pSAT RNA Interference Vectors: A Modular Series for Multiple Gene Down-Regulation in Plants[1][OA]

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RNA interference (RNAi) is a powerful tool for functional gene analysis, which has been successfully used to down-regulate the levels of specific target genes, enabling loss-of-function studies in living cells. Hairpin (hp) RNA expression cassettes are typically constructed on binary plasmids and delivered into plant cells by *Agrobacterium*-mediated genetic transformation. Realizing the importance of RNAi for basic plant research, various vectors have been developed for RNAi-mediated gene silencing, allowing the silencing of single target genes in plant cells. To further expand the collection of available tools for functional genomics in plant species, we constructed a set of modular vectors suitable for hpRNA expression under various constitutive promoters. Our system allows simple cloning of the target gene sequences into two distinct multicloning sites and its modular design provides a straightforward route for replacement of the expression cassette's regulatory elements. More importantly, our system was designed to facilitate the assembly of several hpRNA expression cassettes on a single plasmid, thereby enabling the simultaneous suppression of several target genes from a single vector. We tested the functionality of our new vector system by silencing overexpressed marker genes (green fluorescent protein, DsRed2, and *nptII*) in transgenic plants. Various combinations of hpRNA expression cassettes were assembled in binary plasmids; all showed strong down-regulation of the reporter genes in transgenic plants. Furthermore, assembly of all three hpRNA expression cassettes, combined with a fourth cassette for the expression of a selectable marker, resulted in down-regulation of all three different marker genes in transgenic plants. This vector system provides an important addition to the plant molecular biologist's toolbox, which will significantly facilitate the use of RNAi technology for analyses of multiple gene function in plant cells.

Posttranscriptional gene silencing (PTGS) is a powerful tool for functional gene analysis. PTGS reduces the level of target transcripts in a sequence-specific manner (Agrawal et al., 2003), enabling loss-of-function studies in living cells. PTGS was first discovered in transgenic petunia (*Petunia hybrida*) plants, which, upon overexpression of the chalcone synthase-encoding sequence, resulted in variegated plants instead of the predicted purple flowers (Napoli et al., 1990). This phenomenon was originally termed cosuppression because it suppressed the expression of both the transgene and the native genes (Napoli et al., 1990), and it was thus suggested that interaction between the transgenes and the native transcripts triggers a mechanism that leads to destruction of both transcripts or obstruction of the translation process and to gene silencing.

Today we know that overexpression-mediated gene silencing results from in vivo hybridization between native and transgene RNA molecules and the production of double-stranded (ds)RNA molecules, which are then targeted for degradation by a complex cellular mechanism (for review, see Watson et al., 2005). Whereas the silencing effect can be directly attributed to the degradation of these dsRNA molecules, gene silencing can also occur via a range of epigenetic phenomena, including paramutation, epimutation, DNA methylation, and imprinting (McGinnis et al., 2007).

The mechanism of dsRNA-mediated gene silencing is probably similar in all eukaryotes (for review, see Horiguchi, 2004). Briefly, dsRNA molecules are processed by an RNase III-like enzyme (dicer) into small interfering RNA (siRNA) molecules. These 21- to 28-base-long siRNA molecules (Hamilton and Baulcombe, 1999; Elbashir et al., 2001) mediate the degradation of target RNAs and possibly also the methylation of genomic DNA by RNA-induced silencing-like effector complexes in a process generally known as RNA interference (RNAi; Mette et al., 1999; Hamilton et al., 2002; Zilberman et al., 2003). Interestingly, siRNAs can also move from cell to cell and systematically spread and deliver the silencing signal to the entire organism (Voinnet et al., 2000; Winston et al., 2002). Whereas both sense- and antisense-mediated gene silencing have proven fruitful for PTGS in plant cells (Bruening, 1998; Waterhouse et al., 1998), RNAi

induction can be more efficiently achieved by specialized expression cassettes that produce self-complementary hairpin (hp)-like RNA molecules (Horiguchi, 2004; Watson et al., 2005; Hirai et al., 2007). Such cassettes typically include plant promoter and terminator sequences that control the expression of two inversely repeated sequences of the target genes that are separated by a specific spacer (Fig. 1A). Upon delivery to plant cells, expression of an RNAi cassette will result in a dsRNA molecule composed of two distinct regions: a single-stranded loop, encoded by the spacer region, and a ds stem, encoded by the inverted repeats. It is the stem region that is used as a substrate by the dicer, but, because the spacer itself can potentially be recognized as a substrate as well, intron sequences are often used in the construction of such RNAi vectors (e.g. Meyer et al., 2004; Miki and Shimamoto, 2004; Hirai et al., 2007). RNAi (or hpRNA) plant expression can potentially be delivered to plant cells by various means of transformation but are typically used by mounting on binary plasmids to be delivered to plant cells by *Agrobacterium*-mediated transformation.

RNAi-mediated gene suppression has been highly instrumental in basic plant cell research and biotechnology (for review, see Tenllado et al., 2004; Small, 2007). RNAi vectors have been used, for example, in determining the importance of flavone synthases for nodulation in *Medicago truncatula* (Zhang et al., 2007), in revealing the role of a homeobox gene in rice (*Oryza sativa*) leaf development (Dai et al., 2007), and in engineering transgenic resistance to nematodes in Arabidopsis (*Arabidopsis thaliana*) plants (Huang et al., 2006). Biotechnologically, RNAi has been used, for example, to suppress the caffeine synthase gene, reducing caffeine content in coffee (*Coffea arabica*) plants (Ogita et al., 2003), to knock down a transcription factor that regulates the expression of a set of storage proteins in maize (*Zea mays*) plants while increasing the Lys content in maize (Segal et al., 2003), and to manipulate the expression of a cinnamyl alcohol dehydrogenase to produce tragenic flax (*Linum usitatissimum*) with reduced lignin and improved mechanical properties (Wrobel-Kwiatkowska et al., 2007).

Realizing the importance of RNAi for plant research, different reports have described the development of various vectors for the construction and expression hpRNA in plant cells. These include, for example, the pHANNIBAL, pKANNIBAL, and pHELLSGATE vectors, which were designed for high-throughput gene silencing in plant cells and allow the assembly of hpRNA constructs by conventional cloning or Gateway recombination (Wesley et al., 2001; Helliwell and Waterhouse, 2003), or the pX7 vectors, which were specifically designed as either chemically inducible or constitutive RNAi vector systems (Guo et al., 2003). Other, more specialized vectors have also been developed and include, for example, the pANDA and pANDA-mini vectors, which were developed for stable and transient RNAi-mediated suppression of gene function in rice (Miki and Shimamoto, 2004), and a set

of small, high-replicating pUC-based shuttle and binary RNAi vectors suitable for direct gene transfer and *Agrobacterium*-mediated transformation of poplar (*Populus* spp.) tissues (Meyer et al., 2004). These vectors, as well as many others that have been constructed for more specific tasks or as part of large families of plant vectors (e.g. Earley et al., 2006), have been successfully used for down-regulation and functional studies of various genes in various plant species (e.g. Stoutjesdijk et al., 2002; McGinnis et al., 2005, 2007; Travella et al., 2006).

