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Development of enabling technologies to modify gene expression in *Arabidopsis thaliana*

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Preface

The work presented in this PhD thesis was carried out at the Department of Plant Systems Biology, Flanders Interuniversity Institute for Biotechnology (VIB), University of Ghent, Belgium.

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Goga

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ABBREVIATIONS

3'nos	3' region of the nopaline synthase gene
bar	Bialaphos acetyltransferase
Chs	Chalcone synthase
CRE	CRE recombinase
CycB1;1	B-type cyclin
CycD4 ;1	D-type cyclin
dsRNA	Double-stranded RNA
GFP	Green Fluorescent protein
Gus	β-glucuronidase
hpRNA	hairpin RNA
HSE	Heat shock element
HSF	Heat shock factor
HSP	Gmhsp 17.6L soybean heat-shock promoter
ihpRNA	intron hairpin RNA
IR	Inverted repeat
KRP4	Kip-related-protein, negative regulator of cell division
LB	Left T-DNA border
Lox	34 base pairs recombination sequence of the CRE/lox recombination system
miRNA	Micro RNA
mRNA	Messenger RNA
nptII	Neomycin phosphotransferase gene
P35S	Cauliflower mosaic virus 35S promoter
Pds	Phytoene desaturase gene
Pnos	Nopaline synthase promoter
PTGS	Post-transcriptional gene silencing
rasiRNA	Repeat-associated short interfering RNA
RB	Right T-DNA border
RdRP	RNA-dependent RNA polymerase
RISC	RNA-induced silencing complex
RNAi	RNA interference

siRNAs	small interfering RNA
sRNA	small RNA
T-DNA	Transferred DNA
TE	Transposable element
TSP	Total soluble protein
VIGS	Virus-induced gene silencing
WRKY23	Plant transcription factor

CHAPTER 1

General Introduction

1.1 RNA silencing

1.1.1 Sense Cosuppression in plants

The RNA silencing phenomenon was first described in plants following attempts to overexpress gene constructs encoding enzymes for pigment production, in transgenic petunia (Napoli *et al*., 1990; van der Krol *et al*., 1990). Contrary to the expectation, the flowers of transgenic petunia plants exhibited less and variable, rather than increased pigmentation. As both the expression of the transgenes and the endogenous genes was suppressed, this phenomenon was termed cosuppression (Napoli *et al*., 1990; van der Krol *et al*., 1990). The analysis of the petunia plants transformed with a *chalcone synthase* transgene showed that the efficiency of cosuppression correlates with the strength of the promoter driving the transgene, suggesting a transgene product dose rather than a transgene dose effect (Que *et al*., 1997). Whereas cossupression by a single-copy *Chs* transgene depends on a strong transgene promoter, suppression by an inverted repeat *Chs* transgenes does not have this requirement (Que *et al*., 1997). This observation initially suggested that post-transcriptional gene silencing (PTGS) was due to the overproduction of transgene RNA above a putative threshold level that triggers the irreversible degradation of RNA (Dehio and Schell, 1994). Since the level of transgene transcription was not always found to be significantly higher in silenced lines as compared to non-silenced lines (English *et al*., 1996), the presence of repeats at the transgene locus of the silenced lines was most likely the cause of cosuppression (Que *et al*., 1997; Dalmay *et al*., 2000; Beclin *et al*., 2002). Futhermore, the observations that trancriptionally active genes are better inducers of post-transcriptional gene silencing than transcriptionally inactive genes (Que *et al*., 1997; Vaucheret *et al*., 1997), suggested that an RNA molecule is the sequence-specific trigger of PTGS.

Similarly with transgene-induced suppression of homologous endogenous genes, the silencing can also be initiated by sense transgenes expressing RNA homologous to a viral genome conferring resistance to that virus (Lindbo *et al*., 1993). Over 90% of plant viruses have single-stranded RNA genomes that are replicated by a virus-encoded RNA-dependent RNA polymerase (RdRP). Upon infection of the host cell, the single-stranded virus RNA genome replicates via a dsRNA intermediate, which probably triggers RNA silencing (Ratcliff *et al*., 1997). The induction of post-transcriptional gene silencing by viruses was confirmed by the observation that endogenous genes or transgenes can be silenced after infection with

recombinant viruses carrying part of the (trans)gene sequence (Ruiz *et al*., 1998; Ratcliff *et al*., 2001). This phenomenon is called virus-induced gene silencing (VIGS; Baulcombe, 1999).

There are two models explaining a sense-mediated induction of the post-transcriptional gene silencing. A threshold model has been proposed to explain those cases where a high transcription rate is required to trigger silencing. This model for PTGS induction proposed that there is a surveillance system within plant cells that detects mRNAs expressed above an acceptable level, and induces the RdRP to recognize these molecules as templates. This was also supported by the observation that highly transcribed sense transgenes expressing RNA homologous to a viral genome conferred resistance to that virus. In the threshold model of RNA-mediated silencing, a plant-encoded RdRP would copy small segment or segments of an RNA that had accumulated to high levels. These small RNAs would hybridize with the target RNA, and RNases would target the partially double-stranded messenger or viral RNA for degradation (Dougherty and Parks, 1995). The induction of degradation following overproduction of transgene mRNA fits well with many examples of cosuppression (De Carvalho *et al*., 1992; Elmayan and Vaucheret, 1996). Cosuppression occurs efficiently when transgenes are transcribed from a strong promoter (Jorgensen *et al*., 1996), or when they are present in high copy numbers (Palauqui and Vaucheret, 1995). The model was further supported by the fact that SDE1/SGS2 and QDE-1 mutants, which encode a putative RdRP in plants and fungi, respectively, are defective in sense-mediated induction of the PTGS (Cogoni and Macino, 1999; Dalmay *et al*., 2000; Mourrain *et al*., 2000). However, PTGS is not always associated with highly active transgenes (van Blokland *et al*., 1994), and can be induced by weakly transcribed or promoterless transgenes (van Blokland *et al*., 1994), suggesting that a high transcription rate of the transgene is not always required for induction of this form of RNA silencing (Stam *et al*., 1997; Voinnet *et al*., 1998; van Blokland *et al*., 1994). Many cases of sense-mediated gene silencing, including in *trans* silencing triggered by promoterless constructs have been associated with the transgene loci, which consists of transgene repeats (Depicker and Van Montagu, 1997; Stam *et al*., 1998).

The ‘aberrant RNA’ model of cosuppression has been proposed to explain the ability of these loci to induce cosuppression. In this model, certain transgene loci could interact with the homologous genes, via DNA-DNA pairing, to produce RNAs with certain aberrant features. This aberrant mRNA can be recognized by the RdRP as a template, thus targeting degradation of itself and other complementary RNAs (Stam *et al*., 1997; 1998).

1.1.2 dsRNA as an inducer of RNA silencing

The first indication that double-stranded RNA (dsRNA) might be a trigger of RNA silencing in plants came from experiments by Waterhouse *et al*. (1998), who reported that plants carrying both a sense and an antisense transgene homologous to a viral genome were much more resistant to the virus than plants carrying either a sense or an antisense transgene alone. The importance of dsRNA as a trigger of the PTGS was supported by the discovery of sRNAs, corresponding to both sense and antisense sequence of the targeted mRNAs (Hamilton and Baulcombe, 1999). These findings could explain why inverted transgene repeats are such efficient silencing loci (Muskens *et al*., 2000). When the multiple copies of transgene are integrated as an inverted repeat, dsRNA is likely produced by read-through transcription (Stam *et al*., 1997; De Buck *et al*., 2001). Once a role for dsRNA was suggested (Waterhouse *et al*., 1998), the involvement of the plant-encoded RdRP in sense cosuppression became apparent. The recognition of a sense transcript to serve as a template for the RdRP enzyme would be the initial event in the sense cosuppression mode of silencing. In addition, an RdRP homolog in *Arabidopsis* (SDE1/SGS2) was shown to be required for transgene-induced RNA silencing, but not for RNA virus-induced RNA silencing (Dalmay *et al*., 2000; Mourrain *et al*., 2000). Because viruses replicate via a dsRNA intermediate produced by a virus-encoded RdRP enzyme, a plant-encoded RdRP is not needed to trigger RNA silencing of replicating RNA viruses.

RNA silencing, manifested as transgene-induced gene silencing which operates at the post-transcriptional level, has also been described in the fungus *Neurospora crassa* (quelling; Romano and Macino, 1992; Cogoni *et al*., 1996). Furthermore, gene expression was shown to be suppressible by homologous dsRNA in *C.elegans*, a phenomenon termed RNA interference (RNAi; Fire *et al*., 1998; Sijen and Kooter, 2000). RNAi results in the specific degradation of endogenous RNA in the presence of homologous dsRNA either locally injected or transcribed from an inverted-repeat transgene (Tavernarakis *et al*., 2000). Just a few dsRNA molecules per cell are sufficient to trigger RNAi (Fire *et al*., 1998) and local delivery of dsRNA leads to RNAi in the entire body and even in the progeny (Fire *et al*., 1998).

dsRNA might be derived from inverted-repeat transcripts, from complementary RNAs that anneal by base pairing or, in worms, fungi and plants from single-stranded RNA that is copied into a duplex by an RNA-dependent RNA polymerase (RdRP) (Dalmay *et al.,* 2000). In plants, viruses and transgenes are efficient exogeneous inducers of RNA silencing through the

formation of dsRNA. A dsRNA intermediate of the viral replication by a virus-encoded RdRP in most single-stranded RNA viruses contains the sequence-specific information and guides the plant to protect itself by degrading viral RNAs (Sijen *et al*., 2001). The generation of dsRNA from sense transgenes requires a plant-encoded RdRP. RdRP synthesizes dsRNA intermediates from the template of aberrantly expressed single-stranded RNA (Dalmay *et al*., 2000; Mourrain *et al*., 2000). By contrast, if multiple copies of transgene are integrated as inverted-repeat in the genome, or a transgene is designed to create a dsRNA structure, RNA silencing is triggered in RdRP-independent manner (Waterhouse *et al*., 1998; Muskens *et al*., 2000; Wang and Waterhouse, 2000; De Buck *et al*., 2001). In addition, specific genes in plants and animals encode short forms of fold-back dsRNA, the precursor molecules of miRNAs (Bartel, 2004) (see also in the Biological function of RNA silencing section of this introduction).

1.1.3 Mechanism of RNA silencing

RNA silencing is triggered by double-stranded RNA (dsRNA), which is processed by an Rnase III-like enzyme (Dicer; Bernstein et al., 2001) into small RNAs (sRNAs) of 21-28 nucleotides (nt), which have 5' phosphates, and 2-nucleotide 3' overhangs (Elbashir *et al*., 2001). Several organisms contain more than one Dicer gene, with each Dicer preferentially processing dsRNAs that come from a specific source. For example, *Drosophila melanogaster* has two paralogues: Dicer-1 (DCR-1) which preferentially processes miRNA precursors (Lee *et al*., 2004), and Dicer-2 (DCR-2) which is required for long dsRNA processing (Lee *et al*., 2004; Liu *et al*., 2003). In *Arabidopsis thaliana* four Dicer-like (DCL) proteins (DCL1 to DCL4) have been identified, three of which are involved in processing dsRNA that comes from different sources (Xie *et al*., 2004). Mutation of the *Arabidopsis* Dicer-1 homolog (DCL1) blocks miRNA production (Bernstein *et al*., 2001; Reinhart *et al*., 2002; Schauer *et al*., 2002), but not PTGS or siRNA production induced by self-complementary hairpin RNA (Finnegan *et al*., 2003). DCL1, which is likely to be localized to the nucleus, is more important in the production of miRNAs and could also play a role in degrading any hairpin RNA that is localized within the nucleus (Mette *et al*., 2000). On the other hand, DCL2, which probably resides in the cytoplasm, could be important in siRNA production and in defense against viral infection. Most likely the location of these two Dicer enzymes in different cellular compartments would prevent any potential disruption to developmental

pathways through the saturation of Dicer after virus infection (Finnegan *et al*., 2003). By contrast, in *C. elegans* and mammals, only one Dicer (DCR-1) has been identified. The Dicer-interacting dsRBD-containing proteins, which would allow Dicer to recognize different sources of dsRNA, are not yet identified. DCR-1 in *C. elegans* cooperates with the dsRBD protein RDE-4 during RNAi, although RDE-4 is not required for miRNA function (Tabara *et al*., 2002).

The two strands of these sRNAs are then separated, and one of the two strands is incorporated into the RNA-induced silencing complex (RISC; Hammond *et al*., 2000). This RISC complex contains a member of the Argonaute (Ago) protein family, which probably binds directly to the RNA in this complex (Liu *et al*., 2004). The single-stranded sRNA in RISC guides changes in chromatin state, target degradation or translation repression of complementary single-stranded RNAs, such as messenger RNAs or viral genomic / antigenomic RNAs (Hammond *et al*., 2000; Hammond *et al*., 2001; Zamore *et al*., 2000; Fegard *et al*., 2000; Béclin *et al*., 2002; Morel *et al*., 2002; Zilberman *et al*., 2003). According to their origin or function, three types of naturally occurring small RNA have been described: short interfering RNAs (siRNA), repeat-associated short interfering RNAs (rasiRNA) and microRNA (miRNAs). The dsRNAs produced by RNA dependent RNA polymerase (RdRP), from sense transgenes or viruses or by hybridisation of overlapping transcripts (for example, from repetitive sequences such as transgene arrays or transposons), give rise to siRNAs or rasiRNAs, which generally guide mRNA degradation and/or chromatin modification. In addition, dsRNA can be formed from endogenous transcripts that contain complementary or nearly complementary inverted repeats. These dsRNAs are processed into miRNAs that mediate translation repression, although they may also guide mRNA degradation (reviewed by Baulcombe, 2004) (Fig.1.1).

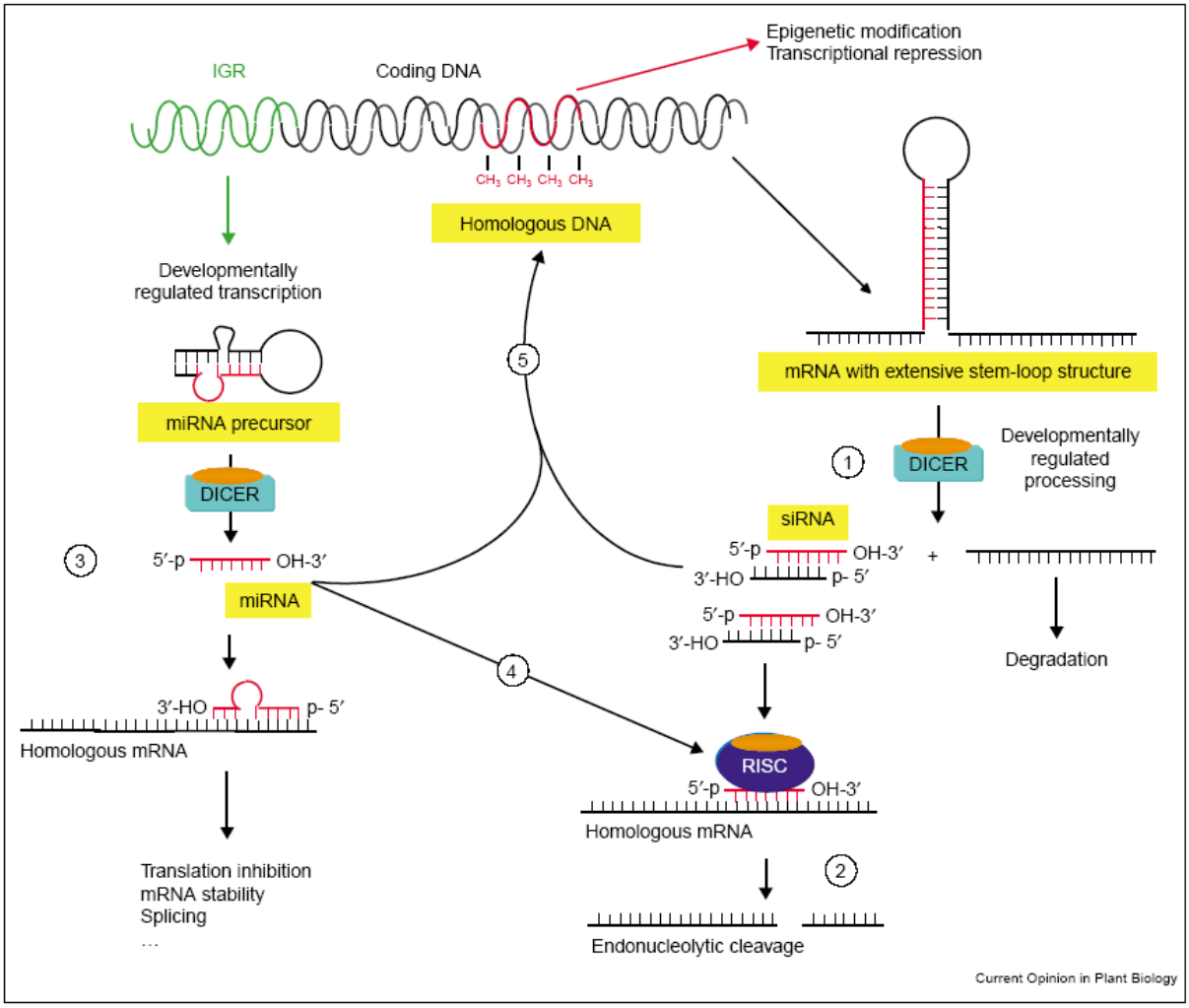

Fig. 1.1 Regulation of gene expression by small RNAs in plants. (1) mRNA with extensive stem-loop structure could be developmentally processed by Dicer, which would eventually lead to degradation. (2) siRNAs produced by such processing could then be involved in endonucleolytic cleavage of related mRNAs. (3) miRNAs produced from intergenic regions (IGR)-encoded precursors could participate in a variety of regulatory processes affecting, for instance, mRNA translation, stability or splicing. (4) miRNAs that are perfectly complementary to the coding region of some mRNA could also be recruited as siRNAs. (5) siRNAs and miRNAs may direct sequence-specific modifications of homologous DNA. For example, homology with promoter sequences could cause transcriptional gene silencing (From Voinnet O., 2002).

1.1.4 Design of constructs for RNA silencing in plants

Since the observation that dsRNAs are efficient inducers of PTGS (Fire *et al.*,1998; Waterhouse *et al.*, 1998), many different transformation vectors capable of dsRNA formation were constructed to obtain efficient RNA silencing in plants (Chuang and Meyerowitz, 2000; Levin *et al.*, 2000; Wesley *et al.*, 2001; Helliwell *et al.*, 2002). The most basic type of

construct is a binary vector harbouring an inverted repeat placed downstream of a strong, constitutive promoter (Waterhouse *et al*., 1998; Chuang and Meyerowitz, 2000). In comparison with conventional cosuppression or antisense constructs that gave in average 13% silenced transformants, these vectors showed improved silencing frequency and strength (Wesley *et al*., 2001). The use of an intron as a spacer (intron hairpin RNA, ihpRNA) further increased the silencing efficiency to nearly 100% of transformants (Smith *et al*., 2000). For the large-scale investigation of gene functions, a high-throughput vector was designed by using Gateway technology, a recombination-based cloning method that does not require restriction digestion and ligation (Wesley *et al*., 2001; Fig. 1.2).

In the Gateway binary vector, the same gene specific fragments can be simultaneously cloned at each end of the intron as an inverted repeat (Wesley *et al*., 2001; Fig. 1.2, upper LR reaction). Although gene fragments ranging from 50 bp to 1000 bp could successfully silence genes, the shorter fragments induced less frequently effective silencing (Helliwell and Waterhouse, 2003). Similar binary vectors, which are also suitable for large-scale investigation of gene function, have been subsequently developed by Karimi *et al*. (2002).

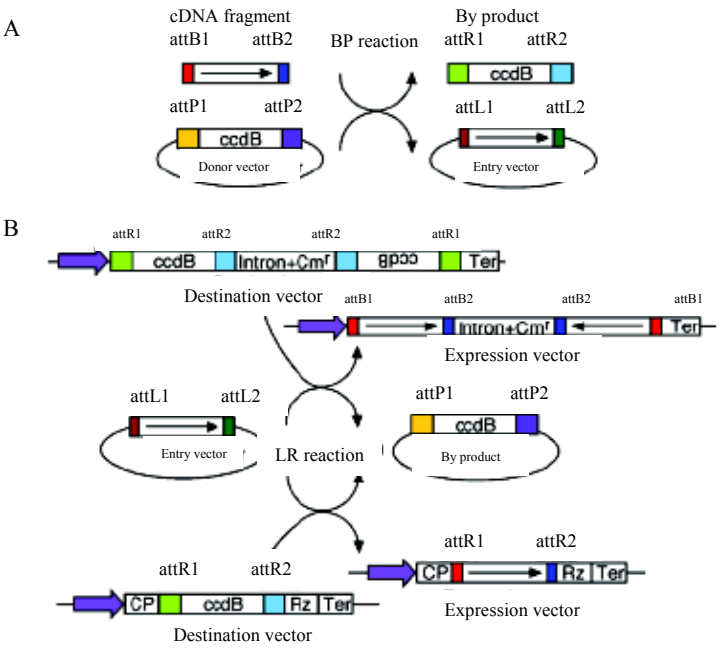

Fig. 1.2 Gateway RNA silencing vector. A, a cDNA fragment is amplified by a polymerase chain reaction so that the attB1 and attB2 sites are attached at both ends. This product is cloned into a donor vector by the BP reaction to yield the entry vector. An *E. coli* strain, such as DH5α, cannot grow if it is transformed with a plasmid containing the negative selection marker *ccdB* gene. B, The entry vector is subjected to the LR reaction with a selected destination vector. In the upper LR reaction, an inverted-repeat vector is shown. The use of the Cm^r gene ensures recovery of the expression vector with the intron. In the lower reaction, a TRV-based VIGS vector is shown. attB1, B2, P1, P2, L1, L2, R1, and R2 represent sequences required for specific recombination reactions. CcdB, bacterial negative selection marker; CP, coat protein; Rz, self-cleaving ribozyme; Ter, terminator; Cm^r, chloramphenicol resistance gene. The BP and LR reactions are the recombination reactions between specific att elements, such as attB1 versus attP1 and attL1 versus attR1 (From Horiguchi, 2004).

The essence of RNA-induced gene silencing is the delivery of dsRNA into an organism or cell, to induce a sequence-specific RNA degradation mechanism that effectively silences a target gene. There are three methods of delivery: by virus infection, particle bombardment and *Agrobacterium* infection. Bombarding cells with dsRNA, sRNAs, or DNA constructs that encode hpRNA can produce transient silencing of target reporter genes (Schweizer *et al*., 2000; Voinnet *et al*., 1998)). Similarly, DNA constructs encoding these types of RNAs, when placed into the T-DNA of *Agrobacterium* and then injected into the intercellular spaces of leaves, induce silencing of reporter genes (Voinnet *et al*., 1998). This has been demonstrated in transgenic *Nicotiana benthamiana* plants expressing the green fluorescent protein (GFP). When these plants were infiltrated with an *Agrobacterium* culture carrying a 35S-gfp construct, the endogenous GFP expression was silenced (Voinnet *et al*., 1998; Voinnet and Baulcombe, 1997). This localized silencing was subsequently spread throughout the plant. Also, silencing has been found to occur when T-DNAs containing hpRNA encoding sequences are used for agroinfiltration (Johansen and Carrington, 2001). However, agroinfiltration-mediated silencing has only been demonstrated in *N. benthamiana*. Another way to induce RNA silencing in plants is by viruses that produce dsRNA during their replication. In plants infected with unmodified viruses the mechanism is specifically targeted against the viral genome. However, with virus vectors carrying inserts derived from host genes the process can be additionally targeted against the corresponding mRNAs. Virus-induced gene silencing (VIGS) is a virus vector technology that exploits RNA-mediated antiviral defence mechanism (Baulcombe, 1999). The conditional nature of VIGS makes this system useful for suppression of genes that are essential for host cell growth and development (Peele *et al*., 2001). In addition, similarly to the construction of high-throughput hpRNA

vectors, Gateway technology has also been used to construct a high throughput VIGS vector (Liu *et al*., 2002; Fig. 1.2 B, lower LR reaction).

Stable transformation with transgenes designed to express the self-complementary hpRNA (Wesley *et al*., 2001) generates a series of independent lines that have different phenotypes and degrees of target mRNA reduction (Helliwell *et al*., 2002). The advantage of stable gene silencing induced by hpRNA constructs is that the induced silencing can be inherited from generation to generation allowing continued study of the phenotype. For example, the silencing of the *Arabidopsis fatty acid desaturase 2* (FAD2) gene by a hpRNA has been maintained for five generations and has been found to be consistent in its effectiveness (Stoutjesdijk *et al*., 2002). Moreover, there are now systems that use animal hormone-inducible or heat-inducible promoters to activate the expression of the hpRNA, making this technology useful for functional analysis of essential genes.

1.1.5 Inducible RNA silencing systems

The first construct for chemically inducible hpRNA production was developed from the XVE system and demonstrated in *Nicotiana benthamiana* and *Arabidopsis thaliana* by targeting both *gfp* transgene and endogenous *phytoene desaturase (pds*) gene (Guo *et al*., 2003). This system contains two steps: the inducible expression of dsRNA resulting from 17β-estradiol-induced DNA recombination and induction of target gene silencing by the dsRNA. In this construct, the CRE recombinase is placed under the control of the chimeric transcription factor XVE, whose activity is strictly regulated by estrogens (Zuo *et al*., 2000; Zuo *et al*., 2001) (Fig.1.3). Upon induction by 17β-estradiol, Cre/lox-mediated recombination led to activation of the hairpin cassette by bringing it immediately downstream of the constitutive G10-90 promoter (Fig.1.3). A detailed characterization of this inducible silencing system demonstrated that the system is stringently controlled and effective at inducing conditional silencing of both *gfp* transgene and endogenous *pds* gene and shows no background expression in the absence of the inducer. Upon induction at seed germination stage, all analysed RNAi lines displayed uniform silencing of the *gfp* transgene. By contrast, uniform estradiol-induced *gfp* silencing at post-germination stage was observed in 10 out of 15 RNAi lines analysed, while the remaining five lines showed varying initiation of *gfp* suppression after 1-week induction. However, further incubation with the inducer resulted in complete

gfp silencing in all these plants. Similarly, efficient silencing of the *pds* gene was observed in all RNAi lines tested, but only upon induction at seed germination stage. Post-germination induction resulted in a uniform photobleaching phenotype in 4 out of 12 RNAi lines analysed. This was the result of complete DNA excision, which reconstituted the G10-90-RNAi transcription unit. By contrast, most of the RNAi lines showed varying photobleaching phenotypes. The observed silenced sectors were the result of incomplete CRE-mediated DNA excision throughout the plant. However, multiple treatments with the inducer could reactivate this RNAi system in these transgenic lines. Nevertheless, the incomplete Cre/lox-mediated DNA excision, wich resulted in genetic chimera in transgenic plants, may provide a useful system to study mechanisms of long-distance signal transduction in gene silencing in *Arabidopsis*, which is difficult to graft (Guo *et al*., 2003).

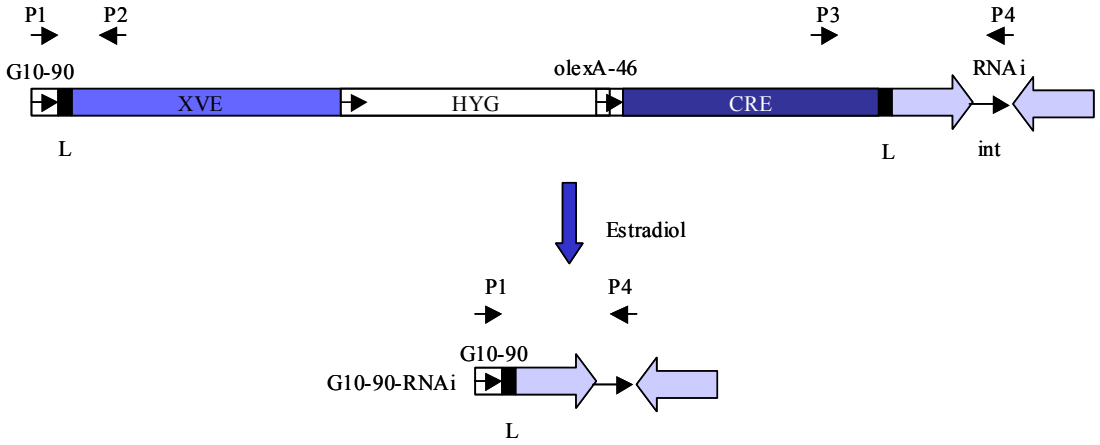

Fig. 1.3 Schematic diagram of the inducible RNAi construct. XVE, a chimeric transactivator containing the regulator domain of an estrogen receptor; HYG, a hygromycin-resistance marker; CRE, bacteriophage P1 CRE recombinase; L, specific recognition sites of CRE; OlexA-46, eight copies of the LexA DNA binding site fused to the –46 CaMV 35S promoter; G10-90, a strong, synthetic, constitutive promoter; int, actin 11 intron; RNAi, DNA sequences encoding the intron-containing inverted-repeat RNAs; G10-90-RNAi, the reconstituted transcription unit derived from CRE/lox-mediated DNA recombination after inducer treatment; P1-4, primers used for PCR analysis. Arrows inside transcription units indicate the direction of transcription (Guo *et al*., 2003).

A reversible knockdown of the expression of the *Magnesium chelatase subunit 1* (Ch1) and *glutamate 1-semialdehyde aminotransferase* (GSA) genes, which are involved in chlorophyll biosynthesis, was reported by Chen *et al*. (2003). In this ethanol-inducible RNAi system, the

alcA promoter did not drive transcription of the hpRNA in the absence of ethanol, but upon induction could efficiently silence the target genes. Only a small proportion of primary transformants exhibited an RNAi phenotype (33% for Ch1 and 13% for GSA), but these were stronger than those observed with constitutive expression of the hpRNA (Chen *et al*., 2003). By repeated ethanol treatments they achieved extended periods of gene silencing which could enable to reveal the full sequence of consequences of reduced gene expression. However, the *alc* system has been shown to have a higher basal expression in *Arabidopsis* growing in tissue culture that may be due to ethanol production from anaerobic metabolism (Roslan *et al*., 2001).

Another inducible silencing system, composed of the heat-shock plant promoter HSP18.2 from *Arabidopsis* and inverted-repeat RNA corresponding to *phytoene desaturase (pds*) gene, was developed by Masclaux *et al*. (2004). Activation of the HSP18.2 promoter is transient since repetitive heat-shock treatments were required to get a stable silencing of *pds* gene in new developing organs of the plant. Compared to chemical-induced systems, heat-shock can be applied easily and uniformly on a large number of plants and does not require the co-introduction of an exogenous transcription factor. However, a less efficient heat-shock induction was observed when applied on mature plants compared with in vitro cultured young plantlets (Masclaux *et al*., 2004).

Recently, Wielopolska *et al*. (2005) described an inducible RNAi system, which combined the dexamethasone-inducible pOp6/LhGR promoter system (Craft *et al*., 2005) with the hpRNA cassette derived from the pHELLSGATE 12 GATEWAY^{TM} silencing vector (Helliwell and Waterhouse, 2003). Again using *pds* as a target, it was shown that a high proportion of primary transformants exhibited silencing phenotypes, following application of dexamethasone. The observed phenotypes were comparable to those obtained with the 35S promoter. Although the pOp6 promoter in the pOpOff vector is not completely off in the absence of dexamethasone, they did not observe effective gene silencing before the application of the inducer. However, in contrast with the reversibility of the *pds* silencing, the silencing of the *luc* expression continued after removal of the dexamethasone, indicating that pOpOff mediated inactivation of target genes is not always reversible (Wielopolska *et al*. 2005).

1.1.6 Biological function of RNA silencing

RNA silencing is a genetic regulatory mechanism that is conserved in most eukaryotic organisms such as plants, animals, and fungi (reviewed by Meister and Tuschl, 2004). RNA silencing, which is active at different levels of gene expression, appears to have evolved to counter the proliferation of foreign sequences, such as transposable elements (TEs) and viruses, many of which produce dsRNAs during replication. A possible biological role for RNA inreference (RNAi) in transposon silencing is supported by the observation that mobilization of some TEs is increased in several RNAi mutants in *C. elegans* (Plasterk and Ketting, 2000; Tabara *et al*., 1999). Similarly, some post-transcriptional gene silencing (PTGS)-defective mutants in plants show high sensitivity to certain RNA viruses (Vance and Vaucheret, 2001). The fact that RNA viruses replicating in the cytoplasm act as both targets and inducers of PTGS further suggested that RNA silencing may have evolved as an anti-viral defense mechanism in plants (Waterhouse *et al*., 2001). RNA silencing in plants prevents virus accumulation through cleavage of the dsRNA intermediate during its replication (Baulcombe, 2004). Accordingly, viruses have evolved a counter defense, which involves suppressor proteins of silencing encoded by their genomes (Moissiard and Voinnet, 2004). Also in animal cells, RNA silencing can act as an antiviral defense. The *Flock house virus* (FHV), an RNA virus that can infect both vertebrate and invertebrate hosts and is both an initiator and target of RNA silencing in *Drosophila* host cells, encodes a protein B2, which also operates as an RNA-silencing suppressor (Li *et al*., 2002).

The general role of silencing in the regulation of gene expression became apparent when it was realised that specific genes in plants and animals encode short forms of fold-back dsRNA, the precursor molecules of miRNAs (Ambros *et al*., 2003; Bartel, 2004). As regulators of gene expression, miRNAs target mRNA either by cleavage or by translational repression (Tang *et al*., 2003; Bartel, 2004). Animal miRNAs are only partially complementary to their target mRNAs, and they regulate the expression of the target genes through the repression of translation rather than through target degradation (reviewed by Hutvágner and Zamore, 2002). In contrast, plant miRNAs base pair with mRNA targets by precise or nearly precise complementarity, and direct degradation of the target mRNA (Llave *et al*., 2002; Tang *et al*., 2003). Many of these miRNA genes are evolutionarily conserved, and play important roles in the regulation of development (Bartel, 2004).

1.1.7 Applications of RNA silencing in plants

The sequencing of the complete genomes of a number of important eukaryotic organisms has opened new challenges and possibilities for understanding gene function. Currently, the double-stranded RNA (dsRNA)-mediated silencing appears to be the best technique available to undertake such gene function analyses. This homology-dependent mechanism results in the knockdown of gene function through the degradation of mRNA transcripts, and results in a phenotype that mimics the loss-of-function phenotype (Baulcombe, 1999; Vaucheret *et al*., 2001; Waterhouse *et al*., 2001).

RNA interference (RNAi) has recently been exploited successfully for whole-chromosome studies in the nematode *Caenorhabditis elegans* (Fraser *et al*., 2000; Gönczy *et al*., 2000). This has been feasible because of the ease with which dsRNA can be delivered to worms, by either soaking them in dsRNA (Maeda *et al*., 2001) or feeding them with bacteria that express dsRNA (Fraser *et al*., 2000).

In animals, RNA silencing has been mainly used to identify gene function (Fraser et al., 2000; Gönczy et al., 2000; Maeda et al., 2001). However, there is also potential for using RNAi for the treatment of viral diseases such as those caused by the hepatitis C virus (HCV) and the human immunodeficiency virus (HIV) (reviewed by Hannon and Rossi, 2004).

The expression of siRNAs under the control of PolIII promoters is a widely used approach to induce RNAi in mammals, and is even being used for large-scale genomic screens (Paddison *et al*., 2004). This approach is relatively unexplored in plants, although it has been shown to work in tobacco using the human H1 promoter and the *Arabidopsis* 7SL RNA gene promoter (Lu *et al*., 2004). This strategy ensures high levels of expression of siRNAs in every cell of the plant and should promote highly specific silencing of target genes, with reduced risk of cross-silencing non-target genes.

Two completed plant genome sequences are now available, the *Arabidopsis thaliana* (The Arabidopsis Genome Initiative, 2000) and *Oryza sativa* (rice; Goff *et al*., 2002; Yu *et al*., 2002). The highly efficient induction of RNA silencing by ihpRNA (or hpRNA) constructs provides an opportunity to investigate plant gene function in a genome-wide manner (Hilson *et al*., 2004). Although vectors based on Gateway cloning technology can facilitate the rapid generation of hpRNA constructs, the stable delivery of these transgene libraries into plants is only practically feasible in *Arabidopsis*, for which an easy method of transformation is available.

