Pleiotropic morphological and abiotic stress resistance phenotypes of the hyper-abscisic acid producing Abo$^-$ mutant in the periwinkle *Catharanthus roseus*

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The pleiotropic properties of a *abo abo* (Abo$^-$) *g*-ray induced mutant of *Catharanthus roseus* cv. Nirmal, selected among the M_2 generation seeds for ability to germinate at 45°C, are described. The mutant produced seeds possessing tricotyledonous embryos, unlike the typically dicotyledonous embryos present in the wild type Abo$^+$ seeds. In comparison to Abo$^+$ adults, the mutant plants had short stature and lanceolate leaves. The vascular bundles in the leaves and stem were poorly developed. Leaf surfaces were highly trichomatous, epidermal, cortex and mesophyll cells were small sized and a large majority of stomata were closed. Besides high temperature, the mutant was salinity and water-stress tolerant. The abscisic acid (ABA) content in the leaves was about 500-fold higher. The genetic lesion *abo* responsible for the above pleiotropy was recessive and inherited in Mendelian fashion. The seedlings and adult plants of the mutant accumulated higher proline than Abo$^+$ plants. The phenotypes of *abo abo* mutants permitted the conclusions that (i) the mutant synthesizes ABA constitutively, (ii) both ABA-dependent and ABA independent pathways for proline and betaine accumulation are functional in the mutant, and (iii) cell division, elongation and differentiation processes in embryo and adult plant stages are affected in the mutant.

1. Introduction

It is established that plants can tolerate and adapt to sub-lethal levels of a variety of stresses, including the abiotic stresses caused by low and high temperature, drought, salinity and injury. The molecular mechanisms of adaptation to abiotic stresses in plants are quite complex. These have been shown to involve a cascade of events starting from the perception of stress to the expression of functions that mitigate the stress (Campalans *et al* 1999). The plants exposed to stress can modify their essential metabolic processes and in addition are able to synthesize enzymes (Mittler and Zilinska 1994; Williams *et al* 1994) and proteins (Rubio *et al* 1995; Close 1996) and metabolites (Delauny and Verma 1993; Sakamoto and Murata 2000) that can regulate the expression of sensitive operons, repair cellular damage, restructure cell organelle(s) and/or help ward off stress. While some of the stress induced gene functions have been found to be common for acclimation to cold, heat, dessication and/or salinity, many are specific to the type of abiotic stress. Besides, the inducible adaptive responses, plants synthesize some of the stress mitigating gene products constitutively (Shinozaki and Yamaguchi-Shinozaki 1996).

Several lines of evidence implicate the phytohormone abscisic acid (ABA), synthesized from isopentenyl phosphate via C_{40} xanthophylls (Campalans *et al* 1999), could have a regulatory role in conferring stress tolerance in plants (Chandler and Robertson 1994; Leung and Giraudat 1998; Campalans *et al* 1999). The endogenous ABA levels are known to be high in plants undergoing cold, drought or salinity stress. The ABA-pretreated plants demonstrate tolerance to environmental stresses (Zeevart and Creelman 1988; Shinozaki and Yamaguchi-Shinozaki 1996). Some similarity has been observed between the protein profiles induced by stresses and those induced by application of ABA in unstressed plants (Söderman *et al* 1996; Hong *et al* 1997; Netting 2000). The ABA-deficient

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(*aba*) mutants iso1ated in several plants are more stress-sensitive than their wild-types (Leotenberg *et al* 1999) because the proteins induced by stress conditions remain largely unexpressed in them (Chandler and Robertson 1994). There is as yet little evidence to relate any of the ABA-induced proteins either to stress tolerance or to recovery from stress.

A small number of ABA-deficient, ABA-insensitive ABA-hypersensitive and/or ABA deregulated mutants have become available in *Arabidopsis thaliana* (Koorneef *et al* 1984; Cutler *et al* 1996; Wu *et al* 1996; Hurry *et al* 1997; Schwartz *et al* 1997; Foster and Chua 1999; Quesada *et al* 2000), *Hordeum vulgare* (Raskin and Ladyman 1988), *Lycopersicon esculentum* (Linforth *et al* 1987; Burbidge *et al* 1998), *Nicotiana plumbaginifolia* (Rousselin *et al* 1992; Marlin *et al* 1996), *Pisum sativum* (Duckham *et al* 1989) and *Zea mays* (McCarty *et al* 1991; Sturaro *et al* 1996; Tan *et al* 1997). Their phenotypes have revealed roles of endogenous ABA in plants at post-embryo development stages seed maturation, onset and maintenance of dormancy (Garello and Le Page-Degivry 1999) and seed germination. The protein products of some of these genes have been identified and a few of them have also been cloned (Marlin *et al* 1996; Schwartz *et al* 1997). The analysis shows that typically ABA interacts with G-box ACGT core motifs and transcription factor at promoter site to activate transcription of gene(s) (Ono *et al* 1996; Busk *et al* 1997; Guan *et al* 2000; Lopez-Molina and Chua 2000). Fuller description of the ABA roles in plants awaits isolation of mutants of ABA-biosynthesis, ABA-metabolism, and signal transduction process possessing new phenotypes and identification of phenotypes of mutants affected in the ABA regulated genes expressed in vegetative tissues in plants.

Periwinkle *Catharanthus roseus* a diploid ($2n = 16$), small sized, self compatible and rapidly recyclable plant and the only source of potent anticancer drugs vinblastine and vincristine, is emerging as a model experimental system for the analyses of developmental and environmental controls on plant secondary metabolism (Vazquez *et al* 1997; St. Pierre *et al* 1999). The chemistry of the indole terpenoid alkaloids has been investigated extensively (Blasko and Cordell 1990; DeLuca 1993). Exposure of the periwinkle cell and organ cultures to a variety of stress conditions or supplementation with ABA is known to increase the expression of the alkaloid biosynthetic pathway (Smith *et al* 1987; Blasko and Cordell 1990; DeLuca 1993). In our investigations to genetically modulate the expression of secondary metabolism, a number of phenotypically different mutants have been isolated in *C. roseus.* The present work reports on the isolation and characterization of a mutant in *C. roseus* in which a recessive allele at a locus confers ABA-overproduction and abiotic stress resistance.

2. Materials and methods

2.1 *Mutagenesis*

The Abo^- mutant was isolated in the background of *C. roseus* cv. Nirmal, which bears pure white flowers (Kulkarni *et al* 1992). About 20,000 seeds were irradiated with 10 to 45 krad *g*-rays, in batches of about 1000 seeds, using a Cobalt 60 source that emitted *g*-rays of 200 rads/min in a gamma cell 200. The Cobalt 60 source was provided by the Bhaba Atomic Research Center, Mumbai, India. The treated seeds were sown over farm yard manure : soi1 :: 2 : 1 mixture in earthen pans. About one month old M_1 seedlings were transplanted in the field. The M_2 seeds were harvested from each individual plant.

