum foil while still attached to the stem. A dramatic increase in fluorescence was obtained by six days of etiolation (Fig. 4.1e). Measurement of relative fluorescence and chlorophyll content showed a proportional increase in fluorescence with decreased chlorophyll content (Fig. 4.1f). The etiolated wild-type leaf control showed only background levels of fluorescence, demonstrating that the etiolation itself does not cause the leaf to fluoresce.

Further evidence that chlorophyll is a major factor in diminishing GFP fluorescence was obtained when *M. truncatula* leaflets were extracted with 95% ethanol (Fig. 4.2a). Although GFP fluorescence diminishes with duration of exposure to ethanol (data not shown), leaves extracted for up to 8 h with 95% ethanol showed a dramatic restoration of fluorescence compared with untreated transgenic control leaves or wild type ethanol-treated control leaves (compare center panels of Fig. 4.2(a) with the left and right panels, respectively). The autofluorescence detected in the control leaves using a 500 nm emission filter (upper panels) was essentially eliminated when a 525 nm emission filter was used (lower panels).



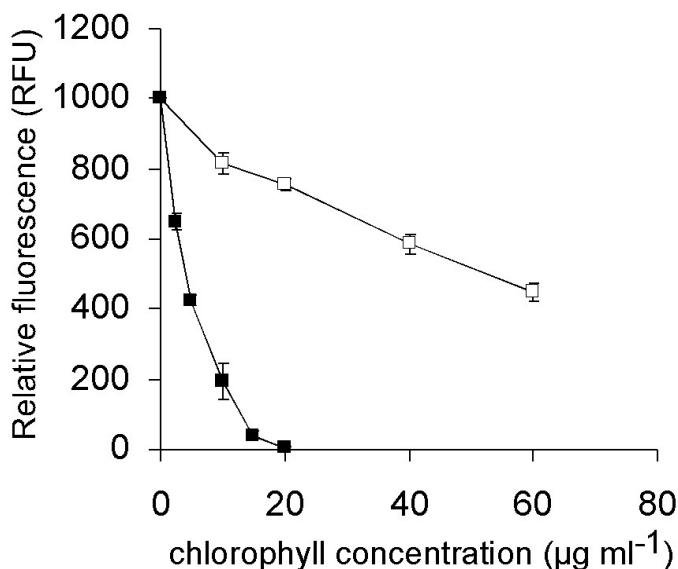
**Fig. 4.2** Recovery of GFP fluorescence in transgenic *M. truncatula* by ethanol extraction. **(a)** Transgenic *M. truncatula* leaves extracted with 95% ethanol (center panel, both rows) show much stronger GFP fluorescence than untreated leaves using either a 500 nm emission filter (top panels) or a 525 nm emission filter (bottom panels). Wild-type leaves extracted with ethanol were used as controls (right). All images shown in **(a)** were obtained by a 5 sec exposure of second trifoliolate leaves. **(b)** An intact, untreated *M. truncatula* plant (left) shows bright green GFP fluorescence in roots and emerging leaves, but not in mature leaves whereas (right) extraction of pigments with 95% ethanol for 8 hr yielded green fluorescence in most mature trifoliolate leaves (right).

A comparison between ethanol-treated and non-treated whole *M. truncatula* plants is shown in Fig. 4.2(b). Whereas strong green fluorescence was evident for roots under both conditions, shoots of the untreated plant (left) showed bright red autofluorescence

(except for the nodal meristem tissue). In contrast, most of the trifoliolate leaves on the ethanol-treated plant (right) fluoresced green.

#### Titration of GFP with chlorophyll strongly decreases fluorescence

The effect of chlorophyll on GFP fluorescence was directly investigated by titrating a solution of EGFP ( $1 \mu\text{g ml}^{-1}$ ) in TE buffer against solution of HPLC-purified chlorophylls a and b (Sigma) solubilized in  $20 \text{ mg ml}^{-1}$  Na cholate (Griffiths 1978). The Na cholate was used to permit the mixing of chlorophyll, which is not soluble in water, with the aqueous solution of GFP. Fluorescence was measured as RFUs using a Bio-Rad VersaFluor fluorometer. Na cholate by itself decreases fluorescence by  $\sim 20\%$ , but was present at the same concentration for all readings. At low concentrations ( $10 \mu\text{g ml}^{-1}$ ), fluorescence loss was fourfold greater for chlorophyll b than for chlorophyll a.(Fig. 4.3), probably indicating their relative interference with GFP excitation and emission. At a concentration of  $60 \mu\text{g ml}^{-1}$ , chlorophyll a alone caused a decrease of more than 50% in GFP fluorescence; at  $20 \mu\text{g ml}^{-1}$  chlorophyll b cut off GFP fluorescence completely (Fig. 4.3).