Biotechnological applications and analysis of various cellular processes sometimes require suppression of more than just a single gene. Suppression of a multigene family or any other collection of related sequences can potentially be achieved using a single transgene construct and a specific design of the hpRNA expression cassette. Efficient simultaneous targeting of four different tospoviruses, for example, was reported by expression of a single hpRNA transgene designed to target the conserved N gene in four major tomato (*Lycopersicon esculentum*)-infecting tospoviruses (Bucher et al., 2006) and several members of the *psb*P gene family from tobacco (*Nicotiana tabacum*) were simultaneously suppressed using a single hpRNA that targeted the 3′-untranslated sequences of the four *psb*P tobacco isogenes (Ifuku et al., 2003). Nevertheless, whereas the suppression of several gene family members can be achieved by a single construct (e.g. Ifuku et al., 2003; Miki et al., 2005), cosuppression of unrelated genes requires using similar strategies for the delivery of multiple transgenes into plants cells. These include, for example, crossing two transgenic lines that are homozygous for each transgene (e.g. Chabannes et al., 2001; Pincon et al., 2001), retransformation (e.g. Zhong et al., 1998), and cotransformation (e.g. Ye et al., 2000). More recently, simultaneous suppression of three genes using a single chimeric construct was achieved by incorporating partial sequences from three different lignin biosynthetic genes under the control of a single promoter (Abbott et al., 2002). Whereas such chimeric constructs can certainly provide a possible technical solution for multiple gene suppression in plant cells, the assembly of several expression cassettes onto a single plasmid can provide a simpler and more efficient route for the delivery of multiple transgenes into plant cells. Such a strategy was used, for example, by Zhong et al. (2007), who assembled two RNAi constructs to simultaneously inhibit *SND1* and *NST1* genes in Arabidopsis plants. Nevertheless, more flexible and modular vectors than those used in many RNAi studies are needed to facilitate the construction of multiple RNAi expression cassette vectors and for the assembly of any complex gene expression system for that matter.

We previously described construction of the versatile pSAT plant expression vector system (Tzfira et al., 2005), which allows the assembly of multiple expression cassettes into specially designed *Agrobacterium* binary plasmids (Goderis et al., 2002). The pSAT plasmid,

Figure 1. Structural features of the pSAT-RNAi vector system. A, The general structure of a pSAT-RNAi plasmid and its cloning compatibility with pRCS2-based binary plasmid. A typical pSAT-RNAi plasmid is composed of a promoter and terminator region, the ChsA intron, and two unique multi-cloning sites, MCS-I and MCS-II, which should be used for cloning the two target gene sequences in reverse orientations. The entire hpRNA cassette is flanked by *Age*I and *Not*I, allowing its transfer between different pSAT plasmid backbones. The hpRNA cassette is also flanked with rare cutters (e.g. PI-*Psp*I), which can be used for its cloning into the pRCS2-based binary plasmid. B, Outline of the different pSAT-RNAi plasmids produced in this study. Expression of hpRNA is controlled by various promoters and terminators and each of the various RNAi cassettes can be transferred to the pRCS2-based binary plasmid using different combinations of rare-cutting endonucleases, as indicated. Both MCS-I and MCS-II are present in all plasmids and restriction recognition sites that are not unique in all plasmids are indicated by asterisks. Plasmid names from top to bottom are: pSAT3.masP.RNAi, pSAT4.35SP.RNAi, pSAT5.nosP.RNAi, pSAT6.masP.RNAi, pSAT6.35SP.RNAi, pSAT6.supP.RNAi, and pSAT6.rbcP. RNAi. 35S, CaMV 35S promoter and terminator; sup, superpromoter; mas, manopine synthase promoter and terminator; rbc1, Rubisco small-subunit promoter and terminator; nos, nopaline synthase promoter and terminator; ags, agropine synthase terminator. One, two, and three asterisks represent *Bsp*EI, *Bam*HI, and *Xba*I restriction sites, respectively.
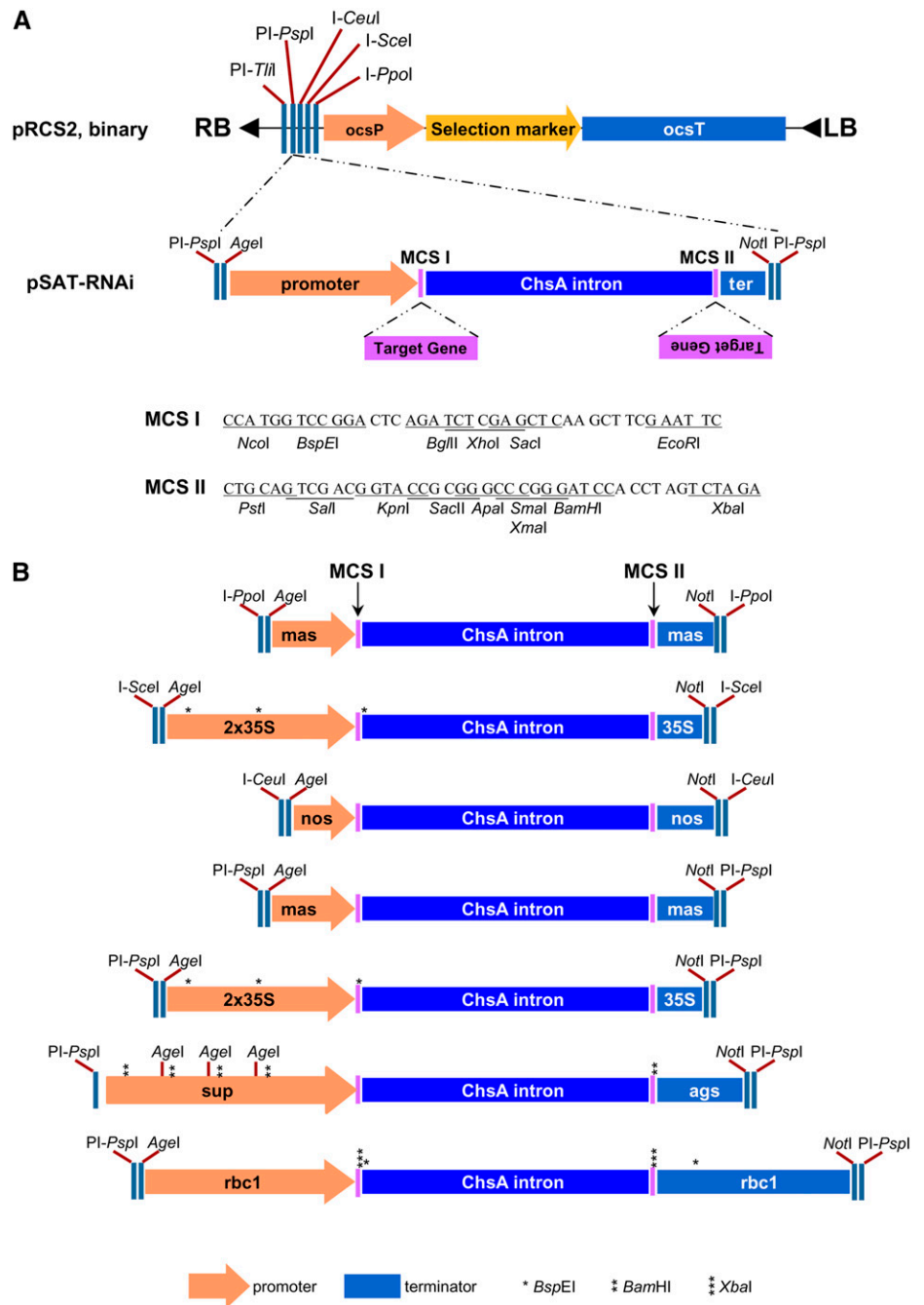
originally constructed as a set of modular satellite vectors in a system that supports fusions to five different autofluorescent tags under the control of the cauliflower mosaic virus (CaMV) 35S promoter (35SP; Tzfira et al., 2005), was later expanded to support the expression of foreign genes under the control of various promoters and terminators (Chung et al., 2005) and with the support of GATEWAY-mediated cloning (Chakrabarty et al., 2007). Relying on the system's flexible design, we sought to expand its cloning possibilities to include the expression of multiple hpRNAs in plant cells. Here, we describe the assembly of our