2.2 *Isolation of mutants*

The M_2 and control seeds were sterilized separately with 0·1% $HgCl_2$ for 1 min, washed thoroughly with distilled water and blotted dry. The seeds were separately sown over sterile double layer of Whatman No. 1 filter paper circles moistened with milli-Q-water kept in petridishes which were incubated at 45°C. No wild type seed, of about 20,500 tested, was found to germinate under these conditions. By screening a total of 35,200 seeds raised from about 700 M_1 plants, a few temperature tolerant seedlings were recovered; one of the selected seedling was transferred to soil in a pot and grown in the glass-house. After about a month the pot was transferred to a greenhouse, and upon maturity of the plant M_3 seeds were harvested. The temperature tolerance phenotype of M_3 progeny seedlings was tested as described above and the seedlings were transplanted in the field to multiply the seeds. M_2 generation onwards the flowers were manually self-pollinated to obtain pure seeds of this line. In the initial stages of the work itself the Abo^- plants had been found to possess pleiotropic phenotype comprising of three cotyledons, dwarf habit, fistulate leaves and heat tolerance.

2.3 *Screening of seeds, seedlings and plants under ambient and stressed conditions*

(a) The effect of light, salinity and temperature on seedlings in Abo^- and Abo^+ stocks was studied using 100 seeds, in triplicate. Seeds were sown on moistened filter paper in a Petridish, and irrigated with deionized water or 200 mM NaCl. The incubation temperatures were 25° and 45°C. The plates were covered with black paper for incubation of seeds in dark. The plates incubated in light received florescent light of 3000 lux for 16 h/day. Germination was scored after 2 weeks of incubation. The Abo^- and

Abo^+ stocks were sown separately on moistened filter paper in Petridishes, using deionized water (0·1 dSm^{-1}) for irrigation and incubating the Petriplates at 25°C. Five days old seedlings were treated variously: (i) some were retained under these very conditions; (ii) some were transferred to 35°C and some to 45°C, and irrigated with deionized water; (iii) some were transferred to new Petridishes for which the irrigation medium was 200 mM NaCl and incubation temperature 25°C.

(b) To compare the adult p1ants of the mutant and wild type, the Abo^+ and Abo^- seedlings were raised in Petridishes as described above. The 15 day seedlings were transferred to vermiculite in pots and grown in the glasshouse. The plants were next transferred to a greenhouse. After 2 months plants were given the varying temperature and salinity treatments for 7 days as described above for seedlings. Several plants of each genotype were retained in the greenhouse to characterize them at the age of 8 months.

(c) To compare the Abo^- and Abo^+ plants for their drought tolerance, plants grown in green house for about 8 months in pots on soil were used. The well watered soil surface (80% soil moisture) and the pot of each plant was covered with polythene to avoid evaporation of soil water. The plants were kept in the described conditions as above in triplicate. The soil moisture and proline contents of leaves were assayed monthly till Abo^- and/or Abo^+ had wilted/dried up.

2.4 *Estimation of endogenous proline, betaine, alkaloid and ABA*

The proline estimations were carried out in triplicate on 2 week old entire seedlings and leaves of two month old plants. For each sample, approximately 0·5 g of fresh tissue was ground in 3% sulphosalicylic acid and the extract was assayed for proline concentration by the method described by Bates *et al* (1973). For betaine estimations the tissue was dried in oven at 75°C, ground in a blender and assayed for betaine by the periodide assay (Grieve and Grattan 1983). For alkaloid analysis leaves sampled from eight month old plant were dried in shade and extracted for alkaloids by following the method already standardized (Anonymous 1966).

The ABA estimations were made on freshly harvested leaves. About 30 g samples of leaves were collected from mature plants grown in green house or field. Each leaf sample was extracted with absolute methanol overnight at 4°C. After filtration, methanol was evaporated in vacuum at 40°C and the aqueous phase was frozen, thawed and centrifuged at 15000 *g* at 2°C for 20 min. Supernatant was further purified by the procedure of Knegt and Bruinsma (1973) as modified by Kamisaka and Larsen (1977). Evaporation of the acidic ether phase in vacuum at 35°C furnished a residue which was dissolved in methanol and chromatographed on preparative thin-layer chromatography (TLC) (20 × 20 cm, 0·4 mm thick silica gel G) with benzene : ethyl acetate : acetic acid :: 70 : 30 : 10 as the solvent system. The authentic (±)-cis, *trans*-ABA (Sigma) was spotted on the plate as a marker. The TLC plates were dried and visualized by exposure to iodine vapours and UV. Silica gel corresponding to marker, was scrapped, suspended in methanol and concentrated. The residue was dissolved methanol and analysed using high performance liquid chromatography (HPLC) (Shimadzu-LC8A HPLC) equipped with photodiode array detector. The chromatography was done with MeOH : H_2O :: 1 : 1 as solvent [flow rate = 0·7 ml/min, column = Lichro CART (124–4 mm) PP-18 HPLC column (5 ~ m)]. The quantitation was done considering specific area of the peak knowing the retention time of a standard ABA at 254 nm.

2.5 *Microscopic studies*

The seeds were germinated on water soaked filter paper in Petridishes at 25°C in the dark and transferred to light after 3 days. Hypocotyls of the 2 weeks old seedlings were hand sectioned. The juveni1e leaves and stems of 2 month-old plants were also sectioned. The fresh sections were mounted in glycerin and examined using a Nikon optiphot-Pol inverted light microscope. The stomatal morphology was studied using leaf epidermal peels. Each experiment was successfully repeated thrice and about 100 stomates were scored each time.

2.6 *Genetic analysis*

To study the inheritance of the phenotypes of Abo^- genotype, the mutant was crossed with parental Abo^+ wild type, reciprocally. The buds were hand emasculated in the morning and pollinated in the evening. The flowers borne on the F_1 plants were self pollinated to produce F_2 seeds. The parent, F_1 and F_2 plant phenotypes were compared.

3. Results

3.1 *Isolation of temperature tolerant mutant(s)*

Plants are known to acquire thermotolerance by prior exposure to elevated temperature (Burke *et al* 2000). It has also been demonstrated that constitutive expression of a heat shock transcription factor increases the level of thermotolerance in *Arabidopsis thaliana* without prior exposure to higher temperature (Prandl *et al* 1998). We wanted to isolate mutants in periwinkle *C. roseus* in which thermotolerance would be expressed without the need for pre-exposure to non lethal temperature. In this regard it

had been shown in the preliminary experiments that the periwinkle *C. roseus* cv Nirmal seeds did not germinate when incubated over moistened filter paper at 45°C. Accordingly it was thought that the seeds of thermotolerant mutants of periwinkle would germinate in the above test at 45°C. To recover temperature tolerant mutants, a mutation experiment was conducted in *C. roseus* cv. Nirmal. About 20,000 seeds were irradiated with varying doses of *g*-rays (10–45 kr). The irradiated seeds gave rise to 700 M_1 plants when sown in the field. The seeds harvested from 10 M_1 plants each were pooled together for ease in screening. The germination test at the high temperature was conducted altogether on about 35,200 M_2 seeds. Among a batch of seeds from ten plants, 4 seeds germinated and produced seedlings on moistened filter paper in Petridish incubated at 45°C. These seedlings were assumed to be progeny of an M_1 plant and therefore one seedling was grown to maturity and seeds were collected, following selfing. The ability of M_3 seeds to germinate at 45°C, confirmed the temperature tolerant phenotype. The mutant has since been maintained as a pure stock for six generations. The seedlings and adult plants of the mutant, since given the designation Abo^- (ABA overproduction), were observed in the early M_2 generation to be morphologically distinct from the Abo^+ wild type plants.

