Research Paper

Regulation of Lifespan by the *LLI* and *EGD* Genes in the Perennial Plant Species *Catharanthus roseus*

RENU KUMARI[a,b], SWATI CHAUDHARY[a,b], RAGHVENDRA KUMAR MISHRA[a], SHASHI PANDEY RAI[a], SANJAY KUMAR RAI[a], VINAY SHARMA[b], BHUMI NATH TRIPATHI[b] and SUSHIL KUMAR[a*]

[a]*Genetical Genomics Laboratory, National Institute of Plant Genome Research (NIPGR), Aruna Asaf Ali Marg, New Delhi 110 067, India*

[b]*Department of Bioscience and Biotechnology, Banasthali University, Banasthali 304 022, Rajasthan, India*

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Contrasting point of views exist about the biology of lifespan in seed producing perennial plant species. One of the view is that the plants of perennial plant species are heritably immortal and die of accidents. The other view is that lifespan of perennial plant species is determinate and plants die from progressive senescence. The present experiment in *Catharanthus roseus* was based on the premise that the demonstration of heritable variation in survival time of its different genotypes will provide evidence for the determinate nature of lifespan in this perennial plant species and intra-genotype differences observed between the apparently normal and senescent plants will reveal the symptoms associated with mortality. The survival frequencies of the plant populations of four genotypes derived by combining the wild type and mutant alleles of the genes *LEAFLESS INFLORESCENCE* (*LLI)* and *EVERGREEN DWARF* (*EGD)*, in a common background and different genetic backgrounds, were compared at 5 and 6 years from germination. Phenotyping showed that the surviving plants were either green leaved (normal) or bore pale leaves (senescent). The normal and senescent plants were investigated genotype-wise for the expression of plastidic and nuclear genes related to photosynthesis and certain nuclear genes involved in mitochondrial expression. The *LLI EGD*, *lli EGD*, *LLI egd* and *lli egd* genotypes were observed to differ in survival. The *lli* mutation decreased lifespan and *egd* mutation increased it and the interaction between *lli* and *egd* was additive. The leaves of senescent plants were deficient in chlorophylls, photosynthesis and expression of nuclear genes associated with plastidic and mitochondrial functions. The lifespan in the perennial plant species *C. roseus* is increased by the *LLI* gene and decreased by *EGD* gene. It is a determinate genetic trait. Degradation of nuclear-organelle interactions, manifested in the form of pale leaves, is a symptom of incipient mortality in senescent plants.

Key Words: *Catharanthus roseus*; Evergreen Dwarf; Genetics of Plant Perenniality; Leafless Inflorescence; Plant Lifespan; Plant Ageing

Introduction

In terms of life history, the seed producing plant species fall into two classes. The species belonging to one of these groups have variable life span (of less than one to many years) but flower only once. The species that comprise the other group experience two to many episodes of flowering and are called perennial. In the one time flowering or so called short-lived species, plant death is a consequence of whole plant senescence resulting from the post-flowering mobilization of nutrients from vegetative organs to the developing seeds. Since the perennial plants can withstand repeat episodes of flowering/fruiting, the reproduction cycles apparently do not induce the acute death causing whole plant senescence in perennial plants. Whether and how the plants of perennial plant species die are questions that are not yet adequately answered.

Two views are in vogue about the lifespan in perennial plant species. One of these considers perennial plants as inherently immortal and provides that perennial plants may die on account of accidental injuries [1]. Some formal evidence, albeit indirect, for the occurrence of mortality in perennial plant

*Senior Author for Correspondence : E-mail: sushil2000_01@yahoo.co.in; Tel. 011-26865494; Fax: 011-26741658

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species, comes from the studies of population structures in plant communities comprising of diverse plant species. The equilibrium between species is maintained by optimization of the intraspecific rates of mortality and progeny emergence; or, within perennial species, death of old plants permits establishment of nascent plants [2]. This interpretation is in consonance with the view that perennial plants may die on account of progressive age-related senescence [3, 4]. There is need for experimental approaches to resolve the issue of lifespan determination in perennial plant species [5].

The eudicot *Catharanthus roseus* (Madagascar periwinkle) species of apocynaceae comprises of perennial (non-annual) plants of small size ($\leq$ 1m height) which bear woody root, woody basal stems and herbaceous upper shoot as an indeterminate inflorescence. The species has short juvenile/ vegetative phase and 10 to 16 weeks old plants begin to bear flowers. Although small in size, *C. roseus* shares its growth traits with trees [6]. In the Indo-Gangetic plain area, such as Lucknow and New Delhi, the *C. roseus* plants bear flowers in spring, summer, autumn and winter, except for a brief period of dormancy in the winter season, and are known to live for many years. *C. roseus* plants harboring monogenetically inherited mutations in different *GLYCOPHYTIC SALINITY RESPONSE (GSR)* genes have been shown to be pleiotropically different from wild type in their morphological, biochemical and stress response properties [7-9]. Several *gsr* mutations produce stress response and developmental features that are distinctly different from those in *GSR* wild types [10, 11] and preliminary observations indicated lifespan differences among the wild type and *gsr* mutants. The availability of this material prompted us to ask the question(s): (a) whether casually observed determinacy of lifespan in the woody perennial plant *C. roseus* is a genetic property, and (b) what are the features associated with longer lifespan in *C. roseus* and *vice versa* ? It has been observed that the lifespan of *C. roseus* is finite and a genetic trait. Lifespan is increased by the *LEAFLESS INFLORESCENCE (LLI)* gene and decreased by *EVERGREEN DWARF* (*EGD)* gene. Continued complimentarity of organelle and nuclear functions in chloroplasts and mitochondria is a requirement for the longer lifespan.

