

Temperature sensitive diphtheria toxin confers conditional male-sterility in *Arabidopsis thaliana*

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Summary

A gene encoding a temperature-sensitive diphtheria toxin A chain (DTA) polypeptide was fused to the *Arabidopsis thaliana* tapetum-specific A9 promoter. Expression of the chimaeric gene in transgenic *A. thaliana* lines resulted in plants that were male-sterile, but female-fertile, when grown at 18 °C, and fully self fertile at 26 °C. No pollen grains were found on the anthers of transgenic plants grown at 18 °C, although aggregated pollen grains were found inside the anthers. Electron microscopy revealed discrete alterations in the tapetal cells of the male-sterile transgenic plants. The strength of the phenotype observed in segregants correlated with the level of expression of the gene and the copy number. The low frequency at which fully male-sterile plants were generated suggests that the temperature-sensitive DTA protein is disabled as a cytotoxin, relative to the wild-type protein activity.

Introduction

The development of a complex organism progresses through a number of cell and tissue specification and differentiation steps that are determined by a cascade of events under the control of spatially and temporally regulated gene expression. The ability to interfere with such a programme is a powerful approach for unravelling its complexity. The specific inhibition of gene expression, either through mutation or reverse genetic techniques, yields information about gene function and sometimes affects the development of the cells in which it takes place. An important adjunct to these techniques involves the specific ablation of cells and the subsequent observation of the consequences on the development of a tissue or organ (van den Berg *et al.*, 1995).

As mechanical (e.g. Manner *et al.*, 1996) and laser ablation (e.g. van den Berg *et al.*, 1997) can only be used in a limited number of situations, the production of a cytotoxin by specific cells offers a valuable alternative. This can be achieved by the expression of a gene encoding a toxin under the control of a transcriptional promoter of known specificity. Such cell ablation experiments were first done in mice (reviewed in

Evans, 1989) by expressing the gene encoding the diphtheria toxin A chain (DTA) polypeptide in lens (Breitman *et al.*, 1987) or pancreatic (Palmiter *et al.*, 1987) cells. The ricin A toxin was also shown to be active when produced in mice lens cells (Landel *et al.*, 1988). In all cases, the production of the toxin by specific cells resulted in cell type or even organ ablation in the transgenic animals.

In plants, the same strategy was applied to the production of male-sterile plants, by the expression of cytotoxins in the tapetum, a specialized tissue of the anthers surrounding the sporogenous cells. The tapetum-specific promoter TA29 has been used to drive expression of a ribonuclease gene (Mariani *et al.*, 1990) and the DTA gene which resulted in dominant male-sterility due to the specific ablation of the tapetum (Koltunow *et al.*, 1990). Collapse of the tapetum was also observed when the A9 and A6 promoters were used to drive expression of the barnase gene in transgenic plants (Hird *et al.*, 1993; Paul *et al.*, 1992). Similarly, when the S-locus glycoprotein gene promoter of *Brassica* was fused to the DTA gene and transferred into tobacco (Thorness *et al.*, 1991) and *A. thaliana* (Thorness *et al.*, 1993) it resulted in self-sterile plants due to expression of the gene in both pistil and

anthers. Stigma-specific cell ablation by DTA protein induced female-sterility in tobacco plants (Goldman *et al.*, 1994). Expression of the DTA gene fused to the pea vicilin promoter induced seed mortality in transgenic plants (Czako *et al.*, 1992), whereas an AP3-DTA fusion resulted in the complete ablation of petals and stamen in transgenic flowers (Day *et al.*, 1995).

These cell ablation experiments all required the use of a characterized promoter coupled to a dominant-acting cytotoxin gene, an approach which has two drawbacks. Firstly, the expression of a toxin under the control of a promoter of unknown specificity may not be informative because of the early death of the organism and secondly, the dominant action of the cytotoxin reveals only the developmental impact of the first instance of gene expression.

One means of avoiding such drawbacks is through the use of an inducible system of cell ablation. This would allow normal development in permissive conditions but could be activated by a shift to non-permissive conditions. Such systems have been developed and used in eye cell ablation experiments in *Drosophila melanogaster* (Kunes *et al.*, 1991). Temperature-sensitive mutants of the DTA (DTA^{ts}) (Bellen *et al.*, 1992) and cold-sensitive ricin A mutants (Moffat *et al.*, 1992) have been isolated. Their expression in *D. melanogaster* cells resulted in temperature-dependent cell death. To date, the application of these systems in plants has remained untested.

Another interesting application of a conditional cell ablation system is in the isolation of regulatory mutants in model plants like *A. thaliana*. Any mutation affecting the expression of the toxin gene would produce a suppression of toxin-induced phenotype which is easily scorable in a large population. However, any mutagenesis screen requires the production of a large quantity of seeds transgenic for the toxin gene, which would not be possible if the expression of the gene affected vital processes such as embryo development, germination or fertility. The use of a toxin with temperature-dependent activity would circumvent this problem. We demonstrate here that a mutant DTA can induce specifically conditional male sterility. This is controllable at temperatures easily obtainable in simple plant growth rooms and has been used in a screen for apomictic mutants in *A. thaliana*, as described in Praekelt and Scott (2001).