3.2 *Morphological features of Abo⁻ mutant*

The M_3 seeds were used to raise many temperature tolerant seedlings which were then taken to maturity and observed throughout their life cycle. The phenotype of the temperature tolerant mutant was dramatically different from that of the wild type, at all stages of development (figure 1). The wild type seedlings typically have two cotyledons while the temperature tolerant mutant seedlings exhibited three distinct cotyledons. The mutant and wild type differed morphologically as well as anatomically (table 1). In comparison to the obovate, elliptic and smooth leaves of the wild type, those of the mutant plant were linear, thicker and curved and small sized, bearing a fistulated shape. The mutant plants were dwarf and had a bushy canopy due to profuse branching at the base. Mutants plants had five-fold fewer flowers than the wild type. The flowers, fruits and seeds were also smaller in the mutant than the wild type. There was high degree of pollen sterility in the mutant. Seed setting per fruit was several fold lower than the wild type.

It will be seen from the observations summarized in the table 2 that the dwarf nature of Abo^- plants was related to reduced development of sclerenchyma and vascular tissues and reduction in the size of cells in all the tissues as compared to the wild type (figure 2). The laticiferous system was also poorly developed in the mutant shoot. The frequency of trichomes on the abaxial and adaxial surfaces of leaves was higher in the mutant. The leaves in the mutant had additional layers of mesophyll cells (figure 3). On the adaxial surface about 50% of stomata were closed and/or semi-closed in Abo^- plants, whereas only 15% stomata were closed or semi-closed in Abo^+ plants. The reduced transparation in the Abo^- plants reflected their xerophytic character.

3.3 *Multi-abiotic stress tolerant behaviour of Abo⁻ mutant*

The temperature tolerant mutant abo^- shared properties which are typical of drought-tolerant plants. Therefore,

Table 1. Morphological characters of Abo^- mutant and Abo^+ wild type plants of periwinkle *C. roseus*.

Characters[a]	Wild type Abo^+	Mutant plant Abo^-
Total plant biomass (g/plant)	69·0 ± 6·3	16·0 ± 1·5
Plant height (cm)	63·4 ± 2·2	9·7 ± 0·4
Main stem diameter (mm)[b]	12·0 ± 0·8	11·1 ± 0·3
Internodal length (cm)	2·1 ± 0·1	0·2 ± 0·1
Number of branches/plant[c]	26·7 ± 3·5	5·0 ± 1·2
Length of petiole (cm)	0·7 ± 0·3	1·4 ± 0·1
Number of leaves/plant	496 ± 31	308 ± 22
Leaf length (cm)	4·7 ± 0·3	2·4 ± 0·1
Leaf width (cm)	2·3 ± 0·2	0·7 ± 0·1
Leaf area (cm^2)[d]	8·9 ± 1·5	1·3 ± 0·1
Leaf biomass (g/plant)	43·0 ± 4·2	12·0 ± 1·3
% alkaloid in leaves	1·3 ± 1·0	1·9 ± 1·0
Number of flowers/branch	121 ± 4·0	20 ± 1·0
Length of corolla tube (cm)[e]	2·9 ± 0·1	2·2 ± 0·1
Length of petal (cm)	2·3 ± 0·2	1·0 ± 0·1
Width of petal (cm)	1·8 ± 0·1	0·4 ± 0·1
Length of sepal (cm)	0·4 ± 0·3	0·4 ± 0·1
Pollen fertility (%)[f]	87 ± 4·0	12 ± 2·0
Pollen germination (%)[g]	45·7 ± 2·3	3·3 ± 0·9
Length of style (cm)	1·9 ± 0·1	1·5 ± 0·1
Length of pods (cm)	2·8 ± 0·1	0·7 ± 0·1
Average number of seeds/siliqua	16 ± 0·6	1·0 ± 0·3
Seed length (mm)	2·2 ± 1·7	2·4 ± 0·1
Seed width (mm)	1·1 ± 0·1	0·9 ± 0·1
100 seeds weight (mg)	81 ± 2·0	41 ± 1·0

[a]All the quantitative observations were taken in triplicate in 8 months old green house grown plants and seeds produced on them.
[b]Main stem diameter was measured in the middle of land surface and site of emergence of first branch from stem.
[c]Total number of branches were counted.
[d]Leaf area was measured by using leaf area meter.
[e]Floral morphology was studied by examination of flowers under dissecting microscope.
[f]Pollen fertility was assessed using acetocarmine and fluorochromatic reaction tests (Heslop-Harrison and Heslop-Harrison 1970).
[g]Pollen germination was assessed in Brewbaker medium (Brewbaker and Kwack 1963).

