Induced mutation to monocotyledony in periwinkle, *Catharanthus roseus*, and suppression of mutant phenotype by kinetin

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Abstract

A recessive EMS-induced mutation inherited in Mendelian fashion caused monocotyledonous embryo formation and seed germination on high salt medium in *Catharanthus roseus.* Availability during embryo development of exogenously supplied cytokinin kinetin suppressed the mutant phenotype. These observations suggest that, in *C. roseus*, (i) insufficiency in endogenous kinetin may lead to monocotyledonous embryo patterning and (ii) dicotyledonous embryo formation requires a critical amount of kinetin in certain cells of early embryos.

[Rai S. P. and Kumar S. 2000 Induced mutation to monocotyledony in periwinkle, *Catharanthus roseus*, and suppression of mutant phenotype by kinetin. *J. Genet:* **79**, 97–104]

Introduction

Natural and certain synthetic cytokinins are able to induce cell division and organogenesis in *in vitro* plant cell and tissue cultures (Miller *et al.* 1955; Mok *et al.* 2000). Also, the endogenous cytokinins are essential hormones for *in vivo* cell division and differentiation processes in plants. Cytokinins have been shown to regulate a variety of plant development events, such as *de novo* bud formation, release of buds from apical dominance, leaf expansion, delay of senescence, maintenance of male fertility, promotion of seed germination, and chloroplast formation (Mok 1994). They are also known to affect DNA replication and RNA transcription and translation, enzyme induction and activation, calcium flux stimulation, microbial pathogenesis control, and stress remediation processes in plants (Barciszewski *et al.* 1999).

Induced-mutagenesis experiments in plants have so far yielded few mutants affected in cytokinin biosynthesis, metabolism and perception. Mutants affected in cytokinin degradation and/or perception are known in *Arabidopsis thaliana*. In one class of these mutants simultaneous auxin and cytokinin resistance is accompanied by poor root growth (Coenan and Lomax 1997). In another mutant cytokinin resistance causes defect in leaf and cotyledon expansion (Diekman and Ulrich 1995). In a third category higher than normal cytokinin levels are associated with hypercotyledony, constitutive photomorphogenesis and precocious flowering (Chowdhary *et al.* 1993). The phenotypes of the mutants are in agreement with the view that cytokinin effects are the result of antagonistic, additive and synergistic interactions with signals transduced by other phytohormones and nutritional and environmental factors (Barciszewski *et al.* 1999; Mok *et al.* 2000). The failure to generate cytokinin biosynthetic (deficient) mutants has been taken to mean that severe impairment of cytokinin action may be lethal to plants. It may also indicate redundancy and tissue-specific differential regulation in the genetic apparatus for cytokinin(s) biosynthesis in plant systems.

Here we report an EMS (0.2% v/v)-induced mutant in periwinkle, *Catharanthus roseus*, in which the embryo in seed has only one cotyledon, but developing embryos upon supplementation with the natural cytokinin kinetin develop two cotyledons. This is perhaps the first plant mutant in which developmental-stage-specific cytokinin deficiency has been demonstrated.

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Keywords. recessive mutation; monocotyledonous embryo; cytokinin; *Catharanthus roseus*; Mendelian inheritance.

Materials and methods

***Plant material and mutagenesis*:** *Catharanthus roseus* ($2n = 16$) is a glycophyte plant. Seeds of *C. roseus* cv Nirmal do not germinate on filter paper soaked with 200 mM NaCl in place of distilled water. To isolate mutants, whose seeds would germinate in the presence of 200 mM NaCl, lots of 2000 seeds of the parent periwinkle cultivar were exposed to 0.2% (v/v) of EMS (ethyl methanesulphonate) and sown in earthen pots containing soil and farmyard manure in equal proportion. The seedlings were later transplanted in the field to obtain M_1 mature plants. The M_2 seeds borne on 10 M_1 plants were harvested together.