Results

Isolation of temperature-sensitive male-sterile plants

The DTA^{ts} gene encodes a mutant diphtheria toxin which is reportedly active at 18 °C and inactive at 25 °C (Bellen *et al.*,

1992). To generate conditional male-sterile *A. thaliana* plants, we placed this gene under control of the *A. thaliana* tapetum-specific promoter A9 (Paul *et al.*, 1992) to create the A9::DTA^{ts} gene.

Two separate *Agrobacterium*-mediated root transformation experiments were performed with the A9::DTA^{ts} construct to assess whether the temperature regime altered the frequency of transformation. Our hypothesis was that transformation performed at 20 °C (Experiment 1) would be less efficient than at 26 °C (Experiment 2) because DTA^{ts} is inactive at 26 °C. In both experiments, primary putative transformants (Generation-1 plants) were allowed to self-pollinate (at either 20 °C in Experiment 1 or 26 °C in Experiment 2) to produce Generation-2 seed. Plants grown from Generation-2 seed were assessed for conditional sterility by first growing the plants at 18 °C until flowering and then increasing the temperature to 26 °C. Generation-2 plants were scored as conditionally sterile if they lacked elongated pods at 18 °C but produced elongated pods at 26 °C. The number of primary transformants resulting from plant transformation at 20 °C was 43 and from transformation at 26 °C was 100.

Analysis of transformants recovered from transformation Experiment 1 (20 °C)

Seeds were successfully harvested from the 43 putative primary transformants (Generation-1 plants) grown at 20 °C. These were sown on soil and the resulting plants were grown at 18 °C. To assess the transformation frequency, a proportion of the Generation-2 plants were analysed for the presence of the neomycin phosphotransferase II (NPTII) gene by polymerase chain reaction (PCR) using specific primers. Of 18 lines tested, 14 produced the expected 524 base pair (bp) PCR product (not shown).

Each of the PCR positive Generation-2 plants were assessed for conditional sterility as described in Experimental procedures. One of the 43 Generation-2 plants failed to produce elongated siliques at 18 °C but did produce seeds at 26 °C. This originated from primary transformant A9::DTA^{ts}-Q.

Generation-3 seeds were harvested from each of the 11 Generation-2 plants originating from transformant A9::DTA^{ts}-Q, which will be referred to as Q1 to Q11. Generation-3 plants Q3, Q4 and Q6 were male-sterile at 18 °C but produced siliques when grown at 26 °C (Figure 1B), whereas untransformed control plants produced siliques at both temperatures (Figure 1A). All plants from the Generation-3 families Q2, Q3, Q4 and Q6 were sterile at 18 °C, whereas other families contained a mixture of conditionally sterile plants, fertile plants and plants with reduced fertility (Table 1). All plants from

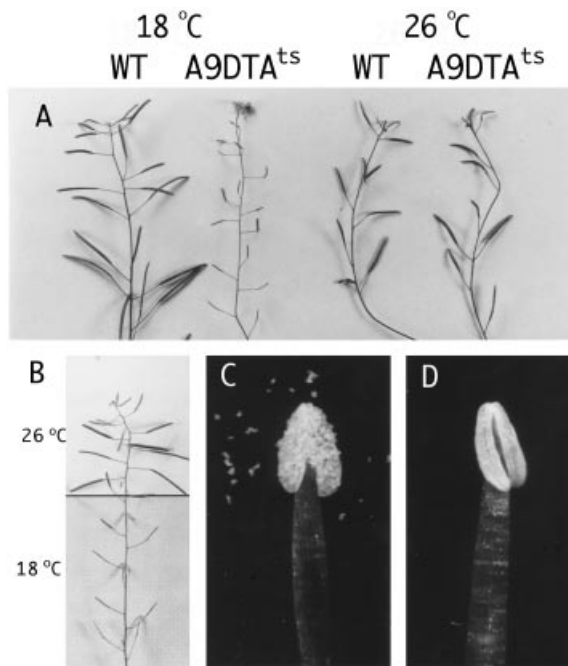


Figure 1 Conditional male sterility in plants expressing the DTA^{ts} gene. (A) Comparison of pod lengths between wild-type *A. thaliana* ecotype C24 (WT) and Generation-3 A9::DTA^{ts}-Q3 plants shows that plants containing the A9::DTA^{ts}-Q3 construct are sterile at 18 °C and fertile at 26 °C. (B) plant A9::DTA^{ts}-Q6 grown during flowering at 18 °C and then transferred to 26 °C showing that the sterility observed is temperature dependent. (C) anthers of wild-type *A. thaliana* ecotype C24 plants grown at 18 °C showing abundant pollen grains. (D) anthers of A9::DTA^{ts}-Q3 plants grown at 18 °C showing absence of free pollen grains.

families Q1, Q7 and Q8 were fertile at 18 °C, although Q1 plants showed a reduced fertility.

To determine the cause of sterility in A9::DTA^{ts}-Q plants, anthers from open flowers were compared from wild-type and A9::DTA^{ts}-Q6 plants grown at 18 °C. No pollen was found on anthers from A9::DTA^{ts}-Q6 plants (Figure 1D) but cross-pollination with wild-type pollen (Figure 1C) resulted in elongated pods (not shown), therefore the plants were female fertile. When the same A9::DTA^{ts}-Q6 plants were transferred to 26 °C, pollen was present on the anthers within 5 days (data not shown). This data suggested that the cause of the observed sterility in line A9::DTA^{ts}-Q was temperature-sensitive male-sterility.

