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Transgene Containment Using Cytokinin-Reversible Male Sterility in Constitutive, Gibberellic Acid–Insensitive (Δ*gai*) Transgenic Tobacco

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Abstract

Mechanisms are needed to prevent gene flow from transgenic crops, and the later establishment of these transgenes in populations of other varieties, weeds, or wild relatives. Such prevention can be achieved by containing the transgene within a crop, and then mitigating the effects of the inherent leakage and unidirectionality of containment systems. Mitigation lowers the fitness of recipients below that of the wild-type so that transgenes cannot spread. Transplastomic and male-sterility systems suppress transgene outflow, but not the influx of pollen from relatives, requiring mitigation. The *Arabidopsis thaliana* Δ*gai* (gibberellic acid–insensitive) gene, driven by its own promoter, induced male sterility in transgenic tobacco (*Nicotiana tabacum*), which is chemically reversible by kinetin applications. Female reproduction was not affected. Kinetin-treated sterile hemizygous and homozygous dwarf tobacco produced viable pollen, becoming self-fertile with copious viable seed, restoring the small amount of seed production needed for such a crop. Thus, Δ*gai,* under its endogenous promoter, can be used as a containment mechanism to prevent transgene outflow. This application is in addition to the previously described highly effective role of Δ*gai* as a dwarfing mitigator gene, which renders the rare transgenic tobacco hybrids unfit and unable to compete with the wild-type in the mixed cultures. Δ*gai* is unique in that it can be used both to prevent transgene outflow and to mitigate the flow should containment fail or should gene influx occur, a dual role for the gene, not previously reported.

Key words: Gibberellic acid–insensitive; Kinetin; Reversible male sterility; *Nicotiana tabacum*; Gene flow; Transgene containment; Transgenic mitigation (TM)

Introduction

Several molecular mechanisms have been proposed for containing transgenes within crops, preventing transgene introgression into other crop varieties or

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related weeds and wild species (Gressel 2002; Stewart and others 2003). All systems have their drawbacks. These include utilization of partial genome incompatibility with crops having multiple genomes derived from different progenitors. It had been suggested that the risk of introgression could be reduced if the transgene was inserted into the unshared genome. This proved ineffectual in the case of oilseed rape (*Brassica napus*) (Tomiuk and others 2000). Another possibility is to integrate the transgene in the plastid or mitochondrial genomes (Daniell 2002; Maliga 2004). The opportunity of gene flow is limited due to maternal inheritance of these genomes. This technology does not preclude the possibility of pollen influx from the relatives to the transgenic crop. Leakage of chloroplast-encoded genes via pollen typically occurs, as greater than 0.03% pollen transmission was found in the field (Wang and others 2004). A novel additional combination that considerably lowers the risk of plastome gene outflow within a field (but not gene influx from relatives) can come from utilizing male sterility (described below) with transplastomic traits (Wang and others 2004). Introducing plastome-inherited traits into varieties with complete male sterility would vastly reduce the risk of transgene outflow. Such a double fail-safe containment method might be considered sufficient where there are highly stringent requirements for preventing gene outflow to other plant varieties (for example, to organically cultivated ones), or where pharmaceutical or industrial traits are engineered into a species (Galun and Galun 2001).

Other approaches including seed sterility utilizing the genetic use restriction technologies (GURT) (Oliver and others 2004) and recoverable block of function (Kuvshinov and others 2004), have been proposed. Such technologies control volunteer seed dispersal, but theoretically if the controlling element of the transgene is silenced, expression would occur, rendering a critical defect in principle and in practice. The frequency of loss of such controlling elements remains unclear, as there have been no large-scale field trials to date. A more complicated technology suggests that the use of a repressible seed-lethal system (Schernthaner and others 2003), which seems to be technically impractical at present, as it requires that a repressor gene and a seed lethal gene be engineered at identical allelic positions on sister chromosomes to prevent recombination (crossing over). Another option would be insertion of the transgene behind a chemically induced promoter so that it will be expressed upon chemical induction (Jepson 2002). Still, there is a possibility of an inducible promoter mutating to become constitutive.

