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Reversible male sterility in eggplant (*Solanum melongena* L.) by artificial microRNA-mediated silencing of general transcription factor genes

Laura Toppino[1], Maarten Kooiker[2,†], Matias Lindner[2], Ludovico Dreni[2], Giuseppe L. Rotino[1] and Martin M. Kater[2,*]

[1]*CRA-ORL Agricultural Research Council, Research Unit for Vegetable Crops, Montanaso Lombardo (Lo) Italy*

[2]*DSBB, Department of Biomolecular Sciences and Biotechnology, University of Milano, Milan, Italy*

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**Correspondence* (fax +39 02 50315044; email martin.kater@unimi.it)
†Present address: CSIRO Plant Industry, 306 Carmody Road, St Lucia, QLD, Australia.

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Summary

Since decades, plant male sterility is considered a powerful tool for biological containment to minimize unwanted self-pollination for hybrid seed production. Furthermore, prevention of pollen dispersal also answers to concerns regarding transgene flow via pollen from Genetically Modified (GM) crops to traditional crop fields or wild relatives. We induced male sterility by suppressing endogenous general transcription factor genes, TAFs, using anther-specific promoters combined with artificial microRNA (amiRNA) technology (Schwab *et al.*, 2006). The system was made reversible by the ethanol inducible expression of an amiRNA-insensitive form of the target gene. We provide proof of concept in eggplant, a cultivated crop belonging to the *Solanaceae* family that includes many important food crops. The transgenic eggplants that we generated are completely male sterile and fertility can be fully restored by short treatments with ethanol, confirming the efficiency but also the reliability of the system in view of open field cultivation. By combining this system with induced parthenocarpy (Rotino *et al.*, 1997), we provide a novel example of complete transgene containment in eggplant, which enables biological mitigation measures for the benefit of coexistence or biosafety purposes for GM crop cultivation.

Introduction

Male sterility, when referred to as the inability of a plant to produce functional pollen (Kaul, 1988), is an economically important trait for commercial hybrid seed production and for possible biological mitigation measures benefiting coexistence or biosafety purposes in GM crop cultivation. With respect to this latter issue, transgene transfer to non-GM crops or even wild relatives has invoked a strong demand for implementing technologies that will allow coexistence of GM and non-GM agriculture (Daniell *et al.*, 1998). Male sterility offers both benefits for biosafety and coexistence demands. In case of biosafety, environmental and health impact of GM crop cultivation can be significantly reduced in terms of pollen and seed exposure to nontarget organisms, adverse effects related to gene flow to other plants, or persistence and invasiveness of such plants if fitness enhancing traits are part of the genetic modification. For the concerns about the coexistence demand, biological containment measures can be addressed to minimize possible economic consequences of admixing GM and non-GM crops.

Concerning commercial hybrid seed production, exploitation of male sterility strategies has often been hampered because either the expression of the trait is insufficient to give a complete male sterile phenotype or the development, maintenance costs of male sterile plants and the exploitation of the male sterile phenotype have discouraged the development of a reliable and cost-effective technique.

For some crops, prevention of pollen flow between target and nontarget plants can be physically impossible, as it is mediated by environmental factors, such as wind, insects and the proximity of sexually compatible weedy relatives, which are beyond the control of the breeder and farmer, thus leading to the necessity to develop tightly controllable alternative strategies for biological containment (Kuvshinov *et al.*, 2001). Among these strategies for biological containment, male sterility provides an effective way to minimize pollen-mediated gene flow from GM plants to crossable crop or wild plants (Daniell, 2002). Male sterility is also considered one of the most efficient ways to prevent unwanted self-pollination in the process of hybrid seeds production (Kaul, 1988). Hybrid seeds play an essential role in agriculture, because they produce uniform plants benefiting from the effect called heterosis (hybrid vigor), which results in a large increase in yield over the inbred lines or comparable lines produced by out crossing (Lippman and Zamir, 2007). Moreover, F1 hybrids enable '*per se*' the protection of the F1 variety produced by the breeder because the seeds from hybrid plants do not produce plants with an identical phenotype, and therefore new seeds must be purchased for each commercial planting and in the case of exploitation of F1 hybrids in a breeding programme, several selection cycles are necessary to extract potential useful lines (Brown and Caligari, 2008). Many naturally occurring cytoplasmic and nuclear male sterile mutants have been identified in crop plants and thereafter incorporated into hybrid breeding programmes (Daskalov *et al.*, 2007).

Research activities aiming at improving the strategies for gene containment focus on the exploitation of mutations causing a male sterile phenotype (Hanson, 1991), the assessment of mechanical and chemical strategies to disturb pollen function and the development of different pollination control systems by genetic engineering (Perez-Prat and van Lookeren Campagne, 2002). However, for some crops that are not usually vegetatively propagated, an irreversible system is undesirable because of the inability to propagate the male-sterile female parent line in a cost-effective way. Therefore, strategies to make the male sterility trait reversible, yet tightly controllable, are needed.

