Plant Physiol. (1991) 95, 174–178
0032-0889/91/95/0174/05/$01.00/0

Received for publication June 29, 1990
Accepted September 10, 1990

Specific Levels of DNA Methylation in Various Tissues, Cell Lines, and Cell Types of *Daucus carota*[1]

Gorm Palmgren*, Ole Mattsson, and Finn T. Okkels

Institute of Plant Physiology, University of Copenhagen, 2A Øster Farimagsgade, DK-1353 Copenhagen K, Denmark (G.P., O.M.); and Biotechnology Research Division, Danisco A/S, 1 Langebrogade, P.O. Box 17, DK-1001 Copenhagen K, Denmark (F.T.O.)

ABSTRACT

The level of DNA methylation in *Daucus carota* was found to be tissue specific, but no simple correlation between developmental stage or age of tissue and the level of DNA methylation was found. Among three different suspension culture lines from the same variety grown under identical conditions, large differences in the level of DNA methylation were observed. The highest and lowest levels were found in two embryogenic cell lines originating from the same clone. Suspension cells from one of the embryogenic cell lines were fractionated into three morphologically defined cell types using Percoll gradient density centrifugation, and the uniformity of these fractions was evaluated by image analysis. The three cell types showed different levels of DNA methylation. The lowest level was found in the fraction containing the precursor cells of somatic embryos.

mdC[2] is present in DNA of all higher organisms investigated (5, 15). DNA methylation is involved in regulation of gene activity and differentiation, and normally methylation of a gene inactivates its transcription (4, 6). In plants, it has been shown that in tissues, where a gene is expressed, it is often hypomethylated in comparison to tissues, where it is not expressed (1, 2, 16).

Large variations in the content of mdC, *i.e.* the level of DNA methylation, have been found among different plant species (9, 14, 17, 19, 20). However, for several species different DNA methylation levels have been reported by different authors. This observation could be due to analysis of different tissues or tissues of different age or differentiated stage.

Here we present data showing tissue-specific as well as cell-type-specific DNA methylation in *Daucus carota*.

MATERIALS AND METHODS

Plant Material and Tissue Culture

Mature plants (carrots [*Daucus carota*] with green leaves) were bought locally. Pollen was obtained from Allergon (Uppsala, Sweden). The suspension culture cell lines NO4, NR, and NR2 were established from seedlings of the "Nobo" variety (Danefeldt, Odense, Denmark). They were initiated and kept as previously described (14). Somatic embryos were obtained by washing cells three times in Gamborg B5 medium (7) without hormones and diluting the cells to 10% settled cell volume. They were then plated on the same medium with 0.7% (w/v) agarose (type I, Sigma) and kept in the light for 7 d or until globular somatic embryos had emerged.

Isolation of DNA and Determination of the Level of DNA Methylation

DNA was isolated and further purified and the level of DNA methylation was determined as previously described (14). Briefly, DNA was digested by nuclease P1 and bacterial alkaline phosphatase and the resulting deoxyribonucleosides were separated and quantified by HPLC.

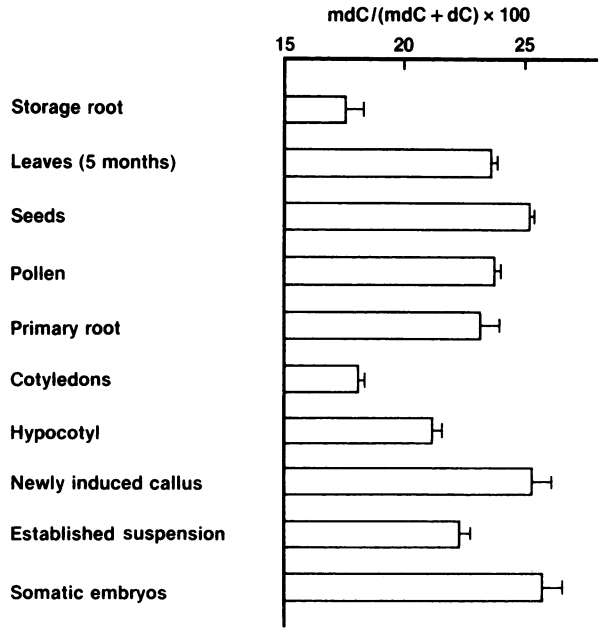

Figure 1. Specific levels of DNA methylation in various tissues of *D. carota*. Storage root and leaves were from a 5 month old plant; pollen was bought commercially; all others were established from seeds of var Nobo.