None of the above containment mechanisms is absolute; they are all unidirectional, preventing either transgene outflow or influx, and none can do both. Thus, the risk can only be reduced by stacking mechanisms to compound the infrequency of gene escape. Still, even at very low frequencies of gene transfer, once it occurs, the new bearer of the transgene could disperse throughout the population if it has just a small fitness advantage. Thus, the concept of ''Transgenic Mitigation'' (TM) was proposed to add mitigator genes to the desired transgene. This would reduce the fitness advantage to weeds and considerably reduce the risk of gene spread (Gressel 1999). In mitigation, the primary transgene of interest (herbicide resistance, disease resistance, and so on) is tandemly coupled with a suitable mitigator gene such as a dwarfing, nonbolting, no secondary dormancy, no seed shattering, or poor seed viability gene, depending on the instance. For example, RNAi of a gene for starch synthesis coupled to a pharmaceutical gene in maize (*Zea mays*) should prevent shattered seed from being able to overwinter and become a volunteer weed the following season (Gressel and Al-Ahmad 2005).

In our previous studies on transgenic mitigation (Al-Ahmad and others 2004, 2005a), we demonstrated the potential utility of the mitigation concept using tobacco (*Nicotiana tabacum*) as a model, with herbicide resistance as the primary gene and dwarfing as the mitigator. We showed that the Δ*gai*-induced dwarf plants were highly productive when grown alone but were weak competitors and unable to reach maturity when grown interspersed with the wild-type tobacco. In addition to the dwarfing effect, the Δ*gai*-transformants were male sterile, failing to produce mature, viable pollen. Male sterility is used in many crop species as a means of producing hybrid seed. It can also be used for transgene containment by preventing transgene outflow from genetically modified flowering plants into non-transgenic varieties and/or related species (Gressel 2002). However, the male sterile plants can be pollinated by the wild-type or by weeds, and the resulting hybrids can spread into the natural populations. The related species can then act as the recurrent pollen parent.

Several male-sterility systems targeting pollen have been developed to limit or control gene outflow. The *barnase* gene driven by a tapetum-specific promoter caused pollen sterility in tobacco and oilseed rape, and fertility restoration was achieved by using the *barstar* gene, which encodes a *barnase*-specific inhibitor (Mariani and others 1992). Another system relies on constitutive expression of the *Agrobacterium rhizogenes rolC* sterility gene. Fertility was restored in the sterile tobacco plants by crossing them with plants

having *rolC* in an antisense orientation (Schmulling and others 1993). An alternative strategy relies on site-specific recombination to remove the transgenes from pollen (Keenan and Stemmer 2002).

Gibberellins (GAs) are essential endogenous regulators of plant growth and reproductive development (Sawhney and Shukla 1994). The semi-dominant Δ*gai* (GA-insensitive) mutant allele with a deletion of the GA-reception DELLA domain, reduces GA responses and causes dwarfism (Koornneef and others 1985; Peng and Harberd 1993). In *A. thaliana* and petunia (*Petunia hybrida*) GAs promote flowering, as well as petal, stamen, and anther development, and their deficiency results in male sterility because of a lack of mature pollen (Blazquez and Weigel 2000; Cheng and others 2004; Huang and others 2003; Hynes and others 2003; Izhaki and others 2002; Wilson and others 1992). Male fertility can be restored by GA application to GA-deficient tomato (*Lycopersicon esculentum*) mutants (*ga*-2 and *gib*-1) (Jacobsen and Olszewski 1991; Nester and Zeevaart 1988), but not to mutants such as Δ*gai* that are deficient in the GA receptor. Cytokinins also regulate many developmental processes in plants, including flower development (Sawhney and Shukla 1994), and they can restore cytoplasmic male sterility in barley (*Hordeum vulgare*) (Ahokas 1982). Exogenous applications of kinetin and thidiazuron restored male fertility in *CKX*1 (cytokinin oxidase) transgenic maize. Kinetin alone restored the fertility in transgenic tobacco expressing Δ*gai* under an anther specific promoter, but thidiazuron and GA failed (Huang and others 2003).

In this study, we tested the effect of the constitutive expression of the *A. thaliana* Δ*gai* gene, driven by its own promoter, on anther and pollen development in primary transformants as well as in later homozygous and hemizygous generations of transgenic tobacco. The dwarf-inducing Δ*gai* caused male sterility, which was restored with exogenous applications of kinetin. These results suggest a dual role for Δ*gai*, both as a containment mechanism by preventing male fertility, and in its role in mitigating the effects of transgene flow by causing dwarfism, which will together preclude transgene spread throughout the population.

