Control of seed germination in transgenic plants based on the segregation of a two-component genetic system

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We have developed a repressible seed-lethal (SL) system aimed at reducing the probability of transgene introgression into a population of sexually compatible plants. To evaluate the potential of this method, tobacco plants were transformed with an SL construct comprising *gene 1* and *gene 2* from *Agrobacterium tumefaciens* whereby *gene 1* was controlled by the seed-specific phaseolin promoter modified to contain a binding site for the *Escherichia coli* TET repressor (R). The expression of this construct allows normal plant and seed development but inhibits seed germination. Plants containing the SL construct were crossed with plants containing the *tet* R gene to derive plant lines where the expression of the SL construct is repressed. Plant lines that contained both constructs allowed normal seed formation and germination, whereas seeds in which the SL construct was separated from the R gene through segregation did not germinate. The requirements of such a method to efficiently control the flow of novel traits among sexually compatible plants are discussed.

One of the major concerns surrounding the widespread use of genetically modified crops is that these crops may transmit their novel traits to wild relatives or that novel traits may contaminate other related crop species. This is of particular concern for crops where seed is saved, because seed formed by cross-pollination from varieties carrying novel genes can lead to populations where the novel gene becomes established. Thus, a mechanism to reduce the incidence of the transfer of novel traits to the seed of wild or domestic relatives can provide a means of restricting the novel traits to the specific variety being cultivated. Examples of current containment techniques include maternal inheritance, male sterility, seed sterility, cleistogamy, apomixis, incompatible genomes, and transgenic mitigation by temporal or tissue-specific control of suicide genes (reviewed in ref. 1).

In this article, we describe an approach to gene containment that relies on the maintenance of a seed-lethal (SL) gene (which can be linked to a novel trait) and a repressor (R) element added by crossing. The rationale of the method is to achieve repression of seed lethality by combining the SL construct with the R construct. The combination of the two elements leads to repression of the SL gene, thus allowing seed propagation of the plant by selfing. However, pollen-mediated incrossing or outcrossing can result in the separation of the two elements, in which case the SL construct is consequently activated in the seed embryo, causing the arrest of germination for seed containing the SL construct and the linked novel trait (Fig. 1). Under optimized conditions (single SL and R loci at the same location on both parental chromosomes) such a method would be simple and efficient and would not require any intervention under managed conditions.

In the example presented, seed lethality is achieved by the embryo-specific overproduction of auxin mediated by the products of *Agrobacterium tumefaciens gene 1*, tryptophan 2-monooxygenase (*iaaM*), and *gene 2*, indole-3-acetamide hydrolase (*iaaH*). Both enzymes combine in an unusual pathway, not known to exist in higher plants, to produce indole-3-acetic acid

