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RESEARCH ARTICLE

Pleiotropic phenotypes of the salt-tolerant and cytosine hypomethylated *leafless inflorescence*, *evergreen dwarf* and *irregular leaf lamina* mutants of *Catharanthus roseus* possessing Mendelian inheritance

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Abstract

In *Catharanthus roseus*, three morphological cum salt-tolerant chemically induced mutants of Mendelian inheritance and their wild-type parent cv Nirmal were characterized for overall cytosine methylation at DNA repeats, expression of 119 protein-coding and seven miRNA-coding genes and 50 quantitative traits. The mutants, named after their principal morphological feature(s), were *leafless inflorescence* (*lli*), *evergreen dwarf* (*egd*) and *irregular leaf lamina* (*ill*). The Southern-blot analysis of *Msp*I digested DNAs of mutants probed with centromeric and 5S and 18S rDNA probes indicated that, in comparison to wild type, the mutants were extensively demethylated at cytosine sites. Among the 126 genes investigated for transcriptional expression, 85 were upregulated and 41 were downregulated in mutants. All of the five genes known to be stress responsive had increased expression in mutants. Several miRNA genes showed either increased or decreased expression in mutants. The *C. roseus* counterparts of *CMT3*, *DRM2* and *RDR2* were downregulated in mutants. Among the cell, organ and plant size, photosynthesis and metabolism related traits studied, 28 traits were similarly affected in mutants as compared to wild type. Each of the mutants also expressed some traits distinctively. The *egd* mutant possessed superior photosynthesis and water retention abilities. Biomass was hyperaccumulated in roots, stems, leaves and seeds of the *lli* mutant. The *ill* mutant was richest in the pharmaceutical alkaloids catharanthine, vindoline, vincristine and vinblastine. The nature of mutations, origins of mutant phenotypes and evolutionary importance of these mutants are discussed.

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Introduction

Phenotypic variation in populations of eukaryotes arises in part from the superimposition of epigenetic variation over genetic variation (Kumar *et al.* 2013). In eukaryotic individuals, the genetic information is contained in the sequences of bases in DNA; the epigenetic information consists of post-translational modifications in histones that comprise nucleosomes together with nuclear DNA in chromatin and methylation of cytosines in DNA. Both histone and cytosine modifications occur by enzymatic mechanisms that are genetic and widely conserved (Law and Jacobsen 2010; Zemach *et al.* 2010; Deal and Henikoff 2011; He *et al.* 2011; Lauria and Rossi 2011; Margueron and Reinberg 2011). In plants, cytosine methylation occurs in three contexts in DNA, CG, CHG and CHH (where H = A, T or C). Present understanding of epigenetic mechanisms in plants is largely due to analysis in *Arabidopsis thaliana, Oryza sativa* and *Zea mays* (Chandler 2010; Garcia-Aguilar *et al*. 2010; Yan *et al*. 2010; Bauer and Fischer 2011; Raissig *et al*. 2011; Schmitz *et al*. 2011; Ikeda 2012; Xiao 2012).

In plants, cytosine methylation is established principally by a small interfering RNA (siRNA) based mechanism termed RNA directed DNA methylation (RdDM) (Kumar *et al.* 2013). In this process, *DOMAINS REARRANGED METHYLTRANSFERASE 2* (*DRM2*), guided by a complex of interacting factors and homology of siRNAs to the target DNA sequences, methylate cytosines in DNA in all of the above-mentioned three contexts (Law and Jacobsen 2010;

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Haag and Pikaard 2011; He *et al.* 2011; Kanno and Habu 2011; Wierzbicki *et al.* 2012). During DNA replication in cell divisions, the newly synthesized strand is cytosine methylated at the symmetric sites CG and CHG by the *METHYLTRANSFERASE 1* (*MET1*) and *CHROMOMETHYLASE 3* (*CMT3*), respectively. The RdDM pathway maintains cytosine methylation in CHH elements (Lindroth *et al.* 2001; Cao *et al.* 2003; Aufsatz *et al.* 2004; Chan *et al.* 2005; Woo and Richards 2008; Saze *et al.* 2012; Zubko *et al.* 2012). There is cooperative interaction between DRM2, CMT3 and MET1 on one hand and specific histone proteins on the other, for the recruitment of methyltransferases to the DNA sites requiring *de novo* or maintenance propagation of methylation marks (Jackson *et al.* 2002; Lindroth *et al.* 2004; Woo *et al.* 2007; Woo and Richards 2008; Chodavarapu *et al.* 2010; Deleris *et al.* 2010; Zubko *et al.* 2012).

Each genetic locus can have many epialleles because only rarely are all the cytosines sensitive to methylation methylated altogether. Epialleles of the various genetic loci arise by gain or loss of methylation at cytosines. The processes of methylation establishment and maintenance at cytosines are imperfect (Zhu 2009). Spontaneous deamination of methylated cytosines leads to base sequence mutations (Pfeifer 2006; Walsh and Xu 2006; Ossowski *et al.* 2010). DNA is also actively demethylated by several demethylases: *REPRESSOR OF SILENCING 1 (ROS1)*, *DEMETER* (*DME*), *DEMETER-LIKE 2* (*DML2*) and *DML3* (Choi *et al.* 2002; Gong *et al.* 2002; Penterman *et al.* 2007; Ortega-Galisteo *et al.* 2008). The glycosylase-cum-lyase activity of the demethylases removes methylcytosine as a free base such that a gap is created in the phosphodiester backbone which is filled up by the DNA repair pathway(s) (Bhutani *et al.* 2011). An epiallele, once established, is inherited through mitoses and meioses until changed (Vaughn *et al*. 2007, Huff and Zilberman 2012). The cytosine methylation marks over specific genes may change tissuewise/organwise as per the developmental programme of the plant, while remaining intact in the germline stem cells (Sha *et al.* 2005; Brown *et al.* 2008; Lu *et al.* 2008; Jullien and Berger 2010; Bauer and Fischer 2011; Schmitz *et al.* 2011; Jiang and Kohler 2012). Cytosine methylation patterns over loci also respond to environmental changes. Exposure to harsh environments may lead to widespread changes in the methylation patterns, affecting the expression of coding-genes and of transposons (Mirouze *et al.* 2009; Dowen *et al.* 2012; Luna *et al.* 2012; Nosaka *et al.* 2012; Slaughter *et al.* 2012). Loss of methylation from transposons leads to their activation, thereby transcription from their promoters leads to read out of adjacent genes and transpositions (Kashkush *et al.* 2003; Vitte and Bennetzen 2006; Slotkin and Martienssen 2007; Lisch 2009; Bennetzen and Zhu 2011; Nosaka *et al.* 2012). Analysis of correlations between changes in cytosine methylation patterns of genes and physiological response by changes in gene expression, following exposure to stress, is an active area of research in plants.

In *A. thaliana*, *met1* and *drm1 drm2 cmt3* mutants have been observed to be heritably tolerant to a virulent strains of *Pseudomonas syringae* and *Hyaloperonospora arabidopsidis*, much like the F_1 progeny of wild-type *A. thaliana* exposed to avirulent or virulent strains of *P. syringae* or β-aminobutyric acid, suggesting correlation of hypomethylation with the synthesis of protective proteins, RNAs and metabolites (Dowen *et al*. 2012; Luna *et al*. 2012; Slaughter *et al*. 2012). Herbivorous damage in *Solanum lycopersicon* and *Taraxacum officinale* (Verhoeven *et al.* 2010; Rasmann *et al.* 2012), salinity stress in *Oryza sativa*, *Glycine max*, *Nicotiana tabacum* and *Laguncularia racemosa* (Wada *et al.* 2004; Choi and Sano 2007; Lira-Medeiros *et al.* 2010; Karan *et al.* 2012; Song *et al.* 2012), heavy metal stress in *Trifolium repens* and *Linum usitatissimum* (Alina *et al.* 2004) and low temperature stress in *Antirrhinum majus* and *Z. mays* (Steward *et al.* 2002; Hashida *et al.* 2006) also led to wide hypomethylation together with adaptive response. Contrarywise, *Pinus silvestris* exposed to ionizing radiations (Kovalchuk *et al.* 2003) and salt-stressed *Mesenbryanthemum crystallinum* (Dyachenko *et al.* 2006) demonstrated hypermethylation. There has been scarcity of comparisons between mutants compromised in cytosine methylation and isogenic wild types stressed biotically or abiotically, outside of *A. thaliana*, in relating transgenerational inheritance of stress response with changes in DNA methylation. The present work extends this area of investigation to *C. roseus*.

C. roseus ($2n$ = 16; 738 Mbp) of Apocynaceae, a medicinal-cum-floricultural plant species, has been developed as a genetic system for the analyses of gene regulatory network involved in secondary metabolism (Mishra and Kumar 2000; van der Heijden *et al.* 2004; El-Sayed and Verpoorte 2007; Guirimand *et al.* 2010; Sharma *et al.* 2012a, b). In this species, certain salt-tolerant mutants displayed conspicuous morphological alterations (Rai *et al.* 2003; Kulkarni *et al.* 2003; Kumar *et al.* 2007, 2012; Kumari *et al.* 2010; Chaudhary *et al.* 2011; Kumar and Sharma 2012). Three of the mutants of this category were *leafless inflorescence* (*lli*), *evergreen dwarf* (*egd*) and *irregular leaf lamina* (*ill*), wherein the salt tolerance cum altered morphology were inherited together in Mendelian fashion. On the basis of earlier work on several plant species on transgenerational inheritance of epigenetic adaptation to stress conditions, it was desired to describe their characteristics in some detail. Questions about the three mutants addressed in the present work were: whether they were (i) deficient in DNA methylation; (ii) altered in the expression of genes involved in the performance of diverse plant functions; and (iii) possessing other phenotypes. The *lli*, *egd* and *ill* single mutants and *lli egd*, *lli ill* and *egd ill* double mutants were compared with wild type with respect to DNA methylation at repeat sequences, expression of 126 genes and phenotypes for 48 traits. It was found that mutants had highly pleiotropic phenotypes, demonstrated differential patterns of gene expression and were relatively demethylated in DNA.

Materials and methods

Plant material

The homozygous genotypes *egd* (*evergreen dwarf*), *lli* (*leafless inflorescence*) and *ill* (*irregular leaf lamina*) are respectively ethyl methanesulphonate and nitrosomethylurea-induced Mendelian recessive mutants of the wild type (WT) medicinal cultivar 'Nirmal' of *C. roseus* (figure 1). The mutants *egd* and *lli* are respectively the *gsr1* and *gsr8* mutants (*gsr* = *glycophytic salinity response* (Rai *et al.* 2003; Kumar *et al.* 2007)); these were isolated as M_2 seedlings that germinated in the presence of 250 mM NaCl. At 250 mM NaCl concentration, in a test none out of 3×10^3 seeds of 'Nirmal' had germinated. The mutants *gsr1* and *gsr8* were renamed after their most conspicuous morphological phenotype (Kumari *et al.* 2010). The *ill* mutant was isolated as a leaf morphology variant (Kulkarni *et al.* 1999, 2003) and subsequently found to share the *gsr* phenotype with *egd* and *lli* mutants. The details of procedures for the isolation of *gsr* mutants on the basis of their salt tolerance phenotype and testing of their drought tolerance are described in Rai *et al.* (2003). The double mutants *egd lli*, *egd ill* and *lli ill* were isolated on the basis of their respective evergreen dwarf-cum-leafless inflorescence, evergreen dwarf-cum-irregular leaf lamina and leafless inflorescence-cum-irregular leaf lamina morphologies from among the F_2 generation segregants in *lli* × *egd*, *egd* × *ill* and *lli* × *ill* crosses. The single and double mutants have been maintained by selfing for several generations before their characterization in the present experiments.

Seedlings of WT 'Nirmal' and each of the three single and three double mutants were raised in nursery and subsequently planted in field in a completely randomized design with five replications ($n = 5$). Nursery, field planting and crop husbandry procedures were the same as described earlier (Mishra *et al*. 2001; Singh *et al*. 2008; Chaudhary *et al*. 2011; Sharma *et al*. 2012a, b). Field experiments were laid in 2008, 2009 and 2010 in the same design and in the same plot at the NIPGR's experimental farm at New Delhi, India. There were 10 plants per replication. All plants were labelled. Three randomly labelled plants/replication from the 2009 experiment served as resource for the leaf material for the DNA and gene expression analyses. For such analysis, young leaves borne at shoot tips were harvested, frozen and used immediately/stored at −80°C as per the experimental requirements. Among the remaining plants of 2009 season and from 2008 and 2010 field experiment, three plants per genotype per replication were sampled for biomass measurements and analysis of K^+ and Na^+ contents. The yearwise observations from field experiments were averaged replication-wise. The remainder of the plants were sampled for organ size measurements.