pSAT-RNAi vectors and demonstrate their use for the simultaneous silencing of multiple transgenes in transgenic plants. Our pSAT-RNAi plasmids feature a ChsA intron sequence that is flanked by two unique multiple cloning sites (MCSs) suitable for insertion of target gene sequences in reverse orientations. Importantly, expression of hpRNA in different pSAT-RNAi plasmids is controlled by various promoter and terminator sequences and several hpRNA expression cassettes can be assembled into a single *Agrobacterium* binary vector. We believe that our vector system not only provides a unique and important addition to the

growing collection of plant RNAi plasmids, but will also significantly facilitate the use of RNAi technology for the analysis of multiple gene function in plant cells.

RESULTS AND DISCUSSION

Design of a Modular Set of Plasmids for Expression of hpRNA

We decided to use the previously described basic design of our pSAT family of plasmids to address the difficulties involved in assembling multiple hpRNAi expression cassettes on a single plasmid (Tzfira et al., 2005). One crucial feature of the cloning system is that each expression cassette, whether it directs the expression of an untagged or autofluorescently tagged protein (Tzfira et al., 2005) under the control of various promoters and terminators (Chung et al., 2005) or whether it directs the expression of a hpRNA sequence (as described below), is flanked by different rare cutter sites that allow its transfer from pSAT plasmids into the corresponding sites of the pPZP-RCS1- or pPZP-RCS2-based (Goderis et al., 2002) *Agrobacterium* binary plasmids. This feature is exemplified in Figure 1A, where the hpRNA expression cassette from the generic pSAT-RNAi vector is flanked by PI-*Psp*I and thus can be cloned directly into the MCS of pPZP-RCS1, pPZP-RCS2 (Goderis et al., 2002), or their descending plasmids pRCS2-ocs-*bar*, pRCS2-ocs-*hpt*, and pRCS2-ocs-*nptII* (Chung et al., 2005). The latter plasmids, exemplified by the generic pRCS2 binary plasmid (Fig. 1A), carry three different plant selection marker expression cassettes cloned near the left border of the pPZP-RCS2, which facilitates their direct use for plant transformation experiments (Chung et al., 2005).

Other key features of individual pSAT-RNAi plasmids are exemplified by the generic pSAT-RNAi illustrated in Figure 1. A complete hpRNA expression cassette is composed of four distinct regions: a promoter and terminator sequence, the ChsA intron sequence, and a dual MCS. The dual MCS results from cloning of the ChsA intron sequence into pSAT6-MCS (Tzfira et al., 2005) and dividing the original MCS into two new, distinct regions, designated MCS-I and MCS-II, which contain the following unique restriction endonuclease recognition sites: *Nco*I, *Bsp*EI, *Bgl*II, *Xho*I, *Sac*I, and *Eco*RI in MCS-I and *Pst*I, *Sal*I, *Kpn*I, *Sac*II, *Apa*I, *Xma*I, *Sma*I, *Bam*HI, and *Xba*I in MCS-II. The two MCS regions allow the successive cloning of the target gene sequence in reverse orientation and assembly of a hpRNA sequence (Fig. 1A). In pSAT6.35S.RNAi, for example (Fig. 1B), expression of hpRNA is controlled by tandem CaMV 35SP and CaMV 35S terminator (35ST), conferring a complete expression cassette that is flanked by PI-*Psp*I.

Another important aspect of our extended family of pSAT plasmids is their flexible design, which allows the simple, one-step replacement of their regulatory elements and mobilization of the hpRNA expression cassettes between different pSAT plasmids. Exemplified by pSAT6.35S.RNAi, the promoter region in this plasmid (i.e. the tandem CaMV 35S) is flanked by *Age*I and *Nco*I and the terminator (i.e. CaMV 35ST) is flanked by *Xba*I and *Not*I, allowing their replacement with different regulatory elements by successive cloning. Furthermore, the entire hpRNA expression cassette can be mobilized between various pSAT plasmid backbones as *Age*I-*Not*I fragments (excluding pSAT6.sup.RNAi.ags, which lost its original *Age*I site during cloning). Indeed, we employed these various cloning strategies while assembling additional pSAT-RNAi plasmids (Fig. 1B). Overall, we produced seven different pSAT.RNAi plasmids (i.e. pSAT3.masP.RNAi, pSAT4.35SP.RNAi, pSAT5.nosP.RNAi, pSAT6.masP.RNAi, pSAT6.35SP.RNAi, pSAT6.supP.RNAi, and pSAT6.rbcP.RNAi; Fig. 1B), in which the expression of hpRNA is under the control of the tandem CaMV 35SP, the super-promoter (supP; Ni et al., 1995), the manopine synthase promoter (masP), the strong Rubisco small-subunit promoter (rbcP) from *Asteraceous chrysanthemum* (http://www.pri.wur.nl/UK/products/ImpactVector), or the nopaline synthase promoter (nosP). In the resulting series of pSAT-RNAi constructs, the hpRNA expression cassettes are flanked by I-*Ppo*I (pSAT3), I-*Sce*I (pSAT4), I-*Ceu*I (pSAT5), and PI-*Psp*I (pSAT6; Fig. 1B), which allow their successive cloning into the MCS of pPZP-RCS1- or pPZP-RCS2-based plasmids (Goderis et al., 2002; Chung et al., 2005) and assembly of multiple hpRNA expression cassettes.