***Isolation of salt-tolerant mutant*:** M_2 seeds and control seeds were surface sterilized separately by soaking in 0.1% $HgCl_2$ for 1 min, washed in running water, and blotted dry. A few control and a large number of M_2 seeds were placed in sterilized petridishes containing Whatman no. 1 filter paper moistened with 200 mM NaCl. The petridishes were placed at 25 ± 2°C under a 16-h light 8-h dark regime for 15 days; when needed 200 mM NaCl was added as an irrigant. Of a small number of seedlings produced, four had a single cotyledon. These were isolated and transplanted in 1 : 1 soil : farmyard manure mix in cups and later in pots to obtain M_2 plants. These were selfed to obtain M_3 seeds. The mutants have now been maintained for five generations in pure state. One of these was characterized in detail. Since the mutant seedling is salinity tolerant and bears one cotyledon, and since seeds formed after flowers and growing fruits had been sprayed with kinetin produced dicotyledonous seedlings which were salinity sensitive, the mutant phenotype and genotype were designated as Cdd^- (cytokinin-dependent dicotyledony) and *cdd cdd*.

***Genetic analysis*:** To study the inheritance of Cdd^- phenotypes the true breeding Cdd^- plants were crossed with the parental Cdd^+ wild-type plants, reciprocally. The buds were emasculated in the morning and pollinated in the evening. The flowers borne on the F_1 plants were self-pollinated to produce F_2 seeds. The F_2 seedlings and plants therefrom were compared with the parents and F_1s.

***Application of cytokinin and microscopic studies*:** Foliar application of cytokinin (6-furfurylaminopurine) at 100 ppm was made on branches of eight-month-old plants undergoing flowering, from which siliquae at various stages of development had been excised. Control plants were sprayed with distilled water. The spraying was done at 24-h intervals and continued for two weeks (~ 2 mg kinetin/plant). Some of the developing pods were harvested at 15 days from fertilization. The growing seeds were dissected to collect embryos for examination using a stereo dissection microscope fitted with bright field optics at 10× magnification. The embryos were photographed at 25× magnification using bright field optics.

***Estimation of proline and glycine-betaine content*:** Approximately 0.5 g of fresh seedling or leaf material was ground in 3% sulphosalicylic acid to extract proline (Bates *et al.* 1973). For the betaine estimations seedling or leaf material was dried in an oven at 75°C. The dried biomass sample was ground in a blender and assayed for betaine by periodide assay of Grieve and Gratton (1983).

Results

Isolation of salt-tolerant mutant

C. roseus is a small perennial herb of family Apocynaceae whose leaves are the only resource of the anticancer drugs vincristine and vinblastine and their precursors. This diploid, self-compatible and rapidly recyclable plant is being increasingly used as a model system to study plant growth and development processes and expression of secondary metabolism. A large population of M_2 seeds from an EMS mutagenesis experiment were screened for germination on a medium containing 200 mM NaCl. Four seedlings that emerged under these conditions were found to have the monocotyledon character. We obtained such seedlings at 0.02% frequency among M_2 progeny and 0.001% among M_1. The monocotyledonous seedlings were raised into plants and selfed seeds were collected. They bred true, and since F_1s of crosses between them had mutant phenotype one of them, designated as Cdd^- (*cdd cdd*), was studied further.

Inheritance of Cdd^- phenotypes

The seeds of the mutant *cdd cdd* were able to germinate on 200 mM NaCl medium and produced monocotyledonous seedlings both in presence and in absence of NaCl. Contrastingly, seeds of the wild-type parent of the mutant did not germinate on 200 mM NaCl and they produced dicotyledonous seedlings when irrigated with distilled water or low concentrations of NaCl solution. The Cdd^- seedling had 2.5-fold and 1.5-fold more proline and glycine-betaine respectively (data not shown) than the Cdd^+ seedlings. In reciprocal crosses between true-breeding Cdd^+ and Cdd^- plants (table 1), cotyledon number on seedlings and germinability of seeds on 200 mM NaCl in the F_1 and F_2 generation progenies were coinherited recessively in Mendelian fashion. This suggests that the same genetic lesion was responsible for the pleiotropic changes in the Cdd^- mutant seedling and adult plant.