Genotypes were also grown in clay pots of 75-cm diameter in the years 2009 and 2010. Nursery and planting

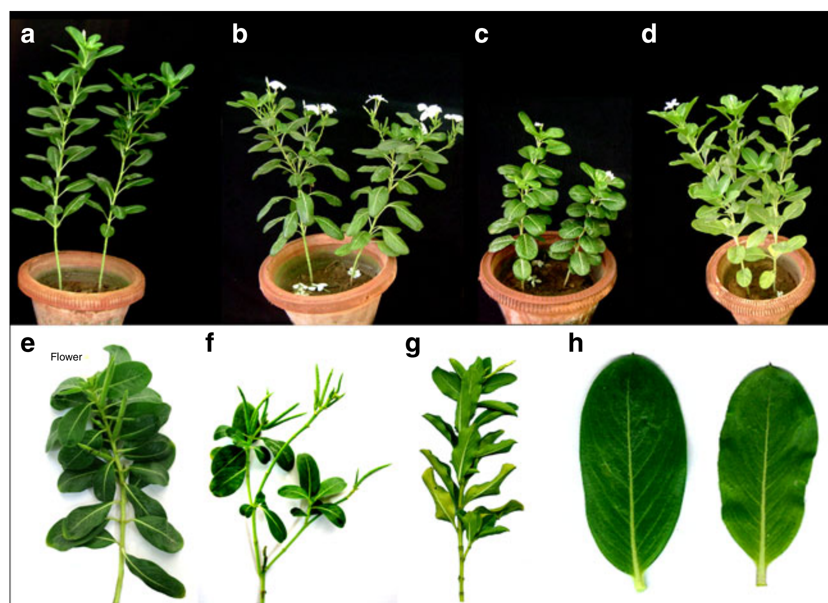

Figure 1. Wild type and salt-tolerant cum morphological mutants of Mendelian inheritance in the *C. roseus* cv Nirmal genetic background. (a), Wild type (WT, cv Nirmal); (b), *leafless inflorescence* (*lli*); (c), *evergreen dwarf* (*egd*); (d), *irregular leaf lamina* (*ill*); (e), a fruiting primary stem of WT; (f), a fruiting primary stem bearing secondary and tertiary branches of *lli*; (g), a stem of *ill* in which the irregular leaf lamina feature is clearly visualized from sides; (h), front views of a wild type (left) and a *ill* leaf (right).

procedures were same as in the field. Number of pots per replication per genotype was 10. Each pot was transplanted with four seedlings. Number of replications varied as per the experiment. The labelled pots were kept experiment-wise in a field plot randomly and were husbanded on alternate days as per the requirements of the experiment. The leaf fresh weight, photosynthetic and leaf histological measurements, water, trehalose, proline, chlorophyll and alkaloid content assays and determinations of time for 50% water loss were conducted on pot grown plants.

Samples were taken from flowering plants that had attained the age of 18–21 weeks from the time of seed germination.

Procedure for Southern blot hybridization

Total genomic DNA was isolated from 500 mg fresh leaves of *C. roseus* using CTAB (cetyl-trimethyl-ammonium bromide) method (Saghai-Maroof *et al.* 1984). The leaf powder was incubated with extraction buffer (100 mM Tris-Cl, 25 mM EDTA, 1.5 M NaCl, 2.5% CTAB, 1% poly vinyl pyrrolidone and 0.2% β-mercaptoethanol) at 60°C for 2 h and this treatment was followed by chloroform:isoamyl purification. After ethanol precipitation, DNA was dissolved in 0.1 M Tris-EDTA buffer (0.1 M Tris-Cl and 0.01 M EDTA, pH 8.0) and checked on 0.8% agarose with ethidium bromide staining and quantified spectrophotometrically. Ten microgram of genomic DNA was digested with 5 μg^{-1} DNA units *Msp*I (New England Biolab, Beverly, USA) enzyme (Wada *et al.* 2004) and the digest was size fractionated by electrophoresis on 0.8% agarose gel. The gel was sequentially treated with each of depurination, denaturation and neutralization buffers (pH 7.4) (Sambrook *et al.* 1989) at room temperature for 10, 30 and 30 min, respectively. The gel was subjected to alkali method of transfer to Hybond N^+ membranes (Amersham Pharmacia Biotech, Sweden). DNA present on the membrane was hybridized with [α-^{32}P-dCTP]-labelled DNA probes (20 μCi/μL; Megaprime DNA labelling system, Amersham Pharmacia Biotech). The membrane was washed under high-stringency condition at 65°C, autoradiographed, incubated at −80°C for 5–6 days and the image was taken with Gel Doc system, Los Angeles, USA. To study methylation in ribosomal DNA, 18S rRNA probe was synthesized by using the 5′-GGCTTCGGGATCGGAGTAAT-3′ (forward) and 5′-CAAATTAAGCCGCAGGCTCC-3′ (reverse) primers (primers had been designed after http://www.ncbi.nlm.nih.gov/tools/primer-blast/). The 294 bp PCR product from 18S rDNA was purified and used as probe. For 5S rDNA the pUC18 plasmid carrying the 5S rRNA gene of *Lupinus luteus* was deployed as the probe (Rafalski *et al.* 1982). The centomeric region was amplified using 5′-CATATTCGACTCCAAAACACTAACC-3′ (forward) and 5′-AGAAGATACAAAGCCAAAGACTCAT-3′ (reverse) primers (Nagaki *et al.* 2003) to obtain a 200 bp probe. Southern blot hybridization experiment was repeated twice.

Gene expression analysis

Total RNA was isolated from leaves using RNeasy plant mini kit (Qiagen, Hilden, Germany). The quality of total RNA was checked on 1.5% denaturing formaldehyde agarose gel (Dutta *et al.* 2005; Tan 2010). The miRNA was isolated with mirVana^{TM} isolation kit (Ambion, USA). The first strand cDNA was synthesized with 2 μg of each total RNA and miRNA using cDNA synthesis kit (Fermentas Life Sciences, Massachusetts, USA). miRNA was subjected to poly(A) tailing kit (Ambion, USA) before cDNA synthesis (Zhu *et al.* 2010). The cDNA from total RNA and microRNA were equalized with *ACTIN* and *UBIQUITIN* as control genes. Primers for expression analysis of hypomethylated genes in *C. roseus* were designed using Pimer-Blast (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) (Ratcliffe *et al.* 2003; Tan 2010). The primer sequences used for miRNA amplification were described in Kim and Sung (2010) and Zhu *et al.* (2010). The PCR cycle conditions for semiquantitative RT-PCR were 95°C for 3 min, 94°C 30 s, 52°C 30 s, 72°C 1 min (35×) and 72°C for 10 min. The amplification conditions (t_m) were varied with primers. After amplification, the PCR product was separated on 1.5% agarose gel with ethidium bromide stain. The image and the intensity of the PCR products in the gel were taken using gel documentation system (Alpha Imager, San Legendra, USA) and quantified by use of image acquisition and analysis software (UVP, Cambridge, UK). The list of primer sequences is given in table 1 in electronic supplementary material at http://www.ias.ac.in/jgenet/. This table also provides PCR conditions optimized genewise for the annealing temperature and number of amplification cycles to obtain PCR products in high intensities.

Microscopy and photography

To estimate their dimensions, the cells, tissues and organs, in sections or whole mounts, were examined and photographed microscopically at 4×, 10× and/or 40× magnification(s). Simultaneously, pictures of a micrometre were also taken. The microscope used was Nikon E100 and the digital camera attached to microscope was Nikon 8400. The pictures of cells/tissues/organs were printed together with those of the micrometre on mm^2 graph paper. The dimensions were determined by counting the squares calibrated by the micrometre.

Germination tests

Ten seeds per replication were germinated on filter paper irrigated with 100 mM NaCl in a Petri dish at 37°C. Experiment was replicated thrice ($n = 3$). After three weeks, seedlings were weighed replication-wise.

Biomass measurements

Field grown plants were excavated along with their root system. From the plants sampled from a replication, roots, stems

and leaves (+ flowers and fruits) were separated and placed in separate paper bags. The material was dried (at 80°C for 30 min, at 37°C for two days and room temperature for several weeks) and weighed organ-wise.

Organ biomass and dimension measurements

Fresh weight of a leaf was determined by weighing 25 leaves/replications taken from pot experiment. The area of the leaf, lamina and length of petiole were measured with the help of scanned pictures taken on mm^2 graph paper. Flower pedicel, whole flowers, sepals, petals, corolla tubes, gynoecium styles, ovaries were traced on mm^2 graph paper to estimate their sizes. The sample size was five leaves of flowers per replication from field grown plants. The microscopic pictures of dry seeds were used to measure the area of the seeds. Seeds were taken from a pot experiment. Sample size was five seeds/replication.

Time period taken for 50% loss of water (h)

To determine the leaf dryness rate, 15 leaves (fresh) were taken and their initial weight was measured. They were allowed to dry at the room temperature and their weight was measured every 3 h. The process of drying was done until the weight of the leaf sample became constant. The two parameters measured were time required for 50% reduction of water content and total water content.

Determination of leaf water content in normal and stressed plants

There were three treatments per genotype–normal irrigation, three weeks withdrawal of irrigation and four weeks irrigation with 100 mM NaCl (saline) water. Twenty-five fresh leaves per replication per treatment per genotype were allowed to dry at 80°C for 30 min followed by 30°C until weight became constant.

Histological measurements

To study the epidermis, leaves fixed in 70% alcohol were incubated in phenol:lactic acid:glycerol:water::1:1:1:1 mixture for 15 min at 90°C, transferred to 20% glycerol and examined microscopically with safranin staining. Pictures taken at different magnifications were used for obtaining the area of the pavement cell and number of stomata per unit area. The leaves were sectioned transversely and safranin stained sections were photographed and pictures were used to estimate the mesophyll parenchyma cell dimensions and adaxial–abaxial thickness. Photographs of micrometre and of epidermis and sections taken at different magnifications were printed on graph paper to estimate the size of cells and tissues and frequencies of stomata etc. (Sharma *et al.* 2012a). Leaf samples were taken from pot experiments.

Methodology of elemental analysis

Leaf sample was dried at 70°C for 48 h and ground in Willey mill, and 0.25 g was digested in concentrated $H_2SO_4 + H_2O_2$ on block digester under controlled temperature (till the plant material + acid became colourless). The digest was cooled and diluted to a volume of 100 mL. The acid digest was subjected to flame photometer (Systronics Model 128) against known standard of Na and K (Piper 1967) for the Na^+ and K^+ content measurements.

Measurement of photosynthetic rate and chlorophyll contents in leaves

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 metre square of leaf area per second ($\mu mol.m^{-2}.s^{-1}$) and total photosynthesis in leaves as μmol CO_2 utilized per second ($\mu mol.s^{-1}$). The latter was calculated by multiplying the rate with leaf area (Sharma *et al.* 2012b). Chlorophyll 'a', chlorophyll 'b' and total chlorophyll contents in the leaves were estimated using the Arnon (1949) method.

Estimation of alkaloid, proline and trehalose contents

Alkaloids present in the *C. roseus* were extracted organ-wise and quantified by the method described by Singh *et al.* (2004, 2008). Proline and trehalose contents in leaf samples were determined respectively by the methods described by Bates *et al.* (1973) and Mahmud *et al.* (2009). Leaf samples were resourced from separate pot experiments.

Statistical procedures

Statistical analyses were carried out by various modules of the software SPSS 16.0 (SPSS, Chicago, USA). Analysis of variance (ANOVA) was used to reveal the genetic and genotype × environment components of phenotypic variation. Associations between traits were examined by Pearson's phenotypic correlation analysis.

Results

Correlation between morphological phenotype and salt tolerance in lli, egd, ill, lli egd, lli ill and egd ill mutants

The principal morphological alterations recorded in the *lli* mutants are extensive terminal branching and absence of leaves from flowering nodes. These are dwarfness, evergreen foliage and late flowering habit in the *egd* mutant. The *ill* mutant demonstrates many undulations in leaf lamina seen from the sides of margin. The seeds of all the three mutants are known to germinate in the presence of up to 250 mM

NaCl. The double mutants were recovered as segregants in F_2 generation from three two-way crosses. Seven plants of *lli* and *egd* morphologies were identified among 103 F_2 progeny plants of the *lli* × *egd* cross. Among 88 F_2 plants from the *lli* × *ill* cross, five plants possessing both *lli* and *ill* morphologies were isolated. Four plants of *ill* and *egd* morphologies became available from among 72 F_2 plants of the *egd* × *ill* cross. All the 16 double mutant plants were selfed to obtain F_3 seeds. A part of F_3 seeds of each double mutant isolate was tested for germination in the presence of 250 mM NaCl. Unlike the wild type, and like *lli*, *egd* and *ill*, all the double mutants proved to be salt tolerant. These results demonstrated correlation between the morphological phenotype and salinity tolerance phenotype in *lli*, *egd*, *ill* and *lli egd*, *egd ill* and *lli ill* segregants isolated from intermutant crosses. One representative double mutant plant isolated from each cross was carried forward via selfing over subsequent generations for further characterization. Observations are presented in table 1 on fresh weight of seedlings germinated in the presence of 100 mM NaCl in Petri dishes under room temperature in dark and K^+/Na^+ ratios in leaves of field grown plants, in wild type and six mutants. It will be seen that mutant leaves generally had higher K^+/Na^+ ratios than wild type. The susceptibility of wild-type seeds to salt stress led to poor growth (lower mass) in their seedlings as compared to mutants. These observations showed quantitative differences in response to salt in wild type and mutants. The correlation between morphological alteration and salinity tolerance in each of the *lli*, *egd* and *ill* mutants could be either due to one or two lesions that are very closely linked to each other, distinct in each mutant.

