Meiotic transmission of epigenetic changes in the cell-division factor requirement of plant cells

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Accepted 9 September 2003

Development 130, 6201-6208
Published by The Company of Biologists Ltd 2003
doi:10.1242/dev.00856

Summary

During the development of tobacco plants, cells undergo epigenetic changes that alter their requirement in culture for the cell-division factor cytokinin. Cultured leaf cells alternate between cytokinin-requiring (C^-) and cytokinin-independent (C^+) states at extremely high rates of approximately 10^{-2} per cell generation by a process called pseudodirected variation. Here we show that plants regenerated from most C^+ clones express the *Habituated leaf* (Hl) trait, i.e., leaf tissues exhibit the C^+ phenotype rather than the wild-type C^- phenotype in culture. This new trait then segregates as a monogenic dominant trait indicating that conversion of C^- cells to C^+ cells is associated with a meiotically transmissible, genetic modification. Two independent mutants, *Hl-2* and *Hl-3*, derived from C^+ variants arising in culture were unstable in planta and reverted gametically at rates roughly comparable to pseudodirected variation in culture. Cells of the *Hl-2* mutant, but not of a stable *Hl-1* mutant, reverted phenotypically at high rates in culture. This revertant C^- phenotype persisted in some plants regenerated from cloned revertant lines, and then showed irregular segregation in two successive sexual generations. These results show for the first time that meiotically transmissible epimutations can occur reversibly and at high rates in culture.

Key words: Cell-division factor, Cytokinin, Epigenetic changes, Epimutation, Habituation, Pseudodirected variation, Tobacco

Introduction

Organisms arise from a germ by epigenesis, i.e., the progressive formation of new structures resulting from selective gene expression. Nevertheless, compelling evidence indicates that certain developmental states of cells can be transmitted mitotically to daughter cells. This led Nanney (Nanney, 1958) to propose two systems of inheritance: a genetic system concerned with the transmission of developmental potentialities between sexual generations of organisms; and, an epigenetic (i.e., developmental) system concerned with the somatic transmission of patterns of gene expression. Thus, the term *epigenetic change* has been used to denote cell-heritable, potentially reversible alterations that do not result from permanent genetic modifications (Meins, 1996).

More recently, it was recognized that some epigenetic changes could even be transmitted meiotically. Examples of this phenomenon, called *epimutation* (Jorgensen, 1993) include paramutation (Chandler et al., 2000), presetting of transposable elements (Fedoroff et al., 1989), transcriptional and post-transcriptional gene silencing (Matzke et al., 2001; Plasterk, 2002; Bird, 2002), and genomic imprinting (Reik, 2001; Baroux et al., 2003). Epimutation is of particular interest because it raises the possibility that some post-zygotic developmental events can be transmitted by sexual reproduction and, hence, could play a role in evolution (Jablonka and Lamb, 1995).

The present study deals with the nature of heritable changes associated with cytokinin habituation, i.e., the epigenetic, cell-heritable loss in the requirement of plant cells for cell-division factors in culture (Meins, 1989). Tobacco cells cultured from explants of leaf exhibit a cytokinin-requiring (C^-) phenotype; they show an absolute requirement for a cell division factor such as cytokinin for continuous growth on an otherwise complete culture medium containing auxin. In contrast, cells cultured from explants of stem cortex exhibit a constitutive cytokinin autotrophic (C^+) phenotype, i.e., they can grow continuously in the absence of added cytokinin. Cultures established from pith consist of a mixture of two types of C^- cells. Inducible C^- cells rapidly habituate, i.e., they shift to the C^+ state in response to cytokinin treatment or when cultured at elevated temperatures. Noninducible C^- cells remain C^- under these conditions. Cloning experiments have shown that both the C^- and C^+ states can be inherited at the cellular level. Nevertheless, tissues of plants regenerated from C^- and C^+ clones exhibit the cytokinin requirement of comparable tissues from seed-grown plants indicating that the two mitotically transmissible states are not permanent. This observation and the finding that the rates of induction and reversion are high – 10^2- to 10^3-fold faster than gametic mutation – and developmentally regulated provide strong evidence that tissue-specific states of cytokinin requirement result from epigenetic changes.

Stable C^+ variants can also be recovered from populations of noninducible C^- cells serially propagated on media containing reduced concentrations of cytokinin (Meins and Foster, 1985; Meins and Foster, 1986). This form of variation has several surprising features (Meins and Seldran, 1994): First, leaf tissues of plants regenerated from these variants,

unlike those from inducible C$^-$ cells, exhibit the constitutive C$^+$ phenotype in culture. This new phenotype, called habituated leaf (Hl), is inherited meiotically as a dominant trait at the *Habituated leaf-2* (*Hl-2*) locus (Meins and Foster, 1986). Second, although C$^+$ cells arise by a random rather than by a directed process, the rate at which they arise is extremely high – approximately 10^{-2} per cell generation. Third, cultured cells alternate between the C$^+$ and C$^-$ states at this high rate in both the forward and back direction. This phenomenon, called *pseudodirected variation*, results from phenotypic changes so rapid that the classical distinction between random and induced events is blurred. Because C$^-$ and C$^+$ cells differ in growth rate in response to cytokinin, cytokinin can act by selection on the alternating population of cells to give changes that appear to be induced when examined at the tissue level.