Materials and Methods

Plasmid Construction

The pPZP212-*ahas*^{R}-Δ*gai*-1 tandem construct (TM 1) includes the *Arabidopsis thaliana ahas*^{R} (acetohydroxy acid synthase) mutant gene for herbicide (imazapyr) resistance as the primary gene of choice, and the dwarfing Δ*gai* (gibberellic acid insensitive) mutant gene as a mitigator. The assembly and verification of the TM 1 construct are described by Al-Ahmad and others (2004). Both genes were tightly linked in the same orientation with a 15-base pair linker. The construct also contained the *kan* gene encoding neomycin phosphotransferase II (NPTII), conferring plant resistance to kanamycin, carried within the native T-DNA of the pPZP212 binary vector (Hajdukiewicz and others 1994).

Plant Material

The TM 1 construct was transformed into *Nicotiana tabacum* cv. Samsun NN leaf discs, as described by Horsch and others (1985). The male sterile primary T_0 transgenic plants were transferred to soil and were backcrossed with wild-type *N. tabacum* as the pollen donor. Hemizygous semi-dwarf and imazapyr-resistant TM T_0 (Line 10), T_1 (= BC_1 with wild-type) (lines 8, and 10), and homozygous TM T_4 (line 7) tobacco plants were transferred to soil and grown to flowering under greenhouse conditions. The length and fresh weight of the whole flower of the wild-type, hemizygous and homozygous TM T_4 (line 7) plants, as well as for the male (stamen) and the female (pistil) reproductive organs were measured.

Reversal of Male Sterility

Three to 6 plants from each of the above transgenic lines were sprayed with 7.5 mg of kinetin (Sigma—batch analysis >99%) per plant. The final aqueous solution contained 1 mM NaOH and 0.1% (v/v) Tween 20 surfactant. Control groups received the 1 mM NaOH and the surfactant solution, or were not treated. All applications were made as foliar sprays every other day for two weeks. Wild-type pollen donors were backcrossed to two plants from each line without kinetin application to check the female fertility of the TM plants. In these cases, the plants were emasculated prior to anthesis and pollinated by rubbing two to three anthers on the stigma surface. When mature plants stopped flowering, the capsules formed were counted and the dry weight of set seed of up to 10 capsules per plant was measured, and the total seed yield per plant was calculated.

Pollen Viability Test

Young anthers were collected from three or more flowers per tobacco plant at least three times during the flowering period. A minimum of three plants