Morphological features of Cdd^- mutant

Relevant characters of the Cdd^- mutant and Cdd^+ *C. roseus* wild-type plants are compared in table 2. The

Table 1. Coinheritance of seedling morphology and salinity tolerance in crosses involving mutants and wild type.

Parents and crosses	Number of monocotyledonous seedlings that were		Number of dicotyledonous seedlings that were		c^2*
	resistant to 200 mM NaCl	sensitive to 200 mM NaCl	resistant to 200 mM NaCl	sensitive to 200 mM NaCl	
Cdd^+	0	0	0	72	
Cdd^-	68	0	0	0	
$Cdd^- \times Cdd^+$ (F_1)	0	0	0	61	
$Cdd^- \times Cdd^+$ (F_2)	207	0	0	586	0.52
$Cdd^+ \times Cdd^-$ (F_1)	0	0	0	81	
$Cdd^+ \times Cdd^-$ (F_2)	140	0	0	425	0.015

*c^2 calculated based on an expected ratio of 3 sensitive to 1 resistant, $P > 0.05$.

Table 2. Qualitative characters of Cdd^+ and Cdd^- plants in *Catharanthus roseus*.

Stage	Character	Cdd^+ (wild type)	Cdd^- (mutant)
Seedling stage	Hypocotyl	Normal hypocotyl, light green in colour (2.1 cm)	Slightly thicker, light green in colour (1.5 cm), shorter than the wild type
	Cotyledon	Two cotyledons, each dark green in colour, elongated oblanceolate with smooth margin and tip, size of cotyledon 0.12 cm^2, shed off after some time (third leaf stage) and does not grow in size when first leaf arises	Single cotyledon, green in colour, larger than the wild type (0.18 cm^2), oval margin curved upward forming a cup shape, persistent for a long time and grows in size
	Root	Normal	Shorter and thicker than the wild type
Adult stage	Habit	Tall, plant height 63.4 cm	Semi-dwarf, plant height 44.1 cm
	Leaf	Green in colour, elliptic in shape with cuspidate apex and acute base, leaf size 8.9 cm^2, normal number of leaves (496), petiole normal	Yellowish green in colour, obovate with acuminate apex and acute base, leaf size 3.7 cm^2, smaller than the wild type, number of leaves less (352), petiole smaller than the wild type
	Flower, pod and seeds	Flower normal, pods normal (2.8 cm), average number of seeds per siliqua 16, seeds black in colour, 100-seed weight 81 mg	Flower smaller than the wild type, pods (siliquae) also small (2.1 cm), average number of seeds per siliqua 12, brownish black in colour, lighter than the wild type (76 mg)

dicotyledonous seedlings of the wild type had two lateral cotyledons borne on the central axis. A terminal bud or plumule formed the apical end of the axis. The lower end of the axis was formed by the root tip of the radical. The axis in between the radical and plumule was occupied by the hypocotyl. The seedlings in the mutant were characterized by bilateral asymmetry; there was only one cotyledon on the axis. The plumule appeared to be lateral on account of absence of one cotyledon, although it was terminal. The mutant seedlings possessed hypocotyl and radical of shorter sizes and normal morphologies.

The monocotyledon formed on the mutant seedling was itself bilaterally symmetrical. It was roundish and cup-shaped and of somewhat larger size than the elongate oblanceolate individual cotyledons of the wild type. The cotyledons from wild-type seedlings/plants were shed off

earlier than the single one from the mutants. A number of differences were noticeable between wild-type and mutant plants of eight months age (table 2). The mutant plants were short-statured and light green. Their leaf petioles and laminae, flowers, siliquae and seeds were of smaller size. The number of leaves borne on the mutant plants was about 40% less than that on wild-type plants. The quantitative differences in growth parameters, leaf shape, number of leaves and monocotyledonous character all indicated that the plants may be deficient in cytokinin (figure 1).

