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carpel, a New *Arabidopsis* Epi-Mutant of the *SUPERMAN* Gene: Phenotypic Analysis and DNA Methylation Status

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The *carpel* (*car*) mutation affects the morphology of reproductive organs in *Arabidopsis thaliana*. *car* flowers have an increased number of carpels, on average 2.7±0.8 instead of two in the wild type. Through allelism test with *fon1-3* and analysis of the methylation state of the *SUPERMAN* (*SUP*) gene in *car* mutants, we show that *car* is an epi-mutation of *SUP*. The methylation pattern of *car* is clearly distinct from that of *fon1-3*, another epi-mutation of the *SUP* gene. Methylation was found predominantly in Cp(A/T)p(A/G) triplets and in CpG pairs. We suggest that the extensive *SUP* methylation in *car* has arisen from an abundant methylation of a single CpG site that was already present in *abscisic acid-insensitive* (*abi3-4*) mutants, from which *car* was segregating.

Key words: DNA methylation — Epi-mutant — *SUPERMAN*.

Our present understanding of flower formation has been gained through a combination of genetic analyses and the molecular characterization of a number of regulatory genes in the two model species *Arabidopsis* and *Antirrhinum* (Weigel 1995, Yanofsky 1995, Ma 1998). In *Arabidopsis*, the shoot apical meristem changes fate upon floral induction to become the inflorescence meristem. On the flanks of the inflorescence meristem a number of floral meristems arise that develop into flowers with typically four concentric whorls consisting of four sepals, four petals, six stamens, and two fused carpels. The cells in the meristem need to integrate temporal and positional information in order to differentiate appropriately into a floral organ. This information is in part provided by floral meristem identity genes (e.g. *LEAFYI, APETALA1, CAULIFLOWER1*), floral meristem-structuring genes (e.g. *CLAVATA1, CLAVATA3, PERIANTHA*), organ identity genes (*APETALA1, APETALA2, APETALA3, PISTILLATA*, and *AGAMOUS*), and cadastral genes (e.g. *SUPERMAN, LEUNIG*). Floral meristem-structuring genes determine the site and the number of floral organ primordia that will develop in each whorl. Subsequently, this pattern of organ primordia is elaborated by the organ identity and the cadastral genes. The organ identity (or homeotic) genes are expressed each in two neighbouring whorls and, through their interactions, determine ultimately the organ type (ABC model, Meyerowitz et al. 1991). The cadastral genes restrict the expression of the organ identity genes to specific regions of the floral meristem.

Mutations in the cadastral gene *SUPERMAN* (*SUP*) result in the development of extra stamens at the expense of carpels (Schultz et al. 1991, Bowman et al. 1992). In *sup-1* mutants, the number of stamens can amount to 26 with a concomitant reduction of the gynoecium (Bowman et al. 1992), whereas *sup-5* is a weaker allele that produces 12.3±0.3 stamens and 2.9±0.1 carpels in the first ten flowers (Gaiser et al. 1995). These differences illustrate the phenotypic plasticity caused by *sup* alleles. *SUP* encodes a zinc-finger transcription factor that is thought to be a negative regulator of the B function genes *APETALA3* (*AP3*) and *PISTILLATA* (Schultz et al. 1991, Bowman et al. 1992, Sakai et al. 1995). Alternatively, *SUP* may be involved in forming a boundary between the third and the fourth whorl, possibly through the repression of cell division at the boundary (Bowman et al. 1992, Sakai et al. 1995). Both hypotheses are consistent with the observation that in *sup* mutants *AP3* is aberrantly expressed in the fourth whorl (Schultz et al. 1991, Bowman et al. 1992). In addition, *sup* mutants have lost the determinacy of the floral meristem and are defective in the growth of the outer ovule integument (Schultz et al. 1991, Bowman et al. 1992, Gaiser et al. 1995). The latter phenotype is independent of AP3 activity and was, therefore, suggested to be unlinked to the cadastral role of SUP during floral development (Gaiser et al. 1995).

A comparable, but weaker Sup phenotype was observed in *clark kent* (*clk*) mutants (Jacobsen and Meyerowitz 1997). The first ten flowers of *clk-3* mutants have 7.8±0.3 stamens and 3.4±0.1 carpels (Jacobsen and Meyerowitz 1997). *sup* mutants could not be complemented with *clk* mutants indicating allelism. Accordingly, bisulphite sequencing of the *SUP* gene demonstrated that *clk*

Abbreviation: 5mC, 5-methylcytosine.

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mutants represent epi-alleles of *sup*. Extensive hypermethylation of the transcribed region leads to a strong reduction of *SUP* mRNA levels (Jacobsen and Meyerowitz 1997). *clk* mutants could be restored to wild type by transformation with a 6.5-kb *SUP* genomic fragment (Jacobsen and Meyerowitz 1997). Also *floral organ number* (*fon1-3*) mutants have Sup-like phenotypes with more stamens, and as in *clk* mutants, extra stamens are not produced at the expense of carpels. Instead, the number of both stamens (8.6 ± 1.6) and carpels (3.6 ± 0.3) has increased (Huang and Ma 1997). However, the double mutant phenotypes of *fon1-3* with other flower mutants were similar, albeit weaker, to those of *sup* double mutants (Huang and Ma 1997). Although the *fon1-3* mutation was originally mapped as a novel gene near *SUP* on chromosome 3 (Huang and Ma 1997), sequencing of the *SUP* locus in *fon1-3* mutants revealed that they have a methylation pattern similar to that of *clk* mutants (Jacobsen and Meyerowitz 1997). Therefore, *fon1-3* is considered as another epi-allele of *SUP* (Huang and Ma 1998).

The latter mutants clearly illustrate that the epigenetic status can have a great impact on gene expression. Generally, epigenetic changes are considered when modifications in gene expression occur that are caused by heritable, but potentially reversible changes in chromatin structure and/or DNA methylation (Henikoff and Matzke 1997). In plants, phenomena such as paramutation, transposition, imprinting in the endosperm, (trans)gene silencing or cosuppression are correlated with epigenetic changes such as methylation (Richards 1997, Finnegan et al. 1998, Paszkowski and Mittelsten Scheid 1998). Methylation plays also a crucial role in genome defence against invading DNA (Martienssen 1998, Matzke and Matzke 1998). A role for methylation in developmentally regulated gene expression was first demonstrated in studies on vernalization. Chemical demethylation could functionally replace the requirement of low temperatures for the promotion of flowering, thus supporting an involvement of demethylation in vernalization (Burn et al. 1993).