Analysis of the number of transformants from Experiment 2 (26 °C)

Of the 100 putative primaries generated in Experiment 2, only three plants showed conditional male sterility in Generation-2 progenies. This suggested that performing the transformation at 26 °C produced no significant improvement in the

Table 1 Segregation analysis of kanamycin resistance (km^r) and kanamycin sensitive (km^s) in A9::DTA^{ts}-Q Generation-3 seedlings, and the ratio of sterile to fertile plant phenotypes at 18 °C

Family	km ^r : km ^s	sterile : fertile
C24 wt	0 : 114	0 : 14
A9::DTA ^{ts} -Q1	131 : 0	0 : 10
– 2	101 : 0	9 : 1
– 3	121 : 0	10 : 0
– 4	144 : 0	10 : 0
– 5	120 : 15	3 : 7
– 6	112 : 0	10 : 0
– 7	123 : 36	0 : 10
– 8	0 : 140	0 : 10
– 9	133 : 0	1 : 9
– 10	100 : 12	1 : 9
– 11	109 : 22	5 : 5

frequency of conditional sterile plants as compared to 20 °C.

Molecular characterization of conditional male-sterile line A9::DTA^{ts}-Q

To determine the number of transgene loci in A9::DTA^{ts}-Q, Generation-3 seeds from segregants Q1 to Q11 were sown on a germination medium containing kanamycin to give the segregation data of kanamycin sensitive plants to resistant plants (Table 1). A plant carrying a single locus when selfed would be expected to give a 3 : 1 ratio of resistant to sensitive plants. A plant carrying two transgenes at separate loci would be expected to give a 15 : 1 ratio of resistant to sensitive plants. A9::DTA^{ts}-Q7 showed a 3 : 1 segregation which indicated the presence of the NPTII gene at a single locus. The ratio of resistant to sensitive plants for A9::DTA^{ts}-Q5, A9::DTA^{ts}-Q10 and A9::DTA^{ts}-Q11 was between these figures (8 : 1, 8.3 : 1 and 5 : 1, respectively) which is consistent with the integration of the transgene at two or more linked loci. Families A9::DTA^{ts}-Q3, Q4 and Q6 appear to contain transgene integrations at more than two independent loci as there are resistant plants but no sensitive plants. Families A9::DTA^{ts}-Q3, Q4 and Q6 account for all of the homogeneously sterile (at 18 °C) families, which suggested a link between the number of transgene loci and the degree of sterility. The A9::DTA^{ts}-Q8 family appeared not to have inherited the NPTII transgene.

To further investigate the link between transgene copy number and sterility, we quantified the number of DTA^{ts} gene loci by Southern analysis. DNA was digested with *Hind*III (which cleaves upstream of the A9 promoter but not

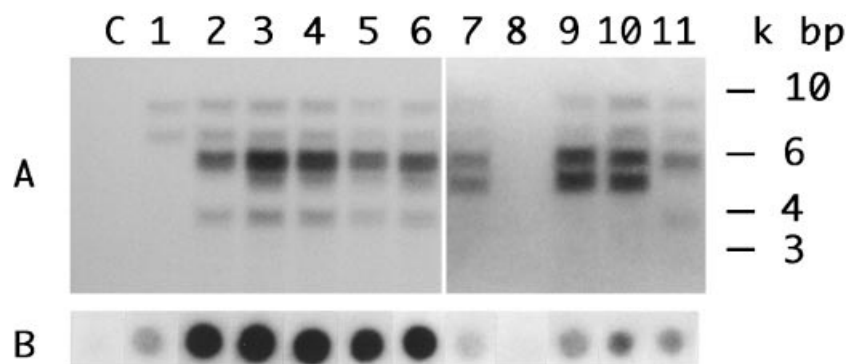


Figure 2 Analysis of DTA^{ts} transgene copy number and expression level in A9::DTA^{ts}-Q plants. (A) Southern blot showing the number of A9::DTA^{ts} gene copies integrated into the genome of A9::DTA^{ts}-Q Generation-3 families 1–11. DNA was extracted from at least eight Generation-3 plants from each of the 11 families. 5 µg of this DNA was digested with *Hind*III. The image from samples 7–11 has been altered by increasing the contrast to enable the bands to be easily visible. This reveals that although family eight is untransformed, the other families have two or more transgene insertions. (B) Northern blot revealing the DTA^{ts} mRNA from corresponding samples where 20 µg of total RNA was loaded. This shows that transcribed RNA is correlated to the number of copies of the transgene in the genome. C: untransformed *A. thaliana* ecotype C24.

between the DTA^{ts} gene and the transformed DNA (T-DNA left border) so that each T-DNA insertion event in the plant genome resulted in the presence of one band of specific molecular weight on the Southern blot which would be different from any other insertion. When probed with the DTA^{ts} coding sequence, it appeared that all but one family contained several copies of the transgene (Figure 2A). No signal was produced by wild-type or A9::DTA^{ts}-Q8 DNA, which confirmed the NPTII segregation data. Results from other plants were inconsistent with the segregation data. For example, the Q7 family showed a 3 : 1 segregation for kanamycin resistance (Table 1) which suggested a single gene insertion, but Southern analysis showed the presence of several DTA^{ts} gene insertions (Figure 2A) which might indicate that there are tandem gene insertions.

