GENETIC AND PHENOTYPIC CHARACTERIZATION

OF COTYLEDON-MUTANTS OF TOMATO

A Thesis Submitted To University of Hyderabad for The Degree of
Doctor of Philosophy

BY

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Certificate

This is to certify that the thesis entitled **"Genetic and Phenotypic Characterization of Cotyledon-mutants of Tomato"** is based on the results of work done by Mr. **Arif Saeed Aqulan Al-hammadi** for the degree of **Doctor of Philosophy** under my supervision. This work has not been submitted for any degree or diploma of any other University or Institution.

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Declaration

I hereby declare that the work presented in this thesis has been carried out by me under the supervision of Prof. R.P. Sharma and that this has not been submitted for a degree or diploma in any other University or Institute.

Supervisor

Candidate

Dedicated
To
My sons, Abdurahman and Abdullah

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LIST OF ABBREVIATIONS

λ_{max}	Maximum wavelength
abphyl	*aberrant phyl*lotaxy
AC	Ailsa Craig
ADP	Adenosine 5-diphosphate
ag	*agamous*
amp	altered *m*eristem program
an	*angustifolia*
AP	*APETALA*
ARF	ADP-ribosylation factor
au	*aurea*
BAP	N^6-benzylaminopurine
CAL	*CAULIFLOWER*
cDNA	Complementary DNA
cop	*co*nstitutive *p*hotomorphogenic
CRY	Cryptochrome
CUC	*CUP-SHAPED COTYLEDON*
Cyp5	Cyclophilin 5
DAP	Days after pollination
DEF	*DEFICIENS*
dem	*d*efective embryo meristem
DET	*DE-ETIOLATED*
dgt	*diageotropica*
dpy	*dumpy*
Ds	Dissociation element.
emb	*embryo-lethal mutant*
EMS	Ethyl methane sulphonate
fa	*falsiflora*
fk	*fackel*
FM	Flower meristem
FON1	*FLORAL ORGAN NUMBER 1*
GA	Gibberellic acid
GEF	Guanine-nucleotide exchange factor
gn	*gnom*
GTP	Guanosine 5-triphosphate
hbt	*hobbit*
hp	*high pigment*
hy	long *hy*pocotyl
hyd	*hydra*
IAA	Indole-3-acetic acid
IM	Inflorescence meristem
kD	Kilodalton
KN	*KNOTTED*
La	*Lanceolate*
lat	*l*ow auxin ttansport

lec	*leafy cotyledon*
LeT	*Lycopersicon esculentum Tomato class 1 Knox gene*
LFY	*LEAFY*
M_2	Mutagenized generation 2
MM	Moneymaker
mp	*monopteros*
NAA	Naphthalene acetic acid
NAM	*No Apical Meristem*
nc	*narrow cotyledons*
NPA	naphthyl phthalamic acid
npc	Harrow petioleless cotyledon
PCIB	p-Chlorophenoxyisobutyric acid
pct	*polycot*
PHAN	*PHANTASTICA*
PHAT	*PHANTASTICA TOMATO*
PHYA	Phytochrome A
PHYB	Phytochrome B
PID	*PINOID*
pin	*pin-formed*
poc	polycotyledon
poc hp1	double mutant of polycotyledon and *high pigment-1*
poc au	double mutant of polycotyledon and *aurea*
SAM	Shoot apical meristem
shy2	*short hypocotyl*
sic	single cotyledon
sin1	*short integument*
stm-	*shoot meristemless*
TAG1	*Tomato Agamous*
TFL1	*TERMINAL FLOWER 1*
TGC	Tomato Genetics Cooperative
TGRC	Tomato Genetics Resource Center
TM	*Tomato –MADS-boxgene*
UFO	*UNUSUAL FLORAL ORGANS*
UV	Ultraviolet (light)
xtc	*extra cotyledon*
YNV2	A hypothetical protein with unknown function in yeast

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1. Introduction

Development of living organisms follows a highly coordinated expression of genetic information to give rise its characteristic phenotype. While each individual species has its characteristics phenotype, species share several characters among themselves, which allows them to be classified into genus, family and so on. Such grouping of species means that in spite of differences in their development program, they have common elements, which allows them to be grouped. The genetic basis of this commonness is yet to be understood completely. One good example, which shows common feature in a range of plants, is the presence of two cotyledons in the seedlings, which forms the basis of classification of angiosperms into two classes, dicotyledons and monocotyledons. While these two groups can also be distinguished on the basis of several other features, such as the numbers of flower parts, the leaf venation, the arrangement of the vascular bundles and the existence of secondary growth, the cotyledon number is the most prominent feature.

During the course of evolution there has been reduction in the cotyledon numbers. The gymnosperms have many cotyledons, which are reduced to two in dicots, and one in monocots. In fact, Burger (1996) suggested that the cotyledons of the monocots and the dicots are not homologous, since in the monocots the cotyledon is apparently a modified leaf. In dicots the cotyledons are the first differentiated organs of the embryo, which, consists of five main segments: the hypocotyl, which is the lower primitive stem; the shoot apical meristem, small dome of undifferentiated cells which give rise to all the above ground portion of the plant; the radicle or primitive root; root apical meristem; two seed leaves or cotyledons, which are symmetrically positioned across the body axis.

The main function of cotyledon in dicots is to help the young seedling after germination to acquire phototrophy. To achieve this function the cotyledon may serve as

repository of nutrients, and in case of epigeally germinating plants also carry out photosynthesis, till the leaves can appear and begin photosynthesis. The mature seeds of dicot can be classified as endospermic (with endosperm) or non-endospermic. Many dicots seeds remain endospermic but, some (e.g. Pea) absorb the endosperm during development redistributing the reserves within the cotyledons. In such nonendospermic seeds, the cotyledons are storage organs of all the nutrients needed for germinating seeds, therefore these cotyledon loose their leaf-like characteristics. Whereas, in the endospermic seeds the cotyledons have proteins and lipids bodies, in addition to their role in mobilization of nutrients from the endosperm in early stages of seed germination. In epigeal species where cotyledons come out of soil on germination and become photosynthetic, the cotyledons also play important role during initial seedling growth by carrying photosynthesis.

Cotyledons are often erroneously described as embryonic leaves. However, both ontogenically and morphologically cotyledons are different from true leaves. Firstly, the leaves arise as primordia on SAM, whereas, the cotyledons can form in the mutants, whose embryos lack SAM, showing independence of cotyledon origin. Secondly, the cotyledons contain embryo specific characters such as the presence of protein and lipids bodies. In comparison to leaf, the cotyledons have simple morphology, less number of cells, uniform size, fixed number and less influenced by the environment.

The genetic regulation of the cotyledon formation and differentiation is currently being investigated in *Arabidopsis.* Since cotyledon forms during embryo development, the study of embryo development has provided a wealth of information about the regulation of cotyledon development. The genetic dissection of embryo development in *Arabidopsis* has provided information about the genes controlling cotyledon number,

expansion, differentiation and cotyledon relationship with the shoot apical meristem. But, above knowledge is still incomplete and has to be expanded for other plants too.

I have used tomato as a model system to initiate a program on genetic regulation of cotyledon development and differentiation. As a part of this study I have isolated and characterized a *po*ly*co*tyledon and a narrow *p*etioleless cotyledon mutants of tomato. Such mutants though have been reported earlier in few dicot species the detailed genetic, morphological and physiological studies have not been carried out. Even in *Arabidopsis*, where several mutants have been reported for the occurrence of the polycotyledony, the mutation has low penetrance, with only 30-40% seedlings showing multiple-cotyledon phenotype. Since tomato is a crop species, the understanding of the mechanism and factors controlling the cotyledon number and differentiation, would add and complement the information currently obtained in *Arabidopsis*. In this study isolation and characterization of two cotyledon mutants in an alternative system, the tomato is presented.

2. Review of Literature

Angiosperms are classified as dicotyledons (dicots) and monocotyledons (monocots), based on the number of cotyledons, which can be seen in seeds and seedlings. Between these two groups, dicots are considered more primitive, they show in mature seed an embryo with two cotyledons, a shoot apex, a hypocotyl, and an embryonic root. On the other hand, monocots differ from dicots in having only one cotyledon called as scutellum on the embryonic axis, which has a laterally placed shoot apex. The embryonic root of monocots lacks a functional root meristem and the root forms from lower region of the hypocotyl.

The differences between the dicots and monocots arises form the differences in their embryogenesis patterns. Embryogenesis in dicots and monocots is similar up to the octant stage and follows different pathways thereafter (Raghavan, 1986). Typical embryo development in dicot manifests several successive stages: globular, heart-shape, torpedo-shape, and cotyledonary stages. The last stage involving seed maturation is marked with distinct physiological changes and accumulation of storage products to prepare the seed for desiccation and dormancy. The mature seed on germination gives rise to a juvenile plant, the seedling.

The organization of embryonic pattern of dicots can be visualized in two different fashions either from apex to base or radially. The main distinct pattern elements of dicot embryo, shoot meristem, cotyledons, hypocotyl, root and root meristem are distributed along the apical-basal axis of the mature embryo. Whereas, the three main tissues: epidermis, ground and vascular tissue are arranged in radial layers perpendicular to the apical-basal axis of the embryo. The embryo structure, which consists of superimposition of apical-basal and radial pattern elements, is relatively uniform in dicots (Johri, 1984).

Descriptive plant embryology has illustrated successive steps of dicot embryogenesis. Figure 2.1 illustrates embryogenesis in a representative dicot plant *Brassica napus*. After fertilization, the zygote undergoes an asymmetric division, producing two unequal cells with different fates. The smaller and cytoplasmically dense apical cell divides further to produce the embryo, and the elongated vacuolated basal cell divides to produce the hypophysis and the suspensor. The suspensor is a transitory organ that plays role in supplying the nutrients from the mother plant to the developing embryo, whereas the hypophysis forms the founder cells for specific cell types in the seedling root meristem. The asymmetric division of zygote is followed by periclinal cell division in the eight-celled octant stage. At this stage, embryo takes globular shape and generates the first embryonic tissue, the protoderm. Later divisions within the globular embryos produce elongated cells that define the procambium and ground meristem. During transition from globular to heart stage, localized cell division generates the cotyledons, the embryonic storage organs. By this stage, the pattern formation process ends with formation of all representative embryo elements. In the following torpedo stage, both shoot and root apical meristems are visible as organized structures. Thereafter, at morphogenic level, the meristem activity is triggered and at physiological level, the processes of growth, storage accumulation, and maturation are initiated. Physiological changes, like desiccation and quiescence complete the processes of seed formation and embryo maturation.

Among the five distinct elements of mature embryo, formation of cotyledon takes place at the transition from globular to heart stage. The cotyledon has an important role to play in the establishment of juvenile plant by acting as repository of nutrition, which is consumed during seedling development till plant acquires phototrophy. Like all other organs in embryo, the process of the cotyledon formation is highly regulated and a set of

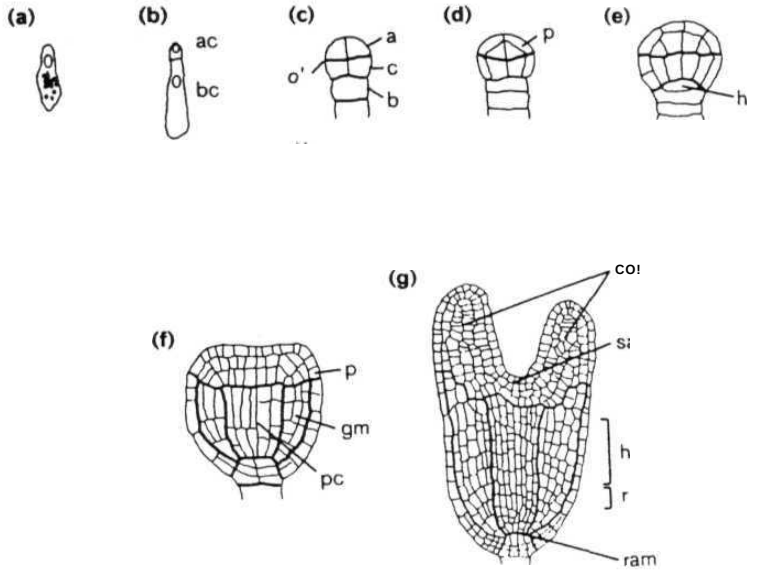


Figure 2.1 Embryo development in a representative dicot plant *Brassica napus* (a) Single-celled zygote (b) two-celled embryo comprising of the apical cell (ac) that give rise to most of the embryo proper and basal cell (bc) that becomes part of the root apical meristem and suspensor, (c) the O' symbol on the left side of octant-stage embryo represents the first transverse division of the embryo proper and separates the apical (a) and central (c) embryonic domains. The basal domain (b) forms the uppermost cell of the suspensor. (d) Periclinal divisions in the octant-stage embryo give rise to the protoderm (p) (e) the hypophysis (h) derived from the uppermost cell of the suspensor in a globular-stage embryo. (0 in this transition-stage embryo, the three major embryonic tissue systems are visible: protoderm (p), ground meristem (gm), and procambium(pc). (g) five major organs/ structures along the apical-basal axis of the early torpedo-stage embryo are cotyledons (cot), shoot apical meristem (sam), hypocotyl (h), root (r) and root apical meristem (ram). Adopted from West and Harada (1993).

specific genes has been shown to affect the cotyledon development in plant model system *Arabidopsis*. In the last decade, the examination of embryo development defective mutants has facilitated the genetic dissection to study pattern formation during *Arabidopsis* embryo development (Jurgens *et al.*, 1994). It is a known fact that controlled expression of many genes is needed to regulate cell differentiation and pattern formation during plant development (Goldberg *et al.*, 1994). While, it is easy to identify mutants defective in the development, it is difficult to assign the regulatory hierarchy, which ultimately determines a particular developmental pathway. This problem is further compounded by the fact that one is searching for regulatory genes, which control very complex developmental pathways with varied level of interactions.