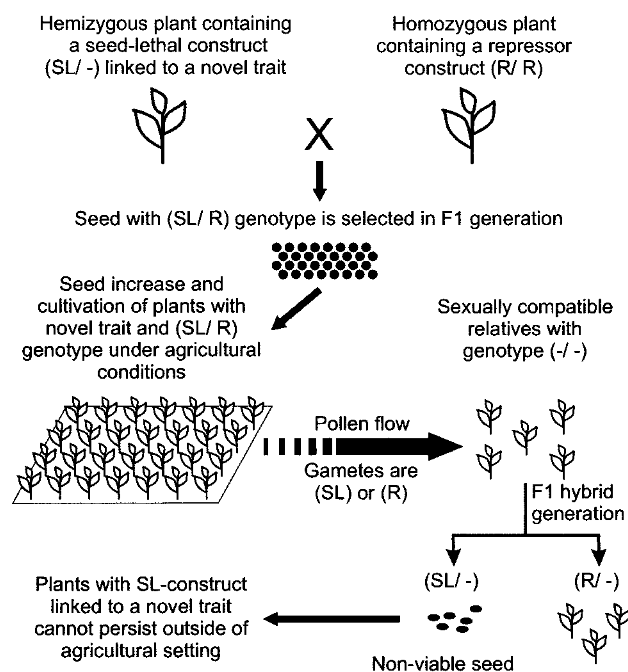

Fig. 1. Principle of a method to control seed germination. Two lines of transgenic plants are produced. One line of primary transformants represents the hemizygous T_0 generation of SL plants, which contains a single locus of the SL construct linked to a novel trait. The SL construct generates an overproduction of auxins in the developing seed, and, as a consequence, seed germination is inhibited. The T_0 SL line is then crossed with homozygous R plants, which produce *TET*R protein that binds to a DNA binding site inserted into the seed-specific phaseolin promoter of the SL construct. In (SL × R) crosses, the *TET*R protein prevents the expression of the SL construct. This enables the production of a viable F_1 progeny in which the SL construct is repressed but the novel trait is not. The F_1 generation of (SL, R) genotype can then be maintained by selfing and a crop with a novel trait can be produced. Provided that single loci of the SL and R constructs are inserted into the same location on the same parental chromosomes, any outcrossing with WT or non-R plants will segregate the SL and R component from each other, leading to viable WT/R (R, −) plants and nonviable (SL/−) seeds. Thus, a novel trait can be maintained and contained within a variety without further intervention.

whereby *iaaM* is the rate-limiting enzyme (2–4). Seed-specific expression is mediated by the embryo-specific phaseolin promoter from French bean, *Phaseolus vulgaris* (5). Repression of the SL construct is conferred by the R element *tet*R that acts on

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Abbreviations: SL, seed-lethal; R, repressor; P-SL, phaseolin promoter–SL; P-TOP-SL, phaseolin promoter with tet operator sequence–SL; MS, Murashige and Skoog.

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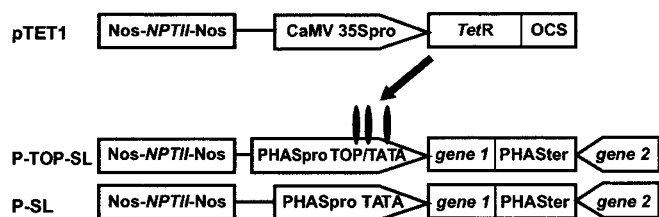

Fig. 2. Binary vector constructs used to transform tobacco W38 cultivar. pTET1, R construct. P-TOP-SL and P-SL, SL constructs. *Tet*R, *E. coli* tet R gene. The interaction of the tet R protein (*TET*R) with its DNA binding element (*tet*O) in P-TOP-SL is shown. TOP/TATA, three copies of the DNA binding element (*tet*O) inserted into the native TATA box sequence of the phaseolin promoter (PHASpro). P-SL is a nonrepressible control construct. Arrows indicate promoters and direction of transcription. *Gene 1*, *Agrobacterium* gene for tryptophan-2-monooxygenase (*iaaM*). *Gene 2*, *Agrobacterium* gene for indole-3-acetamide hydrolase (*iaaH*). The seed-specific phaseolin promoter drives *gene 1*, whereas *gene 2* is controlled by its own promoter. Coexpression of *gene 1* and *gene 2* leads to overproduction of indole-3-acetic acid in the seed and consequently to inhibition of germination. PHASpro and PHASter are the promoter and terminator, respectively, of the *phaseolin* gene. *NPTII*, neomycin phosphotransferase gene conferring kanamycin resistance in transgenic plants. Nos, promoter and terminator of the *Agrobacterium* nopaline synthetase gene. TATA, native TATA-box sequence present in PHASpro. CaMV 35S, promoter of the cauliflower mosaic virus 35S gene. OCS, terminator region of the *Agrobacterium* octopine synthetase gene.

its binding site, *tet*O, which was introduced into the core promoter region of the phaseolin promoter (Fig. 2). Both elements, *tet*R and *tet*O, are derived from *Escherichia coli*, where they are part of an operon regulating tetracycline resistance (6). Functionality of the *tet*R/*tet*O system as a genetic control system in plants has been demonstrated numerous times (7–9). In this study, we used transgenic tobacco to test whether it was possible to (*i*) achieve seed-specific lethality, (*ii*) repress a SL gene, and (*iii*) maintain selfed plants containing both SL and R elements over several generations.