We have combined cell cloning and plant regeneration experiments to show that cell-heritable states of cytokinin requirement generated by pseudodirected variation persist in regenerated plants and can be meiotically transmitted. Unlike most classical mutations, these heritable states undergo rapid reversion in successive sexual generations indicating that pseudodirected variation is a novel form of epimutation.

Materials and methods

Plants and cell lines

Three genotypes of *Nicotiana tabacum* L. cv. Havana 425 were used: wild-type plants exhibiting the C$^-$ leaf phenotype and homozygous *Hl-1*/*Hl-1* and *Hl-2*/*Hl-2* plants exhibiting the C$^+$ leaf phenotype (Meins and Foster, 1986; Meins et al., 1983). The plants were grown from seed in a greenhouse. The cloned C$^+$ lines derived from leaf clone L113H and pith clone P278H have been described previously (Meins and Seldran, 1994).

Culture of tissues, cell cloning and plant regeneration

Methods for isolating tissues, culturing tissues, cloning cells and regenerating plants have been described in detail elsewhere (Binns and Meins, 1973; Meins et al., 1980). In brief, C$^+$ tissues were grown on a basal medium containing agar, salts, sucrose, *myo*-inositol and thiamine at the concentrations recommended by Linsmaier and Skoog (Linsmaier and Skoog, 1965) supplemented with 2.0 mg/l of the auxin α-naphthaleneacetic acid, and 5 mg/l of the pH indicator chlorophenol red. The C$^-$ tissues were grown on a complete medium consisting of basal medium supplemented with 0.3 mg/l of the cytokinin kinetin. Tissue explants, ≈10 mg in weight, were incubated in the light for 21 days at 25°C in shell vials containing 10 ml of medium. Clones were obtained by marking the position of single cells plated in soft agar. Plants were regenerated from cloned lines by incubating tissues on kinetin medium (i.e., complete medium without auxin), and transferring the resultant shoots on a rooting medium. Plants were placed in soil and grown to maturity in a greenhouse. The regenerated plants are referred to as the S_0 generation. Plants obtained by selfing S_0 plants are referred to as S_1, S_2, etc. for each successive generation. Haploid plants were regenerated from cultured anthers as described by Bourgin and Nitsch (Bourgin and Nitsch, 1967).

Measuring cytokinin requirement

Two sets of four replicate tissue explants were subcultured twice, one set on +kinetin (complete) medium and one set on –kinetin (basal) medium and were then weighed. Tissues were classified as C$^-$ or C$^+$ using as the criterion relative growth rate (R) on –kinetin and +kinetin media. R was calculated from the expression $\ln(W/W_0)_{-\text{kinetin}}/\ln(W/W_0)_{+\text{kinetin}}$, where W_0 and W are the fresh weights of the inoculum and the tissue after 3 weeks, respectively. Tissues giving an average R value greater than 0.4 were judged to be C$^+$ (Binns and Meins, 1973). Sampling error in distributions of progeny was estimated by the binomial proportions test (Simpson et al., 1960).

Selection for variants

C$^+$ variants were obtained from cloned lines of C$^-$ cells by subculturing tissues on medium containing 1% of the kinetin concentration in complete medium as described previously (Meins and Seldran, 1994). C$^-$ variants were obtained from cloned lines of C$^+$ cells by subculturing tissues sequentially on media containing 1%, 10% and 100% of the kinetin concentration in complete medium and selecting for rapidly growing colonies after each transfer.

Results

Meiotic transmission of the Hl trait

If pseudodirected variations result in genetic alterations, then both the wild-type C$^-$ phenotype and the variant C$^+$ phenotype should be meiotically transmissible when S_0 plants regenerated from cloned lines are self crossed to give the S_1 generation. The protocol used to test this hypothesis is outlined in Fig. 1. We isolated two cloned C$^-$ lines, L113N and L201N, from leaf explants harvested from different plants in separate experiments. One set of cultures from each line was maintained on complete medium containing cytokinin, which favors the proliferation of C$^-$ cells. A second set of cultures was subcultured on medium containing 1% of the cytokinin concentration in complete medium, which favors proliferation of C$^+$ cells. After three subculture cycles on the 1% cytokinin medium, the tissues were able to grow on cytokinin-free medium. Subclones were then isolated from the C$^+$ variants L113H and L201H and from their respective parent C$^-$ lines L113N and L201N. Plants were regenerated from the C$^-$ and C$^+$ subclones, the resultant S_0 plants were selfed, and the progeny were scored for the Hl trait by comparing the relative growth rate (R value) of cultures established from leaf tissues on medium with and without cytokinin. Additional progeny tests were made with plants regenerated from four C$^-$ leaf clones (L12N, L33N-L35N) and from four C$^+$ clones obtained by low-cytokinin selection from the noninducible C$^-$ clone P278N of pith origin (Fig. 1).