Materials and Methods

Plant Material

The list of genotypes of *C. roseus* used in the study and their origins and properties are shown in the Table 1. The morphologies of about 20 weeks old plants of

Table 1: Origin and properties of the *Catharanthus roseus* genotypes used

Relevant genotype (homozygous for the alleles)			Properties	Origin/Reference
CPC	*LLI*	*EGD*		
+[a]	+	+	Rosypink corolla petal color, tall habit, leaf lamina pigmentation dark green; compound racemose inflorescence, secondary inflorescences borne in the axils of alternate leaves	Floricultural cultivar called 'Delhi Pink'; [52].
–[b]	+	+	White corolla, tall habit, leaf lamina dark green; compound racemose inflorescence, secondary inflorescences borne in the axils of alternate leaves	Medicinal cultivar called 'Nirmal'; [10,12].
-	-	+	Induced *glycophytic salinity response-8* (*gsr-8*) mutant, more drought and salinity tolerant than the parent Nirmal; light green leaves; compound cymose – cum - racemose inflorescence and consequent extensive terminal branching; secondary inflorescences are borne on nodes barren of leaves	[11]
-	+	-	*gsr-1* mutant, more drought and salinity tolerant than the parent Nirmal; dwarf evergreen habit, gibberellin responsive; late flowering; wild type inflorescence, like in 'Nirmal'	[10]
-	-	-	*gsr-1 gsr-8* double mutant, more drought and salinity tolerant than the parent Nirmal; green leaves; inflorescence like in *gsr-8*; dwarfer than *gsr-8* and taller than *gsr-1*	Present study

[a] +, wild-type allele; [b] -, mutant allele.

the five genotypes grown in clay pots are shown in Fig. 1. The F_2 progenies were produced by selfing of F_1 plants from two crosses. The *cpc lli EGD* line was crossed with *CPC LLI EGD* line in the spring of the year 2002 (Cross-1). The line *cpc lli egd* was crossed with *CPC LLI EGD* line in the winter of the years 2002-2003 (Cross-2). One hundred and fourteen F_2 plants from the Cross-1, 106 F_2 plants from the Cross-2, 10-11 plants each of *CPC LLI EGD*, *cpc LLI EGD*, *cpc lli EGD* and *cpc lli egd* were deployed in the study. The morphological marker *CPC* refers to *COROLLA PETAL COLOR*.

Growth Conditions

Each of the experimental plant was labelled and maintained in a pot of 30 cm diameter in the field conditions until transplanted in a field plot of the experimental farm of the institute, in the autumn of 2005. By this time the F_2 plants of the Cross-1 and Cross-2 were of about 30 and 15 months age, respectively. The experimental plants were transplanted from pots to field beds each of 2.1 x 1.5 m^2 size. Among the 30 beds, 29 were planted with 9 plants each and the last bed with 10 plants. The arrangement of 271 plants (of the long term plant populations) between and within the beds was random. The soil used in the pots and of the field plot was sandy-loam with pH about 8.0. The soil was mixed with manure (2:1) to prepare the initial medium for the pots. The pots and field beds were applied with N, P and K fertilizers (80:40:40 kg h^{-1}) twice in a year, in late February and late August. The pots and beds were weeded of any extraneous growth and irrigated as and when required. The plant population