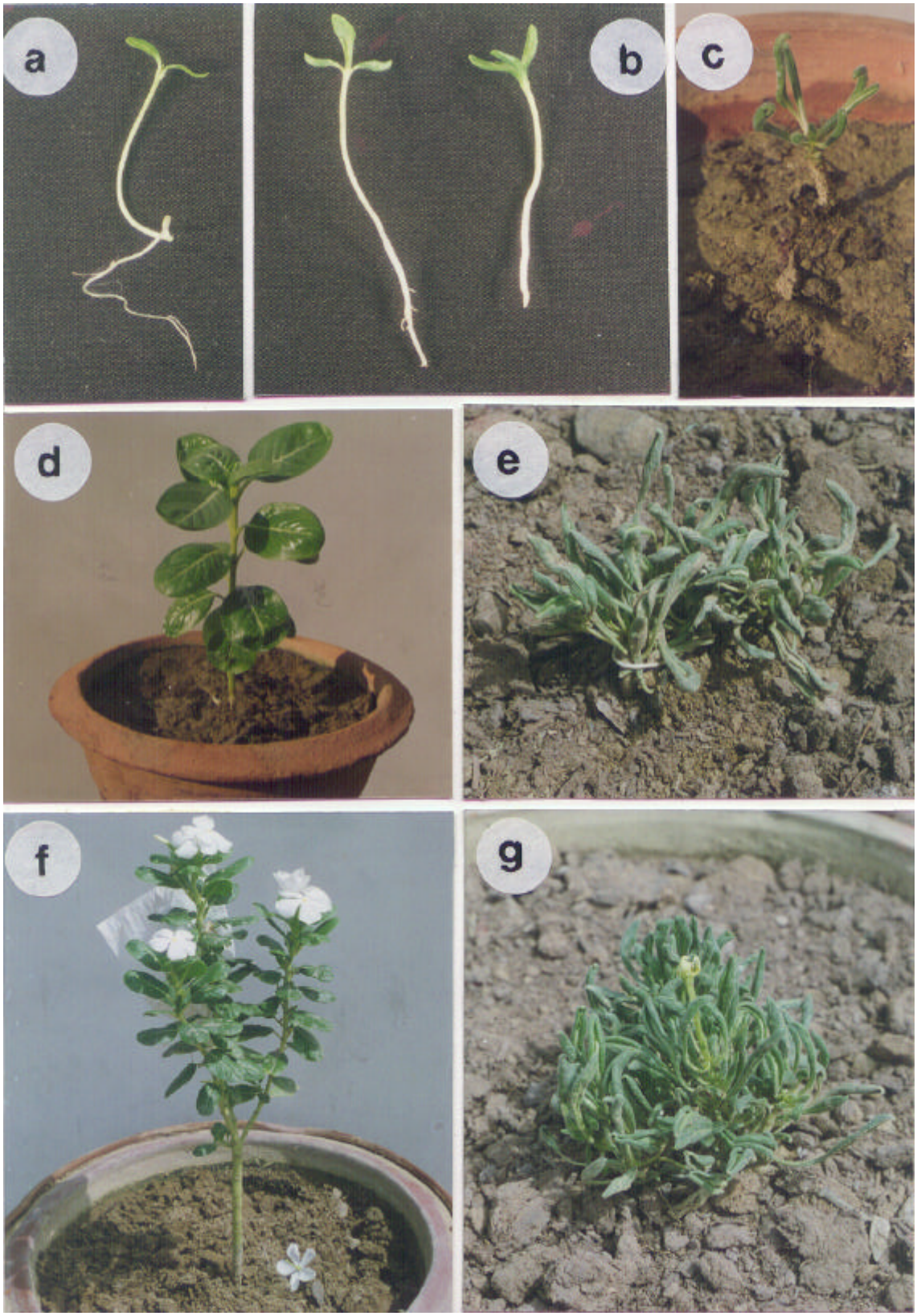

Figure 1. Morphology of the wild type and mutant Abo⁻ plants of *C. roseus.* (**a**) Abo⁺ two cotyledons bearing two weeks old seedling. (**b**) Two week old Abo⁻ seedlings bearing three cotyledons. (**c**) One month old Abo⁻ bearing-plant. (**d**) Two months old Abo⁺ plant. (**e**) Two months old Abo⁻ plant. (**f**), (**g**) Three months old adult reproductive Abo⁺ and Abo⁻ plants.

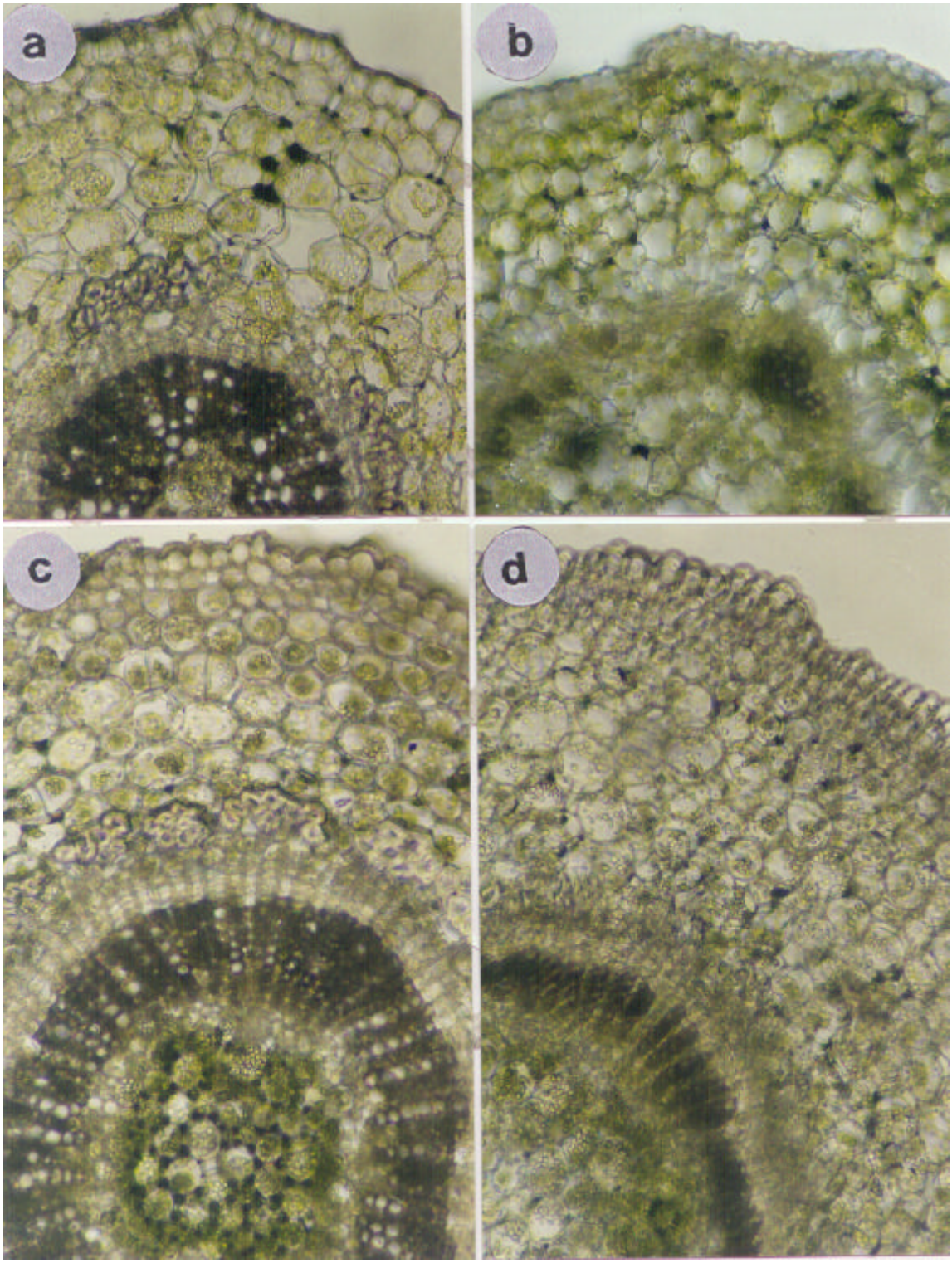

Figure 2. Anatomical features of Abo^{+} and Abo^{-} seedlings and adult plants. Transverse section of hypocotyl of (**a**) Abo^{+} seedling and (**b**) Abo^{-} seedling; transverse section of two month old plants of (**c**) Abo^{+} and (**d**) Abo^{-}.