Here, we describe the isolation of a new mutation, *carpel* (*car*) that affects the morphology of the reproductive organs similar to *fon1-3* and *clk*. By analyzing the methylation state of the *SUP* gene we show that *car* is another epi-mutation of *SUP*. The methylation pattern of *car* is clearly distinct from that of *fon1-3*, as is the strength of the phenotype. We propose that the extensive *SUP* methylation in *car* has arisen from a methylation in a CpG dinucleotide that was already present in *abscisic acid-insensitive* (*abi3-4*) mutants, from which *car* was segregating.

Materials and Methods

Plant material, growth conditions—The *car*, *fon1-3*, and *abi3-4* mutants are in *Arabidopsis thaliana* ecotype Landsberg *erecta* (L*er*). After harvesting, seeds were treated for 2 weeks at 28°C, and stored at 4°C until use (except for green, desiccation-intolerant, homozygous *abi3* seeds). For germination, seeds were surface sterilized and placed on a MS medium supplemented with 10 g liter^{-1} sucrose. After a cold treatment for homogenous germination (overnight for fully stratified and 4 d for freshly harvested seeds), seeds were exposed to 20°C, 50 μmol m^{-2} s^{-1} light intensity, 70% relative humidity, under a 16-h light/8-h dark cycle. The greenhouse conditions for plant growth and crosses were as follows: 40 μmol m^2 s light intensity at plant level (MBFR/U 400 W incandescent lamps; Philips, Eindhoven, The Netherlands), a 16-h light/8-h dark cycle, 40% relative humidity, 23°C, without shielding from incident day light. The morphology of flowers and siliques was examined under a binocular.

DNA preparation and bisulfite conversion—Rosette leaves were harvested from 3-week-old *Arabidopsis* plants growing in the greenhouse. DNA was isolated according to Dellaporta et al. (1983) and 1 μg of DNA was digested with *Hpa*II at 37°C for 1 h. Bisulfite genomic sequencing was carried out as described by Frommer et al. (1992). Briefly, the digested DNA was denatured in 111 μl 0.3 M NaOH (in the presence of 10 μg yeast mRNA) at 42°C for 20 min. For deamination of unmethylated cytosines to uracil, 1,200 μl of freshly prepared sodium bisulfite solution [541 g liter^{-1} (ACS grade reagent; Sigma, St. Louis, MO, U.S.A.) in 10 mM hydroquinone (Sigma), pH 5] were added directly to the denatured DNA. The reaction was overlaid with 200 μl mineral oil (IR grade; Sigma) and incubated at 55°C for 4 h in the dark. The DNA was desalted using the Wizard DNA purification kit (Promega, Madison, WI, U.S.A.) and eluted in 105 μl 1 mM Tris-HCl pH 8.5. Of the DNA solution, 100 μl were desulfonated in 0.3 M NaOH at 37°C for 30 min. For neutralization, ammonium acetate was added to a final concentration of 3 M and the DNA was precipitated with ethanol using 1 μg yeast mRNA as carrier. The DNA was dissolved in 100 μl 10 mM Tris-HCl (pH 8.5) and stored at −20°C until further use.

PCR amplification and sequencing of SUPERMAN—The bisulfite-treated DNA (2.5 μl) was used for PCR amplification of two overlapping *SUP* fragments of the top strand of *SUP*. Throughout the paper, the positions 1 to 1,053 refer to the *SUP* Genbank entry (Accession no. U38946), whereas −59 to −1 refer to sequences upstream of the Genbank entry, as published by Jacobsen and Meyerowitz (1997). The amplification was carried out in two consecutive PCR reactions using a set of five nested primers. Primers were designed following the guidelines of Clark and Frommer (1997). In the first PCR, the primers 5′-AGTTTTAATATTAAAGAGTTTTAA-3′ and 5′-AAAAACAAAATTATATCCATTATCA-3′ were used to amplify a *SUP* fragment spanning the gene from positions −59 to 1,053. In the second PCR, two overlapping fragments were generated: a *SUP* fragment spanning the gene from positions 30 to 692 with the primers 5′-AGATATAGATATATTTTAGATTT-3′ and 5′-ACCTTATAACCTCTCCAACCTCCAC-3′ and a *SUP* fragment spanning the gene from positions 638 to 1,053 with the primers 5′-GTTTGTAAGATTAAGAAATTATTTT-3′ and 5′-AAAAACAAAATTATATCCATTATCA-3′. For PCR reactions, 50 pmol of each primer, 2.5 U Taq-polymerase (Qiagen, Chatsworth, CA, U.S.A.), the standard buffer supplied with the enzyme and 250 μM dNTPs were used in a total volume of 50 μl. Incubation times and temperatures were 94°C for 2 min followed by five cycles (at 94°C for 1 min, at 45°C for 2 min, at 72°C for 3 min), 25 cycles (at 94°C for 0.5 min, at 50°C for 2 min, at 72°C for 1.5 min) and at 72°C for 10 min. Of the first PCR mixture, 2.5 μl were used as template for the second PCR with the following incubation times and

temperatures: 94°C for 2 min, five cycles (at 94°C for 1 min, at 48°C for 2 min, at 72°C for 3 min), 25 cycles (at 94°C for 0.5 min, at 50°C for 2 min, at 72°C for 1.5 min), and 72°C for 10 min. The PCR products were separated in a 1% agarose gel and purified using the QIAquick gel extraction kit (Qiagen). To ensure maximum cloning efficiency, an aliquot of the PCR product was incubated with 2.5 U Taq polymerase (Qiagen) and 200 μM dATP in PCR buffer for 10 min at 72°C and subsequently ligated into the pGEM-T-easy vector (Promega). Sequencing of cloned PCR products was performed using the ABI Prism BigDye Terminator cycle sequencing kit (Perkin Elmer, Norwalk, CT, U.S.A.). The recognition rates of this method have been previously determined to be 99.48±0.39% for unmethylated cytosines and 100.00± 0.00% for 5-methylcytosines (Grunau et al. 1999). The total error rate of the PCR and the sequencing reaction was 0.23% in non-cytosine sites.

Analysis of DNA methylation—The sequences of the PCR products were assembled and aligned to the genomic sequence using the GAP4 computer package (Dear and Staden 1991). Cytosines that were converted to uracils and subsequently amplified as thymines in the PCR products corresponded to unmethylated cytosines whereas the remaining cytosines indicated the occurrence of 5-methylcytosines (5mC). Bisulfite-treated DNA strands are not complementary after the conversion reaction and primers were designed to amplify the top strand only. Thus, the obtained methylation patterns correspond to those of individual single-strand DNA molecules. The alignments were used (i) to calculate the average methylation level for each cytosine position of the top strand, (ii) to visualize the sequences in a context-sensitive manner, and (iii) to determine the methylation density for each clone. The latter was calculated as the portion of methylated cytosines to total cytosines in a window of 100 bp shifted in 1-bp steps over the sequence.