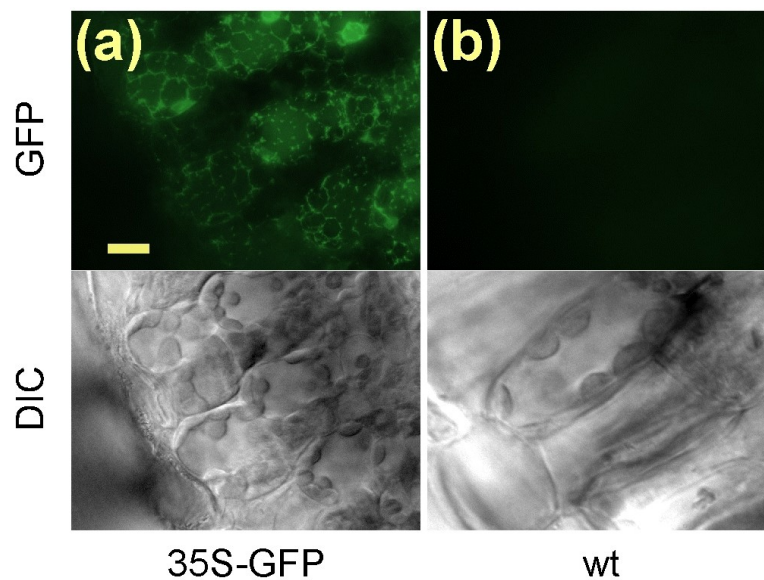


**Fig. 4.3** Titration of EGFP with chlorophylls. 1  $\mu\text{g}$  of EGFP was mixed with increasing amounts of chlorophyll a or b in 1 ml TE buffer containing 20  $\text{mg ml}^{-1}$  Na cholate. Relative fluorescence units (RFU) were measured for both chlorophyll a (*open square*) and b (*closed square*). Error bars indicate SD.

GFP fluorescence is clearly visible with microscopic detection methods

The presence of GFP fluorescence in *M. truncatula* leaves was investigated using fluorescence microscopy with oil-immersion optics. In intact leaves viewed from either the top or the bottom, the ER-targeted GFP was readily detected in the epidermis (in which only the guard cells of the stomata have chloroplasts), but GFP signal intensity quickly decreased when focusing deeper into the tissue (not shown). However, in leaf cross-sections, the typical reticulate pattern of ER-localized GFP fluorescence was observed in all cell layers, including the palisade and spongy mesophyll cells (Fig. 4.4a).

This suggested that the loss of GFP signal when focusing deeper into the tissue was caused by interference from chlorophyll in the chloroplast-rich mesophyll cells as well as spherical aberrations due to the refraction index mismatch between the immersion medium and the aqueous sample. No discernable fluorescence was detected in the wild-type leaves under the same imaging conditions (Fig. 4.4b).