Most of the information about the embryo development has come from the embryo mutants which have been screened using two different strategies; to examine seed development to isolate embryo-lethal mutants (Meinke, 1986; Jurgens *et al.*, 1994), and to examine seed germination stage to isolate the pattern mutants (Mayer *et al.*, 1991). In *Arabidopsis*, many mutants are embryo lethal (Meinke, 1986) and since these mutant embryos die they are maintained as heterozygotes. The embryo development is examined after self-pollinating heterozygous plants which would contain *V** of homozygous lethal mutant embryo. The studies of embryo mutants have also shown that single-gene mutation without causing lethality can modify several developmental process in embryo such as pigment formation; formation of storage material in the cotyledons; establishment of embryo pattern and cotyledon morphology etc. Jurgens *et al.* (1991) estimated that in *Arabidopsis* about 4,000 genes are essential for embryogenesis, out of which 40 are likely involved in pattern formation. Most of the genes identified are involved in pattern formation, especially in the establishment of the apical-basal pattern and radial pattern.

The information on genetic determinant regulating cotyledon development and number is limited. The present review summarizes the information mainly about the embryo development with emphasis on the cotyledon formation. The review also summarizes the other aspects related to cotyledon development and function (Table 2.1 and Figure 2.2).

2.1 Embryo development mutants

2.1.1 Maternal regulation of development

Jurgens *et al.* (1991) proposed that the body plan in *Arabidopsis* embryo could be visualized as two superimposed segmentation pattern: one along the apical-basal axis and another along the radial axis. The apical-basal axis can be demarcated into five segments: the shoot apical meristem (SAM), cotyledons, hypocotyl, radicle, and root apical meristem (RAM) (Laux and Jurgens, 1997). The studies of domains set out by transverse divisions in early embryo development (Scheres *et al,* 1994) lend support to above body plan of *Arabidopsis* embryo. The first transverse division of the proembryo at the four-cell stage produces an upper and a lower tier of cells. The upper tier of cells gives rise to most of the cotyledons and the shoot apical meristem, while the lower tier gives rise to the basal part of the embryo, the part of the cotyledons, hypocotyl and root.

In plants embryogenesis can occur even without the surrounding maternal tissue, for example, in many species somatic embryogenesis can be induced in the tissue culture (Goldberg *et al,* 1994). However, there are evidences accumulating that the maternal genes play an essential pattern formation role in zygotic embryogenesis. In *Arabidopsis thaliana,* a mutation in the *SIN1* gene causes aberrant ovule development and female-specific sterility. Ray *et al.* (1996) reported that the maternal recessive gene *short integument1 (sin])* also influences pattern formation in *Arabidopsis* embryo. The phenotype of the defective embryo depends on the genotype of the maternal tissue, not on

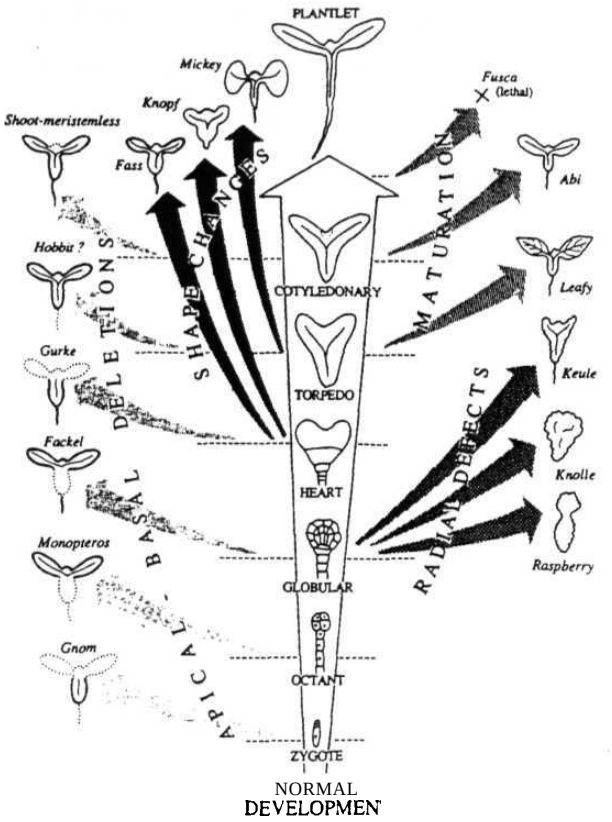


Figure 2.2 A sketch showing different *Ambidopdsis* embryo mutants phenotypes having apical-basal deletion, shape change and defects in maturation. Adopted from Dodeman *et al.* (1997).

the zygotic tissue. Irrespective of the genotype of the endosperm or the embryo, a wild-type *SINl* allele in the maternal sporophyte is essential for normal embryogenesis. A homozygous *sinl/sinl* mutant shows normal embryo when nursed by a *sinl/SINl* heterozygous maternal sporophyte. Surprisingly, a *sinl/sinl* or a *sinl/SINl* embryo if nursed by a *sinl/sinl* homozygous maternal sporophyte shows developmental defects in the apical-basal and radial axis. The most pronounced effects of the mutation was on cotyledon numbers and shape giving rise to seedlings with 0, 1, 3, or a funnel shaped cotyledon(s). Moreover, several seedlings, which had two normal cotyledons, did not develop the primary leaf because they had little or no meristem at shoot apex. These seedlings also lacked root elongation. Thus, it was suggested that *SINl* gene either codes or controls the formation of a diffusible morphogen, which is required for proper zygotic embryogenesis.

The cotyledon formation is also affected in the *gnom* mutant of *Arabidopsis*. This mutant was originally described as a segment deletion mutant lacking the root apical meristem (Mayer *et al.*, 1991). The *gnom* phenotype is caused by a recessive mutation in single zygotically active gene. However, recently Vielle-Calazada *et al.* (2000) showed that *emb30/gnom* has a maternal-effect phenotype, which can be paternally rescued in addition to its zygotic lethality. These results indicate that more work is needed to interpret the function of *GNOM* gene. There may be more genes, which may regulate early embryo and endosperm development by maternal control.

Mutations in the *GNOM* gene disrupt the apical-basal pattern of the seedling. The majority of mutant seedlings lacked root differentiation. The *gnom* seedlings can be grouped in three phenotypes *viz.* 'ball-shaped', 'oblong', and 'cone-shaped'. The 'ball-shaped' seedling showed the strongest modification of phenotype: the seedling lacked cotyledons and root, and no apical-basal axis was apparent. The 'oblong' seedling also

lacked both cotyledons and root but seems to have an axis, as it was evident by formation of vascular tissue. On the other hand, 'cone-shaped' seedlings, though lacked the root, had cotyledons whose shape varied from nearly normal to reduced in size, and at times fused to form a funnel.

The phenotype of *gnom* seedling results from defective embryo development, which can be seen at heart-stage, where embryo displays abnormal development at both apical and basal end. In fact, the *gnom* embryo shows abnormal development at one-cell stage. In wild type the apical cell of embryo is about one-third the size of the basal cell, whereas, in *gnom* embryo the apical cell was only slightly smaller than the basal cell. The apical daughter cell then undergoes abnormal divisions, resulting in an octant embryo with about twice the normal number of cells, while the uppermost derivative of the basal cell fails to become hypophysis, which normally contributes to root development (Mayer *et al*, 1993). The *gnom* mutant embryo at heart stage lacks the cells representing the incipient root primordium and cotyledonary primordia at the apical end. The *GNOM* gene appears to be an essential gene regulating the process of root formation, as the tissue culture of *gnom* mutant does not produce roots.

The cloning of *GNOM* gene, also known as *EMB30* revealed sequence similarity to the yeast vesicle trafficking protein Sec7P, including a central region called the Sec7 domain (Busch *et al.*, 1996). Proteins with Sec7 domains catalyze guanine nucleotide exchange on small GTP binding protein of ADP ribosylation factor (ARF), family required for vesicle coating in membrane trafficking (Springer *et al*, 1999). Steinmann *et al*. (1999) looked for localization of auxin efflux carrier PINl in *gnom* embryo and found that coordinated polar localization of PINl is defective in *gnom* embryos. Thus, *GNOM*-dependent vesicle trafficking may establish cell polarity, resulting in polar auxin transport. It is known that dimerization is needed for the *GNOM* function and in yeast

two-hybrid system GNOM was found to interact with cyclophilin 5 (Cyp5). Moreover, Cyp5 protein accumulates in plant embryos and like *GNOM*, was partitioned between cytosolic and membrane fractions. It was suggested that Cyp5 may regulate the ARF-GEF function of the GNOM protein during embryogenesis (Grebe *et al.*, 2000).

2.1.2 Pattern formation mutants

2.1.2.1 Apical-basal axis mutants:

The formation of cotyledon appears to be a process independent of the formation of the shoot apical meristem (SAM), which is also one of the elements of the apical-basal axis of dicot embryo. Once formed, SAM functions throughout the life of the plant generating organs such as leaves, stem and inflorescence meristems. The SAM consists of a small mound of densely cytoplasmic, undifferentiated dividing cells. Barton and Poethig (1993) identified a recessive mutant of *Arabidopsis,* which lacked the SAM, but was normal in all other aspects of embryo development. The anatomical examination of mutant embryos revealed that this mutation completely blocks the initiation of the SAM, but has no other obvious effect on embryo development. On the basis of this phenotype, the embryo was named as *shoot meristemless-1* (*stm-1*). The root explants of *stm-1* produce only abnormal leaves or shoots and overall gave rise to fewer such structures, than did the wild type tissue. Thus, the *STM1* interferes both with the initiation of the SAM during embryogenesis, as well as with the shoot initiation in culture. The *stm-* is recessive mutation and defines a locus that is required for SAM initiation both embryonically and postembryonically.

Long *et al.* (1996) cloned the *SHOOTMERISTEMLESS (STM)* gene and found it be a member of *KNOTTED 1* class of homeodomain protein. These genes appear to be ubiquitously present in angiosperms. The expression pattern of these genes clearly indicates that they play an important role in shoot meristem function. The *STM* is first

expressed in one or two cells of the late globular embryo and has dynamic expression pattern during embryogenesis. The *STM* is likely to promote SAM formation through transcriptional regulation.

The formation of cotyledon is also altered by a combination of two mutants of *Arabidopsis*, Adia *et al.* (1997) showed that, two recessive mutations at the *cucl* and *cuc2* loci when combined, lead to the formation of cup-shaped cotyledon. The mutant also failed to form SAM during embryogenesis, but the *CUC* genes are not absolutely required for SAM formation, which is evident by the fact that *cucl cuc2* embryos can be induced to form shoot in tissue culture. The shoots of double mutant though do not show defects in phyllotaxy, many flowers on the *cucl cuc2* shoots have incompletely separated third-whorl organs. In spite of the defects observed in double mutants, in single *cucl* and *cuc2* mutants, alteration in the phenotype of embryo or flower is barely discernible. The alteration in cotyledon formation can be seen in *cucl cuc2* mutant at cotyledon initiation stage. At this stage instead of forming two bilaterally placed bulges on the flank of the triangular embryo, *cucl cuc2* cotyledons initiate as a doughnut-shaped ring that surrounds the apex of the embryos. The ring grows further and becomes cup shaped cotyledon.