Reduction of genomic DNA methylation in mutants

Salt tolerance has been earlier reported to be associated with genome-wide DNA demethylation in several plant species. To test whether *C. roseus* morphological-cum-salinity tolerant mutants were also deficient in cytosine methylation, *Msp*I digested DNAs of each of the mutants and wild type were probed with sequences from 5S and 18S rDNA and centromeric DNA using Southern blot analysis. It will be seen from figure 2 that the mutants differed from wild type in intensity and diversity of *Msp*I sensitive sites. The mutants had greater distribution of *Msp*I sensitive sites presumably due to loss of methylation from cytosines at these sites distributed over chromosomes. Both centromere and rDNAs represent major locations of repeat sequences in chromosomes. Thus, the results suggest deficiency of methylated cytosines at global level in the genomes of the single and double mutants.

Comparative gene expression profiles of mutants and wild type

RT-PCR was used to estimate transcript levels for a total of 126 genes in the leaves of seven genotypes. Among the genes, whose expression levels were studied, five were known to be stress response genes, seven were microRNA genes, 17 concerned chromatin modelling and cytosine methylation, 82 were known to participate in the plant development processes and 15 determined the terpenoid indole alkaloid metabolism. The objective of gene expression profiling was to find out whether the *lli*, *egd* and *ill* mutations affected gene expression in positive or negative direction (figure 3).

Table 1. Salinity tolerance characteristics of the wild type, *lli*, *egd*, *ill*, *lli egd*, *lli ill* and *egd ill* homozygous genotypes of common genetic background in *C. roseus*.

Genotype	Fresh weight of seedlings germinated in the presence of 100 mM NaCl in Petri dishes	K^+/Na^+ ratio in the leaves of field grown plants
WT	0.20 ± 0.07^a	2.2 ± 0.3^a
lli	10.32 ± 1.78^b	4.2 ± 0.8^b
egd	8.60 ± 0.78^b	3.7 ± 0.6^{ab}
ill	9.54 ± 0.76^b	3.7 ± 0.8^{ab}
lli egd	9.52 ± 0.53^b	5.5 ± 0.4^b
lli ill	9.44 ± 0.53^b	4.9 ± 0.1^b
egd ill	9.72 ± 0.40^b	4.8 ± 0.4^b
Mean of all genotypes	8.19 ± 1.35	4.2 ± 0.4
F value	17.5**	4.2**
CD 5%	2.47	1.54
CD 1%	3.33	2.08

** Significant at 1% probability level.
[a, b]For a character, the values that do not have the same letter as superscript are different.

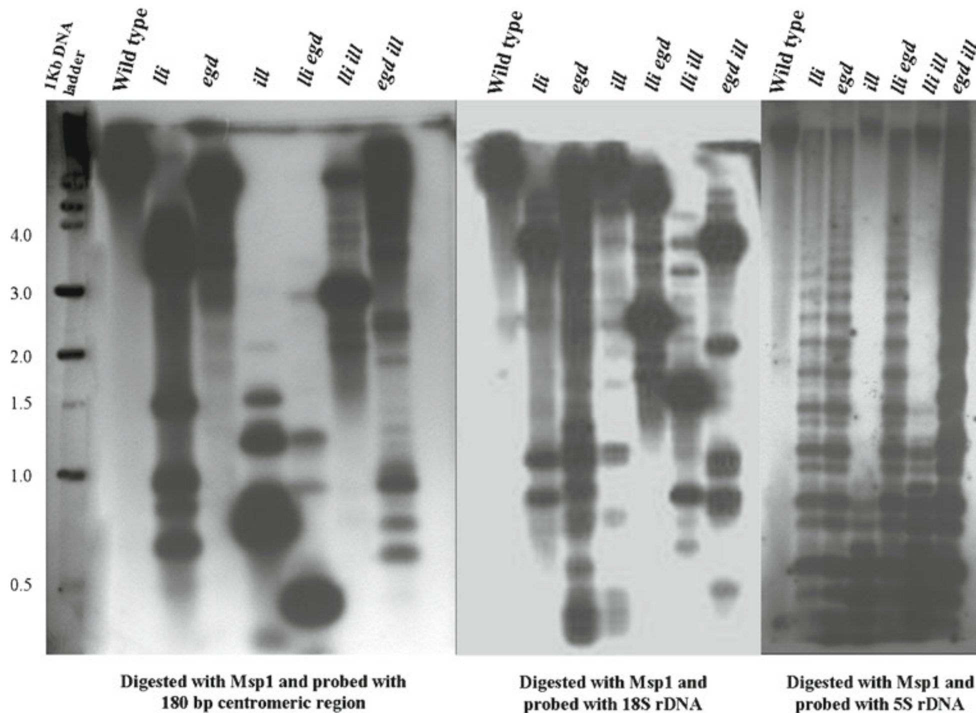

Figure 2. Loss of cytosine methylation at centromere-, 5S- and 18S-rDNA repeat sequences in *lli*, *egd* and *ill* mutants in *C. roseus*. *Msp*I digested DNAs of wild type, three single and three double mutants, were hybridized to each of 5S and 18S rDNA and centromeric DNA probes.

For each of the gene surveyed, the transcript level observed for the wild type was taken as one and the transcript levels in mutants were expressed in relation to this value. In all, 85 genes were observed to be upregulated and 41 genes downregulated in mutants. These observations are presented in tables 2 and 3. In general, mutants demonstrated epistasis over each other in gene expression. Interestingly, all five genes known to respond to abiotic stress were observed to have upregulated expression in mutants (table 2). These genes were orthologs of the *COR15A*, *DREB1A*, and 2*A*, *OSMOTIN* and *RD29A* genes of *A. thaliana*. Nine genes were nearly five-fold or more upregulated in mutant genotypes: *DREB1A*, *RD29A*, *miR171*, *miR159*, *REF6*, *ORCA3*, *LEC2*, *PRF* and *SLS* counterparts of *A. thaliana* in *C. roseus*. The *C. roseus* orthologues of *DRM2*, *RDR2* and *DRD1* genes of *A. thaliana* were found to be downregulated in mutants (table 3).

Gene	Genotype						
	WT	*lli*	*egd*	*ill*	*lli egd*	*lli ill*	*egd ill*
E/L/TH							
LEC2							
miR 395c							
MBD8							
KAN							
RDR2							
ACTIN							

Figure 3. Gene expression levels examined by semiquantitative RT-PCR. Some representative results with upregulated and downregulated genes are shown. *ACTIN* served as the control gene in these experiments. Full names of the genes are given in tables 2 and 3.

Pleiotropic effects of lli, egd and ill mutations on quantitative traits

Morphological changes in each of the *lli*, *egd* and *ill* mutants, extensive loss of cytosine methylation sites and associated large differences in the expression of genes relating to diverse functions, indicated that the mutants may pleiotropically affect a wide variety of traits. Therefore, observations were recorded on 48 traits. To reveal the effect of *lli*, *egd* and *ill* mutations on a trait, the observation on the seven genotypes (wild type, three mutants and three double mutants) were compared genotype-wise as well as mutation-wise (effect of say the mutation *lli* on a parameter = measurements on *lli* + *lli egd* + *lli ill*/3). ANOVA was applied to observations on each trait for deducing the significance of observed effects. For the purposes of ease of presentation, the traits seemingly related to each other were grouped together.

Table 2. Genes whose expression was upregulated in *lli*, *egd*, *ill*, *lli egd*, *egd ill* and *lli ill* mutants as compared to the wild type in *C. roseus*.

Broad function	Gene	Level of upregulation (× fold) with respect to wild type level (1.00) in					
		lli	*egd*	*ill*	*lli egd*	*lli ill*	*egd ill*
Abiotic stress response	COLD REGULATED 15A (COR15A)	1.38	5.23	5.37	4.61	4.68	4.73
	DEHYDRATION-RESPONSIVE ELEMENT-BINDING 1A (DREB1A)	5.02	6.31	6.64	2.17	6.88	6.73
	DEHYDRATION-RESPONSIVE ELEMENT-BINDING 2A (DREB2A)	12.53	8.19	5.14	6.41	8.36	6.54
	OSMOTIN-like protein OSM34 (OSMOTIN)	5.82	2.39	1.76	2.36	2.47	1.78
	RESPONSIVE TO DESSICATION 29A (RD29A)	5.22	5.17	7.39	4.82	10.08	4.61
	Mean ± SE	5.99 ± 1.81	5.46 ± 0.94	5.26 ± 0.97	4.07 ± 0.80	6.49 ± 1.34	4.88 ± 0.89
	Control (ACTIN)	0.96	1.14	1.16	1.16	0.93	1.17
MicroRNA (miR) mediated regulation	miR171	5.09	6.17	5.97	5.79	4.76	8.04
	miR159	10.10	4.43	7.30	10.57	4.32	7.10
	miR156	1.82	1.52	2.06	1.77	2.21	1.52
	miR395	5.63	4.38	3.00	1.29	2.36	1.33
	miR395c	2.41	4.76	4.37	1.43	1.17	3.22
	Mean ± SE	5.01 ± 1.47	4.25 ± 0.76	4.54 ± 0.95	4.17 ± 1.80	2.96 ± 0.68	4.24 ± 1.41
	Control (ubiquitin)	1.08	1.12	1.14	1.09	1.08	1.00
Chromatin remodelling	CHROMATIN REMODELLING PROTEIN 2 (CHR2)	1.26	1.58	1.28	4.31	1.42	1.22
	CHROMATIN REMODELLING PROTEIN 5 (CHR5)	10.08	2.19	6.74	1.16	3.31	3.27
	CHROMATIN REMODELLING PROTEIN 11 (CHR11)	2.90	1.07	4.23	3.17	2.90	3.11
	HISTONE ACETYLTRANSFERASE 3 (HAC3)	3.15	1.00	2.11	2.73	1.65	2.04
	HISTONE ACETYLTRANSFERASE 5 (HAC5)	3.52	2.75	4.62	4.55	3.41	2.29
	HISTONE DEACETYLASE 9 (HDA9)	3.21	1.24	4.96	1.17	8.16	8.28
	HISTONE DEACETYLASE 19 (HDA19)	1.74	4.93	3.02	4.86	3.35	2.21
	HISTONE DEACETYLASE 2A (HD2A)	3.08	3.70	10.92	4.43	5.20	5.47
	NUCLEAR FUSION DEFECTIVE 1 (NFD1)	2.46	3.46	2.38	5.17	3.55	3.83
	Mean ± SE	3.49 ± 0.86	2.32 ± 0.50	4.47 ± 1.00	3.51 ± 0.51	3.66 ± 0.67	3.52 ± 0.72
	Control (ACTIN)	1.11	1.18	1.13	1.14	1.12	1.09
Plant development gene	PHYTOCHROME B (PHYB)	5.85	3.18	2.62	3.14	3.60	3.23
	PHYTOCHROME C (PHYC)	2.65	3.09	1.13	1.14	5.09	5.42
	PHYTOCHROME D (PHYD)	3.24	2.62	2.98	2.86	4.16	2.01
	PHYTOCHROME E (PHYE)	1.74	2.02	1.24	1.36	1.39	4.37
	CRYPTOCHROME 1 (CRY1)	2.18	1.66	2.20	1.47	1.80	1.51
	CRYPTOCHROME 2 (CRY2)	4.76	4.63	3.01	5.71	10.02	6.12
	EARLY FLOWERING IN SHORT DAYS (EFS)	2.66	3.06	5.48	5.63	3.60	1.17
	CUP-SHAPED COTYLEDON 1 (CUC1)	2.92	2.52	4.22	1.25	1.27	2.94
	VERNALIZATION INDEPENDENCE 3 (VIP3)	1.08	1.69	2.85	3.01	1.96	1.17
	LSD1-LIKE1 (LDL1)	1.00	1.68	3.07	1.12	1.18	2.01
	MADS AFFECTING FLOWERING 1 (MAF1)	5.83	5.05	6.04	2.02	5.63	5.71
	MADS AFFECTING FLOWERING 5 (MAF5)	1.14	1.87	2.07	1.73	1.94	1.84
	FLOWERING TIME CONTROL PROTEIN (FPA)	1.96	1.12	1.87	1.56	1.63	1.65
	LEAFY (LFY)	4.01	2.92	5.33	1.23	1.12	3.01
	METAL ION BINDING (FVE)	1.00	1.13	2.26	2.25	0.32	6.54
	EARLY FLOWERING 7 (ELF7)	1.00	1.00	1.31	1.16	1.26	1.21