When RNA dot blots were probed with the DTA^{ts} coding sequence, it became apparent that the level of expression was very variable between families (Figure 2B). The highest levels of RNA were found in plants from families Q2, Q3, Q4 and Q6. This supported a correlation between the gene copy number, as seen by Southern analysis (Figure 2A), and the strength of the sterility phenotype observed in the populations (Table 1). This was consistent with the hypothesis that the expression of the DTA^{ts} gene was the cause of the temperature-dependent male-sterility observed in A9::DTA^{ts}-Q plants. However, the gene copy number in Q5 is inconsistent with this, because although the mRNA levels are high as in Q3, Q4 and Q6, the temperature dependent sterility is 30% for Q5 compared to 100% for the Q3, Q4 and Q6, which indicates that the Q5 mRNAs are not all translated.

Cytological analysis of anthers from pA9::DTA^{ts} transformant

To assess the physiological effect of the DTA^{ts} gene upon pollen development, flower buds were viewed by light and electron microscopy. Although no pollen grains were visible at the time of dehiscence on the surface of the A9::DTA^{ts}-Q anthers grown at 18 °C (Figure 1D), some grains were observed using light microscopy when the anthers were crushed. Pollen grains from wild-type plants (Figure 3A) were different from those from sterile plants that appeared to be abnormally shaped (Figure 3B) and were sometimes aggregated in one mass inside the anther (Figure 3C).

Thin transverse sections taken across flower buds from untransformed control-plants and A9::DTA^{ts}-Q plants at the tetrad stage of development were observed by light microscopy (Figure 4A,C). Dramatic degeneration of the tapetal cells is associated with expression of the barnase gene (Paul *et al.*, 1992), but DTA^{ts} did not appear to produce such acute effects. However, the tapetal cells in male-sterile A9::DTA^{ts}-Q plants (Figure 4C) were less intensely stained than tapetal cells from the control plants (Figure 4A), which indicated a change in cell composition. There was also a reduction in the number of vacuoles in A9::DTA^{ts}-Q plants (Figure 4C, arrows) compared with the control plants (Figure 4A, arrows). Electron micrographs showed that there was a reduced ribosome density between the cells of the control (Figure 4B) and those expressing the DTA^{ts} gene (Figure 4D) which could be the cause of the less intense staining seen in Figure 4C. In addition, dense material was visible between the pollen grains, by light and electron microscopy, which could be callose

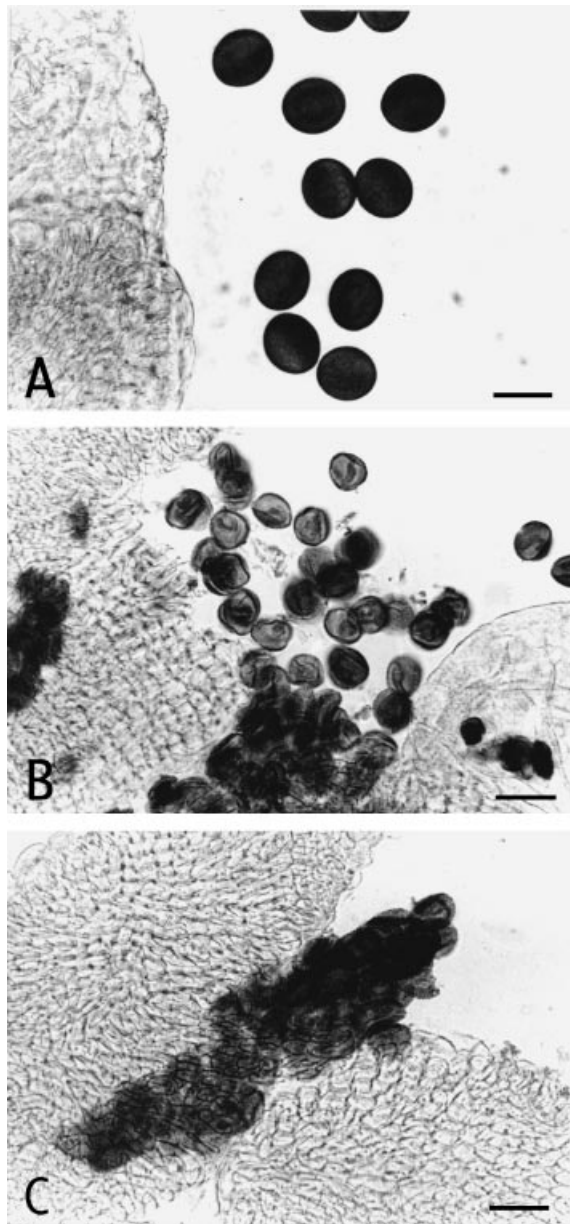


Figure 3 Comparison by light microscopy of pollen grains after dehiscence. (A) Wild-type *A. thaliana* ecotype C24 showing the typical appearance of round separate pollen grains. (B) A9::DTA^{ts}-Q3 transformed with DTA^{ts} revealing pollen grains which are smaller than wild-type and appear collapsed. (C) A9::DTA^{ts}-Q3 transformed with DTA^{ts} revealing pollen grain aggregation. Bars = 25 μ m.

(Figure 4E,F, arrows). Callose is normally digested by a mixture of enzymes (callase) secreted by the tapetal cells shortly after the completion of male cytokinesis. These results suggest that there is a reduction in the ability of the tapetal cells to either synthesize or secrete callase enzyme and this could indicate a general cell dysfunction.