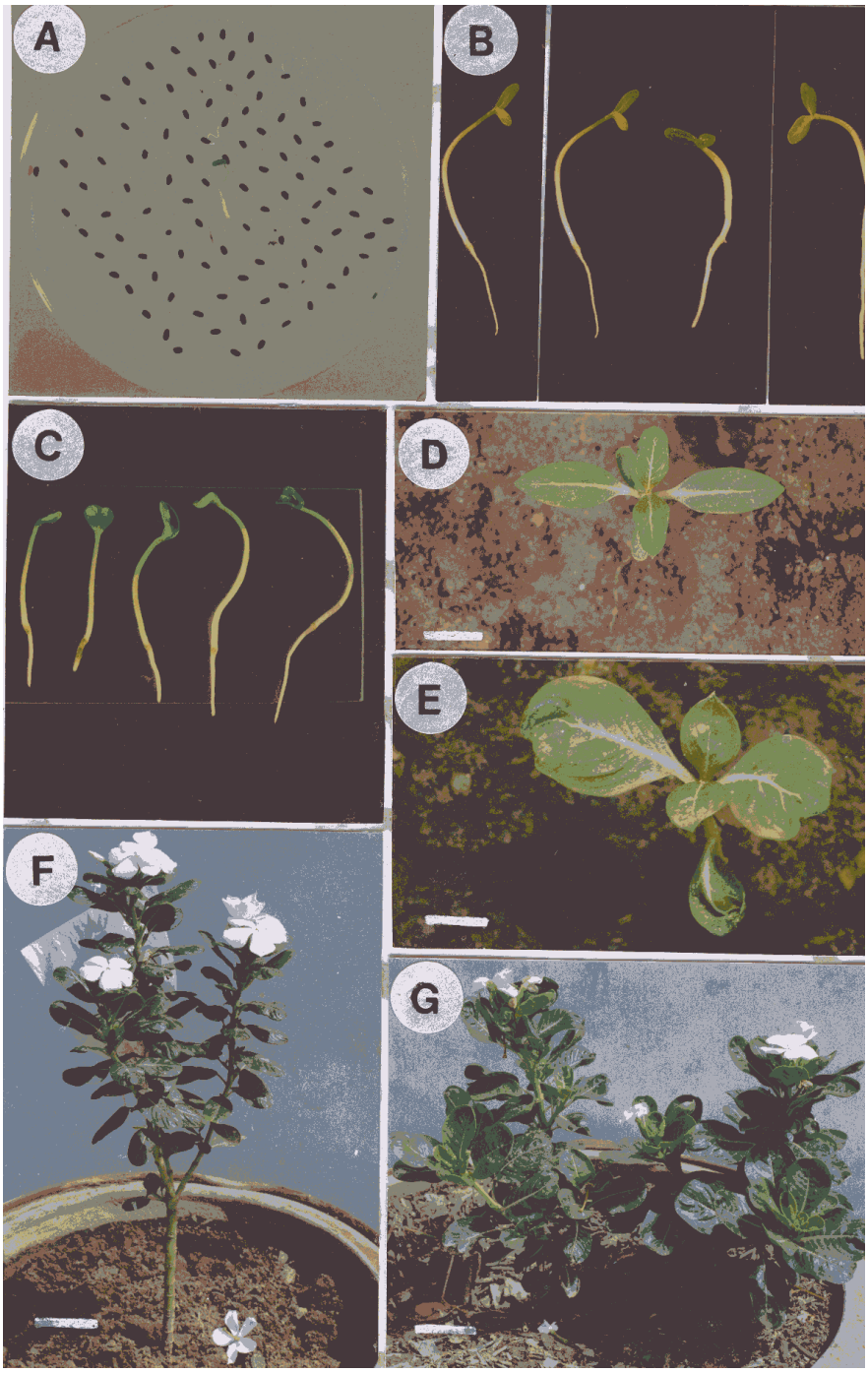

Figure 1. Characteristics of the Cdd^{-} monocotyledonous salinity-resistant mutant compared with its dicotyledonous salinity-sensitive wild-type parent. (A) A monocotyledonous salinity-resistant mutant seedling (centre of dish) seen together with 99 seeds that did not germinate in presence of 200 mM NaCl solution as the irrigant; (B) typical dicotyledonous Cdd^{+} seedlings; (C) the monocotyledonous Cdd^{-} seedlings; (D) four-leaf-stage plant of cdd^{+} cdd^{+} genotype, bar = 1.2 cm; (E) four-leaf-stage plant of cdd^{-} cdd^{-} genotype, bar = 0.4 cm; (F) 12-week-old plant of Cdd^{+} phenotype, bar = 3.6 cm; (G) 12-week-old plant of Cdd^{-} phenotype, bar = 2.4 cm.