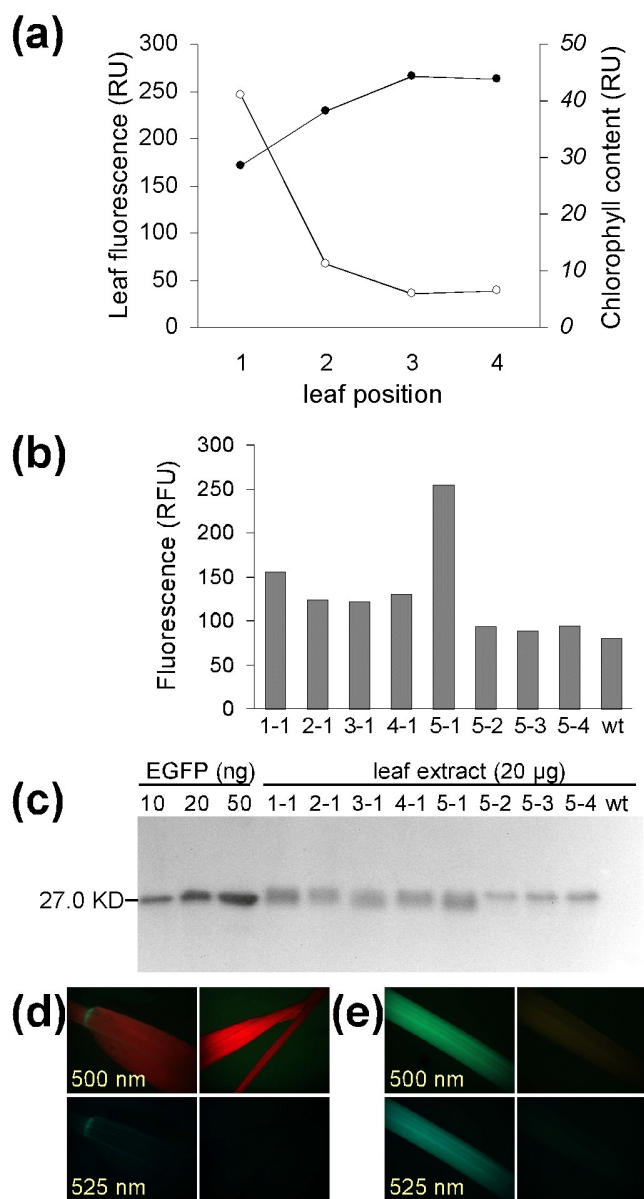
**Fig. 4.4** GFP fluorescence and differential interference contrast (DIC) microscopy of *M. truncatula* leaf cross-sections. **(a)** Transgenic plant expressing *35S-mGFP5er*. GFP fluorescence is shown on top, while the corresponding DIC images are on the bottom. Scale bar = 10  $\mu\text{m}$ . **(b)** Wild-type plant.

*M. truncatula* and rice, but not *Arabidopsis*, leaves show early loss of GFP fluorescence

To explore the generality of our observations, use was made of available rice lines transgenic for the same *35S-mGFP5er* construct as that used for *M. truncatula*. As was

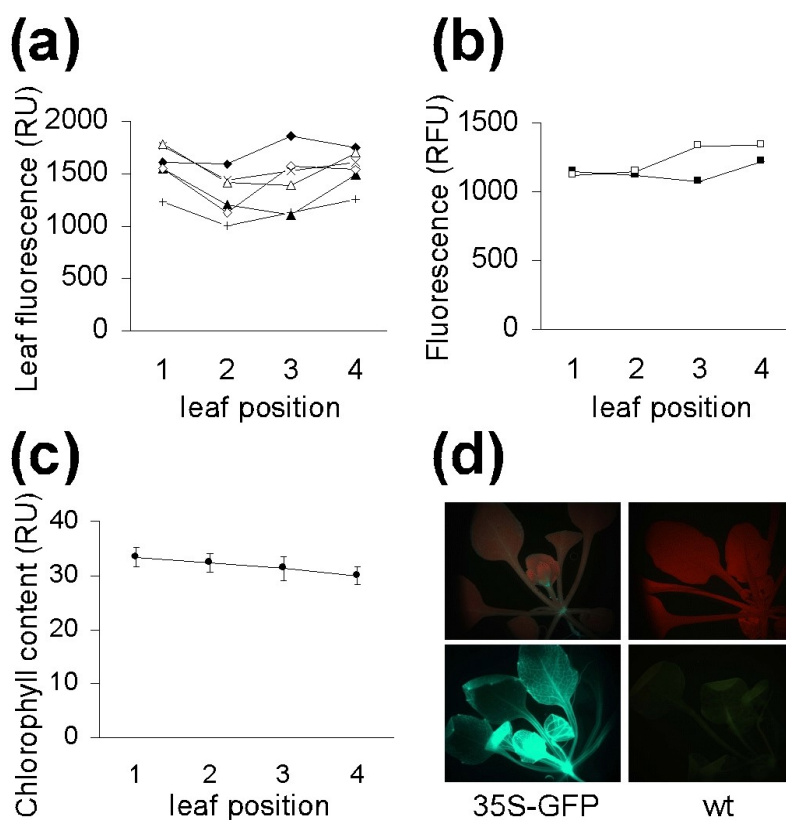
found for *M. truncatula* (Fig. 4.1b), a decrease in fluorescence with leaf age (position) was evident for rice, and was correlated with an increase in chlorophyll content, as shown in Fig. 4.5(a). The relative GFP fluorescence of aqueous extracts was also determined. For line 2, which showed the highest fluorescence for the first leaf, the relative fluorescence of maturing leaves (at positions 2, 3 and 4) was followed. The *in vitro* data closely parallel the *in planta* data (Fig. 4.5b) in that relative fluorescence drops most dramatically from leaf 1 to leaf 2. Western blot analysis (Fig. 4.5c) revealed a substantial (~ 3-fold) decrease in GFP protein from the first to the second leaf (compare lane 2-1 with lane 2-2). Except for the nodes (Fig. 4.5d), GFP fluorescence in light-grown leaves of transgenic rice seedlings (7 day old) was hardly visible, paralleling the situation for maturing *M. truncatula* leaves. Rice and *M. truncatula* were also similar in that etiolation resulted in strong green fluorescence throughout the leaf (Fig. 4.5e). Thus, the diminution of GFP fluorescence with leaf maturity results from both chlorophyll interference and decreased GFP levels.