Among the Solanum genera, different approaches to obtain conditional male sterile plants have been reported in petunia (Napoli *et al.*, 1990; Derksen *et al.*, 1999), tobacco (Kriete *et al.*, 1996;. Fisher *et al.*, 1997; Cho *et al.*, 2001; Bayer and Hess, 2005) and tomato (Mariani *et al.*, 1990 and 1992; Ribarits *et al.*, 2007), and most of them include a system for the recovery of pollen fertility. However, all these approaches are based on the introduction of genes derived from organisms other than plants, which generally raises public concern. In the last few years, efforts were made towards the acceptance of cisgenic plants that differ from transgenic plants by the use of only genes derived from crossable, sexually compatible plants (Schouten *et al.*, 2006). Recently, Li *et al.* (2007) reported the development of a male sterility induction system in *Arabidopsis thaliana* based on the loss of the endogenous *AtMYB103* transcription factor function through a T-DNA insertion in this gene or by expressing AtMYB103 as a fusion to the EAR repressor domain (Hiratsu *et al.*, 2003). Male sterility was restored in the F1 generation by crossing the male sterile lines with plants that express *AtMYB103* at high levels from the TA39 promoter.

Here, we report the development of a reversible male sterility system based on anther-specific artificial microRNA-mediated silencing of endogenous TBP-associated factors (TAFs) encoding genes (Albright and Tjian, 2000), which reduces the use of non-plant genes with respect to other strategies developed in the past. This male sterility system was validated in eggplant (*Solanum melongena*), an important vegetable crop of which the majority of cultivars are F1 hybrids. Production of eggplant F1 hybrid seeds is expensive because F1 seeds are generally obtained through hand emasculation and hand pollination. Moreover, the employment of the only male sterile recessive mutant (*fms*, *functional male sterility*) known in eggplant has been very limited as the presence of viable pollen in the indehiscent anthers is considered risky because it may cause accidental contamination of the hybrid seeds with seeds obtained from selfing of the female parents (Daunay, 2008). Furthermore, the availability of the transgenic parthenocarpic eggplant lines (Rotino *et al.*, 1997) would allow combining together the seedless and the TBP-associated factor (TAF)-based male sterility traits.

TBP-associated factors belong to the TFIID basal transcription complex that is one of the general transcription factor complexes that make up the RNA polymerase II preinitiation complex. Many of these TAF factors have shown to be essential in various organisms (Sekiguchi *et al.*, 1991; Metzger *et al.*, 1999; Chen and Manley, 2000; Shen *et al.*, 2003). The TAF-based male sterility system was made reversible by the ethanol inducible expression of an amiRNA-insensitive form of the *TAF* target genes. By combining this male sterility system with genetically engineered parthenocarpy, we demonstrate that in these eggplants complete transgene containment is established.

Results and discussion

Setting up the reversible sterility system for eggplant

TBP-associated factors have in most organisms essential roles in the tissues where they are expressed. Recently, we showed for instance that in *Arabidopsis* loss of *TAF6* activity is lethal and that pollen tube growth is severely affected in *taf6* mutant pollen (Lago *et al.*, 2005). We characterized the complete TAF family of *Arabidopsis* (Lago *et al.*, 2004), which showed that all TAF encoding genes are broadly expressed. *TAF10* of *Arabidopsis* is highly expressed in anthers and transiently expressed during lateral root, rosette leaf and floral organ development (Gao *et al.*, 2006; Tamada *et al.*, 2007), and its overexpression causes abnormal morphology of inflorescences (Furumoto *et al.*, 2005). In *Arabidopsis*, TAF10 and TAF13 are encoded by single copy genes and they are the smallest TAF encoding genes, which make them more suitable for complex cloning strategies. By aligning the available *Solanaceae TAF* cDNA sequences obtained from the NCBI databank, we revealed that *TAF10* and *TAF13* from tomato, potato, pepper and tobacco are highly conserved (Supplemental Figures S1 and S2). Based on these data, we decided to use the *TAF10* and *TAF13* genes for the development of the male sterility system in eggplant.

TAF10 and *TAF13* cDNA sequences were isolated from eggplant by RT-PCR using RNA extracted from eggplant anthers. Primers to amplify the complete open reading frames of these genes were designed based on the available tomato *SlTAF10* and *SlTAF13* sequences. The amplified cDNA sequences were subsequently cloned and sequenced. Southern blot analysis using the obtained cDNA sequences showed that both *SmTAF10* and *SmTAF13* are single copy genes in eggplant (data not shown). To investigate the expression of *SmTAF10* and *SmTAF13* in anthers, we performed *in situ* hybridization experiments on eggplant flowers using various stages of development (Figure 1). These experiments revealed that *SmTAF10* and *SmTAF13* are mainly expressed in the anthers and in the ovary. During early stages of anther development, *SmTAF10* and *SmTAF13* are expressed in the cells from which the tapetum originates and in the sporogenous tissue (Figure 1a,d). Later, they remain expressed in the tapetum and in the male germline (Figure 1b,c,e,f) until the tapetum degrades and microspores are released from tetrads (Figure 1h). *SmTAF10* and *SmTAF13* expressions in the ovary occur early in the placenta and continue in ovule primordia and developing ovules (Figure 1a,d,g,h).