Effect of cytokinin on Cdd⁻ mutant

The above observations allowed the hypothesis that if the *cdd⁻ cdd⁻* developing embryos were supplemented with cytokinin(s), such embryos may follow normal pattern of development; the seeds formed under these conditions would give rise to saline-sensitive dicotyledonous seedlings. To test this hypothesis the canopy segments of mutant plants that displayed flowers were sprayed with the natural cytokinin kinetin repeatedly. The siliquae formed after the kinetin sprays were harvested at different times to examine the developing embryos and to recover seeds. The data in tables 3 and 4 show that indeed some dicotyledonous embryos/seedlings and salt-sensitive dicotyledonous seeds were recovered upon kinetin application to the mutant plants (figure 2). It is noteworthy that while F_1 plants recovered from the crosses (table 1) were phenotypically wild type, their F_2 progeny did have mutant plants in certain proportions. We infer that, in these mutants, the mother tissue surrounding the embryos could not complement the biochemical deficiency of the mutant embryos. The results given in tables 1, 3 and 4 are consistent with the explanation that the *cdd⁻ cdd⁻* mutant embryos did not possess the required cytokinin (kinetin) in sufficient concentration to allow normal dicotyledonous development.

Discussion

Among the phenotypes that distinguish the Cdd⁻ mutant from wild-type plants, the monocotyledonous nature of Cdd⁻ seedlings is of particular importance in view of the demonstration of suppression of this phenotype by exogenous kinetin supplementation. The periwinkle *C. roseus* being an angiospermic dicotyledonous species, its normal cotyledons, borne as lateral appandages on seedlings, are in structure and ontogeny specialized leaves formed during zygotic embryogenesis, modified in function as a storage organ of seeds (Eames 1961). The cotyledons have their own developmental pathway in the embryo during seed formation, distinct from that of true leaves, which result from apical meristematic activity of seedlings growing into plants (Convey and Poething 1997). During embryogenesis a single zygotic cell develops into a torpedo-shaped mature embryo within the seed coat of mother tissue through programmed cell divisions, cell differentiation and morphogenetic events. The mother tissue has accumulated storage products and is in a state of dormancy. The torpedo-stage embryo mimics seedling structure in that both have apical–basal axis largely in the form of hypocotyl, which at the top has the apical meristem and in its axils the lateral cotyledons, and at the base gets extended into the radical which ends in a root meristem. The torpedo stage of the embryo is preceded by heart and globular stages, in that order. In the triangular heart-shaped embryo, the cotyledons are observed at the upper side and the radical on the lower side, and the apical meristem (plumule) is morphologically apparent between the emerging cotyledons. The cotyledons are thought to be differentiated in early pre-heart-shaped embryo stage by induction of localized cell divisions in different planes in the apical flanks, followed by vascular tissue differentiation from the hypocotyl region into the two cotyledon regions (Maheshwari 1950; Schulz and Jensen 1968; Mayer *et al.* 1991). The Cdd⁻ embryos appear to be deficient in the emergence of bilateral centres for cotyledonary growth; in them only one centre, somewhat lateral to the apical meristem, becomes active for cotyledon development. The rest of the pattern of embryo development does not seem to be affected by the mutation in the Cdd⁻ mutant. As a result a Cdd⁻ embryo forms a seed in which the embryo has an apical shoot meristem, a single lateral cotyledon, and a hypocotyl and a radical like in the wild type.