Discussion

This is the first report of an engineered temperature-dependent phenotype in transgenic *A. thaliana*. The gene encoding the temperature-sensitive protein was expressed under the control of the A9 tapetum promoter and caused discrete alterations in tapetum development leading to pollen malformation and aggregation. As a result, transgenic plants were male-sterile at 18 °C but were fully male-fertile at 26 °C whilst being fully female-fertile at both temperatures.

The activity of the A9 promoter was previously shown to be subject to tight temporal and spatial regulation. Studies of temporal expression with A9::Gus and A9::RNase in tobacco showed expression in the tapetum before sporogenous cell meiosis, increased dramatically reaching a plateau and then fell sharply and ceased in anthers with premitotic microspores (Paul *et al.*, 1992). Transgenic A9 expression patterns closely match the temporal accumulation of *B. napus* A9 transcript determined by Scott *et al.* (1991). The A9 spatial expression was found to be tapetum-specific (Paul *et al.*, 1992; Scott *et al.*, 1991) and was confirmed when the A9 promoter was used to drive the expression of the firefly luciferase reporter gene in *A. thaliana*. No luciferase was detected in pollen grains (A.-M. Sorensen, unpublished results) indicating that the promoter was inactive in male gametophytic cells. This suggests that the phenotypic alterations visible in the A9::DTA^{ts}-Q pollen grains also resulted from tapetal dysfunction and not from the production of the toxin by the pollen grains. Since it has been established that the DTA acts in a cell autonomous manner when expressed in transgenic cells (Evans, 1989), passive diffusion of the toxin from the tapetal cells towards the sporogenous cells can also be ruled out. Therefore, our evidence strongly suggests that temperature-sensitive male sterility resulted from the expression and action of the DTA^{ts} within the tapetal cells and not via non-cell autonomous effects on adjacent cell types. Our results therefore indicate that the DTA^{ts} gene is a potentially useful tool for addressing cell function in plants.

Our data is consistent with previous reports that indicated that the DTA^{ts} toxin was of very low activity compared to the wild-type DTA (Bellen *et al.*, 1992; De Wet *et al.*, 1985). This low activity might account for the low frequency of completely male-sterile transgenic plants in those lines that contained the A9-DTA^{ts} gene. This contrasts with the outcome of experiments where expression of the barnase gene or the wild-type DTA toxin gene under control of the tapetum-specific promoter TA29 resulted in a high number of male sterile plants and a complete collapse of the tapetum (Koltunow *et al.*, 1990; Mariani *et al.*, 1990). This was also the case