[1] Supported by grants from Danisco A/S, Copenhagen, Denmark and the Danish Ministry of Education (G. P.), by the PharmaBiotec Research Center (O. M.), and by the EEC Biotechnology Action Program, contract No. BAP-0078-DK (F. T. O.).

[2] Abbreviations: mdC, 5-methyldeoxycytidine.

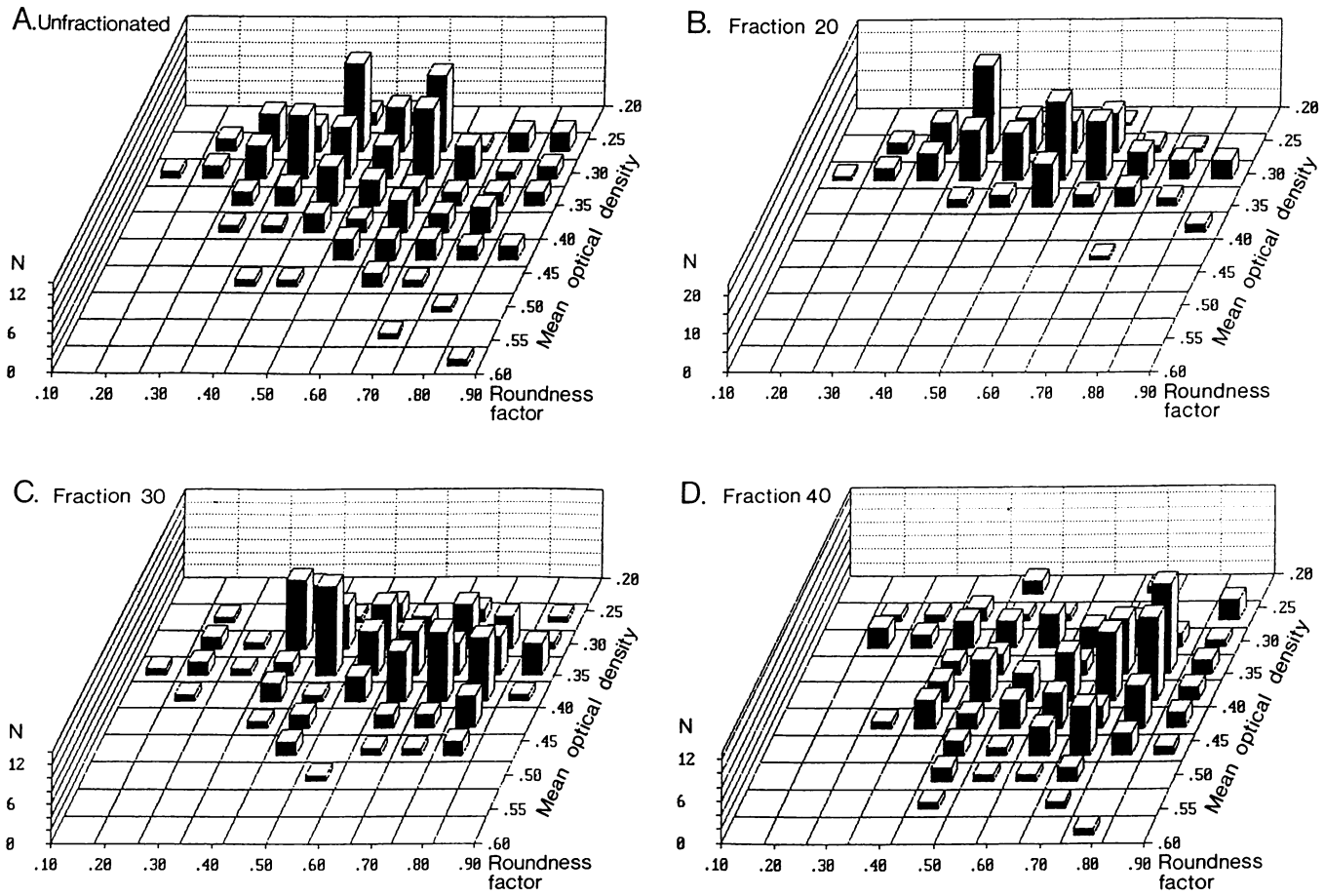

Figure 2. Distribution of cells and cell clusters according to shape and mean optical density in a suspension culture of *D. carota* and in three Percoll fractions of the suspension culture. Cells and cell clusters were analyzed according to their mean optical density and their roundness factor as described in "Materials and Methods." The height of the bars indicate the number of cells or cell clusters with these parameters.