Table 2 *(contd.)*

Broad function	Gene	Level of upregulation (× fold) with respect to wild type level (1.00) in					
		lli	*egd*	*ill*	*lli egd*	*lli ill*	*egd ill*
	EARLY FLOWERING 8 (ELF8)	3.84	2.51	2.87	1.96	3.46	3.52
	PHERES1 (PHE1)	2.15	4.39	3.41	4.26	4.11	2.18
	Arabidopsis thaliana PROTEIN ARGININE METHYLTRANSFERASE 4A (PRMT4A)	1.03	1.31	1.60	2.76	2.24	1.30
	PROTEIN ARGININE METHYLTRANSFERASE 4B (PRMT4B)	1.24	1.26	1.76	2.48	1.51	2.31
	AGAMOUS LIKE MADS-BOX PROTEIN 31 (AGL31)	6.55	2.29	2.86	3.28	2.32	2.40
	ABNORMAL LEAF SHAPE 2 (ALE2)	4.16	4.8	3.54	4.01	4.11	4.12
	PHYTOCHROME AND FLOWERING TIME 1 (PFT1)	2.72	1.95	2.76	3.67	1.88	2.69
	RELATIVE OF EARLY FLOWERING 6 (REF6)	10.00	6.89	10.04	8.05	3.13	3.21
	VACUOLAR PROTON ATPase PROTEOLIPID SUBUNIT-LIKE PROTEIN (*Solanum tuberosum*) (ST)	1.14	1.12	5.01	2.64	3.02	5.19
	CYTOCHROME P450 72C1 (CYP72C1)	2.26	3.12	3.29	4.30	5.58	1.18
	Lipid transfer protein (LTP)	2.41	8.13	8.09	6.63	7.91	2.48
	(EDD1) EMBRYO-DEFECTIVE-DEVELOPMENT 1	2.18	3.17	3.23	3.18	5.45	4.81
	TIMING OF CAB EXPRESSION 1 (TOC1)	2.58	2.89	5.33	4.18	3.47	2.37
	GIBBERELLIN 20-OXIDASE (GA20OX1)	4.27	3.52	3.25	1.71	3.76	4.63
	Homeobox protein LUMINIDEPENDENS (LD)	1.04	1.02	1.16	3.38	2.01	3.43
	DWARF AND DELAYED FLOWERING 2 (DDF2)	3.19	1.15	2.46	1.28	1.30	1.88
	ORESARA 1 (ORE1)	4.93	3.96	6.36	3.13	0.94	4.42
	Structural constituent of cytoskeleton (ATARP6)	2.18	1.29	1.56	1.58	8.23	3.35
	SERRATED LEAVES AND EARLY FLOWERING (SEF)	1.12	1.01	4.12	6.51	2.77	1.09
	GIGANTEA (GI)	1.53	4.33	1.00	1.60	1.20	1.44
	HUA ENHANCER 1 (HEN1)	1.13	2.30	1.79	1.87	1.81	1.85
	ESTERASE/LIPASE/THIOESTERASE (E/L/TH)	2.24	6.94	6.89	5.47	6.76	3.81
	Ribonuclease/transcriptional repressor (R/TR)	4.38	1.14	3.26	1.07	2.08	2.39
	MULTICOPY SUPRESSOR OF IRA1 (MSI)	2.01	2.14	12.38	3.17	6.25	8.26
	AP2-domain DNA-binding protein (ORCA3)	6.47	6.34	5.58	4.86	5.39	7.71
	LEAFY PETIOLE (LEP)	5.48	6.38	7.23	10.36	3.29	5.54
	GLYCINE-RICH PROTEIN 2B (ATGRP2B)	2.74	4.88	3.98	3.66	2.65	4.07
	LEAFY COTYLEDON 2 (LEC2)	8.19	7.61	7.07	6.74	5.32	7.48
	YELLOW-LEAF-SPECIFIC GENE 9 (YLS9)	1.37	1.34	2.39	1.29	1.54	1.33
	TRANSCRIPTION FACTOR IIIA (TFIIIA)	4.71	4.27	5.18	3.36	3.68	4.24
	Proline-rich family protein (PRF)	6.67	5.71	8.15	6.28	10.07	3.11
	Pathogenesis Related (Cr PR)	2.87	3.44	5.36	4.13	8.28	4.82
	ETHYLENE RECEPTOR 1 (Cr ETR1)	1.04	2.43	6.27	1.62	2.17	1.16
	Map Kinase (Cr MAPK)	3.34	3.45	4.91	5.65	2.48	3.97
	FLOWERING LOCUS D (FLD)	2.81	5.22	2.74	8.39	6.26	12.16
	APETALA 2 (AP2)	2.70	5.03	3.03	3.28	3.43	3.13
	Basic-leucine zipper (bZIP) transcription factor family protein (FD)	2.44	5.16	5.00	10.28	5.63	1.42
	CASEIN KINASE II BETA SUBUNIT (CKB2)	2.83	4.18	4.24	4.11	6.13	5.36
	PHAVOLUTA (PHV)	3.46	5.24	8.10	8.14	4.16	5.82
	REVOLUTA (REV)	3.90	3.41	3.00	5.25	2.50	5.28
	Mean ± SE	3.11 ± 0.26	3.32 ± 0.25	4.07 ± 0.32	3.68 ± 0.32	3.65 ± 0.31	3.59 ± 0.29
	Control (ACTIN)	0.96	1.14	1.16	1.16	0.93	1.17

Table 2 *(contd.)*

Broad function	Gene	Level of upregulation (× fold) with respect to wild type level (1.00) in					
		lli	*egd*	*ill*	*lli egd*	*lli ill*	*egd ill*
Terpenoid indole alkaloid accumulation	GERANIOL-10-HYDROXYLASE (G10H)	4.63	4.91	4.58	4.98	5.82	4.99
	GERANYL GERANYL PYROPHOSPHATE SYNTHASE (GGPS)	2.37	4.08	3.96	4.13	3.98	4.11
	1-DEOXY-D-XYLULOSE-5-PHOSPHATE REDUCTOISOMERASE (DXR)	3.34	3.14	3.36	1.12	3.19	1.18
	O-METHYLTRANSFERASE (OMT)	2.61	2.60	2.94	2.58	2.93	2.98
	TABERSONINE 16-HYDROXYLASE (T16H)	1.04	1.13	3.14	3.18	3.43	2.38
	SECOLOGANIN SYNTHASE (SLS)	4.76	6.19	6.09	5.87	6.21	5.82
	TRYPTOPHAN DECARBOXYLASE (TDC)	2.17	3.68	3.51	3.45	3.71	1.59
	ANTHRANILATE SYNTHASE (AS)	2.59	1.91	1.89	1.86	1.99	2.04
	DESACETOXYVINDOLINE-4-HYDROXYLASE (D4H)	3.68	3.57	3.73	1.23	3.71	3.15
	CYTOCHROME P450 REDUCTASE (CPR)	2.26	2.39	2.94	2.42	1.17	2.40
	PHOSPHOENOLPYRUVATE CARBOXYLASE (PEPC)	1.53	6.33	1.64	1.00	1.62	2.42
	STRICTOSIDINE SYNTHASE (STR)	2.23	3.34	3.33	3.16	3.58	3.15
	Mean ± SE	2.77 ± 0.33	3.61 ± 0.46	3.43 ± 0.34	2.92 ± 0.44	3.45 ± 0.43	3.02 ± 0.39
	Control (ACTIN)	1.11	1.18	1.13	1.14	1.12	1.09

Size of vegetative organs: Observations recorded on biomass of roots, stems, leaves and entire plant, and one leaf and root/shoot and stem/leaf ratios in seven genotypes are summarized in table 4. In terms of five of seven parameters of plant vegetative growth studied, the mutants and wild type fell into the following order: *lli* > wild type > *ill* > *egd*. The mutations affected the root/shoot ratio similarly, which was higher in *lli*, *egd* and *ill* genotypes than in the wild type. According to stem/leaf ratio, the genotypes fell in the following order: *lli* > *egd* > *ill* and wild type. On the whole, the *lli* mutation increased the vegetative growth and *egd* and *ill* mutations were some what detrimental to vegetative growth. The *egd ill* combination was most detrimental to tissue growth.

Cell sizes and stomata frequency in leaf tissues: It will be seen from table 5 that the adaxial–abaxial thickness was similar in the wild type and mutants. The differences in the sizes of mesophyll parenchyma cells between mutant and wild type genotypes were marginal—*egd*:*ill*:wild type and *lli*::1:0.84:0.73. The *egd* and *ill* mutations increased the size of pavement cells up to 23% and 83%, respectively. There were more stomata in epidermal tissues of mutants as compared to wild type: *ill* > *egd* > *lli* > wild type. Each of the *ill* and *egd* mutations increased the stomata frequency by 30–50%.

Photosynthesis traits: Total chlorophyll, chlorophyll a and chlorophyll b contents in leaves were respectively 25, 40 and 10% higher in wild type than in mutants which had similar chlorophyll contents (table 6). According to total leaf photosynthesis, the genotypes could be arranged in the following decreasing order: *egd* (1.0) > *lli* (0.87) > wild type (0.76) > *ill* (0.58). Ten per cent increase in *egd* and 10% decrease in *ill* in photosynthetic rate as compared to wild type were significant. The observations indicated complexity of the photosynthetic traits.

Organ dimensions: The observations summarized in table 7 showed that the lamina area-wise, leaves were smaller in mutants: wild type (1.0) > *egd* (0.97) > *lli* (0.87) > *ill* (0.79). However, the mutants differed in leaf petiole length in relation to the wild type: *lli* (1.0) > *egd* (0.90) > wild type (0.89) > *ill* (0.69). The flower pedicels were of larger size in *egd* and *ill* mutants than in wild type. The mutants bore flowers of smaller diameter, in which petals, corolla tube and style of gynoecium were all smaller than in wild type. The length of pods and number of seeds in a pod was also smaller in mutants than in wild type. Contrastingly seeds were of larger size in mutant genotypes: *lli* (1.0) > *ill* (0.89) > *egd* (0.83) > wild type (0.81).

Stress related traits: The parameters of mutant genotypes in respect of the stress-indicative traits demonstrated their innate tolerance towards salinity and drought stresses (table 8). Whereas, the water content in leaves of the mutants

Table 3. Genes whose expression was downregulation in *lli*, *egd*, *ill*, *lli egd*, *egd ill* and *lli ill* mutants as compared to the wild type in *C. roseus*.

Broad function	Gene	Level of downregulation (× fold) with respect to wild type level (1.00) in					
		lli	*egd*	*ill*	*lli egd*	*lli ill*	*egd ill*
MicroRNA (miR) mediated regulation	miR319	0.11	0.53	0.52	0.66	0.58	0.73
	miR166	0.65	0.83	0.56	0.86	0.84	0.17
	Mean ± SE	0.38 ± 0.27	0.68 ± 0.15	0.54 ± 0.02	0.76 ± 0.1	0.71 ± 0.13	0.45 ± 0.28
	Control (ubiquitin)	1.08	1.12	1.14	1.09	1.08	1.00
Chromatin remodelling	CHROMOMETHYLASE 3 (CMT3)	0.32	0.44	0.38	0.50	0.41	0.48
	DOMAIN REARRANGED METHYLTRANSFERASE 2 (DRM2)	0.03	0.04	0.10	0.01	0.30	0.10
	GENERAL TRANSCRIPTION FACTOR GROUP E6 (GTE6)	0.32	0.32	0.13	0.25	0.48	0.41
	HISTONE ACETYL TRANSFERASE 13 (HAC13)	0.39	0.15	0.19	0.24	0.23	0.30
	HISTONE DEACETYLASE 14 (HDA14)	0	0	0	0	0	0
	METHYL-CpG-BINDING DOMAIN PROTEIN 8 (MBD8)	0.32	0.36	0.42	0.65	0.13	0.63
	METHYL-CpG-BINDING DOMAIN PROTEIN 10 (MBD10)	0.09	0.32	0.46	0.32	0.17	0.58
	METHYL-CpG-BINDING DOMAIN PROTEIN 11 (MBD11)	0.81	0.26	0.89	0.31	0.77	0.61
	Mean ± SE	0.29 ± 0.09	0.24 ± 0.06	0.32 ± 0.10	0.29 ± 0.08	0.31 ± 0.09	0.39 ± 0.08
	Control (ACTIN)	1.11	1.18	1.13	1.14	1.12	1.09
Plant development	PHYTOCHROME A (PHYA)	0.52	0.21	0.33	0.8	0.47	0.67
	REDUCED VERNALIAZATION RESPONSE 1 (VRN1)	0.38	0.63	0.47	0.52	0.19	0.93
	LSD1-LIKE2 (LDL2)	0.32	0.51	0.36	0.37	0.26	0.33
	UBIQUITIN CARRIER PROTEIN 1 (UBC1)	0.63	0.83	0.52	0.52	0.63	0.64
	HISTONE MONO-UBIQUITINATION 2 (HUB2)	0.68	0.65	0.7	0.93	0.6	0.53
	FLOWERING TIME CONTROL PROTEIN (FY)	0.93	0.52	0.84	0.84	0.92	0.97
	FLOWERING TIME CONTROL PROTEIN-RELATED/ FCA GAMMA-RELATED (FCA)	0.85	0.83	0.35	0.66	0.51	0.76
	SQUAMOSA PROMOTER BINDING PROTEIN-LIKE 14 (SPL14)	0.58	0.61	0.87	0.70	0.71	0.72
	FLOWERING LOCUS T (FT)	0.11	0.17	0.34	0.17	0.14	0.53
	FERTILIZATION INDEPENDENT SEED 2 (FIS2)	0.45	0.41	0.35	0.32	0.46	0.48
	LATE ELONGATED HYPOCOTYL (LHY)	0.23	0.48	0.54	0.40	0.19	0.57
	ASYMMETRIC LEAVES 1 (AS1)	0.35	0.24	0.33	0.35	0.34	0.26
	PHD FINGER PROTEIN-LIKE PROTEIN (PHD)	0.91	0.28	0.87	0.84	0.80	0.74
	AGAMOUS-LIKE 24 (AGL24)	0.80	0.63	0.30	0.48	0.31	0.78
	KANADI (KAN)	0.22	0.26	0.94	0.64	0.89	0.87
	RNA-DEPENDENT RNA POLYMERASE 2 (RDR2)	0.24	0.36	0.21	0.37	0.53	0.44
	DEFECTIVE IN RNA-DIRECTED DNA METHYLATION 1 (DRD1)	0.04	0.16	0.04	0.13	0.61	0.58
	HIGH EXPRESSION OF OSMOTICALLY RESPONSIVE GENES 1 (HOS1)	0.16	0.68	0.55	0.92	0.11	0.88
	ACTIN-RELATED PROTEIN 4 (ARP4)	0.37	0.49	0.39	0.46	0.63	0.61
	Arabidopsis thaliana SEED GENE 3 (ATS3)	0.48	0.08	0.12	0.52	0.50	0.02
	SWINGER (SWN)	0.42	0.14	0.41	0.37	0.63	0.44
	VERNALIZATION5/VIN3-LIKE (VEL2)	0.16	0.86	0.63	0.95	0.84	0.35
	NUCLEAR RNA POLYMERASE D 1A (NRPD1A)	0.40	0.38	0.09	0.06	0.11	0.08
	NUCLEAR RNA POLYMERASE D 1A (NRPD1B)	0.08	0.54	0.27	0.32	0.68	0.38
	PHENYLALANINE AMMONIA-LYASE (Cr PAL)	0.37	0.19	0.27	0.16	0.53	0.11