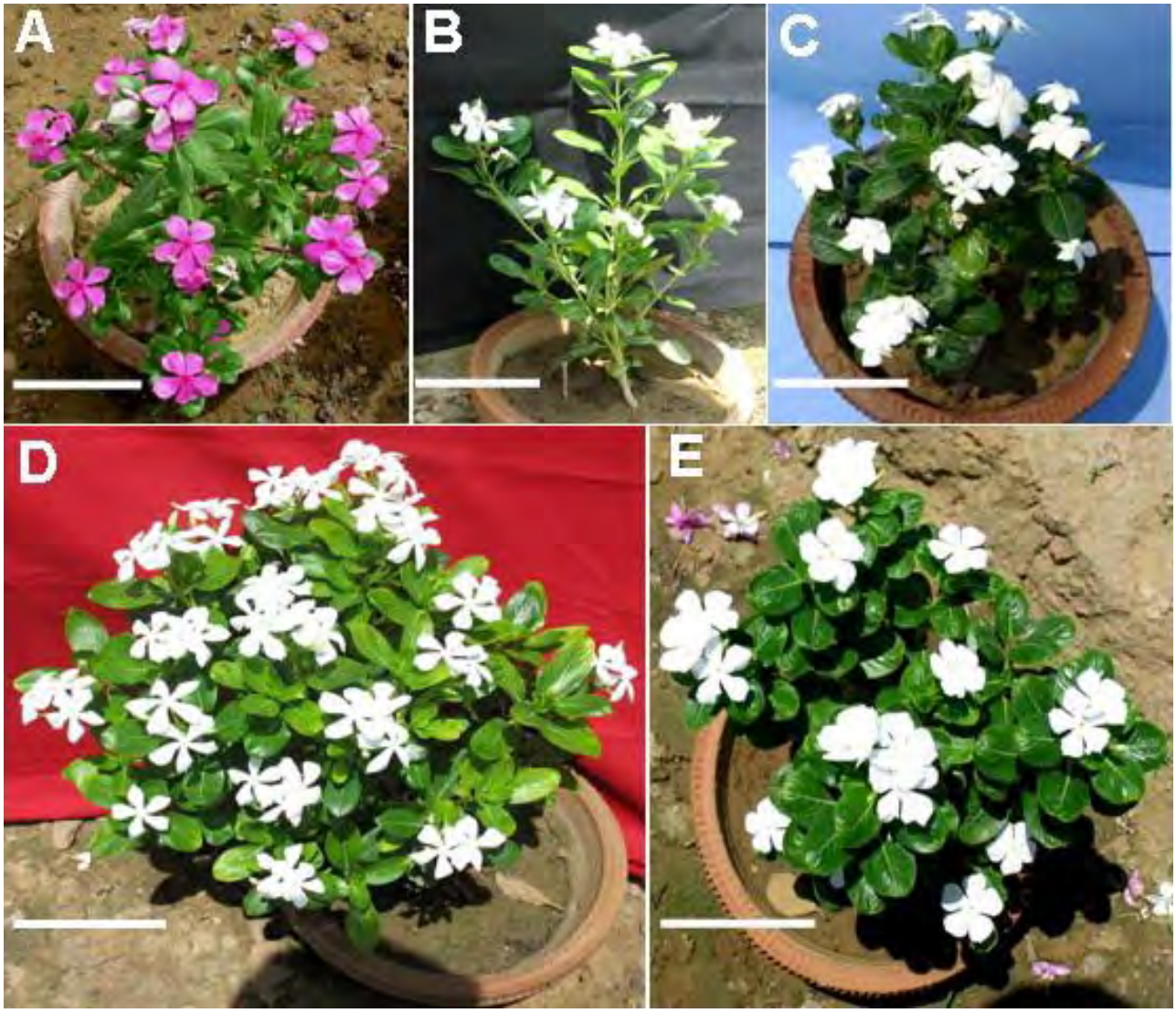

Fig. 1: Morphologies of about 20 weeks old plants of the five parental genotypes of *Catharanthus roseus* used in the present study. (A) *CPC LLI EGD* or 'Delhi Pink'. (B) *cpc LLI EGD* or 'Nirmal'. (C) *cpc lli egd* double *gsr-1 gsr-8* mutant. (D) *cpc lli EGD* single *gsr-8* mutant. (E) *cpc LLI egd* single *gsr-1* mutant. Scale bar = 15 cm.

was sprayed with 0.1 % bavastin and micronutrient solution in February. Further details of the agronomic procedures used were the same as described earlier [12, 13].

For the purpose of inter-genotype comparisons of vigor, the seedlings of *CPC LLI EGD, cpc LLI EGD, cpc lli EGD, cpc LLI egd* and *cpc lli egd* were transplanted in the summer of 2008 in a separate part of the field (short term populations). Ten plants of each genotype were grown 30 cm apart in lines 60 cm apart in plots replicated three times.

Survival, Allometery, Chlorophyll and Photosynthesis related Observations

Short term populations: Leaves borne on third to tenth nodes from apex in the main branch of five plants per replication at the age of 18 weeks were studied for *in situ* photosynthetic rate and total photosynthesis. For the latter parameter, the sampled leaves were plucked to determine their areas. Photosynthesis in individual leaves was studied using GFS-3000 portable gas exchange fluorescence system (Heinz-Walz, Effeltrich, Germany). Photosynthetic rate was expressed in terms of µmol of CO_2 utilized per meter square of leaf area per second (µmol. $m^{-2}.s^{-1}$) and total photosynthesis in leaves as µmol CO_2 utilized per second (µmol.s^{-1}). The latter was calculated by multiplying the rate with leaf area. At 21 weeks of plant age, 5 whole plants/ replication/ genotype were excavated and dried at room temperature in paper bags. Subsequently, their roots, stems and leaves + flowers + fruits (called leaves here) were separated plant-wise and weighed to estimate the biomass accumulated and to reveal partitioning of biomass among organs.

Long Term Populations: Survival was recorded plant-wise, in the spring and autumn seasons in the years 2006-2008 and finally in the spring of 2009. Simultaneously, morphological observations were recorded on the surviving plants. In the late autumn of 2008, *in situ* photosynthetic rates were determined, genotype-wise in the leaves of surviving plants of varying leaf morphologies. In the early spring of 2009, same plants were sampled for leaves for the estimation of leaf area, chlorophyll contents, expression of certain nuclear and organelle encoded genes and PCR amplification of chloroplast (cp) DNA. The genotype/phenotype-wise samples for cpDNA quantification and organelle expression comprised of leaves pooled from three plants. The procedure of Arnon [14] was used to estimate chlorophyll *a* and *b* contents.

RNA Isolation and Semiquantitative RT-PCR

Total RNA was isolated from 1g of leaves (fresh weight) by grinding them to powder in liquid nitrogen and extraction in buffer containing 0.01M EDTA, 0.2 M sodium acetate (pH-5.2) and 1% SDS. After two phenol:chloroform (V:V::1:1) extractions the nucleic acids were precipitated with 10 M lithium chloride. The pellet after washing with 70% ethanol (V/V) was resuspended in sterilized water [15]. Total RNA was quantified using GeneQuant Pro Spectrophotometer (Amersham Pharmacis Biotech, Uppsala, Sweden) and checked on 1.5% denaturing agarose gel. The reverse transcription was accomplished with the use of cDNA synthesis kit (Fermentas Life Sciences, Canada). Using 5µg total RNA, the reverse transcription (RT) reaction was primed with oligo $(dT)_{20\text{-}23}$ primers using 15 units of reverse transcriptase according to the manufacturer's guidelines. The RT with the use of primers for *ACTIN* gene served as the control. RT was carried out at 50ºC for 30 min, followed by initial PCR activation step at 95ºC for 5 min. PCR reactions consisted of 30 cycles of denaturation at 95ºC for 30 sec, annealing at 52ºC for 30 sec, extension at 72ºC for 1 min and a final cycle for 10 min at 72ºC. The primer sequences used in the RT-PCR amplifications of transcription products of nuclear and plastid genes are given in the Table 2.

Semiquantification of Chloroplast DNA

The yield of DNA isolated by the hexadecyltrimethylammonium bromide method [16] was 100-500 ng/g fresh leaves. DNA amplifications were carried out in an iCycler Thermal Controller (Bio-Rad, USA). The PCR reactions were performed in 20 µL volume containing 2.0 µL 10X PCR reaction buffer [200 mM Tris-HCl (pH 8.4), 500 mM KCl], 3 mM $MgCl_2$, 0.1 mM dNTP, 0.8 µM primer, 25 ng of genomic DNA and 1 unit Taq polymerase (Sigma-Aldrich). The chloroplast DNA content was semiquantified by PCR amplifications using three sets of chloroplast DNA-specific universal primers;

trnT- fw 5´-CATTACAAATGCGATGCTCT-3´;
trnL- rv 5´- TCTACCGATTTCGCCATATC-3´;

Table 2: Primer sequences of the nuclear- and plastid- encoded genes deployed in the study of RT-PCR amplifications of their transcription products in the leaves of *Catharanthus roseus* variants