Experimental Protocol

Vectors and Constructs. To facilitate subcloning, a pGEM7Zf(+) plasmid (Promega) was modified by inserting restriction sites for *Nco*I, *Bgl*II, *Pst*I, and *Eco*RV between the *Cla*I and *Bam*HI sites. The phaseolin promoter for the phaseolin promoter–SL (P-SL) construct was amplified by PCR from pAGM219 (obtained from Mycogen, San Diego) and corresponds to nucleotides 128–933 of the GenBank sequence J01263 (10). Restriction sites for *Xho*I and *Hin*dIII were added to the primers. The phaseolin promoter for the construct phaseolin promoter with tet operator sequences–SL (P-TOP-SL) was also amplified by PCR from pAGM219. It corresponds to nucleotides 128–833 of J01263. Restriction sites for *Xho*I and *Csp*45I were added to the primers. Both strands of the triple operator sequence were chemically synthesized, and *Csp*45I and *Cla*I sites were added. The (+) strand sequence is 5′-CGAAGACTCTATCAGTGATAGAGTGTATATAAGACTCTATCAGTGATAGAGTGAACTCTATCAGTGATACAGTAT-3′. The phaseolin terminator was excised as a *Pst*I fragment from pAGM219. The *gene 1* coding region was isolated by PCR from plasmid p202 (obtained from Mycogen) and subcloned as a *Cla*I fragment. The sequence corresponds to nucleotides 5755–8056 of pTi 15955 (11). *Gene 2*, which includes its own promoter and terminator region, was isolated by PCR from p101 (obtained from Mycogen) and subcloned as an *Sph*I fragment. The sequence corresponds to nucleotides 5785–3237 of pTi 15955 (11). For plant transformation, constructs were cloned into the binary vector Bin 19 (12) to give plasmids P-TOP-SL and P-SL, respectively (Fig. 2).

Plant Transformation. Tobacco cv. Wisconsin 38 (W38) was transformed by using the leaf disk method (13) and *Agrobacterium* strain EHA105. Fifty-three transgenic lines containing the P-TOP-SL construct and nine lines with the P-SL construct were kept for further analysis. Transgenic W38 tobacco already transformed with pTet1 (R lines) was obtained from C. Gatz (University of Göttingen, Göttingen, Germany). All transgenic plants were grown in greenhouses with a 16-h light/8-h dark cycle.

Germination Assays. Sterile seeds from each line (88 per plant) were placed on 9-cm Petri dishes on Murashige and Skoog (MS) medium with or without kanamycin. Seedlings were analyzed after 4 weeks of germination. The T_0 SL lines displaying SL phenotypes were kept for crossing with R lines.

PCR Analysis of Transgenic Plants. DNA was prepared from seedlings and mature plants according to the method of Edwards *et al.* (14) with the modification that an additional chloroform extraction was carried out before the DNA precipitation step. PCR was usually carried out in 96-well plates by using the primer pair gctcacccatctcaacccacac and tctaagaaggcatcggaaacc to detect the presence of *gene1*, and the primer pair attgagatgttagataggcac and ccactttcacatttaagttg to detect the presence of the *tet*R gene. Cycling conditions comprised an initial step at 94°C for 3 min followed by 30 cycles of 45 s at 94°C, 1 min at 56°C, 1 min at 72°C, and a final extension step at 72°C for 5 min.

Results

Transformation of Tobacco Plants. Two constructs, P-SL and P-TOP-SL, were prepared to evaluate the effect of *gene 1* and *gene 2* on tobacco seed development and the ability to repress P-TOP-SL with the tet R (Fig. 2). Fifty-three plants transformed with construct P-TOP-SL and 10 plants transformed with construct P-SL (SL lines containing SL genes) were selected for analysis based on the presence of the SL construct as determined by Southern blots (data not shown). All transformed plants showed normal vegetative development and seed production. An average of four flowers per SL line were either self-pollinated (selfed) or cross-pollinated (crossed) with either untransformed tobacco (backcrossed) or plants carrying the *tet*R gene (R lines containing R genes). The resulting seeds were germinated on medium with and without kanamycin (n = 88 per cross). The number of active SL loci was determined by counting the number of SL phenotypes and the kanamycin-resistant and kanamycin-sensitive seedlings obtained from selfed and backcrossed plants (Table 1). The inheritance of the SL and/or R genotype was determined by duplex PCR using gene-specific primer pairs for *gene 1* and *tet*R.