Table 3 *(contd.)*

Broad function	Gene	Level of downregulation (× fold) with respect to wild type level (1.00) in					
		lli	*egd*	*ill*	*lli egd*	*lli ill*	*egd ill*
	TOPOISOMERASE1 (Cr TOPO1)	0.84	0.99	0.53	0.90	0.88	0.93
	Mean ± SE	0.41 ± 0.05	0.43 ± 0.05	0.42 ± 0.05	0.49 ± 0.06	0.48 ± 0.05	0.52 ± 0.06
	Control (ACTIN)	0.96	1.14	1.16	1.16	0.93	1.17
Terpenoid indole alkaloid pathway	2C-METHYL-D-ERYTHROL-2,4-CYCLODIPHOSPHATE SYNTHASE (MECS)	0.94	0.11	0.18	0.83	0.34	0.38
	ACETYL COA:17-O-DEACETYL VINDOLINE 17-O-ACETYLTRANSFERSE (DAT)	0.61	0.42	0.47	0.49	0.87	0.79
	CHORISMATE MUTASE (CMU)	0.86	0.90	0.95	0.83	0.84	1.00
	Mean ± SE	0.80 ± 0.10	0.48 ± 0.23	0.53 ± 0.22	0.72 ± 0.11	0.68 ± 0.17	0.72 ± 0.18
	Control (ACTIN)	1.11	1.18	1.13	1.14	1.12	1.09

was 12% lower on average basis under irrigation water abundance conditions, it was about 70% higher under artificially created drought conditions and following irrigation with saline water. According to drought and salinity tolerance, the mutants fell in the following order: *egd* (1.0) > *lli* (0.92) > *ill* (0.83). The wild-type leaves lost their water about 24% faster than the average rate of water loss from the leaves of mutant genotypes. This parameter suggested relative stress tolerance among the mutant genotypes was: *egd* > *lli* > *ill*. The mutants accumulated osmoprotectants in their leaves in higher concentrations as compared to wild type. There was 36% more proline and 91% more trehalose than in wild-type leaves. The mutant leaves accumulated 25% less Na^+ and 44% higher K^+ than the leaves of wild type.

Terpenoid indole alkaloid (TIA) accumulation: The TIA accumulation was relatively less in the mutant genotypes as compared to wild type, except that *ill* genotypes accumulated 50% more vindoline in leaves and 37% more vinblastine + vincristine in leaves as compared to wild type leaves (table 9).

Effect of salt on leaf histology

Leaves taken from plants of the seven genotypes that had received normal irrigation and from those that were irrigated with saline water were sectioned transversely and their sections stained with safranin were examined microscopically. The observations are presented in table 10. It will be seen that treatment with salt, resulted in reduction of sizes of both palisade and spongy mesophyll parenchyma cells. Salt treatment produced similar effect on the wild type and mutant mesophyll parenchyma. The adaxial–abaxial thickness of leaves was also not reduced by salt treatment. It was similar in all the genotypes.

Discussion

Pleiotropy in leafless inflorescence (lli), evergreen dwarf (egd) and irregular leaf lamina (ill) mutants

The specific morphologies after which the *lli*, *egd* and *ill* mutants were named were recombinable such that double mutants that possessed the name-wise characteristics of the single mutants were identifiable in the segregating populations. With the availability of double mutants, expression of such mutation could be studied in the background of each of the other two mutations. The *lli* and *egd* mutants were isolated as salt-tolerant seedlings and their adult plants were observed to have distinctive morphologies. The *ill* mutant was isolated as a morphological mutant and its seedlings were subsequently noted to be salt tolerant. The single and double mutants were found to be similarly salt tolerant at their seedling stage. Their gene expression patterns were also

Table 4. Expression of the vegetative organ biomass related traits in the wild type (WT) and *leafless inflorescence* (*lli*), *evergreen dwarf* (*egd*) and *irregular leaf lamina* (*ill*) single and *lli egd*, *lli ill* and *egd ill* double mutants in *C. roseus*.

Genotype	Root weight of the plant (g) ($n = 5$)	Stem weight of the plant (g) ($n = 5$)	Leaf weight of the plant (g) ($n = 5$)	Weight of the whole plant (g) ($n = 5$)	Weight of the single fresh leaf (g) ($n = 3$)	Root/Shoot ratio ($n = 5$)	Stem/Leaf ratio ($n = 5$)
WT	2.5 ± 0.2^{b}	16.5 ± 0.7^{c}	12.0 ± 0.5^{d}	30.9 ± 0.7^{d}	0.3 ± 0.1^{c}	0.09 ± 0.01^{a}	1.4 ± 0.1^{a}
lli	5.2 ± 0.1^{d}	30.0 ± 1.9^{e}	14.0 ± 0.6^{e}	49.2 ± 0.2^{f}	0.2 ± 0.1^{b}	0.12 ± 0.01^{bc}	2.2 ± 0.1^{b}
egd	2.0 ± 0.1^{ab}	9.7 ± 0.1^{b}	7.7 ± 0.2^{b}	19.4 ± 0.2^{b}	0.3 ± 0.1^{c}	0.12 ± 0.01^{bc}	1.3 ± 0.1^{a}
ill	2.3 ± 0.1^{b}	12.1 ± 1.1^{b}	9.5 ± 0.5^{c}	23.9 ± 0.8^{c}	0.2 ± 0.1^{b}	0.11 ± 0.01^{b}	1.3 ± 0.2^{a}
lli egd	2.4 ± 0.1^{b}	16.0 ± 0.8^{c}	7.4 ± 0.2^{b}	25.8 ± 0.9^{c}	0.2 ± 0.1^{b}	0.10 ± 0.01^{ab}	2.2 ± 0.2^{b}
lli ill	4.3 ± 0.2^{c}	21.6 ± 1.1^{d}	11.0 ± 0.5^{d}	36.9 ± 0.7^{e}	0.1 ± 0.1^{a}	0.13 ± 0.01^{c}	1.9 ± 0.2^{b}
egd ill	1.8 ± 0.1^{a}	5.8 ± 0.1^{a}	5.7 ± 0.3^{a}	13.3 ± 0.4^{a}	0.2 ± 0.1^{b}	0.16 ± 0.01^{d}	1.0 ± 0.1^{a}
Mean of all genotypes	2.9 ± 0.5	15.9 ± 3.0	9.6 ± 1.1	28.5 ± 4.5	0.2 ± 0.1	0.12 ± 0.01	1.6 ± 0.2
F value	97.4**	63.1**	44.9**	132.2**	18.9**	21.1**	12.9**
CD 5%	0.38	2.93	1.26	2.99	0.04	0.01	0.38
CD 1%	0.51	3.95	1.69	4.04	0.06	0.02	0.52
Mean of individual mutation							
lli	3.9 ± 0.8	22.6 ± 4.1	10.8 ± 1.9	37.3 ± 6.8	0.2 ± 0.1	0.1 ± 0.01	2.1 ± 0.1
egd	2.1 ± 0.2	10.5 ± 2.9	6.9 ± 0.6	19.5 ± 3.6	0.2 ± 0.1	0.1 ± 0.02	1.5 ± 0.4
ill	2.8 ± 0.8	13.2 ± 4.6	8.7 ± 1.6	24.7 ± 6.8	0.2 ± 0.2	0.1 ± 0.01	1.4 ± 0.3
Comparisons							
WT vs *lli*	$F_{1:28} = 289.1$**	$F_{1:28} = 80.2$**	$F_{1:28} = 17.2$**	$F_{1:28} = 84.1$**	$F_{1:14} = 14.6$**	$F_{1:28} = 87.9$**	$F_{1:28} = 65.9$**
WT vs *egd*	$F_{1:28} = 22.3$**	$F_{1:28} = 79.5$**	$F_{1:28} = 306.6$**	$F_{1:28} = 277.8$**	$F_{1:14} = 4.7$*	$F_{1:28} = 131.5$**	$F_{1:28} = 1.2$
WT vs *ill*	$F_{1:28} = 13.4$**	$F_{1:28} = 24.5$**	$F_{1:28} = 127.6$**	$F_{1:28} = 83.2$**	$F_{1:14} = 17.4$**	$F_{1:28} = 186.3$**	$F_{1:28} = 0.4$
lli vs *egd*	$F_{1:28} = 314.8$**	$F_{1:28} = 212.8$**	$F_{1:28} = 119.1$**	$F_{1:28} = 445.0$**	$F_{1:14} = 1.8$	$F_{1:28} = 2.9$	$F_{1:28} = 32.7$**
lli vs *ill*	$F_{1:28} = 118.7$**	$F_{1:28} = 128.9$**	$F_{1:28} = 34.1$**	$F_{1:28} = 223.0$**	$F_{1:14} = 0.1$	$F_{1:28} = 12.2$**	$F_{1:28} = 37.5$**
egd vs *ill*	$F_{1:28} = 46.9$**	$F_{1:28} = 10.5$**	$F_{1:28} = 25.7$**	$F_{1:28} = 37.9$**	$F_{1:14} = 2.7$	$F_{1:28} = 3.2$	$F_{1:28} = 0.2$

*Significant at 5% probability level; **significant at 1% probability level; ^{a,b,c,d,e,f}for a character, the values that do not have the same letter as superscript are different.

Table 5. Expression of leaf histological/anatomical traits in the wild type (WT) and *leafless inflorescence* (*lli*), *evergreen dwarf* (*egd*) and *irregular leaf lamina* (*ill*) single and *lli egd*, *lli ill* and *egd ill* double mutants in *C. roseus*.

Genotype	Area of the pavement cell (μm^2) ($n = 6$)	Total no. of stomata in 25000 μm^2 area ($n = 6$)	Area of spongy cell (μm^2) ($n = 2$)	Area of palisade cell (μm^2) ($n = 2$)	Adaxial–abaxial thickness of lamina next to midrib ($n = 2$)
WT	52.3 ± 5.7^{bc}	91.0 ± 8.1^{a}	$490.7 \pm 51.2^{a,b}$	333.5 ± 50.3^{a}	161.4 ± 10.5^{a}
lli	55.5 ± 5.4^{bc}	85.7 ± 9.5^{a}	$508.9 \pm 32.9^{a,b}$	415.3 ± 31.5^{a}	172.3 ± 21.4^{a}
egd	64.5 ± 9.1^{c}	137.7 ± 20.1^{b}	$560.2 \pm 18.4^{a,b}$	429.9 ± 46.2^{a}	161.4 ± 10.5^{a}
ill	28.6 ± 2.0^{a}	136.5 ± 6.3^{b}	$474.8 \pm 67.1^{a,b}$	383.5 ± 0.2^{a}	150.4 ± 0.4^{a}
lli egd	58.4 ± 7.5^{bc}	103.5 ± 12.3^{ab}	$546.8 \pm 5.0^{a,b}$	373.3 ± 10.4^{a}	122.3 ± 28.5^{a}
lli ill	37.2 ± 5.0^{ab}	109.7 ± 18.9^{ab}	378.3 ± 163.5^{a}	385.5 ± 1.7^{a}	137.9 ± 12.9^{a}
egd ill	45.8 ± 4.4^{b}	115.0 ± 17.2^{ab}	868.1 ± 326.3^{b}	365.2 ± 18.5^{a}	150.4 ± 0.4^{a}
Mean of all genotypes	48.9 ± 4.8	111.3 ± 7.7	546.8 ± 58.1	383.7 ± 12.1	150.9 ± 6.3^{a}
F value	4.5**	2.1	1.2	1.2	1.2
CD 5%	17.11	40.63	474.92	98.58	51.45
CD 1%	22.95	54.52	704.33	146.19	76.31
Mean of individual mutation					
lli	50.4 ± 6.6	99.6 ± 7.2	478.0 ± 53.8	391.4 ± 11.6	144.2 ± 13.5
egd	56.2 ± 5.5	118.7 ± 10.0	658.4 ± 107.4	389.5 ± 18.4	144.7 ± 10.8
ill	37.2 ± 4.9	120.4 ± 8.2	573.7 ± 134.7	378.1 ± 6.3	146.3 ± 4.3
Comparisons					
WT vs *lli*	$F_{1:35} = 0.2$	$F_{1:35} = 0.8$	$F_{1:7} = 0.01$	$F_{1:7} = 8.6$*	$F_{1:7} = 2.8$
WT vs *egd*	$F_{1:35} = 1.0$	$F_{1:35} = 8.6$**	$F_{1:7} = 3.1$	$F_{1:7} = 8.1$*	$F_{1:7} = 2.6$
WT vs *ill*	$F_{1:35} = 14.4$**	$F_{1:35} = 9.7$**	$F_{1:7} = 0.8$	$F_{1:7} = 5.1$	$F_{1:7} = 2.2$
lli vs *egd*	$F_{1:35} = 1.5$	$F_{1:35} = 2.7$	$F_{1:7} = 2.4$	$F_{1:7} = 0.01$	$F_{1:7} = 0.001$
lli vs *ill*	$F_{1:35} = 7.3$*	$F_{1:35} = 3.2$	$F_{1:7} = 0.7$	$F_{1:7} = 0.3$	$F_{1:7} = 0.03$
egd vs *ill*	$F_{1:35} = 15.3$**	$F_{1:35} = 0.1$	$F_{1:7} = 0.5$	$F_{1:7} = 0.2$	$F_{1:7} = 0.02$

*Significant at 5% probability level; **significant at 1% probability level; [a, b, c] for a character, the values that do not have the same letter as superscript are different.