Interestingly, *Arabidopsis*, the third species in which we examined the relationship between GFP fluorescence and leaf age, differed from rice and *M. truncatula*. Six lines of *Arabidopsis* transgenic for the same *35S-mGFP5er* construct used in *M. truncatula* and rice were chosen. In contrast to the dramatic decrease in fluorescence found between leaves 1 and 2 for *M. truncatula* and rice, in *Arabidopsis*, little difference in fluorescence is evident from leaf 1 to leaf 4 at 525 nm emission wavelength. This was the case for lines of both high and low intrinsic fluorescence (Fig. 4.6a). A similar situation was found for leaf extracts (Fig. 4.6b). Intriguingly, in *Arabidopsis*, the chlorophyll content



**Fig. 4.5** Analysis of rice plants transgenic for *35S-mGFP5er*. **(a)** Chlorophyll levels (*closed circles*) and relative fluorescence (*open circles*) for leaves of line 2 at leaf positions 1 to 4. **(b)** Relative fluorescence of leaf extracts (100 µg total protein) from the first leaves of five independent transgenic lines (1-1, 2-1, 3-1, 4-1, 5-1) and the leaves 2 through 4 of line 5 (5-2, 5-3, 5-4), selected based on its relative high fluorescence of its first leaf. **(c)** Western blot analysis of the leaf extracts in **(b)**. **(d)** Leaves of transgenic (left) and wild-type (right) rice seedlings recorded using 500 nm emission filter and 525 nm emission filter (top and bottom panels respectively). **(e)** Leaves of dark-grown transgenic (left) and wild-type (right) rice seedlings recorded using 500 nm emission filter and 525 nm emission filter (top and bottom panels respectively). Pictures in **(d)** and **(e)** were exposed for 5 sec. RU denotes relative units.

for leaves at different developmental stages (positions) shows only a minor decrease from leaf 1 to leaf 4 (Fig. 4.6c). Remarkably, upon extraction of chlorophyll with ethanol, a substantial increase in GFP fluorescence was attained for *Arabidopsis* leaves of all developmental stages, especially young leaves (Fig. 4.6d, left panels).



**Fig. 4.6** GFP fluorescence in *Arabidopsis*. **(a)** Relative GFP fluorescence in intact leaves at leaf positions 1 to 4 for six *Arabidopsis* lines. **(b)** Relative fluorescence of leaf extracts (100  $\mu$ g total protein) from leaves 1 to 4 of two *Arabidopsis* lines. **(c)** Relative chlorophyll content in *Arabidopsis* leaves of leaf positions 1 to 4. **(d)** GFP fluorescence in an intact transgenic *Arabidopsis* plant (top, left panel) and the same plant extracted with 95% ethanol (bottom, left panel). Untreated and ethanol-extracted wild-type plants were shown in right panels (top and bottom, respectively). All pictures in **(d)** were recorded by exposure for 5 sec using a 500 nm emission filter. RU denotes relative units.