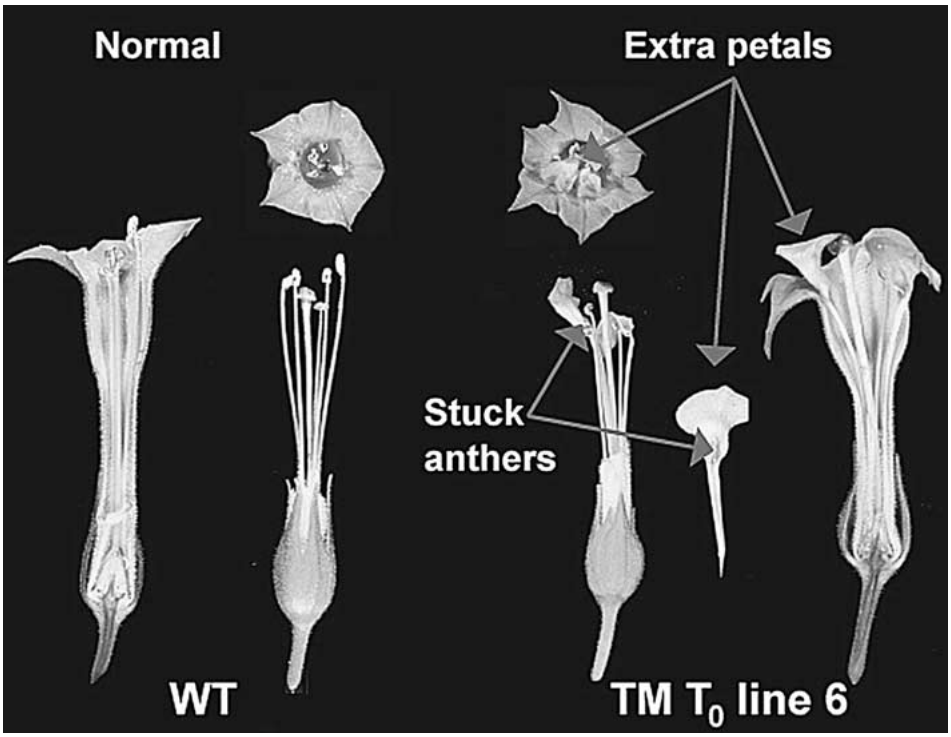

Figure 1. Transgenic expression of Δ*gai* alters tobacco flower development. Almost all the flowers of the primary transgenic line 6 and few from selfed T_2 had extra petals with stuck aborted anthers. The other TM transgenic lines (2, 5, 7, 8, 9, and 10) had normal flower morphology, with the exception of male sterility and smaller size than the wild-type (Figure 2A).

were analyzed per biotype per test. Pollen viability was scored by staining the pollen with cotton blue as outlined by Phillips (1981), and by counting the number of viable, deeply stained grains having normal morphology versus nonviable abnormal shrunken and non-stained ones.

Seed Viability

The viability of seeds obtained from plants with kinetin-restored male fertility was checked. Seeds of untreated wild-type, the treated TM plants, as well as from the untreated TM plants backcrossed with the wild-type, were germinated on wet filter paper in petri-dishes. The performance and the possibility of spontaneous male fertility of the next generation progeny was ascertained by germinating seeds in soil, and five plants from each line were transferred to the greenhouse. Plant growth was monitored, and the TM nature of the progeny was confirmed by testing for imazapyr resistance by the AHAS enzyme assay as described in Al-Ahmad and others (2004).

Statistical Analyses

The data were statistically analyzed using the JMP program (version 4.0.1; SAS Institute 2000) by one way analysis of variance (ANOVA) and comparing the least significant differences (LSD). Probability levels were considered to be statistically significant at $p \leq 0.05$, and highly significant at $p \leq 0.01$. Differences were considered to be statistically not significant at $p > 0.05$.

Results and Discussion

Constitutive Expression of Δ*gai* Causes Male Sterility in Tobacco Transformants

We tested the effect of constitutive expression of the Δ*gai* transgene driven by its own *A. thaliana* promoter in the primary T_0 transformants and each generation progeny through T_5 . This testing was performed to ascertain whether the male sterility achieved when the native constitutive promoter is used can be reversed by cytokinins. Seven independent primary semi-dwarf, kanamycin-resistant and imazapyr-resistant tobacco lines were obtained by transformation with the TM 1 construct (Al-Ahmad and others 2004). The TM T_0 flowers had normal exterior morphological proportions, with the exception of line 6, which had extra petals with stuck aborted anthers (Figure 1), an effect that disappeared in the T_3 selfed progeny. The level of Δ*gai* gene expression affected the length of the floral organs. The wild-type corolla, stamens, and pistils were significantly longer than the transgenic ones (T4 line 7) ($p \leq 0.01$; Figure 2), and the homozygous flowers and stamens were shorter yet than the hemizygous ($p \leq 0.05$; Figure 2A,B). The wild-type