Table 6. Expression of the photosynthesis related traits in the wild type (WT) and *leafless inflorescence* (*lli*), *evergreen dwarf* (*egd*) and *irregular leaf lamina* (*ill*) single and *lli egd*, *lli ill* and *egd ill* double mutants in *C. roseus.*

Genotype	Photosynthetic rate in leaf (μmol/m^2/s) ($n = 10$)	Total photosynthesis in leaf (μmol/m^2/s*10^{-4}) ($n = 10$)	Chlorophyll 'a' (mg/g dw) ($n = 3$)	Chlorophyll 'b' (mg/g dw) ($n = 3$)	Total chlorophyll (mg/g dw) ($n = 3$)
WT	19.0 ± 1.2^{ab}	146.3 ± 16.6^{b}	2.5 ± 0.1^{b}	0.6 ± 0.0^{b}	3.0 ± 0.1^{b}
lli	19.6 ± 1.2^{ab}	140.8 ± 11.6^{b}	1.8 ± 0.1^{ab}	0.5 ± 0.1^{ab}	2.3 ± 0.1^{a}
egd	21.6 ± 0.8^{b}	206.9 ± 12.0^{c}	2.1 ± 0.1^{b}	0.5 ± 0.1^{ab}	2.6 ± 0.1^{ab}
ill	17.3 ± 0.8^{a}	94.7 ± 6.3^{a}	1.9 ± 0.1^{ab}	0.4 ± 0.1^{a}	2.4 ± 0.1^{ab}
lli egd	24.2 ± 1.2^{b}	243.6 ± 25.6^{c}	1.9 ± 0.2^{ab}	0.6 ± 0.1^{b}	2.5 ± 0.2^{ab}
lli ill	19.3 ± 1.6^{ab}	115.0 ± 7.1^{ab}	2.1 ± 0.1^{b}	0.5 ± 0.1^{ab}	2.6 ± 0.1^{ab}
egd ill	16.1 ± 2.3^{a}	122.3 ± 22.3^{ab}	1.5 ± 0.3^{a}	0.5 ± 0.1^{ab}	2.0 ± 0.4^{a}
Mean of all genotypes	19.6 ± 1.0	152.8 ± 20.2	2.0 ± 0.1	0.5 ± 0.1	2.5 ± 0.1
F value	3.6**	11.1**	3.5*	1.3	2.7
CD 5%	3.96	45.32	0.47	0.16	0.60
CD 1%	5.24	60.04	0.65	0.22	0.84
Mean of individual mutation					
lli	21.0 ± 1.6	166.5 ± 39.3	1.9 ± 0.1	0.5 ± 0.1	2.5 ± 0.1
egd	20.6 ± 2.4	190.9 ± 35.9	1.8 ± 0.2	0.5 ± 0.1	2.4 ± 0.2
ill	17.6 ± 0.9	110.7 ± 8.3	1.8 ± 0.2	0.5 ± 0.1	2.3 ± 0.2
Comparisons					
WT vs *lli*	$F_{1:63} = 4.5$**	$F_{1:63} = 3.6$	$F_{1:14} = 2.5$	$F_{1:14} = 0.6$	$F_{1:14} = 2.0$
WT vs *egd*	$F_{1:63} = 2.9$	$F_{1:63} = 17.4$**	$F_{1:14} = 3.7$	$F_{1:14} = 0.3$	$F_{1:14} = 2.7$
WT vs *ill*	$F_{1:63} = 2.4$	$F_{1:63} = 11.1$**	$F_{1:14} = 3.7$	$F_{1:14} = 1.3$	$F_{1:14} = 3.2$
lli vs *egd*	$F_{1:63} = 0.1$	$F_{1:63} = 3.5$	$F_{1:14} = 0.1$	$F_{1:14} = 0.1$	$F_{1:14} = 0.1$
lli vs *ill*	$F_{1:63} = 9.1$**	$F_{1:63} = 18.2$**	$F_{1:14} = 0.1$	$F_{1:14} = 0.1$	$F_{1:14} = 0.1$
egd vs *ill*	$F_{1:63} = 7.1$**	$F_{1:63} = 37.7$**	$F_{1:14} = 0.1$	$F_{1:14} = 0.2$	$F_{1:14} = 0.1$

*Significant at 5% probability level; **significant at 1% probability level; $^{a, b, c}$for a character, the values that do not have the same letter as superscript are different.

Table 7. Expression of the leaf lamina and petiole and flower organ size dimension(s) related traits in the wild type (WT) and *leafless inflorescence* (*lli*), *evergreen dwarf* (*egd*) and *irregular leaf lamina* (*ill*) single and *lli egd*, *lli ill* and *egd ill* double mutants in *C. roseus*.

Genotype	Leaf area (mm^2) ($n = 5$)	Petiole length (mm) ($n = 5$)	Pedicel length (cm) ($n = 5$)	Flower diameter (cm) ($n = 5$)	Sepal length (cm) ($n = 5$)	Petal area (mm^2) ($n = 5$)	Corolla tube length (cm) ($n = 5$)	Length of flower styl (cm) ($n = 5$)	Ovary length (cm) ($n = 5$)	Area of the seed (μm^2) ($n = 3$)	Pod length (cm) ($n = 5$)	Number seeds/ pod ($n = 5$)
WT	657.9 ± 91.1^{b}	8.1 ± 1.1^{b}	0.2 ± 0^{a}	4.1 ± 0.1^{c}	0.3 ± 0.1^{b}	178.4 ± 6.3^{d}	2.5 ± 0.1^{c}	1.8 ± 0.1^{b}	0.2 ± 0.0^{a}	1589.5 ± 133.0^{ab}	2.5 ± 0.1^{cd}	34.0 ± 2.6^{ab}
lli	570.7 ± 124.4^{ab}	9.5 ± 1.5^{b}	0.2 ± 0^{a}	3.1 ± 0.1^{ab}	0.2 ± 0.0^{a}	86.4 ± 11.5^{ab}	2.2 ± 0.1^{b}	1.5 ± 0.1^{ab}	0.2 ± 0.0^{a}	2037.7 ± 69.4^{b}	2.1 ± 0.1^{ab}	27.6 ± 3.4^{ab}
egd	711.1 ± 201.2^{b}	8.6 ± 0.4^{b}	0.3 ± 0^{b}	3.6 ± 0.1^{b}	0.2 ± 0.0^{a}	129.0 ± 16.4^{c}	2.0 ± 0.1^{a}	1.3 ± 0.1^{a}	0.2 ± 0.0^{a}	1653.6 ± 23.7^{ab}	2.0 ± 0.1^{ab}	27.6 ± 1.3^{ab}
ill	536.9 ± 96.8^{a}	5.5 ± 0.5^{a}	0.2 ± 0.1^{a}	3.2 ± 0.1^{ab}	0.3 ± 0.0^{b}	95.4 ± 11.3^{b}	2.4 ± 0.1^{c}	1.7 ± 0.1^{b}	0.2 ± 0.0^{a}	1806.0 ± 133.5^{b}	1.8 ± 0.1^{a}	26.4 ± 3.4^{a}
lli egd	668.6 ± 53.8^{b}	10.2 ± 0.8^{b}	0.2 ± 0.1^{a}	3.5 ± 0.1^{b}	0.3 ± 0.0^{b}	142.4 ± 8.7^{c}	2.1 ± 0.1^{ab}	1.3 ± 0.1^{a}	0.2 ± 0.1^{a}	1866.8 ± 112.3^{b}	2.7 ± 0.1^{d}	34.6 ± 2.5^{b}
lli ill	484.5 ± 27.9^{a}	7.5 ± 0.5^{ab}	0.3 ± 0.1^{b}	2.9 ± 0.1^{a}	0.3 ± 0.1^{b}	46.0 ± 5.7^{a}	2.2 ± 0.1^{b}	1.7 ± 0.1^{b}	0.2 ± 0^{a}	2007.2 ± 152.4^{b}	2.4 ± 0.1^{c}	27.4 ± 1.3^{ab}
egd ill	547.1 ± 37.9^{a}	5.8 ± 0.8^{ab}	0.3 ± 0.1^{b}	3.4 ± 0.1^{b}	0.3 ± 0.0^{b}	116.2 ± 5.9^{bc}	2.4 ± 0.1^{c}	1.5 ± 0.2^{ab}	0.2 ± 0.1^{a}	1418.6 ± 90.2^{a}	2.3 ± 0.1^{bc}	28.0 ± 3.0^{ab}
Mean of all genotypes	596.7 ± 54.9	8.0 ± 0.1	0.2 ± 0.11	3.4 ± 0.2	0.3 ± 0.1	113.4 ± 16.1	2.3 ± 0.1	1.5 ± 0.1	0.2 ± 0.1	1768.5 ± 85.7	2.3 ± 0.1	29.4 ± 1.3
F value	5.9**	5.6**	7.8**	10.8**	14.4**	17.9**	25.5**	4.7**	0.9	4.2**	8.6**	1.7
CD 5%	107.61	2.47	0.04	0.34	0.03	29.18	0.10	0.25	0.05	333.93	0.29	7.60
CD 1%	145.18	3.34	0.06	0.46	0.05	39.37	0.14	0.33	0.07	463.46	0.39	10.25
Mean of individual mutation												
lli	574.6 ± 53.2	9.1 ± 0.8	0.2 ± 0.1	3.2 ± 0.2	0.3 ± 0.1	91.6 ± 27.9	2.2 ± 0.1	1.5 ± 0.1	0.2 ± 0.1	1970.6 ± 52.6	2.4 ± 0.2	29.9 ± 2.4
egd	642.3 ± 49.1	8.2 ± 1.3	0.3 ± 0.1	3.5 ± 0.1	0.3 ± 0.1	129.2 ± 7.6	2.2 ± 0.1	1.4 ± 0.1	0.2 ± 0.1	1646.3 ± 129.4	2.3 ± 0.2	30.1 ± 2.3
ill	522.8 ± 19.4	6.3 ± 0.6	0.3 ± 0.1	3.2 ± 0.1	0.3 ± 0.1	85.9 ± 20.8	2.3 ± 0.1	1.6 ± 0.1	0.2 ± 0.1	1743.9 ± 172.7	2.2 ± 0.2	27.3 ± 0.5
Comparisons												
WT vs *lli*	$F_{1:28} = 0.8$	$F_{1:28} = 0.1$	$F_{1:28} = 10.9$**	$F_{1:28} = 141.4$**	$F_{1:28} = 11.2$**	$F_{1:28} = 167.0$**	$F_{1:28} = 270.8$**	$F_{1:28} = 27.9$**	$F_{1:28} = 0.4$	$F_{1:14} = 3.0$	$F_{1:28} = 1.6$	$F_{1:28} = 5.6$*
WT vs *egd*	$F_{1:28} = 0.8$	$F_{1:28} = 4.0$	$F_{1:28} = 43.8$**	$F_{1:28} = 69.7$**	$F_{1:28} = 2.8$	$F_{1:28} = 53.7$**	$F_{1:28} = 261.3$**	$F_{1:28} = 46.4$**	$F_{1:28} = 3.2$	$F_{1:14} = 0.1$	$F_{1:28} = 2.8$	$F_{1:28} = 5.1$*
WT vs *ill*	$F_{1:28} = 15.7$**	$F_{1:28} = 27.8$**	$F_{1:28} = 35.4$**	$F_{1:28} = 149.6$**	$F_{1:28} = 0.7$	$F_{1:28} = 189.8$**	$F_{1:28} = 96.3$**	$F_{1:28} = 6.1$	$F_{1:28} = 1.4$	$F_{1:14} = 0.5$	$F_{1:28} = 19.7$**	$F_{1:28} = 14.8$**
lli vs *egd*	$F_{1:28} = 2.1$	$F_{1:28} = 2.3$	$F_{1:28} = 7.3$*	$F_{1:28} = 8.4$**	$F_{1:28} = 1.9$	$F_{1:28} = 20.9$**	$F_{1:28} = 0.1$	$F_{1:28} = 1.6$	$F_{1:28} = 0.9$	$F_{1:14} = 1.4$	$F_{1:28} = 0.1$	$F_{1:28} = 0.1$
lli vs *ill*	$F_{1:28} = 15.7$**	$F_{1:28} = 17.7$**	$F_{1:28} = 4.7$*	$F_{1:28} = 0.1$	$F_{1:28} = 11.7$**	$F_{1:28} = 0.5$	$F_{1:28} = 29.4$**	$F_{1:28} = 5.3$*	$F_{1:28} = 0.2$	$F_{1:14} = 0.7$	$F_{1:28} = 6.7$*	$F_{1:28} = 1.5$
egd vs *ill*	$F_{1:28} = 6.3$*	$F_{1:28} = 7.2$*	$F_{1:28} = 0.3$	$F_{1:28} = 10.0$**	$F_{1:28} = 4.2$	$F_{1:28} = 27.8$**	$F_{1:28} = 26.9$**	$F_{1:28} = 12.6$**	$F_{1:28} = 0.2$	$F_{1:14} = 0.1$	$F_{1:28} = 5.1$*	$F_{1:28} = 1.7$

*Significant at 5% probability level; **Significant at 1% probability level; $^{a, b, c}$ for a character, the values that do not have the same letter as superscript are different.