1.2 Site specific recombination systems and their application in eukaryotes

A large number of site-specific recombinases from prokaryotic or yeast origin have been described (review by Odell and Russell, 1994). Recombinases are specialized enzymes that can catalyse precisely defined recombination reactions between specific DNA motifs (recombination sites) by breakage and rejoining of the participating DNA strands. Site-specific recombinases can be classified in two major families based on amino acid sequence homology and catalytic residues, either tyrosine or serine: the family of the integrases (or tyrosine recombinases) and the resolvase/invertase family (review by Groth and Calos, 2004). The bacteriophage P1 Cre/lox system (Hoess and Abremski, 1985; Hoes *et al*., 1982; Dale and Ow, 1990; Russell *et al*., 1992; Odell *et al*., 1990), the *Saccharomyces cerevisiae* FLP-FRT system (Lyznik *et al*., 1993; Kilby *et al*., 1995) and the *Zygosaccharomyces rouxii* R-RS recombinase (Onouchi et al., 1995) belong to the family of the integrases and can carry out site-specific recombination without the need for additional factors. This makes these systems very interesting for their applications in heterologous hosts (reviewed by Kilby *et al*., 1993). In the natural situation these recombinases provide the mechanism for plasmid monomerization, circularization of viral DNA (P1), integration into the host genome and amplification of plasmids (reviewed by Odell and Russell, 1994).

The recombination systems above have in common the property that a single polypeptide recombinase catalyses the recombination between two sites of identical or nearly identical sequences. Each recombination site consists of a short, 2 to 8 bp, asymmetric spacer sequence where strand exchange takes place. This spacer is flanked by an inverted repeat of 12 to 13 bp, where recombinases bind. The asymmetry of the spacer sequence gives an orientation to the recombination site, and dictates the outcome of a recombination reaction. Recombination between directly or indirectly oriented sites *in cis* excises or inverts the intervening DNA (Fig.1.4). Recombination between sites *in trans* causes a reciprocal translocation of two linear DNA molecules, or cointegration if at least one of the two molecules is circular (Fig.1.4).

The bacteriophage P1 Cre/*lox* site-specific recombination system is one of the best characterized and most used recombination systems in plants (reviewed by Gilbertson, 2003). The *cre* gene encodes a 38 kDa recombinase which is able, without any other additional factors, to catalyse the recombination between two *lox* sites. The *loxP* sites (further referred to as *lox* sites) consists of two perfect 13 bp inverted repeats separated by an 8 bp spacer

region (Fig.1.4A). Binding of the CRE molecules to the inverted repeats in the *lox* site, followed by two consecutive breakages and rejoining reactions between the four participating DNA strands, results in recombinant DNA molecules (Hoess and Abremski, 1985).

Figure 1.4 (B-D) shows the different types of recombination events, in which the orientation of the *lox* sites determines the outcome of the reaction. Intramolecular recombination results in the inversion of intervening DNA when the sites are in inverted orientation (Fig.1.4B), while excision results from a directly repeated orientation (Fig. 1.4C). The reverse reaction of excision allows the integration of circular molecules (Fig. 1.4C), whereas recombination between two linear molecules will result in reciprocal translocation (Fig. 1.4D).

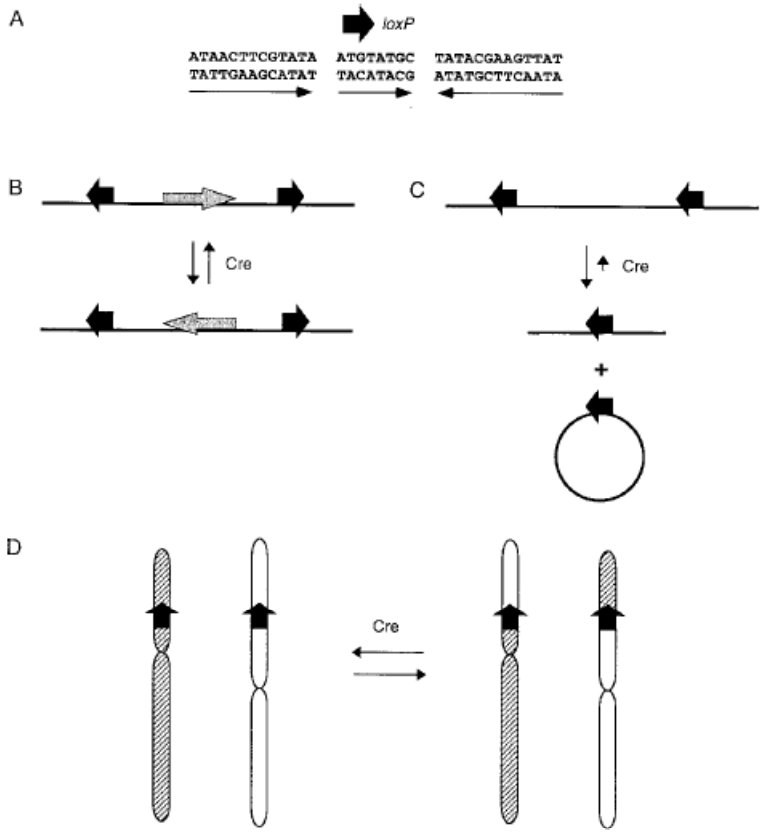

Fig. 1.4 A, DNA sequence of the loxP recombination site. Long thin arrows indicate the two inverted repeats, separated by an 8 bp asymmetric spacer region, giving directionality to the site represented by the direction of the arrow above the DNA sequence; B, CRE-mediated inversion of DNA flanked by two *lox* sites in an inverted orientation; C, CRE-mediated excision/integration reactions; D, Two *lox* sites present on distinct chromosomes can result in CRE-mediated translocation events (From Vergunst and Hooykaas, 1999).

The ability of CRE to catalyse a crossover between directly repeated *lox* sites flanking any DNA fragment has been exploited to remove selectable marker genes from transgenic plants (Dale and Ow, 1991; Russell *et al*., 1992; Sreekala *et al*., 2005). Also, since the process of plant transformation often generates insertions containing multiple copies of the transgenes, the Cre/lox recombination was used to excise these extra copies (Srivastava *et al*., 1999; Srivastava and Ow, 2001a). These authors designed a transformation vector in which the transgene was flanked by *lox* sites in an inverted orientation. Regardless the number of copies integrated between the outermost *lox* sites, recombination between these outermost sites resolves the integrated molecules into a single copy. The best results have been obtained when the CRE recombinase is provided through sexual cross with a CRE-expressing plant line (Srivastava *et al*., 1999), but single copy lines have also been obtained after co-introduction of a recombinase-expressing construct (Srivastava and Ow, 2001a). A nearly 100% recovery of single-copy lines have been obtained by using the Cre/lox site-specific recombination system for the resolving-complex loci in wheat, but whether the decrease in copy number was correlated with an increase in expression was not investigated. Nevertheless, by using the same approach in transgenic mouse, the reduction in copy number resulted in a marked increase in transgene expression, a decrease in chromatin compaction, and decreased methylation of the transgene locus (Garrick *et al*., 1998).

In plants, there is a preference for random integration of the introduced DNA by nonhomologous recombination, which might lead to the inactivation of important genes and to variable and unpredictable expression of the transgene itself. One way to minimize expression variability amongst transgenic lines and to increase the stability of gene expression is targeted integration of transgenes into predetermined chromosomal positions. This can be achieved by the use of Cre/lox recombination system (Day *et al*., 2000; Srivastava and Ow, 2001b). A general strategy of this approach involves two rounds of transformation. In the first round, a single *lox* site is randomly introduced into the plant genome. In the second round, the new DNA construct is integrated into this genomic target site via CRE-mediated site-specific integration. However, integration of identical transgenic inserts into the same genomic location by site-specific integration may still result in variable levels of expression among transformants (Day *et al*., 2000).

A gene can be introduced into a plant in an inactive state, and activated by recombination when expression is desired. Gene expression can be activated either by excising a blocking fragment, which can be inserted between the promoter and the coding region, or by flipping an inverted coding region with respect to the promoter. Although regulation of gene

expression can also be achieved by using tissue-specific promoters, this approach has a disadvantage, as many such promoters are active during the process of regenerating transgenic plants (Weising *et al*., 1998; Cheon *et al*., 1993). However, when site-specific recombination is used to regulate gene expression, the construct is designed in such a way that there is no expression until a recombinase gene is introduced into the same genome. This is especially important in circumstances where transgene expression may block transgenic regeneration (Joubès *et al*., 2004). A frequently used strategy to study the function of genes is to evaluate the consequences of inhibition of a gene of interest on the plant phenotype. Although the constitutive silencing technology allows efficient gene suppression, it cannot be used to study the function of essential genes and to study the role of genes at specific stages in plant development. To extent the application of silencing technology, a Cre/lox recombination system can be used to achieve a conditional functional knockout in plants (Guo *et al*., 2003). The basic principle of this Cre/lox approach is to design a construct in such a way that the invertedly repeated sequences of the target gene are not transcribed because they are separated from the promoter by a blocking cassette flanked by two *lox* sites. To induce silencing by having the hairpin construct expressed, activity of the CRE recombinase would be required. For this kind of studies, a valuable tool would be an inducible promoter system that can tightly control the expression of CRE recombinase at different developmental growth stages of the plant, allowing a CRE-dependent suppression of the target gene.

1.3 Inducible promoter systems

Inducible promoter systems allow the temporal and quantitative control of transferred genes in *vivo*. Such systems would be a valuable tool for functional studies of genes whose inappropriate expression is harmful or lethal during the development of the plant (Guo *et al*., 2003). Additionally, inducible expression of the gene under study at specific stages of development can contribute to the understanding of the correlated phenotype.

The expression level of the inducible genes should, ideally, be very low in the absence of the inducer so that it does not affect the wild-type phenotype. Upon induction, the expression

should increase rapidly to high level to obtain a clear gene related phenotype. In addition, the inducer should be non-toxic and should not induce pleiotropic effects in treated plants.
A number of controllable gene expression systems are available for plants, including systems based on promoters containing regulatory elements from evolutionary distant organisms or systems based on plant promoters (Gatz, 1997; Gatz and Lenk, 1998).

1.3.1 Heterologous inducible promoters

Promoters that contain regulatory elements from non-plant organisms include systems that respond to the antibiotic tetracycline (Gatz *et al*., 1992), the steroids dexamethasone (Aoyama and Chua, 1997), estradiol (Zuo *et al*., 2000), copper (Mett *et al*., 1993, 1996), and ethanol (Caddick *et al*., 1998). This strategy requires the expression of two genes in transgenic plants: the gene encoding the protein responsible for the regulation (transcriptional repressor or activator) and the gene of interest under the control of a target promoter, inducible by a transcriptional regulator.
The concept of using a regulatory protein from a prokaryote to control plant gene expression was first realized through the use of the bacterial tetracycline repressor (TetR). The TetR, encoded by the *E.coli* transposon Tn10, regulates expression of the tetracycline (tc) resistance gene in *E.coli*. TetR binds to the Tet operator (TetO) DNA only in the absence of its inducer, tetracycline (tc). The DNA binding activity of this protein is abolished by very low amounts of the antibiotic tetracycline (Hillen and Berens, 1994). TetR was successfully used to control the activity of a modified CaMV 35S promoter (Gatz and Quail, 1988). The disadvantage of this system is that a stringent repression depends on high intracellular repressor concentrations, because the repressor must compete with at least 40 proteins that assemble around the TATA-box to form a competent transcription initiation complex. However, there are reports that these high repressor concentrations, needed for stringent repression, cannot be tolerated in *Arabidopsis* and tomato (Corlett *et al*., 1996).
The use of transcriptional activators from higher eukaryotes is another approach to construct inducible expression systems in higher plants. Steroids such as the glucocorticoid dexamethasone (dx) are attractive options as chemical inducers because of their high specificity for the transcriptional activator, the glucocorticoid receptor (Fig.1.5). Glucocorticoid-dependent transcription is based on the inhibitory interaction between the heat shock protein HSP90 and the ligand-binding domain of the receptor that occurs in the absence

of the ligand (Picard, 1994). Binding of the ligand leads to dissociation of the receptor from HSP90 and thus to the release of a transcriptional activator. Aoyama end Chua (1997) developed the dexamethasone-inducible system, which consists of two components. The first is a glucocorticoid-regulated transcription factor GVG, which contains the DNA binding domain of the yeast transcription factor GAL4, the transactivation domain of the herpes viral protein VP16 and the receptor domain of the rat glucocorticoid receptor. The second component of the system contains the gene of interest, which is transcribed from a promoter containing six GAL4 DNA binding sites. The dexamethasone-inducible system has been successfully used in transgenic tobacco plants, in which the luciferase activity was induced 100-fold by dexamethasone (Aoyama and Chua, 1997). However, this dexamethasone-inducible system caused severe growth defects and induced defense-related genes in *Arabidopsis* (Kang et al., 1999) (Fig. 1.5).

Based on the same principle, Zuo et al (2000) developed an estrogen receptor-based chemical-inducible system. A chimeric transcription activator, XVE, was assembled by fusion of the DNA binding domain of the bacterial repressor LexA (X), the transactivating domain of VP16 (V) and the regulatory region of the human estrogen receptor (E). In transgenic *Arabidopsis* and tobacco plants, estradiol-activated XVE could stimulate expression of a *gfp* reporter gene controlled by the target promoter, which consists of eight copies of the LexA operator fused upstream of the –46 35S minimal promoter. Upon induction by estradiol, GFP expression levels were eightfold higher than that transcribed from a 35S promoter, whereas the uninduced controls had no detectable GFP transcripts (Zuo *et al*., 2000).

A copper-inducible expression system in plants was established by Mett et al (1993, 1996). It is based on control elements that regulate the expression of copper detoxification genes in *Saccharomyces cerevisiae* in response to elevated copper concentrations. Regulation is mediated by the transcriptional activator ACE1, which binds to specific *cis* elements only when coordinated with copper (Dameron *et al*., 1991). In contrast to dexamethasone, copper plays an important role in plant metabolism. Sufficient copper has to be present in the cell to drive essential biochemical processes, but accumulation to high levels leads to toxic effects.

A system based on the regulatory elements of the *Aspergillus nidulans alc*A promoter that is strongly induced by ethanol, is another example of inducible systems based on the activation principle. The transcriptional activator AlcR binds to its target sequence within the alcA promoter when cells are grown in the presence of ethanol or other inducers such as ethyl methyl ketone (Panozzo *et al*., 1997). The system was adapted for plants by placing the alcR coding region under the control of the CaMV 35S promoter. The target promoter contains the

TATA box as well as upstream sequences of the alcA promoter fused to position –23 of the CaMV 35S promoter (Caddick *et al*., 1998). This promoter system is successfully used in tobacco (Caddick *et al*., 1998), and *Arabidopsis* plants (Roslan *et al*., 2001). However, Roslan *et al* (2001) observed a significant level of basal expression in the absence of an exogenous inducer in *Arabidopsis* plants grown on agar. By contrast, they did not observe detectable GUS activity in soil-grown *Arabidopsis* plants. It is therefore likely that anaerobic respiration of agarose-grown seedlings resulted in the production of an endogenous inducer at sufficient concentration to partially induce the system.

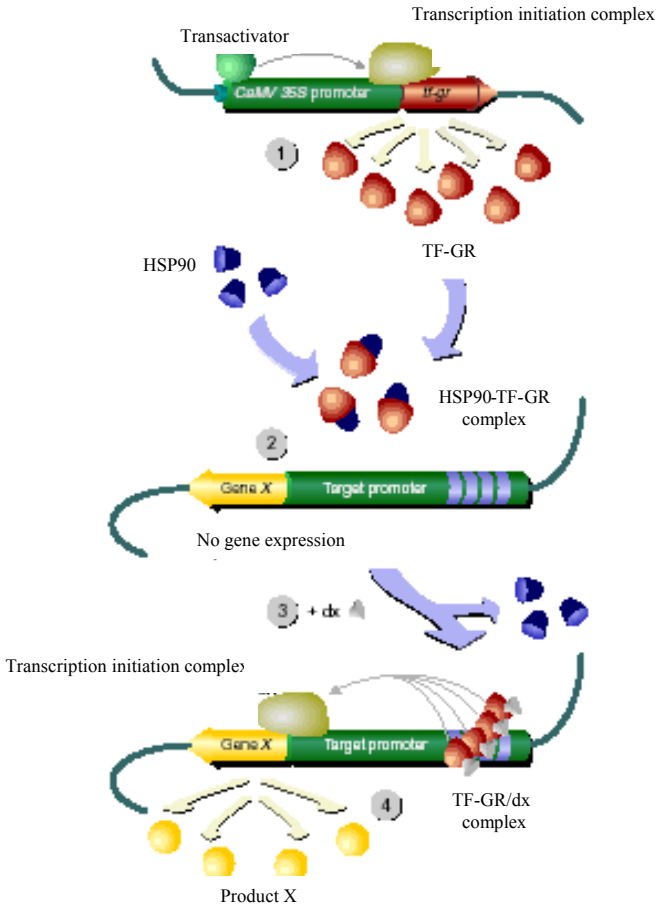

Fig. 1.5 The dexamethasone-inducible expression system. 1, the fusion protein TF-GR, consisting of a transcription factor (DNA binding domain and transcriptional activation domain) and the glucocorticoid binding domain, is expressed under the control of a strong promoter (e.g. CaMV 35Spromoter). 2, in the absence of dexamethasone the activator is trapped by the formation of an inactive complex with HSP90. 3, the binding of dexamethasone mediates dissociation from HSP90 and allows binding of the activator to a target promoter that contains multiple TF-GR binding sites upstream of a short DNA fragment that encodes the TATA box. 4, transcription from the target promoter is induced. TF can be any transcription factor that contains a DNA-binding domain and an activation domain (From Gatz and Lenk, 1998).

A promoter activation system, which allows a gene of interest to be activated in specific plant tissues after a cross between defined transgenic lines, has been developed by Moore *et al*. (1998) (Fig.1.6). The promoter, pOp, consists of *lac* operators cloned upstream of a minimal promoter. Transcription from the promoter was achieved by crossing effector plants with activator lines that expressed a chimeric transcription factor LhG4, which comprised transcription-activation domain-II from Gal4 of *Saccharomyces cerevisiae* fused to a mutant lac-repressor that binds its operator with increased affinity. The LhG4 system offers spatially regulated gene expression in the tissues of whole plants growing under normal conditions without the need for external intervention. It complements inducible expression systems that offer temporal control of gene expression in tissues that can be treated with inducing chemicals (Moore *et al*., 1998). However, whereas the pOp/LhG4 system can offer tissue-specific control over transgene expression through use of tissue-specific promoters, it provides minimal temporal control. To increase the versatility of this system, the existing pOp/LhG4 system was brought under chemical control by adding the ligand-binding domain (LBD) of the rat glucocorticoid receptor (GR) to LhG4 creating a glucocorticoid-dependent transcription factor LhGR (Samalova *et al*., 2005; Craft *et al*., 2005). This modified LhGR transcription factor in combination with the improved pOp promoter, that carries six copies of an *lac* operator sequence, was shown to be efficient and tightly regulated chemically inducible transgene expression system for both tobacco and *Arabidopsis* (Samalova *et al*., 2005; Craft *et al.,* 2005, respectively) (Fig. 1.6).

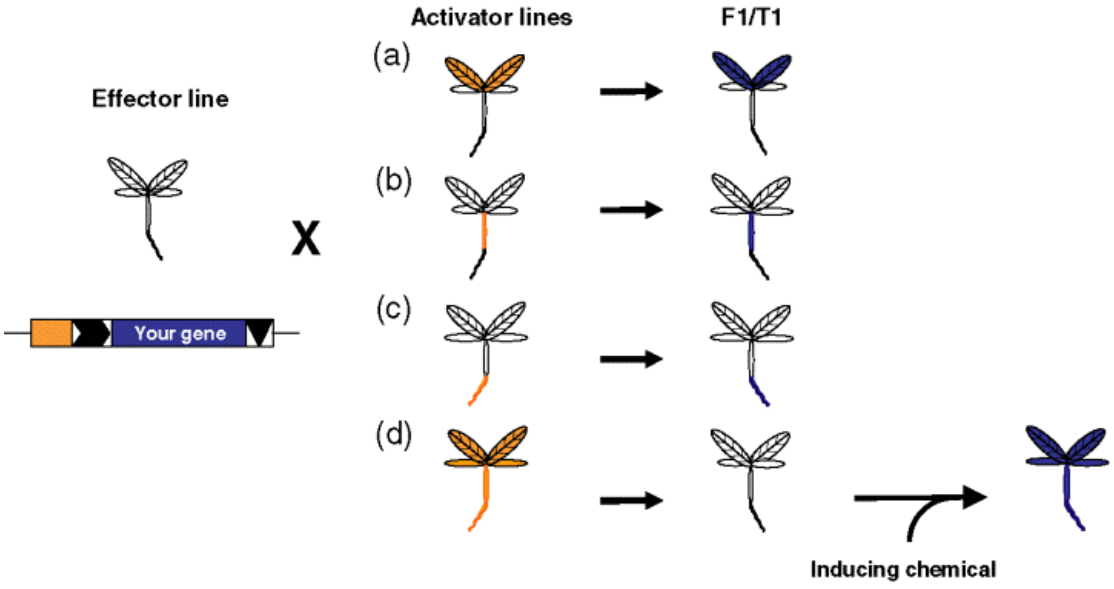

Fig. 1.6 The principle of transactivation and chemically inducible systems. In the effector line, a gene of interest (blue) is introduced under control of expression signals (orange) that are not recognized by wild-type plants. These plants can be maintained without interference from the latent transgene, which can be activated following a cross with activator lines (a–d) or transformation with activator T-DNAs that express an activating molecule in diverse patterns (orange). The gene of interest will be expressed in the corresponding pattern in the F1 or T1 plants (blue). If the activating molecule requires the presence of a chemical ligand that does not exist in the plant (d) the gene of interest will not be expressed until this chemical is applied (From Moore *et al*., 2006).

Each of the chemically inducible systems described above is based on the introduction of a chemically responsive transcriptional activator or repressor that interacts with regulatory sequences from evolutionary distant organisms in a target promoter. The advantage of the heterologous elements of gene regulation in these systems is that they respond to chemical inducers usually not encountered by higher plants (Gatz, 1997). On the other hand, promoters based on plant regulatory sequences are attractive because their use requires only the cloning of the responsive promoter upstream of the coding region of the gene of interest. The disadvantage of this approach is that native genes controlled by these regulatory sequences are also induced upon application of the inducer. Therefore it is important to choose an inducer that affects a set of genes that does not interfere with normal growth and development.

1.3.2 Plant heat-inducible promoters

The exposure of living organisms to elevated temperatures induces the synthesis of a set of proteins known as heat-shock proteins (hsps). The induction of hsps is dependent on the transcriptional activation of a set of genes at the elevated or heat-shock temperature. In plants, the major heat-shock protein accumulation is represented by a complex group of about 20 15- to 18-kDa and approximately 10 20- to 27-kDa proteins. The high-molecular weight hsps of plants, in contrast to those of *D. melanogaster* represent a relatively small fraction of total heat-shock protein accumulation (Nagao *et al*., 1985). The expression of these hsps is primarily controlled at the transcriptional level and this is structurally related to the occurrence of multiple copies of heat-shock elements (HSEs) clustered within about 150 bp upstream from the TATA box sequence. These HSEs have been shown to interact with a regulatory protein,

the heat shock factor (HSF), in yeast (Jakobsen and Pelham, 1988), *Drosophila* (Zimarino and Wu, 1987), and human cells (Goldenberg *et al*., 1988). The occurrence of multiple copies of HSEs in the promoter is necessary and sufficient to confer heat inducibility. These HSEs are binding site for heat-shock transcription factor (HSF). The HSF is present in uninduced cells in an inactive form, and for activation requires phosphorilation, which occurs in response to heat-shock (Sorger and Pelham, 1988). Due to conservation of the *cis*- and *trans*- acting components, heat-shock promoters of plants are recognized in heterologous species (reviewed by Schöffl *et al*., 1990) and can be used for the regulated expression of chimeric genes (Schöffl *et al*., 1989). The optimal temperature for the induction of transcription of hsp genes varies among species. The hsp mRNAs of *Arabidopsis* attain maximal levels at temperatures below 40^0 C, the temperature at which the maximal induction of synthesis of hsps is seen in soybean (Takahashi and Komeda, 1989). The response to the heat-shock temperature may be dependent upon the combined properties of all the various factors involved in the signal transduction and transcription as well as the structure of the heat-shock protein genes.

Inducible systems based on the heat-shock gene promoters have been successfully used for temporal control of gene expression in plants (Masclaux *et al*., 2004 Joubès *et al*., 2004; Kilby *et al*., 2000). Joubès *et al*. (2004) developed an inducible system based on two characterized systems: the Cre/lox site-specific recombination system of bacteriophage P1 and the subcellular targeting of proteins by a mammalian glucocorticoid receptor (GR). By fusing the receptor domain of the rat GR to the carboxyl terminus of the CRE recombinase, a double-lock conditional transcriptional induction system was created. The system was tested in *Nicotiana tabacum* bright yellow-2 (BY-2) cells. In this study, a heat-inducible expression of the CRE recombinase by the HSP18.2 heat-shock promoter from *Arabidopsis* was used to remove the *egfp* gene placed between two *lox* sites, bringing the gene of interest under the direct control of the CaMV 35S promoter. This strategy allows tight control of gene expression and therefore is useful to overexpress genes whose expression might block transgenic regeneration. Only cells that are exposed to the two inducers (dexamethasone and heat-shock) expressed the transgene (Joubès *et al*., 2004).

Similar system for controlled induction of GUS marked clonal sectors in *Arabidopsis* was developed by Kilby *et al*. (2000). In this system, inducible expression of the FLP recombinase was achieved from the soybean Gmhsp 17.6L heat-shock promoter (Severin and Schöffl, 1990). Heat-shock induction of the FLP expression in plants containing the target construct consisted of two directly oriented FLP recognition sites, flanking a hygromycin resistance cassette located between a GUS coding region and an 35S promoter, led to activation of

constitutive GUS expression. They observed both large and small GUS sectors in heat-shocked plants and proposed that this correlated with the time at which GUS activity was initiated. The most extensive clonal GUS sectors were achieved by GUS activation early in the development. Although the Gmhsp 17.6L heat-shock promoter exhibits all structural features assumed to be essential for efficient heat-inducible expression of a fused heterologous gene, the TATA box as a general eukaryotic promoter element, multiple overlapping HSEs and a part of the 5' untranslated mRNA leader which seems to be important for efficient mRNA translation during heat-shock, it may not provide sufficient expression of the FLP recombinase in all developmental stages and in all cell types. Importantly, no GUS activition events were observed in the absence of the heat-shock. Also, by optimizing the experimental conditions, sectors could be induced in up to 100% of plants without any obvious abberant (heat-shock-induced) effects on phenotype (Kilby *et al*., 2000).

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CHAPTER 2

Objectives

2.1 Objectives

It has been shown that constructs that encode a hpRNA against both endogenous genes and transgenes produce efficient post-transcriptional gene silencing (Waterhouse *et al*., 1998; Wang and Waterhouse 2000; Chuang and Meyerowitz, 2000; Levin *et al*., 2000; Smith *et al*., 2000; Wesley *et al*., 2001; Stoutjesdijk *et al*., 2002; Guo *et al*., 2003). The most effective constructs appear to be those that encode an intron-spliced hairpin RNA (ihpRNA; Smith *et al*., 2000; Wesley *et al*., 2001). However, plants transformed with constitutively expressed hpRNA transgenes induce silencing soon after germination. When this type of hpRNA transgene is targeted against genes, which are required for basic cell function or development, it can result in plant lethality, thus preventing recovery of transgenic plants for investigation.

The general objective of this work was to develop an experimental approach by which down-regulation of endogenous plant gene expression could be controlled in a conditional way. The hpRNA construct was designed in such a way that the invertedly repeated sequences of the target gene are not transcribed because they are separated from the promoter by a blocking cassette flanked by two *lox* sites. To induce silencing by expression of the hairpin construct, activity of the CRE recombinase would be required. Therefore, an inducible promoter system that can tightly control the expression of the CRE recombinase at different developmental growth stages of the plant development, would allow a CRE-dependent expression of the hpRNA and concomitantly suppression of the target gene. With respect to these requirements, we evaluated the efficiency of hpRNA-mediated suppression of a *gus* reporter gene expressed under the control of different promoters, the tissue-specificity and inducibility of the Gmhsp 17.6L soybean heat-shock promoter (Severin and Schöffl, 1990) using the green fluorescent protein *(gfp)* and β-glucuronidase (*gus*) marker genes, and the efficiency of CRE-mediated excision of a DNA fragment flanked by directly repeated *lox* sites, after expression from different promoters.

Although work on RNA silencing in plants demonstrated a consistent and profound inhibition of the expression of both endogenous genes and transgenes by hpRNA transgenes, it is not clear whether hpRNA-mediated gene suppression can efficiently occur in all tissues; especially conflicting results have been obtained for highly proliferating cells (Mitsuhara *et al*., 2002; Corrêa *et al*., 2004; Chuang and Meyerowitz, 2000; Wang and Waterhouse, 2000;

Wesley *et al*., 2001). Therefore, the efficiency of hpRNA-mediated suppression was evaluated for a reporter *gus* gene that was under the control of different promoters. As a target gene expressed in the meristems, a β-glucuronidase gene driven by the CycB1;1, CycD4;1 and KRP4 promoters were chosen. As a target gene active in numerous cell types, a WRKY23 promoter driven *gus* target gene was used. Finally, as a control, the cauliflower mosaic virus 35S promoter driven *gus* gene was used as a target gene expressed in practically all plant tissues (Chapter 3).

Inducible promoter systems are a valuable tool for functional studies of genes whose inappropriate expression is harmful or lethal, especially during the development of the plant (Guo *et al*., 2003). To drive expression of the *cre* gene, the promoter of the soybean heat shock gene Gmhsp17.6L (Severin and Schöffl, 1990) was chosen as it allowed induction of transgene expression in *Arabidopsis thaliana* (Kilby *et al*., 2000). However, the activity of the soybean heat shock gene Gmhsp17.6L promoter in uninduced conditions and its induction level had not been determined in *Arabidopsis*. Therefore, the basal level, inducibility and the organ specificity of the Gmhsp 17.6L soybean heat-shock promoter were determined using the green fluorescent protein *(gfp)* and β-glucuronidase (*gus*) marker genes. The obtained expression pattern was compared with the *gfp-gus* expression driven by two commonly used promoters, the CaMV 35S and the nopaline synthase gene promoter (Pnos) (Chapter 4).

In a next step, the Cre/lox recombination system has been evaluated to remove a lox-flanked DNA fragment. By placing a fragment between a promoter and a gene of interest, a tight control of gene expression can be obtained, as only after the expression of the CRE recombinase a gene would become expressed, which otherwise might block transgenic regeneration (Joubès *et al*., 2004). In Chapter 5, we have analysed the efficiency of the CRE recombinase, expressed from the CaMV 35S**,** the nopaline synthase gene promoter (Pnos), and the soybean heat shock gene Gmhsp17.6L promoter, to induce DNA recombination and excision of a *lox* sequence flanked *gus* gene in transgenic *Arabidopsis thaliana*.

Finally, a conditional gene-silencing vector was constructed. The vector contains the Pnos-bar-3'nos expression cassette flanked by two directly repeated *lox* sites, which separates the 35S promoter from a hairpin RNA-encoding cassette. In this way, the transcription of the hpRNA is dependent on the expression of the CRE recombinase. When the *cre* gene is expressed, recombination will eliminate the Pnos-bar-3'nos expression cassette between the

lox sites allowing hpRNA transcription from the 35S promoter. The system was tested with a DNA fragment that targets the *catalase 2* gene of *Arabidopsis thaliana*, as described in Chapter 6.

2.2 References

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CHAPTER 3

35S driven hairpin silencing of a *gus* reporter gene driven by different promoters in *Arabidopsis thaliana* plants

35S driven hairpin silencing of a *gus* reporter gene driven by different promoters in *Arabidopsis thaliana* plants

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3.1 Abstract

The introduction of constructs designed to produce self-complementary hairpin RNA (hpRNA) appears to be the most efficient way to trigger post-transcriptional gene silencing (PTGS) in plants. In this study we investigate the efficiency of such a construct to silence the *gus* gene expressed under the control of the 35S promoter and different cell cycle specific promoters. Efficient suppression by a 35S driven hairpin construct of the last 792 nucleotides of the *gus* coding sequence was observed in the expanding root and leaf tissues. Also, the GUS expression was significantly reduced in the tissues containing highly proliferating cells. However, most hpRNA transformants showed residual GUS activity in root tips, young leaves and callus tissues, suggesting that the silencing machinery is less active or less competitive with downstream expression in meristematic tissues.

3.2 Introduction

Over the last years, much progress has been made in unravelling the mechanism of post-transcriptional gene silencing in plants (PTGS), a process leading to the sequence-specific degradation of target mRNAs**.** This process is termed RNA interference in animals (RNAi) (Fire *et al*., 1998), and quelling in fungi (Cogoni and Macino, 2000). PTGS is triggered by the double-stranded RNA (dsRNA), which is processed into small interfering RNAs (siRNAs) of 21-28 nucleotides in length by an RNase III-like enzyme (Dicer) (Bernstein *et al*., 2001). Subsequently, these siRNAs are incorporated into an RNA-induced silencing complex (RISC) to guide degradation of homologous mRNAs (Hamilton and Baulcombe, 1999; Hammond *et al*., 2000; Beclin *et al*., 2002; Matzke *et al*., 2001). Since the observation that the delivery of double-stranded RNA into an organism or cell induces a sequence-specific RNA degradation mechanism that effectively silences a targeted gene (Fire *et al*.,1998; Waterhouse *et al*., 1998; Tang *et al.,* 2003; Waterhouse and Helliwell, 2003), many different transformation vectors capable of dsRNA formation were constructed to obtain efficient PTGS in plants (Chuang and Meyerowitz, 2000; Karimi *et al*., 2002; Levin *et al*., 2000; Wesley *et al*., 2001; Helliwell *et al*., 2002). The most effective constructs to date appear to be those that encode an intron-spliced hairpin RNA (ihpRNA) (Smith *et al*., 2000; Wesley *et al*., 2001). The highly efficient induction of RNA silencing by hpRNA encoding constructs provides an opportunity to investigate plant gene functions in a genome-wide manner (Hilson *et al*., 2004).

RNA interference (RNAi) (Fire *et al*., 1998) has been exploited to study the function of over 4000 genes on chromosomes I and III in *Caenorhabditis elegans* (Fraser *et al*., 2000; Gönczy *et al*., 2000). This has been feasible largely because of the ease with which RNAi can be delivered to worms, either by soaking them in dsRNA (Maeda *et al*., 2001) or by feeding them with bacteria that express dsRNA (Fraser *et al*., 2000). In contrast, in plants, RNAi was mainly used to validate the already known functions of genes. Although a consistent and profound inhibition of the expression of both endogenous genes and transgenes by hpRNA transgenes has been demonstrated in plants (Wang and Waterhouse 2000; Chuang and Meyerowitz, 2000; Levin *et al*., 2000; Smith *et al*., 2000; Wesley *et al*., 2001; Stoutjesdijk *et al*., 2002; Guo *et al*., 2003), it is not clear whether hpRNA-mediated gene suppression can efficiently silence genes in all types of cells, especially in tissues containing high proliferating cells. It has been reported that silencing cannot be maintained in proliferating tissues such as callus, meristematic tissue, developing young leaf, and flower (Mitsuhara *et al*., 2002). They

showed that the calli induced from leaf pieces of two tobacco plants which contained a sense transgene for overproduction of *luciferase* (*luc*) but exhibit post-transcriptional gene silencing of *luc,* exhibited strong *luciferase* activity similar to nonsilenced leaves. However, it has not been investigated whether genes in meristematic cells may become silenced by a 35S driven hpRNA construct.

To address this question, the efficiency of hpRNA-mediated suppression was determined for the *gus* reporter gene expressed under the control of different promoters. First, as a control, the cauliflower mosaic virus 35S promoter driven *gus* gene was used as a target gene expressed in practically all plant tissues (Odell *et al*., 1985). Second, as a target gene active in numerous but not all cell types, the *gus* target gene driven by a WRKY23 promoter was chosen (for review see Eulgem *et al*, 2000). Finally, a *β-glucuronidase* gene driven by the CycB1;1, CycD4;1 and KRP4 promoters were chosen as a target gene expressed in the meristems (Ferreira *et al*., 1994; Burssens *et al*., 2000; De Veylder *et al*., 1999).

An efficient hpRNA-mediated suppression of all target genes occurred in most of the plant tissues in different independent transformants. However, a residual GUS activity was often observed in the callus tissue, root tips, and young developing leaves and flowers.

3.3 Results

3.3.1 Construction of pHhpUS vector and plant transformation

The pH7GWIWG2 vector is designed for cosuppression of plant endogenes (Karimi *et al*., 2002). This vector takes the advantage of the GATEWAY™ recombination system and allows rapid production of hpRNA constructs. Using the pH7GWIWG2 destination vector (Fig.3.1b), the pHhpUS construct (Fig.3.1c) was generated in a single step from the 3'CS-GUS GATEWAY™ entry clone (Fig.3.1a), which contains the last 792 nucleotides of the *gus* coding sequence (see Material and Methods).