The *CUC2* gene was cloned by using transposon-tagging. It belongs to a small gene family, which also includes *Petunia NAM* (No Apical Meristem) gene (Souer *et al.*, 1996), and shows similarity to rice, but not to any animal system. The *NAM* gene also affects both the initiation and separation of organs in both the embryo and the flower. The deduced amino acid sequence of NAM and CUC2 are quite similar in N-proximal domain and has some shared blocks of sequence towards the C-termini of the proteins (Aida *et al.*, 1997).

Since *CUC1* and *CUC2* genes are required for cotyledon separation and lacks SAM, Aida *et al.* (1999) examined genetic interactions among *CUC1*, *CUC2*, and *STM*

genes. These studies indicated that CUC1 and CUC2 are redundantly required for expression of STM to form the SAM. On the other hand, STM is required for proper spatial expression of CUC2 to separate cotyledons.

The *gurke* mutant of *Arabidopsis* shows seedlings with either highly reduced or totally absent cotyledons. While the mutant shows normal formation of the root and the root meristem, the strong *gurke* alleles eliminate the entire shoot apex and a part of hypocotyl. The mutation affects apical region of embryo by deleting cotyledons and apical meristem in strong alleles. The loss of cotyledons in *gurke* mutant results from abnormal cell divisions within the apical region during the triangular/early-heart stage of embryo leading to formation of no or only rudimentary cotyledon primordia. Histological examination of mutant seedlings showed that the vascular system ends apically without forming a network of veins as present in wild type cotyledons. In *gurke* mutant the effect of mutation is primarily on apical portion of embryo and other effects are secondary in nature.

The role of *gurke* mutation on postembryonic development was examined by *in vitro* culture of the embryo. The strong *gurke* mutants produced a mass of unorganized tissue, which though formed green leaf-like structure lacked orientation. In the leaf-like structure, vascular bundles were disorganized and not connected to the vascular system of the hypocotyl. The intermediate and weak alleles of *gurke* showed vigorous growth in majority of the seedlings. The mutant plantlet partially resembled wild type in appearance forming postembryonic structures such as, expanded cotyledons forming tube-like appendages, a nearly normal leaf like structures with pedicels. However, the mutant formed abnormal flowers with sepals and small petal-like structures but no anthers. In the flower, the thecae without subtending filaments were directly fused to the poorly developed carpels (Torres-Ruiz *et al.*, 1996).

Several mutants of *Arabidopsis* show deviation from dicot phenotype by having variation in the number of cotyledons in the seedlings. The *hydra1* (*hyd1*) mutant of *Arabidopsis* is one such mutant showing extreme variation in the cotyledon number ranging from one to seven. Like other mutants, the variation in the cotyledon number results from abnormal embryo development at globular stage, where embryo lacks the characteristic cell arrangement both in upper and lower tiers of embryo. Subsequently, embryos lack the bilateral symmetry in heart and torpedo stage compared to wild type and did not show incipient vascular tissue or embryo root.

One of the primary defects in the *hydra* mutant is in regulation of cell shape, where cell is unable to expand in correct orientation. Topping *et al.* (1997) proposed that the in *hydra* mutant, multiple cotyledon arises as a secondary effect of the wide hypocotyl and broad shoot apex. Moreover, they also proposed that an inhibitor might be involved in cotyledon initiation and depletion of its level due to widening of shoot apical meristem leading to formation of more cotyledons.

2.1.2.2 Radial axis mutants

The radial axis of embryo consists of three elements *viz.* epidermis, ground and vascular tissues. The mechanism of establishing the radial axis in the embryo is yet to be deciphered, as only few mutants affecting the radial axis have been identified. Mutation in *KNOLLE* gene of *Arabidopsis* affects the radial pattern of the seedlings affecting the overall appearance of the seedling. The seedling looks round or tuber-shaped, and the surface appears rough because of the lack of well-formed epidermal layer. In wild type, at globular stage embryo shows that the outer layer of eight-epidermal precursor cell surrounds an inner group of eight cells. Instead, the embryo of *knolle* mutant lacks oriented cell divisions that initiate protoderm formation and consists of irregularly spaced

enlarged cells. In addition, the upper end of the suspensor is also enlarged as if this region had become part of the embryo (Mayer *et al*, 1991).

The cloning of *Arabidopsis KNOLLE* gene showed that it encodes a protein related to vesicle-docking syntaxins, which is required for cytokinesis (Lukowitz *et al*, 1996). The KNOLLE protein is detected in mitotically dividing cells of plant, including seedling root, inflorescence meristem, floral meristems and ovules. This protein is membrane associated and is specifically expressed during mitosis where it is localized to the plane of cell division during cytokinesis (Lauber *et al,* 1997). The KNOLLE protein appears to be involved specifically in cytokinetic vesicle fusion.

2.2 Hormone mutants

It is known that many defects in organogensis of plants may result due to alteration in synthesis or distribution of hormones. Such a role for hormone transport is evident in *pin-1* mutant *of Arabidopsis* which shows fused or deformed cotyledon. Both *pin1-1* and *pin1-2* show extremely decreased auxin polar transport compared to the wild type. Even the amount of free IAA in the *pinl-* mutant was 10 fold less than the wild type. The *pin* mutant shows pleiotropic effect on vegetative development of plant with altered phyllotaxy in both vegetative and reproductive shoots. It forms wider leaves with major vein branched at the base and time of flowering is delayed. Moreover, mutant may lack floral buds or forms deformed flowers with wide petals, no stamens, and lacks ovules in the ovary. The mutant phenotype can be phenocopied in wild type using inhibitors of auxin polar transport (Okada *et al,* 1991). For example, addition of auxin transport inhibitors to mustard embryo cultured *in vitro* causes fusion of cotyledons to form ring of cotyledon encircling embryo (Liu *et al,* 1993). Based on these results, it is suggested that auxin transport is necessary for the establishment of bilateral symmetry leading to formation of cotyledons (Chasau, 1993).

Similar to *pin* mutants of *Arabidopsis,* the *lat (low* auxin rransport) mutant of tobacco shows cup-shaped cotyledons, cylindrical embryos with fused cotyledons (Naderi *et al.*, 1997). In *lat* mutant too, the polar transport of auxin in the inflorescence axis was 5-10 fold less than the wild type. The mutant shows strong pleiotropic effect throughout plant development. The morphology of the leaf varied between normal to cup-shaped, and occasionally some leaves produced shoots from the leaf midvein. Likewise, flowers ranged from normal to compound with occasional fused floral parts or split petal, or had petal-like stamens. The addition of synthetic auxin NAA to sterile media suppressed few abnormalities of the *lat* mutation.

The *pinoid (pid)* mutant *of Arabidopsis* also shows defects in cotyledon formation. The mutant phenotype resembles very closely to plants, which are grown on auxin transport inhibitors or *pin* mutants *of Arabidopsis.* Like other mutants, defect in cotyledon formation is due to aberrant embryogenesis altering the number, size, and shape of cotyledons. The cotyledon numbers in *pid* mutant range from one to four with about 10-23% dicots. Cotyledons were sometimes small in size, notched or deeply lobed. The *pid* mutant also shows strong pleiotropic effect on structure and development of the inflorescence, floral organs and leaves. Christensen *et al.* (2000) showed that the *PID* gene encodes for a serine-threonine protein kinase, which negatively regulates auxin signaling and play a specific role in promoting primordium development. The *PID* gene is transiently expressed in the embryo and in initiating floral anlagen. The constitutive expression of *PID* induces a phenotype resembling that of auxin-insensitive plants in both shoots and roots.

Though it is still not confirmed, the presence of multiple cotyledons in *amp* (altered meristem program) mutant of *Arabidopsis,* is attributed to six-fold increase in the level of cytokinin (Chaudhury *et al,* 1993). The *amp* show alteration in phenotype altered

in three different aspects of plant development; spatial pattern, photomorphogenesis and initiation of flowering. The homozygous lines of *amp* mutants produce about 20-30% of non-dicot seedlings ranging from monocot to tetracot. It also shows a rosette different from the wild type. The vegetative growth of *amp* mutant is characterized by decreased apical dominance, altered phyllotaxy, higher rate of leaf initiation than wild type (Chaudhury *et al.*, 1993). In *amp]* the pattern of cell division in embryo is perturbed at early globular stage and it also dramatically increases the size of the shoot (Conway and Poethig 1997).

The *fackel* (*fk*) mutant of *Arabidopsis* cotyledons appears to be directly attached to the root, due to extreme reduction of hypocotyl. This mutation particularly deletes the central part of embryo leading to loss of hypocotyl. The defect becomes obvious at the midglobular stage of embryo, when *fk* mutant fails to undergo the asymmetric division that forms the elongated vascular precursor cells of the hypocotyl (Mayer *et al.*, 1991). Jang *et al.* (2000) reported that *fackel-J79* dwarf mutant shows deformed embryos, supernumerary cotyledons, multiple shoot meristems, and stunted roots. In developing embryo, *FK* mRNA is localized to meristematic zones. On the other hand, the morphology of adult plant resembles the brassinosteroid-deficient mutants. The *FACKEL* gene encodes a protein which is similar to a integral membrane protein related to the vertebrate lamin B receptor and sterol reductases across species, including yeast sterol C-14 reductase (Schrick *et al.*, 2000) The mutation causes deficiency in C-14 sterol reductase activity, reduction in brassinosteroids and abnormal sterol composition. The application of exogenous brassinosteroids cannot correct the suppression of hypocotyl indicating that the sterol composition may play a crucial role in regulation of cell division and cell expansion during the embryonic and vegetative development

Mutations in the *MONOPTEROS (MP)* gene of *Arabidopsis* alter the cell division pattern in the basal region of early-globular stage embryo, producing seedling without hypocotyl, radicle, and root meristem (Berleth and Jurgens, 1993). The major effect of this mutation is on basal part of embryonic axis leading to deletion of root and hypocotyl. This mutant also shows variable positioning of cotyledons, which may be the result of the absence of a morphological axis. Alternatively, the basal region of embryo may be playing a role to establish bilateral symmetrical cotyledon position. However, the weak alleles of *mp* form seedlings with short hypocotyl and show normal arrangement of the cotyledons. Since root formation can be induced in culture, it is likely that the *MP* gene is required for organizing the basal body region, rather than for making the root. Interestingly, the developing embryo occasionally produces some adventitious roots allowing studies of mutant traits at post-embryonic stages. The developmental abnormalities of mutants are reminiscent of plants treated with auxin transport inhibitors. Przemeck *et al.* (1996) found that in all organs, the cells within vascular strands appear incompletely differentiated and insufficiently interconnected. Moreover, in the leaf, the vascular system is reduced to higher order veins. The mutant also shows reduction in capacity of auxin transport even in weak alleles which otherwise do not display marked vascular abnormalities.

The *MP* gene encodes for a protein possessing DNA binding domain similar to auxin inducible promoters and is likely localized in nuclei. During embryogenesis and as well as in organ development, initially *MP* expresses in broad regions and gradually gets confined towards the vascular tissues. It is suggested that the *MP* gene plays a role in the establishment of vascular and body pattern in embryonic and post-embryonic development (Hardtke and Berleth, 1998).

The role of auxin in embryogenesis was examined by Hadfi *et al.* (1998) who *in vitro* treated isolated zygotic embryos of *Brassica juncea* with IAA and the auxin transport inhibitors N-(l-naphthyl)thalamic acid (NPA) and antiauxin p-chlorophenoxyisobutyric acid (PCIB). The application of 10-40 μM of auxin to the globular embryos and at late transition stage completely inhibited morphogenesis. The resulting embryos were ball-shaped or egg-shaped or cucumber-shaped with a shortened hypocotyl without any apical structures. On the other hand, inhibition of auxin transport caused duplication of axis leading to the development of twin embryos. The change in auxin distribution at the time of transition from globular to heart stage caused development of either split-collar or collar-cotyledons reminiscent of *cuc* mutant. The cotyledon formation was inhibited by anti-auxin leading to embryos with single or no cotyledons; it also inhibited development of hypocotyl and radicle.

