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IRREVERSIBLE INACTIVATION OF A TRANSGENE IN *ARABIDOPSIS THALIANA*

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Abstract

Plant transformation is now a routine practice in many laboratories around the world. However it has been observed that sometimes the introduced gene becomes inactive. This inactivation could be reversible or irreversible. In this study the genetic behavior of an inactive transgene was observed. For this purpose a wild type chlorophyll gene *CH-42* was introduced into a pale (*ch-42*) mutant *Arabidopsis* by *Agrobacterium* mediated transformation. After transformation most of the plants showed green wild type phenotype. However, some transgenic lines did not show the phenotype of the *CH-42* transgene and appeared pale in color. Four transgenic lines showing inactive *CH-42* transgene were selected to study the segregation of the inactive transgene in the next generations. These transgenic lines with inactive insert were crossed to the wildtype and the *CH-42* mutant plants. The F_1 plants produced from these crosses were allowed to self fertilize and some of the seeds of these F_1 were germinated for analysis of the F_2 generation. The results of the reciprocal crosses of all four inactive lines were similar. The inactivation of transgene *CH-42* was irreversible and the cellular gene at the *CH-42* locus did not have any effect on the expression of the transgene.

Introduction

Transgenic plants are being generated routinely in many laboratories. The methods and techniques employed vary, but the aim is usually the same; stable nuclear integration and expression of foreign DNA sequences. Perhaps the most common method of plant transformation is based on the natural ability of some *Agrobacterium* species to transform plants. The transferred (T) DNA becomes stably integrated into the plant genome (Zambriski, 1992). Once integrated T-DNA genes might be expected to behave as normal cellular genes i.e., show expression in the primary transformant and simple Mendelian dominant behaviour in subsequent generations (Otten *et al.*, 1981). The great majority of transgenes show this behaviour but some do not. Transgenes can become inactive in the primary transformants or show reduced or absent transmission of the trait in subsequent generations (Deroles & Gardner, 1988; Kilby *et al.*, 1992). In some cases it has been observed that when multiple copies of a gene or a transgene homologous to an endogenous gene are introduced into a plant, one or all of the gene copies can become inactive (Napoli *et al.*, 1990; Van der Krol *et al.*, 1990; Goring *et al.*, 1991; Smith *et al.*, 1988; Matzke *et al.*, 1989). The interaction between homologous sequences resulting in suppression of gene expression can involve transgenes and/or endogenous cellular genes. These phenomenon are collectively called "homology dependent gene silencing" (Jorgensen 1992) or "repeat induced gene silencing (RIGS)" (Assaad *et al.*, 1993). However, only some of the resulting transformants exhibit gene silencing. At the same time there are several examples of transgene silencing which do not involve homology generations (Deroles & Gardner, 1988; Kilby *et al.*, 1992). These observations suggest that the presence of homologous DNA sequences is insufficient to initiate gene silencing. Other factor(s) appear to be required. Whatever is the reason the transgene inactivation

can be a problem for the exploitation of transgenic plants in science, industry and agriculture but studies of transgene silencing may provide novel insights into the control of gene expression. The present report describes the genetic behavior of a transgene present on a T-DNA for which a pale mutant *CH-42 Arabidopsis* was transformed with a construct (pCV002GC) containing the wild type copy of the gene *CH-42* and a selectable drug marker (NPTII).

Material and Methods

***Arabidopsis* line and plasmid:** The tagged *CH-42 (CS)* allele was produced in the columbia background (Koncz *et al.*, 1990). Later it was back crossed to landsberg *erecta* and selected for the *ch-42* and *erecta* in the F_2 and is referred as *ch-42* in this paper. This *ch-42* was transformed by the plasmid pCV002GC and kindly provided by Dr. C Koncz. The structure of the T-DNA tag at *CH-42* locus is shown in Fig. 1 and the structure of the pCV002GC T-DNA in Fig. 2.