The patterns of segregation obtained for 17 S_0 plants, each from an independently isolated clone, are shown in Table 1. The Hl trait was found in the S_1 generation derived from 8 of the 9 C$^+$ clones tested. The S_0 plant derived from leaf clone L201H-36 did not give C$^+$ progeny suggesting that reversion had occurred during shoot initiation or subsequent development of the regenerated plant. Although it was not technically feasible to screen large numbers of progeny, the patterns of segregation obtained were not significantly different (P<0.05, binomial proportions test) from 3:1. This is consistent with the hypotheses that the Hl phenotype is inherited as a dominant monogenic trait and that plants regenerated from the cloned C$^+$ variants are heterozygous for the Hl trait. In contrast, none of the progeny tested from 8 C$^-$ clones of leaf origin showed the Hl trait. Taken together, these results indicate that most plants regenerated from C$^+$ variants have undergone a meiotically transmissible genetic modification at a single locus.

Partial characterization of the *Hl-3* mutant of pith origin

Earlier we identified two unlinked *Habituated-leaf* mutants:

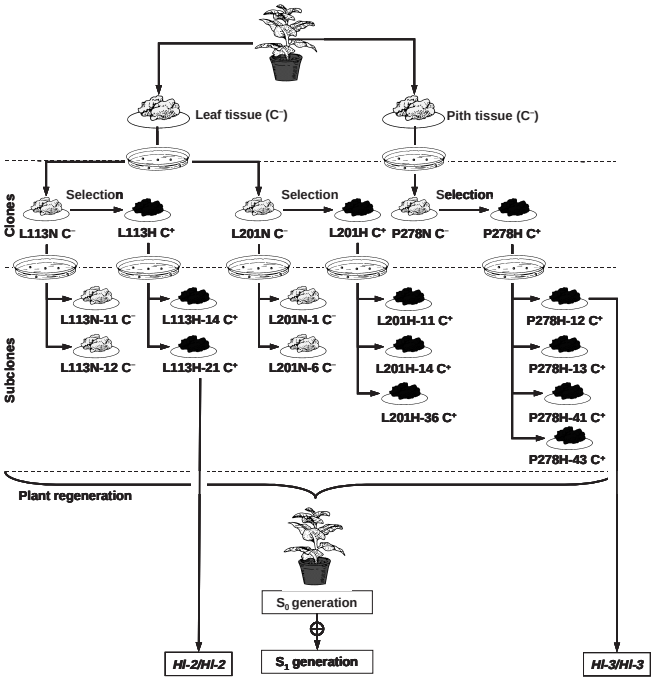

Fig. 1. The lineage of cell clones and plants regenerated from C⁻ leaf and C⁻ pith tissues. Clones were obtained by plating cell suspensions in soft agar and marking colonies of single-cell origin. C⁻ leaf clones L113N and L201N and C⁻ pith clone P278N were derived from different plants in separate experiments. C⁺ variants were obtained by selecting for growth on low-cytokinin medium. S_0 generation plants regenerated from subclones were selfed to obtain the S_1 generation. The patterns of segregation for the C⁺ leaf phenotype are shown in Table 1. The origins of homozygous mutant lines *Hl-1/Hl-2* and *Hl-2/Hl-2* derived from subclones L113H-21 and P278H-12, respectively, are indicated. Clones showing the C⁺ phenotype are shaded.

Hl-1 regenerated from a culture of constitutive C⁺ cortex cells; and *Hl-2* regenerated from a C⁺ variant of cultured, noninducible C⁻ leaf cells (Meins et al., 1983; Meins and Foster, 1986). To detect possible tissue-of-origin effects on the properties of *Hl* mutants, we partially characterized a third mutant, designated *Hl-3*, which is of pith origin. The C⁺ variant P278H was obtained by low-cytokinin selection from the clone P278N of non-inducible C⁻ pith cells (Fig. 1) (Meins and Foster, 1985). A plant regenerated from the C⁺ subclone P278H-12 was selfed, a homozygous Hl plant was selected from the S_1 population, and this plant was crossed with wild-type, seed-grown plants to generate F_1, F_2 and backcross generations. The results of the progeny tests are summarized in Table 2. Except for one of 42 progeny, all progeny obtained from reciprocal crosses of wild-type and Hl plants gave Hl progeny. The Hl trait segregated 3:1 in the F_2 generation and 1:1 in the backcross of the F_1 with a wild-type plant. Thus, this Hl phenotype is usually inherited as a dominant, monogenic trait. One F_1 plant and 5/60 of the progeny from crosses of a F_1 plant showing the Hl phenotype with a homozygous Hl plant unexpectedly showed the wild-type phenotype. This suggests that the Hl trait in homozygous *Hl-3* plants can revert to wild type.