Not all mutants defective in embryo development show alteration in the cotyledon number or formation. The *HOBBIT* (*HBT*) gene of *Arabidopsis* is essential for root meristem formation. The *hbt* embryo shows abnormal hypophyseal cell development starting from quadrant stage of embryogenesis. The adjoining cell tier of *hbt* embryos develops abnormally at the heart stage disturbing formation of a lateral root cap layer. The mutant does not have a mitotically active root meristem and lacks a differentiated lateral root cap. However, the apical portion of the mutant is normal showing normal formation of the cotyledon. (Willemsen *et al.*, 1998)

2.3 Tomato mutants

A systemic study of embryo development and mutant isolation in tomato is yet to be done. However, a mutant with variable number of cotyledons named, as *dem* (*de*fective embryo meristem) was isolated from population of seedling carrying independent transposition of the maize transposon *Ds* (Keddie *et al,* 1998). The *dem* is a

recessive mutant, in homozygous state show seedlings with small, slightly concave, abnormal cotyledons and no shoot apical meristem. In F_2, it segregates into high ratio of tricotyledon seedlings, then tetracot and even about 1% of monocot seedling. The apical growth of *dem* seedling was terminated soon after germination, no shoot apical meristem was found between the cotyledons, and tissue with disorganized cells arrangement formed. The root system of *dem* mutant is also affected, which aborted after a shoot period of extension.

Using transposon-tagging *DEM* gene was cloned form tomato. It encodes a novel protein of 72 KD with significant homology to YNV2, a protein of unknown function in yeast. *In situ* localization of *DEM* mRNA showed that in the shoot apex it was restricted to apical meristem and adaxial side of the leaf primordia and young leaves. The expression pattern *of DEM* indicates that it plays an important role in both shoot and root meristems. Its level is downregulated in mature leaf tissue, but is higher in developing leaflets primordia, floral meristems, developing flowers, as well as in root apex. However, expression of *DEM* is yet to be examined during tomato embryogenesis.

The seedlings of *Lanceolate (La)* mutant of tomato may produce three classes, those with extremely lanceolate leaves, those with one cotyledon and shoot apical meristem, and those that entirely lack both cotyledons and shoot apical meristem (Mathan and Jenkins, 1962). The embryo of the last group fails to undergo the heart stage. In addition, during embryogenesis the shoot apical meristem cells lose their meristematic characters and develop into mature parenchyma, which leads to the determinate growth of the seedling (Caruso, 1968). Dosage analysis showed mutant phenotype cannot be rescued by extra wild type dose of the gene (Stettler, 1964).

It is suggested that *Lanceolate (La)* mutation could be a dominant negative mutation, and the LA^+ gene is required for formation of apical structures. However, a

comparative study of leaf development *of Lanceolate* mutant and wild type showed that despite considerable differences in mature leaf size and form, the dimensions of the shoot apical meristem, arrangement of young leaves in the bud, and pattern of leaf expansion are similar in the three genotypes (Dengler, 1984). Dengler (1984) explained that the differences in leaf shape arises because, in wild type leaves, lateral leaflets formation begins earlier and last longer than the mutant or heterozygous *Lanceolate.* As a consequence of this wild type plant shows more pairs of leaflets.

In tomato application of phenylboric acid simulates the effect of the *Lanceolate* mutant (Mathan, 1965), whereas, the mutant phenotype can be blocked by application of actinomycin-D. Similarly, Avasrala *et al.* (1996) produced phenocopies of the homozygous *Lanceolate* mutant using polar auxin transport inhibitors on geminating seeds and regenerating shoot meristems of tomato. However, no detailed study of embryogenesis has been done in the mutant. The effect of inhibitors of auxin transport on embryo development of mutant or wild type has also not been examined.

2.4 Single cotyledon mutant

Mutations leading to formation of monocot seedlings appear to be rare phenomena in dicots. Some dicots, such as the lotus (*Nelumbo*), have a single cotyledon as the result of the fusion of cotyledons. Some times members of family Brassicaceae may have only a single cotyledon after one of the two original cotyledon aborts. Liu *et al.* (1999) isolated three mutant lines of pea having a *single* cotyledon (*sic*), controlled by a single recessive mutation. Among these only *sicl* and *sic2* lines were viable, whereas *sic3* mutant line was embryo lethal. The study of embryogenesis showed that *sic]* had the least effect on embryo development except that embryo produced a single cotyledon. The position of the shoot and root apex, growth of embryo and mass of seedlings of *sic]* was similar to wild type. The *sic]* mutants germinated normally and showed normal post-embryonic

development similar to wild type. The *sic2* mutants differed from *sic1* in having narrow cotyledon, which showed a notch at the top of the cotyledon. Occasionally, the *sic2* mutant embryo possessed a single cylindrical cotyledon. The *sic3* mutant had a sharply pointed root instead of more rounded root tip of wild type, a shoot apex, and a single narrow cotyledon. In *sic3* mutant, the single cotyledon formed under the shoot apex breaks the vascular connection between root and shoot, thereby causing embryo lethality.

2.5 Mutants defective in cotyledon identity

Cotyledon is a transitory organ in the plant development, which is morphologically different from the true leaves. In comparison to leaf, cotyledons have a very simple morphology. The cotyledons also contain lipid and protein bodies, which are embryo specific markers, the pattern of gene expression in cotyledons and in the leaf is also different. The genetic relationship between cotyledon and leaf is yet to be deciphered. The *leafy cotyledon1* (*lec1*) mutant of *Arabidopsis* suggests that a single regulatory gene may control many of the differences between true leaves and cotyledons in higher plants (Meinke, 1992). The cotyledons of *lecl* mutant produced trichomes characteristic of leaves, lacked embryo-specific protein bodies and exhibited a vascular pattern intermediate between that of the leaves and cotyledons. The *LEC1* gene appears to play a fundamental role in regulating late embryogenesis. The *lecl* mutant fails to accumulate storage proteins and to acquire desiccation tolerance and dormancy. The mutant embryos remain green late in development, the cotyledons are rounded, contain unusual protrusions on the adaxial surface, and often accumulate anthocyanin at low temperatures. Often, the root apical meristem becomes prematurely active resulting in viviparous seeds. The precocious germination is due to entering into germination pathway by the torpedo stage embryo (West *et al.*, 1994). The embryo shows normal development until the torpedo stage where the abnormalities started as pronounced vacuolation of

hypocotyl cells. The trichomes also appeared at this stage. The *LECl* gene encodes a transcription factor homologous to HAP3 subunit of the CCAAT box-binding factor. During seed development, the *LECl* RNA accumulates only in embryo cell types and in endosperm tissue. The post-embryonic ectopic expression of *LECl* gene in vegetative cells induces the formation of embryo-like structures on vegetative tissues. Apparently, the *LECl* is an important regulator of embryo development, which activates the transcription of genes required for both embryo morphogenesis and cellular differentiation (Lotan *et al.*, 1998).

The reverse of *leafy cotyledon* mutant is observed in *extra cotyledon1 (xtc1)* and *extra cotyledon2 (xtcl)* mutants of *Arabidopsis* where the first seedling leaves are transformed into cotyledons. In *xtc* mutants, the leaves partially transform into extra cotyledons and can be distinguished from true cotyledons, as these are smaller than cotyledons and often irregular in shape. Moreover, these cotyledons are located in the position of the first true leaves on the shoot. The venation pattern is similar to that of the cotyledons. The *xtcl* and *xtc2* mutants phenotype show variable penetrance and expressivity. The homozygous lines of *xtcl* and *xtc2* show 30% and 70% extra cotyledon phenotype, respectively. The transformation of first leaf pair into cotyledon is associated with change in timing of events in embryogenesis. In *xtcl* and *xtc2* mutant, while the transition form globular-to-heart stage embryo is delayed, the development of shoot apex is advanced. Apparently, these genes play important regulatory roles in embryogenesis and in the development of the shoot apical meristem (Conway and Poethig, 1997).

2.6 Cotyledon expansion

In epigeally germinating dicot seedlings, the seedling has a hypocotyl hook, which serves to protect the cotyledons from damage, when the seedling is growing through the soil. The cotyledons remain small and expand only when the seedling emerges out of soil

and cotyledons are exposed to light. A phenocopy of soil-grown seedling however, can be seen in the seedlings that are grown in total darkness and allow the study of effect of light on seedling growth and cotyledon expansion. Basically light controls the process of cotyledon expansion by its epigenetic action, where availability of light triggers the process of cell expansion and cell division in the cotyledons. Now it is known that the expansion of cotyledon by light is mediated by photoreceptors, phytochrome and cryptochrome perceiving red/far-red and blue waveband of light respectively.

2.6.1 Cell division and cell expansion **during** cotyledon development

The information on the cell division and expansion of cotyledons in different species of dicots after germination is very limited. In *Arabidopsis* cell division in cotyledons cease before the end of embryo ontogeny, when the cells are highly differentiated (Mansfield and Briary, 1992), it is logical to assume that the same may be the case for other dicot species during seed dormancy. The post-germinative fate of cotyledon cells varies among species; for example, in hypogeally germinating seedlings such as *Phaseolus vulgaris* L. and *Pisum sativum* L. the cotyledons do not enlarge significantly after germination, but serve mostly as storage organs and gradually shrink after germination. However, in most epigeally germinating seedlings the cotyledon area increases dramatically (10-100 fold) after germination, for example in *Cucurbita moschata* the cotyledon size increases from 0.5-1 cm^2 in the dry seeds to 10-15 cm in the seedlings after full expansion. Even in species with very small seeds such as tobacco, the cotyledon area increases from 0.15 mm^2 in the dry seed to 10-15 mm^2 on full expansion (Avery, 1933). Avery (1933) studied cell proliferation during tobacco cotyledon expansion and found that while there was no increase in number of cell layers of the cotyledon, the number of cells per layer increased by 10-fold.

In *Arabidopsis* there are conflicting reports about the cell division and expansion during cotyledon enlargement. In constitutive photomorphogenic *(cop)* mutants of *Arabidopsis*, such as, *cop2*, *cop3*, and *cop4*, *which* have open and enlarged cotyledons in dark resembling those of light-grown wild type seedlings, the enlargement of cotyledons was due to cell-type differentiation, cell enlargement, and lateral cell division (Hou *et al*, 1993). However, in another study Tsukaya *et al.* (1994) reported no cell division in cotyledon expansion and attributed increase in cotyledon area solely to cell expansion. Levy and co-workers (Fridlender *et al*, 1996) examined the post-germinative cell proliferation in the cotyledon of three plant species e.g. *Nicotiana tobacum*, *Petunia hybrida*, and *Arabidopsis*. In these species cotyledon undergoes a significant expansion after germination. The clonal analysis was used to characterize pattern of cell division in the expanding cotyledons of *N. tobacum* after germination. This study showed that during the tobacco cotyledon expansion the initial cells undergoes one to seven anticlinal divisions throughout the surface of the cotyledon. The analysis of shape of clonal sector showed that these initial cells formed clones, which divided more in the length than in the width. The analysis of cell division in tobacco and *Petunia* in time course using cytological methods showed that cell divisions were detected only after emergence of radicle from the second day after sowing. The rate of cell division was maximal between the second and third day after sowing and then the rate declined. Tsukaya *et al.* (1994) reported that in *Arabidopsis* the cotyledon expansion is only by the process of cell expansion leading to increase in size by nearly 100 fold, however a reexamination of *Arabidopsis* cotyledon expansion by Fridlender *et al*. (1996) showed formation of 2-8 cell layer sectors on cotyledon after mutagen treatment indicating formation of new cells during cotyledon expansion.

The above studies indicate that during the cotyledon expansion both cell division and cell expansion plays important role. Since the organ such as cotyledon has a flattened shape, it is logical to assume that there is a co-ordinated cell division in three dimensions to regulate the shape of the organ. The genetic analysis of leaf expansion in dicots has indicated that specific genes may regulate the direction of cell expansion and perhaps the cell division in the plants. The existence of such mechanisms has been indicated by study of specific mutants such as the *angustifolia (an)* mutant of *Arabidopsis*, which is characterized by narrow cotyledons and leaves (Tsukaya *et al*, 1994). The analysis of cotyledon expansion in *an* showed that the mutation causes defects in the polarity of cell-expansion process, which is eventually manifested as reduced width of cotyledons. The cotyledons of *an* mutant were narrow in the width, but at the same time were thicker than the wild type. It is believed that the polarity of cell expansion may be regulated by *ANGUSTIFOLIA* gene during cotyledon and leaf development.