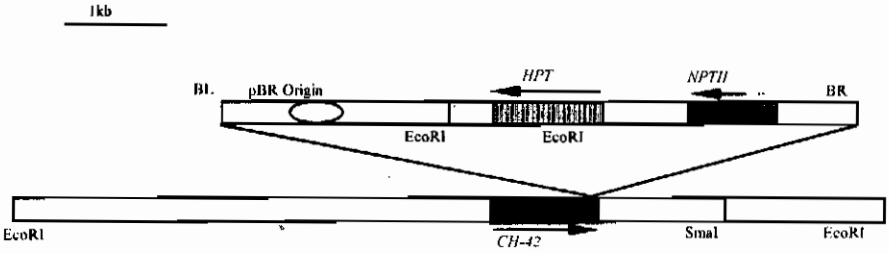

Fig. 1. The structure of the endogenous tagged *ch-42* allele and the structure of its T-DNA insert. The T-DNA contains a hygromycin resistance (*HPT*) gene and an *NPTII* open reading frame without a promoter.

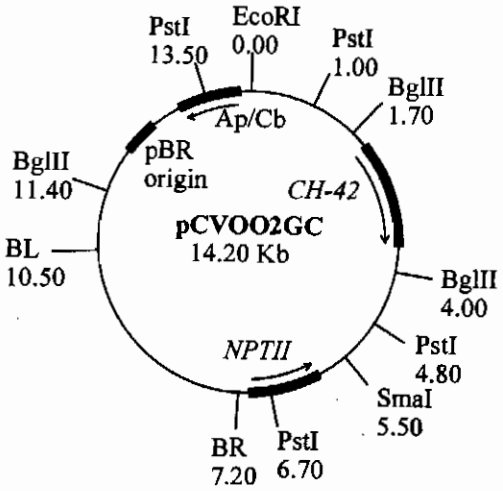

Fig. 2. The plasmid pCVOO2GC kindly provided by Dr C. Koncz.

***Arabidopsis* transformation:** Transformation of *Arabidopsis* roots was carried out as described by Kilby *et al.*, (1992). The NPTII gene was used as selectable maker. The primary transformants were termed the T_1 generation.

Growth of plants in soil: Plants were grown in an autoclaved mix of 75% v/v sand and 25% v/v autoclaved Fisons Levington multi-purpose compost. The plants were maintained at 22°C with 18 hours of light each day.

***Arabidopsis* crosses:** *Arabidopsis* plants normally self-fertilize before their flowers are fully open. In order to perform crosses the flowers were emasculated shortly before they opened. The anthers petals and sepals were removed using fine forceps. Pollen from the desired male parent were transferred to the stigma of the emasculated flower by pulling mature stamens from the male parent and touching them to the recipient stigma.

Extraction and assay of chlorophyll from plant tissues: Chlorophyll was extracted and assayed according to method of Lichtenthaler & Wellburn (1983). Tissue samples of between 10 and 100mg were ground in a pestle and mortar with 3ml of 80% acetone. The cell debris was pelleted and the optical density of the supernatant recorded at 663 and 646nm. The concentration of chlorophyll (mg/L) in the extract was then calculated according to the following formula:

$$\text{Total chlorophyll} = 7.18\ OD_{663} + 17.32 OD_{646}.$$

Result and Discussion

Chlorophyll content of the wildtype and the *ch-42* plants: The plants homozygous for the *ch-42* gene are pale compared to the green wild-type plants. In order to quantify the amount of chlorophyll in the lines at different stages of their life cycle. the chlorophyll content of the *ch-42* mutant and untransformed wild-type plants was assayed. Tissue samples were harvested each week, starting at week two. Fig. 3 shows the chlorophyll content of *ch-42* and wild-type plants over time. At the first point (two weeks) the chlorophyll content of both lines was higher than at later points. At this stage of growth the plants have cotyledons (but no true leaves) and the high values presumably represent a high chlorophyll content of the cotyledons. The chlorophyll content was constant from weeks 3 to 6. The wild-type had approximately 0.5mg/g chlorophyll and the *ch-42* plants only 0.3mg/g chlorophyll showing approximately a 40% reduction in the chlorophyll content.

Transformation of the ch-42 and the chlorophyll content of different transgenic lines: The *Arabidopsis ch-42* mutant plants were transformed by using plasmid pCVOO2GC. The roots of the pale *ch-42* mutant plants were cocultivated with *Agrobacterium* containing the plasmid pCVOO2GC. Transformed plant cells were selected on kanamycin and were regenerated into complete plants. In most of the transgenic lines the green phenotype was restored after transformation with the *CH-42* gene but in some lines this correction failed. It was of interest to quantify the amounts of chlorophyll in the lines. Chlorophyll was extracted from four weeks old plants of *ch-42*, wild-type and all of the transgenic lines (Fig. 4).