S.No.	Gene			Primers:
	Class	Name	Abbreviation	Forward (fw) and Reverse (rv)
1	Nuclear encoded chloroplast/ photosynthesis genes	RUBISCO small subunit	RBCS1B	fw 5′ GTCGCTCCATTCACTGGTTT 3′ rv 5′ ATCCATTTGTTGCGGAGAAG 3′
2		Component of chlorophyll a/b binding protein complex	LHCB1	fw 5′ AGCCATCGTCACTGGTAAGG 3′ rv 5′ ATGTTCAATCACGTTGCTCG 3′
3		Subunit of oxygen evolving system of PSII	PSBO	fw 5′ GAGCACCAAAGAGGCTAACG 3′ rv 5′ GGGTCATGAGCTTGGTGTTT 3′
4		EU135981, Cr gi:159792897 Isopentenyl pyrophosphate	NPP	fw 5′ ATTCCGCTATGGATGCTGTC 3′ rv 5′ AGACCAGGGGAAAGGTCACT 3′
5	Plastid encoded chloroplast genes	RUBISCO large subunit	RBC L	fw 5′ TCTTGGCACCATTCCGAGTAAC 3′ rv 5′ GAAAAAGATAACCGCGAGCACGA 3′
6		Photosystem II protein D1	PSB A	fw 5′ GCAACTGGATAACTAGCACCGA 3′ rv 5′ GAGGGAAGTTGTGAGCATTACG 3′
7		Photosystem II reaction center W protein	PSB W	fw 5′ TGCTCCTTCTGCTCTTGGTT 3′ rv 5′ CGTGCTCATTCTCTCATCCA 3′
8	Nuclear encoded genes for mitochondrial functions	NAD(P)H dehydrogenase B2	NDB2	fw 5′ GAAGAAGAAGGTGGTGCTGC 3′ rv 5′ CAACAACCACAAAATGCAGG 3′
9		Alternative oxidase 1a	AOX1a	fw 5′ TGGACTAGAGCTCCGACGAT 3′ rv 5′ GCGCTCTCTCGTACCATTTC 3′
10		Upregulated by oxidative stress= At2g21640	UPOX	fw 5′ GATTCAGATGGCTTCCCAGA 3′ rv 5′ GCTCCCGAATATCTTGTCCA 3′
11	Nuclear encoded non-photosynthetic and non-mitochondria related gene	ACTIN	ACTIN	fw 5′ TGAAATGTGACGTGGATATC 3′ rv 5′ GCTGGAATGTGCTTAGAGA 3′

trnL-fw 5′-CGAAATCGGTAGACGCTACG-3′; *trnL*-rv 5′- GGGGATAGAGGGACTTGAAC-3′;

trnL-fw 5′-GGTTCAAGTCCCTCTATCCC-3′; *trnF*-rv 5′- ATTTGAACTGGTGACACGAG- 3′

[17]. PCR conditions used for the amplification were: one cycle of 3 min at 95ºC followed by 30 cycles of 30 sec at 95^0C, 30s at 52ºC and 1 min at 72ºC and a final cycle of 10 min at 72ºC. The amplified products were separated by electrophoresis in 1.2 % agarose gels, stained with ethidium bromide and visualized under UV light. The bands were photographed using gel documentation system (Alpha Imager, USA) and quantified by use of image acquisition and analysis software (UVP, Cambridge UK).

Results

Inter-genotype Differences in Lifespan

The survival frequencies for the 5 and/or 6 year old plant populations of the *LLI EGD, lli EGD, LLI egd* and *lli egd* mutant genotypes in the background of cultivar Nirmal, *LLI EGD* Delhi Pink genotype and F_2 populations having origin in the cross *lli EGD* mutant x Delhi Pink (Cross-1) and *lli egd* double mutant x Delhi Pink (Cross-2) are given in the table 3. The Fig. 2 shows the status of some of the beds planted with plants of varying genotypes, at the time of final counting for estimation of survival frequencies; some dark green and green leaf bearing healthy, yellow leaves bearing senescent and dead plants are depicted. Since the individual beds had healthy, senescent and dead plants adjacent to each other, the environmental factors were probably not the cause for their differential health. It will be seen from the Table 3, that the mutant and wild type counterpart genotypes in the Nirmal background could be arranged in the following order in terms of their survival *LLI egd* (60%) > *lli egd* (50%) > *LLI EGD* (18%) >*lli EGD* (0%). Among the two *LLI EGD*

Table 3: Survival of 5 and 6 years old plants of different genotypes under field crop conditions, in *Catharanthus roseus*

S.No.	Plant material age in years (y)	Parameter	Phenotype					
			LLI	lli	LLI EGD	lli EGD	LLI egd	lli egd
1	F_2 of cross-1[a] (6y)	Number planted	86	28[d]				
		Number survived	24	4				
		% Survival	28	14[f]				
2	F_2 of cross-2[b] (5y)	Number planted	[c]		60	18	20	8[e]
		Number survived			21	3	11	5
		% Survival			35	17	55	62[g]
3	*CPC LLI EGD* Delhi Pink	Number planted			10			
	[Wild type (5y, 6y)]	Number survived			3 (4)[h]			
		% Survival			30 (40)			
4	*cpc LLI EGD* (5y, 6y)	Number planted	11					
		Number survived	2 (3)					
		% Survival	18 (27)					
5	*cpc lli EGD* (5y, 6y)	Number planted		10				
		Number survived		0 (1)				
		% Survival		0 (10)				
6	*cpc LLI egd* (5y, 6y)	Number planted					10	
		Number survived					6 (6)	
		% Survival					60 (60)	
6	*cpc lli egd* (5y, 6y)	Number planted						10
		Number survived						5 (5)
		% Survival						50 (50)

[a]Cross-1, *cpc lli EGD* homozygote in Nirmal background x *CPC LLI EGD* wild-type homozygote Delhi Pink, F_1 phenotype was LLI; [b]Cross-2, *cpc lli egd* homozygote in Nirmal background x Delhi Pink, F_1 phenotype was LLI EGD; [c] Empty space means not applicable; [d]χ^2 for 3 : 1 :: LLI : lli = 0.1 ($P \leq 0.1$); [e]χ^2 for *LLI EGD* : *lli EGD*: *LLI egd*: *lli egd* :: 9:3:3:1= 0.5 ($P<0.1$); [f]χ^2 for equal 28% expected survival % of the two phenotypes = 7, ($P < 0.01$); [g]χ^2 for equal 62% expected survival % for the four phenotypes= 45.3, ($P < 0.001$); [h]survival frequencies in parenthesis pertain to observations in the spring of 2008.

wild type genotypes, survival was higher for Delhi Pink (30%) than for Nirmal (18%). In the F_2 populations, the survival of *LLI* plants was 2 and 1.3 fold higher than *lli* plants in the cross-1 and cross-2, respectively. In the cross-2 survival frequency was 31 and 57% for the *EGD* and *egd* plants respectively. The four F_2 genotypes of the Cross-2 could be arranged in the following order in terms of their survival: *lli egd* (62%) and *LLI egd* (55%)> *LLI EGD* (35%) > *lli EGD* (17%). The nature of interaction between the *lli* and *egd* mutations appeared to be additive.