Seed Specificity of the SL Constructs. The P-TOP-SL and P-SL plants displayed the same range of inhibition of seed germination on MS solid medium. The seeds from a single transformed line displayed either one or a combination of four arbitrarily determined phenotypes (Fig. 3). Of the primary transformants, 41 plants containing the P-TOP-SL construct and 9 plants containing the P-SL construct were recovered that segregated SL phenotypes. Based on the results of the germination assays, the number of SL loci ranged from one to four (Table 1).

When seeds of SL lines were placed on soil instead of medium, to reflect natural conditions, the distinction between normal germination and seed lethality was clearly obvious. Only seeds from plants that lost the SL genotype through segregation germinated, with no observable intermediate phenotypes. Under typical soil germination conditions, the SL construct provided a complete inhibition of germination (data not shown). Crucial to our approach was also the demonstration that the introduction of the *tet*O binding sequence into the core phaseolin

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Table 1. Germination analysis of plants transformed with the P-TOP-SL construct

Analysis	No. of SL loci 1	2	3	4	Total
Frequency of SL loci	20	11	6	4	41
SL phenotypes and their frequency per loci					
No SL phenotype					12
a	2	1	2	0	5
a, b	10	6	3	4	23
a, b, c	0	1	0	0	1
a, c	1	0	0	0	1
b	4	0	0	0	4
b, c	0	1	0	0	1
b, c, d	1	0	0	0	1
c, d	1	1	1	0	3
d	1	1	0	0	2
Total					53
No. of SL plants (T_0) crossed with R plants	18	10	6	3	37
No. of F_1 seeds (SL × R) germinated	1,716	924	528	352	3,520
Percent of SL phenotype in F_1 of (SL × R) crosses	46	58	76	50	
SD	19	6	13	4	
Percent of SL phenotype that can be expected in backcrosses	50	75	87.5	93.8	
No. of N/(SL; R) lines recovered in F_1 generation	9	4	5	3	21
No. of N/(SL; R) lines recovered in F_2 generation	7	3	5	3	18
Reoccurrence of SL phenotype in F_2	Yes	Yes	Yes	Yes	

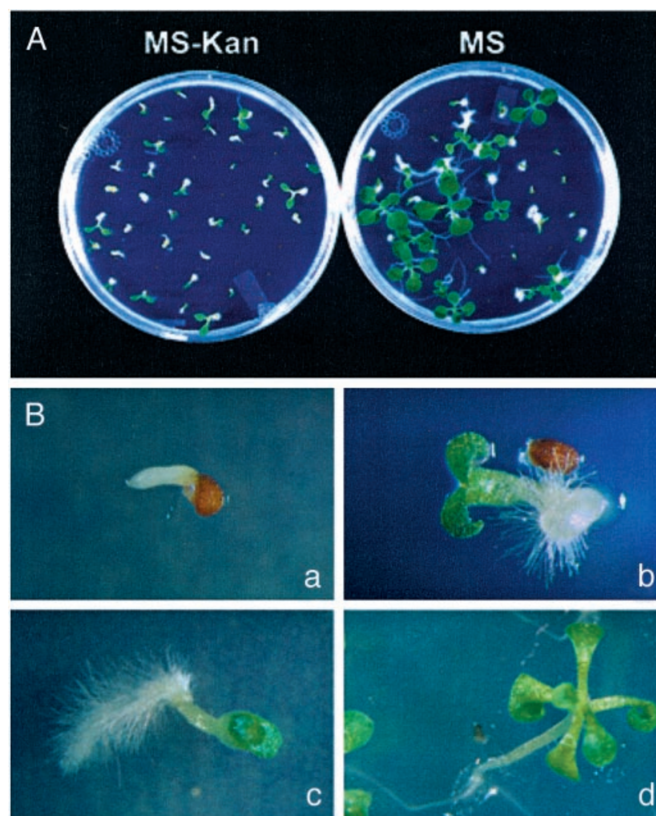

Fig. 3. (*A*) Germination of tobacco seeds from selfed T_0 plants transformed with the SL construct P-TOP-SL on medium with (MS-Kan) and without (MS) kanamycin. The seedlings are ≈4 weeks old. The SL phenotypes are clearly distinguishable. The normally developing seedlings on the MS plate have lost the P-TOP-SL construct through segregation. (*B*) Four typical SL phenotypes, arbitrarily classified as a, b, c, and d, based on their appearance, are shown. These phenotypes were observed only when SL seeds were germinated on MS medium. When SL seeds were put in soil, no germination was observed. The distribution of the SL phenotypes is shown in Table 1.

promoter did not affect tissue specificity and that both SL constructs had no observable effect on pollen viability, and hence normal male fertility could be observed.