The 35S driven hpRNA construct (pHhpUS, Fig.3.1c) was transformed using the floral dip method (Clough and Bent, 1998) in kanamycin resistant *Arabidopsis* lines containing the *gus* gene under the control of the P35S, CycB1;1, CycD4;1, KRP4, and WRKY23 promoters. The number of super-transformants obtained with the pHhpUS construct for each *Arabidopsis* reporter line is shown in Table 3.1.

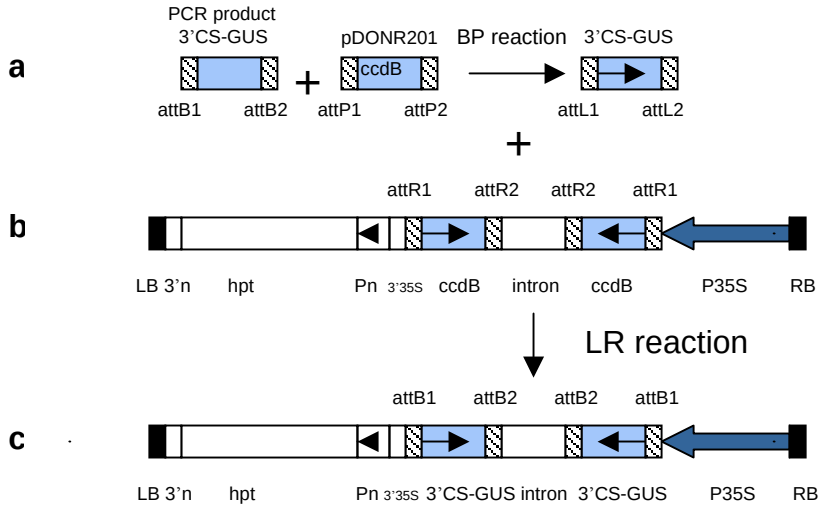


Fig. 3.1 Schematic representation of the pHhpUS vector. A PCR product was amplified from the GUS gene with attB1 and attB2 sites incorporated into the PCR primers (see Material and Methods). This product was inserted into the pDONR201 vector by recombination between attB1/attB2 and attP1/attP2 sites mediated by BP Clonase enzyme (Invitrogen) to yield the 3'CS-GUS-entry clone (a). The expression vector pHhpUS (c) was generated in the LR reaction where the 3'CS-GUS entry clone was incubated with the GATEWAY^{TM}-compatible binary T-DNA destination vector, pH7GWIWG2(I) (Karimi *et al*., 2002) in the presence of LR Clonase enzyme (Invitrogen). The attB3'CS-GUS cassette forms the arms of the hairpin. When the construct is expressed in plants a hairpin RNA (hpRNA) with the intron spliced out is produced. The construction of the 3'CS-GUS entry clone and pHhpUS expression clone is described in Material and methods.
Abbreviations: P35S, cauliflower mosaic virus promoter; 3'35S, 35S terminator; Pn, promoter of the nopaline synthase gene; 3'n, 3' end of the nopaline synthase gene; ccdB, bacterial negative selection marker; hpt, hygromycin phosphotransferase gene; LB, left border; RB, right border. The BP and LR reactions are the recombination reactions between specific att elements, such as attB1 versus attP1 and attL1 versus attR1.

Table 3.1 Overview of the number of the hpUS super-transformants (T1 generation) obtained.

Name	#transformant
P35S::gus/hpUS	75
CycB1;1::gus/hpUS	2
CycD4;1::gus /hpUS	18
KRP4::gus /hpUS	8
WRKY23::gus/hpUS	5

3.3.2 Histochemical analysis of GUS activity in different *Arabidopsis* reporter lines and hpUS super-transformants shows efficient GUS suppression in most of the tissues

In order to determine the extent of GUS suppression induced by the pHhpUS construct, the GUS activity in hpUS super-transformants was compared with the GUS activity in the original lines by histochemical staining of roots, leaves and flowers. Seeds of the original lines and of the T2 segregating hpUS super-transformants were germinated on medium containing kanamycin to select for plants that contain the reporter promoter::gus T-DNA. To identify the progeny plants that also received the HhpUS T-DNA, leaf tissue from all kanamycin resistant plants was assayed for the ability to form callus on medium supplemented with hygromycin. The efficiency of GUS suppression was evaluated in five T2 seedlings of nine independent *P35S::gus/hpUS* transformants, five *CycD4;1::gus/hpUS, KRP4::gus/hpUS,* and *WRKY23::gus/hpUS* and two independent *CycB1;1::gus/hpUS* transformants.

Histochemical analysis of the GUS activity in different Arabidopsis reporter lines

Four-weeks old seedlings of *Arabidopsis* lines containing the *gus* gene under the control of the P35S, CycB1;1, CycD4;1, KRP4, and WRKY23 promoters were stained for GUS activity. As shown in the Figure 3.2, seedlings of FK24/3 transgenic *Arabidopsis* line carrying a *P35S::gus* transgene showed uniform GUS staining in all tissues (Fig.3.2a) (De Buck *et al*., 2004). *Arabidopsis* plants transgenic for *CycB1;1::gus* showed activity of the promoter only in the actively dividing tissues, such as root meristem (root tip and emerging lateral roots) and in the young leaves (Ferreira *et al*., 1994) (Fig.3.2b). In transgenic *Arabidopsis* plants carrying the *CycD4;1::gus* or *KRP4::gus* T-DNA, the GUS activity was restricted to root tips (Fig.3.2c,d). Finally, the WRKY23 promoter in the transgenic *Arabidopsis* line carrying a *WRKY23::gus* T-DNA showed activity in different tissues, including the roots, leaves and vascular tissues (Fig.3.2e).

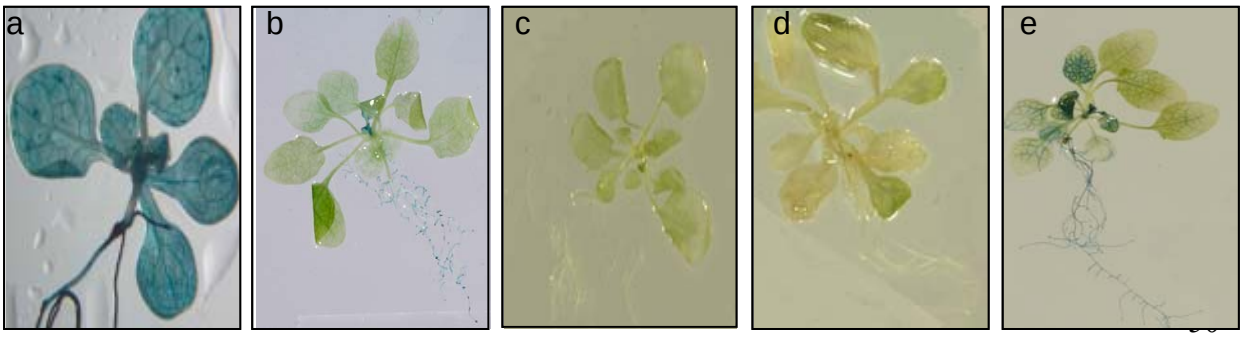


Fig. 3.2 Histochemical analysis of GUS activity in different *Arabidopsis* reporter lines. (a) Seedling of the P_{35S}*::gus* line shows uniform GUS activity in the cotyledons, all rosette leaves and throughout the roots. (b) Seedling of the *CycB1;1::gus* line shows GUS activity in the youngest rosette leaves, emerging and developing lateral roots, and in the root tips. (c-d) Seedlings of the *CycD4;1::gus* and *KRP4::gus* lines show GUS activity only in the root tips. (e) Seedling of the *WRKY23::gus* line shows uniform GUS activity in the main and lateral roots and in the younger rosette leaves. GUS activity is detected in the vascular tissue in some of the expanded leaves (e).

35S driven hairpin silencing of a gus reporter gene in root tissues

Roots of the transgenic FK24/3 *Arabidopsis* line carrying the *P35S::gus* transgene show uniform GUS activity throughout the whole root (Fig.3.3a1), with very high expression in the root tips (Fig.3.3a2). Three of the 9 *P35S::gus/hpUS* super-transformants (*P35S::gus/hpUS*/52, 55 and 57) showed no to very low GUS activity throughout the roots, mainly in the root tips. Four of the 9 (*P35S::gus/hpUS*/28, 51 and 59 and 61) show additional GUS activity in the expanding roots, while the remaining two transformants (60 and 63) showed GUS activity comparable with the GUS activity observed in the FK24/3 line. These different *P35S::gus/hpUS* super-transformants showed a clear difference in suppression of residual GUS activity. The most efficient hpRNA-mediated suppression of the 35S driven *gus* gene was observed in the expanding root tissues (Fig.3.3a3, a4). Next, we investigated whether there is a correlation between the frequencies of root tips in which complete suppression was observed and the level of suppression in the expanding root tissues. Therefore, the roots of ten T2 seedlings of each *P35S:gus/hpUS* transformant were stained, and the number of the GUS positive and GUS negative root tips was determined (Table 3.2). In the *P35S::gus/hpUS* super-transformants with very low GUS activity in the expanding roots (*P35S:gus/hpUS*/52, 55 and 57), more than 60% of the root tips were found to be GUS negative. Accordingly, in the super-transformants with residual GUS activity in the expanding roots (*P35S::gus/hpUS*/28, 51, 59 and 61), 0-50% of the root tips were GUS negative, while in the super-transformants 60 and 63, where GUS activity was comparable with the GUS activity in the original line, more than 80% of the root tips were GUS positive. From this we can conclude that in the *P35S::gus/hpUS* super-transformants showing strong GUS suppression in the expanding roots, a more efficient suppression was also occurring in the proliferating root tissues.

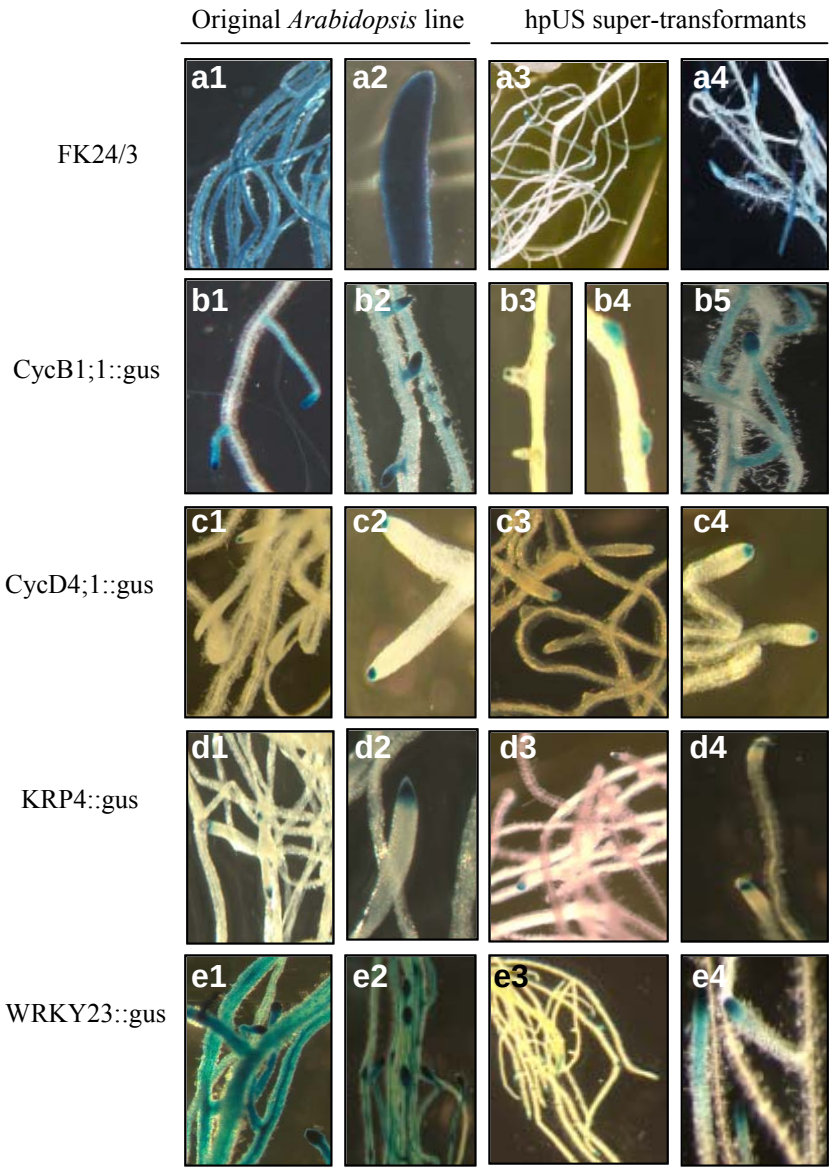


Fig. 3.3 Histochemical analysis of hpUS-mediated GUS suppression in the roots of the original *Arabidopsis* lines and the hpUS super-transformants. (a1, a2) Roots of a *P35S::gus* line are stained uniformly including the root tips. (a3, a4) Most of the *P35S::gus/hpUS* super-transformants showed no to very low GUS activity throughout the roots, mainly in the root tips. (b1, b2) Roots of the *CycB1;1::gus* line and (b3, b4) *CycB1;1::gus/hpUS* super-transformants show GUS activity in the emerging and developing lateral roots, and in the root tips. (c1, c2) In the *CycD4;1::gus* line GUS activity is detected in the root tips. (c3, c4) Representative roots of the *CycD4;1::gus/hpUS* show residual GUS activity in most of the root tips. (d1, d2) In the *KRP4::gus* line GUS activity is detected in the root tips. (d3, d4) Representative roots of the *KRP4::gus/hpUS* show residual GUS activity in most of the root tips. (e1, e2) *WRKY23::gus* transgenic line show uniform GUS activity in the roots, including the root tips (e3, e4) In most of the *WRKY23::gus/hpUS* transformans no to low GUS activity detected in the roots; however, high GUS activity remained present in the root tips.

Table 3.2 Overview of the % of GUS positive and GUS negative root tips of the ten T2 seedlings of *P35S::gus/hpUS* transformants. 100% of the root tips of the FK24/3 transgenic *Arabidopsis* line carrying the *P35S::gus* transgene are GUS positive. The GUS staining was done at 37^{0}C for 15 min.

Transformant		28		51		52 [a]		55		57		59		60		61[a]		63	
		Gus$^+$	Gus$^-$	Gus$^+$	Gus$^-$	Gus$^+$	Gus$^-$	Gus$^+$	Gus$^-$	Gus$^+$	Gus$^-$	Gus$^+$	Gus$^-$	Gus$^+$	Gus$^-$	Gus$^+$	Gus$^-$	Gus$^+$	Gus$^-$
Seedling	1	79	21	100	0	10	90	0	100	0	100	83	17	100	0	35	65	80	20
	2	46	54	100	0	5	95	0	100	0	100	80	20	100	0	50	50	100	0
	3	100	0	73	27	0	100	0	100	0	100	90	10	100	0	0	100	82	18
	4	100	0	100	0	48	52	0	100	0	100	87	13	100	0	61	39	100	0
	5	81	19	100	0	35	65	0	100	12	88	100	0	100	0	100	0	100	0
	6	83	17	100	0	100	0	100	0	0	100	100	0	100	0	100	0	100	0
	8	50	50	80	20			39	61	33	67	100	0	100	0	73	27	100	0
	9	83	17	100	0			40	60	40	60	71	29	100	0			100	0
	10	100	0	100	0			41	59	0	100	100	0	100	0			100	0

[a] Not determined for all seedlings.

The *CycB1;1::gus* line shows GUS activity in all root tips of main and lateral roots and in the emerging lateral roots (Fig.3.3b1,b2). In the two *CycB1;1::gus/hpUS* independent super-transformants, *CycB1;1::gus/hpUS/*1 and 2, GUS activity in the roots was comparable with the activity observed in the roots of the original *CycB1;1::gus* line (Fig.3.3b3-b5). This indicates that in these two transformants the hairpin construct was not capable to significantly suppress the GUS expression in these tissues.

In the transgenic *Arabidopsis* plants carrying the *CycD4;1::gus* transgene, the GUS activity was restricted to the root tips (Fig.3.3c1, c2). Three *CycD4;1::gus/hpUS* super-transformants, *CycD4;1::gus/hpUS/*1, 3 and 5 had some root tips where no GUS activity was detected, while the other two, *CycD4;1::gus/hpUS/*6 and 10, showed GUS activity in all root tips (Fig.3.3c3, c4).

Arabidopsis plants transgenic for *KRP::gus* showed activity of the promoter in all root tips (Fig.3.3d1, d2). Of the five *KRP4::gus/hpUS* super-transformants, two contained some root tips where no GUS activity was observed (*KRP4::gus/hpUS/*3 and *KRP4::gus/hpUS/*4), whereas in the other three (*KRP4::gus/hpUS/*1, *KRP4::gus/hpUS/*2 and *KRP4::gus/hpUS/*5) the GUS activity was detected in all of them (Fig.3.3d3, d4).

The WRKY23 promoter in the transgenic *Arabidopsis* line carrying a *WRKY23::gus* T-DNA showed activity in all root cells, including the root tips (Fig.3.3e1, e2) In three *WRKY23::gus/hpUS* super-transformants, *WRKY23::gus/hpUS/*3, 5 and 6, residual GUS activity was observed only in the root tips (Fig.3.3e3, e4)). However, two of the five analysed

super-transformants, *WRKY23::gus/hpUS*/1 and 4, still showed GUS activity which was comparable with the original *WRKY23::gus* line.

P35S driven hairpin silencing of a gus reporter gene in the leaves

Rosette leaves of the transgenic *Arabidopsis* plants carrying the *P35S::gus* transgene show uniform GUS activity (Fig.3.4a1,a2). Almost all expanded leaves of the *P35S::gus/hpUS*/28, 51, 52, 55, 57, 59 and 61 super-transformants are GUS negative, or show a patchy distribution of the GUS activity (Fig.3.4a3). In the progeny of the remaining two transformants, *P35S::gus/hpUS*/60 and 63, a mixture of GUS negative expanded leaves, leaves with a patchy distribution of the GUS activity and almost uniformly stained leaves was observed.

In the *CycB1;1::gus* line, GUS activity was observed only in the young leaves (Fig.3.4b1, b2). However, leaves of the two *CycB1;1::gus/hpUS* independent super-transformants, did not show any GUS activity (Fig.3.4b3).

Rosette leaves of the *WRKY23::gus* line show GUS activity in the vascular tissue of the expanded leaves and uniform staining in the young leaves (Fig.3.4c1,c2), whereas in all *WRKY23::gus/hpUS* super-transformants residual GUS activity was observed only in the young leaves (Fig.3.4c3).

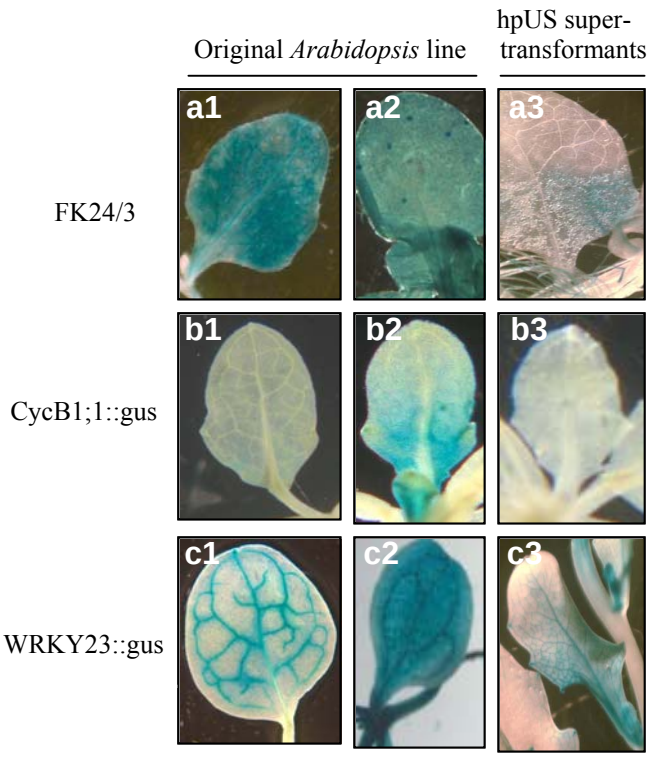


Fig. 3.4 Histochemical analysis of hpUS-mediated GUS suppression in the leaves of the original *Arabidopsis* lines and the hpUS super-transformants. (a1, a2) Leaves (young and expanded) of the *P35S::gus* line are stained uniformly. (a3) In the *P35S::gus/hpUS* super-transformants some of the rosette leaves are GUS negative and some show a patchy distribution of the GUS activity. (b1, b2) In the *CycB1;1::gus* line expanded leaves are GUS negative while young leaves show GUS activity. (b3) In the *CycB1;1::gus/hpUS* super-transformants no GUS activity detected in the leaves. (c1) Expanded leaves of the *WRKY23::gus* line show GUS activity in the veins, while (c2) young rosette leaves are stained uniformly. (c3) *WRKY23::gus/hpUS* super-transformants show GUS activity only in the young leaves.

35S driven hairpin silencing of a gus reporter gene in the flowers

Histochemical staining of the flower of the *P35S::gus* original line show GUS activity in most floral tissues (Fig.3.5a1,a2). The 35S driven hairpin construct induced suppression of the *gus* gene in most of the floral tissues. However, again the variations in the *gus* suppression were observed among different hpUS super-transformants. Most flowers of the progeny plants of *P35S::gus/hpUS* double transformants showed residual GUS activity in the anthers (Fig.3.5a3).

Flowers of the *CycB1;1::gus* line show GUS activity mainly in the ovules. A very weak GUS activity is found in the anthers. The CycB1;1 promoter is active during embryo formation in correlation with mitotic activity (Ferreira *et al*., 1994**;** Fig.3.5b1, b2). In both *CycB1;1::gus/hpUS* super-transformants, GUS remained active in the ovules (Fig.3.5b3).

Flowers of *CycD4;1::gus* (Fig.3.5c1,c2) and *KRP4::gus* line (Fig.3.5d1, d2) show GUS activity in the anthers. In the *CycD4;1::gus/hpUS* (Fig.3.5c3) and *KRP4::gus/hpUS* double transformants (Fig.3.5d3), GUS remained equally active in the anthers.

Flowers of the *WRKY23::gus* transgenic line show GUS activity in most of the floral tissues, especially in the anthers and the carpels (Fig.3.5e1, e2). In the three *WRKY23::gus/hpUS* double transformants (*WRKY23::gus/hpUS/*3, 5 and 6), suppression of the *gus* gene was observed only in the anthers (Fig.3.5e3), while in the progeny of the other two (*WRKY23::gus/hpUS/*1 and 4), GUS activity in the flowers was comparable with the GUS activity observed in the original *WRKY23::gus* transgenic line.

Histochemical analysis of the 35S driven hairpin silencing of a *gus* reporter gene expressed under the control of different promoters showed a difference in the extent of the suppression among different tissues. A less efficient GUS suppression was observed in the proliferating tissues in the different hpUS double transformants. However, when the P35S driven hairpin construct was targeted against a *gus* gene driven by the same promoter, we were able to

demonstrate the silencing in the meristematic tissues. Therefore, it is possible that the efficient silencing of the CycB1;1, CycD4;1, KRP4, and WRKY23 promoter driven *gus* gene in these tissues did not occur because of a differential activity of the 35S promoter which drives the expression of the hairpin construct and promoters used to drive a *gus* target gene.

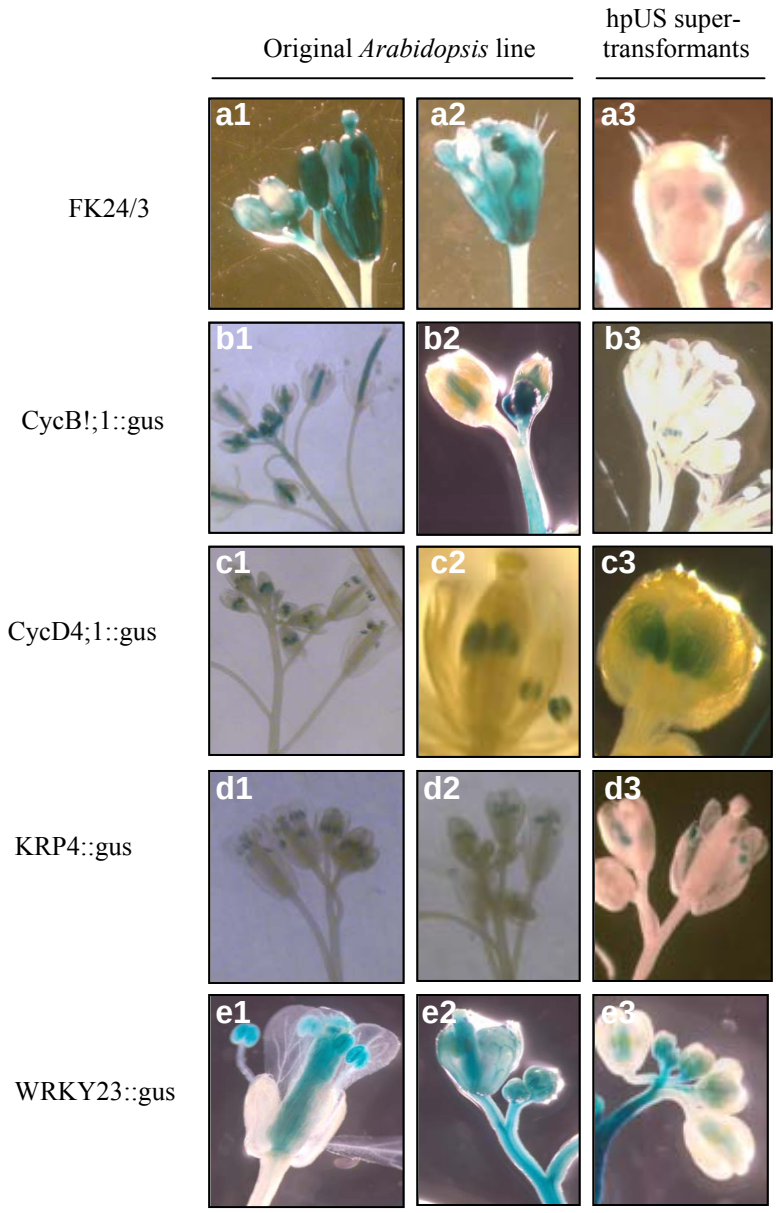


Fig. 3.5 Histochemical analysis of the hpUS-mediated GUS suppression in the flowers of the original *Arabidopsis* lines and the hpUS super-transformants. (a1,a2) Flowers of the FK24/3 line show GUS activity in all floral tissues. (a3) Representative flower of the *P35S::gus/hpUS* transformant in which GUS activity was observed in the anthers. (b1, b2) Flowers of the *CycB1;1::gus* line show GUS activity in the ovules. (b3) In the *CycB1;1::gus/hpUS* super-transformants, GUS remained active in the ovules. Flowers of *CycD4;1::gus* (c1,c2) and *KRP4::gus* line (d1, d2) show GUS activity in the anthers. In the

CycD4;1::gus/hpUS (c3) and *KRP4::gus/hpUS* super-transformants (d3) GUS remained active in the anthers. Flowers of the *WRKY23::gus* transgenic line show GUS activity in most floral tissues (f1, f2). In most of the *WRKY23::gus/hpUS* double transformants, GUS remained active in the young flowers and carpels.

3.3.3 Quantitative evaluation of the hpRNA-mediated GUS suppression in leaf and callus tissues in different *P35S::gus/hpUS* super-transformants

Histochemical staining analysis showed different degrees of GUS suppression in independent *P35S::gus/hpUS* super-transformants. Also, a less efficient hpUS-mediated silencing of the *gus* gene was observed in the meristematic tissues. To further investigate whether an efficient 35S driven hairpin silencing of the *gus* gene under the control of the 35S promoter is possible in the tissues containing highly proliferating cells, GUS activity levels were determined in leaf and callus tissues of the original *P35S::gus Arabidopsis* line (FK24/3) (De Buck *et al*., 2004) and nine independent super-transformants of this line with the pHhpUS construct.

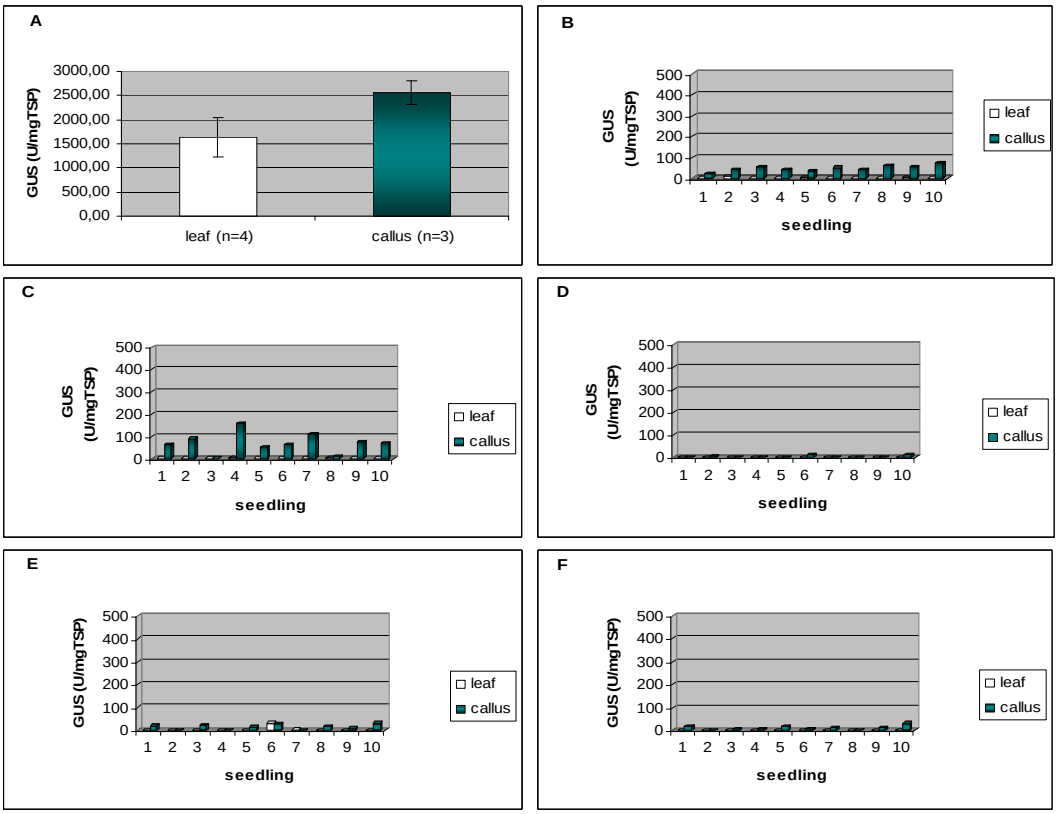
In line FK24/3, GUS activity was high in both leaf (1643 ± 405U GUS mg protein^{-1}) (Fig.3.6 A; white box) and callus tissue (2557 ± 239U GUS mg protein^{-1}) (Fig.3.6A; green box). In contrast, GUS activity measured in the expanding leaves was low in all ten T2 progeny plants of eight of the nine *P35S::gus/hpUS* super-transformants (<25U GUS mg protein^{-1}; Fig.3.6B-G, I, J white boxes). In the *P35S::gus/hpUS* super-transformant 60, half of the T2 plants had low GUS activity (<10U GUS mg protein^{-1}), but the other half showed intermediate GUS activity in the leaves, up to 80U GUS mg protein^{-1} (Fig.3.6H, white box). From this we can conclude that the pHhpUS construct induced an efficient suppression of the *gus* gene in expanding leaves, as previously shown by histochemical staining.

For three of the nine *P35S::gus/hpUS* super-transformants (52, 55 and 57), GUS activity in the callus tissue induced from the leaves of the same ten T2 plants, remained low (<25U GUS mg protein^{-1}; Fig.3.6D-F; green boxes). In three other super-transformants (28, 51, and 61), GUS activity varied among the calli induced on the leaves of the different seedlings, showing low to intermediate GUS activity (<150U GUS mg protein^{-1}; Fig.3.6B, C, I; green boxes).

Finally, in the remaining three *P35S::gus/hpUS* super-transformants (59, 60 and 63), GUS activities were ranging from intermediate to high. For instance, in all analysed calli of super-transformant 59, GUS activity levels varied between 80 and 220U GUS mg protein^{-1} (Fig.3.6 G; green box). In seven induced calli of super-transformant 60, and six calli of super-

transformant 63, GUS activity levels were between 60 and 380U GUS mg protein^{-1}. For the remaining three calli of super-transformant 60, and the remaining four of the super-transformant 63, no detectable to intermediate GUS activity was observed (Fig.3.6H, J; green boxes). These results indicate a strong hpRNA-mediated GUS suppression in the proliferating callus cells in the progeny of three of the nine *P35S::gus/hpUS* super-transformants. In the other double transformants, GUS activity varied among callus tissues, with some showing low and others showing an intermediate GUS suppression. However, these intermediate GUS activities that were measured in some of the callus tissues are still representing a reduction of 7- to 17-fold when compared with the GUS activity measured in the callus cells of the *P35S::gus* FK24/3 line.

The observed variations in the GUS suppression among different *P35S::gus/hpUS* super-transformants could be explained by different T-DNA locus numbers and / or locus structure. A segregation analysis of the T2 progenies of *P35S::gus/hpUS* double transformants revealed the presence of the hpUS T-DNA(s) at one locus in *P35S::gus/hpUS*/28, 51, 52 and 59, and at multiple loci in super-transformants 55, 60, 61 and 63. Based on the single digest of genomic DNAs of *P35S::gus/hpUS* double transformants, the DNA gel blot analysis suggested multiple copy hpUS T-DNA insertions in *P35S::gus/hpUS* super-transformant 28, 52, 57 and 61 (data not shown). Therefore, most likely, the structure of the integrated hairpin T-DNAs at one or multiple loci in different transformants and/or positional effects of particular hpUS transgene events caused the observed variations.



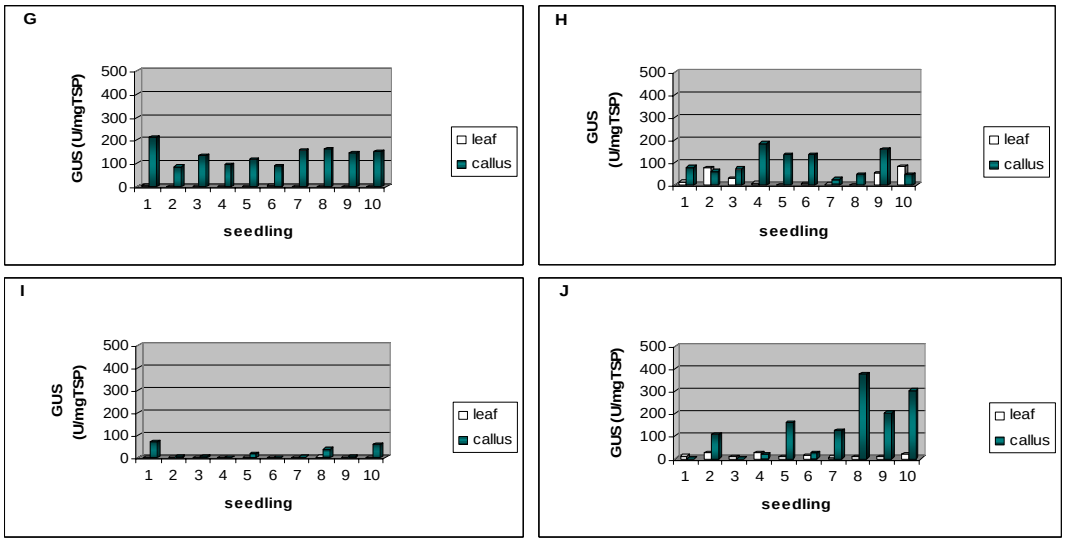


Fig. 3.6 GUS expression analysis of hpUS mediated GUS suppression in leaves and calli of *P35S::gus/hpUS* super-transformants. GUS expression analysis in the leaves and calli of the FK24/3 line (A) and 10 T2 plants of *P35S::gus/hpUS* double transformants 28 (B), 51 (C), 52 (D), 55 (E), 57 (F), 59 (G), 60 (H), 61 (I), 63 (J). GUS expression was measured in the leaves of 4-week old seedlings and 4-week old calli. The GUS activity levels are expressed as units GUS per milligram of total soluble protein (UGUS mg protein^{-1}). The values of 25 units for low and 150 units for high expression were chosen as described in De Buck *et al*. (2004).