In order to identify conserved sequence patterns around methylated cytosines, the frequencies of each nucleotide for positions up to 300 bp up-stream and down-stream of these sites were determined. The information content R of each position (Schneider et al. 1986) was calculated using:

$$R_{(pos.)} = H_{(max.)} - H_{(pos.)} - e_{(pos.)}$$

with e being a correction factor, $H_{(max.)}$ the maximum uncertainty 2.32 (for equal frequency of each base) and H the uncertainty at each position given as:

$$H_{(pos.)} = \sum_{base=A}^{5mC} f(base, pos.) \cdot log_2 f(base, pos.)$$

where f is the base frequency at a position (*pos.*) and *base* corresponds to one of the five nucleotides including 5mC. Because the number of nucleotides (n) per position was usually greater than 50, the correction factor $e_{(pos.)}$ could be approximated with a simplified formula (Schneider et al. 1986, Schneider and Stephens 1990):

$$e_{(pos.)} = \frac{2}{log_2(n)}$$

Despite the fact that the number of analyzed nucleotides per investigated position exceeded usually 50, we observed higher fluctuation in certainty values when the number of nucleotides dropped below 100 (data not shown).

G+C and C densities were calculated using the program WINDOW of the GCG package (Genetics Computer Group, Madison, WI, U.S.A.). The chosen window size was 100 bp and step size 3 bp.

Results

Phenotypic characterization of car—carpel (*car*) plants were first observed as segregants from a homozygous *abi3-4* mutant population and are described as *car/abi3-4* mutants. We will refer to these as *car* mutants throughout the text, as backcrossed single *car* mutants have similar phenotypes. In a number of flowers, *car* plants had more than two carpels, more than six stamens, and chimeric organs that represented fusions of stamen and carpel (Fig. 1A–C). The subsequent development of such flowers was hampered to varying degrees. Part of the carpels appeared curled, or the carpels did not fuse and, as a consequence, no seeds developed (Fig. 1C, D). Other flowers developed a style consisting of three to four carpels giving rise to siliques with mature seeds (Fig. 1A, E). Such siliques had a replum fused to the three placental arrays or a double replum, respectively (Fig. 1G). Additional carpels could also be formed after the "normal" carpel primordia were initiated leading to siliques with partial carpels and a disturbed replum (Fig. 1F).

This phenotype established in a varying percentage of the flowers distributed over the flowering stalk, but was more frequent in the first flowers. The first ten flowers of *car* plants were analyzed in detail to compare them to the phenotypically similar *fon1-3, sup*, and *clk* mutants (Table 1). On average, *car* plants had 6.1±1.0 stamens and 2.7± 0.8 carpels and a number of filamentous organs or chimeric carpel/stamen organs (Table 1). These numbers were clearly lower than for *sup, fon*, and *clk* mutants, particularly with respect to the almost unaffected number of stamens in *car* mutants. Seeds derived from siliques with either two, three, or four carpels had no dramatic effect on the flower phenotype of the resulting plants (Table 1). Additionally, floral development was sometimes arrested after floral meristem formation, but prior to flowering around stage 11 of flower development (according to Smyth et al. 1990, Fig. 1D). Such undeveloped flowers appeared frequently in groups along the shoot; the internodes between these "arrested" flowers were 2–4 mm significantly shorter than the average 1 cm internodes of wild type (Fig. 1D).

The extent to which the phenotypic changes were established varied largely in different experiments. The results of phenotypic penetrance, as expressed as the total percentage of aberrant flowers and siliques, are shown for five generations of self-fertilized *car* mutants in Table 2. Depending on the experiment, between 13% and 62% of all siliques were malformed (Table 2). Also within one experiment, the individual plants varied often tremendously in phenotypic strength. The cause for this variation is yet unknown. The origin of seeds had no consistent influence on the extent of the phenotypic penetrance (data not shown).