To induce male sterility in eggplants, we aimed at the anther-specific silencing of *SmTAF10* and *SmTAF13* genes by an amiRNA-mediated knockdown approach (Schwab *et al.*, 2006). This approach allows triggering in a highly specific manner the silencing of *SmTAF10* and *SmTAF13*. We generated in total four gene constructs (Figure 2a) in which the amiRNAs targeting *SmTAF10* or *SmTAF13* were placed under the control of the tobacco pTA29 and pNTM19 promoters, which are known to rule tissue-specific expression in the tapetum and microspores, respectively (Koltunow *et al.*, 1990; Custers *et al.*, 1997). To make the amiRNA induced male sterility reversible, we developed an inducible system that allows us to complement for the loss of TAF10 or TAF13 activity in anthers. The amiRNAs that target the *SmTAF10* and *SmTAF13* gene transcripts are highly specific for these genes, and *TAF10* and *TAF13* genes that have specific mismatches in the amiRNA target sequences can be

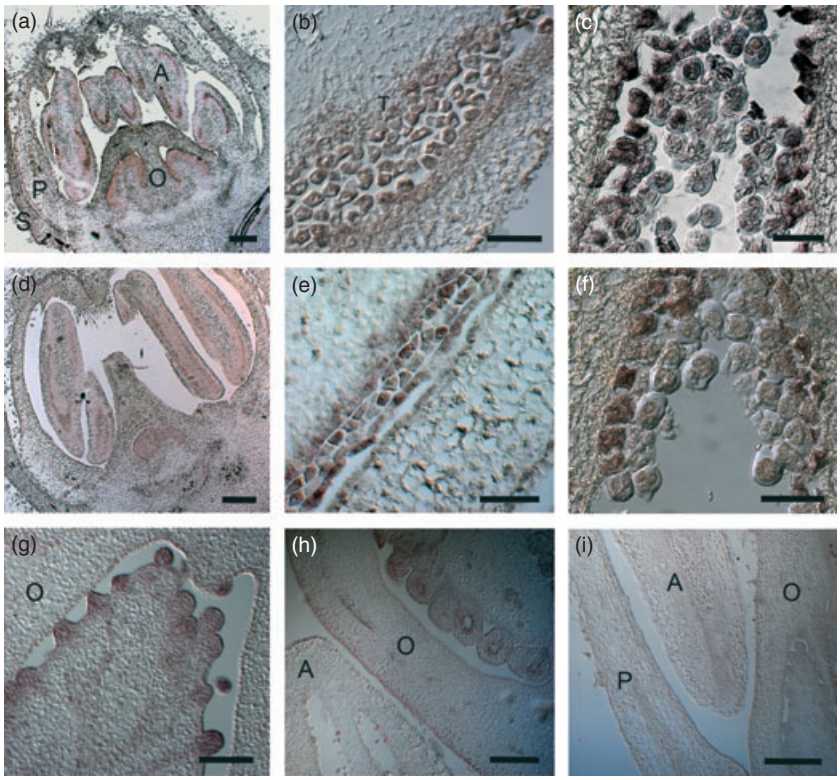

Figure 1 *In situ* analysis of *SmTAF10* and *SmTAF13* expression in eggplant flowers. Both genes are mainly expressed in tapetum and male germ-line cells of the anthers and in the ovary. (a) Expression of *SmTAF10* in eggplant early floral buds. (b) Expression of *SmTAF10* in tapetum and microspore mother cells. (c) Expression of *SmTAF10* in tapetum and tetrads. (d, e, f) Expression analysis of *SmTAF13* in similar stages of eggplant flower and anther development. (g) *SmTAF13* expression in ovule primordia. (h) *SmTAF13* expression decreases in anthers after tapetum degradation, but is still strong in developing ovules. (i) Section of eggplant flower treated with the *SmTAF13* sense probe control showing no hybridization. A, anther; O, ovary; P, petal; S, sepal; T, tapetum. Scale bars represent 200 μm (a, d, h and i), 100 μm (g) and 50 μm (b, c, e and f).

completely insensitive to amiRNA activity (Schwab *et al.*, 2006). This means that amiRNAs can be designed in such a way (according to design principles recently formulated by Schwab *et al.*, 2006 and Ossowski *et al.*, 2008) that they target a specific gene transcript but have no activity towards a closely related mRNA sequence. We designed the amiRNAs in a way that they were expected to be inactive towards the transcripts of the *SlTAF10* and *SlTAF13* genes of *Solanum lycopersicon* (tomato) (Figure 2b,c). Inducing the expression of these tomato TAF genes is expected to complement for the loss of *SmTAF10* and *SmTAF13* activity and will therefore restore male fertility.

To control the expression of the amiRNA-insensitive tomato TAF genes, the ethanol-mediated *alcR/alcA* inducible system of *Aspergillus nidulans* was used (Caddick *et al.*, 1998; Salter *et al.*, 1998). This system allows the activation of gene expression by treating plants with nontoxic low concentrations of alcohol. To prevent segregation of the male sterility system, we combined the anther-specific amiRNA expression constructs with the *alcR/alcA* inducible *SlTAF10* or *SlTAF13* gene constructs (Figure 2a).

Analysis of male sterile transgenic eggplant lines

Agrobacterium-mediated transformation of eggplant with the four pTA29::amiRNA-*SmTAF*/ALC::*SlTAF* and pNTM19::amiRNA-*SmTAF*/ALC::*SlTAF* constructs (targeting either *TAF10* or *TAF13*) was accomplished using the procedure described in Arpaia *et al.* (1997) and resulted in 296 kanamycin-resistant transgenic plants belonging to 77 independent transformed lines. On the average, 19 independent transgenic lines per construct (and an average of four clones/cuttings for each line) were analysed. These plants were indistinguishable from control plants with respect to growth rate, height, morphology and time to flowering, and also morphological evaluation of the anthers did not reveal structural differences with respect to wild-type plants (Supplemental Figure S3). However, all these transgenic plants when left to free pollination produced a reduced number of fruits when compared to the wild-type plants. All transformed lines were weekly evaluated for pollen viability during two successive spring–summer seasons, using at least two cuttings for each line. Pollen viability was first evaluated by staining with acetic carmine. In most of the transgenic lines, pollen staining did not differ from wild type. However, some of the lines (40%) that expressed amiRNAs directed against either *SmTAF10* or *SmTAF13,* under the control of the pTA29 tapetum-specific promoter, showed a large number of uncoloured and shrunken pollen grains (Figure 3b). Subsequently, *in vitro* pollen germination assays were performed (Table 1). Twelve per cent of the plants transformed with the tapetum-specific construct and 21% of the plants with the microspore-specific construct showed throughout the two seasons a dramatic decrease or complete absence in pollen germination (Figure 3b,e) when compared to the untransformed control lines (Figure 3a,d) whose percentage of germination was always above 40%. When using the microspore-specific promoter, a complete