Production of Transgenic Plants Overexpressing Three Marker Genes

To functionally test our pSAT-RNAi plasmids in plant cells, we decided to first produce transgenic plants that overexpress three marker genes: the enhanced green autofluorescent protein (EGFP; CLONTECH), the red autofluorescent protein (DsRed2) from coral (Matz et al., 1999), and the *nptII* gene, which codes for kanamycin (KAN) resistance in transgenic plants. We chose to simultaneously express these three proteins in transgenic plants under the control of strong constitutive promoters, rationalizing that suppressing high levels of protein expression is the best method for evaluating the function of our new RNAi plasmids. We thus constructed the pRCS2-[EGFP][KAN][DsRed2] binary plasmid (Fig. 2), which was composed of three expression cassettes. Expression of the EGFP, KAN, and DsRed2 coding sequences was controlled by nopaline synthase, the tandem CaMV 35S, and the rbcP, respectively. Several KAN-resistant transgenic lines were produced and lines with a putative single T-DNA insertion (as determined by segregation of their offspring on KAN-containing medium) were analyzed for expression of EGFP and DsRed2. Two transgenic lines were selected in which the expressions of both EGFP and DsRed2 were at their highest levels, as determined by the relative expression of the transgene's fluorescence as compared with the chloro-

Figure 2. Outline of the pRCS2-[EGFP][KAN][DsRed2] binary plasmid. The plasmids constructed to carry three cassettes for the stable over-expression of the EGFP, NPTII, and DsRed2 coding sequences. Expression cassettes were cloned as an *Age*I-*Not*I fragment into the different pSAT plasmids and were successively transferred as I-*Ppo*I, I-*Sce*I, and PI-*Psp*I fragments into the pRCS2 binary plasmid. 2x35S, Tandem CaMV 35S promoter; 35ST, CaMV 35S terminator; nos, nopaline synthase promoter and terminator; rbc1, Rubisco small-subunit promoter and terminator.
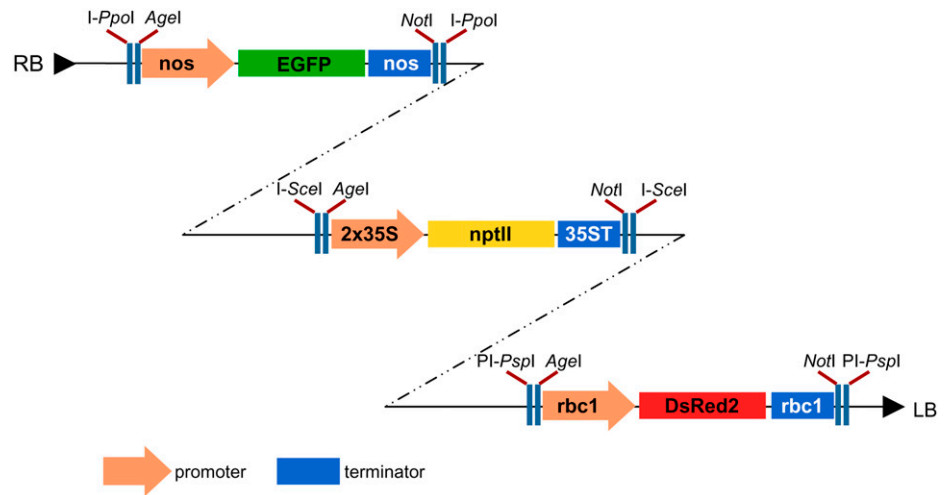

plast's autofluorescence using confocal laser-scanning microscopy. A sample of the confocal imaging of pRCS2-[EGFP][KAN][DsRed2]-transgenic plants, designated KGD (KAN-EGFP-DsRed2), is given in Figure 3, A to D.

Suppression of a Single Marker Transgene

We began analyzing our RNAi system by constructing satellite plasmids for the suppression of EGFP, DsRed2, or KAN in KGD transgenic plants. We first tested the expression of hpRNA under the control of the supP and tandem 35SP by the assembly of functional GFP-RNAi (GFPi), KAN-RNAi (KANi), and DsRed2-

RNAi (DsRed2i) expression cassettes. pSAT6.sup.GFPi, pSAT4.35SP.KANi, and pSAT4.35SP.DsRed2i were constructed by successive cloning of approximately 500-, 300-, and 310-bp-long sequences from the GFP, *nptII*, and DsRed2 coding sequences, respectively, in forward and reverse orientations. The simplicity of assembly of each cassette can be illustrated, for example, by the construction of pSAT6.sup.GFPi, which required only one pair of primers and a single PCR. More specifically, a pair of forward and reverse primers, carrying the *Nco*I and *Xho*I recognition sites, respectively, was designed and used for amplification of the 500-bp partial GFP sequence. The product was

Figure 3. Confocal microscopy analysis of EGFP and DsRed2 expression in single- and double-transformed plants. A to D, Confocal imaging of pRCS2-[EGFP][KAN][DsRed2] (designated KGD plants). E to H, Confocal imaging of KGD transformed by GFPi construct. I to L, Confocal imaging of KGD transformed by DsRed2i construct. M to P, Confocal imaging of KGD transformed by GFPi and DsRed2i constructs. A, E, I, and M, EGFP signal (green, when present). B, F, J, and N, DsRed2 signal (red, when present). C, G, K, and O, Plastid autofluorescence in blue. D, H, L, and P, Merge signals of the corresponding EGFP, DsRed2, and plastid autofluorescence. Images are single confocal sections (bar = 50 μm).
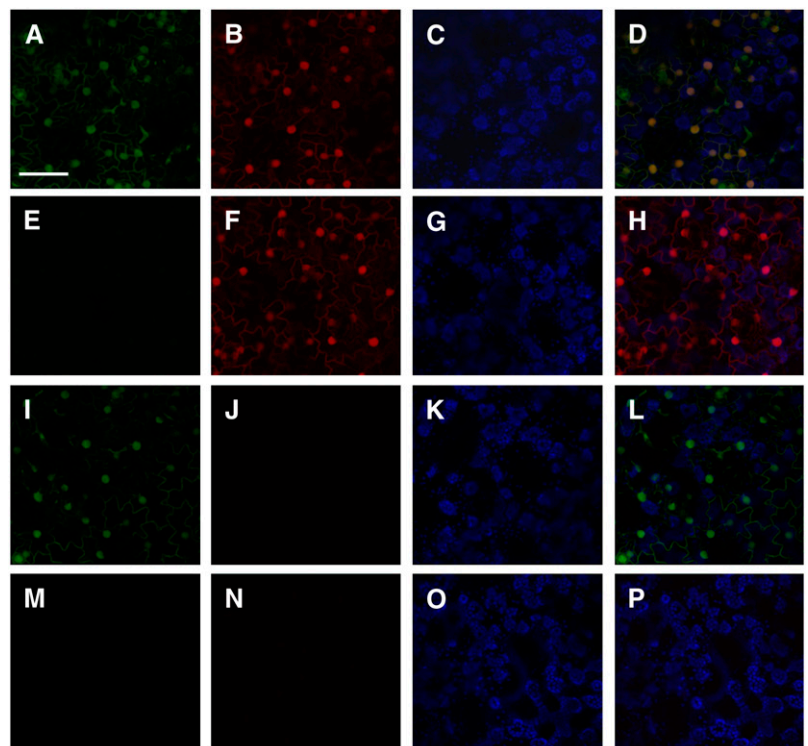
Table I. *Constructs used for analysis of RNAi cassettes in transgenic plants*