Fractionation of Cells

Suspension cells were separated into three fractions enriched in morphologically distinct cell types by Percoll gradient density centrifugation according to Janniche *et al.* (8).

Characterization of Cell Types by Image Analysis

The morphology and optical density of the cultivated cells were characterized by image analysis. Small samples from the nonfractionated suspension culture and from the fractions obtained after Percoll separation were transferred to a haemacytometer with a 0.2 mm chamber depth. From each sample 25 frames were recorded on videotape. The recordings were done in transmitted light with a Zeiss Axioplan microscope with a ×10 objective, a broadband interference filter with maximum transmission at 550 nm and a B/W Uidicon camera. The stored images were analyzed with a Vidas image analysis system (version 2.0, Kontron, FRG).

Several geometrical and densitometric parameters of the cells were calculated from the recordings. The more useful of these were found to be the mean optical density and the "roundness factor" [4 * pi * area/(convex perimeter)^2], which describes the roughness and compactness of the individual cells and cell clusters. To relate previous qualitative observations with the image analysis, typical representatives of cells and cell clusters from the 20 and 40% Percoll fractions were measured selectively.

RESULTS AND DISCUSSION

Tissue Specificity

The level of DNA methylation was determined in 10 different tissues of *Daucus carota* and was shown to vary in the range from 17.8% in the storage root of the mature plant to 25.8% in somatic embryos (Fig. 1). In plants, specific levels of DNA methylation in different tissues have been reported with respect to a gradient in the inflorescens (10), during development of somatic embryos (11), and between leaves and calli (13).

From the data, it can be concluded that the level of DNA methylation can either increase or decrease during differentiation. After differentiation and during ageing and growth, leaves become extensively more methylated, going from 18.5% in the seedling to 24.0% in the adult plant. The opposite is the case for roots, however, which become demethylated, going from 23.1% in the seedling to 17.8% in the