## Discussion

### Chlorophyll significantly interferes with GFP fluorescence

The involvement of chlorophyll in the diminution of GFP fluorescence was convincingly demonstrated both *in vivo* and *in vitro*. Two *in vivo* approaches were employed. In the first, chlorophyll in *M. truncatula* leaves was reduced by etiolation (Fig. 4.1e). A caveat to this approach is the assumption that etiolation does not substantially enhance expression from the *CaMV 35S* promoter. We can find no evidence that this occurs, although light/dark transitions have been shown to alter the transcriptome profile for *Arabidopsis* leaves (Ma et al. 2001). In the second approach chlorophyll was extracted with 95% ethanol. For both treatments, a dramatic recovery of GFP fluorescence was observed (Fig. 4.1e,4.2a). Ethanol *per se* did not cause the increase in GFP fluorescence; indeed prolonged exposure to ethanol results in decreased GFP fluorescence. Further, no appreciable fluorescence was detected for ethanol-treated wild-type *M. truncatula* leaves (Fig. 4.2a, right panels).

As an *in vitro* approach, a solution of GFP was mixed with a solution containing purified chlorophyll a or chlorophyll b. Chlorophyll b exhibited a strong negative effect on GFP fluorescence while a milder effect was caused by chlorophyll a. As chlorophylls, especially chlorophyll b, absorb at the excitation wavelength (488 nm) of GFP, this makes them competitors of GFP for the excitation light. Considering the abundance of chlorophylls in light-grown leaves, it is quite conceivable that the fluorescence from GFP

could be lowered to levels that are barely detectable macroscopically. It is certainly possible that pigments other than chlorophyll may interfere with the perception of GFP fluorescence.

Decreased GFP concentration and increased chlorophyll content combine to diminish fluorescence in developing leaves of *M. truncatula* and rice

In the experiments reported here, GFP was driven by the *CaMV 35S* promoter that is widely considered to be constitutive (Benfey et al. 1989; Battraw and Hall 1990). However, several reports provide evidence for variability in spatial and developmental expression from this promoter. For example, Mitsuhashi et al. (1996) reported that, in tobacco, the highest (youngest) leaf had almost 8-fold greater *CaMV 35S* activity than that of the lowest (oldest) leaf and Nagata et al. (1987) have shown that its expression level varies during the cell cycle. Additionally, Sunilkumar et al. (2002) have detailed variation in *CaMV 35S*-driven expression levels with development and tissue type. Harper and Stewart (2000) also found that *CaMV 35S*-driven GFP fluorescence was higher in leaf primordia than in the lower (older) fully expanded leaves. However, the findings in the two latter papers relied on a direct relationship between GFP fluorescence and promoter activity and may need reconsideration in light of our findings that chlorophyll can substantially disrupt this relationship.

The above observations may partially explain the 2.7-fold difference in relative fluorescence of extracts from leaves 1 and 5 shown in Fig. 4.1(c). However, the

substantial effect of increased chlorophyll concentration with leaf maturity and relative fluorescence (Fig. 4.1f,4.3) must contribute to the significant difference between the respective 7.8-fold and 2.7-fold decreases in fluorescence found for intact leaves and leaf extracts of *M. truncatula* (Fig. 4.1c).

A dramatic decrease of both GFP fluorescence and relative GFP protein content was also observed in rice, accompanied by an increase in chlorophyll level (Fig. 4.5a,b,c). Removal of chlorophyll by etiolation drastically restored GFP fluorescence in transgenic rice seedlings while causing little change in wild-type seedlings (Fig. 4.5d,e). Whereas no green autofluorescence was observed for mature wild type *M. truncatula* or *Arabidopsis* leaves, a low but detectable level was evident in rice (not shown).