Feature Correlated with Longer Lifespan

The short term populations of the five genotypes that served as parents of the F_2 populations were compared for their patterns of biomass accumulation and organ-wise allocation and photosynthetic properties (Table 4). The total biomass accumulation was lower in the genotypes that demonstrated higher survival. Thus slow rate of accumulation of biomass was observed to be a trait related to long lifespan.

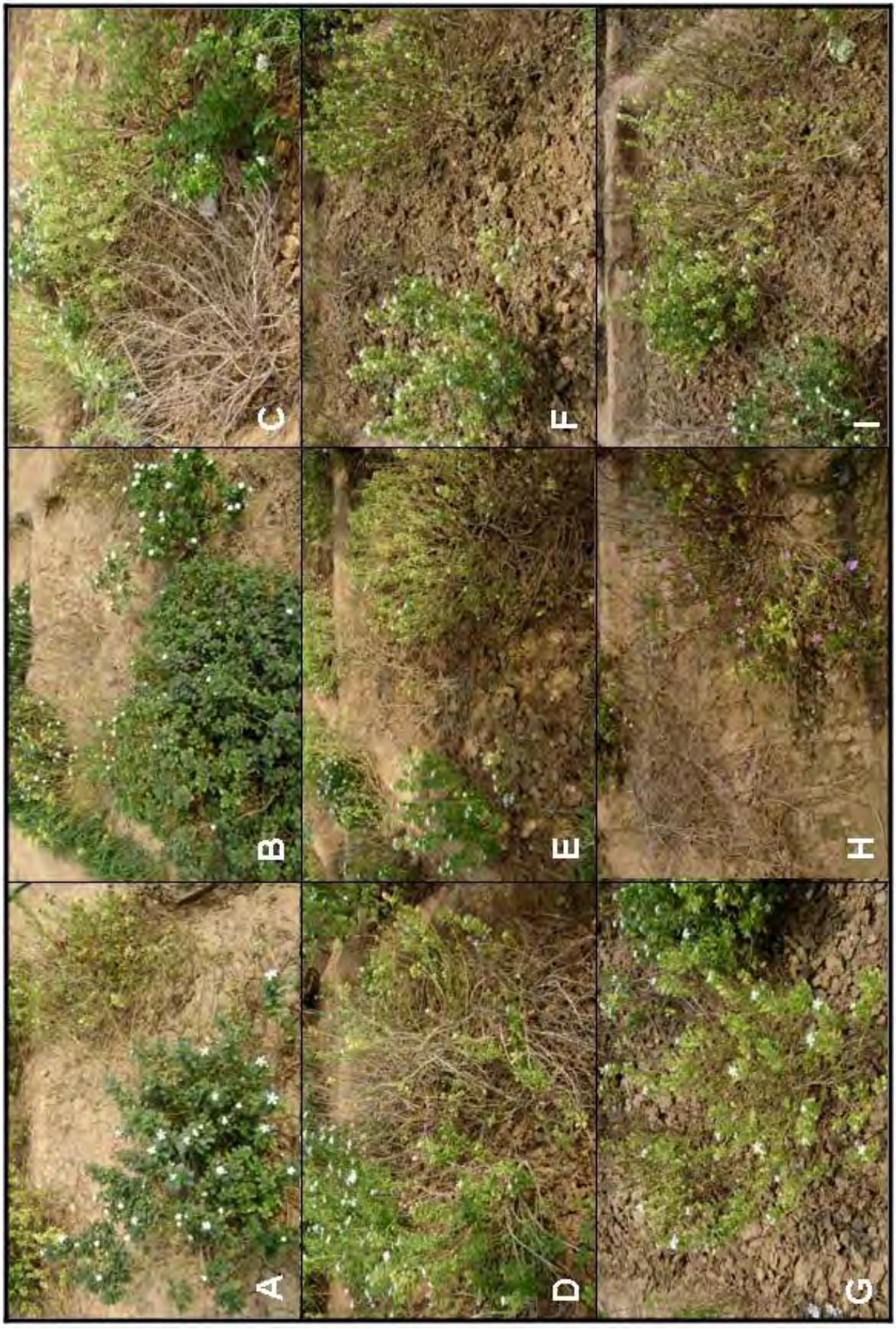

Fig. 2: The surviving and dead F_2 plants of Cross-1 and Cross-2 after 5/6 years of their origin, in *Catharanthus roseus*. (A-C, E, G, I) *LLI egd* and *lli egd* are seen as green to dark green dwarf plants. (B-I) Some dead and dry *LLI EGD* and *lli EGD* plants are seen. (A, C-I) Some yellow leaf bearing plants are seen. (A, B, E-I) Empty spaces that are seen were vacated by plants that had died earlier to the time of taking these pictures. Because the *C. roseus* plantation beds were weeded periodically, *C. roseus* plants competed neither with seeds nor with the seedlings of their own species.