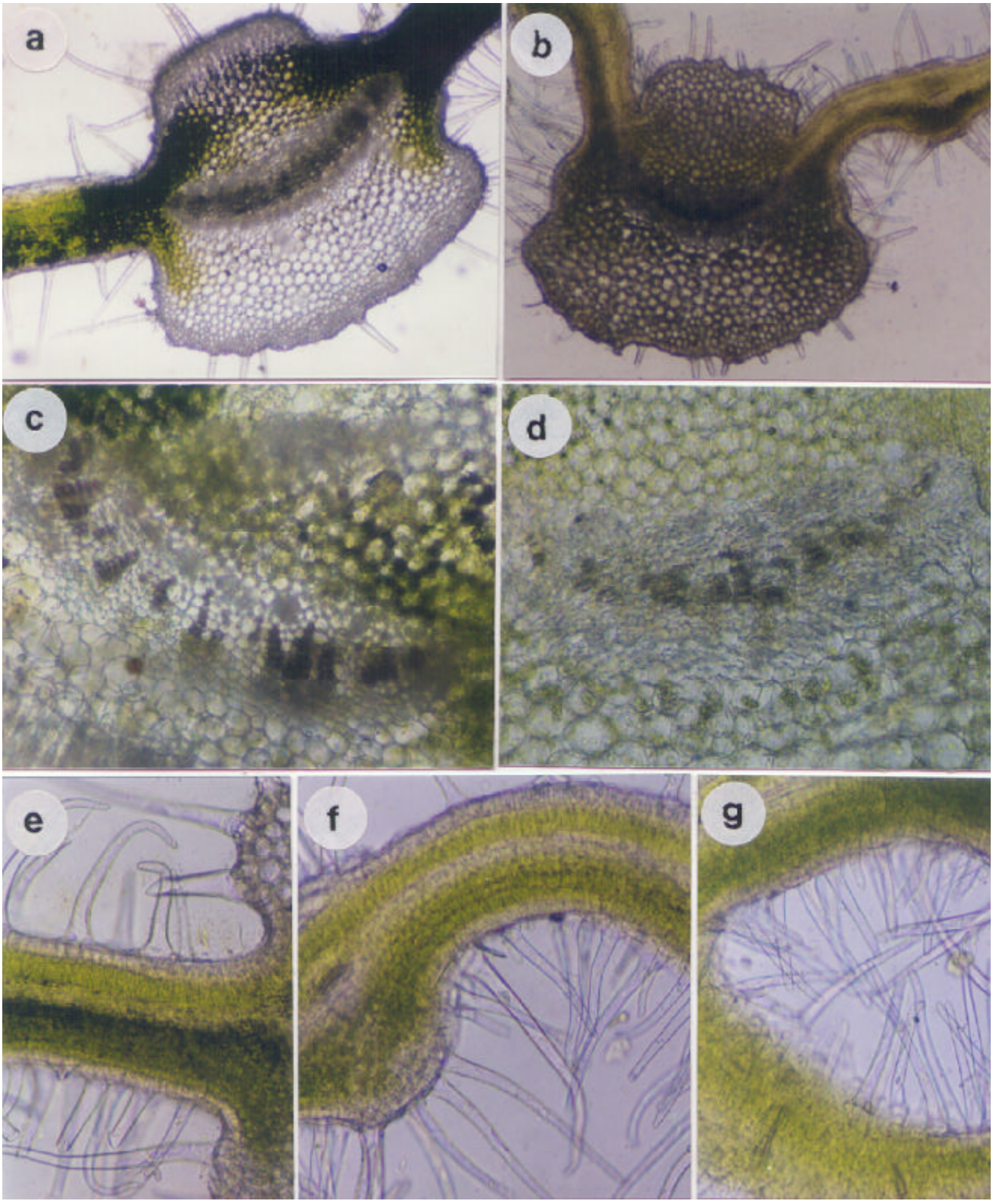

Figure 3. Anatomical features of Abo^+ and Abo^- leaves from on two months old plants. (**a**) Transverse section of Abo^+ leaf. (**b**) Transverse section of Abo^- leaf; corresponding sections at 10 × magnification from midrib region showing vascular tissues. (**c**) Abo^+ and (**d**) Abo^-; leaf lamina at 10 × magnification showing tricomatous epidermis, pallisade and spongy tissues in (**e**) Abo^+, (**f**) Abo^- and (**g**) curved lamina of Abo^- leaf.

the mutant was screened for tolerance against high NaCl and water stress (table 3). The wild type seeds germinate at temperatures up to 39°C and in the presence of 150 mM NaCl but failed to do so at 45°C and 200 mM NaCl. The Abo^- mutant seeds tolerated 45°C and 200 mM NaCl salinity and all of them germinated producing healthy seedlings. When two months old plants of the mutant and wild type were exposed to the above high temperature and high salinity stresses for a period of 1–2 weeks, both types survived for one week. However, later whereas the wild type plants had died, while the mutant plants continued to survive. To find out whether Abo^- and Abo^+ plants differentially accumulated the compatible osmolytes, their proline and betaine contents were compared. Seedlings and leaves of 2 weeks old plants of Abo^- mutant had 50–80% higher level of endogenus proline as compared to the wild type plants. The proline content in the seedlings and the leaves of high temperature stressed Abo^- plants was about 2-fold higher than that in the wild type. Both types of seedlings accumulated similarly high levels of proline. The leaves of Abo^- mutant plants treated with salinity stress showed about 50% more proline content than comparably stressed leaves of the wild type plants. The glycine-betaine content was 25% more in the mutant seedlings germinated in saline water (150 or 200 mM) than the wild type seedlings (figure 4).

Comparison of the adult mutant and wild type plants for their response to irrigation withdrawal showed that wild type lost soil water through transpiration much more rapidly than the mutant plants (table 3); withholding of irrigation for 3 months in potted plants led to the death of wild type, while the mutant type survived. By about two months of drought stress, the leaves of mutant plants had accumulated about 33% more proline than those of the wild type.

The observations described above demonstrated that the mutant plants had the ability to accumulate proline both constitutively and by induction, the latter being more pronounced under the high temperature stress. The wild type plants also accumulated proline under stress albeit at a lower level than the mutant plants.

3.4 *Overproduction of ABA in Abo* $^-$

The results discussed above indicate that the Abo^- mutant plants accumulated higher levels of proline and glycine-betaine in shoot as compared to wild type plants both in the presence and absence of temperature, salinity and drought stresses. It is known that imposition of salinity, drought or cold stresses combined with the application of ABA on the other hand lead to accumulation of compatible solutes such as proline and betaine. Further, the concentration of endogenous ABA rises in plants that have been imposed with these above stresses. Thus Abo^- mutant plants could be over producer of ABA. The results

Table 2. Anatomical characteristics of Abo^- mutant and Abo^+ wild type plants of periwinkle *C. roseus*.