#### How *Arabidopsis* hides its dark side

The results shown in Fig. 4.6 (a,b) reveal that *Arabidopsis* does not exhibit the remarkable decrease in GFP fluorescence level with leaf age found for *M. truncatula* (Fig. 4.1b,c) and rice (Fig. 4.5a,b). However, since extraction of chlorophyll from mature leaves with ethanol results in a 5 to 10-fold increase in GFP fluorescence, there can be little doubt that chlorophyll-induced diminution (the “dark side”) of fluorescence actually occurs in *Arabidopsis* as in *M. truncatula* and rice. Fig. 4.6(c) discloses that a major difference between *Arabidopsis* and the other species studied is that, rather than increasing with age (Fig. 4.1b,4.5a), leaf chlorophyll content in *Arabidopsis* slightly decreases. Further, the data of Fig. 4.6(b) indicate that the proportion of GFP in the total

leaf protein also remains constant, rather than decreasing as in the other species studied. These findings reveal that the chlorophyll content and the proportion of GFP in total protein contribute to loss of fluorescence in maturing leaves and that their relative contribution differs from species to species. In addition, the relative concentrations of chlorophyll a and b, and possibly other pigments, affect the relationship between GFP content and fluorescence.

Leaf optical properties may contribute to diminution of GFP fluorescence

Absorption spectra for green and white segments of variegated *Coleus* leaves (Stahlberg et al. 2000) reveal substantial absorption in green regions at the excitation (488 nm) and emission (509 nm) wavelengths of GFP. This implies that observation of GFP fluorescence in leaves can be hindered by both absorption of the excitation light (thus diminishing the excitation intensity) and reabsorption of the emitted green signal. In contrast, light absorption in non-green leaf segments is significantly lower across the spectrum. Thus, the detectable fluorescence of a unit amount of GFP may be several fold lower in green leaves than in non-green leaves. Since absorption spectra have been shown to vary with the physiological state of the leaf (Gitelson et al. 2001), apparent transgene expression levels based on GFP fluorescence may be affected even if the actual level is unchanged.

Whereas detection of GFP in green tissues using macroscopic methods can lead to substantial underestimation of expression levels, as exemplified by Fig. 4.6(d), GFP

fluorescence in thin sections used for microscopic analysis may be less affected by the presence of chlorophyll (Fig. 4.4a). We speculate that this may reflect the fact that tissues expressing GFP are less likely to be obscured by chlorophyll-rich cell layers in thin sample sections. Similar considerations lead to the possibility that differences in leaf architecture, such as the number of cell layers, cell and organelle shape and cell wall thickness or composition, may also contribute to the observed species differences in apparent diminution of GFP fluorescence.

Our data reveal that considerable deviation may exist between apparent fluorescence from GFP and transgene transcript levels in green tissues, especially leaves, and that the discrepancy can vary significantly between plant tissues and species. Thus, while GFP and other fluorescent proteins clearly have important and increasing roles to play in analysis of gene function, caution should be exercised when relating observed fluorescence to quantitative values.

## CHAPTER V

### SUMMARY

An effective *Agrobacterium*-mediated transformation method has been developed for *M. truncatula* A17 based on a previously reported method (Trieu and Harrison 1996).

Modifications to the original protocol have substantially improved the efficiencies of both regeneration of transgenic shoots and induction of adventitious roots. High reproducibility was also achieved, with PPT-resistant shoots induced in all transformation experiments and fertile transgenic plants regenerated in most of the experiments.

Despite our current success with tissue-culture-based transformation of *M. truncatula*, the *in planta* approach remains of great interest. Although failure to repeat the initial success reported by the Harrison laboratory (Trieu *et al.*, 2000), appears to be experienced by all who have attempted to recapitulate their procedures, we have analyzed progeny (kindly provided by Dr. Harrison) and found strong GUS expression in all tissues (including mature leaves). Genomic blotting confirmed the presence of an integrated *35S-GUS* insert, although an identical pattern was obtained from all seed available to us. Nevertheless, these results support the original work and indicate that the *in vivo* approaches are feasible.