Photosynthesis Related Properties of Senescent Plants

Among the surviving plants, of different genotypes, the senescent plants bore yellow-green to yellow leaves as compared to green to dark green leaves borne on normal/healthy plants. The recurrent observations on individual plants of the seven kinds of populations (five parental genotypes + F_2 segregational populations from two crosses) had shown that in *C. roseus*, senescence in the form of yellow leaves was a prelude to death, which occurred some weeks or months later. In the senescent plants, both the nascent and older leaves were of yellow coloration. The yellow-green or yellow leaves persisted on the senescent host plants for many months. This type of leaf senescence, seen in ageing *C. roseus* plants, is quite distinct from the seasonal leaf senescence of perennial plants whereby all leaves forming the canopy loose their nutrition, breakdown their chlorophyll and fall off within a few weeks [18-20]. Genotype/phenotype-wise comparisons of green, yellow-green and/or yellow leaves for their size, chlorophyll content and photosynthetic rate are presented in the Table 5. It will be seen that on average basis the size of the leaves of senescent plants was reduced to about one third of that of leaves born on healthy plants. Commensurately, the chlorophyll *a* and chlorophyll *b* contents and photosynthetic rates were 4.7, 4.0 and 2.5 fold lower in yellow green and yellow leaved plants as compared to dark green or green leaved plants. Thus, the senescence was reflected in smaller leaf size and lower chlorophyll *a* and *b* contents and photosynthetic rate in senescing plants of all the different genotypes. Because of smaller leaf size and lower photosynthetic rate, the reduction in net photosynthesis in the leaves of senescent plants was magnitudinal.

Chloroplast and Mitochondria Related Nuclear and Organelle Gene Expression

The general loss of chlorophyll and lowering of photosynthetic efficiency in the leaves of senescent plants, seemingly having normal morphological development, indicated defect in chloroplast (cp) activity [21]. The above syndrome of chloroplastic functional deficiency could be related to lowering of cpDNA quantity, in the senescent plants. Therefore cpDNA levels and gene expression patterns of cp-genomes of senescent and healthy plants were compared. The Fig. 3 presents results of the semi-quantitative PCR, using three sets of universal cpDNA primer pairs, on the DNAs extracted from senescent and healthy plants. The DNA levels between the senescent and healthy plants of *lli EGD* and *lli egd* genotypes were indistinguishable. Next, to check the quality of cpDNAs, the expression of selected photosynthesis related genes encoded by cp-genomes were examined in senescent and healthy plants. In parallel, in semi-quantitative RT-PCR assays, the expression patterns of selected photosynthesis related nuclear genes were compared for senescent and healthy plants. The results presented in the Fig. 4A show that the plastid genes *RBCL* (RUBISCO large subunit), *PSBA* (photosystem II (PSII) protein D1) and *PSBW* (photosystem II reaction center W protein) were transcribed similarly in the senescent and healthy plants of *lli EGD* and *lli egd* genotypes. The transcript accumulation levels in the case of nuclear genes for chloroplast functions *NPP* (EU135981, Cr gi:159792897 isopentenyl pyrophosphate), *PSBO* (subunit of oxygen evolving system of PSII), *RBCS* (RUBISCO small subunit) and *LHC B1* (a component of the light harvesting chlorophyll a/b binding protein complex) were found to be (Fig. 4B) lower in the pale leaves of senescent plants than in the leaves of healthy plants of the two genotypes. The transcripts of the non-photosynthesis nuclear gene *ACTIN* appeared to accumulate equally in the leaves of senescent and healthy plants (Fig. 4A, B and 5). Because chloroplast functioning was observed to have been compromised in senescent plants, via subnormal expression of nuclear photosynthetic genes, it was relevant to examine the

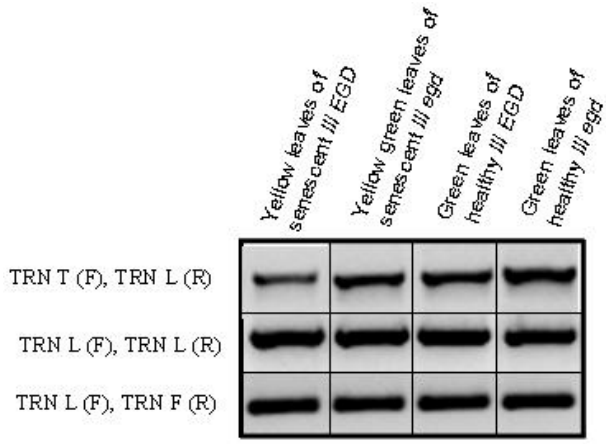

Fig. 3: Semiquantitative PCR analysis of plastid DNA accumulation in the leaves of senescent and healthy plants of *lli EGD* and *lli egd* genotypes. Three pairs of universal plastid DNA primers were used to perform PCR reactions, as given in the material and methods.

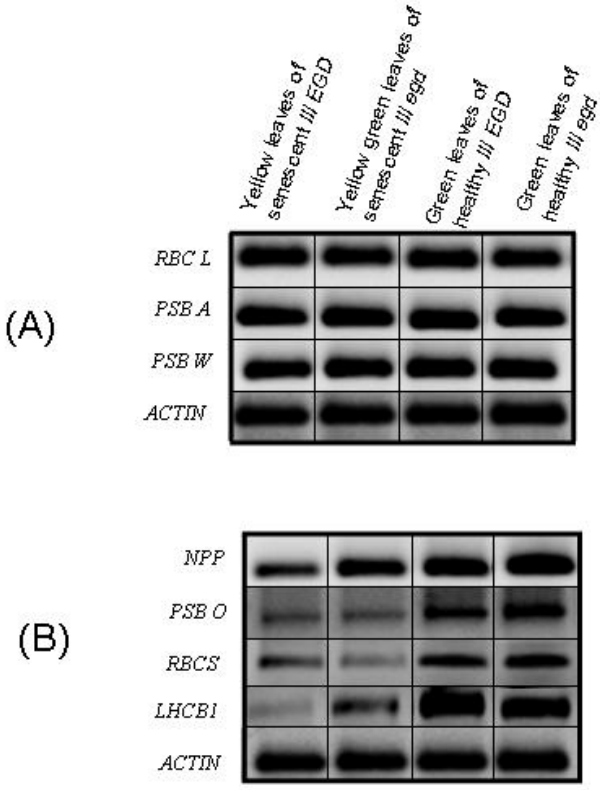

Fig. 4: Semiquantitative RT-PCR analysis of the photosynthesis related transcripts in the leaves of senescent and healthy plants of *lli EGD* and *lli egd* genotypes. The RT-PCR reactions were performed using the primers for the plastidic genes (A) *RBCL, PSBA* and *PSBW* and nuclear genes (B) *NPP, PSBO, RBCS* and *LHCB1* as described in materials and methods section. The *ACTIN* gene primers served as the control. It is seen that the transcript accumulation in respect of the plastidic genes *RBCL, PSBA* and *PSBW* is similar in the pale senescent and healthy green leaves. Contrastingly, the transcript accumulation in respect of the nuclear genes for chloroplast functions, including *PSBO, RBCS* and *LHC B1* is lower in pale senescent leaves as compared to healthy green leaves.