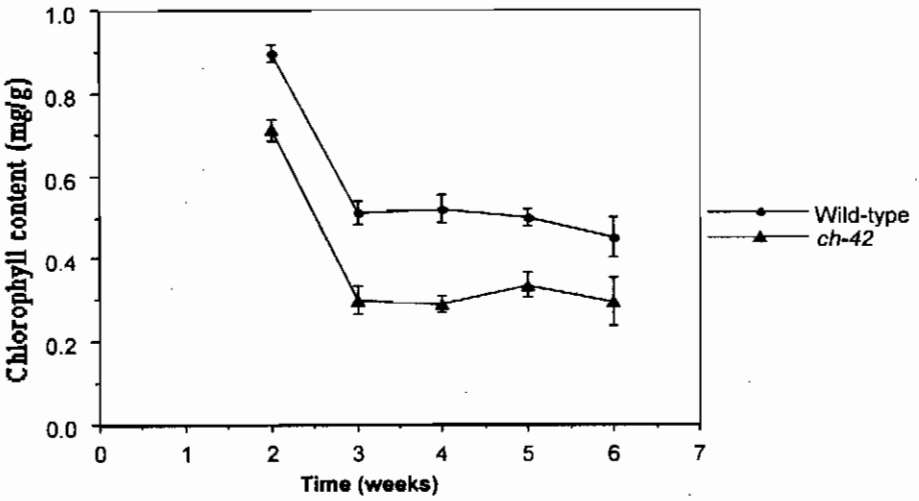

Fig. 3. The chlorophyll content (mg/g of tissue) of *ch-42* plants and wild-type plants over time (the bars indicate standard error).

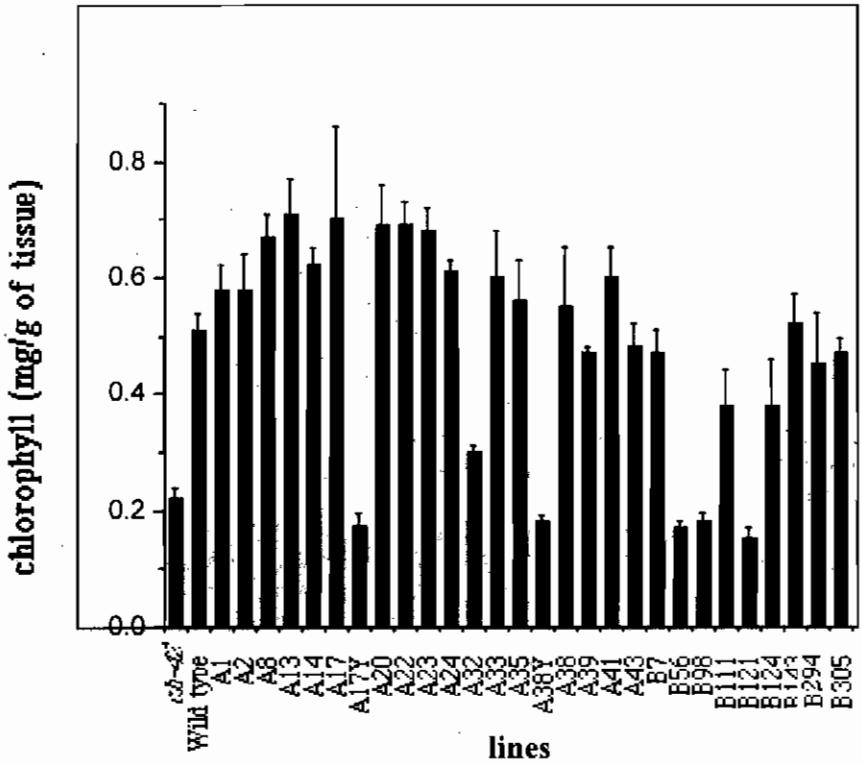

Fig. 4. The chlorophyll content in mg/g of tissue of *ch-42* mutant plants, wild type and transgenic lines. The bars represent standard error.