Table 8. Expression of the traits relating to salinity and drought stress response in the wild type (WT) and *leafless inflorescence* (*lli*), *evergreen dwarf* (*egd*) and *irregular leaf lamina* (*ill*) single and *lli egd*, *lli ill* and *egd ill* double mutants in *C. roseus*.

Genotype	Water content in leaves (g)/g dry weight in the full irrigation water treatment ($n = 5$)	Water content in leaves (g)/g dry weight in the condition of irrigation withdrawal ($n = 5$)	Water content in leaves (g)/g dry weight in the condition of irrigation with salt water ($n = 5$)	Time period for the loss of 50% water from leaves (h) ($n = 3$)	Proline content (μmoles/g dw) ($n = 3$)	Trehalose content (ppm) ($n = 3$)	Per cent Na content ($n = 5$)	Per cent K content ($n = 5$)
WT	5.6 ± 0.2^{b}	2.6 ± 0.1^{a}	3.2 ± 0.2^{a}	19.5 ± 0.3^{a}	32.8 ± 1.3^{a}	41.7 ± 0.2^{a}	0.6 ± 0.1^{bc}	1.2 ± 0.2^{a}
lli	5.2 ± 0.1^{b}	4.2 ± 0.1^{b}	5.0 ± 0.1^{d}	26.0 ± 0.3^{d}	51.1 ± 1.4^{c}	87.6 ± 0.2^{d}	0.4 ± 0.1^{b}	1.7 ± 0.2^{b}
egd	4.8 ± 0.2^{ab}	4.0 ± 0.1^{b}	4.6 ± 0.2^{c}	28.1 ± 0.2^{e}	40.9 ± 0.2^{b}	98.4 ± 0.2^{g}	0.4 ± 0.1^{ab}	1.4 ± 0.1^{ab}
ill	5.5 ± 0.2^{b}	3.4 ± 0.1^{a}	3.7 ± 0.1^{b}	24.9 ± 0.4^{c}	52.8 ± 2.2^{c}	92.8 ± 0.1^{f}	0.6 ± 0.1^{c}	2.0 ± 0.1^{b}
lli egd	4.7 ± 0.2^{a}	5.8 ± 0.3^{d}	4.8 ± 0.1^{cd}	27.9 ± 0.3^{e}	43.2 ± 1.5^{b}	81.5 ± 0.2^{c}	0.3 ± 0.1^{a}	1.6 ± 0.1^{ab}
lli ill	4.9 ± 0.1^{ab}	3.5 ± 0.2^{a}	3.7 ± 0.2^{b}	20.3 ± 0.1^{b}	41.5 ± 0.7^{b}	89.0 ± 0.1^{e}	0.4 ± 0.1^{ab}	2.1 ± 0.1^{b}
egd ill	5.0 ± 0.1^{ab}	5.3 ± 0.2^{c}	4.7 ± 0.1^{cd}	20.5 ± 0.1^{b}	43.0 ± 1.4^{b}	48.3 ± 0.1^{b}	0.3 ± 0.1^{ab}	1.6 ± 0.1^{ab}
Mean of all genotypes	5.1 ± 0.1	4.1 ± 0.4	4.2 ± 0.3	23.9 ± 1.4	43.6 ± 2.5	77.0 ± 8.5	0.4 ± 0.1	1.7 ± 0.1
F value	4.7**	48.8**	25.9**	247.9**	24.5**	19909.9**	5.9**	5.5**
CD 5%	0.46	0.46	0.39	0.72	4.12	0.48	0.13	0.38
CD 1%	0.62	0.62	0.53	0.99	5.70	0.67	0.18	0.51
Mean of individual mutation								
lli	4.9 ± 0.1	4.5 ± 0.7	4.5 ± 0.4	24.8 ± 2.3	45.3 ± 2.9	86.0 ± 2.3	0.4 ± 0.1	1.8 ± 0.2
egd	4.8 ± 0.1	5.0 ± 0.5	4.7 ± 0.1	25.5 ± 2.5	42.4 ± 0.7	76.1 ± 14.7	0.3 ± 0.1	1.5 ± 0.1
ill	5.1 ± 0.2	4.1 ± 0.6	4.0 ± 0.3	21.9 ± 1.5	45.8 ± 3.5	76.7 ± 14.2	0.5 ± 0.1	1.9 ± 0.2
Comparisons								
WT vs *lli*	$F_{1:28} = 39.1$**	$F_{1:28} = 319.4$**	$F_{1:28} = 205.7$**	$F_{1:14} = 123.2$**	$F_{1:14} = 21.2$**	$F_{1:14} = 19236.8$**	$F_{1:28} = 30.7$**	$F_{1:28} = 42.1$**
WT vs *egd*	$F_{1:28} = 51.7$**	$F_{1:28} = 523.9$**	$F_{1:28} = 271.4$**	$F_{1:14} = 161.2$**	$F_{1:14} = 12.6$**	$F_{1:14} = 11561.4$**	$F_{1:28} = 45.6$**	$F_{1:28} = 11.3$**
WT vs *ill*	$F_{1:28} = 19.2$**	$F_{1:28} = 190.3$**	$F_{1:28} = 83.2$**	$F_{1:14} = 25.7$**	$F_{1:14} = 23.0$**	$F_{1:14} = 11998.9$**	$F_{1:28} = 9.8$**	$F_{1:28} = 57.1$**
lli vs *egd*	$F_{1:28} = 0.6$	$F_{1:28} = 16.8$**	$F_{1:28} = 3.0$	$F_{1:14} = 1.7$	$F_{1:14} = 0.7$	$F_{1:14} = 647.8$**	$F_{1:28} = 0.9$	$F_{1:28} = 6.5$*
lli vs *ill*	$F_{1:28} = 2.4$	$F_{1:28} = 11.1$**	$F_{1:28} = 18.2$ **	$F_{1:14} = 24.3$**	$F_{1:14} = 0.1$	$F_{1:14} = 566.8$**	$F_{1:28} = 3.9$	$F_{1:28} = 0.8$
egd vs *ill*	$F_{1:28} = 5.3$*	$F_{1:28} = 55.1$**	$F_{1:28} = 36.1$**	$F_{1:14} = 38.8$**	$F_{1:14} = 1.1$	$F_{1:14} = 2.7$	$F_{1:28} = 8.8$**	$F_{1:28} = 11.8$**

*Significant at 5% probability level; **Significant at 1% probability level; ^{a,b,c,d,e,f,g} for a character, the values that do not have the same letter as superscript are different.

Table 9. Expression of the traits relating to accumulation of terpenoid indole alkaloids in plant organs in the wild type (WT) and *leafless inflorescence* (*lli*), *evergreen dwarf* (*egd*) and *irregular leaf lamina* (*ill*) single and *lli egd*, *lli ill* and *egd ill* double mutants in *C. roseus*.

Genotype ($n = 8$)	Per cent total alkaloid content in roots	Serpentine content in roots (mg/100 g)	Ajmalicine content in roots (mg/100 g)	Catharanthine content in roots (mg/100 g)	Total content of % alkaloids in stem	Serpentine content in stem (mg/100 g)	Per cent content of total alkaloids in leaves	Content of serpentine + ajmalicine in leaves (mg/100 g)	Content of catharanthine in leaves (mg/100 g)	Content of vindoline in leaves (mg/100 g)	Content of VC + VB in leaves (mg/100 g)
WT	2.3 ± 0.4^{ab}	133.0 ± 20.7^{b}	15.0 ± 3.9^{ab}	4.3 ± 1.2^{ab}	1.3 ± 0.2^{a}	43.6 ± 3.7^{ab}	1.9 ± 0.1^{a}	5.4 ± 1.8^{ab}	3.8 ± 1.2^{b}	29.3 ± 4.6^{a}	3.5 ± 0.5^{a}
lli	2.8 ± 0.5^{b}	131.9 ± 22.4^{b}	24.1 ± 5.2^{b}	5.9 ± 1.6^{b}	1.5 ± 0.3^{ab}	34.0 ± 3.0^{ab}	1.6 ± 0.2^{a}	3.4 ± 0.7^{a}	2.0 ± 0.4^{ab}	31.1 ± 4.3^{ab}	3.3 ± 0.7^{a}
egd	2.7 ± 0.5^{b}	87.3 ± 8.2^{a}	18.4 ± 3.5^{ab}	1.6 ± 0.3^{a}	1.8 ± 0.1^{b}	44.5 ± 2.3^{ab}	1.5 ± 0.2^{a}	2.0 ± 0.5^{a}	1.4 ± 0.3^{a}	30.0 ± 3.9^{ab}	4.0 ± 0.9^{a}
ill	1.7 ± 0.1^{a}	147.4 ± 6.7^{b}	24.5 ± 3.3^{b}	5.5 ± 0.9^{b}	1.3 ± 0.1^{a}	64.0 ± 12.1^{c}	1.9 ± 0.2^{a}	8.6 ± 1.9^{b}	3.3 ± 0.5^{b}	63.3 ± 3.9^{c}	6.6 ± 1.1^{b}
lli egd	2.2 ± 0.2^{ab}	110.5 ± 7.4^{ab}	17.8 ± 2.2^{ab}	2.3 ± 0.5^{ab}	1.2 ± 0.2^{a}	43.1 ± 3.8^{ab}	1.6 ± 0.2^{a}	7.6 ± 1.9^{b}	2.6 ± 0.5^{ab}	21.8 ± 2.9^{a}	4.5 ± 0.4^{a}
lli ill	3.0 ± 0.3^{b}	78.8 ± 10.3^{a}	10.9 ± 1.9^{a}	8.0 ± 2.1^{b}	1.4 ± 0.1^{ab}	29.4 ± 5.0^{a}	2.0 ± 0.3^{a}	3.3 ± 0.7^{a}	2.9 ± 0.6^{ab}	30.6 ± 3.8^{ab}	3.0 ± 0.8^{a}
egd ill	2.1 ± 0.2^{ab}	118.4 ± 17.9^{ab}	18.9 ± 2.6^{ab}	5.3 ± 1.5^{ab}	1.4 ± 0.1^{ab}	47.0 ± 5.4^{b}	1.5 ± 0.1^{a}	4.8 ± 0.6^{ab}	3.3 ± 0.5^{b}	41.1 ± 4.0^{b}	4.8 ± 0.5^{ab}
Mean of all genotypes	2.4 ± 0.2	115.3 ± 9.5	18.5 ± 1.8	4.7 ± 0.8	1.4 ± 0.1	43.7 ± 4.2	1.7 ± 0.1	5.0 ± 0.9	2.7 ± 0.3	35.3 ± 5.1	4.2 ± 0.5
F value	1.9	2.9*	2.0	2.9*	1.6	3.5**	1.4	3.3**	1.7	11.9**	2.9*
CD 5%	0.96	41.91	9.65	3.66	0.44	16.79	0.55	3.76	1.78	11.19	2.06
CD 1%	1.29	55.88	12.87	4.88	0.59	22.39	0.73	5.02	2.37	14.91	2.74
Mean of individual mutation											
lli	2.7 ± 0.3	107.0 ± 15.4	17.6 ± 3.8	5.4 ± 1.7	1.3 ± 0.1	35.5 ± 4.0	1.8 ± 0.1	4.8 ± 1.4	2.5 ± 0.3	27.8 ± 3.0	3.6 ± 0.5
egd	2.3 ± 0.2	105.4 ± 9.3	18.3 ± 0.3	3.0 ± 1.1	1.5 ± 0.2	44.9 ± 1.1	1.5 ± 0.1	4.8 ± 1.6	2.4 ± 0.5	30.9 ± 5.6	4.4 ± 0.2
ill	2.3 ± 0.4	114.8 ± 19.9	18.1 ± 3.9	6.3 ± 0.9	1.4 ± 0.1	46.8 ± 9.9	1.8 ± 0.2	5.5 ± 1.6	3.1 ± 0.1	45.0 ± 9.6	4.8 ± 1.0
Comparisons											
WT vs *lli*	$F_{1:49} = 2.16$	$F_{1:49} = 6.97$*	$F_{1:49} = 1.3$	$F_{1:49} = 1.7$	$F_{1:49} = 0.5$	$F_{1:49} = 4.3$*	$F_{1:49} = 0.8$	$F_{1:49} = 0.5$	$F_{1:49} = 9.0$**	$F_{1:49} = 0.3$	$F_{1:49} = 0.1$
WT vs *egd*	$F_{1:49} = 0.03$	$F_{1:49} = 7.89$**	$F_{1:49} = 2.2$	$F_{1:49} = 2.0$	$F_{1:49} = 4.1$*	$F_{1:49} = 0.1$	$F_{1:49} = 6.7$*	$F_{1:49} = 0.4$	$F_{1:49} = 10.2$**	$F_{1:49} = 0.4$	$F_{1:49} = 3.6$
WT vs *ill*	$F_{1:49} = 0.13$	$F_{1:49} = 3.4$	$F_{1:49} = 1.9$	$F_{1:49} = 5.4$*	$F_{1:49} = 1.3$	$F_{1:49} = 0.6$	$F_{1:49} = 0.1$	$F_{1:49} = 0.1$	$F_{1:49} = 2.3$	$F_{1:49} = 36.6$**	$F_{1:49} = 7.2$**
lli vs *egd*	$F_{1:49} = 1.8$	$F_{1:49} = 0.1$	$F_{1:49} = 0.1$	$F_{1:49} = 4.9$*	$F_{1:49} = 1.1$	$F_{1:49} = 3.8$	$F_{1:49} = 1.9$	$F_{1:49} = 0.1$	$F_{1:49} = 0.1$	$F_{1:49} = 0.9$	$F_{1:49} = 1.9$
lli vs *ill*	$F_{1:49} = 2.3$	$F_{1:49} = 0.4$	$F_{1:49} = 0.1$	$F_{1:49} = 0.7$	$F_{1:49} = 0.1$	$F_{1:49} = 5.4$*	$F_{1:49} = 0.3$	$F_{1:49} = 0.5$	$F_{1:49} = 1.5$	$F_{1:49} = 28.6$**	$F_{1:49} = 4.2$*
egd vs *ill*	$F_{1:49} = 0.1$	$F_{1:49} = 0.6$	$F_{1:49} = 0.1$	$F_{1:49} = 9.3$**	$F_{1:49} = 0.5$	$F_{1:49} = 0.2$	$F_{1:49} = 3.7$	$F_{1:49} = 0.5$	$F_{1:49} = 1.9$	$F_{1:49} = 19.1$**	$F_{1:49} = 0.4$