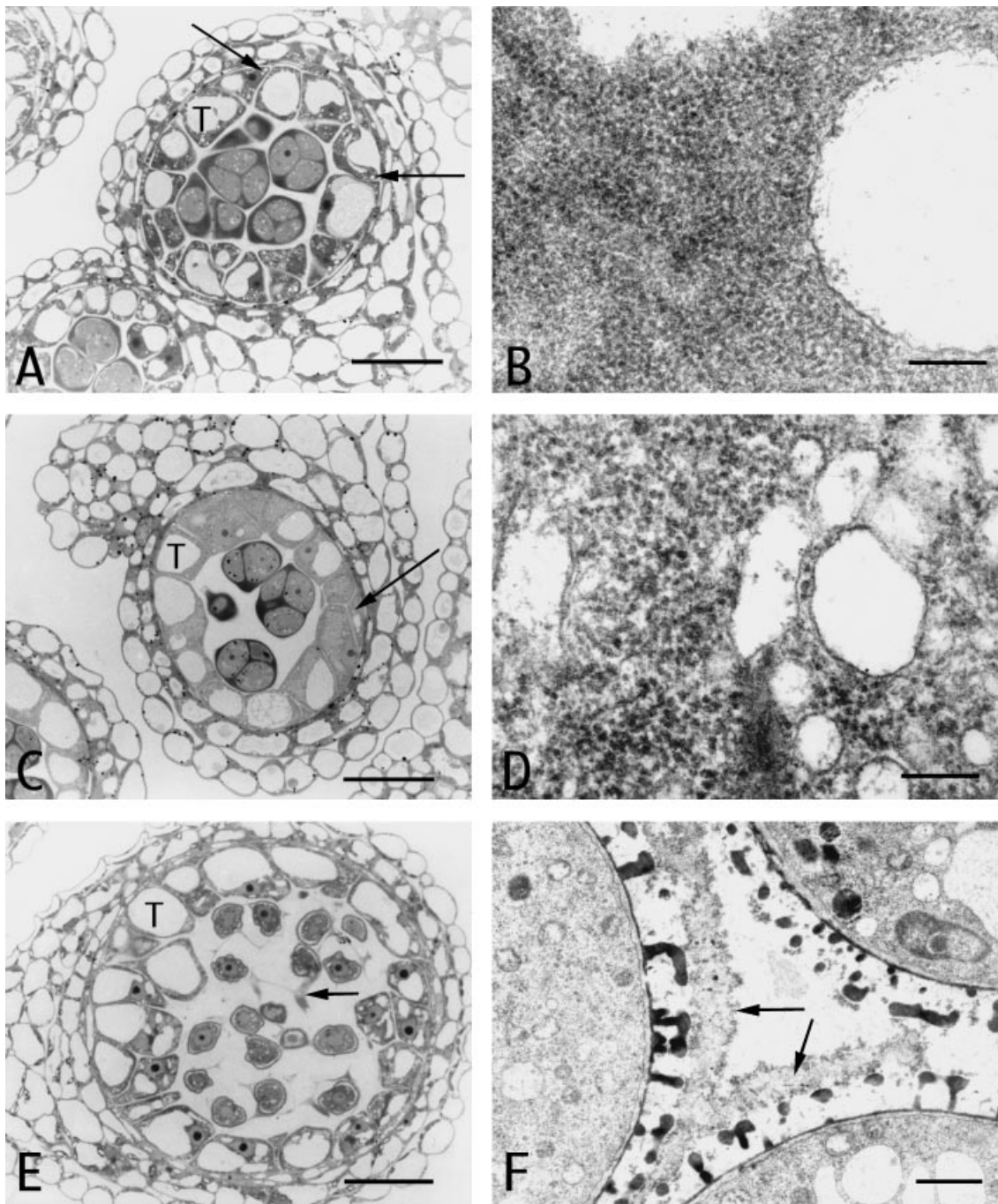


Figure 4 Comparison of tapetal cell ribosome density in anthers of wild-type *A. thaliana* ecotype C24 and A9::DTA^{ts}-Q transformed with DTA^{ts}. Transverse sections through the anthers of C24 (A and B) or A9::DTA^{ts}-Q6 Generation-3 (C–F) observed by light (A, C and E) or electron (B, D and F) microscopy. The wild-type (A) has cytoplasm that stains darkly and has numerous vacuoles (A, arrows). The alterations observed in the transformed plant reveal less intense staining (C and E) of the tapetal cells (T) and fewer vacuoles (C, arrow) indicating the altered cell content. Dense material was visible between the pollen grains which is not normally found at this stage of development and may suggest incomplete digestion of the tetrad callose wall (E and F, arrows). Bars = 25 μm (A, C and E), 0.2 μm (B and D) and 1 μm (F).

when the A9 promoter was used to drive the expression of the barnase gene (Paul *et al.*, 1992). In the A9::DTA^{ts}-Q plants, expression of the DTA^{ts} resulted in discrete alterations in tapetal cells. The lighter Toluidine Blue staining of the cells

expressing the DTA^{ts} gene could be attributed to a lower ribosome density in the cytoplasm, as suggested by electron micrographs (Figure 4D). Such a phenotype was also found in *D. melanogaster* cells expressing the DTA^{ts} gene (Bellen

et al., 1992). A more dramatic phenotype was visible in the A9::DTA^{ts} pollen grains which appeared to aggregate to a greater or lesser extent in the anthers (Figure 3C,B, respectively). This was confirmed by the observation of electron-dense material between the pollen grains (Figure 4E, see arrow). A very similar type of agglutination was described in the *A. thaliana* ms1 mutant (Dawson *et al.*, 1993). A hypothesis for the origin of this material is the incomplete dissolution of the callose wall by the tapetal β -1,3-glucanase as a result of tapetal dysfunction. It has been shown that the timing of the production of this catalytic activity is critical for fertility (Worrall *et al.*, 1992). This aggregation might explain why pollen grains could not be seen on the surface of the A9::DTA^{ts} anthers after dehiscence (Figure 1D). Therefore the DTA^{ts} protein appears to have a chronic rather than an acute effect on anther development and/or function.

Due to the postulated low activity of the temperature sensitive version of the toxin, a high level of expression is probably required for sterility. This was illustrated by several transgenic lines including A9::DTA^{ts}-Q7 (Figure 2), which contained several copies of the T-DNA but was nevertheless male fertile (Table 1).

The poor recovery of transgenic lines capable of complete male-sterility at 18 °C may rest with the technique used to produce the transgenic plants. The Generation-2 seeds were harvested from the primary transformants grown *in vitro*. Since regeneration and seed set are impeded at 26 °C (the non-permissive temperature) the primary transformants were grown at 20 °C. It is possible that some transformants failed to produce seeds because of the activity of the toxin. Thus, the selection could have been biased against the recovery of transgenic lines, giving high expression of the DTA^{ts} gene when hemizygous in Generation-1 and biased towards lines expressing the gene sufficiently highly to cause sterility when homozygous in Generation-2. For future applications, the problem of recovering Generation-2 plants at 26 °C could be easily addressed by using one of the *A. thaliana* transformation methods that avoid *in vitro* regeneration (Chang *et al.*, 1994).

A9::DTA^{ts}-Q has been used in a mutagenesis screen for the isolation of parthenogenetic mutants which involves pollinating large numbers of M1 plants with wild-type pollen; controllable male sterility greatly facilitates this process. These mutants can be switched to permissive temperatures to allow self-pollination and use as males in out-crosses. This mutagenesis screen is further discussed in Praekelt and Scott (2001).

In conclusion, it has been shown that a DTA^{ts} gene induces a temperature-dependent loss of cell function in transgenic

A. thaliana. Since sterility is controllable by applying permissive and non-permissive temperatures, the system is useful in a range of experiments. The transgenic line A9::DTA^{ts}-Q could have applications which include the isolation of regulatory mutants following mutagenesis, the analysis of gene expression patterns or cell fate, or screening of parthenogenetic and apomictic mutants capable of producing seed embryos in the absence of pollination.

Experimental procedures

Plant material and reagents

Restriction endonucleases were purchased from Gibco-BRL and Promega, T4 ligase from Gibco-BRL and *Taq* polymerase (with buffer) from Promega. Antibiotics, media and growth regulators for plant tissue culture were obtained from Sigma. Compounds for cytology were purchased from Agar Scientific Ltd.