Plasmid Name	Target Genes[a,b]		
	gfp	*dsred2*	*nptII*
pRCS2-[bar]	–	–	–
pRCS2-[bar][GFPi]	6, supP, agsT	–	–
pRCS2-[bar][DsRed2i]		4, 35SP, 35ST	
pRCS2-[bar][KANi]	–	–	4, 35SP, 35ST
pRCS2-[bar][GFPi][DsRed2i]	5, nosP, nosT	6, rbcP, rbcT	–
pRCS2-[bar][GFPi][DsRed2i][KANi]	5, nosP, nosT	3, masP, masT	4, 35SP, 35ST

[a]Numbers indicate the pSAT vector serial numbers. [b]The pairs of regulatory elements used are: supP, super promoter; agsT, agropine synthase terminator; nosP, nopaline synthase promoter; nosT, nopaline synthase terminator; rbcP, Rubisco small-subunit promoter; rbcT, Rubisco small-subunit terminator; 35SP, CaMV 35S dual promoter; 35ST, CaMV 35S terminator; masP, manopine synthase promoter; and masT, manopine synthase terminator.

then digested with *Xho*I alone or double digested with *Nco*I and *Xho*I and the fragments were successively cloned into the *Sma*I and *Sal*I sites in MCS-II (*Xho*I-digested fragment) and the *Xho*I and *Nco*I sites in MCS-I (*Xho*I-*Nco*I fragment). The location of the *Xho*I upstream of *Nco*I and the location of *Sal*I (and its compatibility with *Xho*I) downstream of *Sma*I resulted in the inverted alignment of both GFP fragments. Similarly, two pairs of primers were used for successive cloning of the KAN or DsRed2 inverted repeats into the MCS-II and MCS-I for the production of KANi or DsRed2i expression cassettes in pSAT4.35SP.RNAi.

We next assembled the GFPi, DsRed2i, and KANi hpRNA expression cassettes into an *Agrobacterium* binary plasmid. To this end, we removed the GFPi, KANi, or DsRed2i expression cassettes from pSAT6. sup.GFPi, pSAT4.35SP.KANi, or pSAT4.35SP.DsRed2i as PI-*Psp*I or I-*Sce*I fragments and cloned them, individually, into the corresponding sites of pRCS2-ocs-*bar* (Chung et al., 2005), producing pRCS2-[bar][GFPi], pRCS2-[bar][KANi], and pRCS2-[bar][DsRed2i] (Table I). We mobilized these vectors into *Agrobacterium* cells and used them, as well as the empty vector pRCS2-[bar], to transform our KGD transgenic plants. Double-transformed plants were selected on basta-containing medium and were examined for their suppression phenotype. As expected, confocal microscopy analysis of KGD lines, transformed by pRCS2-[bar][GFPi], revealed that these doubly transformed plants (designated KGD-Gi) no longer produced GFP fluorescence (Fig. 3E) but were still capable of emitting red fluorescence from the stable expression of DsRed2 (Fig. 3F). Transformation of KGD plants by pRCS2-[bar][DsRed2i], on the other hand, affected the expression of DsRed2 and not of GFP in these doubly transformed KGD-Di plants (Fig. 3, I and J). No suppression of either GFP or DsRed2 was observed when KGD plants were retransformed by either pRCS2-[bar][KANi] (KGD-Ki plants) or the empty vector pRCS2-[bar] (data not shown). Collectively, these results indicate that the suppression effect of GFPi and DsRed2i was indeed specific and that expression of KANi or the bar selection marker did not have a negative effect on the expression of either GFP or DsRed2 in the KGD transgenic plants.

We next tested the effect of our single-gene RNAi constructs on silencing of the KAN resistance gene. To this end, we set up a regeneration assay in which we placed leaf discs on regeneration medium supplemented with KAN. As expected, whereas leaf discs from wild-type plants failed to regenerate new shoots on this selectable medium, KGD plants, which were originally selected by their resistance to KAN, maintained their resistance capacity and were capable of regeneration in the presence of KAN (compare wild type with KGD in Fig. 4). More importantly, doubly transformed plants, carrying a KANi (KGD-Ki; Fig. 4), but not DsRed2i (KGD-Di; Fig. 4) or GFPi (KGD-Gi; Fig. 4) expression cassette, failed to regenerate in the presence of KAN, thus indicating that the expression of hpRNA for *nptII*, but not for *gfp* or *dsred2*, suppresses the constitutive expression of the *nptII* expression cassettes in the KGD transgenic lines. Transformation of KGD plants with the empty vector pRCS2-[bar] did not affect their regeneration capacity (KGD-bar; Fig. 4), further indicating that expression of bar has no specific effect on the expression or suppression of our target transgenes.

Northern-blot analysis of various transgenic plants revealed the molecular connection between the KANi expression cassette and the resistance to KAN in our transgenic plants. As expected, and in accordance with the KGD plant's resistance to KAN, the *nptII*-specific band could be observed in KGD but not in wild-type plants (Fig. 5). Furthermore, the *nptII* transcript was also visible in KGD-Gi, but not KGD-Ki transgenic plants. Here, again, *nptII* expression was directly linked with KGD-Ki's inability to regenerate on KAN-containing medium (Fig. 4) due to silencing of the *nptII* transcript by the KANi expression cassette.