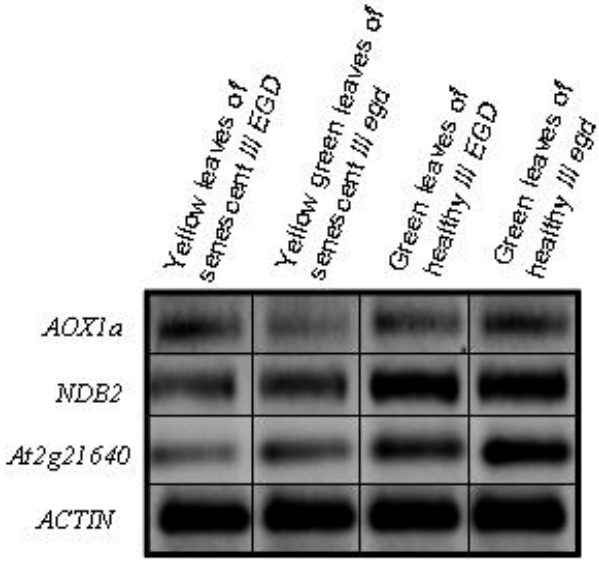

Fig. 5: Semiquantitative RT-PCR analysis of the mitochondria related nuclear transcripts in the leaves of senescent and healthy plants of *lli EGD* and lli egd genotypes, using the primers for the genes *AOX1a, NDB2* and *UPOX* as shown in the material and methods section. The *ACTIN* gene primers served as the control. It is seen that the transcript accumulation in respect of nuclear genes for the mitochondrial functions *AOX1a, NDB2* and *UPOX* is lower in pale senescent leaves as compound to healthy green leaves.

expression of nuclear encoded genes for mitochondrial functions [22], since mitochondria and chloroplasts are both energy converting systems. It was found that the transcripts of mitochondria specific nuclear genes *AOX1a* (alternative oxidase 1a), *NDB2* [NAD(P)H dehydrogenase B2] and *UPOX* (up-regulated by oxidative stress or At2g21640) were accumulated in decreased amounts in the leaves of senescent plants as compared to the leaves of the healthy plants, in *lli EGD* and *lli egd* genotypes (Fig. 5).

Discussion

Out of a total of 271 plants of about 245 overall genotypes, only 84 (31%) plants survived over the 5-6 year period of experiment. These results show that lifespan in the perennial species *C. roseus* is finite. When allelic status at the *LLI* and *EGD* loci is taken into consideration, the lifespan was observed to be least in plants of *lli EGD* genotypes, much longer among plants of *LLI egd* and *lli egd* genotypes and medium in plants of *LLI EGD* genotype. The *LLI* plants survived longer than *lli* plants and *egd* plants had longer lifespan than *EGD* plants. Altogether results showed that in *C. roseus*, *LLI* lengthens lifespan and *EGD* has a shortening effect on lifespan. The effects of *LLI* and *EGD* genes are additive. This observation, that lifespan in perennial plant species is a genetic character, finds support from (a) several instances of hybrids between annual and perennial species having acquired perennial habit [23] and (b) genetical modification of lignin concentration affected fitness and lifespan of the perennial pasture plant species, *Bromus inermis*, *Dactylis glomerata*, *Medicago sativa* and *Panicum virgatum* [24]. The present experiment has identified the two genes *LLI* and *EGD* that regulate lifespan in opposite directions, in *C. roseus*. It is surmised that all the genes that impact fitness, under the variety of environments in which *C. roseus* evolved, may be involved in the determination of lifespan. Accordingly it is further invoked that the degree of perenniality in various perennial plant may be a multigenic, environmental affected and quantitatively determined trait.

Table 4: Vigor reflecting properties of field grown plants of the five genotypes that served as parents of F_2 populations (Table 2), in *Catharanthus roseus*

Genotype[a]	Dry weight (g) in				Photosynthesis[d]	
	root	stem	leaves	whole plant[b]	rate ($\mu mol.m^{-2}.s^{-1}$)	total in a leaf ($\mu mol.s^{-1}.10^{-4}$)
CPC LLI EGD	3.2 ± 0.9	22.6 ± 2.1	22.4 ± 4.8	48.2	26.3 ± 3.0	303 ± 47
cpc LLI EGD	2.8 ± 0.8	23.2 ± 4.5	23.7 ± 5.6	51.7	19.9 ± 3.6	147 ± 52
cpc lli EGD	2.8 ± 1.0	27.0 ± 7.2	26.1 ± 9.3	55.9	19.9 ± 4.5	141 ± 32
cpc LLI egd	2.5 ± 0.5	15.0 ± 4.8	21.5 ± 6.2	39.0	28.3 ± 3.5	298 ± 77
cpc lli egd	2.4 ± 0.4	15.2 ± 6.1	12.0 ± 4.4	29.6	22.1 ± 4.4	177 ± 86
Mean	2.7	20.6	21.1	44.9[c]	23.3	213

[a]Genotype carrying the wild type and mutant alleles of genes whose effects were studied in Cross-1 or Cross-2.
[b]Observations taken on plants of 21 weeks of age (mean ± standard deviation);
[c]Mean of all genotypes for whole plant dry matter;
[d]Observations taken on plants of 18 weeks age.