To test for linkage between the known *Hl* loci, homozygous

Table 1. Segregation of the Habituated-leaf trait in the S_1 generation obtained from cloned C⁻ lines and C⁺ variants

			S_1 generation			
			C⁻ progeny		C⁺ progeny†	
Source tissue	Cloned line*	Clone phenotype	*n*	R value‡	*n*	R value
Leaf	L12N	C⁻	15	0.30±0.02	0	–
	L33N	C⁻	15	0.28±0.02	0	–
	L34N	C⁻	15	0.34±0.02	0	–
	L35N	C⁻	15	0.24±0.02	0	–
	L113N-11	C⁻	15	0.05±0.01	0	–
	L113N-12	C⁻	15	0.09±0.01	0	–
	L113H-14	C⁺	7	0.09±0.03	8	0.97±0.02
	L113H-21§	C⁺	5	0.15±0.03	10	0.93±0.03
	L201N-1	C⁻	15	0.15±0.02	0	–
	L201N-6	C⁻	15	0.17±0.04	0	–
	L201H-11	C⁺	4	0.17±0.03	11	0.83±0.06
	L201H-14	C⁺	4	0.24±0.01	11	0.76±0.06
	L201H-36	C⁺	15	0.14±0.02	0	–
Pith	P278H-12¶	C⁺	5	0.22±0.02	10	1.10±0.06
	P278H-13	C⁺	6	0.19±0.05	8	0.87±0.06
	P278H-41	C⁺	1	0.21	13	1.01±0.03
	P278H-43	C⁺	3	0.33±0.03	12	0.95±0.03

*C⁺ variants were obtained by growth of C⁻ clones on low-cytokinin medium. Properties of P278H S_0 plants are described by Meins and Foster (Meins and Foster, 1985).
Data for L113H lines are from Meins and Foster (Meins and Foster, 1986).
†R value >0.4.
‡Mean±s.e.m. for *n* progeny.
§Origin of the mutant *Hl-2*.
¶Origin of the mutant *Hl-3*.

Table 2. Inheritance of the *Habituated leaf-3* (*Hl-3*) mutant derived from C⁺ pith clone P278H-12

	Phenotype of leaf tissue			
	C⁻ progeny		C⁺ progeny‡	
Cross	*n*	R value	*n*	R value
Wt*×	16	0.15±0.01§	0	–
Hl-3/Hl-3† ×	0	–	21	0.93±0.03
wt × *Hl-3/Hl-3*	1	0.22	20 (21)§	0.90±0.05
Hl-3/Hl-3 × wt	0	–	21 (21)	0.93±0.03
(*Hl-3/Hl-3* × wt) ×	21	0.26±0.02	43 (48)	0.76±0.03
(*Hl-3/Hl-3* × wt) × wt	10	0.23±0.03	20 (15)	0.78±0.04
wt × (*Hl-3/Hl-3* × wt)	15	0.21±0.02	15 (15)	0.71±0.06
(*Hl-3/Hl-3* × wt) × *Hl-3/Hl-3*	3	0.24±0.03	27 (30)	0.75±0.03
Hl-3/Hl-3 × (*Hl-3/Hl-3* × wt)	2	0.24±0.02	28 (30)	0.66±0.04

*Wild-type, seed-grown plant not derived from tissue culture.
†S_2 generation, homozygous C⁺ plant T65-2 derived from P278H-12 (Meins and Foster, 1985).
‡R value >0.4.
§Mean±s.e.m. for the number of progeny (*n*) recovered with the phenotype indicated.

Hl-1/Hl-1, *Hl-2/Hl-2* and *Hl-3/Hl-2* plants were crossed to give the three possible F_1 generations. The F_1 plants were then crossed to wild type. Table 3 shows that the Hl trait segregated 3:1 in the progeny. This is consistent with the hypothesis that the three loci are not linked.

Gametic reversion of *Hl-2* and *Hl-3*

Crosses of homozygous *Hl-2* and *Hl-3* plants with wild-type

Table 3. Test for linkage of the Hl-1, Hl-2, and Hl-3 traits

	Phenotype of leaf tissue			
	C^- progeny		C^+ progeny†	
Cross	*n*	R value	*n*	R value
wt* × (*Hl-1*/*Hl-1* × *Hl-3*/*Hl-3*)	6 (5)§	0.28±0.06‡	14	0.81±0.05
wt × (*Hl-3*/*Hl-3* × *Hl-2*/*Hl-2*)	7 (5)	0.15±0.02	13	0.76±0.05
wt × (*Hl-2*/*Hl-2* × *Hl-1*/*Hl-1*)	8 (5)	0.18±0.08	12	0.67±0.04

*Wild-type, seed grown plant not derived from tissue culture.
†R value >0.4.
‡Mean±s.e.m. for the number of progeny (*n*) recovered with the phenotype indicated.
§Number of progeny expected for unlinked, dominant traits in parentheses.