Multiple Marker Gene Suppression

To demonstrate the suitability of our pSAT-RNAi vector system for the suppression of multiple genes from a single construct, we assembled pair and triplet
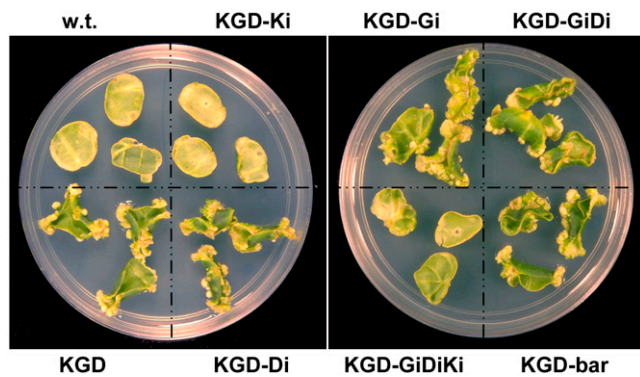
Figure 4. KAN resistance regeneration assay of single- and double-transformed plants. Leaf discs of diverse transgenic tobacco plants were placed on shoot regeneration medium supplemented with KAN and documented following several weeks in culture. w.t., Wild type, nontransformed plants; KGD, single-transgenic plants produced by overexpressing the KAN, EGFP, and DsRed2 coding genes; KGD-Ki, KGD plants transformed with an *nptII* silencing cassette (KANi); KGD-Di, KGD plants transformed with a *DsRed2*-silencing cassette (DsRed2i); KGD-Gi, KGD plants transformed with a *gfp* silencing cassette (GFPi); KGD-GiDi, KGD plants transformed with GFPi and DsRed2i; KGD-GiDiKi, KGD plants transformed with GFPi, DsRed2i, and KANi; KGD-bar, KGD plants transformed with empty binary vector.

hpRNA expression cassette onto binary plasmids. We first constructed the pSAT5.nosP.GFPi and pSAT6.rbcP.DsRed2i, in which the GFPi and DsRed2i hpRNA expression cassettes were driven under the control of the nosP and rbcP, respectively (Table I). We also constructed pSAT3.masP.DsRed2i in which the expression of DsRed2i hpRNA was driven under the control of the masP (Table I). Next, we mounted both GFPi and DsRed2i hpRNA expression cassettes as I-*Ceu*I and PI-*Psp*I fragments from pSAT5.nosP.GFPi and pSAT6.rbcP.DsRed2i, respectively, onto pRCS2-ocs-*bar* (Chung et al., 2005), producing pRCS2-[bar][GFPi][DsRed2i] (Table I). We also mounted all three (GFPi, DsRed2i, and KANi) hpRNA expression cassettes as I-*Ceu*I, I-*Pop*I, and I-*Sce*I fragments from pSAT5.nosP.GFPi, pSAT3.masP.DsRed2i, and pSAT4.35SP.KANi, respectively, onto pRCS2-ocs-*bar*, producing pRCS2-[bar][GFPi][DsRed2i][KANi] (Table I). We then used these constructs in transformation experiments of our KGD transgenic plants. As expected, confocal microscopy analysis of KGD lines transformed by pRCS2-[bar][GFPi][DsRed2i] revealed that these double-transformed plants (designated KGD-GiDi) fail to produce either green (Fig. 3M) or red (Fig. 3N) fluorescence, but are still capable of regenerating on KAN-containing medium (compare wild type with KGD-GiDi in Fig. 4). Similarly, KGD lines transformed with pRCS2-[bar][GFPi][DsRed2i][KANi] failed to produce green or red fluorescence (data not shown). Furthermore, the latter plants (designated KGD-GiDiKi) failed to regenerate in the presence of KAN (compare wild type and KGD-Ki with KGD-GiDiKi in Fig. 4). Northern-blot analysis of KGD-GiDiKi and KGD-GiDi transgenic plants revealed the correla-

tion between the absence (Fig. 5, lane KGD-GiDiKi) or presence (Fig. 5, lane KGD-GiDi) of a stable *nptII* transcript and their ability to regenerate in the presence of KAN. These results illustrate the suitability of our pSAT-RNAi system for assembly of multiple hpRNA expression cassettes and for suppression of multiple, unrelated genes in plant cells.

Inheritance of RNAi Silencing Cassettes

To further explore the relationship between our suppression cassettes and the phenotypes observed in our doubly transformed plants, we let some of our transgenic plants self-fertilize and set seed. We then analyzed the segregation of their progeny by their ability to germinate in the presence of KAN and/or basta. As shown in Table II, while our wild-type plants failed to germinate in the presence of either of these selection agents, 72% of the offspring of self-fertilized T1 KGD plants were able to grow on KAN-, but not basta-containing medium, indicating a ratio of 3:1 with respect to the KGD transgene. Segregation analyses of T1 KGD-Di plants revealed that while 73% or 74% of the seedlings germinated in the presence of KAN or basta, respectively, only 52% of the offspring germinated when both antibiotics were present in the medium (Table II). These data indicate that these two traits were inherited separately by their offspring at a ratio of 3:1 for each transgene and at a ratio of 9:7 when both selection agents were present in the medium. These segregations clearly fit dihybrid segregation with a complementary interaction between both selection genes and thus with both transgenes.

Next, we analyzed the segregation pattern of T1 KGD-Ki plants and found a 3:1 segregation ratio (with a germination rate of 73%) of the basta resistance gene (Table II). The rate of germination on KAN-containing
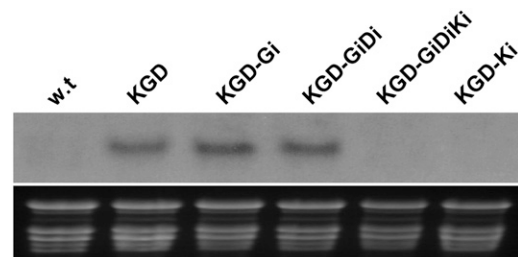
Figure 5. Northern-blot analysis of *nptII* expression in transgenic tobacco plants. Top, Detection of the *nptII* transcript by digoxigenin-labeled *nptII* probe; bottom, total RNA loading. w.t., Wild-type, nontransformed plants; KGD, single-transgenic plants produced by overexpressing the KAN, EGFP, and DsRed2 genes; KGD-Gi, KGD plants transformed with a *gfp* silencing cassette (GFPi); KGD-Ki, KGD plants transformed with an *nptII* silencing cassette (KANi); KGD-GiDi, KGD plants transformed with GFPi and *dsRed2* silencing cassette (DsRed2i); KGD-GiDiKi, KGD plants transformed with GFPi, DsRed2i, and KANi.

Table II. *Progeny segregation in single- and double-transformed transgenic plants*

Plant[a]	Resistant Plants[b,c]			
	B	K	BK	No Selection
	%, survived plants/total seeds			
Wild type	0 (0/134)	0 (0/167)	0 (0/145)	98 (155/158)
KGD	0 (0/156)	72 (125/174)	0 (0/178)	96 (159/166)
KGD-Di	73 (156/214)	74 (115/155)	52 (70/135)	99 (183/185)
KGD-Ki	73 (123/168)	17 (36/213)	0 (0/172)	95 (157/165)
KGD-DiGiKi	72 (133/186)	16 (32/199)	0 (0/144)	96 (148/154)

[a]KGD, GFP, DsRed2, and KAN; Di, DsRed2-RNAi; Ki, KAN-RNAi, Gi, GFP-RNAi. [b]B, Basta-resistant plants; K, KAN-resistant plants; BK, Basta- and KAN-resistant plants. [c]Numbers in parentheses represent surviving plants out of the total planted seeds.

medium, however, was much lower than that with KGD-Di plants and resulted in a 3:13 ratio (17%), fitting dominant epistasis-like segregation. This altered segregation ratio of the KAN resistance trait was due to the presence of the basta resistance gene (which is physically linked to the KANi expression cassette), which masks the expression of the KAN resistance gene in double-transformed plants. Similar ratios were also observed when the segregation of KGD-DiGiKi plants was analyzed (Table II), indicating the independent inheritance of both transgenes and the stability of the KANi expression cassette in the second generation. Confocal microscopic analyses of several T2 selected plants revealed a direct link between the presence of the basta resistance gene and the silencing of GFP- and/or DsRed2-encoding genes by their corresponding hpRNA expression cassettes (data not shown), indicating the integrity of the transgene inheritance by the offspring.