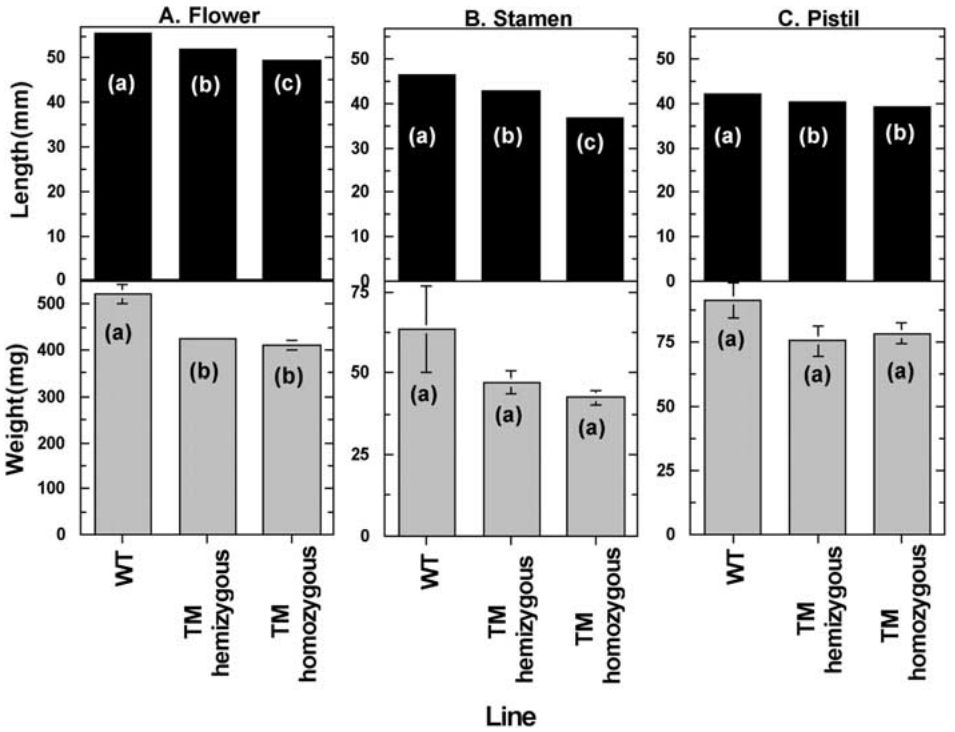

Figure 2. Differences in floral development between selfed wild-type and TM T_4 hemizygous and homozygous tobacco plants bearing the constitutively expressed Δ*gai* gene. Differences in organ length and fresh weight were measured once at the mid-flowering period. The selfed hemizygous TM T_4 plants were almost completely male fertile, whereas the homozygous ones were male sterile. Stamen fresh weight represents the collective weight of all five filaments and anthers per flower. Data bars represent the mean of eight measurements ±SEs. SEs were smaller than the data bars where there are no error bars. Different letters appearing within a panel indicate significant differences among treatments based on LSD values at $p \leq 0.05$.

flowers were heavier than the transgenics ($p < 0.05$), and this may be due to the longer wild-type flower tube, as the fresh weights of separated pistils and stamens of all biotypes were not significantly different (Figure 2). However, when hemizygous and homozygous plants were statistically analyzed together, the TM plants had lighter stamens than the wild-type ($p \leq 0.05$; Figure 2B).

The TM T_0 flowers had abnormal growth and development of the anthers, which were sterile, with almost 0% pollen viability (Figure 3), similar to the results of Huang and others (2003). Female fertility was not affected, as the TM plants set seed normally when pollinated by the wild-type. The T_1 generation also had to be backcrossed with the male wild-type to obtain sufficient seeds. The progeny of these plants shed sufficient pollen to allow selfing to achieve the T_2 generation. T_2 (S_1) progeny of lines 2, 5, 6, 7, and 8 had greater than 50% pollen viability compared to approximately 99% in wild-type plants (Figure 3). Later hemizygous generations (T_3, T_4, and T_5) were sufficiently male fertile that backcrossing with wild-types was no longer necessary. The selfed hemizygous plants (lines 6 and 7) had higher pollen viability (82%–90%; Figure 3) and produced fertile seeds. However, all T_3, T_4, and T_5 homozygous dwarf plants (lines 6 and 7) were male sterile (Figure 3), which had not been previously demonstrated. The reason for the partial restoration of fertility in later generations is not known. Interestingly, male sterility was not found in T_0 oilseed rape plants transformed with the same TM 1 construct (Al-Ahmad, and others 2005b). The reason for the differences is unknown. Oilseed rape, unlike tobacco, is a rosette species, requiring a specific GA as a signal for bolting of the flower stalk, which may require a specific receptor, with which Δ*gai* may not interact.

Exogenous Kinetin Applications Restore Fertility of Male-Sterile TM Transgenic Dwarf Tobacco Plants

Exogenous application of cytokinins could overcome Δ*gai*-induced GA-insensitivity effects in transgenic dwarf plants and restore male fertility.