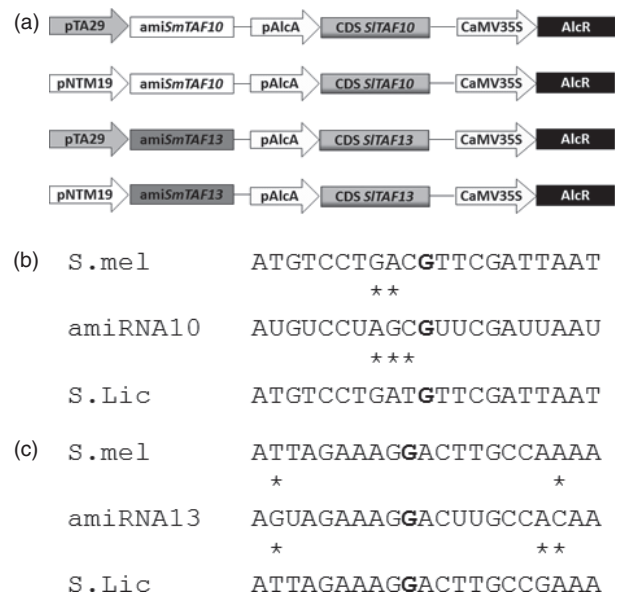

Figure 2 Details of the constructs introduced in eggplant. (a) Graphical representation of the four male sterility constructs that were introduced in eggplant. (b) Alignment showing the mismatches between the *SmTAF10* amiRNA sequence and the corresponding target sequences in *Solanum melongena* (S. mel) and *S. lycopersicum* (S. lic). (c) Same alignment as in b but here showing sequences relative to the *SmTAF13* amiRNA, *S. melongena* and the *S. lycopersicum TAF13* sequences.

sterile line is only expected when multiple T-DNA insertions are present unlinked in the genome. Southern blot analysis indeed confirmed at least three independent insertions in these lines (data not shown). In contrast, for the tapetum-specific TA29 promoter constructs three of the best sterile lines had single insertions. Expression analysis of the target *SmTAF10* and *SmTAF13* genes in anthers of the best male sterile plants transformed with each of the four constructs revealed an anther-specific reduction in the expression of the targeted *TAF* gene (Figure 3g,h), while the expression of the *TAF* gene in nontarget tissues was unchanged. These analyses show that silencing of *SmTAF10* or *SmTAF13* in eggplant anthers is associated with the observed male sterile phenotype suggesting that these two general transcription factors play an important role during eggplant pollen development and function.

We performed selfings and crosses between these transgenic male sterile and nontransgenic eggplants to estimate the ability of the TAF silenced plants to trigger fruit set and seed formation when employed as male or female parent. At least ten crosses or selfings have been performed for each transgenic line. For each of the four constructs, we obtained transgenic lines that did not give offspring in selfings or when employed as male in the backcrosses to wild type. However, all transgenic lines did set fruits with viable seeds when they were hand-pollinated using pollen from wild-type plants, confirming that the female reproductive organs were unaffected.

To assess the stability of the male sterility phenotype, we analysed 19 plants of the T1 backcrossed offspring from 11 best T0 lines using 1–3 plants/T0 line (Table 2). PCR assays were performed for the presence of the transgene, and pollen germination was evaluated. In all the considered T1 plants, the percentage of pollen germination was not different (i.e. from 0 to 2%) from those of their corresponding T0 male sterile line (Table 2). Crossing experiments using pollen from these T1 lines to pollinate emasculated wild-type flowers never resulted in fruit set despite several ($n > 10$) attempts, demonstrating that the male sterility trait was stably transmitted to the progeny.

Male fertility restoration by induction of the *SlTAF10* and *SlTAF13* genes

To rescue the male sterility phenotype, we placed the *SlTAF10* and *SlTAF13* genes of tomato under the control of the ethanol inducible *alcA/alcR* system (Caddick *et al.*, 1998; Salter *et al.*, 1998). These genes have specific mismatches with respect to the amiRNAs that target *SmTAF10* and *SmTAF13* transcripts, which should make them insensitive to the activity of these small RNAs. Flowers at different stages of development of wild-type, T0 and T1 plants were sprayed twice a day with an 8-h interval using a 30% ethanol solution for 2 days. This ethanol concentration and frequency of treatments applied to closed flower buds, well before petals begin to be visible through the sepals, was shown to be the most effective dosage. This ethanol treatment did not hinder the wild-type pollen germination ability, while enabled a complete recovery of pollen germination in all the selected transformed lines with pTA29::amiRNA-*SmTAF* and pNTM19::amiRNA-*SmTAF* (Figure 3c,f). Flowers of wild-type and transgenic male sterile plants when sprayed with ethanol produced viable pollen grains displaying a similar percentage of germination (i.e. >40%). In lines with tapetum- or microspore-specific promoters, sterility seemed to be equally restored after ethanol induction. Pollen from ethanol-treated flowers of male sterile plants, employed for selfing and backcrosses to wild-type eggplants, gave fruits with viable seeds from which we raised TAF-based male sterility construct containing lines, thus confirming the complete recovery of fertility. Using nine of the best T0 male sterile ethanol-treated lines, 17 T1 plants were evaluated for pollen germination. All these plants were male sterile, and pollen germination was not different from their corresponding T0 male sterile line.