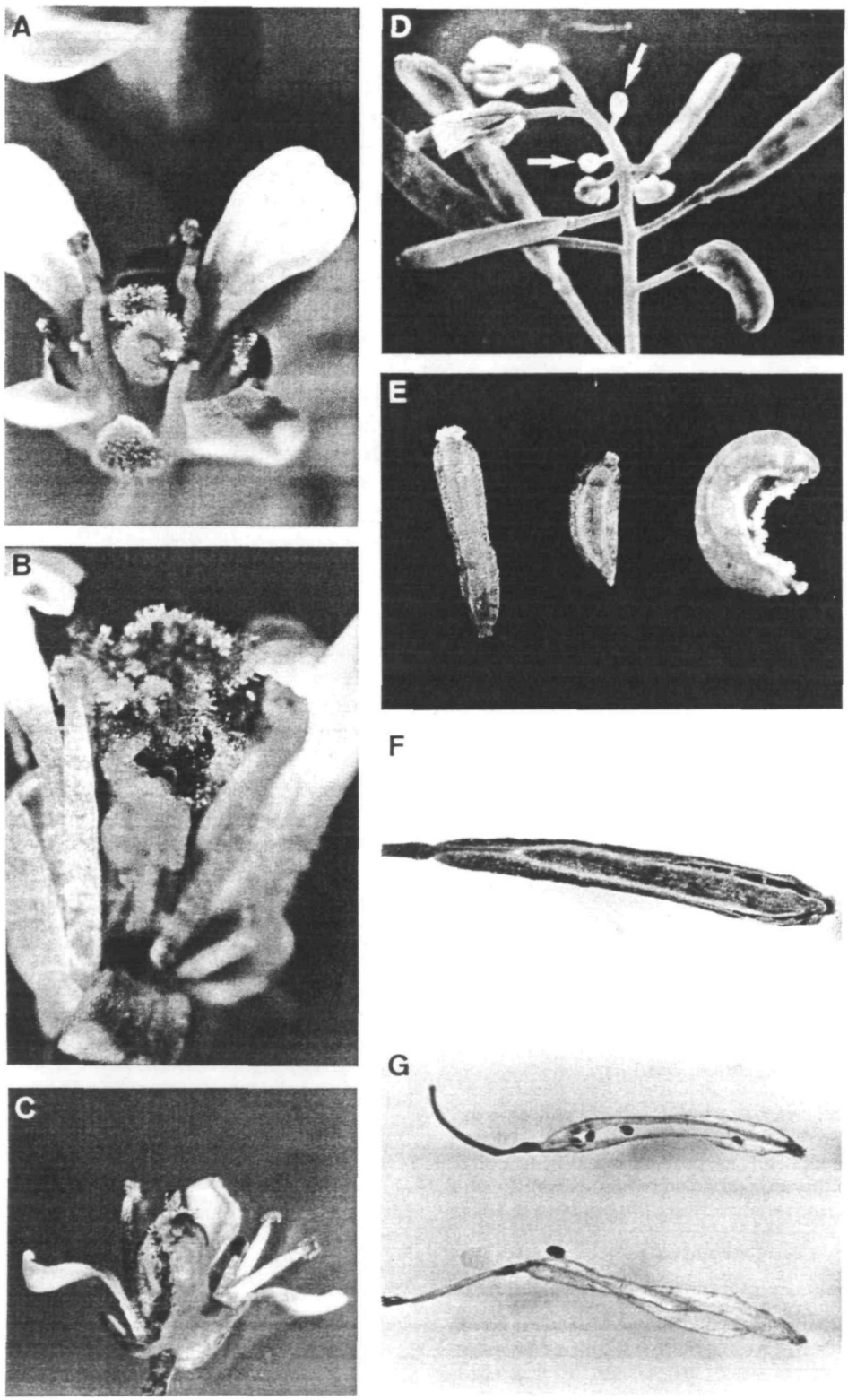

Fig. 1 Phenotypes of the *car* mutant. (A) Flower with a double style and stigma. (B) Flower with carpel/stamen chimeric organs. (C) Flower with unfused carpels. (D) Inflorescence with undeveloped flowers (arrows). (E) Abnormal siliques composed of three carpels, four carpels, and of more than four unfused carpels (from left to right). (F) Abnormal silique with an incomplete additional carpel. (G) Empty siliques having a double or disturbed replum.

Table 1 Number of reproductive organs in the first ten flowers of *car* mutants

Group	Plants	Flowers	Anthers	Filamentous organs[a]	Chimeric organs[b]	Carpels	Undeveloped flowers
2C	33	298	6.1±1.0	0.1±0.3	0.2±0.6	2.7±0.8	14 (4.5%)
3C	24	224	5.9±0.5	0.1±0.2	0.1±0.3	2.4±0.7	10 (4.3%)
4C	37	333	6.3±1.2	0.1±0.7	0.3±0.7	2.9±0.9	12 (3.5%)
Total	94	855	6.1±1.0	0.1±0.5	0.2±0.6	2.7±0.8	36 (4.0%)

[a] That replace stamens.
[b] Such as stamens that grow in association with carpels.
From a total of 94 *car* plants, the first ten flowers were examined under a binocular. The number of anthers, staminodia, carpel/stamen chimeras and carpels is given as average with standard deviation. Undeveloped (arrested) flowers were recorded separately and are not included in the number of total flowers. The percentage of undeveloped flowers was calculated on the sum of flowers and undeveloped flowers. The scored *car* plants were derived from seeds originating in siliques with either two, three, or four carpels (indicated by 2C, 3C, 4C, respectively). In wild-type plants, neither filamentous and chimeric organs nor additional carpels were observed.

Allelism with fon1-3 and inheritance of car—The increase in carpel number is not exclusive to the *car* mutant, but has been observed in *sup-5, fon1-3*, and *clk*, which all represent mutant alleles of the *SUP* gene (Gaiser et al. 1995, Huang and Ma 1997, Jacobsen and Meyerowitz 1997). As investigated by PCR, the *SUP*-coding region was present in *car* mutants (data not shown). To compare the phenotypic differences of *car* with *fon1-3*, we studied the distribution of aberrant siliques in *fon1-3* plants, for which only data on the first ten flowers were available (Huang and Ma 1997, Table 2). The *fon1-3* carpel phenotype was very pronounced in the first flowers and severe with respect to the increase in organ number. Later in development, however, additional organs were formed only in a portion of the flowers. On the whole plant, a comparable number of siliques was affected in *fon1-3* and *car* (Table 2). However, undeveloped flower buds were never observed in *fon1-3*.

In F_1 complementation tests, *car* failed to fully complement *fon1-3*. Among 130 F_1 plants, 92 plants (or 71%) showed the typical increase in carpel number. F_1 plants of back-crosses of *car* with wild type have no phenotype (see below). Therefore, this result suggests allelism of *fon1-3* and *car*. The lack of full allelism might arise from (i) the weak semi-dominance of *fon1-3* (Huang and Ma 1998), and (ii) the unstability of epigenetic mutants.

During maintenance of the *car* (i.e. *abi3-4/car*) mutants, variable and sometimes unstable phenotypic effects were observed, although the mutation was generally heritable. *car/abi3-4* mutants were also backcrossed with Landsberg *erecta*. No phenotype was observed in the F_1 genera-

Table 2 Silique phenotypes in several generations of *car* mutants

Generations	Plants scored	Average number of siliques					
		Total	2 carpels	3 carpels	4 carpels	Unfused carpels	Total aberrant siliques
M1	44	49.4±30.9	40.2±27.5 81.3%[a]	7.1± 8.0 14.4%	2.1± 3.8 4.3%	N.D.[b]	9.2±10.8 18.7%
M2	134	75.8±43.5	62.3±39.8 82.1%	6.5± 7.4 8.6%	1.4± 2.6 1.9%	5.6± 9.8 7.3%	13.8±15.6 17.9%
M3	45	96.9±58.7	84.6±51.2 87.2%	8.0±11.8 8.3%	4.3± 6.5 4.4%	N.D.	12.4±16.7 12.8%
M6	35	207.7±72.7	129.7±70.7 62.4%	34.1±26.8 16.4%	20.3±20.2 9.8%	23.6±33.1 11.4%	78.0±72.3 37.6%
M7	125	53.3±16.7	20.6±13.9 38.6%	11.9± 6.9 22.3%	11.1± 7.2 20.9%	9.8± 8.6 18.3%	32.8±16.4 61.4%
fon1-3	10	104.0±32.2	60.7±14.7 58.4%	27.5±11.5 26.4%	15.8± 9.9 15.2%	N.O.	43.3±20.2 41.6%

[a] Percent of total siliques analyzed.
[b] N.D., not determined; N.O., never observed.
The average number of siliques per plant is given with standard deviation. The percentages are calculated on the total sum of siliques in the experiment. M1 through M7 indicate subsequent generations of self-fertilized *car* mutants.