In contrast to *an* mutant, *rotundifolia3* mutant of *Arabidopsis* also had the same number of cells as the wild type in the leaf but showed reduced cell elongation in the direction of leaf-length. The analysis of double mutants of *angustifolia rotundifoliai* mutant indicated that these two genes independently regulate the leaf expansion. The molecular cloning of *R0TUNDIF0LIA3 (ROT3)* gene indicated that it encodes a cytochrome P450 which might be involved in steroid biosynthesis, as it has domains homologous to regions of steroid hydroxylases of animals and plants (Kim *et al*, 1998). Although, the *ROT3* transcript is ubiquitously present, the *ROT3* gene appears to specifically function to regulate the polar elongation of leaf cells (Kim *et al.*, 1999).

The process of cell expansion and elongation may be regulated by a common mechanism in plants for both leaf and cotyledon development, which may work downstream of light signaling mechanisms. Such a suggestion has emerged from the

observation that a narrow-leaved mutant of *Arabidopsis angustifolia (an)*, that has a defect in the polarity of the cell-expansion process, also shows reduction in the width of cotyledons.

2.6.2 Light regulation of cotyledon expansion

The examination of role of light in regulating the cotyledon expansion has revealed that multiple photoreceptors regulate the process of cotyledon expansion in *Arabidopsis*. Since these photoreceptors may act independently as well as co-operatively, the relative role of each individual photoreceptor is difficult to examine in the wild type seedlings as exposure of light activates all of the photoreceptors. In *Arabidopsis* physiological and genetic studies revealed that at least three photoreceptor systems: the red/ far-red-absorbing phytochromes, the blue/UV-A-absorbing cryptochromes, and unknown UV-B photoreceptors (Quail *et al.*, 1995; Fankhauser and Chory, 1997) perceives light to regulate development. The action spectroscopy studies have indicated that while the individual photoreceptors can independently regulate many developmental responses, in several instances their effect is modulated by other photoreceptor systems (Mohr, 1994).

In *Arabidopsis* the process of cotyledon expansion is controlled by blue, red, and far-red light. The availability of mutants lacking photoreceptors such as phytochrome A (Nagatani *et al.*, 1993) and phytochrome B (Reed *et al,* 1993) which sense red/far-red region of spectrum and cryptochrome 1 which senses blue light (Ahmad and Cashmore, 1993), have allowed examination of relative role of these photoreceptors on cotyledon expansion modulated by other photoreceptor systems (Chory, 1997).

Genetic screening for *Arabidopsis* seedling with undeveloped cotyledon in high-fluence light has identified null mutant lacking blue light photoreceptor *cry1* (previously *hy4*). This mutant was identified as long *hy*pocotyl mutant in continuous white light

(Koornneef *et al.*, 1980). The monochromatic light analysis showed that the mutant was primarily deficient in its response to blue and UV-A light, the decreased cotyledon expansion was observed in red and blue light. This mutant showed normal suppression of hypocotyl elongation in red and far-red light and has normal level of immunologically detectable and spectrally active phytochrome (Koornneef *et al*, 1980; Somers *et al.*, 1991). In addition to cryptochrome 1, phytochrome B also plays a role in cotyledon expansion in *Arabidopsis* under bright-red light (Neff and Van Volkenburgh, 1994).

A distinction between roles of different photoreceptors regulating cotyledon expansion was made by analysis of cotyledon expansion using combination of photoreceptor mutants (Neff and Chory, 1998). In *Arabidopsis* cotyledon expansion involved all three photoreceptors in white light. Among specific photoreceptors, phytochrome B and cryptochrome 1 were the major photoreceptors regulating cotyledon expansion in an additive manner. Between these two photoreceptors, the phytochrome B contributed more than cryptochrome 1 to this growth response, since the cryptochrome 1 deficient mutant caused a 50% reduction in cotyledon area compared to the wild type, whereas the phytochrome B mutant caused a 64% reduction in the area. In fact the cotyledons in the triple mutant were nearly of the same size as that of dark-grown cotyledons. It was suggested that phytochrome A, phytochrome B and cryptochrome 1 together are sufficient to account for the white-light mediated cotyledon expansion. However, in *Arabidopsis* cotyledon expansion phytochrome B act as modulator of cotyledon expansion dependent on the presence of cryptochrome 1.

One of the major factors that regulate cotyledon expansion in dicots is the light. The far-red rich light, which is usually found under plant canopies, induces shade avoidance response in plants including strong induction of *ATHB-2*(*ARABIDOPSIS THALIANA HOMEBOX*) gene encoding an homeodomain-leucine zipper protein.

Steindler *et al.* (1999) showed that the elevated *ATHB-2* levels inhibit cotyledon expansion by restricting cell elongation in the cotyledon-length and -width direction. However at the same time it enhances longitudinal cell expansion in the hypocotyl that is dependent on the auxin transport. The effect of far-red light on cotyledon expansion also argues a strong case for participation of phytochrome A in this response.

Table 2.1 The embryo development defective mutants of dicots showing alteration in pattern formation and the defects in cotyledon formation.

Mutant	Major effect on cotyledons	Gene product
Arabidopsis		
short integument (sin1)	Funnel-shaped cotyledons	Unknown
gnom (gn)	Deletion of cotyledons	The gene product catalyzes guanine nucleotide exchange on small GTP binding protein of ADP ribosylation factor (ARF) family required for vesicle coating in membrane trafficking
shoot meristemless (stm)	Completely lacks the shoot apical meristem, no effect on cotyledons	Encodes a member of *KNOTTED 1* class of homeodomain protein
gurke (gk)	Cotyledons absent or reduced to small knot-like structure	Unknown
cup-shaped cotyledon *(cuc1, cuc2)*	Cup-shaped cotyledon only in double mutants	*CUC2* was sequenced and it is similar to *NAM* of *Petunia*. *CUC1* unknown
pin-formed (pin1-1)	Fused cotyledon or polycot.	The gene encodes protein for auxin transport
pinoid (pid)	Polycot, with low penetrance	*PID* encodes a serine-threonine protein kinase, which negatively regulates auxin signaling.
hydra	1-7 cotyledons	Unknown
altered meristem program (amp)	Produce 20-30% of polycot	Unknown
fackel (fk)	Cotyledons appear to be directly attached to the root	Encodes a protein with similarity to sterol reductases.
monopteros (mp)	No effect on cotyledons, but lacks roots and hypocotyl	Encodes a nuclear localized protein which binds to auxin inducible promoters
Hobbit (hbt)	Normal cotyledons, but lacks root	Unknown
leafy cotyledon (lec)	Cotyledons transformed into leaf like structure	Encodes a transcription factor homolog to the CCAAT box-binding factor HAP3 subunit.
extra cotyledon (xtc)	Transformation of first pair leaf into cotyledon	Unknown
Tomato		
defective embryo and roeristem *(dem)*	No shoot apical meristem, root aborted after a short period of extension, polycot.	Encodes a novel protein of 72 KD with homology to YNV2, a hypothetical yeast protein.
Lanceolate (La)	One cotyledon or no cotyledons. No SAM	Unknown
Pea		
jingle cotyledon *(sic)*	Single cotyledon	Unknown
Tobacco		
low auxin transport *(lat)*	Cup-shaped or fused cotyledons,	Unknown

3. Materials and Methods

3.1 Plant materials

EMS-mutagenized M_2 seeds of tomato *(Lycopersicon esculentum,* cv. Ailsa Craig) were screened for putative cotyledon mutants. We also used the monogenic *aurea (au)* mutant, deficient in all phytochromes species due to a block in chromophore biosynthesis pathway (Koornneef *et al.,* 1985; Terry and Kendrick, 1996) and monogenic *high pigment-] (hp-1)* mutant, which show exaggerated phytochrome responses (Peters *et al.,* 1992). In addition, we used *narrow cotyledons (nc)* [LA 3178] obtained mutant obtained from Tomato Genetics Resource Center, California, USA to compare with mutants isolated in the present study. All the above mutants and wild type were in Ailsa Craig background. The cultivar VF 145 [LA 816] obtained from Tomato Genetics Resource Center, California, USA was also used in this study. The money maker cultivar seeds were also used which were available in the lab and originally obtained from R. E. Kendrick (The Netherlands).

3.2 Light sources

White light (100 μmole $m^{-2}s^{-1}$) was obtained by using four tube-lights (40 W each), three of which were white (Phillips no. 6500 K) and one was brown (Phillips no. 2700 K). The green safe light (<0.01 μmole m^{-2} s^{-1}) was obtained from a coolwhite fluorescent tube light wrapped in six layers of green cellophane paper (λ_{max} 530 nm). The dark manipulations were done under this green safe light. The intensity of the light was measured with a Skye radiometer unit with SKP-215 and SKP-110 probes (Skye Instruments, Powys UK).

3.3 EMS-treatment

To induce mutations, the tomato *{Lycopersicon esculentum* Mill) cultivar Ailsa Craig seeds [harvested in 1996] were imbibed in distilled water for 24 hours at 25 ± 2° C.

The imbibed seeds were then submerged in freshly prepared unbuffered 60 mM ethyl methane sulphonate (EMS) solution for 24 hours in darkness at 25 ± 2° C (Koornneef *et al.*, 1990). Thereafter, the seeds were washed off with tap water for 8 hours to remove the traces of EMS. The mutagenized seeds were then sown in vermiculate (Vermiculite and peat mixture, Karnataka Explosive Limited, Bangalore, India) in plastic trays (40 cm / x 30 cm *b* x 7 cm *h)* and grown at 25 ± 2° C under continuous white light (100 μmoles m^{-2} s^{-1}) for 10-14 days. Seedlings were then transferred to red loam sandy soil in the open experimental field of University of Hyderabad, Hyderabad, India. Seeds of M_2 generation were harvested in bulk. The fruits were first squashed and fermented for 24 hours to ease the removal of the mucilaginous materials from the seeds. Seeds were thoroughly washed with tap water and were dried in shade at an open place for three days. Finally, the seeds were stored in polythene bags with a desiccant at 25° C till further use.

3.4 Mutant isolation

To isolate the mutants, M_2 seeds from two populations, the first raised in 1995 (A. Srinivas, thesis in preparation), and the second populations raised in 1997, for present work by myself were used. The seeds were surface sterilized with 0.1% (v/v) sodium hypochlorite solution for 10 min. Thereafter, seeds were washed in distilled water and sown on filter papers moistened with distilled water in transparent plastic boxes (9.5 cm / x 9.5 cm *b* x 5 cm *h*). When the seeds germinated (showing radicle emergence), they were grown in vermiculate in plastic trays (40 cm / x 30 cm *b* x 7 cm *h*). The above two M_2 populations (about 50,000 seeds) were screened for seedling with altered cotyledon number and size after one week of germination under continuous white light. The polycotyledon mutants isolated were transferred to plastic cups (9 cm *h* x 7 cm *d)* filled with vermiculate. A total of 25 polycotyledon mutants were isolated during the screening.

The mutants were transferred to the net house after one week into pots filled with red loam sandy soil.

Similarly, seventy-one narrow petioleless cotyledon mutants were isolated from the above M_2 population. During the screening putative mutants isolated were assigned tentative numbers in the order of isolation. After the stability of the mutant phenotype in subsequent generations and its genetic characterization, it was given the locus name (*poc* in M_4 segregating generation and *npc* in M_3 generation). The temperature in the field at Hyderabad as between 20-30° C from mid June till mid February (See appendix for meteorological data). In the summer of 1998, the mutants were grown at Faculty of Sciences, University of Sana'a, Republic of Yemen, under similar conditions.