Repression of the SL Phenotype by *TETR*. SL lines containing either the P-SL or the P-TOP-SL construct were crossed with an established R line (obtained from C. Gatz), which had been transformed with pTet1 (8). One R line displaying the highest *tet*R expression, as determined by Northern blots (results not shown), was used for cross-pollination. The seeds resulting from these crosses were germinated on MS medium containing kanamycin ($n = 88$ per plant). The frequency of kanamycin-resistant and SL phenotypes was scored after 4 weeks of germination. If *TET*R represses the expression of the SL construct, normally developing plants containing both the SL and R genes can be expected [referred to as N/(SL; R) for plants of normal phenotype and SL and R genotypes]. Normal kanamycin-resistant seedlings were tested by duplex PCR for the presence of the SL and R genes. An average of two N/(SL; R) plants per cross were kept and grown for further crossing. Based on the ratio of kanamycin-resistant to kanamycin-sensitive plants, the R line used for the crosses had more than four *tet*R loci.

Of the 37 different primary transformant P-TOP-SL plants that were crossed with the R line, 21 produced N/(SL; R) plants, indicating repression of the SL phenotypes in the F_1 (Table 1). However, the percentage of repression varied among individual crosses, and seedlings with SL phenotype occurred in all crosses. The remaining 16 F_1 plants did not produce N/(SL; R) plants, indicating that no repression occurred in these lines. The normally developing seedlings of these plants were of N/(R; R) genotype only.

The PCR analysis of the F_1 generation revealed that 40% of the normally developing seedlings from (SL × R) lines were of N/(SL; R) genotype, whereas 60% were N/(R; R). No N/(SL; SL) genotype was detected (Table 2).

Because a silenced or nonfunctional *gene 1* can also explain the normal development of a plant with a (SL; R) genotype, N/(SL; R) lines were propagated to follow the segregation of the SL phenotype (Table 1). The 21 F_1 lines producing N/(SL; R) progeny were propagated by selfing and backcrossing with nontransformed W38 tobacco. All F_2 lines, selfed and backcrossed, produced seedlings with observable SL phenotypes as a result of segregation of the SL construct from the R gene (Table 1). This finding is an indication that the repression of *gene 1*, as opposed to the silencing of the SL construct, generated the N/(SL; R) plants in the previous generation. Normally developing seedlings were again analyzed by duplex PCR. For all F_2 lines, the N/(SL; R) genotype was present in 29% of the samples with 71% being of N/(R; R). In this generation, one selfed line also produced N/(SL; SL) plants, which made up 0.4% of the total sample (Table 2). The results in Table 2 show that the backcrosses, as expected, resulted in considerably fewer plants with the N/(SL; R) genotype compared with the selfed F_1 because of the segregation of the SL construct from the R. In total, 18 of the 21 lines produced N/(SL; R) plants in the F_2

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Table 2. PCR analysis of normally developed plants obtained from SL × R crosses

Construct/generation	No. of lines	No. of samples	% of N/(SL; R)	% of N/(SL; SL)	% of N/(R; R)
P-TOP-SL construct					
F_1	21	710	40	0	60
F_2 selfed	15	861	19	0.4	44
F_2 backcrossed	16	763	10		27
F_2 total	17	1,624	29	0.4	71
P-SL construct					
F_1	9	296	1.6	0	98.4

generation, indicating that a more consistent repression of the SL phenotype may be achieved in stabilized transgenic SL lines.

The N/(SL; SL) Genotype Is Not Present in F_1 Plants. The repression/derepression of the SL construct is further supported by the analysis of the segregation of the SL genotype. The frequency of normally developing N/(SL; SL) seedlings is an indicator of the functionality of the SL construct, as N/(SL; SL) plants would indicate loss of SL function. Of the 37 SL plants that were initially crossed with R lines, none produced seedlings with only the (SL; SL) genotype in the F_1 (Table 2). In normally developing seedlings of these lines, the SL construct could, without exception, be detected only in combination with the *tet*R gene. In the F_2, however, one line produced plants of N/(SL; SL) genotype, although this is more likely caused by a segregated, inactive SL loci because the same line also displayed functional SL loci. Nonetheless, no line displayed a complete loss of SL function.