3.4 Discussion

Constructs designed to produce double-stranded or self-complementary hpRNA transcripts are an efficient way of inducing targeted gene silencing (Waterhouse *et al*., 1998; Chuang and Meyerowitz, 2000; Smith *et al*., 2000; Levin *et al*., 2000; Wesley *et al*., 2001; Stoutjesdijk *et al*., 2002). In this study we investigated the efficiency of such a hairpin construct to target the *gus* gene expressed in different tissues of *Arabidopsis thaliana*. The hairpin construct to silence a *gus* gene contains the last 792 nucleotides of the *gus* coding sequence (hpUS) and was generated from the pH7GWIWG2 vector, designed for cosuppression of plant endogenes (Karimi *et al*., 2002).

When the 35S driven *gus* gene was used as a target, all hpUS transformants showed silencing. This high proportion of silenced transformants with an intron-containing hairpin construct might be explained by the ability of the construct to produce steady-state levels of duplex RNA in excess of the threshold levels considered necessary to activate PTGS in plants. The process of intron splicing of the ihpRNA could be responsible for more efficient duplex formation through alignment of the complementary arms (Waterhouse *et al*., 2001; Wesley *et*

al., 2001; Stoutjesdijk *et al*., 2002). However, evaluation of the *gus* suppression in the progeny plants of these hpUS double transformants revealed variations in the efficiency among different double transformants. Recently, similar observations were made by Hilson *et al*. (2004). They reported three examples in which the 35S promoter driven hairpin construct produced phenotypic series in T1 *Arabidopsis* lines, that might result from different T-DNA copy numbers or positional effects. It has been shown that multiple copy hpRNA transformants show much variation in the level of reduction of the target RNA, probably because multiple copy lines are subject to some degree of transcriptional silencing of the hpRNA transgene, which can reduce its effectiveness (Kerschen *et al*., 2004). If so, this might be an explanation for the variations in GUS activity reduction in independent transformants, because multi copy hpUS T-DNA insertions were found in most of them (data not shown). In addition, we showed that the efficiency of *gus* suppression also varied between different tissues in which the *gus* gene was expressed. The 35S driven hairpin construct was less effective at inducing silencing of a *gus* gene in meristematic tissues. However, the *P35S::gus/hpUS* super-transformants in which a strong GUS suppression was observed in the expanding tissues, showed a more efficient suppression in the proliferating root and callus tissues. Whereas all *P35S::gus/hpUS* double transformants showed undetectable to very low GUS activity in the expanding leaves, the GUS activity in the callus tissues remained low in six of the nine hpUS transformants. In others, the hpUS-mediated GUS suppression in the leaves was somewhat released in proliferating callus cells. Indeed, instead of 100-fold reduction, some callus tissues showed only a reduction of 7- to 17-fold when compared with the GUS activity measured in the callus cells of the FK24/3 line. This could mean that the silencing machinery is less active in these tissues and more sensitive to the amount of dsRNA produced. The release of PTGS in proliferating cells was previously observed by Mitsuhara *et al*. (2002). Calli were induced from leaf pieces of two transgenic tobacco plants, which contained a sense transgene for overproduction of *luciferase* (*luc*) but exhibited PTGS of *luc*. The induced calli exhibited strong *luciferase* activity similar to nonsilenced leaves. They proposed that proliferating cells might be protected against PTGS, as some elements indispensable for PTGS might be downregulated in these cells. However, we showed that the hpRNA construct is able to induce silencing in the proliferating root and callus tissues. Efficient silencing in tissues containing high proliferating cells may require a more efficient silencing locus such as a hpRNA locus, which is capable to produce a high amount of dsRNA. An intron-containing hpRNA construct not only gave an increased proportion of silenced transformants, but also resulted in the most profound levels of silencing (Wesley et al., 2001).

A correlation between the abundance of siRNAs and the severity of silencing was also found in plants in which RNA silencing was triggered by the expression of siRNAs (Lu *et al*., 2004). This is further supported by the observation that an hpRNA construct driven by the nopaline synthase promoter was less effective than those driven by a stronger promoter, such as the P35S (Chuang and Meyerowitz, 2000). Another explanation for the lack of complete silencing in reproductive and meristematic cells such as root tips and callus is that in these cells the balance between translation and hpRNA-mediated degradation of the *gus* mRNA is shifted towards expression. This could be, for instance, due to a more active recruitment of the mRNAs into the ribosomes.

This analysis demonstrates that a hairpin construct produces transformants with varying degree of silencing, which may be a useful feature for functional analysis of genes required for basic cell function or development. In this case, weakly suppressed lines could give viable plants with phenotypes indicative of the role of the target gene.

When the pHhpUS construct was targeted against the *gus* gene driven by the CycB1;1, CycD4;1 and KRP4 promoters, which activities are restricted to tissues in which an active cell division occurs (Ferreira *et al*., 1994; Burssens *et al*., 2000; De Veylder *et al*., 1999), only a limited effect of the hpUS-mediated GUS suppression was observed. To further test the ability of the 35S driven hairpin construct to induce sequence-specific degradation of the complementary *gus* mRNAs, the WRKY23 driven *gus* gene was targeted. Here, an efficient GUS suppression was observed in the expanding root tissues, but again, tissues containing highly proliferating cells such as root tip and young leaves and flowers showed less profound hpUS-mediated suppression. Although root tips with no detectable GUS activity were detected in some *P35S::gus/hpUS*, *CycD4;1::gus/hpUS*, *KRP4::gus/hpUS* and *WRKY23::gus/hpUS* super-transformants, it seems that root tips are less susceptible to PTGS effect. An explanation for the less efficient hpUS-mediated suppression of the *gus* gene in the meristems could be a differential activity of the 35S promoter used to drive the expression of the hpUS construct as compared to the tissue-specific expression patterns of the CycB1;1, CycD4;1, KRP4, and WRKY23 promoters in these tissues. However, it is possible that the level of reduction obtained with a 35S driven hairpin construct would be informative for some genes as some phenotypes could be less sensitive to the level of gene activity. Also, as efficient silencing of the 35S driven *gus* gene in the meristematic tissues was observed in only a limited number of hpUS super-transformants, screening of more independent transformants might be needed to see an hpRNA effect in the tissues containing highly proliferating cells.

In summary, we showed that a 35S driven hairpin construct produces a series of independent *Arabidopsis* transformants showing a different degree of silencing. In the weakly suppressed hpUS transformants, the silencing of a 35S driven *gus* gene was not uniform within tissues in which the hpUS is expressed. Whereas efficient suppression of the *gus* gene occurred in the expanding leaf and root tissues, a 35S driven hairpin silencing was less efficient in the tissues containing highly proliferating cells**.** The fact that the variations in efficiency of *gus* suppression in different hpUS transformants were mostly observed in the meristems could mean that the meristematic tissues are more sensitive to the level of hpRNA expression. However, we obtained hpUS transformants whose progeny plants showed dramatically reduced levels of a *gus* target gene in all tissues, but only when both the hpRNA and the *gus* target gene were regulated by the 35S promoter.

3.5 Materials and methods

Plasmid construction

We have used the pH7GWIWG2 vector which was developed to convert a polymerase chain reaction product into a dsRNA structure that includes an intron by using an in vitro recombinase system (Karimi *et al*., 2002). The 792 bp fragment of the 3' end of the coding sequence of the *gus* gene from an entry clone was recombined into the destination vector pH7GWIWG2 to produce an expression clone named pHhpUS.

A 792 bp fragment of the 3' end of the GUS coding region was amplified by PCR using the pK2L610 plasmid (De Buck *et al*., 2000) as template and 2 sets of primers to form *att*B1 and *att*B2 recombination sites. In the first step, template specific primers containing 12 bases of *att*B sites were used in 30 cycles of PCR to amplify the target sequence. PCR conditions were as follows: initial denaturation at 94^0C for 5 min, followed by 30 cycles of: denaturation at 94^0C for 1 min, annealing at 55^0C for 1 min and elongation at 68^0C for 1.30 min; and a final elongation step at 68^0C for 15 min. This product was subsequently used as a template in the second PCR reaction with universal *att*B adapter primers to amplify the full *att*B1 and *att*B2 recombination sites (Invitrogen) following the protocol described in the instruction manual. Primers used were: forward template specific primer 5'aaaaagcaggcttgctggactgggcagatgaa 3', reverse template specific primer

5'agaaagctgggtttgcctccctgctgcggttt 3', attB1 adapter primer 5' ggggacaagtttgtacaaaaaagcaggct 3, attB2 adapter primer 5'ggggaccactttgtacaagaaagctgggt 3'.

To obtain the 3'CS-GUS entry clone, a PCR product flanked by *att*B sites was recombined into the pDONR 201- Km^r vector containing *att*P1 and *att*P2 recombination sites in the BP reaction using BP Clonase enzyme (Invitrogen).

The expression vector pHhpUS was generated in the LR reaction where 3'CS-GUS entry clone was incubated with the pH7GWIWG2(I) destination vector (Karimi *et al*., 2002) in the presence of LR Clonase enzyme (Invitrogen). The orientation of the intron after double LR reaction was determined by restriction digest.

Plant material - overview of the target lines

For the pHhpUS transformation the following transgenic *Arabidopsis* lines were used: a transgenic *Arabidopsis thaliana* C24 line carrying a *CycB1;1::gus* transgene with a kanamycin selectable marker at a single locus in homozygous conditions (Ferreira *et al*.,1994); a transgenic *Arabidopsis thaliana* C24 line carrying a *CycD4;1::gus* transgene with a kanamycin selectable marker at a single locus in homozygous conditions (De Veylder *et al*., 1999); a transgenic *Arabidopsis thaliana* C24 line carrying a *KRP4::gus* transgene with a kanamycin selectable marker at a single locus in homozygous conditions (Ph.D Caroline Richard, 2001-2002); a segregating seed stock of *Arabidopsis thaliana* C24 transgenic for *WRKY23::gus* with a kanamycin selectable marker (Ph.D. Mansour Karimi,1998-1999); a transgenic *Arabidopsis thaliana* ColO line carrying a *P35S::gus* transgene with a kanamycin selectable marker at a single locus in a homozygous conditions (FK24/3 line; De Buck *et al*., 2004).

***Agrobacterium* strain, plant transformation and selection and callus induction**

The transformation vector pHhpUS was transformed into the *Agrobacteriom tumefaciens* strain $C58C1Rif^R$, containing pMP90 (Koncz, C. and Schell, J. 1986).

Transformation of the above listed *Arabidopsis* lines was performed by the floral dip method (Clough and Bent, 1998). Seeds of the dipped plants were harvested and sown on K1 medium supplemented with hygromycin (20mg/l) for selection of *CycB1;1::gus/hpUS*,

CycD4;1::gus/hpUS, *KRP4::gus/hpUS*, *WRKY23::gus/hpUS* and *P35S::gus/hpUS* primary transformants. Selected primary transformants were self-fertilized and T2 seeds were collected. All plants were grown under a 16 h light / 8 h dark regime at 21^0C.
Callus was induced by placing leaf pieces on medium containing 1X Murashige and Skoog salts, 1X MS vitamin mixture, 3% sucrose, 0.5 g/l MES, 0.1 mg/l naphthaleneacetic acid, 1mg/l benzylaminopurine and 0.7% agar (De Neve *et al*., 1997).

Histochemical β-glucuronidase assay

Four-week old seedlings were fixed in 90% cold acetone for 30 minutes with continuous shaking. The seedlings were washed three times with 0.1M Na_2HPO_4/NaH_2PO_4 buffer (pH7) and incubated overnight at 37^0C in 0.1M Na_2HPO_4/NaH_2PO_4 buffer (pH7) containing 10mM EDTA, 0.5mM K3[$Fe(CN)_6$)], 0.5mM $K_4[Fe(CN)_6)]$ and 1% DMSO containing 50mg/ml 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid (X-gluc). After that, the seedlings were washed with 0.1M Na_2HPO_4/NaH_2PO_4 buffer (pH7), destained in 90% ethanol and stored in 70% ethanol. Photographs were taken using a digital camera (AxioCam HRc, Zeiss) connected to a Zeiss Stemi SV11 microscope.

Preparation of protein extracts and determination of the β-glucuronidase levels

Grinded leaf (4 week old plants) and callus material (3 week old) was resuspended in 100µl buffer containing 50mM phosphate buffer (pH7), 10mM β-mercapthoethanol, 10mM Na_2-EDTA and 0.1% Triton X-100, centrifuged twice at 4^0C for 10 min to remove insoluble material. The total amount of soluble protein in the protein extracts was determined with the Bio-Rad Protein Assay (Bradford, 1976) using bovine serum albumin as a standard. The GUS activity was determined as described by Breyne *et al*, 1993. GUS activity levels were expressed as units GUS protein relative to the total amount of soluble extracted protein (UGUS mg $protein^{-1}$).

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CHAPTER 4

Heat-regulated GFP-GUS expression by the Gmhsp 17.6L soybean heat-shock promoter in *Arabidopsis thaliana*

Heat-regulated GFP-GUS expression by the Gmhsp 17.6L soybean heat-shock promoter in *Arabidopsis thaliana*

Gordana Marjanac, Sylvie De Buck and Anna Depicker

4.1 Abstract

The ideal inducible system should not have detectable expression in uninduced state. However, upon induction, the level of expression should be comparable to the levels achieved by a strong constitutive promoter such as the CaMV 35S. In this study, we have analysed the expression profile of the Gmhsp 17.6L soybean heat-shock promoter (Severin and Schöffl, 1990) in *Arabidopsis thaliana*. For this analysis, three plant transformation vectors were constructed, which are all identical except for the promoter sequences used to drive the *gfp-gus* fusion gene. The basal level, organ specificity and the strength of the Gmhsp 17.6L soybean heat-shock promoter were determined and compared with the strength of the commonly used P35S and Pnos promoters.

4.2 Introduction

The ability to control the expression of a gene via a highly specific mechanism, offers unique opportunities to study the physiological functions of certain gene products at different stages during development. In addition, a stringently regulated promoter is absolutely required if the expression of a gene product of interest interferes with the regeneration process. Ideally, an inducible promoter should show extremely low or no basal levels of expression in the absence of an inducer. In contrast, upon induction, which should not alter the physiology of the plant, a high level of expression should be achieved.

In most organisms, including plants, temporal control of transgene expression is commonly achieved through the use of an inducible promoter that specifically expresses a transgene in response to an exogenous inducer (reviewed by Gatz and Lenk, 1998; Zuo and Chua, 2000; Tang *et al*., 2004).

The heat stress of plants causes the heat shock response, a biological phenomenon, which is conserved among virtually all organisms. In plants, an increase of the temperature leads to the expression of a set of different heat-shock proteins, which are probably involved in processes protecting the cell from detrimental effects of heat stress. Additionally, expression of the heat-shock proteins in the absence of environmental stress is restricted to certain stages of development (Prandl and Schoffl, 1996). The induction of heat-shock protein synthesis is rapid after temperature elevation and declines shortly after return to normal temperature (Schöffl *et al*., 1986). In all eukaryotes, heat-shock gene promoters contain conserved sequence elements (HSE), which have been shown to interact with a regulatory protein, the heat shock factor (HSF), in yeast (Jakobsen and Pelham, 1988), *Drosophila* (Zimarino and Wu, 1987), and human cells (Goldenberg *et al*., 1988). Due to conservation of the *cis*- and *trans*-acting components, heat-shock promoters of plants are recognized in heterologous species (reviewed by Schöffl *et al*., 1990) and can be used for the regulated expression of chimeric genes (Schöffl *et al*., 1989). It has been previously shown that the promoter of the soybean heat shock gene Gmhsp17.6L conferred heat regulated antibiotic resistance in transgenic tobacco plants (Severin end Schöffl, 1990). Also in *Arabidopsis*, this promoter was highly induced in a temperature-dependent manner (Kilby *et al*., 2000).

Here, the expression profile of the Gmhsp 17.6L soybean heat-shock promoter (Severin and Schöffl, 1990) was analysed in transgenic *Arabidopsis* plants, using the green fluorescent protein *(gfp)* and β-glucuronidase (*gus*) marker genes. These experiments were carried out to

test the inducibility and the organ specificity of the Gmhsp 17.6L soybean heat-shock promoter in *Arabidopsis thaliana*. The basal and induced expression level of the heat-shock promoter were investigated and compared with the activity of two constitutive promoters, the CaMV 35S and the nopaline synthase gene promoter (Pnos).

4.3 Results

4.3.1 Construction of T-DNA vectors and generation of transgenic *Arabidopsis* lines

In order to analyse the expression profile of the Gmhsp 17.6L soybean heat-shock promoter (Severin and Schöffl, 1990), the pKGWFS7 vector, designed for promoter analysis was used (Karimi *et al*., 2002; Fig.4.1a). In this vector, an in frame fusion between the regions coding for the green fluorescent protein (*gfp*) and the β-glucuronidase (*gus*) genes was cloned downstream of the GATEWAY^{TM} cassette. The *gfp-gus* fusion allows efficient monitoring and quantification of the transgene expression, regulated by sequences cloned upstream of this fusion. The GATEWAY^{TM} cassette present in the vector enables efficient cloning of different promoters into the pKGWFS7 vector. Three different vectors were made. The cauliflower mosaic virus promoter (CaMV) 35S, the nopaline synthase gene promoter and the soybean Gmhsp17.6L heat-shock promoters were cloned into the pKGWFS7 "Destination" vector by replacing the negative selectable marker flanked by attR1 and attR2 sites. The resulting "Expression" vectors, pKSFS7, pKNFS7 and pKHSFS7 contain the P35S, Pnos and heat-shock promoters, respectively (Fig.4.1b,c). The construction of these plant transformation vectors is described in more detail in Material and Methods.

The obtained plant transformation vectors were used for transformation of wild type *A. thaliana* plants ecotype Columbia O (ColO) by the floral dip method (Clough and Bent, 1998). Primary transformants (T1 generation) were selected on the Murashige and Skoog medium containing 50 mg/l kanamycin.

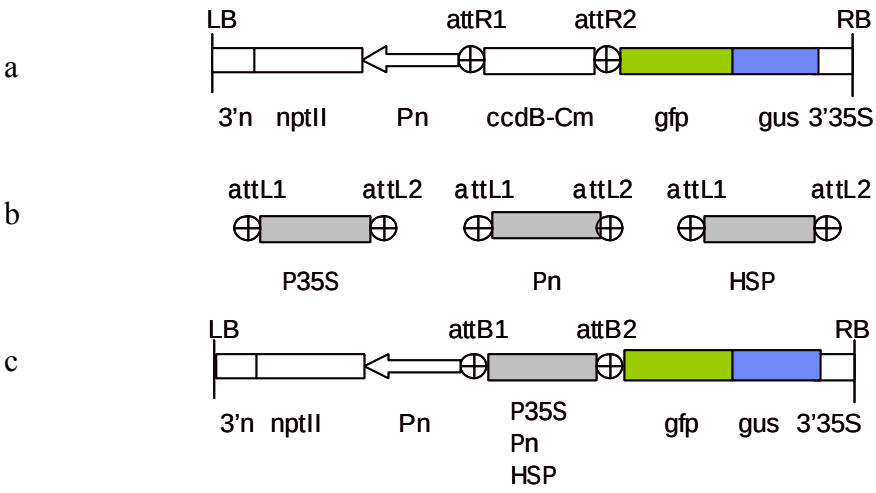

Fig. 4.1 Schematic representation of the T-DNA constructs for promoter analysis. (a) pKGWFS7 GATEWAY™ compatible construct for promoter analysis; (b) P35S, Pnos and HSP-entry clones; (c) pKSFS7, pKNFS7 and pKHSFS7 expression vectors, where S, N, and HS stands for P35S, Pnos and heat-shock promoter, respectively.
Abbreviations: 3'n, 3' end of the nopaline synthase gene; nptII, neomycin phosphotransferase II gene; Pn, promoter of the nopaline synthase gene; gfp, green fluorescent protein; gus, β-glucuronidase gene; 3'35S, 35S terminator**;** P35S, cauliflower mosaic virus promoter; HSP, Gmhsp 17.6L heat-shock responsive promoter; attR1, R2, L1, L2, B1, and B2 represent sequences required for specific recombination reactions; LB, left border; RB, right border.

4.3.2 GFP expression in T2 generation of *Arabidopsis thaliana* transformed with T-DNA vectors containing different promoters

To determine the background activity and the induction of the inducible heat-shock promoter, the GFP and GUS expression was analysed in the T2 progeny of five transgenic FKHSFS7 *Arabidopsis thaliana* transformants, before and after heat induction. The expression level and the organ specificity of the heat-shock promoter were compared with the expression profile of the 35S and Pnos promoters in T2 progeny plants of five FKSFS7 and five FKNFS7 transformants, respectively.

T2 seeds of five different transformants harboring the K35SFS7, KPnosFS7 and KHSFS7 T-DNAs, were germinated on medium containing kanamycin. The GFP expression was analysed in two weeks old plants. All seedlings of *P35S::gfp-gus* transformants FKSFS7/1 and FKSFS7/4 showed uniform green fluorescence in the roots, indicating GFP expression. Whereas progeny of the transformant FKSFS7/1 displayed a mixture of red/green fluorescence in the leaves, T2 seedlings of the transformant FKSFS7/4 mostly showed

uniform green fluorescence in the leaves, probably due to higher GFP expression levels in them (Fig.4.2a). All T2 plants of the remaining three FKSFS7 transformants showed uniform red fluorescence in both the root and leaf tissues that was caused by chlorophyll autofluorescence in the absence of GFP accumulation (Table 4.1A). In the T2 seedlings of four *Pnos::gfp-gus* transformants, GFP accumulation was observed only in the roots (Fig.4.2b), whereas leaves displayed a uniform red fluorescence. The progeny of the transformant FKNFS7/2 did not show a detectable GFP expression in both leaves and roots (Table 4.1A).

Table 4.1 (A) Overview of the GFP and GUS expression in the roots and leaves of five independent *P35S::gfp-gus* (FKSFS7) , *Pnos::gfp-gus* (FKNFS7) and *HSP::gfp-gus* (FKHSFS7) transformants ; (B) GFP and GUS expression in the roots and leaves of five *HSP::gfp-gus* (FKHSFS7) independent transformants after heat induction.

A

Transformant	GFP expression		GUS expression (by histochemical staining)	
P35S::gfp-gus	root	leaf	root	leaf
FKSFS7/1	+	+	+	+
FKSFS7/2	-	-	-	-
FKSFS7/3	-	-	-	-
FKSFS7/4	+	+	+	+
FKSFS7/5	-	-	-	-
Pnos::gfp-gus				
FKNFS7/1	+	-	+	+
FKNFS7/2	-	-	-	-
FKNFS7/3	+	-	+	+
FKNFS7/4	+	-	+	+
FKNFS7/5	+	-	+	+
HSP::gfp-gus				
FKHSFS7/1	-	-	-	-
FKHSFS7/2	-	-	-	+
FKHSFS7/3	-	-	-	-
FKHSFS7/4	-	-	-	+
FKHSFS7/5	-	-	-	+

B

Transformant	GFP expression		GUS expression (by histochemical staining)	
HSP::gfp-gus	root	leaf	root	leaf
FKHSFS7/1	-	-	-	-
FKHSFS7/2	+	-	+	+
FKHSFS7/3	+	-	+	+
FKHSFS7/4	+	-	+	+
FKHSFS7/5	+	-	+	+

Seedlings of five independent *HSP::gfp-gus* transformants did not show detectable GFP expression before heat induction (Fig.4.2c, Table 4.1A). Seedlings were subsequently heat-shocked in a microbiological incubator at 37^0C for four hours. Upon heat-induction, in the majority of the seedlings of four independent transformants (FKHSFS7/2, 3, 4, and 5), GFP expression was induced and green fluorescence was observed in the roots within 4 hours of induction (Table 4.1B). In all cases GFP activity in the root cells continued to increase for the next 3 days, while leaves continued to show red fluorescence caused by chlorophyll autofluorescence in the absence of GFP accumulation (Fig.4.2d). The progeny of transformant FKHSFS7/1 did not show detectable GFP expression, neither in the leaves nor in the root tissues (Table 4.1A,B).

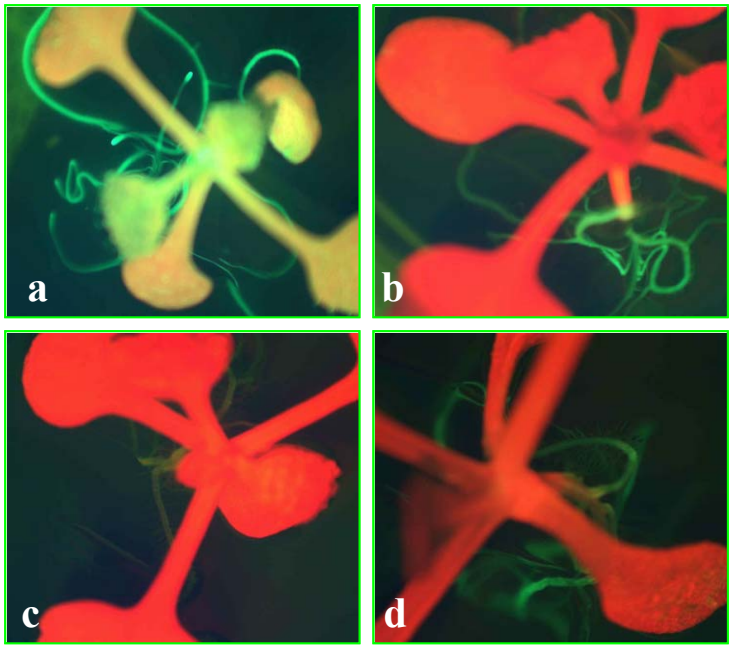

Fig.4.2 GFP expression in the T2 progeny plants of *P35S::gfp-gus* (FKSFS7), *Pnos::gfp-gus* (FKNFS7) *and HSP::gfp-gus* (FKHSFS7) transformants. (a) Representative T2 plant of FKSFS7/4 line showing uniform green fluorescence in the roots and leaves; (b) representative T2 plant of FKNFS7/1 line showing green fluorescence in the roots and red fluorescence in the leaves; (c) representative T2 plant of FKHSFS7/2 line showing red fluorescence in uninduced conditions and (d) green fluorescence in the roots after heat treatment.

4.3.3 GUS activity in the T2 generation of *Arabidopsis thaliana* plants transformed with different promoter *gfp-gus* fusions

In order to determine the GUS activity patterns in transgenic *A. thaliana* plants transformed with T-DNA vectors comprising different promoters, the T2 seedlings that were used for GFP expression analysis were subsequently stained for GUS activity. Incubation of seedlings in a GUS assay buffer for two hours allowed the visualization of GUS activity only in the *P35S::gfp-gus* transformants, indicating strong GUS expression in them. To allow the detection of GUS activity expressed by the weaker Pnos and inducible heat-shock promoter, the seedlings were incubated in the GUS assay buffer for 16 hours. The roots always stained first, even in the *Pnos::gfp-gus* and *HSP::gfp-gus* transformants. Furthermore, the GUS expression could be detected in the leaves of plants in which GFP was detected only in the roots, probably due to high sensitivity of the staining method.

In accordance with the GFP analysis, the progeny of *P35S::gfp-gus* transformants FKSFS7/1 and FKSFS7/4 showed a uniform GUS activity throughout the seedlings (Fig.4.3a), whereas in the remaining three transformants, in which no GFP was detected, also no GUS activity was observed (Table 4.1A).

In four *Pnos::gfp-gus* transgenic lines, GUS activity in the roots was uniform, while the leaves showed a patchy distribution of GUS activity (Fig.4.3b). The progeny of the *Pnos::gfp-gus* transformant FKNFS7/2 did not show detectable GUS activity in both the leaves and roots (Table 4.1A).

Five T2 plants of the five independent *HSP::gfp-gus* transformants, which did not show detectable GFP expression before heat-induction, were stained for GUS activity. Among them, progeny plants of FKHSFS7/1 and FKHSFS7/3 did not show detectable GUS activity in uninduced conditions (Fig.4.3d1), whereas the progeny of the remaining three transgenic lines displayed very low background activity in some leaves (FKHSFS7/2, 4 and 5; Fig.4.3c1,

Table 4.1A). There was no detectable background activity in the roots (Fig.4.3c1, d1). Two days after the heat-shock was applied, another five seedlings were stained for GUS activity. Upon heat induction, uniform GUS activity was observed throughout the roots and patchy to uniform GUS activity in the leaves in the progeny of four independent transformants (Fig.4.3c2, d2). The progeny of the transformant FKHSFS7/1 did not show detectable GUS activity in induced conditions (Table 4.1B).

Fig.4.3 Histochemical analysis of *P35S::gfp-gus, Pnos::gfp-gus and HSP::gfp-gus* transformants. (a) representative T2 plant of *P35S::gfp-gus* transformant FKSFS7/4; (b) representative T2 plant of *Pnos::gfp-gus* transformant FKNFS7/1; (c1, c2) representative T2 plants of the *HSP::gfp-gus* transformant FKHSFS7/2, showing some background activity of the heat-shock promoter in uninduced condition (c1) and uniform GUS activity after heat shock (c2); (d1, d2) representative T2 plants of the *HSP::gfp-gus* transformant FKHSFS7/3, which did not show background activity of the heat-shock promoter in uninduced condition (d1) and GUS activity observed after heat shock (d2).

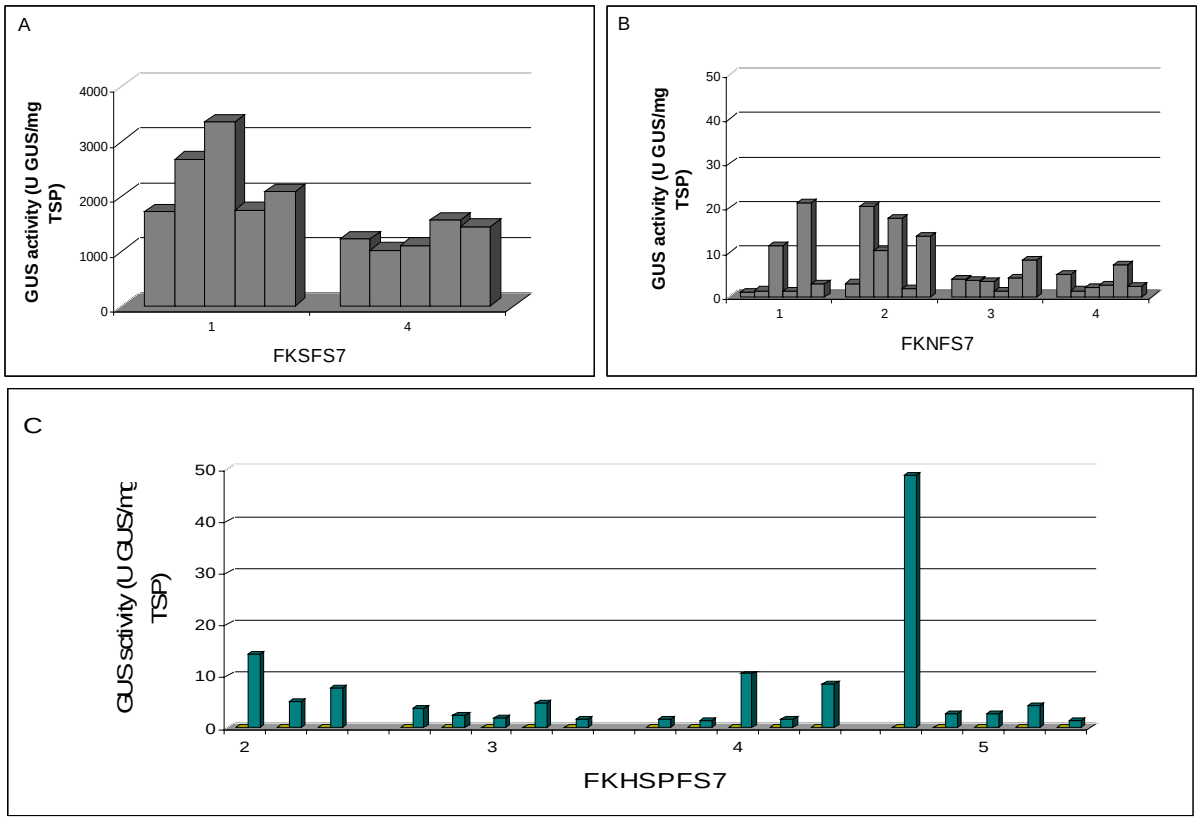

Fig 4.4 GUS expression analysis of *P35S::gfp-gus, Pnos::gfp-gus,* and *HSP::gfp-gus* transformants. (A) *P35S::gfp-gus* transformants FKSFS7/1 and FKSFS7/4; the *P35S::gfp-gus* transformants FKSFS7/2, 3, and 5 did not show detectable GUS expression and are not shown in the Figure; (B) *Pnos::gfp-gus* transformants FKNFS7/1, 3, 4, and 5; the *Pnos::gfp-gus* transformant FKNFS7/2 did not show detectable GUS expression and in not shown in the Figure; C, *HSP::gfp-gus* transformants FKHSFS7/2, 3, 4, and 5, before (grey blocks) and after heat-shock (green blocks); The *HSP::gfp-gus* transformant FKHSFS7/1 with undetectable GUS activity is not shown in the Figure. GUS expression was measured in the extracts prepared from 4-5 leaves of five 4-week-old T2 plants of five independent transformants per construct. The GUS activity levels are expressed as units GUS per milligram of total soluble protein (UGUS mg protein^{-1}).

4.3.4 GUS expression analysis in the T2 generation of *Arabidopsis thaliana* plants transformed with T-DNA vectors containing different promoters

To compare the strength of the soybean Gmhsp17.6L heat-shock promoter with the CaMV 35S and nopaline synthase gene (Pnos) constitutive promoters, quantitative fluorimetric GUS assays were performed on T2 progeny plants of the five *P35S::gfp-gus*, *Pnos::gfp-gus* and *HSP::gfp-gus* transformants previously analysed for GFP fluorescence and by histochemical staining for GUS activity. T2 seeds were germinated on a medium containing kanamycin and the GUS activity was measured in the extracts prepared from a mixture of 4-5 leaves of five four-week old plants per transformant. The results of these assays are shown in Figure 4.4. The *P35S::gfp-gus* lines FKSFS7/1 and FKSFS7/4 showed high GUS activity, ranging from 1000 to 3000 U GUS mg protein-1 (Fig.4.4A). The remaining three *P35S::gfp-gus* lines did not show detectable GUS activity (data not shown). The majority of the T2 *Pnos::gfp-gus* progeny plants showed GUS activity of about 2 to 10 UGUS mg protein-1 (Fig.4.4B). The maximal GUS activity measured in some T2 plants of the *Pnos::gfp-gus* transformants FKNFS7/1 and FKNFS7/3 was 20U GUS mg protein-1 (Fig.4.4B). In the *Pnos::gfp-gus* FKNFS7/2 T2 plants, no detectable GUS activity was observed in the plants analysed.

To analyse the basal activity and the strength of the Gmhsp17.6L heat-shock promoter, GUS expression was measured in the extracts prepared from leaves of five T2 plants of the five *HSP::gfp-gus* transformants, taken before and after the heat-shock (37^0/4h). None of the T2 plants of the five *HSP::gfp-gus* transformants had detectable GUS activity in the leaves before induction. Two days after the heat-shock, all plants of the four *HSP::gfp-gus* transformants FKHSFS7/2, 3, 4 and 5, contained between 2 and 10 U GUS mg protein-1. In one T2 plant of the *HSP::gfp-gus* transformant 5, a 50 units GUS mg protein-1 was detected

(Fig.4.4C). However, no detectable GUS activity was observed in the progeny of the transformant FKHSFS7/1 neither without nor with induction (Table 4.1A, B). In conclusion, the GUS activity level achieved by a strong 35S promoter was about 50 to 150-fold and 100 to 300-fold higher than the GUS activity measured in the *Pnos::gus-gfp* and *HSP::gus-gfp* transformants, respectively.

4.4 Discussion

The potential of the soybean Gmhsp17.6L heat-shock promoter (Severin and Schöffl, 1990) to tightly regulate gene expression in *Arabidopsis* plants was evaluated by analysing the expression of the *gfp-gus* fusion gene under non-induced and induced conditions. Also the strength of this heat shock promoter upon induction was compared with the strength of the commonly used 35S and Pnos promoters. For this analysis, the three promoters were fused to the *gfp-gus* reporter gene in the GATEWAY^{TM} compatible pKGWFS7 vector, constructed for promoter analysis (Karimi *et al*., 2002). Thus, the obtained plant transformation vectors were all identical except for the promoter sequences used to drive the *gfp-gus* fusion gene.