Comparison of the features of Cdd⁻ embryos, seedlings and adult plants of *C. roseus* with embryonic mutants known in other plants reveals that the monocotyledonous character in *C. roseus* Cdd⁻, which is expressed in all the seeds/seedlings, reversed by the presence of exogenously supplied kinetin in developing embryos, and accompanied by normal phyllotaxy and flowers, is indeed novel. Genetic dissection of seed embryogenesis has advanced considerably in *Arabidopsis thaliana*. The induced *gurke, fackel, monopteros* and *gnom* mutants are all seedling lethals characterized by absence of or defect in cotyledons and shoot meristem, hypocotyl and root, and all

Table 3. Effect of kinetin on embryo development in mutant plants.

Strain	Embryo type recovered (number)			
	after supplementation with kinetin*		without kinetin supplementation	
	monocotyledonous	dicotyledonous	monocotyledonous	dicotyledonous
Wild type (*cdd⁺ cdd⁺*)	0	250	0	250
Mutant (*cdd⁻ cdd⁻*)	195	55	250	0

*6-Furfurylaminopurine (100 ppm) was used and 250 embryos were studied.

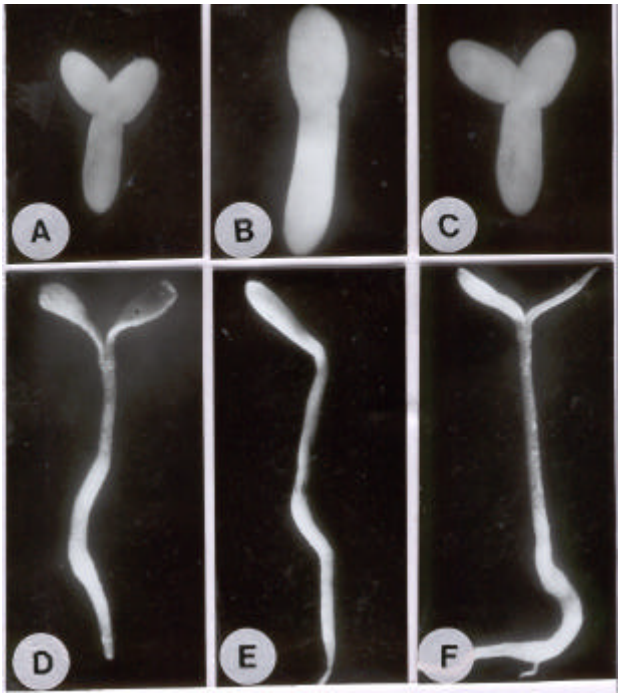

Figure 2. Morphology of the Cdd$^-$ and Cdd$^+$ pre-mature embryos and seedlings obtained with and without cytokinin supplementation to the developing seeds. (A) A typical dicotyledonous embryo as found in the developing seeds of wild-type Cdd$^+$ plants; (B) a typical monocotyledonous embryo as found in the developing seeds of the Cdd$^-$ mutant plants; (C) dicotyledonous embryo dissected from *cdd cdd* siliqua from *cdd cdd* plant that had been sprayed with kinetin; (D) a seedling from seeds borne on *cdd*$^+$ *cdd*$^+$ plant progeny of Cdd$^+$ plants; (E) a typical monocotyledonous seedling as found in the progeny of Cdd$^-$ plants; (F) a dicotyledonous seedling formed by the seed of *cdd cdd* plant sprayed with kinetin.

Table 4. Salinity tolerance of seedlings after kinetin application.

	Resistant*		Sensitive	
	Dicot	Monocot	Dicot	Monocot
Cdd$^+$	0	0	30	0
Cdd$^-$	0	26	4	0

*200 mM NaCl was used.