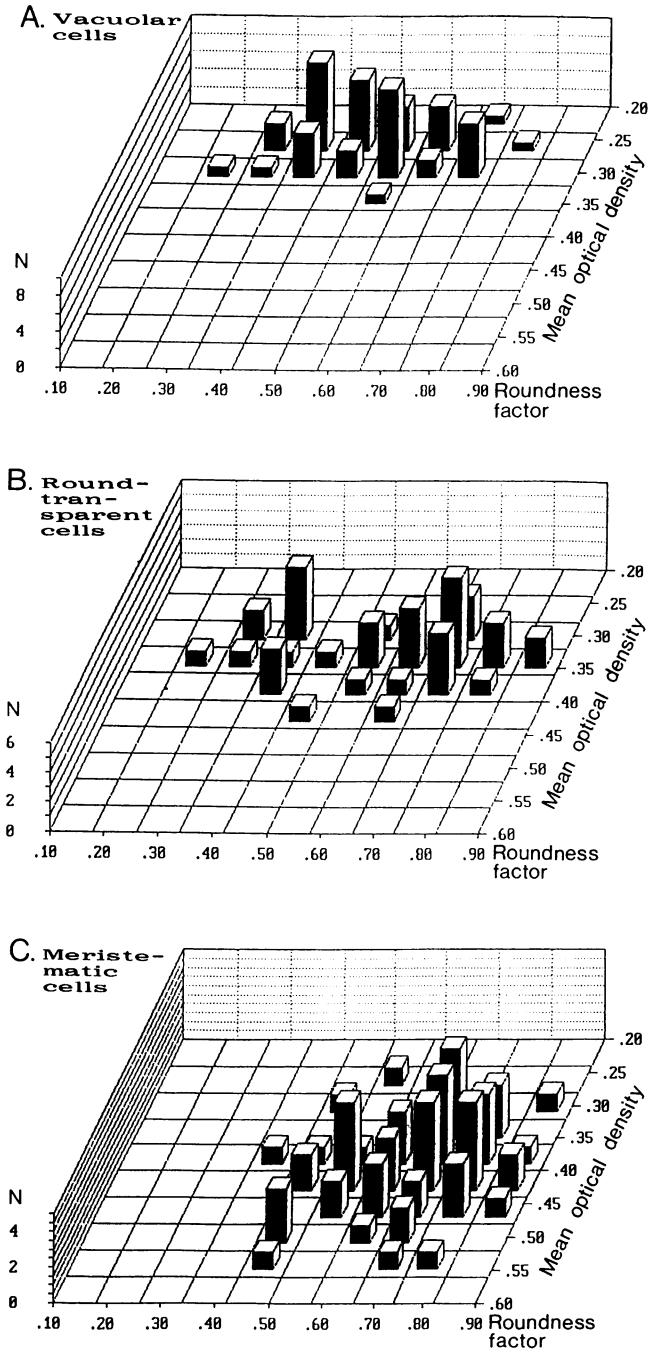

Figure 3. Distribution of three morphologically distinct cell types according to shape and mean optical density. Typical representatives of vacuolar, round-transparent, and meristematic cells and cell clusters were selected and each of the three cell types was then analyzed as described in the legend to Figure 2. The height of the bars indicate the number of cells or cell clusters with these parameters.

adult plant. It is also noteworthy that both the formation of callus from hypocotyl segments, which is generally referred to as a process of dedifferentiation, and the formation of highly organized somatic embryos from suspension cultured cells were accompanied by an increase in the level of DNA methylation (Fig. 1).

There seems not to be any straightforward correlation between the age or the differentiated stage of a tissue and its level of DNA methylation. The magnitude of the tissue specific differences in the level of DNA methylation shown in Figure 1 suggests that the change in methylation pattern is considerable during development (*e.g.* somatic embryos are relatively 45% more methylated than roots), and in this way DNA methylation may be involved in regulation or maintenance of differentiation.

Cell Line Specificity

If the tissue-specific levels of DNA methylation were reflections only of the differences in the differentiated stage of these tissues, one would expect that each cell type in the tissue had a characteristic level of DNA methylation and that differences between apparently similar tissues could be caused by differences in the distribution of cell types in the tissues. To test this hypothesis, the level of DNA methylation was determined in three different cell lines of *D. carota* cultured *in vitro*. All three cell lines were of the same variety and were grown under identical conditions. Large differences in the level of DNA methylation were observed, ranging from 14.5% (n = 1) in NR to 22.6% (SD = 0.3%, n = 6) in NR2. Interestingly, these two cell lines, both being embryogenic, were originally from the same genotype but NR2 had been taken through one cycle of regeneration/callus-induction about a year earlier. A nonhabituated, nonembryogenic cell line, NO4, had an intermediate level of DNA methylation of 20.0% (SD = 0.5%, n = 6). These cell suspensions are seen in the microscope to consist

Table I. *Distribution of Cell Types in an Unfractionated Suspension Culture of D. carota and in Two Percoll Fractions of the Suspension Culture*

Suspension culture cells from a embryogenic cell line (NR2) grown with 0.1 mg/L 2,4-D were harvested on d 7 after subcultivation and fractionated by Percoll density gradient centrifugation. The distribution of two clearly distinct types of cells or cell clusters—vacuolar and meristematic, respectively—in the unfractionated suspension culture and in two Percoll fractions of the suspension culture were calculated using image analysis

Selected Cell Type	Fraction[a]		
	Total	20	40
Vacuolar	57.1%	81.4%	18.4%
Meristematic	34.1%	17.5%	67.4%
Others	8.8%	1.1%	14.2%
n[b]	170	183	178

[a] 'Total' is the unfractionated suspension culture, while the numbers refer to the percentage of Percoll in the fraction on top of which the cells were recovered. [b]Number of cells or cell clusters counted.

Table II. *Level of DNA Methylation in an Unfractionated Suspension Culture of D. carota and in Three Percoll Fractions of the Suspension Culture*

Suspension culture cells from a embryogenic cell line (NR2) grown with 0.1 mg/L 2,4-D were harvested on d 7 after subcultivation and fractionated by Percoll density gradient centrifugation. The level of DNA methylation was then determined in the unfractionated suspension culture and in three Percoll fractions of the suspension culture.