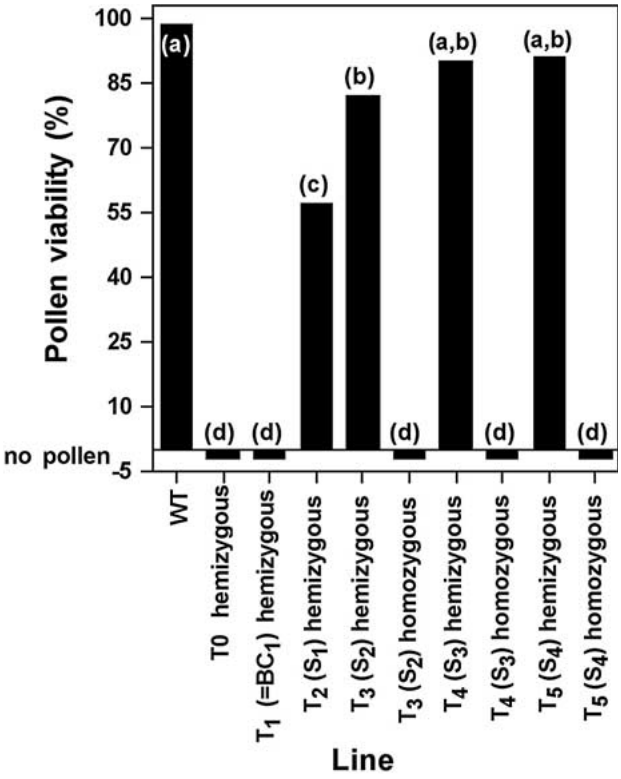

Figure 3. Spontaneous restoration of male fertility in selfed hemizygous, but not homozygous transgenic tobacco constitutively expressing Δ*gai*. ''No pollen'' denotes less than one viable pollen grain measured per anther. Viability is presented as the percentage of pollen stained by cotton blue, as described in Materials and Methods. The different letters above the data bars indicate significant differences among treatments based on LSD values at $p \leq 0.05$.

Sterility caused by anther-specific expression of Δ*gai* in F_1 hemizygous tobacco was reversible by exogenous application of 15 mg kinetin per plant (Huang and others 2003), which had not been checked by Hynes and others (2003) using Δ*gai* with its native promoter.

Our preliminary experiments (data not shown) indicated that kinetin at 7.5 mg per plant was preferable to other concentrations, including 15 mg per plant, which caused severe necrosis as well as early abortion and abscission of almost all flower buds. Lower concentrations of 2 and 5 mg kinetin per plant were less optimal than the 7.5 mg per plant in fertility restoration, and the treated plants had no symptoms of damage. Kinetin applications of 7.5 mg per plant were optimal for fertility restoration of both hemizygous and homozygous without visible phytotoxicity to the flowers, and this amount also resulted in normal fertilization and seed development. The only negative effect of kinetin application was the appearance of necrotic lesions on leaves, which were absent on the leaves of the formulant-treated (aqueous solution containing 1 mM NaOH and 0.1% (v/v) Tween 20 surfactant) control plants. The kinetin treatment did not reverse dwarfism; the height of the treated plants was the same as that of the transgenic control plants.

There was a 2-week lag between kinetin application and the appearance of the first male fertile flowers, suggesting that kinetin could not reverse the effect after a certain point in bud differentiation. After this lag, anthers of the kinetin-treated transgenic plants visibly shed viable pollen (Figure 4). Pollen viability of the restored transgenic lines was greater than 92%, compared to approximately zero in untreated TM plants, and approximately 98% ($p \leq 0.05$) in the untreated wild-type (Figure 4, Table 1). Flowers with kinetin-restored fertility were morphologically normal, and they appeared identical to self-pollinated wild-type flowers (Figure 4). Fertility restoration was observed in all kinetin-treated plants.

Biosynthesis of GA and cytokinins in anthers is required for normal development (described in the introductory paragraphs), but the ability of kinetin to interplay with GA in plants with a perturbed GA-signaling system had not been well established. It might mean that the GA signals induce higher cytokinin levels, which are needed for normal stamen development, and this puts cytokinins closer to fertility control.