Table 4. Estimated gametic reversion rates (μ) of *Hl-2* and *Hl-3* alleles

Genotype	Cross	Frequency (f) C^- progeny/ total	Estimated μ value*
Hl-1	*Hl-1*/*Hl-1* × wt	0/60	–
Hl-2	*Hl-2*/*Hl-2* × wt	3/15	20×10^{-2}
Hl-3	*Hl-3*/*Hl-3* × wt	1/21	4.8×10^{-2}
	Hl-3/*Hl-3* × *Hl-3*/*hl-3*	5/60	14.5×10^{-2}
Wild type (seed grown)	wt ×	244/244	–
Wild type (S_0 from cloned lines in culture)	wt ×	114/114	–

*Reversion rates estimated from the frequency of C^- progeny using as models μ=f for crosses of homozygous plants with wild type and $\mu=((1+8f)^{1/2}-1)/2$ for crosses of homozygous with heterozygous plants.

plants should give exclusively Hl progeny. Unexpectedly, a low frequency of progeny exhibited the wild-type phenotype (Meins et al., 1983) (Table 2). Table 4 shows the rates of gametic reversion estimated from several different crosses. The high rates obtained, 10^{-2} to 10^{-1} per gamete, are comparable with those estimated for phenotypic reversion of heterozygous C^+ variants in culture (Meins and Seldran, 1994). No reversion was detected with the stable mutant *Hl-1* included as a control; and no Hl plants were found in a total of 358 progeny of selfed wild-type plants, which included 114 progeny from five plants regenerated from cloned C^- lines.

Gametic reversion was confirmed by analyzing haploid plants of microspore origin. Haploid plants were regenerated from anther cultures established from two sibling, homozygous *Hl-2* plants and one wild-type plant. The genotype of the plants used was confirmed by selfing: all 20 progeny tested from each *Hl-2* plant showed the Hl trait, whereas none of the 20 progeny of the control wild-type plant showed the Hl trait (Table 5). Of 15 haploid plants regenerated from the *Hl-2* anthers, three plants were wild type. In contrast, all of the nine haploid plants regenerated from wild-type anthers showed the wild-type phenotype. These results show that the frequency of revertant microspores, $\approx19\times10^{-2}$ is roughly comparable to rates of gametic reversion estimated from crosses of *Hl-2*/*Hl-2* with wild-type plants (see Table 4) and that expression of the Hl phenotype does not depend on allelic interaction at the *Hl-2* locus.

Reversion of *Hl-2*/*Hl-2* cells in culture

We also examined the frequency of revertant C^- clones obtained by selection from cultured C^+ *Hl-2* tissues. Leaf cultures designated line A and line B were started from homozygous (*Hl-2*/*Hl-2*) and heterozygous (*Hl-2*/*hl-2*) plants, respectively. These cultures, which expressed the C^+ phenotype, were subcultured successively on medium containing 1%, 10% and 100% of the cytokinin concentration in complete medium. Under these conditions there is strong selection for C^- cells (Meins and Seldran, 1994). After selection, clones were isolated from tissues growing rapidly on complete medium and the R value was determined. The distribution of C^- and C^+ clones obtained and the average R values of the clones are shown in Table 6. C^- clones were recovered from both the homozygous and heterozygous tissue lines. The frequency obtained with the heterozygous line was significantly lower (6.3%) than that of the homozygous line (35.6%) ($P<0.0001$, binomial proportions test). In both cases, the average R values of the C^+ clones were similar to the R value of the parent tissue, indicating that the selection procedure did not significantly change the degree of cytokinin autotrophy of non-revertant C^+ cells.

As a control, similar experiments were performed with leaf tissues of the unlinked mutant *Hl-1*, which is stable in breeding tests (Table 4) (Meins and Foster, 1986). No C^- revertant clones were recovered from the 60 homozygous clones and 120 heterozygous clones tested (Table 6). Taken together, the results indicate that cells derived from plants carrying the dominant *Hl-2* allele show a high incidence of phenotypic reversion in culture. Moreover, the incidence of reversion to the recessive C^- phenotype was ≈5-fold higher in lines from the homozygous *Hl-2*/*Hl-2* plant than in lines from the heterozygous *Hl-2*/*hl-2* plant.

Table 5. Reversion of *Hl-2* in anther-derived haploid plants

	Segregation of progeny				Haploid anther-derived plants			
	C^-		$C^{+‡}$		C^-		C^+	
Genotype	*n*	R value	*n*	R value	*n*	R value	*n*	R value
Wild type*	20	0.12±0.18§	0	–	9	0.16±0.03	0	–
Hl-2/*Hl-2*†	0	–	20	0.73±0.03	1	0.31	6	0.71±0.04
Hl-2/*Hl-2*†	0	–	20	0.88±0.02	2	0.24±0.08	7	0.87±0.07

*Seed grown plant.
†Replicate sibling plants.
‡R value >0.4.
§Mean±s.e.m. for the number of plants (*n*) recovered with the phenotype indicated.

Table 6. Incidence of revertant clones obtained with *Hl-2* and *Hl-1* leaf tissues after selection for growth of tissues on cytokinin-containing medium

			Phenotype of clones after selection†				
			C⁻ clones		C⁺‡ clones		
Tissue line	Genotype	Leaf tissue R value*	*n*	R value	*n*	R value	% Revertant
A	*Hl-2*/*Hl-2*	0.90±0.12 (6)	21	0.22±0.03	38	0.99±0.10	35.6
B	*Hl-2*/*hl-2*	1.84±0.25 (3)	4	0.19±0.10	59	1.27±0.08	6.3
C	*Hl-1*/*Hl-1*	0.73±0.02 (4)	0	–	60	0.86±0.02	0
D	*Hl-1*/*hl-1*	0.58±0.05 (4)	0	–	60	0.80±0.02	0

*Mean±s.e.m. for *n* replicate leaf samples from the plant used to start tissue lines.
†Mean±s.e.m. for *n* clones assayed.
‡R value >0.4.