tion of three independent backcrosses (n = 124) suggesting that *car* was a recessive mutation. Among 338 F_2 plants, excluding the discarded *abi3-4* homozygous progeny, *car* single mutants appeared at a frequency of 6–8% in two

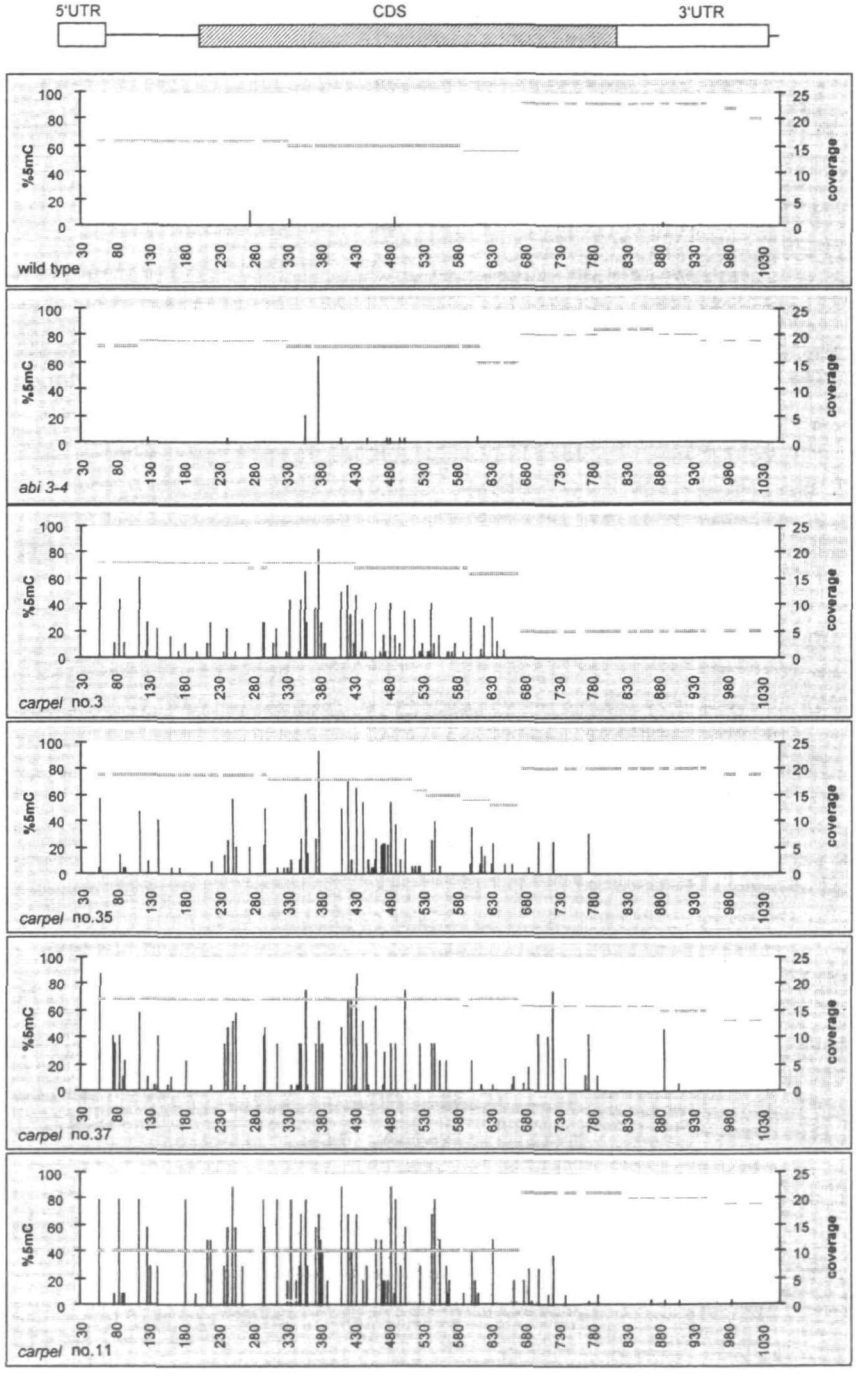

Fig. 2 Methylation profiles of the top strand for the *SUP*-transcribed region in wild type, *abi3-4* mutants, and four individual *car* plants. On top, the *SUP* gene is schematically represented corresponding to the positions on the abscissa in the panels below. On the abscissa the genomic sequence of *SUP* is given in base pairs, corresponding to the *SUP* sequence (GenBank U38946; Sakai et al. 1995). The vertical bars represent the percentage of methylation at individual cytosine positions of the top strand and correspond to the left ordinate. If each PCR clone had a cytosine at a given position, the methylation level would be 100%; if no PCR clone had a cytosine, the average methylation would be 0%. The horizontal lines indicate the total number of clones that were analyzed for methylation at individual cytosine positions and correspond to the right ordinate.

independent backcrosses. This number suggests that the *car* mutation was linked with the *ABI3* locus that in turn is 3.4 cM distant to the *SUP* locus. The deviation from the expected single mutant frequency has probably to be attributed to the small number of progeny and to variabilities in phenotypic penetrance and expressivity.

Testing for the presence of *car* in the seed stock, from which the *abi3-4* homozygotes were originally derived, revealed that the mutation was also present both in segregating *abi3-4* heterozygous and wild-type seeds. Among 68 germinating seeds, two wild types and six *abi3-4* heterozygotes had a *car* phenotype, corresponding to 11.8%.

Because of the phenotypic similarity with *fon1-3* and *clk*, the variability of the *car* phenotype and the partial allelism of *fon1-3* with *car*, we decided to study whether *car* was another epi-allele of *SUP* using molecular tools.

Epigenetic status of SUP in abi3-4, fon1-3, and car— Bisulfite genomic sequencing of the entire transcribed region of the *SUP* gene revealed that the wild-type gene is practically unmethylated in the leaves of young *Arabidopsis* seedlings. In *abi3-4* mutants, almost no difference to the unmethylated wild type was found, except for an abundant CpG methylation at position 375, the only CpGpG of the investigated region (12 5mC in 18 individual clones, Fig. 2).