SUMMARY

Suppression of multiple unrelated genes in transgenic plants poses a technical challenge that we addressed here by constructing a new set of plant RNAi plasmids. The design of our vectors offers an easy cloning route for the assembly of hpRNA expression cassettes and the flexibility of being able to replace the plasmids' regulatory elements and to simply shuffle hpRNA expression cassettes among various pSAT vector backbones. Furthermore, our system provides the user with the ability to mount several hpRNAi expression cassettes onto a single *Agrobacterium* binary plasmid and use them in a single transformation event to silence several target genes. The compatibility of our system with previously described plasmids (Chung et al., 2005; Tzfira et al., 2005; Chakrabarty et al., 2007) further expands the user's flexibility because it potentially allows the assembly of complex constructs carrying gene-expressing, fluorescence-tagging, and gene-silencing cassettes. We thus believe that these vectors will further facilitate the discovery of gene functions in plant cells.

MATERIALS AND METHODS

Construction of pSAT-RNAi Vectors

The approximately 1.4-kb-long ChsA intron was PCR amplified from pFGC5941 (Kerschen et al., 2004) using the primers 5′-GGAATTCGTGTAA-GAATTTCTTATGTTAC and 5′-TCCCCGCGGTACCGTCGACTGCAGA-CCTGCAAATTGACCAAAAAAGATG and cloned into the *Eco*RI-*Sac*II sites of pSAT6-MCS (Tzfira et al., 2005), which resulted in construction of the pSAT6.35S.RNAi plasmid. Insertion of the ChsA intron resulted in splitting of the original single MCS of pSAT6-MCS and assembly of two new MCSs, designated MCS-I and MCS-II, which contained the following unique restriction endonuclease recognition sites: *Nco*I, *Bgl*II, *Xho*I, *Sac*I, and *Eco*RI in MCS-I and *Pst*I, *Sal*I, *Kpn*I, *Sac*II, *Apa*I, *Xma*I, *Sma*I, *Bam*HI, and *Xba*I in MCS-II. In this plasmid, the intron plant gene expression cassette was controlled by the tandem CaMV 35SP and the tobacco etch virus leader sequence from pRTL2-GUS (Restrepo et al., 1990), which was located between the *Age*I and *Nco*I recognition sites and the CaMV 35ST sequence from pRTL2-GUS (Restrepo et al., 1990), which was located between the *Xba*I and *Not*I recognition sites. The entire expression cassette was flanked by the PI-*Psp*I recognition sites.

To produce pSAT6.supP.RNAi, we first PCR amplified the approximately 1.2-kb supP sequence from pMSP-1 (Ni et al., 1995) using the primers 5′-TCC-CCCGGGGCGTTGGCCGATTCATTAATGC and 5′-CATGCCATGGCTA-GAGTCGATTTGGTGTATC and cloned it as an *Xma*I-*Nco*I fragment into the *Age*I-*Nco*I sites of pSAT6-MCS, replacing the tandem CaMV 35SP with the supP and producing pSAT6.supP.MCS.35ST. We next PCR amplified the 406-bp agropine synthase gene poly(A) terminator (agsT) signal from pMSP-1 (Ni et al., 1995) using the primers 5′-GCTCTAGAGAATTAACAGAGGTGGATG-GACAGACCC and 5′-ATAAGAATGCGGCCGCATCATCAATATTATAA-TAAAAATATCC and cloned it as an *Xba*I-*Not*I fragment into the same sites of pSAT6.supP.MCS.35ST, replacing the CaMV 35ST with agsT and producing pSAT6.supP.MCS.agsT. We finally cloned the ChsA intron as an *Eco*RI-*Kpn*I fragment from pSAT6.35SP.RNAi into the same sites of pSAT6.supP.MCS.agsT, producing the final plasmid pSAT6.supP.RNAi.

We transferred the masP and manopine synthase terminator (masT)-controlled expression cassette from pSAT3.masP.MCS.masT (Chung et al., 2005) as an *Age*I-*Not*I fragment into the same sites of pSAT6-MCS (Tzfira et al., 2005; replacing the CaMV 35S-controlled expression cassette) to produce pSAT6.masP.MCS.masT. We also transferred the nosP- and nopaline synthase terminator (nosT)-controlled expression cassette from pSAT2.nosP.MCS.nosT (Chung et al., 2005) as an *Age*I-*Not*I fragment into the same sites of pSAT5-MCS (Tzfira et al., 2005; replacing the CaMV 35S-controlled expression cassette) to produce pSAT5.nosP.MCS.nosT.

To make pSAT3.masP.RNAi, pSAT5.nosP.RNAi, pSAT4.35SP.RNAi, pSAT6.rbcP.RNAi, and pSAT6.masP.RNAi, we cloned the ChsA intron as an *Eco*RI-*Kpn*I fragment from pSAT6.35SP.RNAi into the same sites of pSAT3.masP.MCS.masT, pSAT5.nosP.MCS.nosT, pSAT4.35SP.MCS.35ST (Tzfira et al., 2005), pSAT6.rbcP.MCS.rbcT (Chung et al., 2005), and pSAT6.masP.MCS.masT (Chung et al., 2005), respectively.

All PCRs were performed using a high-fidelity *Pfu* DNA polymerase (Stratagene) and their products were verified by DNA sequencing. Full DNA sequences of pSAT6.35S.RNAi, pSAT6.supP.RNAi, pSAT3.masP.RNAi, pSAT5.nosP.RNAi, pSAT4.35SP.RNAi, pSAT6.rbcP.RNAi, and pSAT.masP.RNAi vectors were deposited in GenBank and assigned the following accession

numbers: EU049862, EU049861, EU049860, EU049865, EU049859, EU049864, and EU049863, respectively.