Organ	Organ/tissue characteristic	Abo^- mutant	Abo^+ wild type
Stem	Epidermis	Cell round of small size (20 μm × 18 μm)	Cells elongated of large size (25 μm × 15 μm)
	Cortex	Small cells (176 μm^2), compactly arranged	Large cells (314 μm^2), loosely arranged
	Scelerenchyma	Absent or reduced around the vascular bundles	Fully developed around the vascular bundles
	Phloem	Less developed	Well developed forming a ring around the xylem
	Xylem	Xylem cells occur scattered	Xylem cells are arranged in the shape of a ring
	Pith	Well developed and large	Less developed
	Laticifers, idioblast tissue	Infrequent	Frequent
Leaf	Epidermal layer	Cells small (18 μm × 15 μm), large trichomes (143 on abaxial and 256 on adaxial surface), frequency of trichomes high (12 on abaxial surface and 25 on adaxial surface); 18% stomata on adaxial surface semiclosed and 40% closed	Cells large (22 μm × 19 μm), small trichomes (110 on abaxial surface and 167 adaxial surface), less frequent (4 on abaxial and 18 on adaxial surface); 15% stomata of adaxial surface semiclosed, nil closed
	Hypodermal Sclerenchyma	Absent or poorly developed below the epidermis	Well developed
	Xylem and phloem	Poorly developed	Well developed
	Laticifers and idioblast tissue	Infrequent	Frequent

shows that the leaves of the adult plants of Abo⁻ contained more than 500-fold ABA per unit biomass than the wild type (table 4).

3.5 *Inheritance of Abo⁻ pleiotropic phenotype*

The Abo⁻ mutant differed from the wi1d type in its being relatively more tolerant to high temperature, salinity and drought and also accumulated more proline and glycine-betaine in its leaves. The morphological differences between Abo⁻ and Abo⁺ plants included the presence of three cotyledon, thicker and hypertrichomated leaf lamina and poorly developed vascular and sclerenchymatous tissues in the mutant as compared to the wild type. The wild type (2 cotyledons, normal leaf, sensitivity to 45°C and 200 mM NaCl solution) phenotype of the F_1 plants from reciprocal crosses between *abo⁻ abo⁻* (Abo⁻) mutant

Table 3. Seed germination, seedling and plant growth and proline content characteristics of the stress tolerant Abo⁻ and wild type genotype in periwinkle *C. roseus.*

Parameter	Conditions of growth	Genotype (phenotype)	
		abo abo (Abo⁻)	Wild type (Abo⁺)
Hypocotyl length (cm)	Distilled water[a]	1·6 ± 0·1	2·1 ± 0·06
	Saline water[b]	1·2 ± 0·1	ND[j]
	35°C temperature	2·2 ± 1·1	2·1 ± 1·0
	45°C temperature	1·1 ± 0·2	ND[i]
Proline content in two weeks old seedlings (µmol/gFw)[c]	Distilled water[a]	1·8 ± 0·1	1·1 ± 0·0
	Saline water[b]	2·6 ± 0·1	2·4 ± 0·0
	35°C temperature	2·8 ± 0·1	1·6 ± 0·6
	45°C temperature	5·3 ± 0·4	2·7 ± 0·5
Shoot length (cm)[d]	Distilled water[e]	3·2 ± 0·4	10·0 ± 0·7
	Saline water[f]	2·1 ± 0·1	6·7 ± 0·2
	35°C temperature	3·6 ± 0·2	11·1 ± 0·2
	45°C temperature	1·7 ± 0·2	4·4 ± 0·4
Proline content in two months old plants (µmol/gFw)[d]	Distilled water[e]	3·8 ± 0·1	2·6 ± 0·1
	Saline water[f]	4·8 ± 0·2	3·1 ± 0·1
	35°C temperature	3·7 ± 0·1	2·6 ± 0·1
	45°C temperature	7·0 ± 0·1	3·4 ± 0·4
Soil water taken up during further growth by adult plants initially irrigated with measured amount of water (%)[g]	After one month from irrigation	8·9 ± 2·4	31·3 ± 4·1
	After two months from irrigation	34·2 ± 6·9	73·0 ± 4·2
	After three months from irrigation	68·3 ± 3·4 (plants were alive)	80·0 ± 4·2 (plants had dried)
Proline content in above treated plants (µmol/gFw)[h]	At one month	35·1 ± 0·4	21·6 ± 3·1
	At two months	40·4 ± 1·5	35·2 ± 1·0
	At three months	42·7 ± 1·2	ND[j]

[a]Filter paper was soaked in distilled water and the incubation temperature was 25°C.
[b]Filter paper was soaked with 200 mM NaCl solution and the incubation temperature was 25°C.
[c]Measured in two weeks old seedlings grown in Petridish(es) over moistened filter paper, Fw, fresh weight.
[d]Measured on 2 months old plants grown in soil.
[e]Plants were grown at 25°C and irrigated with distilled water.
[f]Plants were grown at 25°C and irrigated with 200 mM NaCl solution.
[g,h]Green house grown 8 months old plants in pots were irrigated such that soil water value was 80%, subsequently the pots and soil surfaces were covered with polythene sheet.
[i]Could not be determined as the seedlings/plants had wilted and dried.
[j]ND, not detected.

plants with *abo*$^+$ *abo*$^+$ (Abo$^+$) parental wild type plants showed that mutation(s) carried in the Abo$^-$ plants was recessive. The Abo$^-$ plants recovered in the F_2 generation had 3 cotyledons, fistulate leaves and were tolerant to abiotic stresses. Anatomical studies on some of the Abo$^-$ F_2 plants showed that they had hypertrichomatous epidermis, defective vascular and sclerenchymatous tissues like the Abo$^-$ parent. The F_2 Abo$^-$ p1ants showed elevated ABA levels and exhibited anatomy similar to the Abo$^-$ parent. The proportion of the Abo$^-$ and wild type plants in the F_2 population shows that the Abo$^-$ phenotype was due to a single gene inherited in Mendalian fashion.

To test whether Abo$^-$ and Abo$^+$ plants recovered in F_2 generation also accumulated proline like the respective parents, the F_2 population was raised at 35° and 45°C and two months old plants were examined for their morphology and the proline content (figure 5). It will be seen from the results summarized in the figure 6 that the F_2 tricotyledonous 2 months old plants had proline contents similar to the plants of Abo$^-$ parent and dicotyledonous wild type F_2 plants had proline content similar to the plants of wild type (table 5). The above results demonstrated that the entire pleiotropic phenotype identified here for Abo$^-$ mutant was inherited together as a recessive allele of a Mendelian locus (gene) in crosses with the wild type.