*Significant at 5% probability level; **significant at 1% probability level; ^{a,b}for a character, the values that do not have the same letter as superscript are different.

Table 10. Effect of salinity on the sizes of leaf mesophyll parenchyma cells.

Genotype	Treatment	Area of spongy cell (μm^2)	Area of palisade cell (μm^2)	Adaxial–abaxial thickness of lamina next to midrib
WT	Control	490.7 ± 51.2	333.5 ± 50.3	161.4 ± 10.5
	Salt	197.6 ± 0.10	247.1 ± 12.7	158.3 ± 2.0
lli	Control	508.9 ± 32.9	415.3 ± 31.5	172.3 ± 21.4
	Salt	182.9 ± 14.5	254.4 ± 5.4	134.8 ± 25.5
egd	Control	560.2 ± 18.4	429.9 ± 46.2	161.4 ± 10.5
	Salt	231.8 ± 34.3	293.4 ± 33.7	150.4 ± 9.8
ill	Control	474.8 ± 67.1	383.5 ± 0.2	150.4 ± 0.4
	Salt	175.7 ± 21.9	272.7 ± 12.9	259.8 ± 99.6
lli egd	Control	546.8 ± 5.0	373.3 ± 10.4	122.3 ± 28.5
	Salt	202.5 ± 5.0	255.4 ± 4.3	148.9 ± 11.4
lli ill	Control	378.3 ± 163.5	385.5 ± 1.7	137.9 ± 12.9
	Salt	189.1 ± 8.4	260.5 ± 0.7	127.0 ± 33.3
egd ill	Control	868.1 ± 326.3	365.2 ± 18.5	150.4 ± 0.4
	Salt	237.9 ± 40.4	234.9 ± 24.9	142.6 ± 17.6
'F' for genotypes		1.34	1.75	1.09
'F' for treatments		37.2**	84.91**	0.29
'F' for interaction		0.8	0.4	1.1
CD for genotypes 5% P		228.1	54.4	70.6
CD for salt 5% P		121.9	29.1	37.7
CD for interaction 5% P		322.6	76.9	99.8

**Significant at 1% level of probability.

largely similar. Their pleiotropy shared several other features. In comparison to the wild type, their average expression over 48 traits revealed the following as their pleiotropy: slower rate of water loss, higher content of water under drought and saline conditions and lower water content under conditions of abundant irrigation water; higher frequency of stomata on epidermis, bigger palisade parenchyma cells, smaller leaves, higher total photosynthesis in leaves and chlorophyll in lower concentration; lower content of Na^+ and higher contents of K^+, proline, trehalose and terpenoid indole alkaloids; smaller flower organs, smaller pods and larger seeds; and higher root/shoot ratio. Lower water loss despite abundance of stomata in mutants seems to indicate a mechanism in them for the negative control of stomata opening. Each mutation was associated with some distinctive features in addition to their name-wise unique morphology. The *lli* mutation was associated with largest biomass in stems, leaves and root, seeds of biggest size and very high content of trehalose. The *egd* mutation bestowed the plants with highest rates of total photosynthesis in leaves, and pavement cells and spongy mesophyll parenchyma cells of largest sizes; and highest content of water and least rate of loss of water from leaves. The *ill* mutation led to highest increase in vindoline and vincristine and vinblastine contents in leaves and catharanthine content in roots, least content of chlorophylls and photosynthetic rate in leaves; and least accumulation of biomass in roots, stems and leaves. It will be seen from figure 4 that the wild type and mutants have distinctive pleiotropies based on the quantitation of the 48 traits studied here. It is possible to conclude that single site mutations at *lli*, *egd* and *ill* loci that are not linked to each other resulted in a very wide range of changes, some similar, some distinctive. The *lli*, *egd* and *ill* mutations are thought to be in loci/genes that have very large and wide regulatory roles in the regulatory gene network of *C. roseus* for metabolism, development and adaptation to environment.

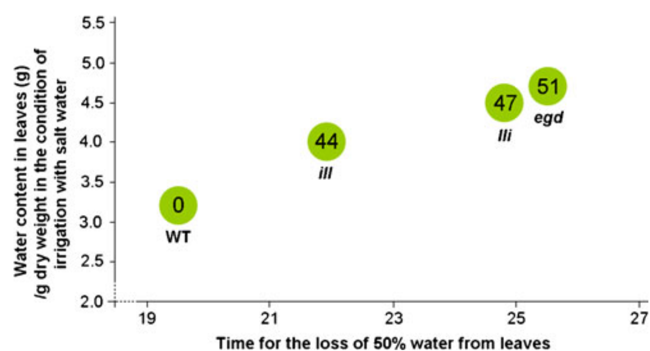

Figure 4. Distribution of wild type, *irregular leaf lamina* (*ill*), *leafless inflorescence* (*lli*) and *evergreen dwarf* (*egd*) mutants of *C. roseus* against the trait called time period for the loss of 50% water from leaves (abscissa) and the trait called water content in leaves of plants irrigated with saline water (ordinate). The value given in the genotype circle is the total score based on all the 50 traits studied. For each trait, the wild type value was given a score of 0. The ascending/descending values of the mutants were given 1, 2 or 3 score. The values that were significantly not different were given the same score.

Relationship of pleiotropies in mutants with their DNA hypomethylation characteristic

The repeat sequences in rDNA arrays and centromeric DNAs of *lli*, *egd*, *ill*, *lli egd*, *lli ill* and *egd ill* mutants were found to be more or less similarly more digestable by *Msp*I as compared to the corresponding DNA sequences in the wild type. These observations are indicative of widespread demethylation at cytosine residues in the genomes of *C. roseus* mutants. In *A. thaliana*, widespread cytosine demethylation is known in *ddm1* and *drm1 drm2 cmt3* mutants and selectively in the coding regions of genome in *met1* mutants (Zilberman and Henikoff 2007; Saze and Kakutani 2011; Pecinka and Mittelsten Scheid 2012) and like the latter in *vim/orth* mutant (Woo and Richards 2008). These mutants also demonstrate a variety of altered phenotypes related to plant organ development and differential expression of protein-coding genes as compared to their counterpart wild types. Genome-wide demethylation is also known to occur, following exposure to biotic or abiotic stress conditions and this adaptive response is associated with upregulation and downregulation in expression of several to many genes (Labra *et al*. 2002; Alina *et al*. 2004; Wada *et al.* 2004; Akimoto *et al.* 2007; Choi and Sano 2007; Lisch and Bennetzen 2011; Dowen *et al.* 2012; Karan *et al.* 2012; Luna *et al*. 2012; Slaughter *et al*. 2012). The upregulation of expression in protein-coding genes following cytosine demethylation in genomes is known to result from three consequences of demethylation: (i) removal of methylation marks at promoter or adjacent sequences that hindered the binding of transcription factors at these sites; (ii) removal of cytosine methylation from gene bodies such that transcription could now occur without premature interruption; and (iii) read through from promoters in transposons (especially retrotransposons located upstream of the genes) activated because of their demethylation (Henderson and Jacobsen 2007; Aceituno *et al.* 2008; He *et al.* 2011). The protein-coding and miRNA-coding genes expression changes, especially upregulation, in gene expression in the *lli*, *egd* and *ill* mutants are thought to result from above described three consequences of demethylation at the gene sites.

Of the 126 genes whose transcription was investigated in the *C. roseus* mutants, 85 genes were upregulated and 41 were downregulated. The downregulation of coding genes could occur on account of one or more of the following kind of events. (i) Due to upregulation of repressive transcription factor(s) or miRNA(s), the target gene(s) may undergo downregulation. (ii) Demethylation at genes may be associated with repressive chromatin remodelling. (iii) Read through of antisense strand from transposon located downstream of the gene may lead to underestimation/repression of transcription.

Together with the principal morphological feature(s) after which the *lli*, *egd* and *ill* mutants were named, mutants differed from the wild type in many of the 50 traits for which they were quantitatively surveyed. The traits were reflections of the interactions between functions of genes concerned with metabolism, organ development and response to environment. Such genes are thought to be under the control of regulatory gene networks. Demethylation in the mutants caused widespread changes in the expression of genes either directly by removal of methylation marks from the operons or indirectly by causing such change(s) at the sites of regulatory genes. The pleiotropies displayed by the mutants are thought to result from gene expression changes affecting various kinds of functions responsible for achievement of plant morphology.

Mechanism of hypomethylation in lli, egd and ill mutants?

Inheritable demethylation at cytosine sites in the nuclear DNA can occur via deficiency in the active DNA methylation and maintenance DNA methylation pronounced demethylation. Active methylation via RdDM is a process in which a very large number of gene functions are involved (Wierzbicki *et al.* 2012). RdDM and maintenance methylations by MET1 and CMT3 methyltransferase functions are also integrated with nucleosome remodelling functions (Johnson *et al.* 2007; Woo and Richards 2008; Greenberg *et al.* 2011). There is considerable redundancy in the demethylation functions (Zhu 2009). Thus decrease in activity of one or more methylation related functions or increase in activity of demethylation functions or both together can produce the demethylation phenotype observed in *lli*, *egd* and *ill* mutants. Differences in the morphologies of mutants and their Mendelian inheritance indicate that mutational events occurred at different locations on the genome of *C. roseus*. Perhaps insertion of some activated transposable element(s) was involved in each case. It is thought that methylation and demethylation processes themselves must be regulated enabling their coordinated expression. Transposon insertions may have disrupted the regulation of methylation and demethylation processes such as to reduce methylation and increase demethylation. The observed downregulation of *CMT3*, *RDR2* and *DRM2* genes in mutants provides partial support to this explanation. The *lli*, *egd* and *ill* are morphological-cum-salinity-tolerant mutants. Following the genetic change, the genome of each of these three mutants was heritably (permanently) altered to stress response. The *lli* and *egd* mutations are perhaps illustrations of single site mutation led morphologically distinctive and fertile changes of evolutionary consequences. The *lli*, *egd* and *ill* mutants of *C. roseus* may share some casual properties of altered morphology with some well known epigenetic variants such as of *Linaria vulgaris* (Cubas *et al.* 1999) and *Solanum lycopersicon* (Schmitz *et al.* 2011). Further work on the *C. roseus* mutants studied here may be helpful in advancing knowledge about coordination between DNA methylation and demethylation processes.

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