Construction of pA9::DTA^{ts}

As a first step to constructing pA9::DTA^{ts}, the 850 bp DTA^{ts} gene was isolated from pLAT59-DTM (Worrall *et al.*, 1996), using *Nco*I and *Eco*RI and cloned into the *Nco*I and *Sma*I sites of pAF0, creating pAF78. pAF0 was a pUC-derived plasmid carrying the firefly luciferase gene (De Wet *et al.*, 1985) inserted between 1 k bp of A9 promoter sequence (Paul *et al.*, 1992) and the 750 bp CaMV polyadenylation sequence of pJIT60 (Guerineau *et al.*, 1992). The *Nco*I-*Sma*I treatment of pAF0 removed the luciferase gene, which was replaced by the DTA^{ts} gene in pAF78. To construct the binary vector (pA9::DTA^{ts}) the A9 promoter-DTA^{ts}-polyadenylation sequence (2.6 k bp) was extracted from pAF78 using *Hind*III and *Xho*I, and inserted into the pBIN19 (Bevan, 1984) site created by digestion with *Hind*III and *Sal*I. In pA9::DTA^{ts} the A9 promoter was such that the transcription of the DTA^{ts} gene was towards the T-DNA left border.

Bacterial and plant transformation experiments

E. coli competent cells (strain JM101) were prepared and transformed as described by Nishimura *et al.* (1990): a 50 mL culture inoculated with 0.5 mL of overnight culture was grown in medium A (LB supplemented with 10 mM MgSO₄·7H₂O and 0.2% (w/v) glucose) to mid-logarithmic phase. The cells were kept on ice for 10 min, then pelleted at 1500 g for 10 min at 4 °C. The cells were resuspended gently

in 0.5 mL of medium A pre-cooled on ice, then 2.5 mL of storage solution B (36% (v/v) glycerin, 12% (w/v) PEG (M_w 7500), 12 mM $MgSO_4 \cdot 7H_2O$ added to LB broth (pH 7.0) and sterilized by filtration) was added, and mixed without vortexing. The competent cells were divided in aliquots of 0.1 mL each and stored at $-80^\circ C$. For transformation, the frozen cells were thawed on ice, mixed immediately with 5 μL (100 pg) of plasmid, and incubated at $4^\circ C$ for 30 min. The cells were then subjected to a heat pulse at $42^\circ C$ for 60 s, chilled on ice for 1 min, diluted 10-fold into pre-warmed L broth, and incubated at $37^\circ C$ for 1 h. Samples (10 μL and 50 μL) were plated on agar plates containing 50 $\mu g/mL$ antibiotic (sodium salt of ampicillin for pAF0 and pAF78). *A. tumefaciens* strain LBA4404 (Hoekema *et al.*, 1983) was transformed in an electroporation cuvette (Bio-Rad) across which 2.5 kV was discharged using an electroporator power unit (Bio-Rad, capacity 25 μF , resistance 200 Ω). *A. thaliana* (ecotype C24) root transformation experiments were conducted as described by Clarke *et al.* (1992) either at $20^\circ C$ (Transformation Experiment 1) or $26^\circ C$ (Transformation Experiment 2). Generation-2 seeds were harvested from the *in vitro* grown primary transformants and sown on soil. Plants were grown under continuous light at $18^\circ C$ or $26^\circ C$. For the segregation analysis, Generation-3 seeds were sterilized (10% v/v bleach, 10 min) and placed on germination medium (Clarke *et al.*, 1992) containing kanamycin at 35 $\mu g/mL$ and grown in a growth chamber prior to segregation analysis. A separate batch of seed was grown in soil in a growth chamber at $18^\circ C$ to assess the ratio of sterile to fertile plants.

Nucleic acid preparation and analysis

Plant DNA mini-preparations for analysis by PCR were done as described by Edwards *et al.* (1991). Centrifugation was performed at 16 060 *g* in a bench-top centrifuge unless otherwise stated. Leaf material was collected using the lid of a sterile Eppendorf tube to pinch out a disc of material into the tube. The tissue was macerated in the tube with a disposable grinder (Bel-Art Products) without buffer for 15 s. A 400 μL aliquot of extraction buffer (200 mM Tris(Tris[hydroxymethyl]aminomethane)-HCl pH 7.5, 250 mM NaCl, 25 mM EDTA (ethylenediaminetetraacetic acid), 0.5% (w/v) sodium dodecyl sulphate (SDS) was added and the tube vortexed for 5 s and centrifuged for 1 min. 300 μL of the supernatant was transferred to a fresh tube and precipitated with 300 μL isopropanol and left at room temperature for 2 min. Following centrifugation for 5 min, the pellet was air dried and dissolved in 100 μL TE buffer (10 mM Tris-HCl