Stability of the revertant C⁻ phenotype in regenerated plants

The protocol for studying the stability of the revertant C⁻ phenotype in S_0, S_1 and S_2 generation plants is illustrated for the *Hl-2/Hl-2* line A in Fig. 2. One to three replicate plants were regenerated from individual revertant and non-revertant clones. Leaf tissues cultured from these plants were then assayed for their cytokinin requirement. Table 7 shows that the phenotype of the non-revertant C⁺ clones persisted in the regenerates: the seven S_0 plants regenerated from the three non-revertant C⁺ clones A7, A29 and A43 expressed the C⁺ phenotype. In contrast, the seven S_0 plants regenerated from the three C⁻ revertant clones A9, A10 and A11 varied widely in phenotype. For example, all plants regenerated from clones A9 and A11 showed the C⁺ phenotype and only one of the two plants regenerated from clone A10 retained the C⁻ phenotype. A similar range of R values was obtained for plants regenerated from different clones and for sister plants regenerated from the same clone.

Phenotypic instability was also a feature of the S_0 generation derived from the heterozygous *Hl-2*/*hl-2* line B. For example, among the plants regenerated from a C⁺ clone, three showed the C⁺ phenotype and one showed the C⁻ phenotype (data not shown). These results and those obtained with homozygous line A indicate that leaf tissues cultured from S_0 plants usually show a C⁺ phenotype independent of the phenotype of the cloned line from which the plants were derived. Therefore, the revertant C⁻ phenotype is usually not stably expressed in the S_0 generation.

Meiotic transmission of the revertant C⁻ phenotype

S_0 generation plants regenerated from clones of line A and B origin were selfed and leaves of the progeny were assayed for their cytokinin phenotype. Progeny showing the C⁻ leaf trait were recovered from S_0 plants derived from both C⁻ and C⁺ clones of homozygous line A (Fig. 2, Table 7). Segregation of the C⁻ leaf trait in the S_1 generation was variable, *viz*., 26:4, 10:5, 14:1. Moreover, the C⁺ progeny showed a 'weak' C⁺ phenotype, with R values in the range 0.4-0.5, which is far lower than the values of about 0.8-1.0 that are typical of leaves from homozygous and heterozygous *Hl-2* plants. These results show that the revertant C⁻ phenotype arising in culture from homozygous *Hl-2*/*Hl-2* cells can be transmitted meiotically, but that its inheritance is irregular. Similar conclusions can be drawn from the results obtained with the heterozygous B line. In this case, the S_0 plants would be expected to be heterozygous for *Hl-2* and the C⁻ leaf trait should segregate 1:3 in the S_1 generation. Instead, two of the three S_0 plants tested gave an unexpectedly large proportion of C⁻ progeny (data not shown).

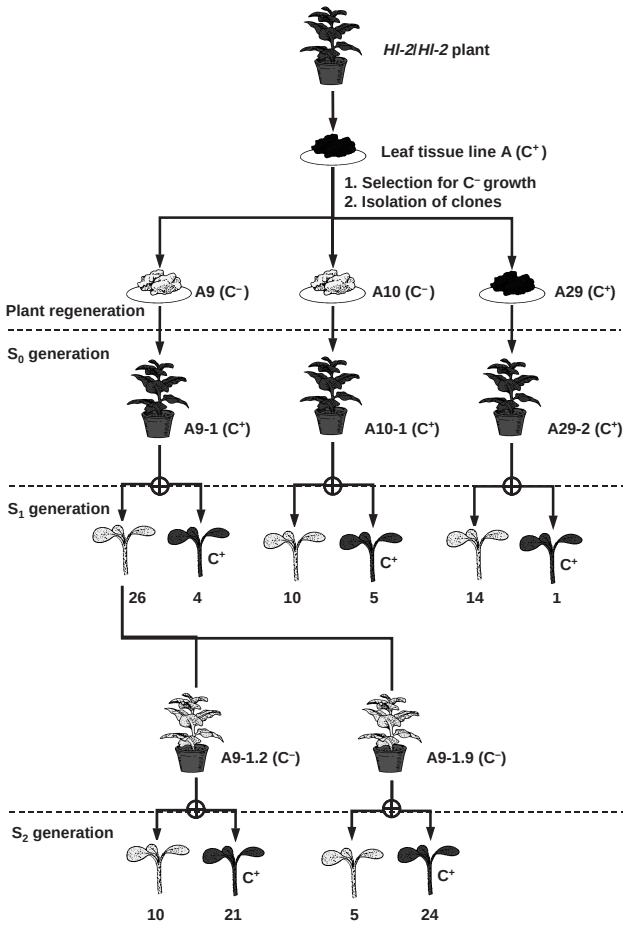

Fig. 2. Segregation of the Hl trait in plants derived from clones of Hl-2/Hl-2 leaf tissues cultured on high-cytokinin medium. Results are shown for three representative S_0 plants derived from three clones. C⁺ cloned lines and S_0, S_1 and S_2 plants showing the Hl trait are shaded. The numbers of C⁻ and C⁺ progeny obtained in crosses are indicated below the seedling. The R values obtained and results for additional regenerates are shown in Table 7.