4. Discussion

The experimental results presented here show that the Abo$^-$ mutant of periwinkle (*C. roseus*) has a unique pleiotropic phenotype that is inherited together recessively in Mendalian fashion. The results permit the inference that due to mutational knocking out of a function, the *abo abo* mutant plants overproduce ABA. As a result the seeds formed have tricotyledonous embryos, adult plants are dwarf, bear lanceolate and hypertrichomated leaves on stem which has poorly developed vascular bundles; many

Table 4. ABA content in Abo$^-$ and Abo$^+$ plants of periwinkle *C. roseus.*

	Genotype	
Parameter	Abo$^+$ (wild type)	Abo$^-$ (mutant)
ABA content (µg/gFw)	0·009 ± 0·004	5·4 ± 1·9

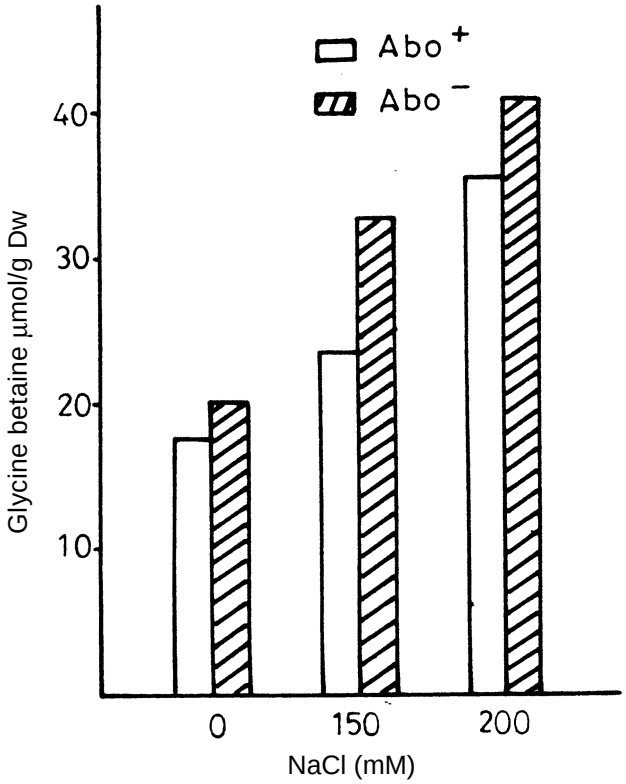

Figure 4. Concentrations of glycine-betaine in the leaves of Abo$^-$ and Abo$^+$ plants.

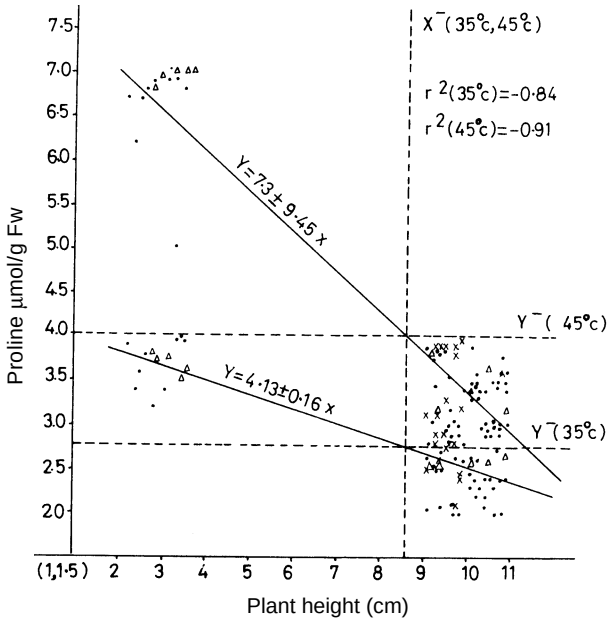

Figure 5. Relationship between plant height and leaf proline content in the Abo$^-$ and Abo$^+$ plants grown at 35° and 45°C. The observations taken on the *abo abo* Abo$^-$ and Abo$^+$ abo$^+$ abo$^+$ or abo$^+$ abo$^-$ plants have been plotted and regression equations estimated at the 2 temperatures of incubations; 35°C, $Y = 4{\cdot}13 - 0{\cdot}16X$; and 45°C, $Y = 7{\cdot}9 - 0{\cdot}45X$.

of the stomata on the leaves are constitutively closed. The mutant seedlings and plants are tolerant to 45°C temperature, 200 mM NaCl and prolonged withdrawal of irrigation water. Under abiotic stress conditions they accumulate higher levels of proline and betaine. The phenotype of the mutant appears to provide some direct evidence in favour of the involvement of ABA in the determination of abiotic stress tolerance and control of morphogenetic development of plant shoot in terms of structural organization and functioning of stem, leaves, flowers, fruits and seeds. Some aspects of the above inferences are discussed below.

There have been a few reports of ABA-overproducing plant mutants. The *pew-l* mutant of *N. plumbaginifolia* which is defective in the synthesis of phytochrome chromophore had been observed to accumulate about 1·4 times more ABA in leaves than its wild type parent (Kraepiel *et a1* 1994). In the present work, the ABA content in the leaves of Abo^- plants of *C. roseus* was observed to be very high, over 500-fold of that in Abo^+ plants. The ABA content in a plant tissue basically represents the sum total of ABA which is synthesised and lost due to photolability or converted into metabolites such as phaseic acid and ABA glucose ester. The available evidence shows that ABA is synthesized from C_{40} xanthophyll precursors in many steps which occur in plastids and cytosol of plant cells in tissue specific manner (Tan et al 1997). The synthesis and degradation of ABA have been reported to be regulated developmentally and by abiotic stress (Liotenberg *et a1* 1999). The observations are consistent with the notion that the *abo⁻* gene of *C. roseus* is responsible for a protein product whose presence is required for the synthesis of ABA in normal amounts. In the absence of Abo protein, the negative control of ABA synthesis does not seem to operate and consequently ABA seems to be overproduced. In the *pew-l* mutant of *N. plumbaginifolia* the high level of ABA is due to a higher level of ABA-degradation and not due to an enhancement of ABA biosynthesis (Kraepiel *et a1* 1994). It is suggested that the loss of negative control over ABA synthesis must be is largely responsible for the over production of ABA in Abo^- mutant.

In the *abo abo* plants of *C. roseus,* the leaves, besides being richer in ABA, had curvaceous fistulate shape, hyper-trichomated surfaces and about 7-fold reduced size as compared to the obovate shaped relatively smooth leaves of Abo^+ plants. The sizes of ce1ls of various tissues in stem and leaves were smaller and xylem poorly developed in Abo^- plants as compared to Abo^+ plants. The small leaf phenotype of ABA rich *abo abo C. roseus* mutant is in agreement with earlier studies which showed that ABA inhibits leaf area in plants (Creelman *et a1*