parts of embryo. The *keule, knolle, fass, knopf, mickey, dem, cyr, aux* and *axr* mutants have variously deformed embryos and seedlings (Mayer *et al.* 1991; Jurgens *et al.* 1994; Sheridon 1995; Fisher *et al.* 1996; Hobbie *et al.* 2000) but not akin to Cdd$^-$ mutant of *C. roseus* . The *lec* mutant has embryonic cotyledons transformed into foliage (Meinke 1992; Keddie *et al.* 1998). The *AMP* and *HPT* mutants have supernumerary cotyledons in their embryos (Hardtke and Berleth 1998). The *dem* (Keddie *et al.* 1998), *monopteros* (Hardtke and Berleth 1998), *abruptus* (Ezhova *et al.* 1999) and *pin* (Bennett *et al.* 1995) mutants produce pseudomonocotyledonous embryos in which cotyledons are fused to different lengths from base to apex. The *pin* mutants mimic the behaviour of *Brassica juncea* somatic embryos developed in the presence of auxin transport inhibitors and themselves demonstrate deficiency in auxin transport (Okada *et al.* 1991; Liu *et al.* 1993; Sieburth 1999). The *C. roseus* Cdd$^-$ mutant appears to be similar to the monocotyledonous species of the angiospermic families Trapaceae and Umbelliferae, in which other member species are dicotyledonous (Eames 1961). In these monocotyledonous plants the embryo forms only one cotyledon owing to defective embryogenesis patterning.

The Cdd function in *C. roseus* seems to be required for bilateral cotyledon formation and not for apical shoot meristem, hypocotyl, radical and root meristem, whose development is largely normal. The plants formed from Cdd$^-$ seedlings had typical morphology, possessed stomata and were green and photosynthetically active, indicating that cell differentiation was left intact by the *cdd* mutation. The primary defect in the Cdd$^-$ mutant appeared to concern initiation of the second cotyledon at early stage(s) of embryogenesis. It is visualized that in plants such as *A. thaliana* and *C. roseus*, normally two cells placed diametrically opposed to each other in the early embryo turn into primordial cells for the growth and development of two cotyledons. We surmise that in each of these cotyledon primordial cells, induction of subsequent division and/or progression and plane of the division, and continuation of divisions among progeny cells coordinately in different planes for the concomitant differentiation and morphogenetic development of cotyledons must be under the control of the *cdd*$^+$ gene.

Kinetin has been implicated in the expression of the genes that encode cycD3 and cdc2 cyclin proteins, which are important for G1/S transition in the plant cell cycle (Zhang *et al*. 1996; Riou-Khamlichi *et al.* 1999); other cyclins may also be involved. Kinetin is also involved in ribosomal RNA synthesis and maturation specific to cotyledons (Gaudino and Pikkard 1997; Yamamoto *et al.* 2000). In *Arabidopsis* overexpression of a mutated *cdc2a* gene in transgenic embryos resulted in, besides several kinds of the apical–basal disturbances observed during embryogenesis, aborted development of one cotyledon with some frequency (Hemerly *et al.* 2000). In embryos missing one cotyledon in various plant species, a cotyledon primordial cell must have failed to prepare for further development for one or other reason(s). Our observations suggest that cytokinin deficiency must be responsible for the preclusion of a cotyledon primordium in the *C. roseus* Cdd$^-$ mutant.

Kinetin has been shown to form intracellularly as a product of repair of oxidative damage to DNA (Pratviel *et al.* 1991; Barciszewski *et al.* 1997). The chance of hydroxy radical damage to DNA is expected to be lower in the Cdd$^-$ early embryos than in Cdd$^+$ embryos. The

Cdd⁻ embryos are salinity resistant and Cdd⁻ seedlings and plants have been found to accumulate the compatible solutes such as proline and glycine-betaine in high amounts (Netting 2000; Zhang *et al.* 2000). These solutes have been shown to have protective effect against active oxygen species (Albinsky *et al.* 1999). We conclude that in the preglobular stage Cdd⁻ zygotic embryo, only one cell synthesizes or accumulates kinetin in the critical amount necessary for it to serve as cotyledon primordium for the initiation of coordinated cell divisions for cotyledon growth and morphogenesis; in the Cdd⁺ embryo and in Cdd⁻ embryo exogenously supplemented with kinetin, two primordia are formed which lead to the dicotyledonous condition in the mature embryo.

Acknowledgements

The work was partially supported by the Department of Biotechnology, Government of India. G. D. Bagchi and R. Luthra helped in the photography of embryos and determination of proline and betaine contents in seeds, seedlings and plants.

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Received 24 November 2000