Quantitative real-time PCR assays were used to analyse the expression of the tomato *SlTAF10* and *SlTAF13* genes in the eggplant anthers obtained from open flowers treated with ethanol. This revealed that the tomato *TAF* genes were strongly induced after ethanol treatment, whereas no significant difference was observed in *SmTAF10* and *SmTAF13* expression in ethanol-treated wild-type flowers. No adverse effect on plant or flower development was observed in these lines upon ethanol induction.

The use of a *TAF* gene that is insensitive to the silencing seems to be particularly efficient because of the use of the highly specific amiRNAs. In fact, even the modification of a single base pair at a precise location in the target site of the recovery gene is expected to make this gene insensitive to the amiRNA activity (Schwab *et al.*, 2006). The amiRNA-insensitive form that we used to restore male fertility in eggplant was obtained from tomato. However, any other amiRNA-insensitive form comprising an artificially mutated sequence will be equally suitable, provided that the gene can complement for the loss of the activity of the endogenous gene. The present method allows the recovery of the blocked function only upon an external stimulus, applicable when desired to obtain seeds for commercial or breeding purposes in a cost-effective way. The male sterile plants will not spread functional pollen in the environment, as the blocked function is not expected to be recovered under natural condition. Furthermore, as the whole system is

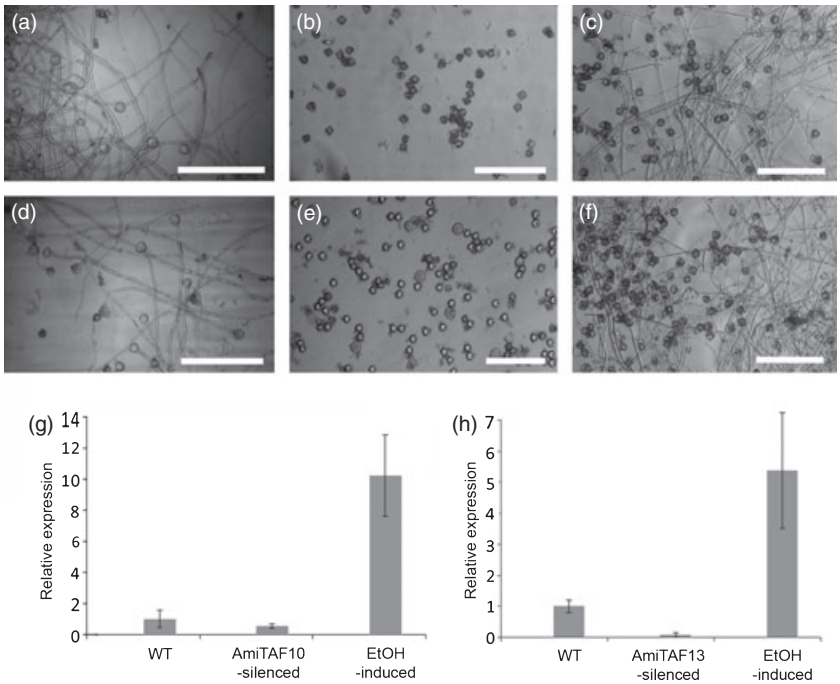

Figure 3 Phenotypic and molecular evaluation of the eggplant lines transformed with amiRNAs targeting *SmTAF10* and *SmTAF13*. (a–c) Representative images of a pollen germination assay for wild-type and pTA29::ami*TAF10* containing eggplant lines. (a) Wild-type germinating pollen. (b) Pollen of an eggplant transformed with the pTA29::ami*TAF10* construct, showing that pollen grains are shrunken and do not germinate. (c) Pollen of the same plant as shown in panel b, but after treatment of developing flowers with ethanol, pollen development and germination is restored. (d–f) Representative images of a pollen germination assay for wild-type and pNTM19::ami*TAF13* containing eggplant lines. (d) Wild-type germinating pollen. (e) Pollen of an eggplant transformed with the pNTM19::ami*TAF13* construct, showing that pollen grains do not germinate. (f) Pollen of the same plant as shown in panel e, but after treatment of developing flowers with ethanol, pollen development and germination are restored. Scale bars represent 200 μm. Real-time PCR analysis showing the (g) *SmTAF10* wild-type expression, the pTA29::ami*TAF10*–mediated silencing of *SmTAF10* and the *SlTAF10* induction upon EtOH treatment in the same line; (h) the *SmTAF13* wild-type expression, the pNTM19::ami*TAF13*–mediated silencing of *SmTAF13* and the *SlTAF13* induction upon EtOH treatment of the same line. Transcript levels were normalized to *18S* ribosomal RNA; standard deviation of triplicate quantification is shown.