Seed Production on Kinetin-Restored Δ*gai* Male-Sterile, Dwarfed Plants

The untreated as well as the kinetin-solvent solution–treated control TM dwarfed plants were completely sterile and thus produced no seed (Table 2). The production of capsules and seed was normal on the TM transgenic plants backcrossed with a wild-type pollen donor (Table 2), suggesting that female reproduction was not affected by Δ*gai*. Selfed wild-type and kinetin-restored hemizygous plants of line 8 were not significantly different in capsule number or seed set per plant (Table 2). As expected, these TM seeds and those of the other homozygous TM lines treated with kinetin gave rise to dwarf plants that were male sterile. Even though the restored hemizygous (line 10) and the homozygous (line 7) produced less seed than the wild-type ($p < 0.05$), the plants were sufficiently productive for agricultural use, as would be similar crops cultivated for leaves or other non-reproductive tissue. The leaf necrosis after kinetin treatment did not prevent seed pro-

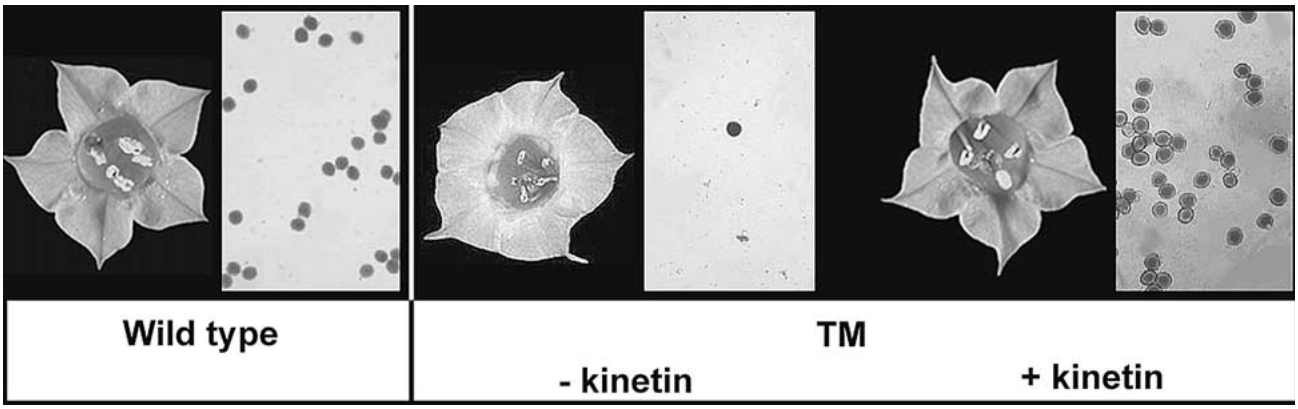

Figure 4. Kinetin-restored male fertility on Δ*gai*-transformed tobacco plants. Restored flowers of the hemizygous TM T_0 line 10, were morphologically normal and had pollen viability similar to the self-pollinated wild-type. Note shriveled anthers and mainly non-viable pollen in TM-transformed tobacco without kinetin and normal anthers and pollen when kinetin is used for fertility restoration.

Table 1. Pollen Viability and Seed Germinability of Kinetin-restored Fertility of Self-pollinated Δ*gai*-transgenic Tobacco Flowers

		Pollen counted			
Plant type	Line	Total	Viable	% Pollen viability	% Seed germinability
Untreated					
Selfed WT		2324	2269	98[a]	82[a]
TM T_0 × WT as pollen donor	10	0	—[d]	—	78[a]
Treated selfed hemizygous TM					
T_0-10	10	438	425	97[a]	84[a]
T_1 (= BC_1)	8	1998	1696	85[c]	78[a]
T_1 (= BC_1)	10	2701	2480	92[b]	85[a]
Treated selfed homozygous TM T_4	7	2540	2422	95[a]	88[a]

BC_1: hemizygous transgenic mitigation (TM) T_0 lines were backcrossed (BC) with the wild-type (WT) as a pollen donor, and their seeds were used to develop the hemizygous TM T_1 (= BC_1) plants that were treated with kinetin.
Different letters within a column indicate significantly different LSD values at $p < 0.05$.

duction, and it would not be a constraint under production of the actual leafy crop, as it occurs only when plants are treated for seed production. Additionally, there was no significant difference between seed germinability obtained from the wild-type (82%) and the kinetin-restored TM plants (84%) (Table 1).

In this study, constitutively expressed Δ*gai* under its native promoter caused male sterility in dwarf tobacco lines, which was reversible by exogenous applications of kinetin. This had been shown with an anther specific promoter (Huang and others 2003), but not checked when constitutive promoters were used (Hynes and others 2003).