3.5 Crossing

The first step in crosses was to select the flower, which has not opened, and due to open the next day, to ensure that the flower is not yet selfed. The emasculation was done by carefully removing the anther cone by pinching the side of the anther cone with tweezers by holding the flower at the pedicel and pulling straight out to avoid damaging the pistil. The emasculated flower was covered with cheesecloth or butter paper bags to protect from insect pollinator or cross-pollination due to wind. Care was taken not to damage the stigma of the flower. After 24 hours of emasculation when the stigma becomes receptive, the pollen collected from 2-3 days old open flowers of the desired pollen donor were carefully applied to the receptive stigma. To help the anther releases all the pollen, anthers were kept in warm morning sunshine for about 10-15 minutes, and then pollen were carefully applied to the stigma using a small brush. The pollinated flowers were covered again with breathable bags and the process of pollination was repeated 3-4 times for 2-3 consecutive days to ensure large number of seeds (G. Kalloo personal communication, Directorate of Vegetable Research, Varanasi, India.). The

recommended time for emasculations is 4 PM and the best time for cross-pollination at Hyderabad is 8-9 AM.

3.6 Pollen germination

Pollens were collected from ten wild type as well as ten *polycotyledon* mutant flowers that opened at the same time. The pollen were collected by opening the anthers manually, the collected pollen were germinated in pollen germination media (Brewbaker and Kwack, 1963). The germination media contained sucrose 10% (w/v), boric acid 100 mg/l, calcium nitrate 300 mg/l, magnesium sulfate 200 mg/l and potassium nitrate 100 mg/l. Samples of five replicates for each genotype were grown in cavity slide and with sufficient media in 1.5 ml Eppendorf tube for ten hours at 25° C. The pollen were then stained with 1% (w/v) acetocarmine and observed under microscope. From each sample the total number of pollen grains and number of germinated pollen were counted and the percentage of pollen germination were calculated.

3.7 **Fertility estimation in *poc* mutants**

Hundred flowers from each of the wild type and the three-*poc* mutant classes were emasculated and pollinated in same way under the same field condition. Fruit setting was scored two weeks after pollination. The number of seeds in 25-30 fruits was used as criterion of seeds setting in each genotype, either in cases of crosses or in naturally selfed or manually selfed fruits.

3.8 Construction of double mutants

For double mutants construction, the crosses were made between the monogenic mutants; *au* and *poc*; *hp* and *poc;* and *npc* and *poc*. Double mutants were scored in F_2 and F_3 generations of segregating populations on the basis of phenotype. The putative double mutant was also crossed with each single mutant to confirm its phenotype. (See Fig. 3.1)

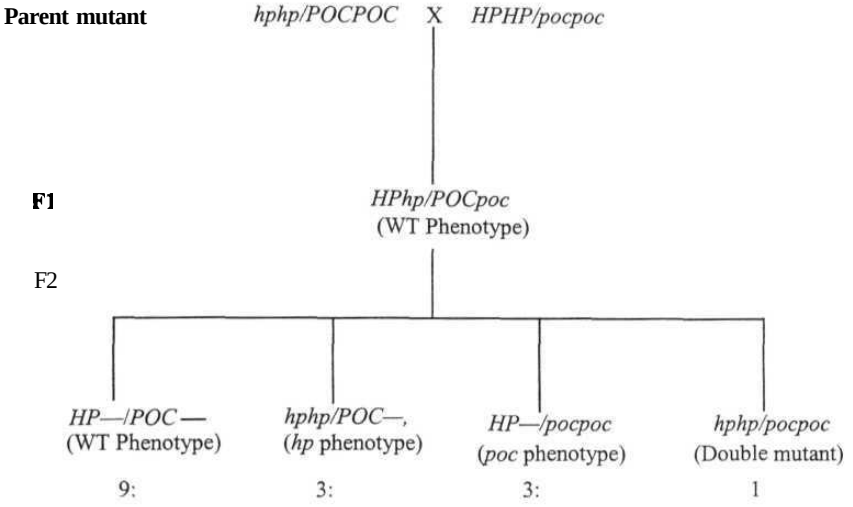


Figure 3.1 Scheme describing the isolation of double mutant *poc hp (hphp/pocpoc)* of tomato in F_2 generation. The *hphp* mutant was crossed with *pocpoc* mutant. The phenotype of F_1 was similar to the wild-type (WT). In F_2 the segregation showed WT, *hp*, *poc* and *hp poc* phenotype in 9:3:3:1 ratio.

3.9 **Embryogenesis**

The pollination was regulated in both wild type and *poc* mutant and fruits were harvested in time course starting ten days after pollination (DAP). Ovules were extracted from the fruits of wild type and *poc* mutant immediately after harvest and fixed in ethanol/acetic acid (6:1, v/v) overnight at room temperature. After several washes for few seconds in 100% ethanol and finally in 70% ethanol, ovules were preserved in 70% ethanol. The ovules were then cleared in mixture of chloral hydrate/glycerol/water (8:1:2, w/v/v) overnight (Berleth and Jurgens, 1993). These whole-mount preparations of ovules were stored at room temperature and ovules were dissected under a stereo dissection microscope. The embryos were mounted with a cover slip, and observed on a Zeiss Axioskop microscope under DIC optics using 40X, 20X, 10X objectives. Photography was done using low speed black and white film (15-25 ASA) which give better details and contrast (Siddiqi *et al.,* 2000)

3.10 Cotyledon area determination

Fully expanded cotyledons of wild type, *poc* and *npc* mutants were excised from two weeks old seedlings under continuous white light. The cotyledons were sandwiched between two transparent sheets and scanned using HP 4C scanner at 600 dpi resolution. The images were saved as TIFF files and analyzed using Image Tool package (Neff and Chory, 1998). The area of the cotyledon was determined in square centimeter (cm^2). For *npc* mutant, *poc* mutant and wild type the mean total cotyledon area of a single cotyledon was calculated.

3.11 Observing cotyledon epidermal cells

The epidermal cells of 10 days old wild type and *npc* mutant cotyledons were observed. The peelings from the epidermal layer were observed under microscope without any processing. The photographs were taken using Kodak 100 ASA film.

3.12 Estimation of germination

Germination behavior of wild and *npc* mutant was scored as a percentage of germination quantified by the emergence of radicle from seed. About 500 seeds from wild type and *npc* mutant were surface sterilized in 0.1% (w/v) $K_2 MnO_4$ solution for five minutes (Atanassova *et al.,* 1997) and rinsed with distilled water several times and then blotted on germination paper. Seeds were germinated in dark at 25 ± 1° C in germination boxes, for each genotype in three replicates. The observation of germination was done under safe green light at every twelve hours interval..

3.13 Clearing of cotyledons and leaves

To observe the venation pattern of the cotyledons, ten days old light grown seedlings were used. In case of leaves, the leaves from the sixth node onwards were used. The pigments were removed by submerging organs in acidified methanol (2% HC1, v/v) for 24 hours at room temperature. Thereafter clearing was done using the in mixture of chloral hydrate/glycerol/water (8:1:2, w/v/v). The cotyledons and leaves were stained using p-rosaniline hydrochloride.

3.14 Rooting assay.

To examine the formation of adventitious roots in the *poc* mutant, one-week light grown wild type and *poc* mutant seedling were used. The taproots of these seedlings were excised above the base of the hypocotyls. The rootless seedlings were the immediately planted on either plain agar or agar with 10 μM kinetin. The seedlings planted on agar plate were grown in light and appearance of the lateral roots was observed after one week by naked eye.

3.15 Estimation of root length

The germinated seeds of both wild type and *poc* mutant were transferred into cups containing the vermiculate. Seedlings were grown in light till the time of measurements of root length. Other group of seeds were transferred after germination into petridishes containing plain agar or agar supplemented with 10 µM kinetin. The agar plates were placed in vertical position under light. The root measurements were taken one week after germination using a ruler.

3.16 Phenotypic characterization

The *poc* and *npc* mutants of tomato were grown in the field and the net house and also in the green house. The regular observations of the mutants and the wild type morphology were made at all stages of seedling, vegetative and reproductive developments. Similarly, the F_1 of the crosses were also grown to detect all the morphological features during its life cycle.

4. Results

4.1 Isolation and characterization of *polycotyledon (poc)* mutant

4.1.1 Mutant isolation

A population of 50,000 M_2 seedlings was screened, which descended from ethyl methane sulfonate (EMS) mutagenized seeds of tomato {*Lycopersicon esculentum* Mill cultivar Ailsa Craig) to isolate mutants with *polycotyledon (poc)* phenotype. Seedlings with altered number of cotyledons were isolated from population after one week of germination under continuous light. Twenty-five putative mutants were isolated in the initial screen, of which about fifteen mutants also showed alteration in plant morphology during subsequent vegetative and reproductive development. Moreover, out of the fifteen mutants only nine mutants survived and produced F_1 seeds, because these mutants were totally male sterile and were rescued by crossing with wild type as pollen donor (Fig. 4.1, Table 4.1).

4.1.2 Genetic characterization

4.1.2.1 Segregation analysis

The M_2 plants of *poc* mutant were totally male sterile, therefore it was rescued by crossing using *poc* as female parent and wild type as pollen donor. The M_3 heterozygous plants (F_1 of M_2 *poc* x WT) showed phenotype similar to wild type both at seedling stage and also during subsequent vegetative and reproductive development and were fertile. However, at seedling stage about 0.5 % of the F_1 seedlings showed polycotyledons. The M_4 (F_2) seedlings segregated to 3:1 ratio of wild type and the *poc* mutant. Although *poc* mutant is male sterile, few of *poc* plants make viable pollen in the anther sac. The pollen were collected from these plants and used for reciprocal crosses using wild type as female parent. The result of reciprocal crosses was similar and showed 3:1 segregation in F_2 generation. These results indicate that the *poc* mutation is controlled by a single recessive

Figure 4.1 Variation in the cotyledon numbers in seedlings of *poc* mutant. Comparison between phenotype of six days old wild-type seedling (top right) showing typical dicot phenotype with one week old seedlings of *poc* mutant showing different number of cotyledons, three cotyledons, four cotyledons, two cotyledons with one of which showing two midveins [1 +(2)], three cotyledons of which two are partially fused [l+(2)] and a *poc* seedling showing two halves of cotyledons which are curled.

Table 4.1 polycotyledon *(poc)* mutants isolated in M_2 from two populations of EMS mutagenized seeds of tomato *(Lycopersicon esculentum*, cultivar Ailsa Craig*)*.

Population	C/N	Mutant Number	Phenotype	Remarks
First Population	1	AR1-46	altered [a]	*poc1-1*
	2	AR1-47	altered	Lost
	3	AR1-113	altered	*poc 1-2*
	4	AR1-114	altered	*poc]-3*
	5	AR1-116	altered	*pocl-4*
	6	AR1-122	altered	*poc1-5*
	7	AR1-124	altered	*pocl-6*
	8	AR1-125	normal[b]	
	9	AR1-127	normal	*poc 1-7*
	10	AR1-128	altered	*poc1-8*
	11	AR1-129	altered	Lost
	12	AR1-130	altered	*poc1-9*
	13	AR1- 131	normal	
	14	AR1-132	altered	Lost, flowers were induced by GA but they were totally malformed. No fruit setting was observed.
	15	AR1-133	altered	Lost
	16	AR1-134	normal	
	17	AR1-137	normal	
Second population	18	AR2-9	altered	Lost, similar phenotype to above group, lost in flowering stage.
	19	AR2-10	altered	Lost
	20	AR2-82	normal	
	21	AR2-83	normal	
	22	AR2-84	normal	
	23	AR2-86	altered	Lost
	24	AR2-91	normal	
	25	AR2-92	normal	

[a]-altered vegetative and reproductive development in addition to altered cotyledon numbers.

[b]-normal in all aspects of development, which was similar to wild-type, and the polycotyledon seedlings were not detected in M_3

nuclear gene (Table 4.2). The test cross using heterozygous *poc (POC/poc)* line as pollen donor and homozygous *poc (poc/poc)* as female parent showed typical 1:1 ratio supporting the reciprocal crosses, that *poc* is a recessive nuclear mutation.

4.1.2.2 Genetic complementation of *poc* mutant

A total of nine lines having *poc* phenotype were isolated and all these lines showed monogenic recessive inheritance. These lines were crossed for complementation analysis (Table 4.3). The results showed that all the lines were allelic, as all of them produced 100% of *poc* phenotype in F_1 generation, on crosses with homozygous lines. Also these lines showed a typical 3:1 ratio, when crossed with heterozygous lines. The results obtained reinforce the view that *poc* mutation in all these lines is in an identical locus and these lines are likely to represent alleles of same gene.