Construction of Binary Plasmid for Transgenic Overexpression

We cloned the EGFP-encoding sequence as an NcoI-XbaI fragment from pSAT6-EGFP-C1 into the same sites of pSAT2.nosP.MCS.nosT (Chung et al., 2005), producing pSAT2.nosP.EGFP.nosT. We cloned the DsRed2-encoding sequence as XhoI-XbaI from pSAT6-DsRed2-N1 into the same sites of pSAT6. rbcP.MCS.rbcT (Chung et al., 2005), producing pSAT6.rbcP.DsRed2.rbcT. We next transferred the EGFP and DsRed2 expression cassettes as AscI-I-PopI and PI-PspI-PI-PspI fragments from pSAT2.nosP.EGFP.nosT and pSAT6.rbcP. DsRed2.rbcT into the corresponding sites of pRCS2-nptII (Tzfira et al., 2005), producing pRCS2-[EGFP][KAN][DsRed2].

Cloning of Tested Genes into pSAT-RNAi Vectors

We constructed several vectors capable of expressing long hp-dsRNAs suitable for suppressing the expression of GFP, DsRed2, and KAN in plant cells. More specifically, about 500 bp of the GFP open reading frame (ORF) were cloned as a PCR-amplified fragment from pSAT6-EGFP-C1 using the primers 5′-CATGCCATGGGGGATCCAAGTTCAGCGTGTCCGGCG and 5′-CCGCTCGAGGGGGCCGTCGCCGATGGGGG. This fragment was digested by either NcoI-XhoI or XhoI alone, and the fragments were successively cloned, first into the NcoI-XhoI sites of MCS-I and then into the SmaI-SalI sites of MCS-II of pSAT6.sup.RNAi, producing pSAT6.sup.GFPi. A similar strategy was employed using the plasmid pSAT5.nosP.RNAi to produce pSAT5. nosP.GFPi. In addition, about 310 bp of the DsRed2 ORF were cloned as a PCR-amplified fragment from pSAT6-DsRed2-C1 (Tzfira et al., 2005) using the primers 5′-CATGCCATGGGGATCCCCTGGGTCACGGTCGC and 5′-CCG-CTCGAGAACGTCATCACCGAGTTCATGC. This fragment was digested by either BamHI-XhoI or NcoI-XhoI, and the fragments were successively cloned, first into the BamHI-SalI sites of MCS-II and then into the NcoI-XhoI sites of MCS-I of pSAT4.35SP.RNAi, producing pSAT4.35S.DsRed2i. A similar strategy was employed using the plasmids pSAT3.masP.RNAi and pSAT6.rbcP. RNAi to produce pSAT3.masP.DsRed2i and pSAT6.rbcP.DsRed2i, respectively. Finally, about 310 bp of the nptII ORF were cloned as a PCR-amplified fragment from pSAT4-nptII (Tzfira et al., 2005) using the primers 5′-CATGC-CATGGGGGATCCAAGCGGGAAGGGACTGGC and 5′-CCGCTCGAGCGAC-GAGATCATCGCCGTC. This fragment was digested by either BamHI-XhoI or NcoI-XhoI alone, and the fragments were successively cloned, first into the BamHI-SalI sites of MCS-II and then into the NcoI-XhoI sites of MCS-I of pSAT4.35SP.RNAi, producing pSAT4.35S.KANi.

Construction of Binary RNAi Plasmids

For down-regulation of GFP alone, we transferred the GFPi expression cassette from pSAT6.sup.GFPi as a PI-PspI-PI-PspI fragment and cloned it into the corresponding site of pRCS2-ocs-bar (Chung et al., 2005), producing pRCS2-[bar][GFPi].

For down-regulation of DsRed2 alone, we transferred the DsRed2i cassette from pSAT4.35S.DsRed2i as a I-SceI-I-SceI fragment and cloned it into the corresponding site of pRCS2-ocs-bar, producing pRCS2-[bar][DsRed2i]. For down-regulation of nptII alone, we transferred the KANi cassette from pSAT4.35S.KANi as a I-SceI-I-SceI fragment and cloned it into the corresponding site of pRCS2-ocs-bar, producing pRCS2-[bar][KANi]. For the combined down-regulation of GFP and DsRed2, we transferred the GFPi and DsRed2 cassettes from pSAT5.nosP.GFPi and pSAT6.rbcP.DsRed2i as I-CeuI-I-CeuI and PI-PspI-PI-PspI fragments, respectively, and cloned them into the corresponding sites of pRCS2-ocs-bar, producing pRCS2-[bar][GFPi][DsRed2i]. For the combined down-regulation of GFP, DsRed2, and nptII, we transferred the GFPi, DsRed2, and KANi cassettes from pSAT5.nosP.GFPi, pSAT3.masP.DsRed2i, and pSAT4.35S.KANi as I-CeuI-I-CeuI, I-PopI-I-PopI, and PI-PspI-PI-PspI fragments, respectively, and cloned them into the corresponding sites of pRCS2-ocs-bar, producing pRCS2-[bar][GFPi][DsRed2i][KANi].

Production and Analysis of Transgenic Plants

Binary plasmids were introduced into Agrobacterium tumefaciens strain EHA105 using the calcium chloride transformation protocol (Tzfira et al., 1997).

The resulting bacterial cultures were used to genetically transform tobacco (Nicotiana tabacum) turk leaf discs according to Horsch et al. (1985). Transgenic plants were first produced using the pRCS2-[EGFP][KAN][DsRed2] plasmid and selected in the presence of 50 μg/mL KAN. Selected lines were retransformed using various RNAi binary plasmids and double-transformed plants were selected in the presence of 20 μg/mL basta. Plants were maintained in tissue culture on basal Murashige and Skoog medium (Murashige and Skoog, 1962) with no exogenous growth regulators. An in vitro regeneration assay was performed by placing aseptic leaf discs from in vitro-grown plants on regeneration medium (Horsch et al., 1985) supplemented with KAN. Selected lines were cultivated in commercial compost in a regular growth chamber. Transformants were allowed to self-pollinate and seeds from individual plants were pooled, surface sterilized, and allowed to germinate on one-half-strength Murashige and Skoog medium (0.8% [w/v] agar and 3% [w/v] Suc) supplemented with the appropriate combinations of KAN and basta. Resistant plants were counted and the transgene segregation ratio was calculated.

Confocal Microscopy

Plant tissues were viewed directly under a Leica confocal microscope. Two to five independent clones were analyzed for expression of EGFP and DsRed2 and four to five leaves were analyzed from each individual plant. In addition, 15 to 20 seedlings were randomly analyzed for expression of EGFP and DsRed2 for each self-fertilized clone.

RNA Gel-Blot Analysis

Total RNA was isolated from approximately 200 mg of leaf tissue as previously described. RNA samples (10 μg/lane) were electrophoresed on a 1.2% formaldehyde/agarose gel, blotted onto Hybond N$^+$ membranes, and probed with digoxigenin-labeled nptII probe followed by autoradiography using standard hybridization and detection protocols. rRNA within the analyzed RNA preparation was detected by ethidium bromide staining of agarose gels and served as an internal control for equal loading of the lanes.

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