In contrast, *SUP* was highly methylated in the young

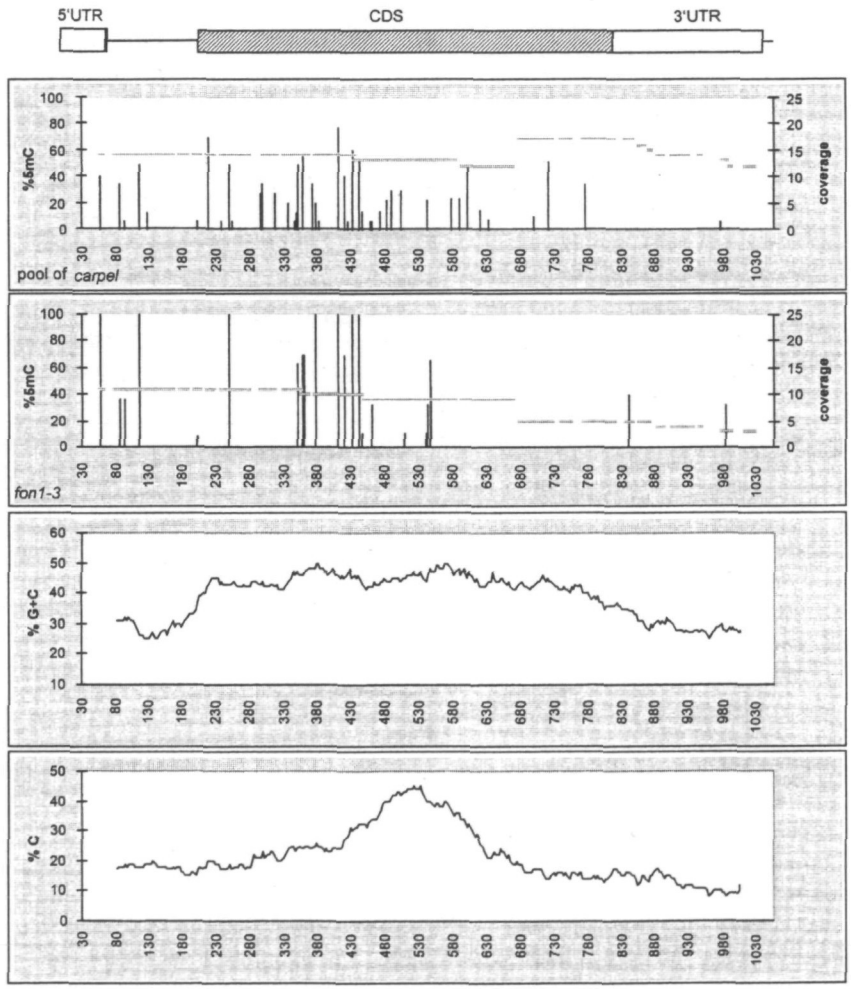

Fig. 3 Methylation profiles of the top strand for the *SUP*-transcribed region in a pool of *car* plants and *fon1-3* mutants. On top, the *SUP* gene is schematically represented corresponding to the positions on the abscissa in the panels below. On the abscissa the genomic sequence of *SUP* is given in base pairs, corresponding to the *SUP* sequence (GenBank U38946; Sakai et al. 1995). The vertical bars represent the percentage of methylation at individual cytosine positions and correspond to the left ordinate. The horizontal lines indicate the total number of clones that were analyzed for methylation at individual cytosine positions of the top strand and correspond to the right ordinate. The methylation level would be 100% or 0%, if each or no PCR clone had a cytosine at a given position, respectively. In the lowest panels, the G+C content and the C content of the *SUP* gene are plotted, showing that the distribution of G and C residues cannot be the cause for the higher density of methylated cytosines (5mC) in the 5′ part as compared to the 3′ part.

leaves of four individual *car* mutants (Fig. 2). Interestingly, methylation was most pronounced in the vicinity (about 100 bp upstream and 250 bp down-stream) of the CpG at position 375 (Fig. 2). The 5′ transcribed region of the gene was found to be variably, but strongly, methylated, whereas little methylation was found down-stream of position 730 (Fig. 2). The average methylation density in the four plants analyzed never exceeded 60% and was usually below 50% (density plots at http://genome.imb-jena.de/PublicationSupplements). The sequences of individual clones showed heterogeneous methylation, an aspect that is usually referred to as a mosaic-like methylation pattern and indicates different methylation patterns for individual cells. Altogether, only one out of 78 clones of the *car* mutant was completely unmethylated upstream of position 667. The four investigated individual *car* plants showed slightly different methylation profiles (Fig. 2). However, these small differences were not correlated with the phenotypic strength: the individuals #11 and #35 had more severe flower phenotypes than the individuals #3 and #37 (data not shown). The average methylation pattern of a pool of randomly harvested *car* plants was similar to that of the individual plants (Fig. 2, 3).

The methylation profile of the *SUP* gene in *fon1-3* was similar to the *car* epigenotypes with respect to the observation that the CpG at position 375 was methylated in all clones and that the immediate vicinity of this site showed a high degree of methylation (Fig. 3). Likewise, the 5′ part of the sequence was highly methylated and only weak methylation was found in the 3′ part of the gene, although the G+C content was not correspondingly lower down-stream of position 730 (Fig. 3). In contrast to the *car* mutants, the methylation in *fon1-3* mutants was generally less dense (Fig. 3). However, some cytosines were methylated in all clones analyzed (Fig. 3). In addition, solitary cytosines (e.g. at position 440, 838, and 979) were highly methylated (100%, 40%, and 33%, respectively) in *fon1-3,* whereas few or no methylation was found at these positions in *car* (Fig. 3).

The calculation and visualization of the information content has been suggested as a useful and improved tool to disclose conserved sequence patterns (Schneider et al. 1986, Schneider and Stephens 1990). Here, we used the method for the first time to determine the consensus for methylation (Fig. 4). The search for regular over- or under-representation of nucleotides 300 bp up- and down-stream of methylated cytosines in *car* mutants revealed no characteristic distribution patterns except a bias towards T and A at position +1 and towards A and G at position +2 (Fig. 4). An under-representation of C was observed in position −8 and +8, but it remains to be elucidated whether this finding is of biological significance (Fig. 4). Similar results were found for *fon1-3*, except for a more pronounced bias towards G at position +2 (Fig. 4).