Table 5: Size, chlorophyll content and photosynthetic rate, of the leaves borne on F_2 generation plants, of different phenotypes/ genotypes, of *Catharanthus roseus* surviving in the field after 5-6 years of planting

S.No.	Genotype : phenotype of sampled plants[a]	Leaf area (mm^2)	Content ($\mu g.g^{-1}$) of dry leaf matter		Photosynthetic rate ($\mu mol.m^{-2}.s^{-1}$)
			Chlorophyll a	Chlorophyll b	
1	*LLI EGD*: green leaves	626 ± 151	1.53 ± 0.21	0.32 ± 0.08	8.8 ± 1.7
2	*LLI EGD*: yellow-green leaves	109 ± 27	0.33 ± 0.13	0.07 ± 0.01	5.0 ± 0.5
3	*lli EGD*: green leaves	405 ± 123	1.48 ± 0.24	0.30 ± 0.09	7.5 ± 1.9
4	*lli EGD*: yellow-green leaves	87 ± 18	0.33 ± 0.03	0.07 ± 0.01	4.3 ± 0.2
5	*lli EGD*: yellow leaves	214 ± 57	0.28 ± 0.04	0.07 ± 0.01	2.8 ± 0.5
6	*LLI egd*: dark-green leaves	348 ± 36	2.30 ± 0.50	0.31 ± 0.11	11.9 ± 1.6
7	*LLI egd*: green leaves	320 ± 42	1.63 ± 0.06	0.28 ± 0.08	8.6 ± 1.9
8	*LLI egd*: yellow-green leaves	79 ± 18	0.44 ± 0.11	0.12 ± 0.02	3.6 ± 1.9
9	*lli egd*: green leaves	199 ± 76	1.30 ± 0.52	0.27 ± 0.12	12.6 ± 2.2
10	*lli egd*: yellow-green leaves	81 ± 20	0.41 ± 0.11	0.04 ± 0.01	4.2 ± 1.1

[a]sample size was 5 or more leaves from 1 to 3 plants depending on availability (mean ± standard deviation).

Our experiment results support the view that perennial plants die of progressive decline of fitness or senescence [3]. The morphological symptoms of severe senescence *in C. roseus* plants, leading to mortality, was that they bore yellow, chlorophyll- and photosynthesis-deficient leaves of small size. The healthy plants bore green, chlorophyll-rich and photosynthetically proficient leaves of large size. The transcripts of the plastid encoded genes *RBCL* and *PSBA* accumulated to the same levels in pale leaves of senescent plants and green leaves of healthy plants. A sharp decrease was observed in the expression of the photosynthesis related nuclear genes *RBCS* and *LHCB1* in the leaves of senescent plants. The observed regression of nuclear genes encoding plastid localized proteins is expected to compromise the development of chloroplasts to their fully functional state in senescent plants. It is known that perturbation in photosynthesis, arising out of multitude of genetical and environmental factors, disturbs retrograde

regulatory signals from chloroplasts that are perhaps essential for the expression of nuclear genes encoding all of the vast majority (> 90%) of the proteins present in the chloroplasts [25-33]. Thus senescence in *C. roseus* plants was accompanied by uncoupling of nuclear and plastid genomes.

The leaves of senescent *C. roseus* plants demonstrated reduced transcript abundance of some of the important nuclear genes for mitochondrial functions that ameliorate oxidative stresses, namely *AOX1a* and *UPOX*. There is evidence for mitochondrion retrograde to nucleus, much like that of chloroplast to nucleus, especially noted when plants are under stress [34-40]. It appears that retrograde mitochondrion signaling to nucleus was also disturbed in the *C. roseus* senescent plants.

In the senescent plants of *C. roseus* the functions of chloroplasts and mitochondria, the two energy converting systems, were apparently subnormal due to uncoupling of the mitochondria-to-nucleus and chloroplast-to-nucleus signaling processes. The onset of senescence leading to mortality may itself be related to the progressive deterioration of coordination between the three genomes-nuclear, mitochondrial and chloroplastic- in *C. roseus*. The consequences will include downturn in nutrient availability, respiration and transpiration adversely affecting the plant health and survival pathways. The downregulation of microRNA miR164, which targets NAC family genes that function in a variety of developmental processes, including boundary formation in separation of primordia for lateral organs in shoot meristem and in lateral root development, has been implicated along with up- and down-regulation of other genes, in senescence related cell death in aged leaves [41].

The general malfunctioning of growth and developmental processes may also be involved in undermining of plant survival. Since lifespan determinancy is a nearly universal feature, genetic factors that modulate lifespan in other organisms might also be involved in lifespan regulation in perennial plants. Genes involved in energy metabolism, protein translation, cell cycle and development and other cellular processes in animals may contribute to lifespan determinacy in plants [42-44]. Regulation of chromatin histone acetylation has been found to be a critical function in ageing pathways of yeast, worms, flies and mouse [45, 46]. Deacetylation of the histones H4K16 and H3K9 by sirtuins (*SIR* gene products) promotes genetic stability and thereby longer lifespan and histone acetyltransferases (*SAS* gene products), which antagonize the activity of sirtuins, reduce lifespan in eukaryotic systems [45, 47-49]. The epigenetic regulators of gene expression may indeed determine lifespan in perennial plants. Whether *lli* and *egd* mutations affect chromatin structure in *C. roseus* remains to be investigated.

Conclusions

Present work shows that lifespan of *C. roseus* is finite. It is increased by the *LLI* gene and decreased by the *EGD* gene. Senescence in the dying plants is related to the uncoupling of chloroplastic, mitochondrial and nuclear functions. The phenotypes of the genotypes with longer lifespan on the one hand and shorter lifespan on the other hand indicated that slow growth and limited branching of racemose inflorescence favoured longer lifespan.

C. roseus shares several perenniality related features with forest trees: developmental and seasonal transitions, branched indeterminate inflorescence [11], ability to respond to biotic and abiotic stresses [10, 50, 51] and radial secondary growth from vascular cambium leading to formation of secondary phloem and xylem tissues (bark and wood) [13; unpublished observation]. Therefore the question arises whether the conclusions arrived at about perenniality in the present case study with *C. roseus* can be extended to the perennial plant species in general? The observations on *C. roseus* in the present study suggest the following overall idea about the perennial plant species in general - the lifespan is finite and its length and genetic regulation are species specific.

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