Table 1 Pollen germination data obtained from all the kanamycin PCR-positive-independent transformed lines and wild-type plants. Plants are grouped in 7 classes of pollen germination efficiency (0–2% to >40%)

Construct	N° of independent lines	Germination ratio (%)						
		>40	40–30	30–20	20–10	10–5	5–2	2–0
pTA29::amiR-*SmTAF10*; pALC::*SlTAF10*	22	1	4	4	1	6	3	3
pNTM19::amiR-*SmTAF10*; pALC::*SlTAF10*	21	2	2	1	7	3	4	2
pTA29::amiR-*SmTAF13*; pALC::*SlTAF13*	19	1	3	2	1	4	3	5
pNTM19::amiR-*SmTAF13*; pALC::*SlTAF13*	15	0	1	1	2	3	6	2
DR2 wild type	1	1	0	0	0	0	0	0

included in one single construct, no segregation of the two different functions (silencing and complementation) is expected in the progeny of male sterile lines.

Combining male sterility with parthenocarpy to obtain complete transgene containment

Crosses between *DefH9-iaaM* parthenocarpic lines of eggplant (Rotino *et al.*, 1997), employed as male parent, and nine of the best T0 male sterile lines (i.e. pollen germination 0–2%) were made to obtain F1 hybrid plants with both the traits of interest. Two plants from each of these male sterile/parthenocarpic hybrids were grown in the greenhouse and compared for pollen germination and fertility with the correspondent progenies obtained from crosses between male sterile and wild-type plants. Open flowers from the male sterile/parthenocarpic hybrids contained pollen grains unable to germinate and, similarly to those obtained from crosses between male sterile and wild-type plants, were unable to generate progeny when employed as male parent in crosses with wild-type eggplants. Also in these plants, male fertility could be restored by ethanol treatments. However, unlike the male sterile/wild-type hybrid, all the male sterile/parthenocarpic hybrids produced a high number of normal-sized seedless fruits (Figure 4). Both pollen and seed dispersal were inhibited in these male sterile/

Table 2 Pollen germination data obtained from the T1 progenies of the 11 best male sterile lines for each of the four constructs selected from the T0 lines and wild-type plants. Plants are grouped in 7 classes of pollen germination efficiency (0–2% to >40%)

Construct	N° of T1 progenies	Germination ratio (%)						
		>40	40–30	30–20	20–10	10–5	5–2	2–0
pTA29::amiR-*SmTAF10*; pALC::*SlTAF10*	4	0	0	0	0	0	1	3
pNTM19::amiR-*SmTAF10*; pALC::*SlTAF10*	3	0	0	0	0	0	1	2
pTA29::amiR-*SmTAF13*; pALC::*SlTAF13*	2	0	0	0	0	0	0	2
pNTM19::amiR-*SmTAF13*; pALC::*SlTAF13*	2	0	0	0	0	0	0	2
DR2 wt	1	1	0	0	0	0	0	0

Figure 4 Fruits of the offspring of a cross between male sterile and parthenocarpic transgenic eggplant lines. (a) There appears to be no difference between the transgenic (1 and 2) and wild-type (3) fruits. (b) There are no seeds developing in fruits of the male sterile parthenocarpic lines.

parthenocarpic hybrid plants; hence, the combination of these two engineered traits may allow the achievement of a complete transgene containment system in eggplant.

With regard to the transmission of the recoverable male sterility trait to the subsequent progeny and considering the earlier reported data, we can summarize that a total of 55 T1 plants (regardless they derived from backcross, selfing after ethanol induction or cross with parthenocarpic eggplant) were utilized to assess the stability of TAF-based male sterility induction strategy. For TAF10, we studied in detail T1 progenies from 7 independent T0 lines (two promoters), while for TAF13, we studied the progenies of four selected (two promoters) lines. For each of these lines, an average number of five plants were used to perform both phenotypical evaluations and pollen germinations assays several times throughout the season. All these lines confirmed the male sterile phenotype of the correspondent T0 line from which they derived. Moreover, in all the T1 offspring lines, the male sterile phenotype was rescued by ethanol induction.

In conclusion, we present a new strategy to obtain reversible male sterility in plants based on artificial microRNA. We demonstrate that amiRNA-mediated silencing of *TAF10* and *TAF13* genes using tapetum- or microspore-specific promoters causes complete male sterility in eggplant.

Pollen-mediated gene flow between GM and non-GM crops depends on several factors (Beckie and Hall, 2008), and even when occurring at low frequencies, considerable distances can be reached by the pollen (Rieger *et al.*, 2002; Busi *et al.*, 2008), thus causing the necessity to adopt severe segregating measures, which suffer from compliance costs for coexistence between GM plants and non-GM crossable crops. This work represents a potential solution for this transgene flow problem. Artificial microRNAs are a very useful tool for gene silencing, which was recently shown to work also in rice, a very important monocot crop (Warthmann *et al.*, 2008). Furthermore, the TAF-based recoverable male sterility system that we present here is highly efficient in eggplant but may also be more widely applicable because TAF gene functions are expected to be conserved between plant species. Taking into account all these aspects, the strategy that we present here may be suitable for a wide use in other important crops, both monocot and eudicot. In eggplant, we have shown that combining male sterility with parthenocarpy results in a very efficient containment system to prevent transgene flow, which might grant the possibility of using transgenic approaches for improvement of this important crop.