Firstly, the induction and the organ specificity of the soybean Gmhsp17.6L heat-shock promoter were evaluated by analysing the GFP expression in transgenic plants. The 35S-driven GFP expression was detected in both the root and leaf tissues. By contrast, in the *Pnos::gfp-gus* and *HSP::gfp-gus* transformants, GFP expression was detected only in the roots. Staining of the whole seedlings for GUS activity allowed a better analysis of the expression profile of the soybean Gmhsp17.6L heat-shock promoter. Also, GUS expression could be detected in the leaves of plants in which GFP was detected only in the root cells, probably due to high sensitivity of this method. On the whole plant level, the soybean Gmhsp17.6L heat-shock promoter showed a low background expression in the leaves of some but not in all *HSP::gfp-gus* transformants in non-induced conditions (Fig.4.3c1, Table 4.1A). This analysis showed that upon heat-induction, the heat-shock promoter confers a uniform GUS expression in the roots and a patchy to uniform distribution of GUS activity in the leaves (Fig.4.3c, d). This observed staining pattern was comparable with the staining pattern of plants containing the *Pnos::gfp-gus* transgene. By contrast, the staining of plants carrying the *P35S::gfp-gus* construct showed a much stronger and uniform expression in all plant tissues.

Secondly, the strength of the soybean Gmhsp17.6L heat-shock promoter was compared with the two constitutive promoters, P35S and Pnos. The 35S promoter yielded the highest GUS

activity, ranging from 1000 to 3000 U GUSmg protein^{-1} in average, similar to many other reports (Butaye *et al*., 2004; De Buck *et al*., 2004). Although the Pnos promoter is used frequently, not many reports have been published on the analysis of promoter strength in plants. It has been reported for transgenic petunia plants that the 35S promoter yields 30-fold higher transgene expression levels in comparison to Pnos (Sanders *et al*., 1987). However, in the work presented here, the GUS activity achieved by the 35S promoter was about 50 to 150-fold higher than the GUS activity measured in the *Pnos::gfp-gus* transformants. The induced levels reached by the heat-shock promoter were similar to those with the Pnos promoter and 100 to 300-fold lower when compared with the GUS activity levels achieved by the 35S promoter.

The experiments described here show that the heat-shock inducible transcription is based on a conserved signalling system in soybean and *Arabidopsis*. The signal sequences in the promoter of the soybean Gmhsp17.6L heat-shock gene are correctly recognized in *Arabidopsis*, by *trans*-acting factors. The optimal temperature for the induction of transcription of heat-shock protein genes varies among species. In *Arabidopsis*, the expression of the heat-shock protein genes obtains maximal levels at temperature below 40^{0}C (Takahashi and Komeda, 1989), while optimal temperature for the induction of soybean heat-shock promoter driven transgene expression was reported to be above 40^{0}C (Severin and Schöffl, 1990; Kilby *et al*., 2000). However, in our experiments, the heat-shock treatment of the *Arabidopsis* plants containing the *HSP::gfp-gus* transgene was carried out at 37^{0}C, since incubation at 40 and 42^{0}C caused lethality of the plants.

We conclude from the experiments that the induction of the soybean heat-shock promoter can be used to obtain heat-regulated transcription in *Arabidopsis*, that there was very little background expression in uninduced conditions and that the induced transgene expression levels are similar to those of the Pnos promoter.

4.5 Materials and methods

Plasmid construction

The pKGWFS7 vector has been described by Karimi et al. (2002) and is ideally suited for promoter analysis. In this vector a in-frame fusion between the regions coding for green fluorescent protein (*gfp*) and β-glucuronidase (*gus*) was cloned downstream of the

GATEWAY™ cassette (Karimi *et al*., 2002). The pKGWFS7 vector is shown in Figure 4.1a. The P35S, Pnos end HSP-entry clones are shown in Figure 4.1b. The P35S sequence (800bp) was amplified by PCR using pXD610 (De Loose *et al*., 1995) as a template. Similarly, the Pnos sequence (280bp) was amplified by PCR using pK2L610 plasmid (De Buck *et al*., 2000), and the Gmhsp 17.6L soybean heat-shock responsive promoter (HSP, 390bp) was amplified by PCR from pNJK18 plasmid (Kilby *et al*., 1995). To form attB1 and attB2 recombination sites, two sets of primers were used. In the first PCR reaction, the template specific primers containing 12 bases of the attB sites were used in 30 cycles to amplify the target sequence. This PCR product was subsequently used as a template in the second PCR reaction with universal attB adapter primers to amplify the full attB1 and attB2 recombination sites (Invitrogen) following the protocol described in the instruction manual. Primers used were: forward P35S specific primer 5'aaaaagcaggctcctttgccccggagatcaa3', reverse P35S specific primer 5'agaaagctgggtatagtaaattgtaatgttgtttgt3', forward Pnos specific primer 5'aaaaagcaggctgatcatgagcggagaatt3', reverse Pnos specific primer 5'agaaagctgggtgagactctaattggat3', forward heat-shock specific primer 5'aaaaagcaggctgaattctgaaattgggtctt3', reverse heat-shock specific primer 5'agaaagctgggtttgaaagttgcaaaattgta3', universal attB1 adapter primer 5'ggggacaagtttgtacaaaaaagcaggct3', universal attB2 adapter primer 5'ggggaccactttgtacaagaaagctgggt3'.

To obtain the P35S-entry clone, Pnos-entry clone and HSP-entry clone (Fig.4.1b), the PCR products flanked by attB sites were recombined into the pDONR201-Km^r vector containing attP1 and attP2 recombination sites in the BP reaction using BP clonase enzyme (Invitrogen). The expression vectors, pKSFS7, pKNFS7 and pKHSFS7 (Fig.4.1c) were generated in the LR reaction where P35S, Pnos and HSP-entry clones were incubated with the pKGWFS7 destination vector in the presence of LR Clonase enzyme (Invitrogen).

Agrobacterium strain, plant transformation and selection

Transformation vectors pKSFS7, pKNFS7 and pKHSFS7 were introduced by electroporation into *Agrobacterium tumefaciens* strain C58C1Rif^R, containing the nopaline vir plasmid pMP90 (Koncz, C. and Schell, J. 1986).

Transformation of wild type *Arabidopsis thaliana* plants ecotype Columbia O (ColO) was performed by the floral dip method (Clough and Bent, 1998). Seeds of the dipped plants were

harvested and sown on K1 medium supplemented with kanamycin (50 mg/l) for selection of the FKSFS7, FKNFS7 and FKHSFS7 primary transformants. Selected primary transformants were self-fertilized and T2 seeds were collected. Plants were grown under a 16 h light / 8 h dark regime at 21^0C.

Heat-shock conditions

Sterilized *Arabidopsis* seeds, sown on medium containing kanamycin, were grown for two weeks under a 16 h light / 8 h dark regime at 21^0C. Heat-shock of two-week old seedlings in closed Petri dishes was performed in an incubator at 37^0C for four hours. After that, they were grown for two days under a 16 h light / 8 h dark regime at 21^0C. Plants were assayed two days after the heat-shock was applied.

Histochemical β-glucuronidase assay

Whole seedlings were fixed in 90% cold acetone for 30 minutes with continuous shaking. The seedlings were washed with 0.1M Na_2HPO_4/NaH_2PO_4 buffer (pH7) and incubated in 0.1M Na_2HPO_4/NaH_2PO_4 buffer (pH7) containing 10mM EDTA, 0.5mM K3[Fe(CN)$_6$)], 0.5mM K_4[Fe(CN)$_6$)] and 1% DMSO containing 50mg/ml 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid (X-glu) for 2 or 16 hours at 37^0C. After that, the seedlings were washed with 0.1M Na_2HPO_4/NaH_2PO_4 buffer (pH7), destained in 90% ethanol and stored in 70% ethanol. Photographs were taken using a digital camera (AxioCam HRc, Zeiss) connected to a Zeiss Stemi SV11 microscope.

Preparation of protein extracts and determination of the β-glucuronidase levels

Protein extracts were prepared from 4-5 rosette leaves from 4-week old plants, frozen in liquid nitrogen, by grinding in 100μl buffer containing 50mM phosphate buffer (pH7), 10mM β-mercapthoethanol, 10mM Na_2-EDTA and 0.1% Triton X-100. The extracts were centrifuged twice at 4^0C for 10 min to remove unsoluble material. The total amount of

soluble protein in the protein extracts was determined with the Bio-Rad Protein Assay (Bradford, 1976) using bovine serum albumin as a standard. The GUS activity was determined as described by Breyne et al. (1993). GUS activity levels were expressed as units GUS protein relative to the total amount of soluble extracted protein (UGUS mg protein^{-1}).

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CHAPTER 5

CRE-mediated gene excision in *Arabidopsis thaliana*

CRE-mediated gene excision in *Arabidopsis thaliana*

Gordana Marjanac, Sylvie De Buck and Anna Depicker

5.1 Abstract

The Cre/lox recombination system has been shown to function in plant cells (Dale and Ow, 1990; Russell *et al*., 1992; Odell *et al*.,1990). The ability of the CRE recombinase to catalyze the excision of any fragment of DNA flanked by directly repeated *lox* sites has been exploited to modify gene expression in transgenic plants (Odell *et al*.,1990; Guo *et al*., 2003; De Buck *et al*., 2001). In the present study, we evaluated the efficiency of CRE-mediated recombination, when CRE is controlled by the 35S promoter, the nopaline synthase gene promoter and the promoter of the soybean heat shock gene Gmhsp17.6L (Severin and Schöffl, 1990). The CRE activity was assessed by the excision efficiency of a *gus* gene between in tandem oriented *lox* sites in transgenic *Arabidopsis thaliana*. Efficient CRE-mediated excision of a *lox* flanked *gus* gene was only obtained in some transformants with the 35S regulated *cre* gene.

5.2 Introduction

Site-specific recombinases are becoming an increasingly important tool for manipulating DNA in higher eukaryotes. There are no known endogenous site-specific recombination systems in plants. In contrast, several recombination systems from bacteriophage and yeast have been well characterized (reviewed by Odell and Russell, 1994). An important feature of these recombination systems is that they are functional in a wide range of organisms. Site-specific recombination in plants has been reported for several of the simplest recombination systems. These are the Cre/*lox* system from bacteriophage P1 (Dale and Ow, 1990; Russell *et al*., 1992; Odell *et al*., 1990), the FLP-FRT system of *Saccharomyces cerevisiae* (Lyznik *et al*., 1993; Kilby *et al*., 1995), and the R-RS system of *Zygosaccharomyces rouxii* (Onouchi *et al*., 1995). The bacteriophage P1 Cre/*lox* site-specific recombination system is the most used recombination system in plants (reviewed by Gilbertson, 2003). The fact that the CRE recombinase has a high recombination efficiency in higher eukaryotes allows the development of this recombination system as a research tool for a wide range of studies and applications. In plants, the Cre/*lox* recombination system has been mostly used for targeted insertion and precise deletion of DNA from transgenic plant chromosomes. The ability of the CRE recombinase to catalyze the excision of any fragment of DNA flanked by directly repeated *lox* sites has been exploited to remove selectable marker genes from transgenic plants (Dale and Ow, 1991; Russell *et al*., 1992), and to reduce the copy number of DNA inserts in the genome (Srivastava *et al*., 1999). Also genes containing *lox* sites have been designed such that CRE-mediated excision or inversion of *lox*-flanked DNA sequences lead to their activation or inactivation. A *lox*-flanked coding region placed in inverted orientation with respect to the regulating promoter was expressed after CRE-mediated inversion (Dale and Ow, 1990). A *lox*-flanked fragment placed between a promoter and the gene to be expressed, blocking gene expression, was excised after CRE expression resulting in restoration of gene activity (Odell *et al*, 1990). De Buck *et al*. (2001) described a silenced transgenic locus, in which two transgene copies are arranged as an inverted repeat. CRE-mediated excision of one of the two copies resulted in a highly expressing single copy transgene locus. In the above described examples, the 35S regulated *cre* gene was introduced either by transformation of tissue containing the *lox*-flanked sequence or by genetic crossing of a *lox*-containing and *cre*-containing plants. A high degree of control over the timing of gene expression can be achieved by making use of inducible promoters to switch CRE on at the desired time point. Hoff *et al.* (2001) used a heat-shock-inducible CRE to excise the *bar* marker gene flanked by two *lox* sites, resulting in

constitutive GUS expression under the control of the CaMV 35S promoter. A similar system was developed and tested in *Nicotiana tabacum* bright yellow-2 (BY-2) cells (Joubès *et al*., 2004). In this study, a heat-inducible expression of the CRE recombinase was used to remove the *egfp* gene placed between two *lox* sites, bringing the gene of interest under the direct control of the CaMV 35S promoter. This strategy allows tight control of gene expression and therefore is useful to overexpress genes whose expression might block transgenic regeneration (Joubès *et al*., 2004).

Although variations in excision efficiency, which is dependent upon the specific *lox* and CRE transformants used as parents had been observed (Odell *et al*., 1990; Russell *et al*., 1992), a direct correlation between recombination efficiencies and the level of recombinase mRNA and protein has not been determined. The objective of the work presented in this chapter was to analyse the effectiveness of CRE-mediated recombination, after expression from different promoters. The CRE activity was assessed upon the excision efficiency of a *gus* gene between in tandem oriented *lox* sites in transgenic *Arabidopsis thaliana*. Two different methods of combining the *lox* flanked *gus* gene and the *P35S::cre* gene were evaluated, namely transformation and genetic cross.

5.3 Results

5.3.1 Strategy for CRE-mediated excision of GUS expression cassette

The experimental strategy used in this study is described in Figure 5.1. The presence or absence of the *gus* gene between two *lox* sites was used as a reporter system for CRE-mediated recombination. Therefore, the transgenic *Arabidopsis* plant lines CK_2L6 (Theuns *et al*., 2002; De Buck *et al*., 1998) and FK24/3 (De Buck *et al*., 2004) containing the K T-DNA were used. The K T-DNA contains a kanamycin resistance gene as selectable marker and an active GUS expression cassette flanked by two directly repeated *lox* sites (Fig.5.1a). These two *lox* sites provide the substrate for the CRE-mediated recombination, leading to the excision of the *gus* gene. Two different methods were used to combine the *lox*-flanked *gus* gene in the K T-DNA and the CRE recombinase expression cassette in the CRE T-DNA, transformation and genetic cross. The excision efficiency of the *gus* gene was then analysed in the progeny plants and demonstrated by histochemical staining, fluorimetric GUS assay and by PCR analysis. First, the histochemical staining assay allows to discriminate between the

plant cells in which the recombination event has occurred and plant cells in which it did not. The plants of the CK_2L6 and FK24/3 lines contain a constitutively expressed *P35S::gus* gene resulting in a uniform blue staining (Fig.5.3 and Fig.5.6). Upon CRE-mediated deletion the *gus* gene is lost and this can be visualised by the lack of blue staining in corresponding cells, sectors or plants.

Secondly, PCR analysis was performed to examine the excision status of the GUS expression cassette. For this analysis, primers amplifying a fragment that is either specific for the excised sequences or for the parental T-DNA were used (Fig.5.1a). The 3 / 4 primer pair is predicted to yield a PCR fragment of 4431 bp for a non-recombinant T-DNA (Fig.5.1a). By contrast, following CRE recombination and deletion of the GUS expression cassette, the 3 /4 primer pair should produce a PCR product of 834 bp (Fig. 5.1c). Since the 3 /4 primer set did not always produce a band of 4431 bp, probably due to its length, primer 5 was included to yield a K T-DNA specific fragment of 1200 bp. The PCR reaction was optimised so that the 3 primers together in one PCR reaction could produce two PCR products: a PCR product of 1200 bp indicating the presence of the non-recombinant T-DNA, and a PCR product of 834 bp, indicating the excision of the *gus* gene (Fig. 5.1a, c).

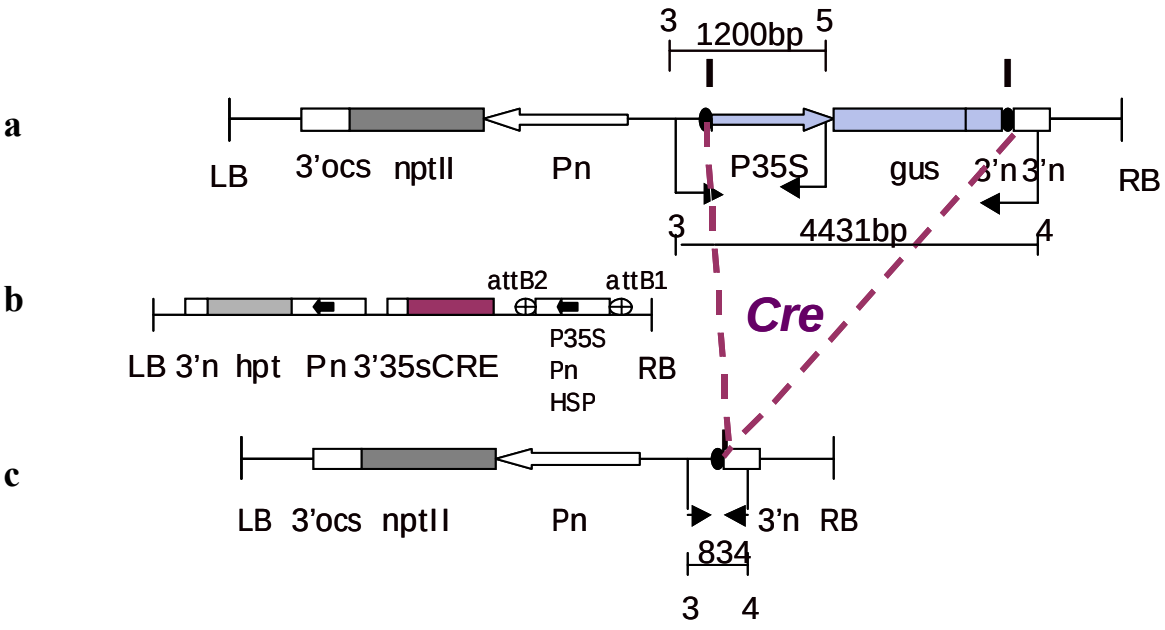


Fig. 5.1 Strategy for CRE-mediated excision of GUS expression cassette. a, Schematic representation of the K T-DNA in the CK_2L6 and FK24/3 lines; the K T-DNA consists of a kanamycin selectable marker and P35S-GUS-3'nos expression cassette; b, the CRE T-DNA consists of a hygromycin selectable marker and P35S, Pn or HSP–Cre-3'35S expression cassette; c, the recombined form of the K T-DNA where CRE-mediated recombination resulted in a deletion of the GUS expression cassette.
Abbreviations: 3'ocs, 3'end of the octopine synthase gene; nptII, neomycin phosphotransferase II gene; Pn, promoter of the nopaline synthase gene; l, *lox* sequence recognition site of the Cre/lox recombination system; P35S, cauliflower mosaic virus promoter; gus, β-glucuronidase gene; 3'n, 3' end of the nopaline synthase gene; hpt, hygromycin phosphotransferase gene; 3'35S, 35S terminator; CRE, cre recombinase; HSP, soybean Gmhsp 17.6L heat-shock responsive promoter; LB, left border; RB, right border; 3,4,5, primers used for PCR analysis.

5.3.2 Construction of CRE-expressing T-DNA vectors and generation of transgenic *Arabidopsis* lines

In order to analyse the effectiveness of a CRE recombinase expressed from different promoters to induce DNA recombination and excision of the *gus* gene in transgenic *Arabidopsis thaliana* plants, we made three different T-DNA constructs on the basis of the binary pHGWC T-DNA vector (Fig.5.2a). The T-DNA contains the hygromycin selectable marker gene and the CRE recombinase coding region placed downstream of the GATEWAY™ cassette. The GATEWAY™ cassette present in the vector enables efficient cloning of promoters into the pHGWC vector. The cauliflower mosaic virus (CaMV) 35S promoter, the nopaline synthase gene promoter and the soybean Gmhsp17.6L heat-shock promoter (Severin and Schöffl, 1990) in an entry clone flanked by attL1 and attL2 sites (Fig. 5.2b) were cloned into the pHGWC "Destination" vector containing a negative selectable marker flanked by attR1 and attR2 sites. The resulting "Expression" vectors, pHSC, pHNC and pHHSC contain the P35S, Pnos and heat-shock promoters flanked by attB1 and attB2 sites in the expression context of the original Destination vector (Fig.5.2c). The construction of these plant transformation vectors is described in more detail in Material and Methods.
The obtained plant transformation vectors were used for transformation of wild type *A.thaliana* plants ecotype Columbia O (ColO) and lox-gus CK_2L6 *Arabidopsis* plants by the floral dip method (Clough and Bent, 1998). The lox-gus *Arabidopsis* line CK_2L6 is homozygous for a transgenic locus containing a single copy of the K T-DNA (Theuns *et al*., 2002; De Buck *et al*., 2004). Primary transformants of the different CRE-expressing T-DNAs

selected on Murashige and Skoog medium containing 20mg/l hygromycin were grown to maturity and T2 seeds were collected (Table 5.1). 22 FHSC primary transformants resistant to hygromycin and carrying the *cre* recombinase gene were obtained (Table 5.1). Hygromycin resistance assays were carried out on the progeny of ten primary transformants to determine how many independently segregating T-DNA loci, capable of conferring hygromycin resistance were present. In the T2 generation, five transformants out of these ten tested (FHSC4, FHSC6, FHSC7, FHSC9, and FHSC12), were selected with a segregation ratio close to 3:1 of hygromycin-sensitive/resistant plants. The 3:1 segregation ratio indicates the insertion of the *P35S::cre* transgene (pHSC) at a single locus, but does not exclude multiple insertions at the same locus. A randomly chosen FHSC6 transformant, in which the *P35S::cre* transgene is integrated at a single locus, was used for further analysis.

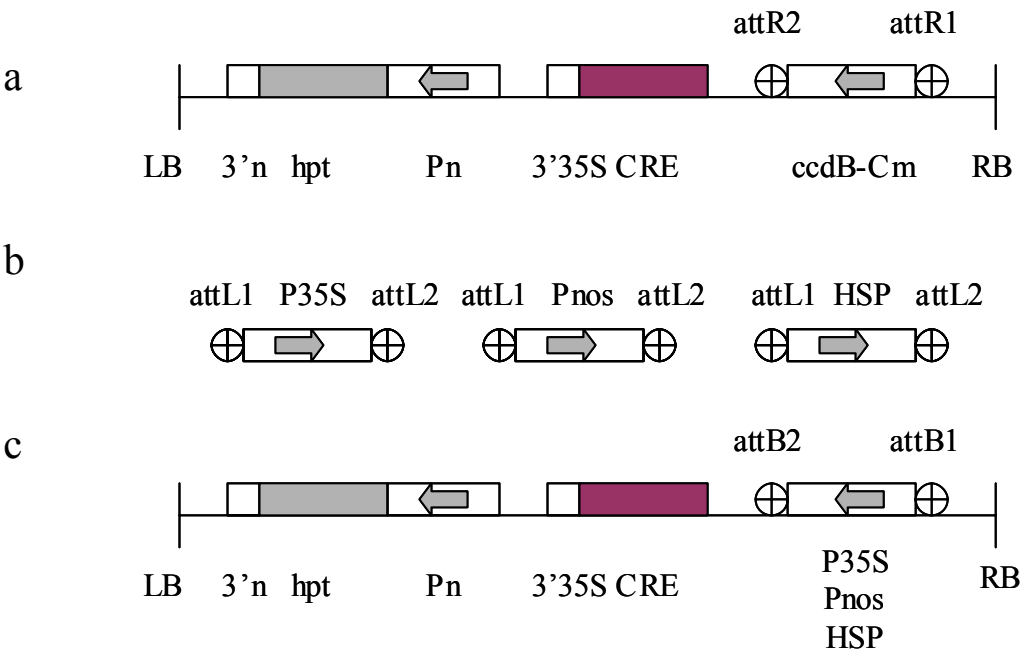


Fig. 5.2 Schematic representation of the the T-DNA of the pHGWC destination vector (a), P35S, Pnos and HSP-entry clone (b) and pHS(N OR HS)C expression vector, where S, N, and HS stands for P35S, Pnos and heat-shock promoter, respectively. For details see Material and methods.
Abbreviations: 3'n, 3' end of the nopaline synthase gene; hpt, hygromycin phosphotransferase gene; Pn, promoter of the nopaline synthase gene; 3'35S, 35S terminator; Cre, cre recombinase; P35S, cauliflower mosaic virus promoter; HSP, soybean Gmhsp 17.6L heat-shock responsive promoter; LB, left border; RB, right border

Table 5.1 Overview of the vectors, CRE-expressing transformants and CK_2L6 super-transformants.

Destination vector	Entry clone	Expression vector	CRE-expressing transformants	CK_2L6 super-transformants
pHGWC	P35S	pHSC	FHSC	CK_2L6-SC
pHGWC	Pnos	pHNC	FHNC	CK_2L6-NC
pHGWC	HSP	pHHSC	FHHSC	CK_2L6-HSC

5.3.3 Introduction of a *P35S::cre* locus into *Arabidopsis* plants of the FK24/3 line by cross pollination

A first way to bring the *lox*-flanked *gus* gene together with the *cre* gene was by genetic crossing, resulting in F1 hybrids in which recombination can occur. Therefore, reciprocal crosses were made in which the CRE-expressing T2 plants of the FHSC6 transformant and homozygous FK24/3 plants (De Buck *et al*., 2004) were used as either the male or female parent. F1 seeds were germinated on medium containing hygromycin to identify those that had received the CRE T-DNA. All F1 progeny plants should inherit a lox-flanked GUS expression cassette, as homozygous lox-gus FK24/3 plants were used.

To determine whether the introduction of the 35S driven CRE recombinase resulted in precise deletion of the GUS expression cassette, three weeks old F1 seedlings were stained for GUS activity. The GUS activity in these F1 hybrids was compared with the activity in the FK24/3 lox-gus parental line. The FK24/3 lox-gus parental line showed uniform GUS staining throughout the seedling, while the plants containing only the *P35S::cre* transgene did not show any blue staining (Fig.5.3a, b). Photographs of representative progeny plants of reciprocal crosses along with the control parental plants are shown in Fig.5.3 and Fig.5.4.

Histochemical analysis of F1 hybrids, derived from crosses between lox-gus plants as the female and cre-expressing plants as the male parent, showed that all F1 plants still retained some degree of GUS activity. While most of the rosette leaves were GUS-negative, the staining pattern throughout the rest of the plant was variable: some plants showed GUS activity only in the cotyledons, whereas others showed a range of GUS-negative sectors in some rosette leaves. Various GUS-negative sectors in rosette leaves were the result of the continuous CRE-mediated excision process. Leaves in which one half was GUS-positive and one half GUS-negative were frequently observed (Fig. 5.3 i). Analysis of the root structures

showed that GUS-positive and GUS-negative sectors were generated throughout the entire length of the root (Fig.5.3c-l). The primary roots showed GUS activity in all F1 hybrids analysed, while almost all adventitious and lateral roots were GUS-negative throughout the entire length (Fig. 5.3.k,l). Most of the F1 plants showed GUS activity in the shoot apical meristem (Fig.5.3 e,f,j).

The staining of the F1 hybrids, generated from a cross between the CRE-expressing plants as the female and lox-gus plants as the male parent, showed similar patterns of GUS activity with the progeny derived from the reciprocal cross. As shown in Figure 5.4, in the rosette leaves various GUS-negative sectors were present (Fig.5.4i, j). Also, most of the mature leaves lost GUS activity in all their cells (Fig.5.4c, d). The primary roots of all F1 plants showed GUS activity in all cells, whereas the adventitious and lateral roots lost the *gus* gene in almost all cells. In some lateral and adventitious roots small GUS-positive sectors were observed (Fig. 5.4e-h).

In conclusion, these results indicate that a 35S driven CRE recombinase mediated the excision of the GUS expression cassette, but the excision was not complete. The F1 progeny plants were mosaic, having lost the *gus* gene in a portion of their cells. Residual GUS activity was observed in the cotyledons, hypocotyls and primary roots in all F1 plants analysed. By contrast, in lateral and adventitious roots, which are formed during post-embryonic development of plants, no or very rare GUS activity was observed. There was no obvious difference in recombination efficiency when the CRE-expressing plants were used as female or male parent, indicating that the CRE activity in the female gametocyte is either absent or not sufficient for CRE-mediated recombination early upon fertilization. CRE-mediated excision resulted in the formation of GUS negative sectors throughout all F1 plants that received the CRE T-DNA.

To quantify the residual GUS activity, a fluorimetric GUS assay was performed on the F1 hybrids of crosses between CRE-expressing plants and lox-gus plants, and compared with the GUS activity in the lox-gus FK24/3 parental line. Protein extracts were prepared from 4-5 rosette leaves of ten F1 hybrids of reciprocal crosses, grown on the medium containing hygromycin (the collected leaf material was used for the preparation of both protein extracts and DNA for PCR analysis). The GUS activity levels in the F1 hybrids were ranging from 15 to 55 U GUS mg protein^{-1}, while those in the parental plants were about 1484± 306 U GUS mg protein $^{-1}$. This represents a 27 to 99-fold decrease of GUS activity in the rosette leaves when compared with GUS activity measured in the FK24 parental line, confirming the

histochemical staining pattern which suggests the deletion of the GUS expression cassette by the CRE recombinase (Fig.5.5a).

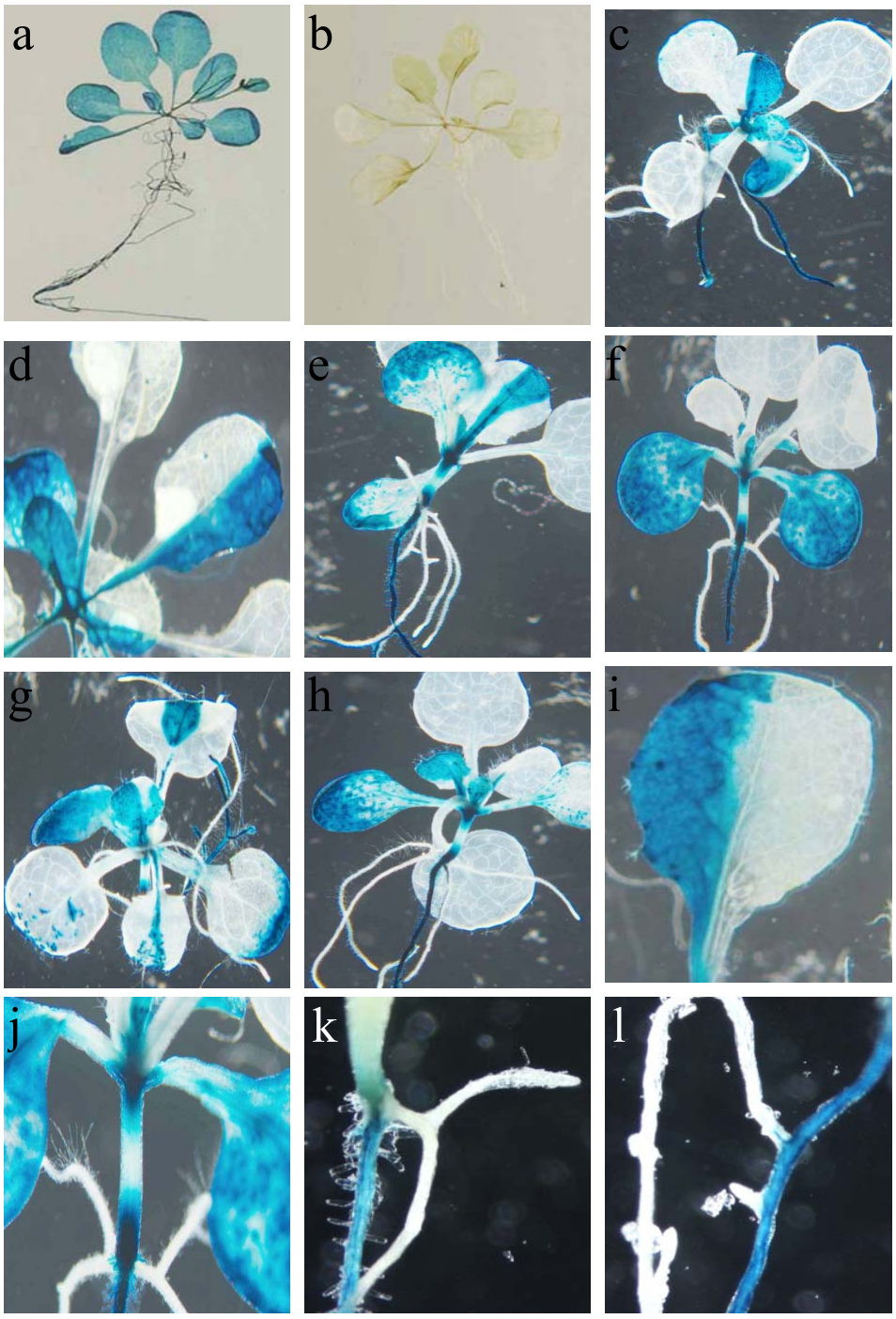


Fig. 5.3 Histochemical analysis of F1 seedlings from the cross FHSC6 X FK24/3, with the CRE-expressing plant as the male parent. a, lox-gus FK24/3 parental line; b, CRE-expressing FHSC6 transformant; c-h, representative F1 plants showing the range of staining patterns; i, leaf with a half GUS-negative sector; j, the hypocotyl and shoot apical meristem consisting of cells where no efficient CRE-mediated deletion of the *gus* gene occurred; k,l, examples of GUS-positive primary roots and GUS-negative lateral and adventitious root.

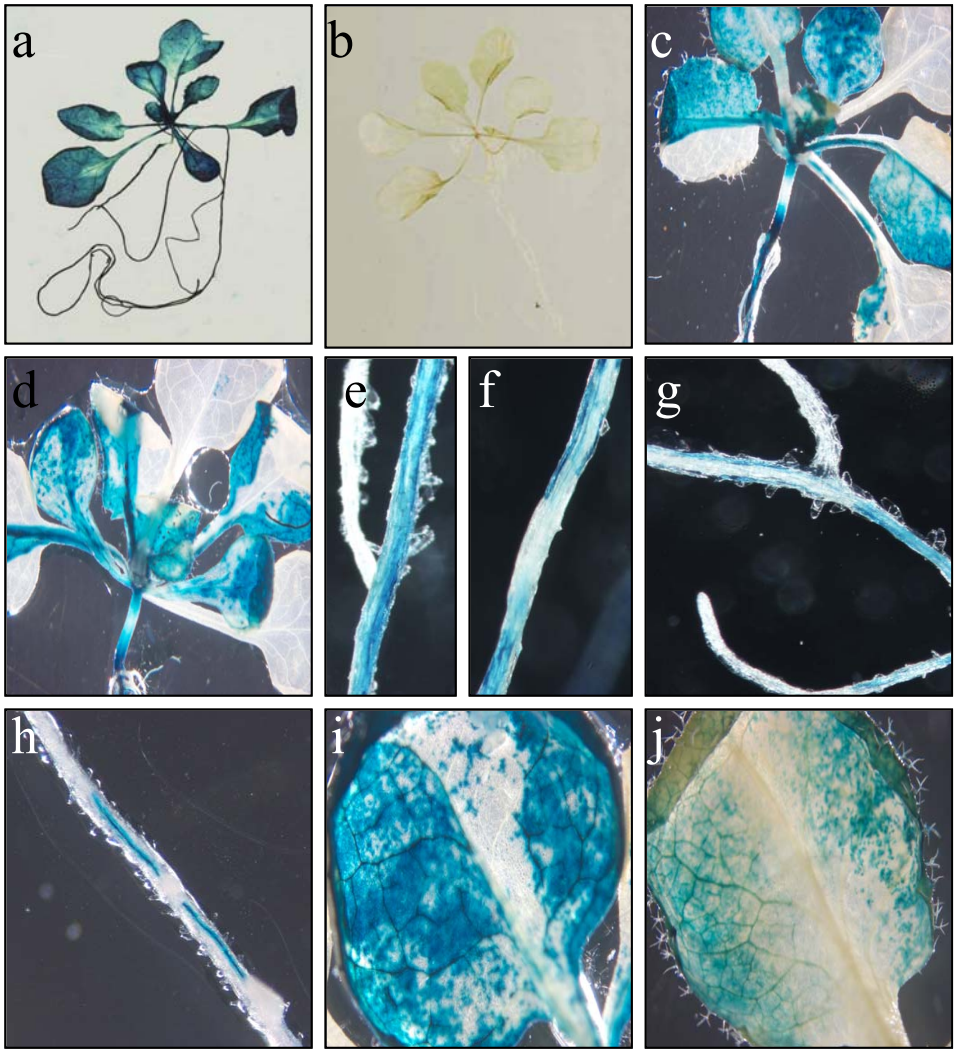
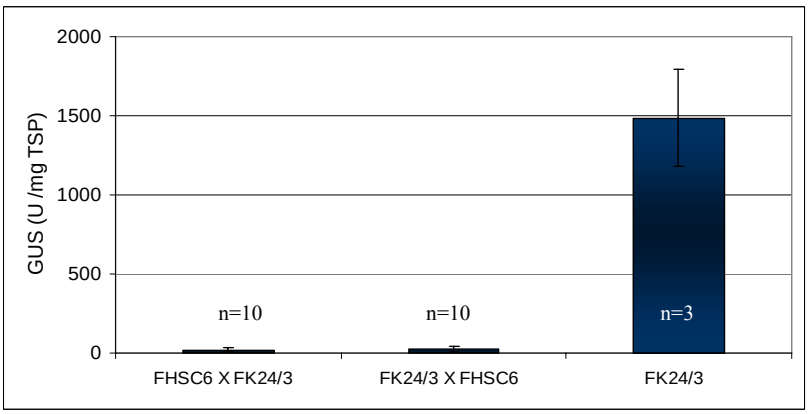


Fig. 5.4 Histochemical analysis of F1 seedlings from the cross FHSC6 X FK24/3, with the CRE-expressing plants as the female parent. a, lox-gus FK24/3 parental line; CRE-expressing FHSC6 transformant; b,c,d, representative F1 plants showing the range of staining patterns; e-h, examples of primary roots and lateral roots showing different GUS-positive sectors; i,j, leaves showing various GUS-negative sectors.