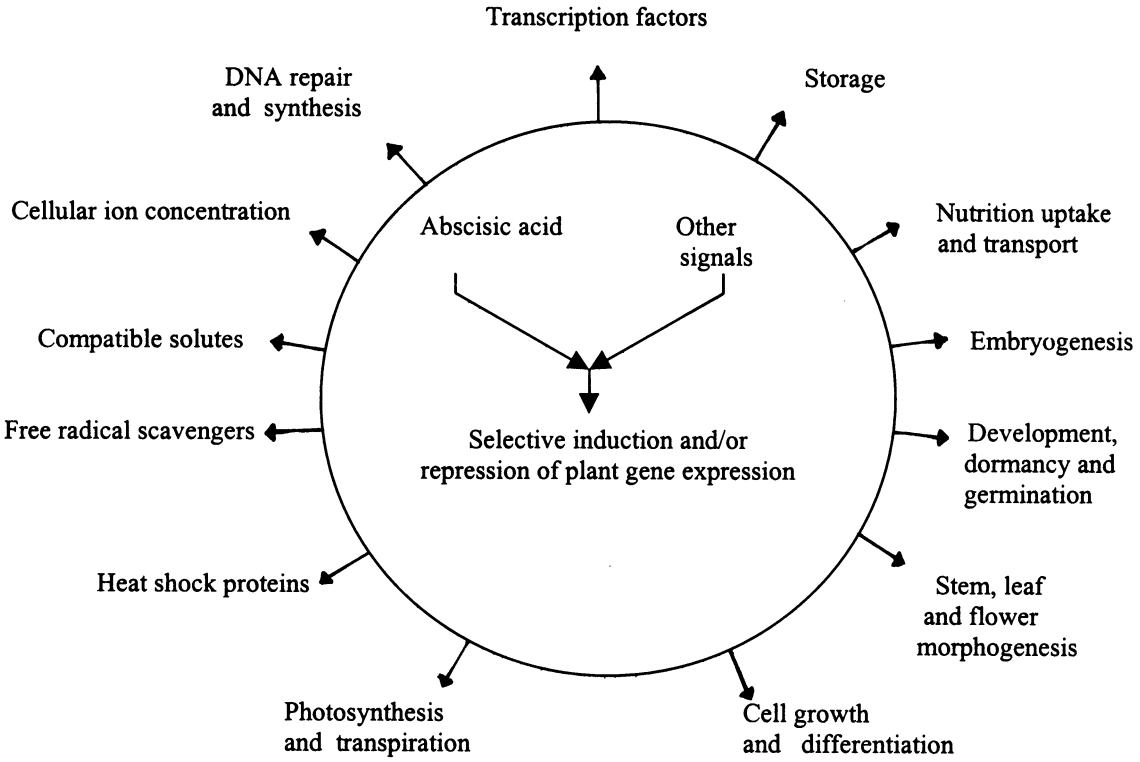

Figure 6. Roles of abscisic acid deduced from the phenotype of Abo^- ABA over producer) mutant identified in *C. roseus.*

Table 5. Co-inheritance of seedling and adult plant morphological, multi abiotic stress tolerance and high ABA content characters in Abo⁻ *abo abo* plants and in the reciprocal crosses between *abo abo* and wild type Abo⁺ plants of periwinkle *C. roseus.*

	Number of trycotyledonous fistulate leaf bearing seedlings that were				Number of wild type seedlings that were				$X^{2(b)}$ test for	
Parents and crosses[a]	resistant to 200 mM NaCl	sensitive to 200 mM NaCl	resistant to 45°C	sensitive to 45°C	resistant to 200 mM NaCl	sensitive to 200 mM NaCl	resistant to 45°C	sensitive to 45°C	response to NaCl	response to 45°C
Abo⁻	36	0	36	0	–	–	–	–		
Wild type	–	–	–	–	0	72	0	72		
Abo⁻ X wild type (F_1)	0	0	0	0	0	20[c]	0	09[c]		
Abo⁻ x wild type (F_2)	17[d,h]	0	15[e,h]	0	0	49[d,h]	0	48[e,h]	0·02	0·05
Wild type x Abo⁻ (F_1)	0	0	0	0	0	18[c]	0	12[c]		
Wild type x Abo⁻ (F_2)	11[f,h]	0	22[g,h]	0	0	39[f,h]	0	65[g,h]	0·24	0·004

[a]The parents were homozygous Abo⁻ *abo abo* (mutant) and Abo⁺ *abo*⁺ *abo*⁺ (wild type).
[b]X^2 calculated on an expected ratio of 3 sensitive to 1 resistant; X^2 $P > 0.05$.
[c]Separate seed samples from the same lot were screened for the two characters.
[d,e]Separate randomly formed seed samples from the bulk seeds produced on the same F_1 plant were screened for the two characters.
[f,g]Like *d* and *e*.
[h]Some randomly picked plants from among these upon checking for proline, betaine and ABA contents in leaves were found to be like the original *abo abo* mutant parental plants in their expression.

1990; Lecocur *et al* 1995). Similar effect has also been well documented in large sized leaf bearing cassava (*Mainhot esculenta*) plant. These results suggest that ABA at high concentrations has repressive effects on the genes related to cell division and cell expansion processes. It is already known that ABA adversely affects the expression of certain plant protein kinases involved in cell division process (Wang *et al* 1998; Covic *et al* 1999). The properties of *abo abo* mutant provide the genetic evidence on the role of ABA in leaf and stem growth, development and morphogenesis in plants.

The *abo abo* (Abo⁻) mutant described in this work was isolated on the basis of its thermotolerant phenotype. Among the M_2 generation seeds, its seeds was the only one to germinate and form seedling at 45°C. While Abo⁺ and Abo⁻ seeds of *C. roseus* germinate at 35°C and lower temperatures, seeds of only the Abo⁻ mutant form seed-1ings at 45°C. It is possible that the constitutive thermotolerance property of Abo⁻ could be related to the ABA over-expression. Induction of high levels of heat shock proteins (Hsps) is believed to confer adaptation to high temperatures in plants (Burke *et al* 2000; Queitsch *et al* 2000). The phenotype of Abo⁻ mutant supports the idea that ABA is indeed a signal for the expression of Hsps in *C. roseus.*

The role of proteins other than the known Hsps in thermotolerance in plants, analogous to that in yeast (Smith and Yaffe 1991), is not known. The Abo⁻ mutant offers opportunities to dissect heat inducible Hsps, constitutive Hsps and non-Hsps involved in thermotolerance in *C. roseus.*

The *abo abo* mutant of *C. roseus* is drought tolerant. Unlike the Abo⁺ seeds, Abo⁻ seeds germinated in a medium containing 200 mM NaCl. The adult plants of Abo⁻ withstood stresses imposed by high salinity and irrigation withdrawal much better than Abo⁺ plants. The *C. roseus* Abo⁻ and Abo⁺ seedlings and adult plants increased their proline and betaine contents upon exposure to salinity, high temperature and water deficiency stresses by about 1·1- to 1·9-fold. Interestingly, increased in *C. roseus* plants. The level of proline upon exposure to osmotic stress as well as heat stress. Under the salinity, drought and temperature stress conditions, the contents of proline were higher in the Abo⁻ and Abo⁺ seedlings and plants than unstressed plants of *C. roseus.* The results are consistent with the idea that although proline and betaine accumulation were inducible by abiotic stress in Abo⁻ and Abo⁺ plants; however, the Abo⁻ plants accumulated additional proline and betaine constitutively, the latter on account of the constitutive synthesis of ABA. These results suggested indicate that in *C. roseus,* the role of ABA is insignificant in the proline and betaine accumulation in response induction caused by abiotic stresses. It is suggested that stress and ABA imposed signal transductions for compatible solute synthesis operate independently in *C. roseus.*

The addition of Abo⁻ mutant of *C. roseus,* to the available collection of plant mutants related with the synthesis/degradation and functions of ABA, should help us in understanding the ro1e of ABA in the regulation of cellular dynamics and morphogenesis in plants (figure 6).

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