Table 7. The leaf phenotype of S_0, S_1 and S_2 plants derived from clones of the C^- *Hl-2*/*Hl-2* leaf-tissue line A

				S_1 generation						S_2 generation			
Cloned line		S_0 generation		C^- progeny		C^+ progeny				C^- progeny		C^+ progeny	
Clone	Phenotype*	Plant†	Phenotype‡	*n*	R value§	*n*	R value	Plant	Phenotype	*n*	R value	*n*	R value
A7	C^+ (0.56)	A7	C^+ (0.57±0.07)										
A9	C^- (0.09)	A9-1	C^+ (0.64±0.05)	26	0.13±0.03	4	0.51±0.03	A9-1.2	C^- (0±0.0)	10	0.15±0.05	21	0.77±0.03
								A9-1.9	C^- (0±0.0)	5	0.13±0.08	24	0.71±0.03
		A9-2	C^+ (0.56±0.06)										
A10	C^- (0.36)	A10-1	C^+ (0.53±0.05)										
		A10-2	C^- (0.37±0.06)	10	0.15±0.05	5	0.47±0.02						
A11	C^- (0.25)	A11-1	C^+ (0.82±0.11)										
		A11-2	C^+ (0.49±0.05)										
		A11-3	C^+ (0.66±0.10)										
A29	C^+ (0.55)	A29-1	C^+ (0.71±0.06)										
		A29-2	C^+ (0.47±0.20)	14	0.14±0.05	1	0.43						
		A29-3	C^+ (0.78)										
A43	C^+ (0.63)	A43-1	C^+ (0.74±0.06)										
		A43-2	C^+ (0.81±0.12)										
		A43-3	C^+ (1.22±0.14)										

*R value of cloned line in parenthesis; tissues with R value > 0.4 were scored as the C^+ phenotype.
†Replicate plants regenerated independently from the same cloned line, e.g., A9-1 and A9-2, are indicated.
‡Mean R value±s.e.m. of 4 replicate leaf explants from the same plant in parenthesis.
§Mean R value±s.e.m. of leaf explants for the number of progeny (*n*) with the phenotype indicated.

Two S_1 plants, A9-1.2 and A9-1.9, descended from plant A9-1 regenerated from C^- revertant clone A9, were selfed (Table 7). Even though the two parent plants exhibited the recessive C^- phenotype, plants exhibiting the dominant C^+ trait were recovered at frequencies of about 67% and 82% in the S_2 generation. In our standard assay, tissues from only one leaf of each plant were scored. Thus, the irregular segregations observed could reflect chimerism for the Hl trait within individual plants. We confirmed that plants descended from A9-1 were variegated by comparing the R value of two different leaves from the same plants. The results showed that 3/12 of S_1 plants, 4/15 of S_2 progeny from plant A9-1.2 and 8/13 of S_2 progeny from plant A9-1.9 exhibited different cytokinin phenotypes in the two leaves tested.

Discussion

Plant cells in culture show high rates of phenotypic variation (Scowcroft et al., 1987). This variation can result from classic genetic alterations including point mutations, deletions, somatic recombination and chromosomal rearrangement, epigenetic modifications and combinations of epigenetic and genetic events (Meins, 1983; Lee and Phillips, 1988; Kaeppler et al., 2000). The stability of these events is also highly variable. Many variant phenotypes are lost during the plant regeneration process; others persist in the primary regenerants; and, less frequently, some are transmitted to subsequent generations.

Although the distinction between genetic and epigenetic changes has been debated, it is generally accepted that epigenetic changes result from cell-heritable, but potentially reversible alterations in gene expression (Meins, 1996; Wu and Morris, 2001; van de Vijver et al., 2002). As judged by these criteria, the present study provides strong evidence that epimutations, i.e., meiotically transmissible epigenetic changes (Jorgensen, 1993), can occur reversibly and at high rates in culture. Most C^+ clones resulting from pseudodirected variation gave rise to plants showing the Hl phenotype, which then segregated as a monogenic trait when the plants were selfed. Therefore, the conversion of C^- to C^+ cells is associated with a meiotically heritable modification of a wild-type *hl* allele to give a dominant *Hl* allele. Moreover, two independent *Hl-2* and *Hl-3* mutants derived from C^+ variants arising in culture were unstable in planta and reverted gametically at rates roughly comparable to pseudodirected variation in culture, indicating that the meiotically heritable changes we observed are potentially reversible.