pH 8.0, 1 mM EDTA). PCR was carried out as described previously (Guérineau and Waugh, 1993) in a Perkin-Elmer thermo-cycler 480. The final volume of 50 μL buffer contained 0.2 mM dNTPs, 1 ng of each primer, 10–300 ng plant DNA and 2.5 units of *Taq* polymerase. Thirty-five cycles were performed at $96^\circ C$ for 40 s, $60^\circ C$ for 1 min, $72^\circ C$ for 1 min, with extension at $72^\circ C$ for 6 min in the last cycle. The primers used were 5'-CTTCGTACCACGGGACTAAACC-3' and 5'-CCTGACACGATTCTCTGCACAG-3', which amplified a 524-bp region. For Southern analysis, DNA was extracted from at least eight Generation-3 plants from each of the 11 A9::DTA^{ts}-Q families, and from C24 wild-type plants as a control. DNA was extracted using a protocol derived from Dellaporta *et al.* (1983): 100 mg of leaf tissue were ground in 300 μL buffer (100 mM Tris-HCl pH 8.0, 50 mM EDTA, 500 mM NaCl, 0.5% (w/v) diethyldithiocarbamate), 40 μL 10% (w/v) SDS was added and then incubated at $65^\circ C$ for 10 min 100 μL 5 M $KC_2H_3O_2$ was added and then incubated on ice for 30 min. Following centrifugation for 10 min, 400 μL of the supernatant was added to 240 μL isopropanol, cooled at $-20^\circ C$ for 30 min then centrifuged for 10 min. The pellet was re-suspended in 200 μL of TE buffer and centrifuged for 5 min to eliminate the insoluble material. 190 μL of the supernatant was added to 12 μL 5 M NaCl and 410 μL ethanol, cooled on ice for 5 min and centrifuged for 5 min. The pellet was washed with 70% (v/v) ethanol and dissolved in 100 μL TE buffer and purified as follows: one volume (100 μL) of a solution containing 50 mM Tris-HCl pH 8.0, 10 mM EDTA, 2% (w/v) cetyltrimethylammonium bromide (CTAB), 2 M NaCl were added to the DNA solution. The mixture was incubated at $65^\circ C$ for 10 min. 200 μL of 50 mM Tris-HCl pH 8.0, 10 mM EDTA, 1% (w/v) CTAB were added to the previous DNA/CTAB solution and the mixture was left at room temperature for 10 min, before being centrifuged in a microfuge for 2 min. The CTAB-DNA pellet was re-dissolved in 400 μL of TE buffer containing 1 M NaCl. A chloroform/isoamyl alcohol (24 : 1, v/v) extraction was performed and to the upper phase obtained after centrifugation two volumes of ethanol were added for 3 min. The DNA was cooled on ice for 5 min, and the precipitant was collected by centrifugation for 5 min, and dissolved in 100 μL of TE buffer. 5 μg DNA was digested with *Hind*III and electrophoresed through a 0.8% (v/v) agarose gel. For Southern analysis the gel was blotted onto a Hybond-N blotting membrane (Amersham) as described by Maniatis *et al.* (1982). Pre-hybridization was carried out for 3 h at $65^\circ C$ in $5 \times SSC$ ($1 \times SSC$ is 15 mM sodium citrate, 150 mM NaCl), 25 mM sodium phosphate buffer pH 6.5, 0.1% (w/v) SDS, 5 mM EDTA, $5 \times$ Denhardt's solution ($1 \times$ Denhardt's solution is 0.02% (v/v) Ficoll, 0.02% (v/v) PVP,

0.02% (v/v) (BSA), 100 µg/mL denatured calf thymus DNA). The DNA probe was labelled with [³²P] dCTP using the random oligonucleotide priming method (Feinberg and Volgelstein, 1983) to a specific activity higher than 10⁹ c.p.m./µg. Hybridization was carried out overnight at 65 °C in the pre-hybridization solution. The membrane was washed in 2 × SSC, 0.1% (w/v) SDS for 30 min at room temperature, then in 2 × SSC, 0.1% (w/v) SDS for 30 min at 65 °C, then twice in 0.1 × SSC, 0.1% (w/v) SDS, for 30 min at 65 °C. The membrane was exposed to pre-flashed X-ray film for 3 days.

RNA was extracted from flower buds taken from at least eight Generation-3 plants from each of the 11 A9::DTA^{LS}-Q families. RNA was prepared (Guerineau *et al.*, 1991) by grinding leaf samples in TLES buffer (50 mM Tris-HCl pH 9.0, 150 mM LiCl, 5 mM EDTA, 5% (w/v); SDS). The lysate was twice treated with phenol/chloroform (1 : 1 v/v) followed by centrifugation for 1 min. The supernatant was removed and total nucleic acids were precipitated with two volumes of ethanol. After 5 min centrifugation the pellet was dissolved in 50 µL of sterile distilled water. One volume of 4 M LiCl was added and RNA was precipitated for 30 min on ice. After centrifugation for 5 min, the pellet was washed with 70% (v/v) ethanol, air dried and re-dissolved in 20 µL of sterile distilled water. 20 µg of each RNA sample was dotted onto Hybond-N membrane as recommended by the manufacturer.

Light and electron microscopy

For observation by light microscopy, flower buds were fixed in glutaraldehyde, dehydrated in an ethanol series and embedded in araldite. Sections of 0.2–0.5 µm were stained with 0.5% (w/v) Toluidine Blue. Electron microscopy samples were fixed using glutaraldehyde and osmium tetroxide 2% (w/v), dehydrated in ethanol and embedded in Spurr resin (Spurr, 1969). Sections were stained with uranyl acetate 2% (w/v) in 50% (v/v) ethanol and 80 mM lead citrate in 120 mM sodium citrate (pH 12 with NaOH). Observations were carried out on a JEOL (Tokyo, Japan) 100CX transmission electron microscope operating at 80 kV.

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