4.1.3 Phenotypic characterization

The effect *of poc* mutation was not restricted to presence of multiple cotyledons in the seedlings alone. The *poc* mutation showed strong pleiotropic effect on all the stages of plant development right from the embryogenesis to flowering. This necessitated examination of morphology of *poc* mutant right from germination to flowering and fruit setting. The morphological features observed are grouped under three categories

1- Presence of multiple cotyledons

2- Vegetative development

3- Reproductive development

The following morphological characterization was made using *poc1-1* and *poc1 -2* as model lines as all the nine lines of *poc* were allelic and showed the same phenotype.

Table 4.2 Inheritance pattern in genetic crossing experiments between *poc* mutant and wild type plants.

Crosses	Generation	No. of seedlings	Seedlings phenotype		P
			WT	Mutant	
pocl-1 x WT	**F,** F_2	**101** 161	101 119	0 42	0.5-0.8
poc1-2 x WT	F, F_2	**47** 185	**47** 141	0 44	0.5-0.8
pocl-3 x WT	F_1 F_2	220 310	220 225	0 85	0.99
poc1-4 x WT	F, F_2	107 160	107 114	0 **46**	0.2-0.5
poc1-5 x WT	F, F_2	103 **186**	103 116	0 70	0.01
WT x *poc1-2*	F, F_2	104 170	**104** 122	0 **48**	0.2-0.5
WT x *pocl-5*	F_1 F_2	117 153	**117** 118	0 35	0.5-0.8
pocl-1 x *POC1/poc1-1*	F_1	183	**90**	93	**0.99**

Table 4.3 Complementation analyses of *poc* mutants.

Parental genotype	Generation	No. of seeds	No. of seedlings	
			Wild type	Mutant
pocl-5/pocl-5 x *poc1-2/poc1-2*	F_1	95	0	95
poc1-5/poc1-5 *xpocl-l/pocl-1*	F,	55	0	55
poc1-2/poc1-2 *xpocl-l/pocl-1*	F_1	90	0	90
pocl-7/pocl-7 x *poc1-1/poc1-1*	F_1	23	0	23
pocl-9/pocl-9 *xpocl-l/pocl-1*	F_1	55	0	55
POC1/poc1-3 x *POC1/poc1-1*	F_1	13	10	3
POC1/poc1-3 x *POC1/poc1-4*	F_1	25	19	6
POC1/poc1-3 x POC1/poc1-6	F_1	20	16	4
POC1/poc1-4 x *POC1/poc1-8*	F_1	26	20	6
POCl/pocl-4 x *POCl/pocl-1*	F,	21	17	04

4.1.3.1 Presence of multiple cotyledons

In the cultivar Ailsa Craig as well as in other cultivars, seedlings with more than two cotyledons were not found in wild type. We randomly screened nearly 5,000 seedlings of Ailsa Craig but did not find any polycotyledonous seedling. Similarly, random screening in other cultivar VF 145, out of 1,826 seedlings only one was tetracot, but showed normal vegetative and reproductive development. Moreover, none of its 175 progeny seedlings showed the polycotyledonous trait again. Evidently, the cotyledon number is hereditably fixed to two in tomato similar to other dicot species. In contrast, the *poc* mutant in homozygous state showed 98.5% seedlings as polycots (Table 4.4 and Fig. 4.1, 4.2). This phenotype was followed till M_7 generation, and similar results were observed indicating that polycotyledonous phenotype is heritable and controlled by *poc* mutation.

The *poc* seedlings showed large variation in their cotyledon numbers, which ranged from 1.5% of seedlings being dicots with slightly rounded cotyledons, to rest being polycots. Figure 4.1 and 4.2A shows variation in the phenotype of cotyledons in *poc* seedling. These variations ranged from two cotyledons with one cotyledon showing two midveins, three cotyledons-two of them separated at the tip [l+(2)], three cotyledons-two of them separated midway between tip and petiole[l+(2)](Fig. 4.2B), three totally separated cotyledons, four cotyledons each two partially fused [(2)+(2)], and four totally separated cotyledons. The range of variation in cotyledon number is quantitively presented in Table 4.4. It is evident from the results presented that, among the seedlings with multiple cotyledons highest percentage was that of tetracotyledon seedlings. Even though, about 1.5% of progeny of *poc* showed dicot seedlings, they could be distinguished from the wild type dicot seedlings by its unique cotyledon morphology, which is significantly different from the wild type (Fig. 4.3).

Table 4.4 Cotyledon numbers in homozygous lines of *poc* mutants.

Numbers of cotyledon	No. of Plants			
	Line		Total	%
	poc1-1	*poc1-2*		
1+1	10	**5**	15	1.5
l+(2)	4	**13**	17	1.7
3	109	**234**	343	34.4
2+(2)	57	**62**	119	11.9
(2)+(2)	8	**5**	13	1.3
4	107	382	489	49.1
Total	295	701	996	100%

Numbers in brackets indicate that the cotyledons are splitted into two partially or totally fused.

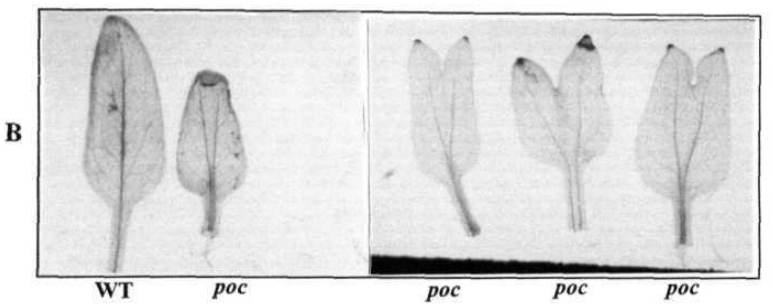
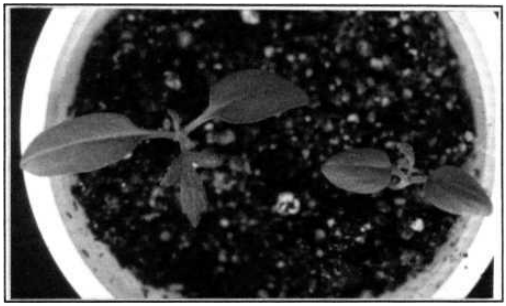


Figure 4.2 A-Variations in cotyledon numbers of six day old seedlings of *poc* mutant. The photograph shows *poc* seedlings with four cotyledons of which two are separated only near cotyledon tip, four cotyledons with two cotyledons partially separated, four cotyledons with two cotyledons partially separated (note their big size), three cotyledons **with** unequal size, four cotyledons with two cotyledons separated only at the tip (note **their** small size), three cotyledons with two cotyledons fused.

B-The pattern of venation in cotyledon of wild-type and *poc* mutant of tomato. The clarified*poc* cotyledon with two midveins. In some *poc* cotyledons two midveins totally separates right in petiole and run parallel through the petiole and also continue to hypocotyl.



WT *poc*

Figure 4.3 Comparison of the cotyledon morphology of a dicot *poc* seedling to wild-type. The *poc* dicot seedling shows reduced cotyledon size with more round cotyledons, and was less tall than the wild-type.

The question that *poc* phenotype leads to an overall increase in cotyledon area was examined by determining the total area of the cotyledons in wild type seedling as well as in the *poc* mutant seedling (Fig. 4.4). No significant differences were found between the total cotyledon area of the wild type and mutant seedlings. In fact, there was a slight increase in cotyledon area of *poc* mutant compared to wild type.

4.1.3.2 Root lengths in seedling stage

The root length was compared between wild type and *poc* mutant by growing germinated seeds after radicle emergence in cups containing vermiculate. Seedlings were grown under continuous light for one week and thereafter the root length was measured. The seedlings showed significant differences in root length of wild type and *poc* mutant. The wild type root reached a length of about 8.5±0.38 cm while the *poc* mutant root was only 3.78+0.19 cm long (Fig. 4.5).

4.1.4 Rooting Assay

The regeneration capacity of wild type and *poc* mutant, which likely reflects endogenous hormonal level, was examined by observing root phenotype in the presence of kinetin. The germinated seeds were transferred to agar media containing 10 uM of kinetin. In the presence of kinetin, both the wild type as well as the *poc* mutant showed reduction in the root length. The reduction in the root length was more in *poc* mutant than the wild type. The root length *of poc* mutant showed a reduction by 73.8% compared to 55% reduction in the wild type (data not shown).

Similarly, the capacity of detached hypocotyls to root was examined in both wild type and *poc* mutant. One-week-old light-grown seedlings were excised close to the base of the hypocotyls to detach root. The excised hypocotyls were placed in agar containing 10 uM of kinetin to examine rooting. Strikingly, the *poc* mutant rooted albeit with reduced number and length compared to *poc* control seedlings on agar media without

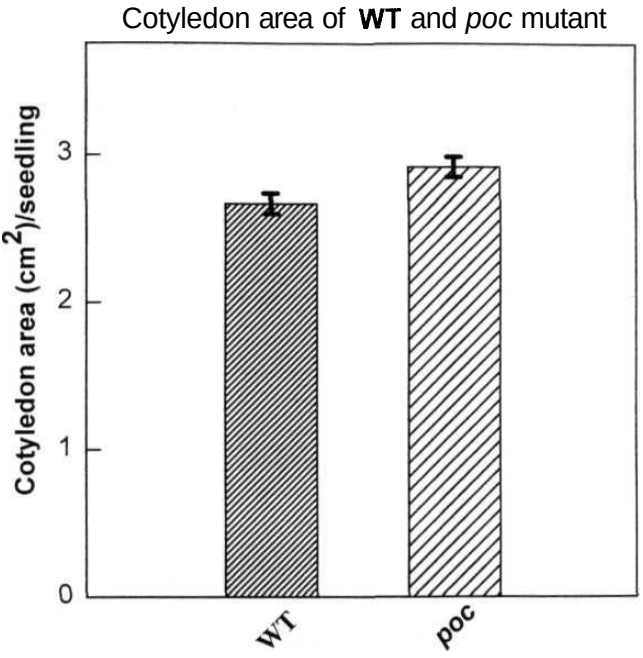


Figure 4.4 The cotyledon area was calculated as the total area of cotyledons of an individual seedling (n = 30 seedlings) and was expressed in square centimeters. The increase in the cotyledon numbers in *poc* mutant seedling does not cause a significant increase in total cotyledon area.

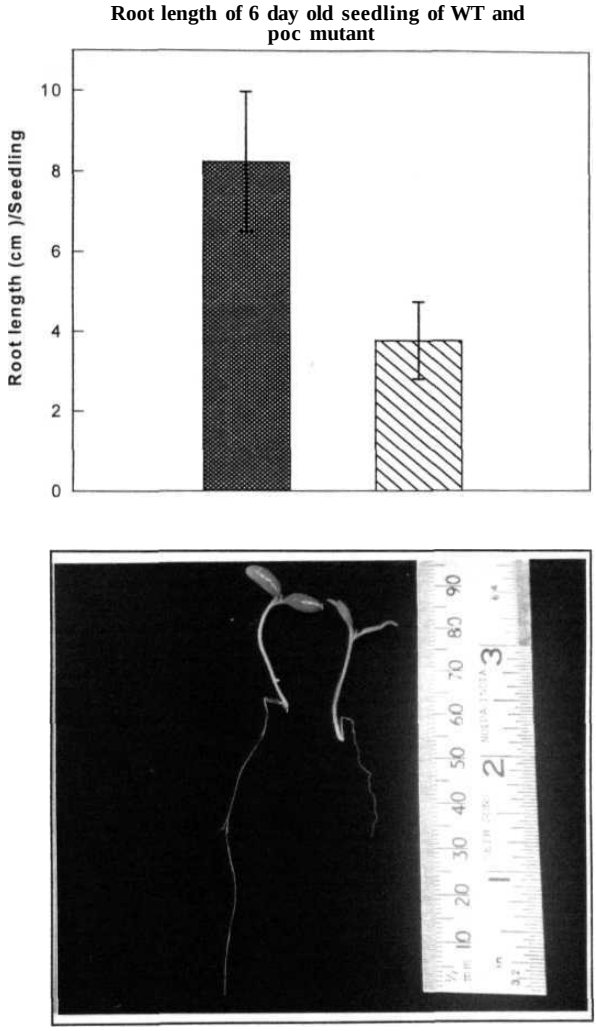


Figure 4.5 The root length of six day old wild type and *poc* mutant seedlings grown on vermicalate.