The P-SL Construct Does Not Mediate Repression. The control crosses performed with the P-SL plants that contain the phaseolin promoter without the *tet*O-binding element provided further evidence that *TET*R is responsible for the loss of the SL phenotype. Of the nine P-SL lines that were crossed with R lines, all lines produced the same SL phenotypes as the P-TOP-SL construct. In lines where normal seedlings appeared, they were of the N/(R; R) genotype, with one exception (Table 2). The only line that produced N/(SL; R) plants contained several SL loci and showed a high number of less severe SL phenotypes in the progeny of the selfed T_0 generation. It is possible that the segregation of these weak SL loci in the F_1 generation gave rise to the normal phenotype observed.

Discussion

The increasing scope of cultivation of crops with novel traits will eventually require systems to ensure that genetic combinations within these plants are maintained and not easily transferred to other crop species or wild relatives. It is therefore not surprising that considerable effort has been put forward to develop methods aimed toward either the containment of transgenes or the restriction of gene flow in general. These technologies can be divided into either conditional genetic use restriction technologies whereby containment is usually achieved through application of a chemical inducer (15–20) or nonconditional methods that require no intervention, such as the restriction of the transgene to maternal inheritance by the use of chloroplast transformation (21).

The system of repression/derepression we have described here is simple and could provide a mechanism to control the unwanted spread and establishment of novel traits within sexually compatible plant species without the need for further intervention. The method takes advantage of the fact that most cultivated crops represent specific gene combinations that are maintained during cultivation. By combining potentially contentious genes, such as transgenes, within a genetic combination that is deleterious in the absence of R, the transgene combination will be rapidly eliminated from a wild or unmanaged population without a managed R locus. The use of this system under typical agricultural conditions, where seed is harvested and new varieties are sown and crops are rotated, may prohibit the establishment of novel traits in unintended populations.

The repressible SL system evaluated here is effective under experimental conditions; nonetheless, it is obvious that for successful field application, the system has to be optimized. During the evaluation of the system, no preselection of primary transformants with respect to parameters such as phenotype, copy numbers of inserts, or number of SL or R loci was carried out. This somewhat complicated the ensuing genetic analysis; on the other hand, it did provide a better insight of the minimal requirements needed to achieve seed lethality and its complete repression after a succession of crosses. The analysis of the data presented here leads to a number of conclusions as to how the system could be optimized. The most critical parameter is to ensure a tight repression. In an ideal system, only one R locus and one SL locus should be present; therefore, the efficacy of the R and the activity of the seed-specific promoter have to be optimized, so as to function reliably in combination. This could be achieved by making the *tet*O binding sites and/or the *TET*R more effective. For example, the TOP sequence used in this study has been shown in other studies to show a low basal level of activity (22) and tighter repression has now been achieved by higher degrees of oligomerization of R binding sites (23). Because *gene 1* is the key component in providing lethality, emphasis should be directed to the use of a weaker, easier-to-repress seed-specific promoter for *gene 1* expression, because the SL effect was so clearly evident with the phaseolin promoter, particularly under soil conditions. Lastly, to achieve a true containment of all transgenes, the R construct itself, although it should not provide any genetic advantage or disadvantage, would have to be controlled as well. This could be accomplished by using a double repression system and associating an additional lethal component to the R construct (24). In such a double repression system, a site-specific insertion of the complementary SL and R constructs on the same parental chromosomes would be necessary to prevent cosegregation of the R and to achieve a complete segregation of the SL and R components upon incrossing or outcrossing.

In summary, we have shown that *gene 1* and *gene 2* from *Agrobacterium* in combination with the *tet*O/*tet*R components, regulating a phaseolin promoter, can be used to promote and repress seed lethality. Although the bacterial Tet-repressible system has been shown to function in plants (7–9), our example shows a tissue-specific repressible system in plants based on the *tet*R. It will be interesting to see whether this approach can be applied to other tissue-specific or inducible plant promoters. The system presented here could be used as a model to address the issue of transgene management and preservation of specific genetic combinations in general.

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