The stability and the efficiency of this recoverable system make it interesting also to be adopted for commercial hybrid seed production. In fact, the ethanol-mediated male fertility induction pertains only the multiplication of the male sterile parent. Therefore, the ethanol treatment will only be used to

obtain a sufficient number of seeds to generate homozygous male sterile plants to be employed as female lines in a field devoted to hybrid seeds production. Considering the application of this technology in crops desired for their seeds, a recovery of male fertility is essential except for the highly allogamous species where it could be sufficient to use hybrid seeds of which 50% will generate male fertile plants (F1 seeds obtained from a cross using a heterozygous male sterile parent) able to ensure successful pollination of all the field-grown plants. For the other crops and especially in the case of autogamous species, we provide an example of the use of ethanol as inducer for seed production. The ethanol inducible system presents several advantages: low cost inducer, nontoxic at the concentration of use and easy to handle in field conditions. In contrast to many products used by farmers that contain chemical compounds of certain toxicity, a short application of a 30% ethanol solution is not expected to be toxic for nontarget animals feeding on the plant. The additional application of ethanol field sprays need to be considered in any cost-benefit analysis, as well as the proposed technology needs to be adopted and implemented in crop species under field conditions, which are beyond the scope of this proof-of-concept study. However, the main economic benefits are the reduced separation distances and the resulting compliance costs for coexistence (Koziolek, 2009).

Recovered viable pollen from ethanol-induced plants might spread in the environment and eventually outcross with non-GM crops or wild relatives; however, the absence of ethanol-mediated induction in the eventually produced progeny will block the transgene flow. On the other hand, undesired activation can be caused under particular conditions such as anoxia or in the presence of endogenous metabolites able to activate Alc system components (Roberts *et al.*, 2005). Nevertheless, it should be clear that the use of the Alc system as we report here just represents a proof of concept for the strategy that we developed to obtain reversible male sterility in crops and other inducible systems that might be more suitable could easily replace it.

Experimental procedures

TAF cDNA isolation

Total RNA was extracted from anthers of both *Solanum melongena* line TAL1/1 and *Solanum lycopersicum* line microtom, according to Verwoerd *et al.* (1989) or using the RNA-easy plant mini kit from Qiagen. Complementary DNA (cDNA) was synthesized after DNase treatment using the Bio-Rad i-Script cDNA synthesis kit. *TAF10* and *TAF13* cDNA sequences were isolated from total cDNA by using the primers: TAF10fw 5′-CACCTATACTCCCACTATTCCTGATG-3′ and TAF10rev 5′-TCATTCCTCTCTTGAAGCAGG-3′; TAF13fw 5′-CACCATGAACAACTCTTCTGCAGG-3′ and TAF13rev 5′-ACTTCCCCTTCACTGATGTG-3′. PCR fragments were cloned in pENTR^{TM}/SD/D-TOPO (Invitrogen, Carlsbad, CA). Accession numbers for the EMBL sequence database: TAF 10 BankIt1346381 HM151875; TAF 13 BankIt1346369 HM151874.

In situ hybridization

In situ hybridizations were performed as described by Lopez-Dee *et al.* (1999). Digoxigenin-labelled gene-specific antisense and sense RNA probes were generated using the *in vitro* transcription kit of Roche. cDNA templates for RNA transcription were PCR-amplified using the primers TAF10fw 5′-TATACTCCCACTATTCCTAGATG-3′, TAF10rev 5′-CCATACTCTCGTAGAGATTTTG-3′, TAF13fw 5′-ATGAACAACTCTTCTGCAGG-3′and TAF13rev 5′-CCTCTGTCAAAAGCTTCC-3′. The T7 promoter was placed in front of the forward or reverse primers to generate the sense or antisense RNA probes, respectively.

Preparation of the constructs

By BLAST comparison between *SmTAF10* and *SlTAF10* coding sequences, we identified regions for designing amiRNAs that will target *SmTAF10* mRNA but not *SlTAF10*. The same was performed for *SmTAF13* and *SlTAF13*. Among the options for amiRNA targeting *SmTAF10* and *SmTAF13,* which were generated following the algorithm and the protocol described at http://wmd.weigelworld.org we selected the best artificial miRNA sequences directed against *SmTAF10* (5′-AUGUCCUAGC**G**UUCGAUUAAU-3′) and *SmTAF13* (5′-AGUAGAAAG**G**ACUUGCCACAA-3′). Primers to create the complete amiRNA-*SmTAF* precursor sequences were retrieved following the algorithm at http://wmd.weigelworld.org. Overlapping PCR on an amiRNA template (plasmid pRS300, containing the *Arabidopsis* miR319a precursor) using the protocol provided by weigelworld. PCR products were cloned in pGEM-T Easy (Promega Corporation, Madison, WI).

Four constructs were prepared, which differ for the use of two different anther-specific promoter, pTA29 or pNTM19, and for the two targets, *SmTAF10* or *SmTAF13* genes to which the amiRNAs are specifically directed. The two promoters pTA29 and pNTM19 were amplified with the primers TA29fw 5′-GTCGACTCTAGACTTTTTGGTTAGCGAATGCA-3′, TA29rev 5′-CCATGGTCGACTAGCTAATTTCTTTAAGTAAAAAC-3′; NTM19fw 5′-GTCGACTCTAGAGCCATGGTTAATCACTAAG-3′ and NTM19rev 5′-CCATGGTCGACGGATCGATGTTGGTTATC-3′ containing restriction sites for *XbaI* and *SalI* and cloned in pGEM-T Easy vector. The promoters were inserted in *XhoI*-linearized amiRNA-*SmTAF* precursors vectors after digestion with *SalI*. The recovery constructs contain an inducible system driving the expression of an amiRNA-insensitive version of the targeted *TAF10* or *TAF13* genes isolated from *S. lycopersicum* (as described earlier). The Gateway cassette, reading frame A (Life Technologies, Carlsbad, CA), was inserted into the *Sma*I site of pL4 vector (Syngenta Ltd, Jeolotts Hill, UK) between the p*AlcA* promoter and the terminator of the 35S CaMV, generating a pL4_pALCA-Gateway vector (NOB419). *SlTAF10* and *SlTAF13* were recombined in NOB419 by LR reaction (Invitrogen). Each of the four blocking constructs was inserted into the *XbaI* sites of the respective recovery construct obtaining four different 'two components' constructs. AmiRNA silencing and recovery sequences were inserted into *HindIII* sites of a pBIN19 binary vector with NPTII selection cassette flanked by T-DNA border sequences containing 35S::ALCR. The four binary vectors were used to transform *Agrobacterium tumefaciens* LB4404::PBI121.