	Fraction[a]			
	Total	20	30	40
Level of DNA methylation	23.0% ± 1.0	25.4% ± 0.7	22.3% ± 0.7	22.1% ± 0.2

[a] 'Total' is the unfractionated suspension culture, while the numbers refer to the percentage of Percoll in the fraction on top of which the cells were recovered.

of several different cell types and the distribution of the cell types are not identical in the suspensions (data not shown). This means that the difference in the level of DNA methylation in the suspensions could be caused by two very different mechanisms: (a) somatic alterations in the genome induced during prolonged *in vitro* culturing, and/or (b) alterations in the distribution of cell types having different levels of DNA methylation.

Fractionation of Cell Types

The cells of one of the embryogenic cell lines (NR2) were fractionated on Percoll density gradient centrifugation to three fractions enriched in either of three morphologically defined cell types. These cell types have been shown to follow distinct developmental pathways (8) and therefore represent distinct stages of differentiation.

The cells and cell clusters in the unfractionated material and in each of the three fractions were analyzed according to their mean optical density and their "roundness factor" as described in "Materials and Methods." The unfractionated material represented great variation with no apparent single-peak distribution (Fig. 2A), while the fractionated cells seemed more homogenous (Fig. 2, B–D). Fraction 20 showed a distribution centered around a relatively low roundness factor (*i.e.* diverging from a circle) and low mean optical density (Fig. 2B). Fraction 40 showed a distribution centered around a high roundness factor (*i.e.* coming close to a circle) and a relatively high mean optical density (Fig. 2D). Fraction 30 showed an intermediate and somewhat unclear distribution using these parameters (Fig. 2C).

Typical representatives of three morphologically clearly distinct cell types (*i.e.* vacuolar, meristematic, and round-transparent) were selected and their distribution according to shape and optical density were analyzed (Fig. 3). The distributions of vacuolar and meristematic cells had virtually no overlap (Fig. 3, A, C) and, accordingly, shape and optical density can be used for distinguishing between these two cell types. The distribution of vacuolar cells closely resembled that of the cells recovered in fraction 20 (Figs. 2B and 3A), while the distribution of meristematic cells resembled that of fraction 40 (Figs. 2D and 3C). This confirmed the visual impression that fractions 20 and 40 were enriched in vacuolar and meristematic cells, respectively. To quantify the purity of the cell-type preparations, the proportion of the two clearly distinct cell types, vacuolar and meristematic, in the unfractionated material and in the fractions 20 and 40 was counted (Table I). It must be noted that meristematic cells were counted as clusters of 10 to 100 cells while vacuolar cells were counted as individual cells or small clumps with very few cells. This means that the proportion of vacuolar cells given in Table I are overestimates.

Cell Type Specificity

The levels of DNA methylation in the unfractionated suspension culture and in each of the three fractions were determined (Table II). Fraction 20, enriched in vacuolar cells, had the highest level of DNA methylation of 25.7%, while fraction 40, enriched in meristematic cells, had the lowest level of 21.9% (Table II). The unfractionated suspension culture had, as expected, an intermediate level of DNA methylation. Because neither of the fractions were pure (Table I), we expect the actual difference in the level of DNA methylation between vacuolar and meristematic cells to be higher than that measured. It is possible that the large differences in the level of DNA methylation between the three cell lines could be due to a different composition of cell types with specific levels of DNA methylation. This could explain why NR and NR2, originating from the same genotype, have a relative difference in the level of DNA methylation of approximately 50%.

Fraction 40 contains the meristematic cells, which are precursors of somatic embryos (3, 8). Such precursors have been shown to have a lower level of DNA methylation than the suspension as a whole (11), and their presence in fraction 40 might be indicative for the low level of methylation in this fraction. In animals it has been shown that in an early, transient stage of zygotic embryogenesis the zygote becomes almost completely demethylated (12). Whether this is also the case in plants remains to be analyzed.

CONCLUSION

In conclusion, our investigations show that in plants different tissues have different levels of DNA methylation and that these levels are changing during growth and aging. Considerable variation in the level of DNA methylation exists between cell types found in unorganized suspension cultures, indicating that the relative distribution of the different cell types is a major factor determining the overall level of DNA methylation in the population or tissue at a certain age.