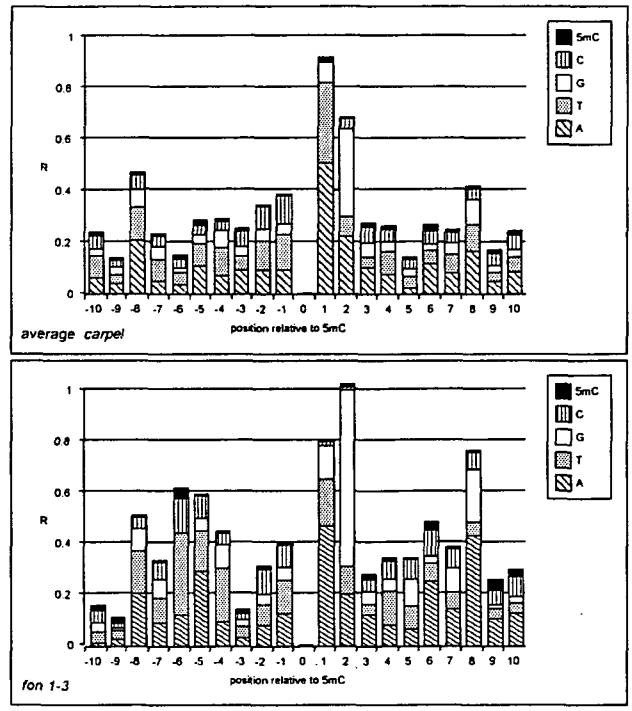

Fig. 4 Occurrence of nucleotides relative to the position of methylated cytosines (5mC) in the *SUP* gene, in *car*, and *fon1-3* mutants. Sequences of 600 bp (300 bp up- and down-stream of an individual methylated C) were analyzed for the information content as well as for the occurrence of one out of the five possible nucleotides (C, A, T, G, 5mC) at positions immediately up- and down-stream of the 5mC. The information content at a given position augments with the increasing difference from the expected equal distribution of all nucleotides at a given position relative to the 5mC. The ordinate represents the amount of information R in bits, the abscissa the relative position to the 5mC at position 0 in bp. The length of each bar represents the frequency of the respective base multiplied with the total information at this position (for details see Materials and Methods). The information content R would be 0, if all bases were equally distributed at a given position. The information content R would be 2.32, if only one base always occurs at a given position. The standard errors were between 0.001 and 0.158 with an average of 0.0184. Errors were omitted from the figure for sake of clarity.

CpGpN and, to a lesser degree, CpNpG were under-represented in the *SUP* sequence (Table 3). By consequence, their share in information content of position +1 was low compared with CpA and CpT (Fig. 4). The low number CpGpN makes it impossible to attribute a preferential methylation for CpGpN other than CpG.

Interestingly, all analyzed epi-mutants (*car, fon1-3*) and *abi3-4* mutants (95 of 96 sequenced PCR clones covering this site) showed a G-to-A exchange at position 668 (position 466 from the start codon) presumably leading to a methionine instead of the wild-type valine. All 23 wild-type clones have a G at this position, identical to the

Table 3 Degree of cytosine methylation of the *SUP* gene in different nucleotide contexts (given in %)

Nucleotide context	Potential sites	Wild type	*abi3-4*	*car*	*fon1-3*
5mC	207	0.17	0.65	9.18± 2.36	7.21
5mCG	12	0.00	5.17	13.51± 3.99	28.36
5mCA	75	0.00	0.22	13.89± 3.31	10.79
5mCT	75	0.23	0.57	8.89± 3.07	4.59
5mCC	39[a]	0.32	0.28	0.44± 0.91	0.91
5mCAG	16	0.00	0.34	37.66± 4.27	39.19
5mCAA	23	0.00	0.47	16.69± 7.49	6.70
5mCAT	22	0.00	0.00	1.38± 0.74	1.02
5mCAC	14	0.00	0.00	1.39± 1.37	0.00
5mCTG	5	0.00	4.04	36.01± 7.52	47.22
5mCTA	19	0.58	0.56	15.01± 5.62	5.33
5mCTT	30	0.00	0.18	2.91± 1.64	0.40
5mCTC	20[b]	0.31	0.28	5.32± 2.59	0.66
5mCGG	1	0.00	66.67	56.07±21.89	90.91
5mCGA	2	0.00	0.00	1.25± 2.50	0.00
5mCGT	6	0.00	0.00	13.82± 2.23	29.41
5mCGC	3	0.00	0.00	1.54± 3.08	0.00

[a] Including six CCC sites.
[b] Including one CTCTC site.
The total number of cytosines in a respective nucleotide context is given for the *SUP* gene. The degree of cytosine methylation is calculated as the percentage of methylated cytosines (5mC) among all cytosines in the respective nucleotide context. For the recognition and error rates of nucleotides, see Materials and Methods. The number of individually analyzed PCR fragments corresponds to the values given in Figures 2 and 3. For wild type, *abi3-4* and *fon1-3*, sequences derived from DNA of pooled plants were analyzed. The analysis for *car* included DNA from four individually harvested plants and a pool of plants. Therefore, the values for the *car* mutant are the average with standard deviation of the data shown in Figures 2 and 3.

database entry (U38946; Sakai et al. 1995). This single base exchange, although not explicitly mentioned by Jacobsen and Meyerowitz (1997), was also present in the *clk* epi-mutants. A single nucleotide polymorphism is improbable, because wild-type and mutant plants belong to the same ecotype and, moreover, the substitution occurred similarly in different mutants. Because flower formation is not affected in the *abi3-4* plants, this base substitution did not account for the Sup phenotype. The importance of this substitution that occurred independently at precisely the same base in *clk* and *car* mutants remains to be investigated. It is possible that this substitution is involved in mediating subsequent methylation of *SUP*.

Discussion

The *car* mutation primarily leads to an increased number of carpels, 2.7±0.8 in the first ten flowers. It has only minor effects on the number of stamens and stamen/carpel chimeras (Fig. 1, Table 1). Similar phenotypes have been described for *sup* mutations and epimutations of *SUP*. However, *sup*, *clk*, and *fon1-3* mutations lead to alterations in both carpel and stamen number (Schultz et al. 1991, Bowman et al. 1992, Huang and Ma 1997, Jacobsen and Meyerowitz 1997). Compared to these mutants that affect the correct elaboration of the third and the fourth whorl of the flower, *car* is a relatively weak mutant with a predominant effect on the fourth whorl. Notwithstanding, the *car* mutant is a new epi-allele of *SUP*, because of allelism with *fon1-3* and methylation of the *SUP* locus (Fig. 2, 3, Table 3). The epigenetic nature of *car* could furthermore be the reason for the phenotypic unstability observed in the experiments and crosses (Table 1, 2). Also, in an individual plant, *car* and *fon1-3* epi-mutant effects can become restored in some, but not all, flowers (Table 2).