The finding that shoots regenerated from genetically mosaic *Su/su* callus tissue are usually homogeneous in phenotype strongly suggests that regenerated tobacco plants are clonally derived from single cells (Lörz and Scowcroft, 1983). While leaf tissue from most of our regenerates showed the same cytokinin phenotype as the clone from which they were derived, some, e.g., plants A10-1, A11-1, A11-2, and A11-3 (Table 7) showed the alternative phenotype. We believe that these plants are derived from a subpopulation of cells that arose by rapid variation in culture subsequent to cloning. Our finding that some regenerated plants were variegated in cytokinin phenotype suggests, moreover, that rapid variation also occurs during the regeneration process and the later development of the plant. This could account for the irregular segregation of the Hl trait in the progeny of selfed plants as reported for stable somatic mutations in tobacco (Dulieu, 1974; Dulieu, 1975; Lörz and Scowcroft, 1983).

Gametic revertants from *Hl-2* and *Hl-3* plants exhibit a stable C^- phenotype indistinguishable from wild type. Selfing of these progeny consistently gave exclusively C^- progeny (Table 4) indicating that the meiotically heritable C^+ state has an epigenetic basis. In striking contrast, revertant C^- plants obtained by high-cytokinin selection of homozygous *Hl-2*/*Hl-2* cells are unstable: they show mosaicism, irregular segregation of cytokinin phenotypes and high rates of reversion to the C^+ state in the S_2 and S_3 generations (Table 7). This suggests that C^+ *Hl-2* cells can also revert incompletely to a

metastable C^- state, which is distinct from the wild-type C^- state.

Cytokinins play a key role in regulating growth, differentiation and morphogenesis (Schmülling, 2002). For example, acting in concert with auxins, cytokinins induce shoot formation and inhibit root formation in undifferentiated cultures of tobacco tissue (Skoog and Miller, 1957). Recent studies with cytokinin-deficient transgenic tobacco suggest cytokinins have a similar function in planta (Werner et al., 2001). Organized structures arising under inductive conditions in culture are derived from a subpopulations of committed, competent cells (Meins, 1986; Merkle et al., 1995). The incidence of these committed cells depends on the concentration of cytokinin and other factors in the culture medium as well as the internal epigenetic and genetic state of the cells. Tobacco cells competent to form shoots in response to cytokinin appear to arise reversibly in culture at rates roughly comparable to that of pseudodirected variation (Meins et al., 1982). Thus, in principle, cytokinins might promote organogenesis by selecting for a subpopulation of committed, cytokinin-responsive cells that arise and are lost by a continuous process of pseudodirected variation. It is often claimed that plant regeneration from species in which regeneration is difficult in culture depends on selection over many transfer generations to produce special morphological types of callus. We speculate that pseudodirected variation provides a general explanation for this phenomenon.

The molecular basis for the rapid variation we observed is not known. Possibilities include positive autoregulation (Meins and Binns, 1978), reversible recombination switches (Silverman et al., 1980), RNA silencing (Matzke et al., 2001) and stable chromatin modification (Li et al., 2002). Another possibility is DNA methylation, which is known to be the basis for well-characterized epimutations affecting the *Arabidopsis SUPERMAN* gene (Jacobsen and Meyerowitz, 1997) and the *Lcyc* gene of *Linaria vulgaris* (Cubas et al., 1999). Increased DNA methylation has been shown to decrease the capacity for cytokinin-independent growth of T-DNA transformants (Amasino et al., 1984; van Slogteren et al., 1984; Sinkar et al., 1988) and tissues of tumor-prone interspecific GGLL *Nicotiana* hybrids (Durante et al., 1989; Ahuja, 1996). Finally, changes in DNA methylation frequently occur in cultured plant tissues and are believed to be a major cause for genetic as well as epigenetic forms of variation that are sometimes meiotically transmissible (Kaeppler et al., 2000).

The few cases studied in detail suggest that methylation of specific genes decreases in cultured plant tissues (Kaeppler et al., 2000). Our working hypothesis is that cytokinin requirement is epigenetically regulated at loci such as *Hl-2* or *Hl-3* that are methylated and transcriptionally inactive in C^- leaf and pith cells. According to this hypothesis, these loci are demethylated at low rates in culture to generate C^+ cells heterozygous for the methylated epiallele. This results in a dynamic equilibrium between the unmethylated C^+, hemimethylated C^+ and methylated C^- states. The state of methylation can also change at low rates in planta; but in this case it appears that transitions to the methylated state are favored since we have never found Hl progeny of wild-type plants (Table 4). DNA methylation can gradually and reversibly spread from an initial site to other sites along the DNA leading to gradual, progressive epigenetic modifications in gene expression (Bird, 2002). As judged by changes in R value, cells can show different degrees of stable alteration; and, during prolonged culture, cells progressively increase in their capacity for cytokinin-independent growth (Meins and Binns, 1977). Graded differences in cytokinin requirement were also evident in progeny obtained by selfing revertant C^- plants regenerated from Hl-2/Hl-2 cells (Table 7). We speculate that these metastable C^- states might represent intermediate states of partial DNA methylation.

We thank our Ortrun Mittelsten Scheid and Todd Blevins for useful criticism and the Novartis Research Foundation for financial support.

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