Plant material and genetic transformation

The procedure for eggplant transformation was essentially as described previously (Rotino and Gleddie, 1990 and Rotino *et al.*, 1997). Leaf and cotyledon explants from the *in vitro* grown DR2 genotype were precultured for 2 days in preculture medium (Arpaia *et al.*, 1997). For explants infections, an overnight *A. tumefaciens* liquid culture was centrifuged and the pellet resuspended at 0.1 OD600 in MS basal medium, 2%

glucose, 200 mM acetosyringone pH 5.5. All the explants were infected by dipping in bacteria suspension for 5 min, blotted dry onto sterile filter paper and placed back in the same plates. After 48 h, the explants were transferred to selective medium without acetosyringone and supplemented with 30 mg/L kanamycin and 500 mg/L cefotaxime. Calli with compact green nodules were transferred to regeneration medium. Regenerated shoots were rooted and propagated in V3 medium (Chambonnet, 1985) without antibiotics. Kanamycin-resistant transgenic plantlets were confirmed by PCR for the presence of the insert, using the primers: NPTII fw: 5′-TGCTCCTGCCGAGAAAGTAT-3′ + NPTII rev: 5′AGAACTCGTCAAGAAGGCGATAG-3′. The PCR-positive plants were potted and grown under greenhouse conditions for evaluation.

Analysis of *S. melongena* transformants

Male sterility was monitored by evaluation of the pollen viability and germination as described by Ribarits *et al.* (2007). Pollen viability was evaluated under light microscope after staining with acetic carmine. *In vitro* pollen germination assays were performed by collecting pollen grains from open eggplant flowers and germinating them in PEG 800 medium overnight at 25 °C in the dark (Touarev and Heberle-Bors, 1999). Pollen tube growth and germination frequencies were observed under a light microscope. Two or more analyses for each transformed line were carried out once a week during the spring–summer season for 2 years.

Histological evaluation

Anthers were collected from wild-type and best male sterile lines and fixed overnight in FAA (50% ethanol, 5% glacial acetic and 3.7% formaldehyde, v/v) and kept in 70% ethanol until further processing. Fixed samples were embedded using the Kulzer technovit 7100 kit (Kulzer, Werheim, Germany). Sections of 5 mm were stained in toluidine blue for 15 min and observed using a Zeiss Axiophot D1 microscope (http://www.zeiss.com/) equipped with differential interface contrast optics. Images were recorded with an Axiocam MRc5 camera (Zeiss) using the Axiovision program (version 4.1).

Fertility restoration of male sterile lines

A 30% ethanol solution was sprayed twice a day for 1–5 days on to leaves and flower buds of greenhouse-grown wild-type and transformed plants. *In vitro* pollen germination frequencies were evaluated as described before. Transgenic and wild-type plants were manually selfed and cross-pollinated with rescued pollen, and seeds were collected and subjected to segregation analysis of the kanamycin resistance trait.

Real-time PCR analysis

Expression of *TAF* genes was assayed through quantitative real-time PCR analysis. RNA was extracted from anthers of wild-type and transgenic male sterile lines both without and after the 2-days ethanol treatments. Real-time PCR was performed using the iQ SYBR-Green Supermix (Biorad Laboratories, Hercules, CA) and a iCycler iQ real-time PCR detection system (Bio-Rad). The primers used for *TAF10* are as follows: fw 5′-TATACTCCC ACTATTCCTGATG-3′ and rev: 5′-TGTCAAGGTAAGGCGTTTG TC-3′; and for *TAF13*: fw 5′-GGAGATGATAGCAATCCACTTCC-3′ and rev 5′-AAGCCTTCCGAGCTTGTTTCAG-3′. These primers allowed the amplification of both the eggplant *SmTAF* genes (10 or 13) in the wild-type and in the male sterile plants, and the tomato *SlTAF* (10 or 13) genes whose expression, in the same plants, was mediated by the alcA/alcR system after ethanol induction. The data were normalized to *18S* ribosomal RNA. All PCRs were performed twice in triplicates. The standard curves were constructed using serial cDNA dilutions. The PCR efficiency was close to 100%; relative gene expression was determined using the 2-DDCt method (Livak and Schmittgen, 2001).

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Supporting information

Additional Supporting information may be found in the online version of this article:

Figure S1 *TAF10* cDNA and protein alignment between *Solanum melongena*, *Arabidopsis thaliana* and other *Solanaceous* species reveals a high sequence conservation.

Figure S2 *TAF13* cDNA and protein alignment between *Solanum melongena*, *Arabidopsis thaliana* and other solanaceous species reveals a high sequence conservation.

Figure S3 Morphological evaluations of anthers of wild-type and eggplant lines transformed with the TAF silencing constructs.

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