Bent (2000) has suggested that the success of *in planta* transformation approaches can be affected by events such as the planting configuration and the developmental stage

(mature plant vs. seedlings) at which the tissues are exposed to *Agrobacterium*. GFP has proven to be a valuable screenable marker, especially in the presence of a selectable marker. Its fluorescence can indicate sites of infection and, possibly, how successful the infection has been. We propose to use *mGFP5er* driven by *Arabidopsis* or soybean ubiquitin (*sUbi*) promoter as the screenable marker for this purpose.

While Trieu *et al.* (2000) used a commercial herbicide solution for selection, it is conceivable that the concentration of active ingredient (PPT) differed from that reported in the protocol. Indeed, the levels reported for the first and second applications (80 and 560 mg/L, respectively) appear to be too high to allow survival of true transformants. For selection in tissue culture media, we find that 1.6 mg/L PPT is very effective. Careful determination of the effective concentration of PPT could be very important to the recovery of transgenic seedlings. Chemicals (DTT and L-cysteine) proven to be beneficial for the tissue-culture-based approach will be tested for their value for *in planta* transformation. For example, the infiltration medium will be supplemented with 1 mM DTT and 400 mg/L L-cysteine to suppress defense responses to pathogen infection (Olhoft *et al.*, 2001).

A constant relationship between GFP content and detected fluorescence is intrinsic to its use as a reporter of promoter function. However, when characterizing transgene expression in *M. truncatula*, a dramatic decrease in fluorescence from *35S-GFP* occurred shortly after leaf emergence, despite the presence of GFP transcript and protein. To evaluate the generality and causes of this phenomenon, fluorescence during leaf development from the same *35S-GFP* transgene was compared in *M. truncatula*, rice and

*Arabidopsis*. A substantial decrease in fluorescence early in the development of *M. truncatula* and rice leaves was found to correlate with chlorophyll accumulation. Initial observations indicated that fluorescence from GFP in *Arabidopsis* did not wane with leaf age. However, whereas the chlorophyll content increased during development of *M. truncatula* and rice leaves, in *Arabidopsis* it declined slightly. Several approaches showed that chlorophyll is causally involved in the loss of GFP fluorescence. Removal of chlorophyll from leaves of transgenic *M. truncatula*, rice or *Arabidopsis* through etiolation or by extraction with ethanol yielded up to a tenfold increase in fluorescence. Direct evidence that chlorophyll is implicated in the loss of fluorescence from GFP was obtained by mixing solutions of chlorophyll and GFP. Thus, substantial errors in estimating promoter activity from GFP fluorescence can occur if pigment interference is not considered.

While chlorophyll has been proven to be an important interfering factor, we do not exclude the possibility that other factors may be involved. Our preliminary experiment showed that carotenoid can also interfere with GFP fluorescence *in vitro* in a concentration dependent manner similar to that of chlorophyll. A more thorough investigation of the effects of carotenoid and possibly other pigments should be done to better identify all the casual factors of diminished GFP fluorescence in the plant tissue.



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### Education

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1994	Field research of intertidal marine communities at Qingdao, China
1996-1997	Molecular screening, cloning and sequencing of thrombin-like enzyme gene from c-DNA library of <i>Agkistrodon acutus</i>
2000-2005	Establishment and optimization of tissue-culture-based transformation system for model legume <i>M. truncatula</i> A17
2003-2005	Functional analysis of transgene expression of GUS and GFP driven by <i>CaMV</i> 35S and <i>phaseolin</i> promoter in transgenic <i>M. truncatula</i> A17
2004-2005	Analysis of the diminution of GFP fluorescence in <i>Medicago</i> , <i>Arabidopsis</i> and rice tissues

### Teaching Experience

2000-present Graduate Teaching Assistant of Introductory Biology Lab

### Publications

Xin Zhou, Mahesh B. Chandrasekharan and Timothy C. Hall. 2004. High rooting frequency and functional analysis of GUS and GFP in transgenic *Medicago truncatula* A17. *New Phytologist* 163 (3): 813-822.

Xin Zhou, Raul Carranco, Stanislav Vitha and Timothy C. Hall. 2005. The dark side of GFP. *New Phytologist* (in press).

### Awards

2004	Second prize of Texas A&M University Student Research Week Poster Competition
2005	First prize of Texas A&M University Student Research Week Poster Competition