Δ*gai*, with its concomitant chemically reversible male sterility, can readily be used as a containment mechanism to prevent gene outflow from the crop to wild species, but not to prevent hybrids due to gene influx, requiring further stacked containment or mitigation. Δ*gai* would be useful as a containment mechanism in crop plants where fruit or seed are not the commercially harvested products. Isolated plants can be treated with kinetin for reproducing varietal material, or they can be crossed with a wild-type pollinator line to produce hybrids, for the small amount of seed needed for planting. For example, it might be useful for containing transgenic herbicide resistance, needed in lettuce (*Lactuca sativa*), which readily intercrosses with a problem weed, *Lactuca seriola* (Mallory-Smith and others 1990).

Interestingly, Δ*gai* did not affect male fertility in transgenic dwarf oilseed rape plants, which had a higher yield than the non-transgenic offspring when grown alone (Al-Ahmad and others 2005b).

Table 2. Kinetin-mediated Male Fertility Restoration and Seed Set in Self-pollinated Δ*gai*-transgenic Tobacco Plants

	Plant biotype	Line (no. of plants)	No. of capsules/plant	Seed set/capsule (mg)	Seed set/plant (mg)
Selfed	Wild-type	(5)	34.2 ± 3.8[a]	120.0 ± 10.0[a]	2566.0 ± 417.6[a]
TM hemizygous					
Untreated	T_1 (= BC_1)	8 (3)	0.0 ± 0.0[c]	0.0 ± 0.0[c]	0.0 ± 0.0[c]
	T_1 (= BC_1)	10 (3)	0.0 ± 0.0[c]	0.0 ± 0.0[c]	0.0 ± 0.0[c]
BC with WT as pollen donor	T_1 (= BC_1)	8 (3)	34.8 ± 2.1[a]	110.0 ± 10.0[a,b]	2376.3 ± 102.42[a,b]
Solvent control	T_1 (= BC_1)	10 (3)	0.0 ± 0.0[c]	0.0 ± 0.0[c]	0.0 ± 0.0[c]
Treated with kinetin	T_1 (= BC_1)	8 (3)	33.3 ± 2.0[a]	107.0 ± 12.0[a,b]	3010.0 ± 105.5[a]
	T_1 (= BC_1)	10 (6)	18.5 ± 2.4	85.0 ± 6.5[b]	1390.0 ± 287.2[b]
TM homozygous					
Untreated	T_4	7 (3)	0.0 ± 0.0[c]	0.0 ± 0.0[c]	0.0 ± 0.0[c]
BC with WT as pollen donor	T_4	7 (3)	21.5 ± 3.3[b]	117.5 ± 11.7[a]	1734.0 ± 177.3[b]
Treated with kinetin	T_4	7 (4)	7.0 ± 0.9[c]	99.6 ± 10.5[a,b]	702.5 ± 116.3[c]

BC_1: hemizygous TM T_0 lines were backcrossed with the wild-type as a pollen donor, and their seed was used to develop the hemizygous TM T_1 (= BC_1) plants.
[a,b,c]Different letters within a column indicate significantly different LSD values at $p \leq 0.05$.
The solvent control was sprayed with aqueous solution containing 1 mM NaOH and 0.1% (v/v) Tween 20 surfactant.

We have demonstrated that the Δ*gai* acts as a transgene outflow containment mechanism in a tobacco model, as it prevents out-crossing to other related species, but it will not prevent influx of pollen from relatives. Influx would not be a problem with crops such as lettuce, which are harvested before they flower, except in the seed-production fields. Δ*gai*, with its second important effect as an efficient mitigator, can prevent the establishment and spread of hybrid progeny from such gene influx (Al-Ahmad and others 2004). Δ*gai* also precludes the crop itself from becoming a voluntary weed. This technology should be useful for many crops cultivated for their leaves, where the production of pollen and selfed-set seeds is prevented in leaf-production fields. As with the other technologies, this technology is not absolute, as genes can mutate or be silenced. Still, it is a stacked system, and stacking of fail-safe measures reduces risks. In situations with exceedingly high risks, this technology could be further stacked with other containment mechanisms and/or other mitigator genes can be added into the tandem construct. This is the first report, to the best of our knowledge, where one gene can have a dual role in transgene containment and in transgene mitigation.

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