To obtain molecular evidence of the CRE-mediated excision event, PCR analysis was performed as described in the section 5.3.1 (Fig.5.1). DNA was prepared from the same leaf material that was collected and used for the preparation of protein extracts for the GUS expression analysis. The 3/ 4/ 5 primer set produced only in the parental FK24/3 *Arabidopsis* line a 1200 bp fragment indicating the presence of the non-excised form of the K T-DNA (Fig.5.5b, lane 21 and 23). No fragment was amplified from WT Columbia (Fig.5.5 lane 24).

All F1 plants showed complete or nearly complete excision of the GUS expression cassette in the leaves used for the DNA preparation, as the PCR primers 3, 4 and 5, produced a band of 834 bp in all of them (Fig.5.5b, lane 1-20).

a



b

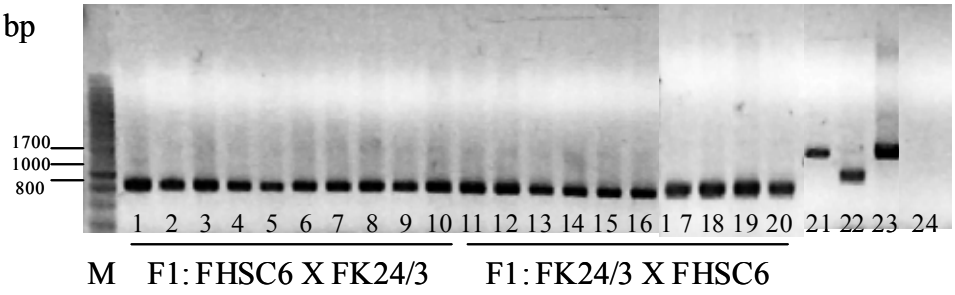


Fig.5.5 (a) GUS expression analysis of F1 progeny from reciprocal crosses. F1 plants were grown on the medium containing hygromycin, to select for the CRE T-DNA. GUS activity was measured in the leaves from 10 F1 hybrids derived from the cross FHSC6 X FK24/3, with CRE-expressing plant as the male parent, in the leaves from 10 F1 hybrids derived from the cross FK24 X FHSC6, with CRE-expressing plant as the female parent, and in the FK24/3 parental line. The GUS activity levels are expressed as units GUS per milligram of total soluble protein (UGUS mg protein^{-1}). Error bars represent standard deviation.
(b) PCR analysis on genomic DNA prepared from rosette leaves of F1 plants derived from cross FHSC6 X FK24/3, with CRE-expressing plant as the male parent (lines 1-10), and F1 plants derived from cross FK24/3 X FHSC6, with CRE-expressing plant as the female parent (lane 11-20). Lane 21 and 23, FK24/3 parental line, control for the presence of the lox-flanked *gus* gene; lane 22, deletion line, control for the excision event; lane 24, WT Columbia. Primers 3, 4 and 5 were used as illustrated in Fig.5.1.

The histochemical staining analysis (Fig.5.3 and 5.4) and fluorimetric GUS assay (Fig.5.5a) showed that the CRE-mediated excision was not efficient enough to cause complete loss of the *gus* gene during early development and therefore, hybrids derived from reciprocal crosses

were mosaic for the excision event. The majority of rosette leaves did not show a lot of GUS staining. This was confirmed by PCR analysis, which demonstrated that the excision occurred in the majority of cells in the leaves of the F1 hybrids, as only a band specific for the excision was amplified (Fig.5.5b).

To investigate whether the excised form of the lox-gus T-DNA could be transmitted to the next generation, F2 progeny from one F1 plant was analyzed by staining for GUS activity. Selfing of the F1 plant, heterozygous for both *cre* and *lox*, produced F2 seed in the following ratios of genotypes: 9 with both T-DNA loci, 3 with only the lox-gus T-DNA locus, 3 with only the CRE T-DNA locus, and 1 with neither of them. To identify the plants that contained the lox-gus T-DNA, F2 seeds were selected on medium containing kanamycin. A total of 111 kanamycin resistant F2 plants were obtained and analysed for GUS activity by staining of some isolated roots (Table 5.2). Next, to assess the presence of the CRE T-DNA, leaf tissue from all kanamycin resistant plants was assayed for the ability to form callus on medium containing hygromycin. Of 86 that were found to be hygromycin resistant, 76 were GUS negative, while the remaining ten showed a mosaic pattern of the CRE-mediated excision event. Among 25 hygromycin sensitive F2 progeny plants, 22 were found to be GUS negative, whereas the remaining 3 expressed the *gus* gene in all cells.

Table 5.2 Overview of the numbers of the 111 kanamycin resistant F2 progeny plants from the FHSC6 X FK24/3 cross which are GUS negative, showing a mosaic pattern of the CRE-mediated excision event (GUS^+/GUS^-) and GUS positive.

	# F2 plants	# GUS^- F2 plants	#GUS^+/GUS^- F2 plants	# GUS^+ F2 plants
#KAN^R / HYG^R F2 plants	86	76	10	-
#KAN^R / HYG^S F2 plants	25	22	-	3

In conclusion, the absence of *cre* in the genome of GUS-negative F2 plants implies that DNA excision took place during the F1 generation in both gametes, and that the excised form of the K T-DNA can be transmitted to the F2 plants.

5.3.4 Introduction of a *P35S::cre* gene into lox-gus CK_2L *Arabidopsis* plants by transformation

Another way of bringing the lox-flanked GUS expression cassette together with the *cre* gene is by *Agrobacterium*-mediated transformation. Therefore, lox-gus CK_2L6 *Arabidopsis* plants were transformed with the P35S, Pnos and heat-shock promoter driven *cre* gene (pHSC, pHNC and pHHSC vectors, Fig.5.2c) by the floral dip method (Clough and Bent, 1998). The experimental strategy used to demonstrate the CRE-mediated excision event in the T2 progeny plants of the CK_2L6 super-transformants is as described in section 5.3.1 (Fig.5.1).

The efficiency of CRE-mediated excision of the GUS expression cassette in the CK_2L-SC super-transformants

Ten independent hygromycin resistant CK_2L6-SC super-transformants (Table 5.1) were grown to maturity, selfed, and seeds were collected. Two-week old T2 progeny plants that showed resistance to hygromycin, implying the presence of the CRE T-DNA, were stained for GUS activity. Photographs of representative progeny of ten independent lox-gus-SC super-transformants along with the control CK_2L6 parental line are shown in Fig.5.6. As shown in Table 5.3, all T2 seedlings derived from transformant CK_2L6-SC/13 were GUS negative (Fig. 5.6l). By contrast, among the progeny of nine other CK_2L6-SC transformants, a different number of GUS negative and seedlings showing a mosaic pattern of the excision event was observed (Table 5.3). For example, 9 of the 10 T2 seedlings of the transformant CK_2L6-SC/6 were GUS-negative, whereas in one of them, a patchy distribution of GUS activity was observed, mainly in the hypocotyl and primary root cells (Fig.5.6 f). In the transformants CK_2L6-SC/3, 7 GUS-negative seedlings were observed. The remaining 3 showed GUS activity in the root hairs of the primary root (Fig.5.6c). Furthermore, 4 to 6 of the progeny plants of the CK_2L6-SC/5, 7, 9, 10, 12 super-transformants showed a patchy distribution of the GUS activity in the cotyledons, hypocotyls and the primary root cells (Fig.5.6e, g, h, i, k). In the transformants CK_2L6-SC/4 and 11, only 2 of the T2 seedlings were GUS-negative, while the remaining 8 seedlings showed larger GUS-positive sectors in the cotyledons, hypocotyls, primary roots and in some lateral roots (CK_2L6-SC/4, Fig.5.6d, j; Fig.5.7g).

Table 5.3 Overview of the number of T2 progeny plants of CK_2L-SC super-transformants showing a mosaic pattern of the CRE-mediated excision event (GUS^+/GUS^- seedlings) and GUS negative T2 progeny of ten independent CK_2L6-SC super-transformants.

	#seedlings	#GUS^+/GUS^- seedlings	#GUS^-seedlings
CK_2L6-SC3	10	3	7
CK_2L6-SC4	10	8	2
CK_2L6-SC5	10	5	5
CK_2L6-SC6	10	1	9
CK_2L6-SC7	10	5	5
CK_2L6-SC9	10	4	6
CK_2L6-SC10	10	6	4
CK_2L6-SC11	11	8	2
CK_2L6-SC12	10	5	5
CK_2L6 -SC13	10	0	10

In conclusion, the efficiency of CRE-mediated excision of the GUS expression cassette varied between different CK_2L6-SC super-transformants. The most efficient CRE-mediated excision of the *gus* gene occurred in the super-transformant CK_2L6-SC/13, since none of the T2 progeny showed detectable GUS activity (Fig.5.6l). On the other hand, the other nine super-transformants gave rise to T2 progeny that consisted of GUS negative seedling and seedlings with mosaic pattern of the excision event (Fig.5.6 c-k, Fig.5.7). The partial excision of the *gus* gene in the T2 progeny plants suggest the inheritance of a non-excised form of the K T-DNA.

The molecular evidence of the CRE-mediated excision event was provided by PCR analysis, performed as described in the section 5.3.1 (Fig.5.1). DNA was prepared from the pooled rosette leaf material of ten four-week old T2 plants. The 3 / 4 / 5 primer set produced 834 and 1200 bp fragments in the progeny from CK_2L6-SC super-transformants 5, 6, 7, 11 and 12 (Fig.5.8 lane 11, 12, 13, 16 and 17). The progeny from CK_2L6-SC super-transformants 4 and 10 showed the 834 bp PCR fragment and a very weak band of 1200 bp (Fig.5.8 lane 10 and 15). In the progeny from CK_2L6-SC super-transformants 3, 9 and 13, only a band of 834 bp was detected, indicating efficient CRE-mediated excision of the *gus* gene in leaves used to prepare DNA. For CK_2L6-SC/13 progeny plants, only a PCR fragment of 834 bp was expected, as no GUS activity was detected in none of them (Fig.5.6l). No fragment wasamplified from WT C24 (Fig.5.8 lane 36).

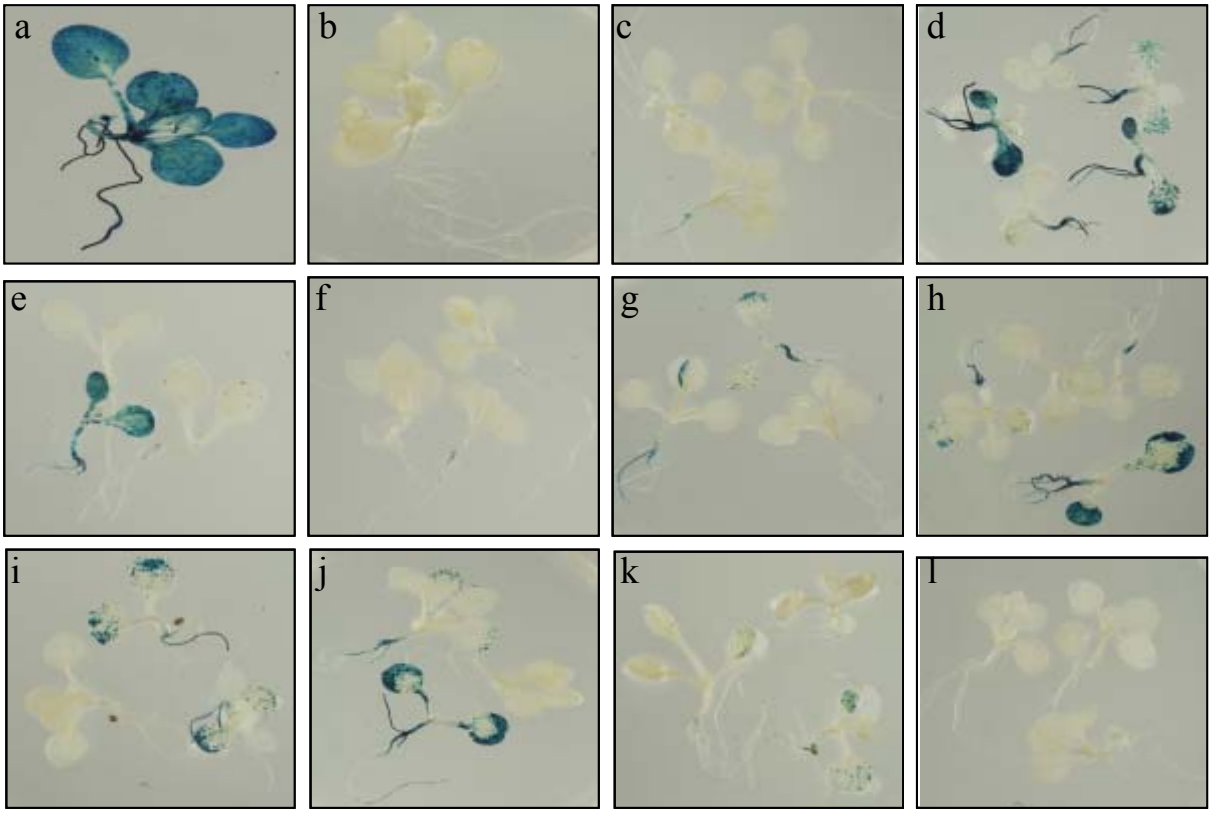


Fig. 5.6 Histochemical analysis of T2 progeny from ten independent CK_2L6-SC super-transformants. a, lox-gus parental line; b, CRE-expressing line; c-l, representative examples of T2 progeny from lox-gus-SC re-transformant 3(c), 4 (d), 5(e), 6(f), 7(g), 9(h), 10(i), 11(j), 12(k), 13(l).

To determine whether the presence of the two bands in CK_2L6-SC/5, 6, 7, 11 and 12 transformants was also reflected in the quantitative GUS measurements, GUS activity was determined in the leaves of the same ten T2 plants used for PCR analysis. As a control, the GUS activity of the CK_2L6 parental line was determined. None of the progeny of ten independent super-transformants tested, showed detectable GUS activity (data not shown). For the CK_2L6 parental line, GUS activity was 920 ± 49 U GUS mg protein $^{-1}$. These results indicate very efficient CRE-mediated excision of the *gus* gene in the leaves used for the preparation of protein extracts. Histochemical staining was performed on two-week old seedlings, while the GUS activity was measured only in rosette leaves of four-week old plants, implying that a lot of excision took place through further growth of the plant. This could be one possible explanation for this seemingly contradictory observation. However, the

presence of the 1200 bp PCR fragment in some transformants with undetectable GUS activity indicate differences in the excision efficiency among the leaves of the same T2 plants.

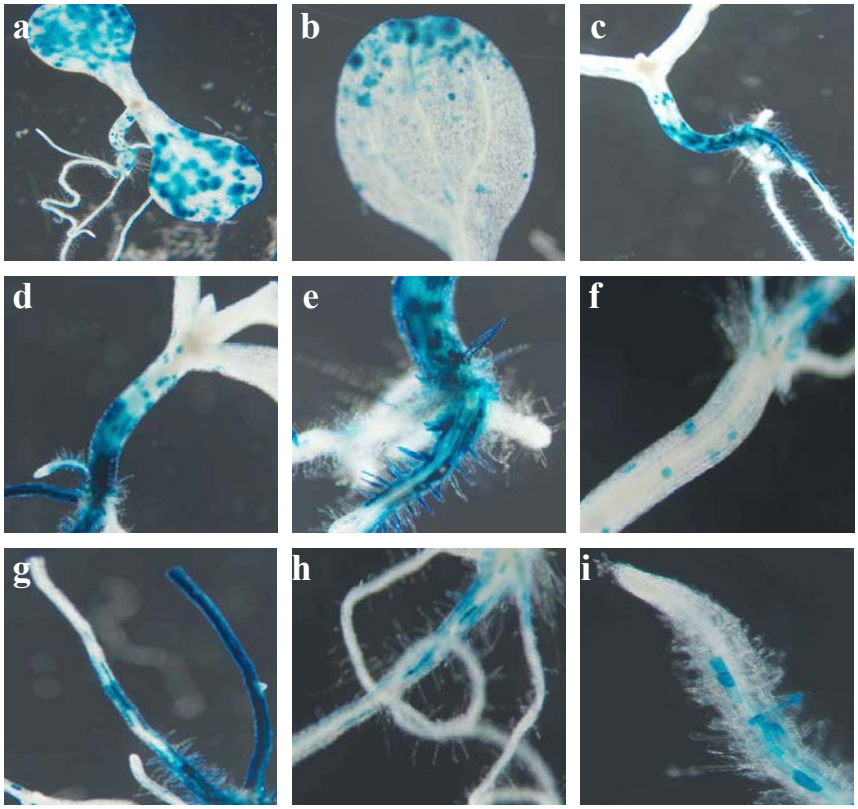


Fig. 5.7 Histochemical analysis of T2 progeny plants from different transformants with the *P35S::cre* gene. a,b, representative photographs showing different GUS-negative sectors in the cotyledons; c-f, representative photographs showing different GUS-negative sectors in the hypocotyls; g-I, representative photographs showing small GUS-positive sectors in the primary roots and completely GUS-negative lateral roots, except for some GUS-positive lateral roots observed in the progeny from lox-gus-SC/4 (g).

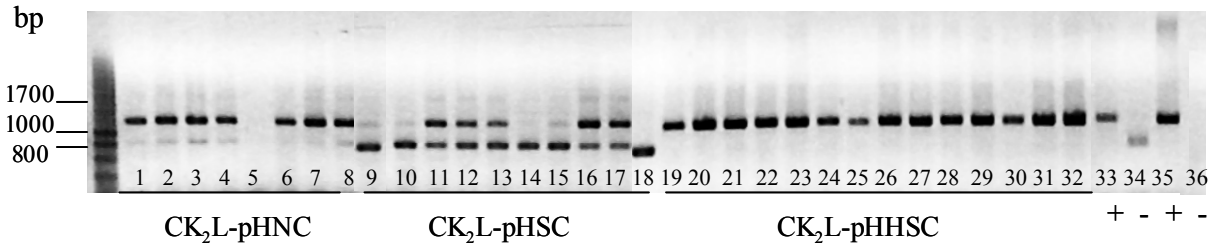


Fig. 5.8 PCR analysis of genomic DNA prepared from T2 progeny from 8 independent CK_2L-NC super-transformants.(lane 1-8), 10 CK_2L-SC super-transformants (lane 9-18), and 7 CK_2L-HSC super-transformants (progeny grown under normal conditions, lane 19-25; heat-shocked progeny, lane 26-32); lane 33 and 35, CK_2L plants; lane 34, positive control for the excised form of the K T-DNA; lane 36, WT C24. Primers 3, 4 and 5 were used as illustrated in Fig.5.1.

The efficiency of CRE-mediated excision of the GUS expression cassette in the CK_2L6-NC super-transformants

Eight randomly chosen independent transformants with a *Pnos::cre* containing T-DNA construct into a plant containing a *lox* cassette with a *gus* gene were selected for detailed analysis (Table 5.1). For each transformant, two-week old T2 progeny plants grown on selective medium for the CRE T-DNA were stained for GUS activity. All plants tested showed a comparable staining as the parental plants and in none of them, GUS-negative sectors were observed (data not shown).

To further determine whether the CRE recombinase, regulated by the Pnos promoter, mediated excision of the *gus* gene at a low level, a fluorimetric GUS assay was performed allowing to detect a decreased GUS activity as compared to the parental plants. The GUS activity was measured in the protein extracts prepared from the leaves of ten selected T2 plants of different CK_2L6-NC super-transformants. In none of the extracts a significant decrease in GUS activity was detected (data not shown).

To test on the molecular level whether CRE-mediated excision of the GUS cassette occurred, a PCR analysis was performed as described in section 5.3.1. The band of 1200 bp was still present in all plants, confirming that all plants still contained non-excised form of the K T-DNA (Fig.5.8 lane 1-8). In the progeny of 5 out of 8 transformants, a very weak 834 bp PCR fragment was amplified (CK_2L6-NC super-transformants 1, 2, 3, 4, 8, Fig.5.8 lane 1,2,3,4,8). This indicates that CRE-mediated excision of the *gus* gene occurred in a very limited number of cells of the leaves used for DNA preparation.

From these experiments it can be concluded that the CRE recombinase regulated by the weak Pnos promoter did not mediate efficient excision of the *gus* gene in any of the eight transformants analyzed.

The efficiency of CRE-mediated excision of a lox flanked GUS cassette in transformants with a cre gene under the control of a heat-shock inducible promoter

To evaluate the efficiency of a heat-shock inducible Cre/lox recombination system, seven independent transformants with a heat-shock promoter regulated *cre* gene were selected for detailed analysis (Table 5.1). The segregating T2 seeds were sown on medium containing hygromycin to select for plants that contained the CRE T-DNA. One set of T2 progeny plants from seven independent transformants were grown under normal, uninduced conditions. A second set of plants was selected in the same way but they were heat shocked during the first week as described in Material and Methods. Subsequently, two week-old seedlings were analyzed by staining for GUS activity.

Two to three of the ten T2 seedlings of transformants CK_2L6-HSC/1, 3 and 4 showed GUS negative sectors in the leaves in induced conditions, whereas none of the progeny plants of these same transformants showed GUS negative sectors when grown in the normal, uninduced conditions. Representative seedlings of transformant 1, 3 and 4 grown in normal, uninduced conditions are shown in Fig.5.9 a1, b1, c1. Heat-shocked seedlings from the same double transformants with a range of GUS-negative sectors in the leaves are shown in Fig.5.9 a2, a3, b2, b3, c2, c3. None of the four other transformants showed evidence for CRE activity.

To see whether the CRE-mediated excision of the *gus* gene in heat-treated plants resulted in a decreased GUS activity in CK_2L6-HSC super-transformants, GUS fluorimetric analysis was performed. Also, to obtain molecular evidence for CRE-mediated excision of the *gus* gene in heat-treated plants, a PCR analysis was used. Leaves from ten four-week old CRE T-DNA selected T2 plants were pooled, and used to prepare the DNA and the protein extracts**.** A fluorimetric GUS assay performed on heat-shocked progeny showed similar levels of GUS activity as progeny grown in normal, uninduced conditions and it was in the range of the GUS activity observed in the CK_2L6 parental line (data not shown). This was not surprising, since only two to three seedlings out of ten stained for GUS activity showed GUS-negative sectors in some of their leaves. By PCR**,** only a band of 1200 bp was amplified in both the progeny grown under normal conditions (Fig.5.8 lane 19-25) and the heat-shocked progeny (Fig.5.8 lane 26-32). Thus, although histochemical staining suggested a partial excision of the GUS expression cassette in the leaves of transformants 1, 3 and 4, the excision could not be detected by PCR. In conclusion, the above analysis demonstrated that heat-shock induction of

a *HSP::cre* gene did not result in deletion of a *lox* cassette in a significant amount of seedling cells.

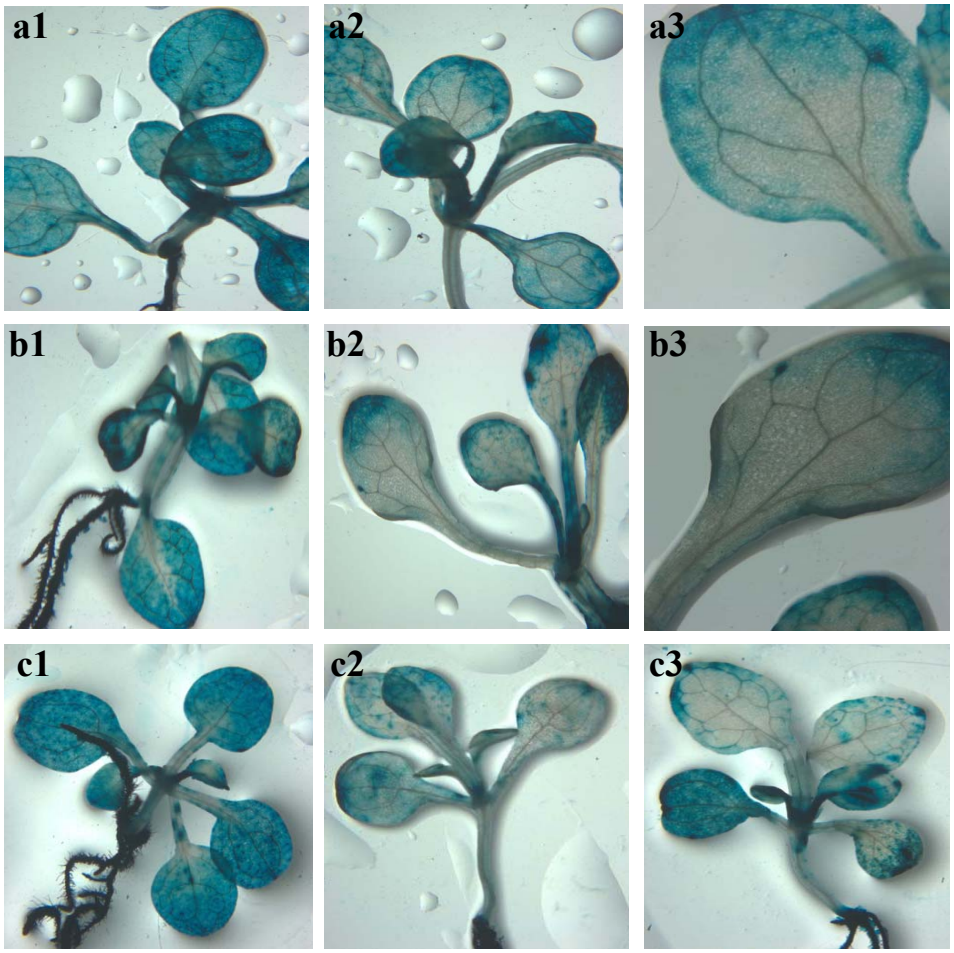


Fig. 5.9 Histochemical analysis of T2 progeny from CK_2L6-HSC super-transformants. Representative T2 seedlings from CK_2L6-HSC super-transformant 1, 3 and 4 grown under normal, uninduced conditions (a1, b1, c1) and induced conditions (a2, a3, b2, b3,c2, c3).

5.4 Discussion

We analysed the CRE-mediated excision efficiency of a *lox* flanked GUS expression cassette in transgenic *Arabidopsis thaliana* plants. Firstly, the *P35S::cre*-mediated recombination was demonstrated in the progeny obtained by two different methods of combining the *lox*-flanked *gus* gene and the CRE recombinase, genetic cross and transformation.

A randomly selected transformant FHSC6, with a single *P35S::cre* transgene locus, was used for reciprocal crossing with the FK24/3 *Arabidopsis* plants (De Buck *et al*., 2004). From each

of the reciprocal crosses, ten F1 hybrids were selected for GUS analyses. The F1 hybrids, grown on selective medium for the CRE T-DNA, showed a mosaic pattern of the excision event. The GUS activity levels in the F1 hybrids were ranging from 15 to 55 U GUS mg protein-1 for both reciprocal crosses, representing a 27 to 99 fold decrease in GUS activity when compared with the GUS activity measured in the FK24/3 parental line. These results indicate incomplete excision of the GUS expression cassette in the F1 hybrids. However, the observed decrease in the GUS activity also indicates that the excision of the GUS expression cassette occurred in the majority of cells in the leaves used for analysis. This was further supported by molecular analysis, as only a DNA fragment corresponding to the CRE-mediated deletion product was detected. In addition, there was no obvious difference in recombination efficiency when the CRE-expressing plants were used as female or male parent, indicating that the CRE activity in the female gametocyte is either absent or not sufficient for CRE-mediated recombination early upon fertilization. Our results demonstrated germinal transmission of the excised form of the lox-gus T-DNA, as 88% of the F2 progeny plants were GUS negative. Since 22 of them did not contain the CRE T-DNA, we could conclude that DNA excision took place during the F1 generation, and that the excised form of the K T-DNA was transmitted to the F2 plants.

Following transformation of CK_2L6 plants with the P35S::cre construct, a different excision efficiency was observed among different independent transformants. One out of ten independent CK_2L6-SC transformants gave 100% GUS-negative progeny. Molecular analysis of these plants confirmed that the absence of GUS staining was due to CRE-mediated excision of the *gus* gene, as only the excised form of the K T-DNA was detected. In other *P35S::cre* transformants, the percentage of GUS-negative and GUS-mosaic plants varied. Based on the assays used to demonstrate CRE-mediated excision of the *gus* gene, all ten transformants thus showed functional CRE activity. However, different excision efficiency is probably related to different expression level of the CRE recombinase caused by the different number and structure of the CRE T-DNA loci in these transformants. Indeed, upon transformation, the CRE T-DNA is randomly integrated in the plant genome. Also, transgenic loci frequently harbor multiple copies of the integrated DNA, which are implicated in silencing, especially transgenic loci harboring inverted T-DNA structures (De Buck *et al*., 2001; Muskens *et al*., 2000; Van Houdt *et al*., 2000). The variable CRE activity levels in different transformants can explain the partial CRE-mediated excision of the *gus* gene in the hybrid plants obtained by crossing the FHSC6 CRE T-DNA containing plants with a *lox* flanked *gus* cassette

containing plants. It remains to be determined how efficient other FHSC transformants are at directing the excision of the *gus* gene.

Several reports have been published on variations in excision efficiency, which is dependent upon the specific *lox* and CRE transformants used as parents (Odell *et al*., 1990; Russell *et al*., 1992; Hoa *et al*., 2002). It has been shown that the efficiency of recombination was related primarily to the CRE parent and that differences were most likely due to CRE expression levels (Russell *et al*., 1992). They also found that the amount of CRE protein in developing embryos, germinating seedlings and young plants could be different from those in mature leaves. Also, different methods of combining the *lox*-flanked gene and the CRE recombinase were found to produce different degrees of excision in the resulting plant (Russell *et al.*, 1992). The expression of the *35S::cre* gene in different cell types and/or the ability of different cell types to respond to the Cre-mediated recombination could explain these differences.

Transformation of the CK_2L6 plants with the the CRE recombinase, controlled by the Pnos and Gmhsp 17.6L soybean heat-shock promoter did not result in the efficient excision of the *lox* flanked *gus* gene. GUS negative sectors were observed with a very low frequency in the leaves of three CK_2L6-HSC super-transformants by histochemical staining. However, the CRE-mediated excision, which could cause the loss of blue staining in these transformants, could not be detected at the DNA level. We postulate that the low efficiency of the CRE-mediated recombination regulated by the weak Pnos and the weak heat-inducible promoter as compared with the strong 35S promoter, is due to the low expression level of the CRE recombinase. We previously showed that the GUS activity level in *P35S::gfp-gus* transformants was about 50 to 150-fold higher than the GUS activity measured in the *Pnos::gfp-gus* transformants. Also, the induced levels reached by heat-shock promoter were 100-300-fold lower when compared with the GUS activity levels achieved by the 35S promoter (Chapter 4). Further optimisation of the heat-shock conditions, that includes the temperature, the duration of heat-shock treatment and developmental stage at which heat-shock is applied, could increase the activity of the promoter resulting in an increased frequency of the excision events.

In conclusion, although the efficiency of CRE-mediated excision of the *lox* flanked *gus* gene was variable, we obtained CRE expressing *Arabidopsis* transformants in which deletion of a *lox* flanked *gus* gene occurred for 100%, and showed that the deleted locus is transmitted to the next generation. The fact that this efficient CRE-mediated recombination was achieved

only with the CRE recombinase regulated by the strong 35S promoter, suggests that high expression levels of the CRE recombinase are required.

5.5 Materials and methods

Plasmid construction

The pHGWC construct is shown in Figure 5.2(a) and construction details are given in Figure 5.10. Cloning of pHGWC was as follows: The CRE coding region was amplified by PCR from the pMM23 plasmid (Dale and Ow, 1990,1991) using primers in which SacII and NcoI sites were included. The primers were: forward *cre* specific primer 5'tccccgcgggttgacatgtccaatttactgacc3' and reverse *cre* specific primer 5'catgccatgggaattcttactaatcgccatcttcc3'. The CRE fragment of 1049bp was purified from gel (Qiaquick gel extraction kit), digested with NcoI and SacII and cloned into the same sites of pT35S plasmid which contained the 35S terminator cloned in the SphII site of the pGEM-5Zf(+) vector, resulting in the pCreT35S. To place the *cre* coding region in a binary vector, the pCreT35S plasmid was digested with SacI/ApaI and the CreT35S fragment of 1276 bp was purified from gel (QIAquick Gel Extraction Kit Protocol) and cloned into the same sites of the pPZP200 binary vector (Hajdukiewicz *et al*., 1994), carrying the hygromycin selectable marker cloned into the EcoRI site. This step resulted in a pHCreT35S plasmid. To create the GATEWAY^{TM} compatible vector, the GW cassette, rfA (Invitrogen) was cloned upstream of the CRE recombinase. First, we designed the oligo that contained two blunt end restriction sites (PmlI, SwaI) flanked by two HindIII sites (NHPN 5' agcttcacgtgccatttaaata3' and NHPNc 5'agcttatttaaatggcacgtga3') to allow the insertion of the oligo into the HindIII site of the pHCreT35S vector. Next, we cloned the GW cassette (1700 nt EcoRV fragment) into the PmlI site to create the pHGWC vector. The transcriptionally correct insert orientation was confirmed by restriction analysis.

The P35S sequence was amplified by PCR using pXD610 DNA (De Loose *et al*., 1995) as a template. The Pnos sequence was amplified by PCR using the pK2L610 plasmid (De Buck *et al*., 2000), whereas the Gmhsp 17.6L soybean heat-shock responsive promoter (HSP, 390bp) was amplified by PCR from the pNJK18 plasmid (Kilby *et al*., 1995). To form attB1 and attB2 recombination sites, two sets of primers were used. In the first step, template specific primers containing 12 bases of attB sites were used in 30 cycles to amplify the target

sequence. PCR conditions for the P35S promoter were as follows: initial denaturation at 94^0C for 5 min, followed by 30 cycles of: denaturation at 94^0C for 1 min, annealing at 55^0C for 1 min and elongation at 68^0C for 1 min; and a final elongation step at 68^0C for 15 min; for Pnos and heat-shock promoter the annealing was done at 50^0C for 1 min. This PCR product was subsequently used as a template in the second PCR reaction with universal attB adapter primers to amplify the full attB1 and attB2 recombination sites (Invitrogen) following the protocol described in the instruction manual. Primers used were: forward P35S specific primer 5'aaaaagcaggctcctttgccccggagatca3', reverse P35S specific primer 5'agaaagctgggtatagtaaattgtaatgttgtttgt3', forward Pnos specific primer 5'aaaaagcaggctgatcatgagcggagaatt3', reverse Pnos specific primer 5'agaaagctgggtgagactctaattggat3', forward heat-shock promoter specific primer 5'aaaaagcaggctgaattctgaaattgggtctt3', reverse heat-shock promoter specific primer 5'agaaagctgggtttgaaagtttgcaaaattgta3', universal attB1 adapter primer 5'ggggacaagtttgtacaaaaaagcaggct3', universal attB2 adapter primer 5'ggggaccactttgtacaagaaagctgggt3'.

To obtain the P35S-entry clone, Pnos-entry clone and HSP-entry clone (Fig.5.2b), the PCR product flanked by attB sites were recombined into pDONR201-Km^r vector containing attP1 and attP2 recombination sites in the BP reaction using BP clonase enzyme (Invitrogen).

The T-DNA vectors, pHSC, pHNC and pHHSC (Fig. 5.2 c) were generated by an LR reaction in which P35S, Pnos and HSP-entry clones were incubated with pHGWC destination vector in the presence of LR Clonase anzyme (Invitrogen).

Agrobacterium strain, plant transformation and selection

Transformation vectors pHSC, pHNC and pHHSC were introduced by electroporation into *Agrobacterium tumefaciens* strain C58C1Rif^R, containing the pMP90 vir plasmid (Koncz, C. and Schell, J. 1986).