The chlorophyll content of the inactive lines (A17Y, A32, A38Y, B56, B98 and B121) was found to be similar to the chlorophyll content of the *ch-42* mutant plants (about 0.2-0.3mg/g). Most of the lines classified as successful correction events (A1, A2, A41, B143, B305) have chlorophyll level similar to the wild-type (about 0.45-0.55mg/g). Several lines appear to be slightly greener than wild-type e.g., A8, A13, A14. Given the size of the error bars these increases are significant only in some lines (A14, A20, A22) but not in other lines (A17 and A33). As the inserts classified as inactive had essentially the same amount of chlorophyll as the mutant there is no evidence that the inactive inserts contribute to the chlorophyll content at all and they may be completely silent. In the active lines the chlorophyll content is essentially the same as wild-type suggesting the levels of expression from the transgenes was sufficient to completely restore the mutant phenotype to wild-type.

Table 1. Crosses of inactive lines to wild type and Ch-42.

	no. of green plants		no. of pale plants		Interpreted
	F_1	F_2	F_1	F_2	ratio (χ^2)
A32 X *ch-42*	0	0	157	75	0:1
A32 X wild-type	36	71	0	26	3:1 (0.169)
A17Y X *ch-42*	0	0	30	68	0:1
A17Y X wild-type	15	66	0	21	3:1 (0.035)
A38Y X *ch-42*	0	0	23	72	0:1
A38Y X wild-type	22	64	0	19	3:1 (0.196)
B121 X *ch-42*	0	0	30	81	0:1
B121 X wild-type	60	79	0	24	3:1 (0.158)

Crosses of inactive lines to wild type and *ch-42*: The inactive lines A17Y, A32, A38Y and B121 each homozygous for an inactive insert were crossed to the wild-type and the *ch-42* mutant plants. The F_1 seeds were scored on soil for the colour phenotype (Table 1). Some of the seeds from the self-fertilised F_1 plants were germinated in soil for analysis of the F_2 segregation (Table 1). The results from crosses of all four inactive lines were similar. When inactive lines were crossed to the parental *ch-42* line, only pale plants were found in the F_1 and F_2 generations. When the inactive lines were crossed to the wild-type, green plants were produced which subsequently segregated 3:1 for green to yellow in the F_2 generation due to the segregation of the tagged *ch-42* allele at the *CH-42* locus. The result showed that the transgene *CH-42* insert was inactive in the homozygous and the hemizygous state. The *CH-42* allele at *CH-42* locus was epistatic to the inactive *CH-42* insert. It seemed that the suppression of the *CH-42* insert was independent of the presence of *ch-42* or *CH-42* allele at *CH-42* locus. No evidence was found in these studies that the *CH-42* insert in the inactive lines either contributed to chlorophyll production or in any way interacted with the cellular *ch-42* allele. The simplest explanation is that these inserts were just inactive. These results are in contrast to a previous report where a low expression insert resulted in suppression of an active insert present at another locus (Hobbs *et al.*, 1993), or a report of an inactive transgene becoming active upon out-crossing. Schied *et al.*, (1991) working on *Arabidopsis* transformed with a hygromycin resistance gene identified transformants with inactive transgene copies. Two inactive lines studied gave a stable inactive hygromycin sensitive phenotype on self-pollination.

However when plants of one of the inactive lines were out-crossed to the wild-type, 6% of the F_2 seedlings grew normally on hygromycin containing medium. Similar results were found when this inactive line was crossed to the other inactive line. In the present study such interaction between the homologous sequences was not observed. The inactivation of the *CH-42* gene was irreversible and presumably did not involve homology. Silencing of the transgene in these lines could be because of some other factors like position of the insert within genome or genetic/epigenetic changes in the transgene during integration. Some reports have been made showing the influence of position of the transgene on its expression level (Prole & Meyer 1992; Meyer & Heidmann, 1994). In addition to these primarily genetic factors, some environmental and developmental factors may also affect transgene expression.

Given the diversity of reports on transgene inactivity, there is no single clear picture. Some examples of silencing are homology dependent while others do not involve homology. The quantitative level of transgene silencing as well as number of transformants showing transgene inactivation varies from system to system. The apparently similar features observed in different reports of transgene silencing may be result of a wide variety of independent mechanisms or there may be one or a few similar mechanisms associated with apparently different features. At the moment there is not enough evidence to draw any firm conclusions. However the correction of the *CH-42* mutant is an elegant and sensitive way to examine transgene activity/inactivity. The assays are simple and convenient as the trait can be assayed at an early stage of growth without destroying the plants. The inactive lines could potentially be used to isolate mutants. Such lines could be selected by the virtue of reversion to a green phenotype. Further research in this system can help to understand transgene inactivation mechanism.

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