Interestingly, the degree of *SUP* methylation does not correspond to the strength of mutant phenotype. Generally, the effects of *fon1-3* on carpel development are more severe as compared to *car*, however, less cytosines are methylated in *fon1-3* than in *car* (Fig. 3, Table 3). Whereas a given C residue was found to be never or only moderately methylated in all analyzed clones of *car*, several C residues were always methylated in *fon1-3* mutants (Fig. 3). This

could reflect a more stochastic methylation state of *SUP* in individual cells of *car* mutants that consequently could lead to a weaker phenotype. Nevertheless, the methylation in the 5′ region of the gene (positions 180 to 580) is nearly identical in *car* and *fon1-3* mutants (Fig. 3). Methylation of the coding region results in the expression of truncated mRNA fragments in fungi or gene silencing in plants (Barry et al. 1993, Finnegan et al. 1993, Bender and Fink 1995, Rountree and Selker 1997). Silencing was found to be stronger when the methylation was preferentially located near the 5′ end of the coding region (Hohn et al. 1996). Whereas our results confirm these earlier observations, they do not exclude that important methylations occur in the *SUP* promoter region. Because the methylation in the promoter region is crucial for transcriptional silencing of not only transgenes but also endogenous genes in plants (Oakeley and Jost 1996, Diéguez et al. 1998, Jeddeloh et al. 1998), this aspect of *SUP* methylation will need further investigation in the future.

Besides methylation at CpG and CpNpG, *car* has a relatively strong methylation at CpApA and CpTpA (Table 3). The methylation at these triplets is 2- to 3-fold higher than in *fon1-3* and reaches the absolute values for the CpNpG counterparts. Methylated cytosines in combinations of CpG and CpNpG have been shown to be abundant in plant DNA (Naveh-Many and Cedar 1982, Kovařík et al. 1997), but Cp(A/T)pA methylation has usually been overlooked because no methylation-sensitive enzyme is available for this site.

Strikingly, a hot spot for methylation was found at position 375, where the sole methylated CpGpG of the *SUP* sequence is located (Table 3). Whereas the C at position 375 is already methylated in 67% of the sequenced clones in *abi3-4* mutants, it did not yet lead to a phenotype (Fig. 2). In both *car* and *fon1-3*, additional C residues are methylated and phenotypic alterations are observed (Fig. 2, 3). Whether the C residue at position 375 is of particular importance for the regulation of the gene or whether this position is simply more accessible to the DNA methyltransferase or guiding components than other regions cannot be clarified here. The search for transcription factor-binding sites or other regulatory elements using MatInspector and BIMAS provided no conclusive results (Prestridge 1991, Quandt et al. 1995). Methylation at this position could, however, be the starting point for methylation spreading, a phenomenon that has been better studied in mammalian systems. In vitro studies with murine DNA methyltransferase revealed that de novo methylation was higher in oligonucleotides that contain adjacent 5mC than in non-methylated controls (Tollefsbol and Hutchison 1997). Indeed, spreading of methylation has been described in the case of a reporter gene construct that had been methylated in vitro and re-analyzed after transfection and propagation in mammalian cell lines (Toth et al. 1989). Similarly, spreading of methylation was found after the integration of adenovirus DNA into the DNA of hamster cell lines (Toth et al. 1990, Orend et al. 1991, reviewed by Doerfler 1990). Our data suggest that a similar methylation spreading from a particularly methylated C to the adjacent DNA sequences is active in *Arabidopsis*.

Another distinct feature of the investigated *SUP* region is the 3-fold underrepresentation of CpGs as compared to GpCs (12 versus 39). The global underrepresentation of CpG pairs has been observed in many species and it was suggested to be due to spontaneous deamination of 5mC to thymine (Duncan and Miller 1980, Mazin 1992). The corresponding overrepresentation of TpG and CpAs is observed in *SUP*: 50 TpG vs. 33 GpT and 150 CpA vs. 49 ApC. Besides, in the case of *SUP* methylation in *car* mutants, the relative share of methylated CpApG (37.7%) and CpTpG (36.0%) is higher than methylated CpG (13.5%) (Table 3). Neither CpApG (16) nor CpTpG (5) are underrepresented in the *SUP* sequence compared to GpApC (19) or GpTpC (2), respectively. Under the chosen conditions, the wild-type *SUP* gene is practically unmethylated. If the hypothesis holds that underrepresentation of CpG is a result of methylation and provided that the deamination rate of methylated cytosines in CpG and Cp(A/T)p(A/G) contexts is identical, *SUP* must have been densely and exclusively methylated in CpG pairs earlier at some point of the phylogenetic development. The disequilibrium between CpG and GpC can, however, have origins other than methylation. CpG frequencies have been suggested to be kept low, because they are important components of regulatory functions other than methylation (Karlin and Burge 1995). Comprehensive investigations of relative dinucleotide abundance in different genomes (including those free of 5mC) showed that dinucleotide properties such as stacking energies, charge interaction, conformational tendencies, amino acid composition, codon choices, and perhaps stability of the DNA helix can underlie the suppression of CpG (Boudraa and Perrin 1987, Karlin and Burge 1995). If the under-representation of CpG is, however, indeed due to methylation, different mechanisms for the CpG and Cp(A/T)p(A/G) methylation have to be assumed because methylation in CpGs would have preceded the methylation in non-CpGs. Accordingly, two different enzymes that catalyze either the methylation of CpG or Cp(A/T)pG have been biochemically characterized in pea and tobacco (Kovařík et al. 1994, Pradhan and Adams 1995). In *Arabidopsis*, two classes of DNA methyltransferases have been identified (Finnegan and Dennis 1993).

Altogether, we suggest that transient methylation as a direct or indirect consequence of the exposure to mutagens could be different from the hypothetical methylation that led to the loss of CpG pairs. *car* was isolated as an epi-mutation in the ethyl methanesulfonate-generated *abi3-4*

mutant (Ooms et al. 1993). Similarly, several *clk* mutants have been isolated after exposure to ethyl methanesulfonate, diepoxybutane, fast neutron, X rays, or insertional mutagenesis (Jacobsen and Meyerowitz 1997). The methylation profiles in *fon1-3, car*, and *clk* as well as the preferentially methylated sites in *fon1-3* and *car* differ from each other and give rise to slightly different phenotypes. Even between different *car* individuals, the methylation profiles vary. We suggest that exposure to mutagens can result in a globally impaired methylation status of the genome. The loss of functional methylation may be visible at discrete loci where the corresponding epi-mutants display a particular phenotype. Of these, the phenotype of *clk, fon1-3*, or *car* mutants can be easily recognized. Surprisingly, in all epi-mutants at the *SUP* locus known to date, a G-to-A transition is observed. It will be most interesting to clarify whether this point mutation is a cause or a consequence of the methylation status of the *SUP* gene. Further research also needs to firmly establish the nature and causes of the described epigenetic changes.

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