Transformation of wild type *Arabidopsis thaliana* plants ecotype Columbia O (ColO) and CK_2L plants (Theuns et al., 2002) was performed by the floral dip method (Clough and Bent, 1998). Seeds of the dipped plants were harvested and sown on K1 medium supplemented with hygromycin (20mg/l) for selection of FHSC, FHNC, FHHSC, CK_2L-SC, CK_2L-NC and CK_2L-HSC primary transformants. Selected primary transformants were self-fertilized and T2 seeds were collected. Hygromycin resistance assays were carried out on progeny of the FHSC primary transformants, to identify lines with segregation ratios close to 3:1 of

hygromycin-sensitive/resistant plants. Transformant FHSC6 containing *P35S::cre* transgene (pHSC) at single locus was selected for further experiments. Transgenic *Arabidopsis* plant lines CK_2L6 (Theuns *et al*., 2002; De Buck *et al*., 1998) and FK24/3 (De Buck *et al*., 2004) were used containing the K T-DNA. The K T-DNA contains a kanamycin resistance gene as selectable marker and an active GUS expression cassette flanked by two directly repeated *lox* sites. All plants were grown under a 16 h light / 8 h dark regime at 21^0C.

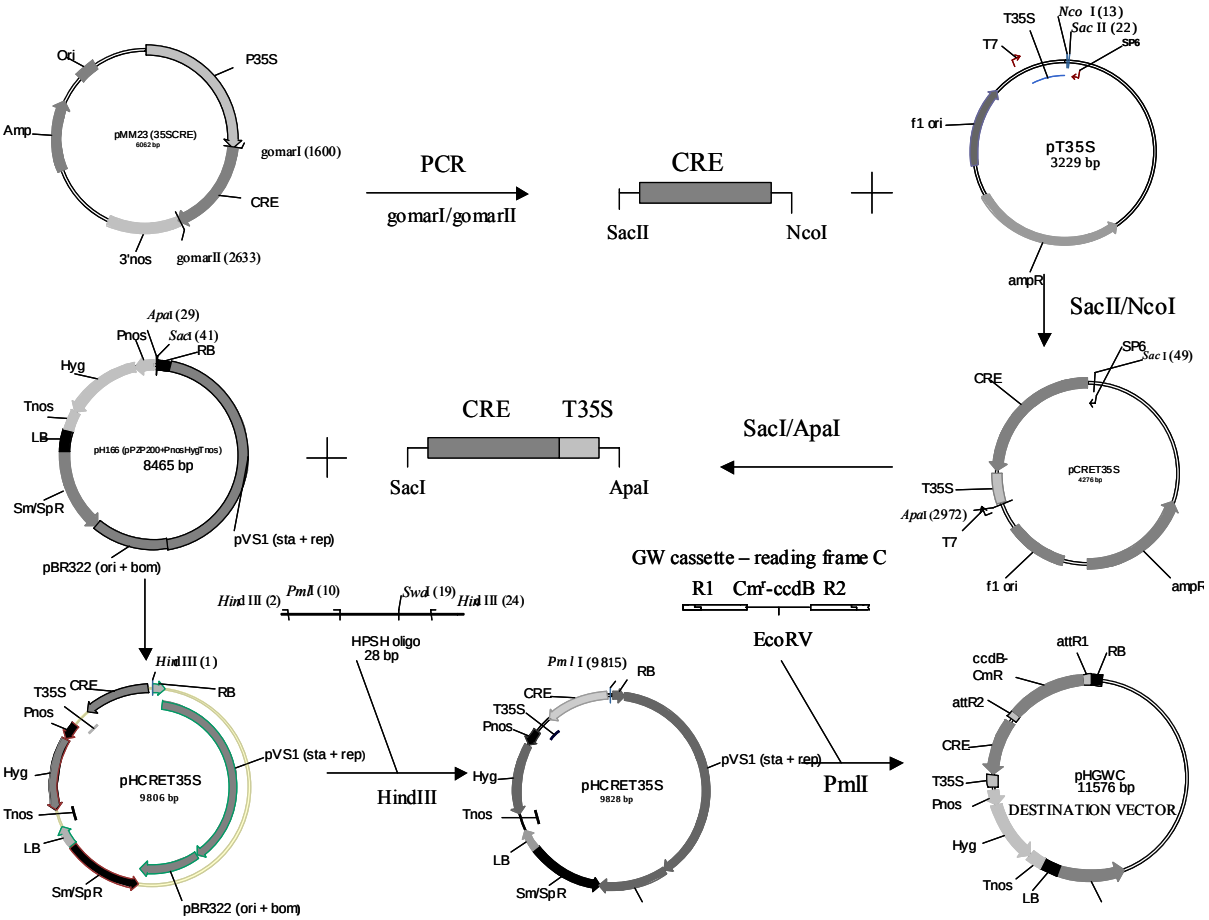


Fig. 5.10 Construction of the pHGWC vector.

Heat-shock conditions

Sterilized seeds were sown on medium containing 20 mg/l hygromycin and placed for one day under a 16 h light / 8 h dark regime at 21^0C. After that, they were heat-shocked in closed Petri dishes in a microbiological incubator for 3 hours at 37^0C on each of five consecutive

days. After each heat-treatment they were allowed to recover under a 16 h light / 8 h dark regime at 21^0C.

Histochemical β-glucuronidase assay

Two-weeks old seedlings were fixed in 90% cold acetone for 30 minutes with continuous shaking. The seedlings were washed with 0.1M Na_2HPO_4/NaH_2PO_4 buffer (pH7) and incubated in 0.1M Na_2HPO_4/NaH_2PO_4 buffer (pH7) containing 10mM EDTA, 0.5mM K3[$Fe(CN)_6$)], 0.5mM $K_4[Fe(CN)_6]$ and 1% DMSO containing 50mg/ml 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid (X-glu) overnight at 37^0C. After that, the seedlings were washed with 0.1M Na_2HPO_4/NaH_2PO_4 buffer (pH7), destained in 90% ethanol and stored in 70% ethanol. Photographs were taken using a digital camera (AxioCam HRc, Zeiss) connected to a Zeiss Stemi SV11 microscope.

Preparation of protein extracts and determination of the β-glucuronidase levels

Protein extracts were prepared from 4-5 rosette leaves of ten F1 hybrids of reciprocal crosses, and from pooled leaf material of ten T2 plants per CK_2L double transformant. Grinded leaf material was resuspended in 100µl buffer containing 50mM phosphate buffer (pH7), 10mM β-mercapthoethanol, 10mM Na_2-EDTA and 0.1% Triton X-100, centrifuged twice at 4^0C for 10 min to remove unsoluble material. The total amount of soluble protein in the protein extracts was determined with the Bio-Rad Protein Assay (Bradford, 1976) using bovine serum albumin as a standard. The GUS activity was determined as described by Breyne *et al*, 1993. GUS activity levels were expressed as units GUS protein relative to the total amount of soluble extracted protein (UGUS mg $protein^{-1}$).

PCR analysis

Genomic DNA was prepared from 4-5 rosette leaves of ten F1 hybrids of reciprocal crosses, and from pooled leaf material of ten T2 plants per CK_2L double transformant, according to the protocol for DNA purification from 10-30 mg frozen plant tissue (Puregene^{TM} DNA

Purification system, Gentra). PCR analysis was performed using 50ng of DNA and primers specific for the non-excised and excised fragment. The primers used were: primer 3 5'tgatcctgtttcctgtgtgaaatt 3', primer 4 5' ttgtaaggagatgcactgatttat 3', primer P35S3 (5) 5' atttgcggccgctttaatagtaaattgtaatgttgt 3'. The PCR reaction conditions were as follows: initial denaturation at 94^0C for 5 min, followed by 35 cycles of: denaturation at 94^0C for 45 s, annealing at 55^0C for 1 min and elongation at 72^0C for 1 min; and a final elongation step at 72^0C for 5 min.

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CHAPTER 6

Conditional approach to down regulate gene expression in *Arabidopsis thaliana*

Conditional approach to down regulate gene expression in *Arabidopsis thaliana*

Gordana Marjanac, Sylvie De Buck, Mansour Karimi and Anna Depicker

6.1 Abstract

RNA silencing induced by hairpin RNA (hpRNA) constructs has been shown to be a highly effective method for targeted suppression of specific genes (Wesley *et al*., 2001; Chuang and Meyerowitz, 2000; Waterhouse and Helliwell, 2003; Hilson *et al*., 2004; Chapter 3). Because of the high degree of silencing, which can be achieved by this method, functional analysis of a particular gene product required for basic cell function or development can be limited, as viable plants might not be recovered. To allow inhibition of a target gene at specific developmental stages, we developed a Cre/lox based approach for the conditional expression of hpRNA. In this vector, the hpRNA-encoding cassette is separated from the 35S promoter by a blocking cassette with the selectable marker flanked by two *lox* sites, which should keep the background expression of hpRNA to minimal levels. In this way, transcription of the hpRNA and suppression of the target gene should occur only after the introduction of the CRE recombinase (Dale and Ow, 1991).

However, our results demonstrated a suppression of the target gene in the absence of the CRE recombinase. Therefore, it was evaluated whether a promoterless hairpin construct also induces silencing, but found that this was not the case. Thus, the presence of the 35S promoter 1200 base pairs upstream of the hairpin is sufficient to mediate low levels of hpRNA expression resulting in a 3 to 10-fold reduction of the hairpin homologous *catalase* endogenes. Upon CRE-mediated deletion of the *lox* flanked blocking cassette, the 35S promoter was fused directly to the cat2hpRNA cassette, resulting in a further 2 to 3-fold reduction of endogenous *catalase* expression.

6.2 Introduction

Post-transcriptional gene silencing in plants is a form of RNA silencing by which target RNA is degraded in a sequence-specific manner. The general model for RNA silencing consists of three steps. First, RNA silencing is initiated by the formation of double-stranded RNA (dsRNA). Second, Dicer processes dsRNA into 21 to 28 nucleotide (nt) dsRNA fragments called small interfering RNAs (siRNAs). Finally, in the last step, the RNA-induced silencing complex (RISC) incorporates these siRNAs and cleaves a target RNA that has a region complementary to the siRNA sequence (Hamilton and Baulcombe, 1999; Matzke *et al*., 2001; Hannon, 2002; Hutvágner and Zamore, 2002).

RNA silencing induced by hairpin RNA (hpRNA) constructs has been shown to be a highly effective method for the targeted suppression of specific genes (Wesley *et al*., 2001; Waterhouse and Helliwell, 2003; Chapter 3). The use of hpRNA to reveal phenotypes through RNAi could be increased if expression of hpRNA is inducible. The first construct for chemically inducible RNAi was developed in transgenic *Arabidopsis thaliana* and *Nicotiana benthamiana* plants by Guo *et al*. (2003). In this system, a chemical-inducible Cre/lox recombination system was used to trigger the expression of an intron-containing inverted-repeat RNA. Application of 17-ß-oestradiol drives expression of the CRE recombinase from the O_{lexA}-46 promoter causing site-specific recombination at two *lox* sites and bringing the hpRNA under the control of the G10-90 promoter. The system is stringently controlled and effective at inducing conditional silencing of both a *gfp* transgene and an endogenous *pds* gene and shows no background expression in the absence of the inducer. This silencing system is irreversible and since CRE-mediated excision did not occur in every cell, silenced sectors were formed (Guo *et al*., 2003). A reversible knock-down of the expression of the *Magnesium chelatase subunit 1* (Ch1) and *glutamate 1-semialdehyde aminotransferase* (GSA) genes, which are involved in chlorophyll biosynthesis, was reported by Chen *et al*. (2003). In this ethanol-inducible RNAi system, the alcA promoter did not drive transcription of the hpRNA in the absence of ethanol but upon induction could efficiently silence the target genes. Only a small proportion of primary transformants exhibited an RNAi phenotype (33% for Ch1 and 13% for GSA), but these were stronger than those observed with constitutive expression of the hpRNA (Chen *et al*., 2003). Recently, Wielopolska *et al*. (2005) described an inducible RNAi system, which combined the dexamethasone-inducible pOp6/LhGR promoter system (Craft *et al*., 2005) with the hpRNA cassette derived from the

pHELLSGATE 12 GATEWAY™ silencing vector (Helliwell and Waterhouse, 2003). Again using *PDS* as a target, they showed that a high proportion of primary transformants exhibited RNAi phenotypes following transfer to agar medium containing dexamethasone. The observed phenotypes were comparable to those obtained with the 35S promoter. While *PDS* silencing was reversible after transfer from dexamethasone, the silencing of the *P35S::luciferase* transgene did not recover, although the pOp6/LhGR system was not active. However, it is not clear whether this reflects the difference between silencing a transgene and silencing an endogenous gene or whether it indicates that the siRNA-mediated suppression of target genes is not always reversible. This system showed weak activity in the uninduced conditions, but this small amount of siRNA was not sufficient to give effective gene silencing (Wielopolska *et al*., 2005).

The work described in this chapter was initiated before the first inducible silencing system was published by Guo et al. (2003). The aim was to develop a Cre/lox based approach for the conditional expression of the hpRNA. The hpRNA construct was designed in such a way that the invertedly repeated sequences of the target gene are not transcribed because they are separated from the promoter by a blocking cassette flanked by two *lox* sites. To induce silencing and to express the hairpin construct, activity of the CRE recombinase is required. By conditional or cell type specific expression of the CRE recombinase, conditional or cell type specific silencing can thus be obtained.

6.3 Results

6.3.1 Strategy for CRE-dependent production of hpRNA

The experimental strategy used in this study is described in Figure 6.1. The pBchpGWIWG construct for conditional suppression of plant genes was constructed as a GATEWAY™ compatible vector (Fig.6.7, see Material and Methods), and its ability to trigger silencing of an endogenous gene in a conditional way was tested with a DNA fragment that targets the *Arabidopsis catalase 2* gene, which is the most abundant catalase in the leaves. The last 800 nucleotides of the cat2 coding sequence were used to generate the 3'CS-CAT2 GATEWAY™ entry clone (Fig.6.7a), and subsequently the expression pBchpAT T-DNA vector (Fig. 6.7c). The BchpAT T-DNA contains the Pnos-bar-3'nos expression cassette flanked by two directly repeated *lox* sites, which separates the 35S promoter from a hairpin RNA-encoding cassette.

In this way, the transcription of the hpRNA is dependent on the expression of the CRE recombinase. When the *cre* gene is expressed, recombination will eliminate the Pnos-bar-3'nos expression cassette between the *lox* sites, allowing hpRNA transcription from the 35S promoter (Fig.6.1).

The pBchpAT plant transformation vector was used for transformation of wild type *A.thaliana* plants ecotype Columbia O (ColO) by the floral dip method (Clough and Bent, 1998). The two obtained hpcat2 primary T1 transformants, called FBchpAT1 and FBchpAT2, selected on Murashige and Skoog medium containing 10 mg/l phosphinothricin were grown to maturity and T2 seeds were collected.

To test the efficiency of the CRE-mediated deletion, crosses were performed with plants expressing the CRE recombinase constitutively to presumably high levels (FHSC6, chapter 5), and the catalase activity was compared in wild type plants, hpcat2 transformants and hpcat2 X CRE hybrids. Therefore the catalase activity was measured in the rosette leaves of 23 T2 plants of the hpcat2 transformants FBchpAT1 and FBchpAT2, of 5 wild type *Arabidopsis* plants and of 20 FHSC6 X FBchpAT1 and 10 FHSC6 X FBchpAT2 plants.

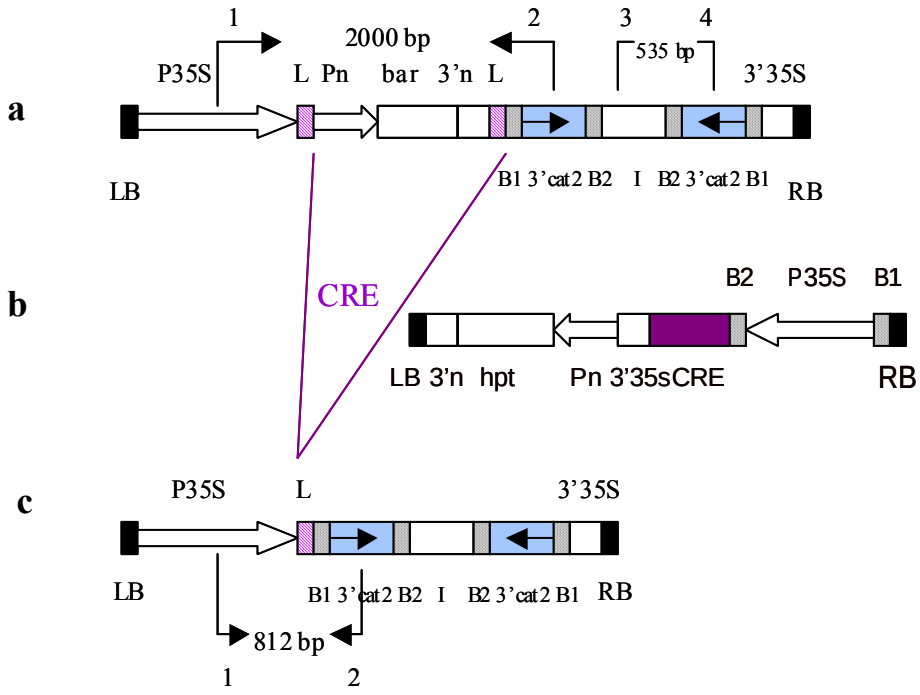

Fig. 6.1 Strategy for CRE-dependent production of hpRNA. a, the hpcat2 BchpAT T-DNA consists of a Pnos-bar-3'nos expression cassette which separates the hpcat2 cassette from the 35S promoter; b, the CRE T-DNA consists of the hygromycin selectable marker and a P35S–cre-3'35S expression cassette; c, The recombined form of the hpcat2 BchpAT T-

DNA where CRE-mediated recombination resulted in a deletion of the Pnos-bar-3'nos expression cassette.
Abbreviations: P35S, cauliflower mosaic virus promoter; 3'35S, 35S terminator; Pn, promoter of the nopaline synthase gene; bar, phophinothricin-N-acetyltransferase coding sequence; 3'n, 3' end of the nopaline synthase gene; 3'cat2, last 800 nucleotides from catalase2 coding sequence; L, lox sequence recognition site of the Cre/lox recombination system; hpt, hygromycin phosphotransferase gene; CRE, cre recombinase; LB, left border; RB, right border; 1, 2, 3, and 4, primers used for PCR analysis.

6.3.2 *Arabidopsis* plants transformed with the pBchpAT constructs show reduced total catalase activity

To determine whether transformation of the wild type *Arabidopsis* plants with the hpcat2 pBchpAT construct had an influence on the expression of the catalase gene, catalase activity was measured in the T2 progeny plants of hpcat2 transformants FBchpAT1 and FBchpAT2. As shown in Fig. 6.1a, the hpcat2 construct was designed in such a way that the blocking Pnos-bar-3'nos cassette should prevent transcription of the hairpin cassette in the absence of the CRE recombinase. Thus, the catalase activity in the hpcat2 transformants was expected to be comparable to the catalase activity measured in the wild-type *Arabidopsis* plants. However, T2 progeny plants of both hpcat2 FBchpAT1 and FBchpAT2 transformants showed an average total residual catalase activity of 22% and 19%, respectively (Fig. 6.3). These results indicate that the hpcat2 construct is able to trigger silencing of the target gene even with a *bar* gene of 1200 bp separating the 35S promoter from the hairpin cassette.
To investigate whether the efficiency of silencing could be increased by the CRE-mediated excision of the Pnos-bar-3'nos blocking cassette, the catalase activity assay and the PCR analysis were performed on the F1 hybrid plants derived from crosses between a CRE-expressing plant (T2 progeny plants of the FHSC6 transformant; Table 5.1, Chapter 5) and the progeny plants of both hpcat2 transformants. F1 seeds were germinated on medium selective for the CRE T-DNA, while the presence of the hpcat2 cassette was checked by PCR with the 3 / 4 primer set (Fig.6.1a). Protein extracts and DNA were prepared from rosette leaves of F1 hybrid plants derived from both crosses. PCR analysis was performed to examine the excision status of the Pnos-bar-3'nos expression cassette. For this analysis, primers amplifying a fragment that is either specific for the excised sequences or for the parental T-DNA were used (Fig.6.1). The 1 / 2 primer pair was expected to yield a PCR fragment of 2000 bp for a non-recombinant T-DNA (Fig.6.1a), while following CRE recombination and

deletion of the Pnos-bar-3'nos expression cassette, the 1 /2 primer pair should produce a PCR product of 812 bp (Fig.6.1 c). With the 3 / 4 primer set a PCR fragment of 535 bp was amplified in 13 out of 20 F1 hybrid plants derived from the FHSC6 X FbchpAT1 cross, indicating the presence of the hpcat2 cassette (Fig.6.2a). The total residual catalase activity, measured in the extracts prepared from these 13 hybrids was ranging from 1% to 48%. The 1 / 2 primer pair produced in 12 of the 13 F1 hybrids a 812 bp fragment, indicating an efficient CRE-mediated excision of the Pnos-bar-3'nos blocking cassette in them (Fig.6.2b). These 12 F1 hybrid plants showed an average total residual catalase activity of 6% as compared to wild type plants and 50% as compared to the parental hpcat2 transformant, indicating some decrease in catalase activity after the expression of the CRE recombinase (Fig. 6.3). In parallel, the same analysis was performed on 10 F1 hybrid plants derived from the FHSC6 X FBchpAT2 cross. Whereas in all of them efficient CRE-mediated deletion of the Pnos-bar-3'nos expression cassette occurred (Fig.6.2c), an average total residual catalase activity was in the same range as the catalase activity detected in the parental FBchpAT2 plants (Fig. 6.3). From this we can conclude that although the CRE-mediated excision of the Pnos-bar-3'nos blocking cassette occurred rather efficiently in the F1 hybrids derived from both crosses, it only resulted in a small further decrease in total residual catalase activity in the F1 hybrid plants derived from the FHSC6 X FBchpAT1.

To analyse the catalase activity in more hpcat2 transformants, a second transformation series by the floral dip method with the pBchpAT construct was performed. Of in total 30 tested T1 FBchpAT transformants, 28 showed a total residual catalase activity of 1 to 20% compared to total catalase activity in wild type *Arabidopsis* plants, further supporting the observation that the hpcat2 construct is quite efficient in triggering silencing of the catalase gene (Fig.6.4). The remaining 2 out of 30 hpcat2 primary transformants FBchpAT50 and 51, showed an increase in the total catalase activity, which could indicate that the blocking *bar* gene containing cassette was efficient in these two transformants (Fig.6.4). However, while the *bar* gene was present, the hpcat2 cassette was not. By PCR analysis with the 3 / 4 primer pair, which is specific for the hairpin cassette (Fig. 6.1a), the band of 535 bp could not be amplified (data not shown). This indicates that the hairpin cat2 cassette was not present, probably due to truncation of the hpcat2 T-DNA. We can conclude that the suppression of the catalase gene occurred in all hpcat2 FBchpAT *Arabidopsis* transformants with the intact hpcat2 T-DNA even before the *cre* gene was introduced.

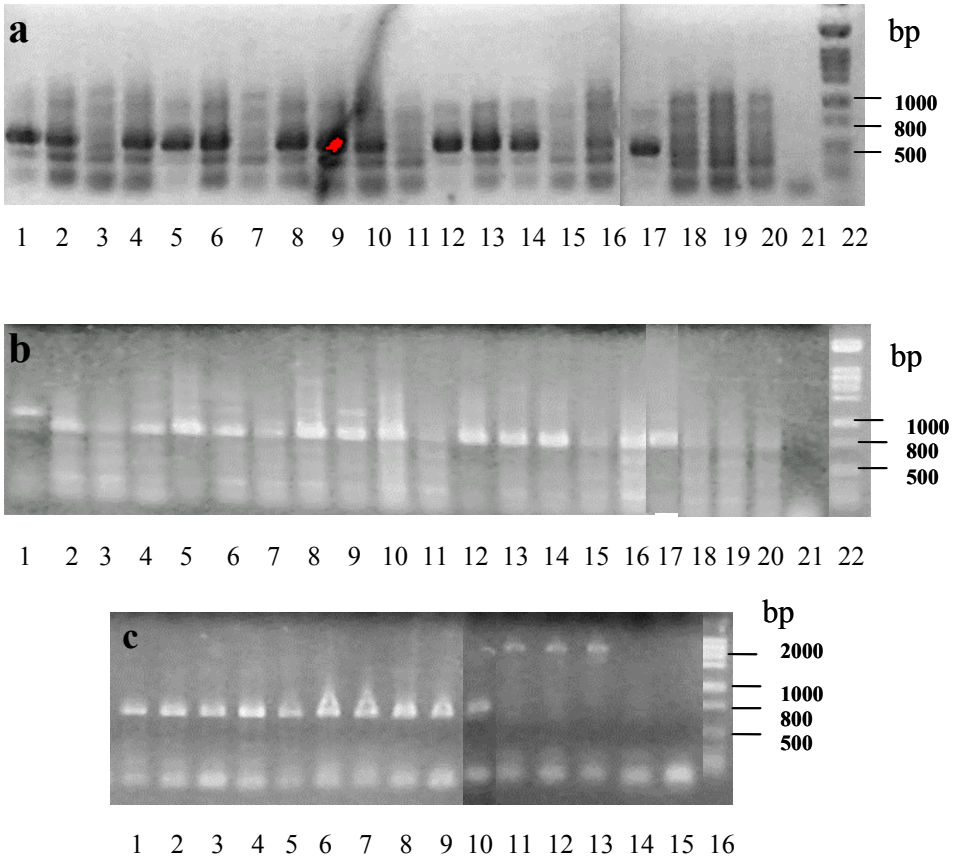

Fig. 6.2 PCR analysis on genomic DNA of F1 hybrid plants derived from FHSC6 X FBchpAT1 and FHSC6 X FBchpAT2 cross. (a) PCR analysis on genomic DNA prepared from 20 F1 hybrid plants derived from FHSC6 X FBchpAT1 cross. PCR reaction with the 3 / 4 primer pair and (b) with the 1 / 2 primer pair. Lane 1-20, F1 hybrid plants, lane 21, WT ColO. (c) PCR analysis on genomic DNA prepared from 10 F1 hybrid plants derived from FHSC6 X FBchpAT2 cross (lane 1-10) and T2 progeny plants of the hpcat2 FBchpAT2 transformant (lane 11-13). PCR reaction was performed with the 1 / 2 primer pair. Lane 14, WT ColO, lane 15, H_2O. Primers 1, 2, 3, and 4 were used as illustrated in Fig.6.1.

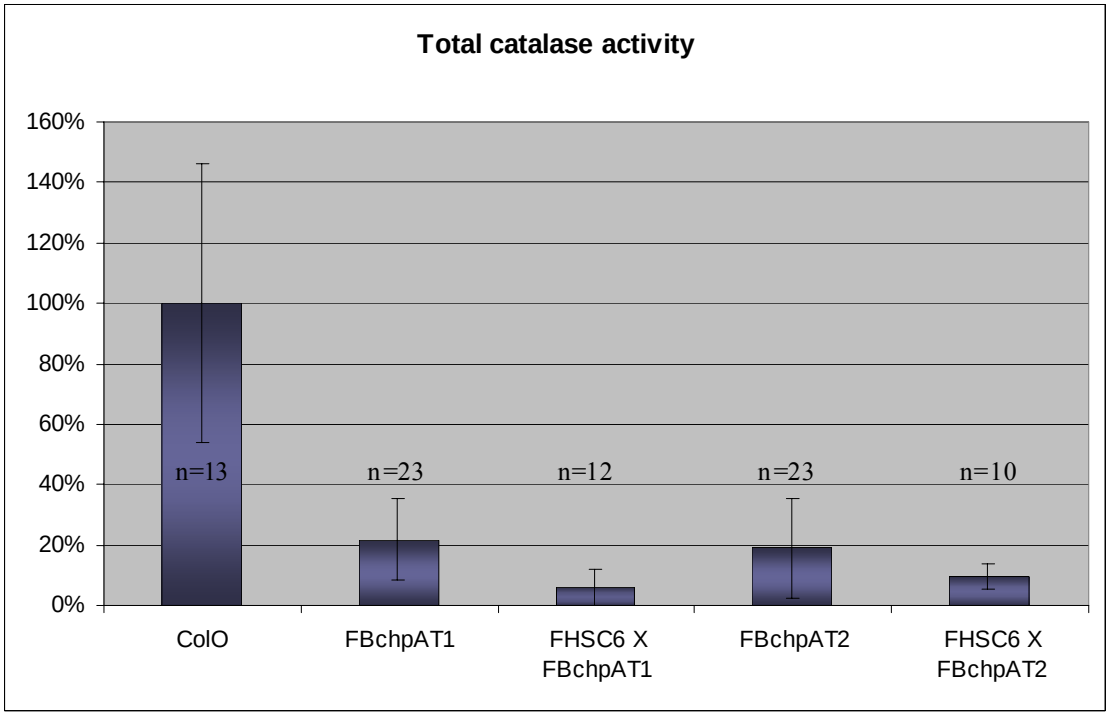

Fig. 6.3 Total catalase activity in the WT *Arabidopsis thaliana* plants (ColO), in T2 progeny of the hpcat2 FBchpAT1 and FBchpAT2 transformants and in the F1 hybrid plants derived from FHSC6 X FBchpAT1 and FHSC6 X FBchpAT2 cross. Catalase activity in protein extracts of leaf tissue was measured with a spectrophotometric assay. The total catalase activity in T2 progeny plants of the hpcat2 FBchpAT1 and FBchpAT2 transformants and F1 hybrids derived from FHSC6 X FBchpAT1 and FHSC6 X FBchpAT2 cross is relative to the average catalase activity of 13 wild type (ColO) *Arabidopsis* plants which is set at 100%**.** Error bars represent standard deviation; n, number of ColO, T2 and F1 progeny plants analysed.

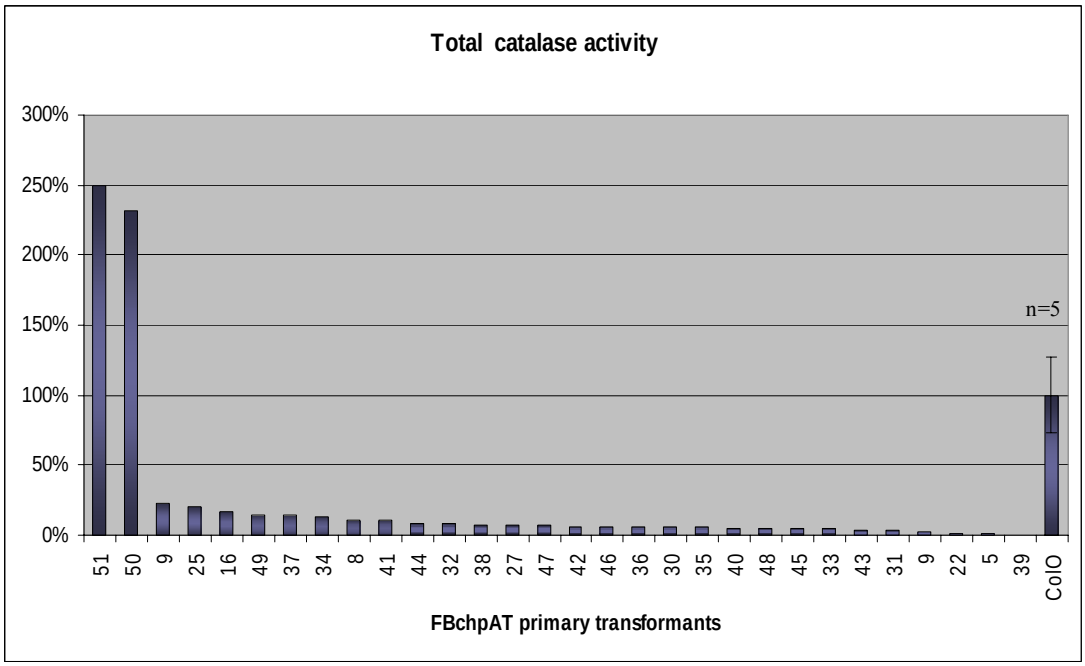

Fig. 6.4 Total catalase activity in wild type and transgenic T1 *Arabidopsis* plants containing the pBchpAT construct. Catalase activity in protein extracts of leaf tissue was measured with a spectrophotometric assay. The total catalase activity of 30 FBchpAT primary transformants is relative to the average catalase activity of five wild type (ColO) *Arabidopsis* plants which is set at 100%**.** Error bars represent standard deviation; n, number of ColO plants analysed.

6.2.3 Catalase activity in *Arabidopsis* plants transformed with the cat2 hairpin construct without the promoter

To investigate whether the hairpin cassette without an upstream promoter is able to induce silencing of the *catalase* gene, we constructed a promoterless cat2 hairpin T-DNA (pBhpATL, Fig. 6.5). This T-DNA was introduced into wild type *Arabidopsis* plants by the floral dip method (Clough and Bent, 1998). Catalase activity was measured in extracts prepared from rosette leaves of 40 independent FBhpATL *Arabidopsis* primary transformants selected on medium containing 10mg/l phosphinothricin. The results are presented in Fig. 6.6. The total catalase activity in these transformants varied from 11 to 219% compared to the total catalase activity in wild type *Arabidopsis* plants. Since 18 out of the 40 FBhpATL T1 transformants (45%) showed total catalase activity that was in the range of the catalase activity measured in the wild type *Arabidopsis* plants and 16 out of these 40 (40%) even higher, we conclude that promoterless hpcat2 construct is not able to induce silencing of the endogenous catalase gene. However, the reason for the observed variations between different FBhpATL T1 transformants is not known. This is in contrast with the results obtained with the pBchpAT construct, which clearly demonstrated suppression of the endogenous *catalase* gene in all transformants.

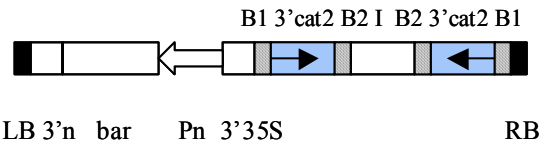

Fig. 6.5 Schematic representation of the pBhpATL expression vector.

Abbreviations: 3'35S, 35S terminator; Pn, promoter of the nopaline synthase gene; bar, phophinothricin-N-acetyltransferase coding sequence; 3'n, 3' end of the nopaline synthase gene; 3'cat2, last 800 nucleotides from catalase2 coding sequence; LB, left border; RB, right border**.**

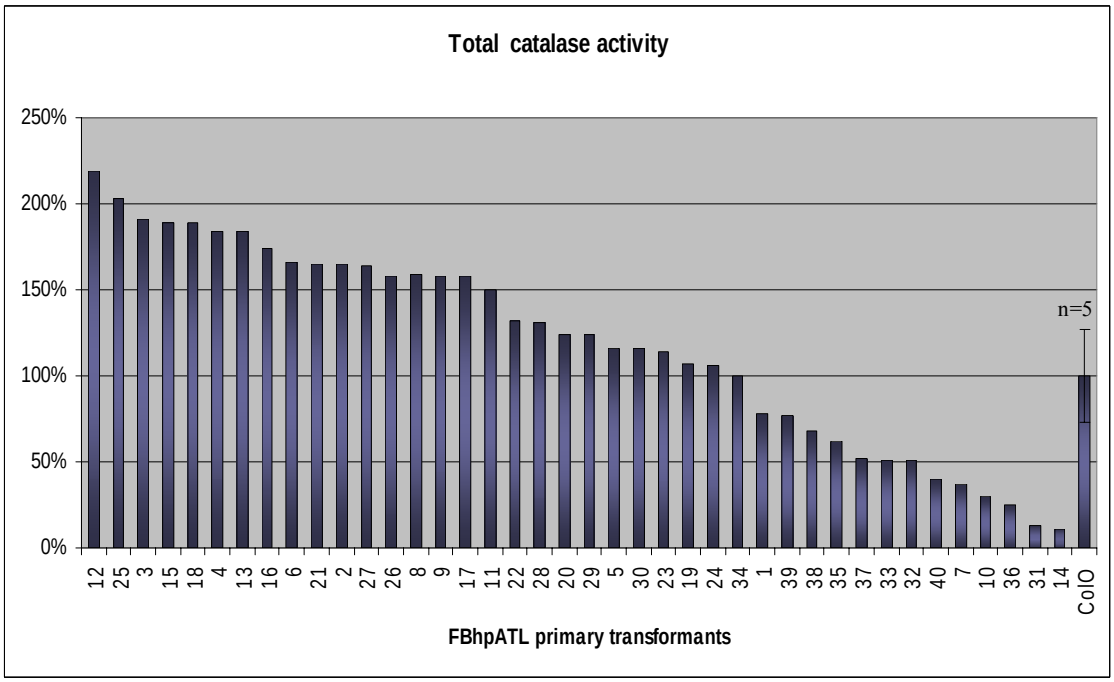

Fig. 6.6 Total catalase activity in wild type and transgenic T1 *Arabidopsis* plants containing the pBhpATL construct. Catalase activity in protein extracts of leaf tissue was measured with a spectrophotometric assay. The total catalase activity of 40 FBhpATL primary transformants is relative to the average catalase activity of five wild type (ColO) *Arabidopsis* plants which is set at 100%**.** Error bars represent standard deviation; n, number of ColO plants analysed.