In animals DNA methylation has been shown to be involved in regulation of differentiation (12, 18). The considerable differences in the level of DNA methylation we have found in different tissues and cell types support that DNA methylation is involved in regulation of differentiation in plants, since this finding fulfill the prerequisite that different tissues exhibit differences in DNA methylation patterns if DNA methylation is involved in their regulation.

LITERATURE CITED

1. **Amasino RM, Powell ALT, Gordon MP** (1984) Changes in T-DNA methylation and expression are associated with phenotypic variation and plant regeneration in a crown gall tumor line. Mol Gen Genet **197:** 437–446
2. **Bianchi MW, Viotti A** (1988) DNA methylation and tissue-specific transcription of the storage protein genes of maize. Plant Mol Biol **11:** 203–214
3. **de Vries SC, Booij H, Meyerink P, Huisman G, Wilde HD, Thomas TL, van Kammen A** (1988) Acquisition of embryogenic potential in carrot cell-suspension cultures. Planta **176:** 196–204
4. **Doerfler W** (1983) DNA methylation and gene activity. Annu Rev Biochem **52:** 93–124
5. **Ehrlich M, Wang RY-H** (1981) 5-Methylcytosine in eucaryotic DNA. Science **212:** 1350–1357
6. **Felsenfeld G, McGhee J** (1982) Methylation and gene control. Nature **296:** 602–603
7. **Gamborg OL, Miller RA, Ojima K** (1968) Nutrient requirements of suspension cultures of soybean root cells. Exp Cell Res **50:** 151–158
8. **Janniche L, Okkels FT, Rajagopal R** (1988) Purification and characterization of the cell types involved in somatic embryogenesis and the cycles of unorganized growth. Physiol Plant **73:** 10A
9. **Kemp JD, Sutton DW** (1976) A chemical and physical method for determining the complete base composition of plant DNA. Biochim Biophys Acta **425:** 148–156
10. **Khvoyka L, Sulimova GE, Bulgakov R, Bashkite EA, Vanyushin BF** (1978) Changes in content of 5-methylcytosine in plant DNA in connection with floral gradient. Biokhimiya **43:** 996–1000
11. **LoSchiavo F, Pitto L, Giuliano G, Torti G, Nuti-Ronchi V, Marazitti D, Vergara R, Orselli S, Terzi M** (1989) DNA methylation of embryogenic carrot cell cultures and its variations as caused by mutation, differentiation, hormones and hypomethylating agents. Theor Appl Genet **77:** 325–331
12. **Monk M, Boubelik M, Lehnert S** (1987) Temporal and regional changes in DNA methylation in the embryonic, extraembryonic and germ cell lineages during mouse embryo development. Development **99:** 382–391
13. **Morrish FM, Vasil IK** (1989) DNA methylation and embryogenic competence in leaves and callus of napiergrass (*Pennisetum purpureum* Schum.). Plant Physiol **90:** 37–40
14. **Palmgren G, Mattsson O, Okkels FT** (1990) Employment of hydrolytic enzymes in the study of the level of DNA methylation. Biochim Biophys Acta **1049:** 293–297
15. **Razin A, Riggs AD** (1980) DNA methylation and gene function. Science **210:** 604–610
16. **Spena A, Viotti A, Pirrotta V** (1983) Two adjacent genomic zein sequences: Structure, organisation and tissue-specific restriction pattern. J Mol Biol **169:** 799–811
17. **Sulimova GE, Mazin AL, Vanyushin BF, Belozerskii AN** (1970) Content of 5-methylcytosine in DNA fractions of higher plants, differing in their nucleotide composition. Dokl Akad Nauk SSSR **193:** 1422–1425
18. **Vanyushin BF** (1984) Replicative methylation in animals and higher plants. Curr Top Microbiol Immunol **108:** 99–114
19. **Vanyushin BF, Kadyrova DKh, Karimov KhKh, Belozerskii AN** (1971) Minor bases in DNA of higher plants. Biokhimiya **36:** 1251–1258
20. **Wagner I, Capesius I** (1981) Determination of 5-methylcytosine from plant DNA by high-performance liquid chromatography. Biochim Biophys Acta **654:** 52–56