6.3 Discussion

In this work, the pBchpGWIWG vector, constructed for conditional suppression of plant genes, was tested using the endogenous *Arabidopsis catalase2* gene. The vector is designed in such a way that the catalase hairpin cassette should be transcriptionally silent because it is separated from the 35S promoter by the *lox* flanked Pnos-bar-3'nos cassette. In this respect, silencing of the *catalase2* gene was not expected to occur before the introduction of the CRE recombinase, which would mediate the recombination at the DNA level resulting in the

excision of the blocking cassette. In this irreversible conditional gene silencing system, the cat2 hpRNA would be produced from the 35S promoter that has been shown to be an efficient way of inducing gene silencing (Hilson *et al*., 2004; Wesley *et al*, 2001; Guo *et al*., 2003; Chuang and Meyerowitz, 2000; Chapter 3). Examination of the catalase activity in the T1 plants containing the FBchpAT T-DNA showed a 3 to 10-fold reduction in total catalase activity in all tested transformants. This suggests that the hpcat2 cassette in the pBchpAT vector is able to trigger silencing of *catalase2* endogene without being transcribed. However, when a promoterless hpcat2 construct was transformed into wild type *Arabidopsis* plants, 85 % (34 out of 40) of the transformants showed catalase activity that was similar or even higher as compared with the total catalase activity in the wild type plants. Although a reduction in catalase activity level was observed in 6 out of 40 transformants with the promoterless hpcat2 T-DNA (15%), these results indicate that transcription of the hpcat2 cassette is probably required for efficient suppression of the target gene. We postulate that the most likely cause of *catalase* suppression in the pBchpAT transformants is readthrough transcription from the 35S or Pnos promoter beyond the 3'nos transcription termination into the hpcat2 cassette (Fig. 6.1a).

Many cases of PTGS have been associated with loci carrying T-DNAs integrated as inverted repeats (IRs) (De Buck *et al*., 2001; Depicker *et al*., 1996; Stam *et al*., 1997; Van Houdt *et al*., 2000). In all these cases transcription of invertedly repeated genes led to the production of dsRNAs, which are potent triggers of gene silencing (Fire *et al*., 1998; Waterhouse *et al*.,1998; Chuang and Meyerowitz, 2000). Even promoterless constructs can induce silencing of the homologous gene when integrated as inverted repeat (IR) and transcribed at a very low level (Van Blokland *et al*., 1994; Stam *et al*., 1998). In the work of Van Blokland *et al*. (1994), a chsA cDNA was placed behind the *nptII* gene of the T-DNA vector, which contained a nopaline synthase polyadenylation or termination signal. With this construct, 15 independent transformants were obtained and the flowers of three transformants showed a reduced pigmentation. These three transformants contained a locus carrying T-DNAs integrated as inverted repeats (IRs) (Stam et al., 1997). The promoterless *chs* sequences of the IR were transcribed at a low level, probably by readthrough transcription of the *nptII* gene (Muskens et al., 2000). This has been taken as evidence that a little amount of dsRNA is enough to trigger degradation of complementary RNAs. Accordingly, Lechtenberg *et al*. (2003) did not see evidence for gene silencing associated with IR T-DNA structures in lines unlikely to produce read-through transcripts as the transgenes were located at a considerable distance from the RB and as two sets of polyadenylation signals downstream of the transgenes

were used. On the other hand, an inducible RNAi system, based on the dexamethasone-inducible pOp6/LhGR promoter system showed weak activity in uninduced conditions, but this small amount of siRNA was not sufficient to give effective gene silencing (Wielopolska *et al*., 2005).

The presence of the hpcat2 pBchpAT construct results in silencing most likely due to inability of the 3' end of the nopaline synthase gene to efficiently terminate the transcription of the 35S and/or Pnos-bar-3'nos expression cassette. The introduction of the *P35S::cre* transgene into the hpcat2 transformants had little impact on the expression level of the *catalase* gene in the leaves, although the Pnos-bar-3'nos blocking cassette was efficiently removed.

In conclusion, the *lox* flanked blocking cassette in the pBchpAT construct was shown to be leaky to block expression from the 35S and/or the Pnos promoter, as the endogenous *catalase* gene was efficiently silenced in all hpcat2 *Arabidopsis* transformants. Other experiments are required to obtain evidence for the proposed readthrough transcription and production of the hpcat2 RNA in the absence of the CRE recombinase. Also, other endogenous target genes should be tested, as each target sequence possesses an inherent degree of susceptibility to RNAi (Kerschen *et al*., 2004). Not only endogenous transcript accumulation of the target gene but also sequence composition, spatial and temporal gene expression patterns and the normal RNA turnover rate was shown to be target specific determinant of RNAi (Kerschen *et al*., 2004).

6.4 Material and methods

Plasmid construction

The pBchpGWIWG construct is shown in figure 6.7b. The vector contains the 35S promoter, which is separated from the hairpin cassette by the *lox* flanked *bar* gene fused to the Pnos promoter and the 3'nos terminator of the *Agrobacterium* nopaline synthase gene. The *lox* flanked Pnos-bar-3'nos cassette and the 35S promoter were first cloned into the pGEM-5Zf(+) vector. The cloning was as follows: The 1200 bp Pnos-bar-3'nos fragment, isolated after EcoRV digestion of the Pnos-bar-3'nos plasmid (provided by dr.Mansour Karimi) and purified from gel using the QIAquick Gel Extraction Kit Protocol, was cloned into the EcoRV site of the pGEM-5Zf(+) vector, resulting in the intermediate construct pGEMbar. Proper orientation of the Pnos-bar-3'nos cassette was confirmed by restriction digest. Next,

the Pnos-bar-3'nos fragment was flanked by two *lox* sites in direct orientation. The *lox* sites were inserted as synthetic oligonucleotides. The NotI-SpeI restriction sites (underlined) flanked the first *lox* sequence: sense strand, GGCCGCATAACTTCGTATAATGTATGCTATACGAAGTTATA and complementary strand CTAGTATAACTTCGTATAGCATACATTATACGAAGTTATGC. This *lox* sequence was ligated into the same sites of the pGEMbar plasmid, yielding the pGEMloxbar plasmid. The second *lox* sequence was flanked with the NcoI restriction site at 5' end of the *lox* sequence, whereas the restriction sites HindIII, EcoRI, PmlI, and ApaI (underlined) were located at the 3' end: sense strand CATGGATAACTTCGTATAATGTATGCTATACGAAGTTATCCCAAGCTTGGGGGAA TTCCCACGTGGGGCC and comlementary strand CCACGTGGGAATTCCCCCAAGCTTGGGATAACTTCGTATAGCATACATTATACGA AGTTATC. This *lox* site was subsequently ligated into the NcoI – ApaI restriction sites of the pGEMloxbar plasmid, yielding in pGEMloxbarlox plasmid. The P35S sequence was amplified by PCR using pXD610 DNA (De Loose et al., 1995) as a template. P35S specific primers, which include the SacI and NotI restriction sites (underlined), were: forward P35S5 specific primer 5' CGAGCTCGCCTTTGCCCCGGAGATCACCAT3' and reverse P35S3 specific primer 5' ATTTGCGGCCGCTTTAATAGTAAATTGTAATGTTGT3'. This P35S PCR product was purified from gel (Qiaquick gel extraction kit), digested with SacI and NotI and cloned into the pGEMloxbarlox vector that had been digested with the same enzymes, and the resulting plasmid was named pGEMP35Sloxbarlox.

In parallel, the hairpin cassette, which consists of two GATEWAY^{TM} cassettes in inverted orientation separated by an intron was constructed using the plasmids pI, pWG7 and pGW7, provided by dr. Mansour Karimi. The construction was as follows: the intron sequence (650bp) was isolated from the pI plasmid as an SacII-NdeI fragment. The WG cassette was isolated as an NdeI-ApaI fragment (1940 bp) from the pWG7 plasmid. These two isolated fragments were introduced into the pGW7 plasmid that was digested with ApaI and SacII enzymes. This three-fragment ligation resulted in the pGEMGWIWGT35S vector. In order to obtain a plant transformation vector, which would contain the P35Sloxbarlox and the GWIWGT35S fragments from the two plasmids described above, the pPZP200 binary vector (Hajdukiewicz et al., 1994) was modified for cloning purposes by inserting the ApaI restriction site within the T-DNA borders. The oligo, which contains HindIII, ApaI, and XbaI restriction sites, was inserted into the pPZP200 vector that was digested with the HindIII and XbaI enzymes, and the resulting modified pPZP200 binary vector was named pPZP200I.

The pGEMP35Sloxbarlox plasmid was digested with the SacI-PmlI restriction enzymes and P35Sloxbarlox fragment was purified. Similarly, the GWIWGT35S fragment was isolated from the pGEMGWIWGT35S plasmid, which was digested with the ApaI-Ecl136II restriction enzymes. These two fragments were introduced into the pPZP200I binary vector, which was digested with the SacI-ApaI restriction enzymes. This three-fragment ligation resulted in the pBchpGWIWG destination vector (Fig. 6.7b).

800 bp of the 3' end of the *catalase2* coding region were amplified by PCR using the 2 sets of primers to form *att*B1 and *att*B2 recombination sites. In the first step, template specific primers containing 12 bases of *att*B sites were used in 30 cycles of PCR to amplify the target sequence. PCR conditions were as follows: initial denaturation at 94^0C for 5 min, followed by 30 cycles of: denaturation at 94^0C for 1 min, annealing at 55 ^{0}C for 1 min and elongation at 68 ^{0}C for 1.30 min; and a final elongation step at 68 ^{0}C for 15 min. This product was subsequently used as a template in the second PCR reaction with universal *att*B adapter primers to amplify the full *att*B1 and *att*B2 recombination sites (Invitrogen) following the protocol described in the instruction manual. Primers used were: forward template specific primer 5'AAAAAGCAGGCTGGAAACCAACTTGTGGAGTC 3', reverse template specific primer 5'AGAAAGCTGGGTAGATGCTTGGTCTCACGTTC 3', attB1 adapter primer 5' GGGGACAAGTTTGTACAAAAAAGCAGGCT 3, attB2 adapter primer 5'GGGGACCACTTTGTACAAGAAAGCTGGGT 3'.

To obtain the 3'CS-CAT entry clone (Fig. 6.7a), the PCR product flanked by *att*B sites was recombined into pDONR 201- Km^r vector containing *att*P1 and *att*P2 recombination sites in the BP reaction using BP Clonase enzyme (Invitrogen).

The expression vector pBchpAT (Fig. 6.7c) was generated in the LR reaction where 3'CS-CAT entry clone was incubated with the pBchpGWIWG destination vector in the presence of LR Clonase enzyme (Invitrogen). The orientation of the intron after double LR reaction was determined by restriction digest.

The promoterless hairpin expression vector containing the last 800 nucleotides of the catalase2 coding region (pBhpATL, Fig. 6.5), was generated in the LR reaction where 3'CS-CAT entry clone was incubated with the pBGWIWGL destination vector in the presence of LR Clonase enzyme (Invitrogen). The orientation of the intron after double LR reaction was determined by restriction digest. The pBGWIWGL destination vector was provided by dr. Mansour Karimi.

Transformation vectors pBchpAT and pBhpATL were introduced by electroporation into *Agrobacterium tumefaciens* strain C58C1Rif^{R}, containing the pMP90 vir plasmid (Koncz, C. and Schell, J. 1986).

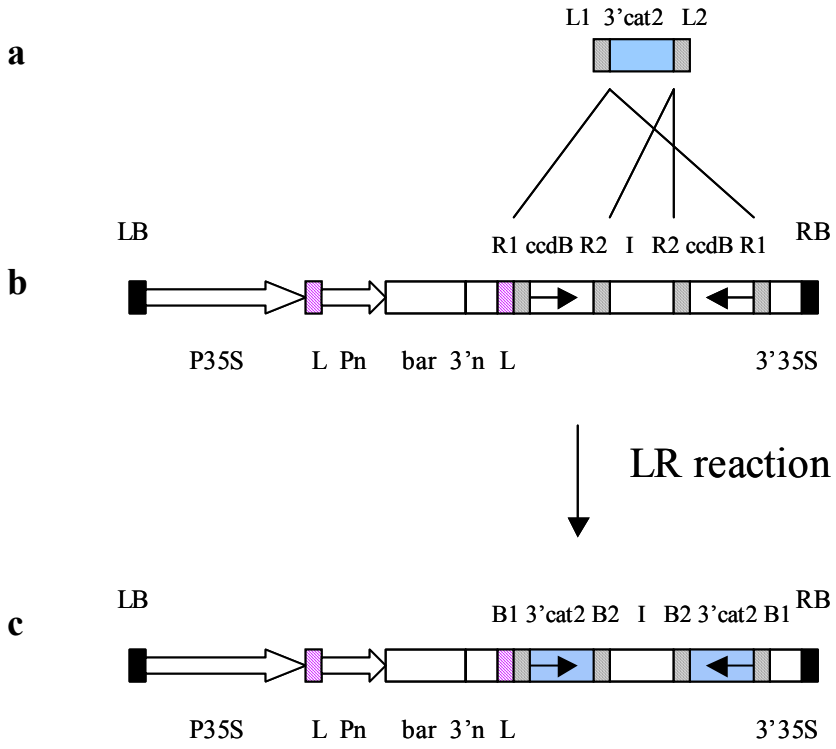

Fig. 6.7 Schematic representation of the pBchpAT vector. (a) A PCR product was amplified from the *catalase 2* gene of *Arabidopsis thaliana* with attB1 and attB2 sites incorporated into the PCR primers. This product was inserted into the pDONR201 vector by recombination between attB1/attB2 and attP1/attP2 sites mediated by BP Clonase enzyme (Invitrogen) to yield the 3'CS-CAT-entry clone. (b) LR reaction in which the 3'CS-CAT entry clone was incubated with the GATEWAY^{TM}-compatible binary T-DNA destination vector pBchpGWIWG in the presence of LR Clonase enzyme (Invitrogen), resulted in the expression vector pBchpAT (c). The attB3'CS-CAT cassette forms the arms of the hairpin. When the construct is expressed in plants a hairpin RNA (hpRNA) with the intron spliced out is produced.
Abbreviations: P35S, cauliflower mosaic virus promoter; 3'35S, 35S terminator; Pn, promoter of the nopaline synthase gene; bar, phophinothricin-N-acetyltransferase coding sequence; 3'n, 3' end of the nopaline synthase gene; L, *lox* sequence recognition site of Cre/lox recombination system; ccdB, bacterial negative selection marker; 3'cat2, last 800 nucleotides from catalase2 coding sequence; LB, left border; RB, right border. The LR reaction is the recombination reactions between the attL and attR elements.

Plant material and crosses

Transformation of the wild type *Arabidopsis thaliana* plants ecotype Columbia O with the hpcat2 pBchpAT and pBhpATL constructs was performed by the floral dip method, as described by Clough and Bent (1998). Seeds of the dipped plants were harvested and sown on medium supplemented with phosphinothricin (10 mg/l) for the selection of hpcat2 FBchpAT and FBhpATL transformants. Crosses of T2 CRE-expressing progeny plants (FHSC6, Chapter 5) with the T2 progeny plants of the hpcat2 FBchpAT1 and 2 transformants were performed with the CRE-expressing plants as the male parent. The resulting hybrid seeds were grown on medium selective for the presence of the CRE T-DNA (hygromycin). The presence of the hpcat2 T-DNA was checked by PCR, since the selectable marker (bar) is excised from the F1 hybrids by CRE-mediated recombination. All plants were grown under a 16 h light / 8 h dark regime at 21^0C.

Catalase activity assay

Protein extracts were prepared from 2-3 rosette leaves of, frozen in liquid nitrogen, by grinding in 120µl extraction buffer (60mM Tris-HCl (pH 6.9), 1 mM PMSF, 10 mM DTT, 20% glycerol). The homogenate was centrifuged twice at 4^0C for 10 min to remove insoluble material. The supernatant was used for spectrophotometric catalase analysis according to Clare *et al*. (1984) after determining the total amount of soluble protein with the BioRad Protein Assay (Bradford, 1976) using bovine serum albumin as a standard.

PCR analysis

Genomic DNA was prepared from 2-3 rosette leaves of 13 wild type *Arabidopsis* plants (ecotype ColO), from 2-3 rosette leaves of 23 T2 progeny plants of both hpcat2 FBchpAT1 and FBchpAT2 transformants, and from 2-3 rosette leaves of 20 and 10 F1 hybrids from crosses of CRE-expressing plants and T2 progeny plants of hpcat2 FBchpAT1 and hpcat2 FBchpAT2 transformants, respectively, according to the protocol for DNA purification from 10-30 mg frozen plant tissue (Puregene^{TM} DNA Purification system, Gentra). PCR analysis was performed using 50ng of DNA and primers specific for the catalase hairpin cassette

(GWIN2R (3)/ G7 (4)) and non-excised and excised fragment (G2 (1)/ G5(2)). The primers used were: primer1: 5' ATTCCATTGCCCAGCTATCT 3', primer2: 5' ATTGCAGGACAGAAAGCAAGTT 3', primer3: 5'CTTGATGGTTGAATAAGGTGGC 3', and primer4: 5' GCATGAAATCCGCAGTATCT 3'. The PCR reaction conditions were as follows: initial denaturation at 94^0C for 5 min, followed by 30 cycles of: denaturation at 94^0C for 50 s, annealing at 53^0C for 50 sec and elongation at 72^0C for 1 min; and a final elongation step at 72^0C for 5 min.

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CHAPTER 7

Summary

7.1 Summary

Double-stranded RNA (dsRNA) induces sequence-specific inhibition of gene expression at a post-transcriptional level in most eukaryotic organisms such as plants, animals, and fungi. This natural phenomenon which has evolved to counter the proliferation of foreign sequences, such as transposable elements (TEs) and viruses, has been developed into a tool for studying gene function in several model organisms, including plants. The most efficient method to suppress a plant gene by post-transcriptional silencing is to express a hairpin RNA (hpRNA) made of a double-stranded stem with homology to the transcript targeted for degradation (Waterhouse *et al*., 1998; Chuang and Meyerowitz, 2000; Smith *et al*., 2000; Levin *et al*., 2000; Wesley *et al*., 2001; Stoutjesdijk *et al*., 2002).

The objective of this work was to construct an hpRNA silencing vector, which would allow down-regulation of endogenous plant gene expression in a conditional way. The conditional silencing approach we designed is based on the Cre/lox recombination system, allowing to switch the expression of the hpRNA by conditional expression of the CRE recombinase. Thus, the hpRNA encoding cassette was separated from the 35S promoter by a *lox* flanked blocking sequence containing the selectable marker of the T-DNA. Upon expression of the CRE recombinase, which mediates the excision of a *lox* flanked blocking sequence, expression of the downstream hpRNA is permanently activated. We anticipated that the efficient expression of the hpRNA and subsequent down-regulation of a plant target gene would depend on (1) the tissue-specificity and inducibility of the *cre* regulating promoter, (2) the efficiency of the CRE-mediated excision of the *lox* flanked DNA fragment and (3) the efficiency of the 35S driven hpRNA-mediated suppression. Therefore, we analysed each of these events in separate model systems.

As it is not clear whether hpRNA-mediated gene suppression can efficiently occur in all tissues, and especially not in highly proliferating cells (Mitsuhara *et al*., 2002; Corrêa *et al*., 2004; Chuang and Meyerowitz, 2000), the efficiency of 35S driven hpRNA-mediated suppression was evaluated for a reporter *gus* gene that was under the control of different promoters (Chapter 3). Therefore, a hairpin construct was generated containing the last 792 nucleotides of the *gus* coding sequence (hpUS) in the pH7GWIWG2 vector, designed for cosuppression of plant endogenes (Karimi *et al*., 2002). This construct was transformed in plants containing a *gus* gene driven by different promoters.

We showed that a P35S driven hairpin construct produces a series of independent *Arabidopsis* transformants with different degree of silencing of a 35S driven *gus* gene. In the weakly

suppressed transformants, the silencing of a P35S driven *gus* gene was not uniform. Whereas efficient suppression of the 35S driven *gus* gene occurred in the expanding leaf and root tissues, P35S driven hairpin silencing of the 35S driven *gus* gene was less efficient in the tissues containing highly proliferating cells**.** The fact that the variations of efficiency of *gus* suppression in different hpUS transformants were mostly observed in the meristems could mean that the meristematic tissues are more sensitive to the level of hpRNA expression. Another explanation for the lack of complete silencing in meristematic cells such as root tips and callus, is that in these cells the balance between translation and hpRNA-mediated degradation of the *gus* mRNA is shifted towards expression. This could be due to a more active recruitment of the mRNAs into the ribosomes. In strongly suppressed hpUS transformants, all progeny plants showed dramatically reduced levels of a *gus* target gene in all tissues. When the same construct was combined with a *gus* gene driven by the CycB1;1, CycD4;1and KRP4 promoters, only a limited effect of the hpUS-mediated GUS suppression was observed. As the CycB1;1, CycD4;1 and KRP4 promoters express in cells in which active cell division occurs, we postulate that silencing is less efficient in meristematic cells. An alternative explanation for the less efficient hpUS-mediated suppression of the *gus* gene in the meristems could be a differential activity of the P35S promoter used to drive the expression of the hpUS construct as compared to the tissue-specific expression patterns of the CycB1;1, CycD4;1, and KRP4 promoters in these tissues. In line with the previous observations, an efficient suppression of the WRKY23 promoter driven *gus* gene was observed in the expanding root tissues, but again, tissues containing highly proliferating cells such as root tip and young leaves and flowers showed less profound hpUS-mediated suppression.

Chapter 4 describes the experiments that were carried out to test the inducibility and the organ specificity of the Gmhsp 17.6L soybean heat-shock promoter in *Arabidopsis thaliana*. We chose this promoter to drive the *cre* gene because it was previously shown that it is efficiently induced by a heat-shock treatment (Kilby et al., 2000). For the analysis, the Gmhsp 17.6L soybean heat-shock promoter was cloned in front of the *gfp-gus* fusion reporter gene. The basal level, organ specificity and the strength of the Gmhsp 17.6L soybean heat-shock promoter was determined and compared with the strength of the commonly used P35S and Pnos promoters, cloned in the same vector in front of the *gfp-gus* sequence. The results showed that the induction of the soybean heat-shock promoter could be used to obtain heat-regulated transcription in *Arabidopsis*, that there was very little background expression in

uninduced conditions and that the induced transgene expression levels were similar to those of the Pnos promoter, but 100 to 300-fold lower than the P35S promoter.

The ability of the CRE recombinase to catalyze the excision of any fragment of DNA flanked by directly repeated *lox* sites has been exploited to modify gene expression in transgenic plants (Odell *et al*.,1990; Guo *et al*., 2003; De Buck *et al*., 2001). In Chapter 5, we evaluated the efficiency of the CRE-mediated recombination. The CRE activity was assessed based on the excision efficiency of a *gus* gene between in tandem oriented *lox* sites in transgenic *Arabidopsis thaliana*. Two different methods of combining the *lox* flanked *gus* gene and the *P35S::cre* gene were evaluated, namely transformation and genetic cross. Following transformation of CK_2L6 plants with the *P35S::cre* construct, a different excision efficiency was observed among different independent transformants. One out of ten independent CK_2L6-SC transformants gave 100% GUS-negative progeny. The majority of transformants, however, showed a chimeric pattern with the *gus* gene removed from most of the leaf cells but not from the meristematic regions. The variations in excision efficiencies are probably related to the different expression level of the CRE recombinase, caused by the different number and structure of the CRE loci in these transformants. There was no obvious difference in recombination efficiency when the CRE-expressing plants were used as female or male parent in the cross, indicating that the CRE activity in the female gametocyte is either absent or not sufficient for CRE-mediated recombination early upon fertilization. No CRE-mediated excision was observed when *cre* was expressed by the Pnos or the Gmhsp 17.6L soybean heat-shock promoter. The fact that the complete CRE-mediated recombination was achieved only with the CRE recombinase regulated by the strong P35S promoter, suggests that higher expression levels of the CRE recombinase are required. We postulate that the low efficiency of the CRE-mediated recombination regulated by the weak Pnos and the heat-inducible promoter as compared with the strong P35S promoter, is due to the low expression level of the CRE recombinase.

Evaluation of the pBchpAT conditional gene silencing vector is described in Chapter 6. The ability of this construct to trigger silencing of an endogenous gene in a conditional way was tested with a hairpin construct that targets the *Arabidopsis catalase 2* gene, which is the most abundant catalase in the leaves. Therefore, the catalase levels were measured in several transformants with the hairpin construct separated from the 35S promoter by a 1200 bp sequence. Surprisingly, we found that pBchpAT construct was able to silence the *catalase* gene in all obtained hpcat2 transformants. This could be due to ability of the hairpin construct to trigger silencing without being transcribed, or due to readthrough transcription

from the 35S promoter across the blocking cassette. Therefore, it was evaluated whether a promoterless hairpin construct also induces silencing. However, this was found not to be the case. Thus, the presence of the 35S promoter 1200 base pairs upstream of the hairpin is sufficient to mediate low levels of hpRNA expression, resulting in 3 to 10-fold reduction of the hairpin homologous *catalase* endogenes. Upon CRE-mediated deletion of the *lox* flanked blocking cassette, the 35S promoter was fused directly to the cat2hpRNA cassette, resulting in a further 2 to 3-fold reduction of endogene *catalase* expression. We conclude that the conditional gene silencing system needs to be further improved, with respect to the background expression of the hpcat2 cassette and then it will become a valuable tool for conditional suppression of plant genes.

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CHAPTER 8

Samenvatting

8.1 Samenvatting

Dubbelstrengig RNA (dsRNA) induceert sequentie-specifieke remming van gen expressie op een post-transcriptioneel niveau in de meeste eukaryotische organismen zoals planten, dieren en schimmels. Dit natuurlijk fenomeen, dat zich ontwikkeld heeft om de proliferatie van vreemde sequenties zoals transposons (TEs) en virussen tegen te gaan, is verder ontwikkeld als een middel voor de studie van genfuncties in meerdere model organismen, inclusief planten. De meest efficiënte methode om een plantengen via post-transcriptionele silencing te onderdrukken, is in transgene individuen een haarspeld RNA (hpRNA) tot expressie te brengen. Dit haarspeld RNA is gemaakt uit een dubbelstrengige stam waarvan de sequentie homoloog is met het transcript dat dient gedegradeerd te worden (Waterhouse *et al*., 1998; Chuang and Meyerowitz, 2000; Smith *et al*., 2000; Levin *et al*., 2000; Wesley *et al*., 2001; Stoutjesdijk *et al*., 2002).

Het doel van dit werk was de constructie van een hpRNA silencing vector, die de uitschakeling van endogenen op een geconditioneerde manier toelaat. De geconditioneerde silencing aanpak die we ontworpen hebben, is gebaseerd op het Cre/lox recombinatie systeem, dat toelaat de expressie van het hpRNA te veranderen door geconditioneerde expressie van het CRE recombinase. De hpRNA coderende cassette is namelijk gescheiden van de 35S promotor door een *lox* geflankeerde blokkeringsequentie, die de selecteerbare merker van het T-DNA bevat. Volgend op expressie van het CRE recombinase, die de excisie van de blokkeringssequentie bewerkstelligt, is expressie van de stroomafwaartse hpRNA permanent geactiveerd. We voorspelden dat de efficiënte expressie van het hpRNA en daaropvolgende uitschakeling van een plant endogen afhankelijk zou zijn van (1) de weefsel specificiteit en de induceerbaarheid van de *cre* regulerende promotor, (2) de efficiëntie van de CRE-gemedieerde excisie van het *lox* geflankeerde DNA fragment en (3) de efficiëntie van de 35S-hpRNA-gemedieerde suppressie. Daartoe analyseerden we elk van bovenstaande gevallen in afzonderlijke modelsystemen.

Gezien het niet duidelijk is of hpRNA-gemedieerde gen supressie efficiënt kan voorkomen in alle weefsels, in het bijzonder in hoog proliferende cellen (Mitsuhara *et al*., 2002; Corrêa *et al*., 2004; Chuang and Meyerowitz, 2000), werd de efficiëntie van een 35S gedreven hpRNA-gemedieerde supressie geëvalueerd voor een reporter *gus* gen dat onder de controle van verschillende promotoren stond (Hoofdstuk 3). Hiervoor werd een hairpin construct gegenereerd, door de laatste 792 nucleotiden van de *gus* coderende sequentie (hpUS) in de

pH7GWIWG2 vector te cloneren (Karimi *et al*., 2002). Dit hpUS T-DNA werd binnengebracht in verschillende transgene lijnen die elk een *gus* gen, gedreven door een andere weefselspecifieke promotoren, bevatten.

Onze resultaten toonden aan dat een P35S gedreven hpRNA construct verschillende graden van silencing van een 35S gedreven *gus* gen kan induceren in een serie van onafhankelijke *Arabidopsis* transformanten. In transformanten met een zwakke geïnduceerde silencing was deze silencing niet uniform. Terwijl efficiënte onderdrukking van het 35S gedreven *gus* gen voorkwam in de uitzettende blad en wortel weefsels, was hpRNA geïnduceerde silencing minder efficiënt in de weefsels die sterk proliferende cellen bevatten. Het feit dat de variatie in efficiëntie van *gus* silencing in verschillende hpUS transformanten vooral geobserveerd werd in de meristemen kon betekenen dat de meristematische weefsels gevoeliger zijn voor het niveau van hpRNA expressie. In sterk onderdrukte hpUS transformanten toonden alle nakomelingen dramatisch verminderde niveaus van het *gus* doelgen in alle weefsels. Toen hetzelfde hpRNA construct gecombineerd werd met een *gus* gen dat door een CycB1;1, CycD4;1 of KRP4 promotor gedreven werd, werd slechts een beperkt effect van de hpUS-geïnduceerde GUS onderdrukking waargenomen. Gezien de CycB1;1, CycD4;1 en KRP4 promotoren tot expressie komen in cellen waar actieve celdeling voorkomt, postuleren wij dat silencing in meristematische cellen minder efficiënt is. Een alternatieve verklaring voor de minder efficiënte hpUS-geïnduceerde silencing van het *gus* gen in meristemen zou te wijten kunnen zijn aan een differentiële activiteit van de P35S promotor, gebruikt om het hpRNA tot expressie te brengen, in vergelijking tot de weefsel-specifieke uitdrukkingspatronen van CycB1;1, CycD4;1, en KRP4 promotoren kunnen zijn. Overeenkomstig met de vorige observaties werd een efficiënte uitschakeling van een WRKY23-*gus* gen waargenomen in de elongatie zone en in gedifferentieerde cellen van de wortel, terwijl de weefsels die meest proliferende cellen bevatten, zoals worteluiteinde en jonge bladeren en bloemen, opnieuw een minder diepgaande hpUS-gemedieerde silencing vertoonden.

Hoofdstuk 4 beschrijft de experimenten die werden uitgevoerd om de induceerbaarheid en de orgaanspecificiteit van de Gmhsp 17.6L sojaboon hitte-schok promotor in *Arabidopsis thaliana* te testen. Wij verkozen deze promotor om het cre gen tot expressie te brengen omdat eerder werd aangetoond dat deze promotor efficiënt geïnduceerd wordt door een hitte-schok behandeling (Kilby et al, 2000). Voor de analyse werd de Gmhsp 17.6L sojaboon hitte-schok promotor voor het *gfp-gus* fusie reporter gen in de pKGWFS7 vector gecloneerd. Het expressieniveau en de orgaan specificiteit van de Gmhsp 17.6L sojaboon hitte-schok

promotor werden bepaald en vergeleken met de sterkte van de algemeen gebruikte P35S en Pnos promotoren. Ook de P35S en Pnos promotoren werden in dezelfde vector voor de *gfp-gus* sequentie gecloneerd. De resultaten toonden aan dat de inductie van de sojaboon hitte-schok promotor zou kunnen gebruikt worden om hitte-geïnduceerde transcriptie van genen in *Arabidopsis* te verkrijgen, aangezien (i) er zeer weinig achtergrondexpressie van de *gus* en *gfp* genen was binnen ongeïnduceerde voorwaarden en (ii) dat de geïnduceerde niveaus van de transgenexpressie na een hitteschok gelijkaardig waren aan die van de Pnos promotor, maar 100 tot 300 maal lager dan de P35S promotor.

De capaciteit van het CRE recombinase om de excisie van om het even welk DNA fragment, geflankeerd door direct herhaalde lox sequenties, te katalyseren, werd aangewend om genexpressie in transgene planten te wijzigen (Odell et al, 1990; Guo et al, 2003; De Buck et al, 2001). In Hoofdstuk 5 evalueerden wij de efficiëntie van de CRE-bemiddelde recombinatie. De CRE activiteit werd beoordeeld op basis van de excisie-efficiëntie van een *gus* gen gelegen tussen in tandem georiënteerde *lox* sequenties in transgene *Arabidopsis thaliana* planten. Twee verschillende methodes om het lox geflankeerde *gus* gen en het *P35S::cre* gen samen te brengen in een trangene plant werden geëvalueerd, namelijk transformatie en kruising. Na transformatie van CK_2L6 planten met het P35S::cre construct werd een verschillende excisie-efficiëntie waargenomen tussen verschillende onafhankelijke transformanten. Eén van de tien onafhankelijke CK_2L6-SC transformanten gaf 100% GUS-negatieve nakomelingen. De meerderheid van de transformanten vertoonden echter een chimerisch patroon waarbij het *gus* gen verwijderd was in de meeste bladcellen, maar niet uit de meristematische zones. De variaties in excisie-efficiëntie zijn waarschijnlijk te wijten aan de verschillende expressieniveaus van het CRE recombinase, welke veroorzaakt worden door het verschillende aantal en de structuur van de CRE loci in deze transformanten. Er was geen duidelijk verschil in recombinatie-efficiëntie toen de CRE-planten als vrouwelijke of mannelijke ouder werden gebruikt tijdens kruising. Dit wijst erop dat de CRE activiteit in de vrouwelijk gametocyt óf afwezig óf niet voldoende is voor CRE-bemiddelde recombinatie vroeg na fertilisatie. Er werd geen CRE-bemiddelde excisie waargenomen toen het *cre* gen onder controle stond van de Pnos of de Gmhsp 17.6L sojaboon hitte-schok promotor. Het feit dat de volledige CRE-bemiddelde recombinatie slechts bereikt werd toen het CRE recombinase gen door de sterke P35S promotor wordt gereguleerd, suggereert dat hoge expressieniveaus van het CRE recombinase vereist zijn en dat de lage efficiëntie van de CRE-

bemiddelde recombinatie door zwakke Pnos en de hitte-induceerbare promotor toe te schrijven was aan het lage expressieniveau van het CRE recombinase.

De evaluatie van de pBchpAT conditionele gen silencing vector wordt beschreven in Hoofdstuk 6. De capaciteit van dit construct om een endogen te inactiveren op een conditionele manier werd getest met een hpRNA construct dat homologie vertoont met het *Arabidopsis catalase 2* gen. Dit *catalase 2* gen is het sterkst tot expressie komende catalase gen in de bladeren van *Arabidopsis thaliana*. Daarom werden de catalase expressieniveaus in verschillende transformanten gemeten. Deze transformanten bevatten allemaal het pBchAT construct waarin de hairpin sequentie van de promotor 35S gescheiden wordt door een 1200 bp blokeringsequentie. Tegen alle verwachtingen in vonden wij dat het pBchpAT construct het *catalase* gen in alle hpcat2 transformanten kon silencen. Dit zou toe te schrijven kunnen zijn aan de capaciteit van het haarspeld construct om silencing teweeg te brengen zonder dat het afgeschreven werd, of aan doorlees transcriptie van de promotor 35S over de blokkerende cassette. Daarom werd nagegaan of een promoterloos haarspeld construct ook het endogene *catalase* gen kon silencen. Dit bleek echter niet het geval te zijn. Dit suggereert dat de aanwezigheid van de 35S promotor 1200 basisparen stroomopwaarts van de haarspeld cassette volstaat om lage niveaus van hpRNA expressie te induceren, welke op zijn beurt een 3- tot 10-voudige vermindering van de haarspeld homologe catalase endogenen kan induceren. Na CRE-bemiddelde verwijdering van de *lox* geflankeerde blokkerende cassette werd de 35S promoter rechtstreeks gefusioneerd met de cat2hpRNA cassette, wat resulteerde in een verdere 2- tot 3-voudige vermindering van de endogene *catalase* expressie. Wij besluiten dat het conditionele gen silencing systeem verder moet worden verbeterd met betrekking tot de achtergrondexpressie van de hpcat2 cassette om een waardevol hulpmiddel voor gecondicioneerde inactivatie van plantengenen te worden.

8.2 Referenties

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