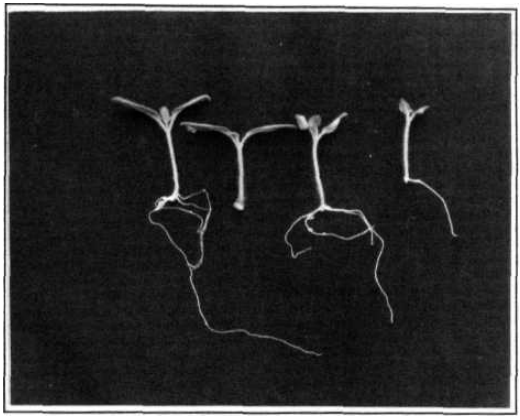
kinetin. In contrast, the wild type hypocotyls showed no rooting of hypocotyl in kinetin containing media while it rooted normally in plain agar media (Fig. 4.6).

4.1.5 Hypocotyl elongation in dark

The dark-grown dicot seedling is characterized by elongated hypocotyl, small folded cotyledons, a prominent plumular hook, this pattern of growth is called skotomorphogenesis. In contrast, the light grown seedling shows reduced hypocotyl elongation, absence of plumular hook, expanded cotyledons and chlorophyll accumulation, this pattern of growth is called photomorphogenesis. In tomato, the dark-grown wild type seedling shows rapid elongation of hypocotyl reaching a height of about 11.5±0.28 cm after one week. In comparison, seedling of dark-grown *poc* mutant attain a height of only 6.3±0.35 cm. Comparing wild-type to *poc* mutant by the t-test indicated highly significant differences in hypocotyl elongation between these plants. However, the opening of the hypocotyls hook in *poc* mutant, which is an ethylene, mediated response, did not show any difference from wild type. Cotyledon expansion was not observed due to the difficulty in removing the seed coat by the seedling, which is retained even by seedlings grown in light (Fig. 4.7).

4.1.6 Double mutants study

To investigate the effect of *poc* mutation in plants that lack phytochrome, *poc* mutant was crossed with *au* mutant to make *poc au* double mutant. The *au* mutant is deficient in phytochrome chromophore and consequently likely possesses all phytochrome species at reduced level. Due to absence of phytochrome, *au* mutant has longer hypocotyl when grown in light compared to wild type. The *poc au* double mutant did not show reduction in hypocotyl length, and was similar to *au* showing hypocotyl elongation. Similarly in mature *poc au* plant, no interaction was detected between the two



WT **WT+K** ***poc*** ***poc*+K**

Figure 4.6 One week old seedlings of wild type (WT) and *poc* mutant *(poc)* grown on vermiculate were excised close to root base and were grown on agar for one week. Both wild type and *poc* mutant produce normal adventitious roots when grown in plain agar. On the other hand, wild type (WT+K) seedling produce no adventitious roots on agar supplemented with 10μM of kinetin (K). The *poc (poc+K)* mutant produce adventitious roots on agar media supplemented with 10 uM of kinetin after one week albeit with reduced length and number.

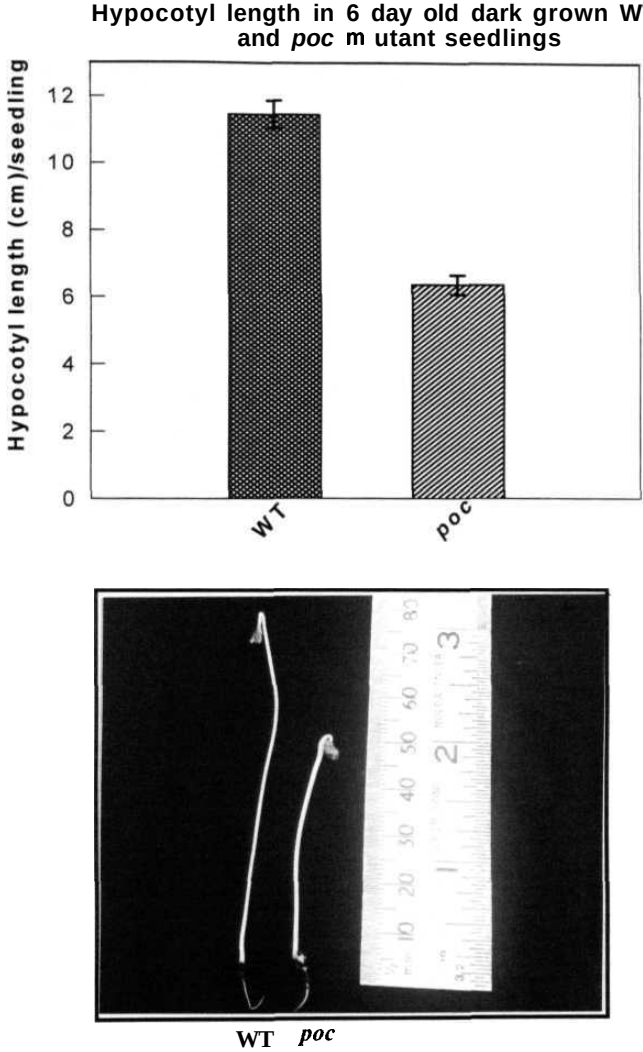


Figure 4.7 Comparison of hypocotyl length of dark grown seedling of wild type and *poc* mutant. The germination of wild-type and *poc* was synchronized and germinated seeds showing radical emergence were grown in dark for one week for making comparison. The wild-type seedlings showed typical elongated hypocotyls, while *poc* mutant seedlings show stunted growth in dark.

genes and plants showed typical morphology of *poc* with yellow color of *au* (data not shown).

The double mutant was also constructed by crossing *poc* with *hp,* which shows exaggerated phytochrome responses. Similar to *poc au* double mutant, the *poc hp* mutant too showed no significant change in the seedling or mature plant phenotype where both genes are expressed (of *poc* and that of *hp).* The results using double mutants showed no interactions between *poc* and *au* as well as *poc* and *hp1-1* genes (Fig. 4.8, 4.9). The F_2 segregation ratio of *AU/au, POC/poc* and *HP1-1/hp1-1POC / poc* double heterozygotes were typical dihybrid segregation, where both showed 9:3:3:1 ratio of WT, *poc, au* or *hp\,* and the double mutants *(au, poc* and *hp, poc).* Since *au* is mapped, to chromosome 1 and *hpl-1* to chromosome 2, the segregation indicates that the *poc* gene is not linked to either the *au* or *hp1* loci.

4.1.8 Alteration of phyllotaxy

Phyllotaxy is the arrangement of organs around the axis of plant. In the wild type Ailsa Craig cultivar, the leaves arrangement is spiral, and leaves appear at an angle of 137° to each other. In contrast, about 30% of *poc* seedlings show deviation from this spiral arrangement of leaves. In these *poc* seedlings, three or four leaves emerge from one single node, making the angle of divergence about 90°-120° between the leaves. However, the observed phyllotactic alteration was seen only in the first node of *poc* mutant and rarely in other nodes (Fig. 4.10).

4.1.9 Splitting of shoot

In few of the *poc* mutant seedlings four weeks after germination, the shoots show splitting of the apical meristem leading to formation of two identical shoots. Both these shoots show similarity in morphology with respect to positioning of leaves and branches up to the sixth leaf, and then they adopt different pattern (Fig. 4.11).

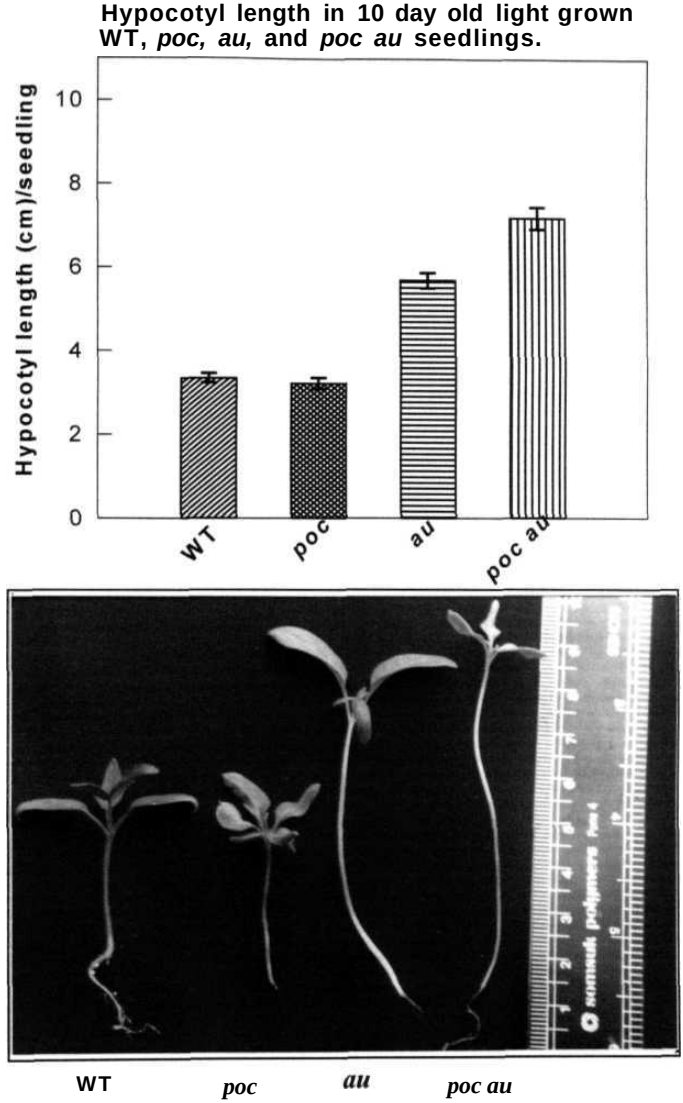


Figure 4.8 Comparison of hypocotyls length of 10 day old light-grown seedlings of wild-type, *poc*, *au*. and *poc au* double mutant. While *poc* hypocotyl was of nearly same length was WT, the *au* hypocotyl was longer. The *poc au* double mutant shows hypocotyl length similar to *au*.

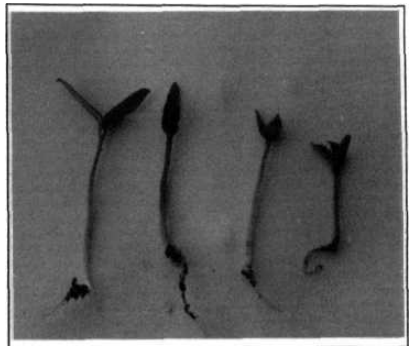


Figure 4.9 The phenotype of three day old light-grown seedlings of wild type, *hpl-1*, *poc*, and *poc hpl-1* double mutant. The *hpl-1* seedling shows more pigmentation in hypocotyl and in cotyledons compared to wild type and *poc* mutant. The double mutant *poc hpl-1* retained the pigmentation of *hpl-1* and polycotyledonous phenotype of *poc*.

Figure 4.10 Comparison of phyllotactic arrangement of leaves on wild-type and *poc* mutant plants. One month old net-house grown plants were examined and shoot was cut off to facilitate observation and photography. Note, while wild-type showed normal spiral phyllotaxy one leaf at each node and angle of divergence between leaves is 137°. In contrast *poc* mutant showed four leaves at one node showing drastic alteration in phyllotaxy.

Figure 4.11 Six weeks old *poc* mutant plant showing splitting of main shoot into two identical shoots, with similar morphology in positioning of leaves and branches. The three cotyledons are still attached to the plant and can be seen at its base.

4.1.10 Leaf morphology and plant height.

The *poc* mutation shows strong pleiotropic effect on vegetative development of plant. This effect on plant morphology becomes apparent at the transformation period from juvenile to more mature development i.e., after 6-7 weeks from germination. This is manifested in the form of strong alteration in vegetative development, and the most distinct modification being the leaf morphology. The close examination of morphology of *poc* mutants particularly that of leaf revealed that the plants on the basis of morphological alteration could be classified into three classes (Fig. 4.12). However, in order to understand the changes in leaf morphology, the morphology of wild type leaf is described for comparative purpose. In tomato, leaves after the fifth or sixth node above the cotyledons reach full size and final shape, whereas the leaves at lower nodes produce one to four pairs of small entire leaflets. The mature leaf of wild type Ailsa Craig cultivar is broadly triangular and imparipinnate with three pairs of large lateral leaflets and one terminal leaflet. Each large leaflet has about six lobes, some of which may be deeply incised, and often the basal leaflet is compound. Usually, leaves also bear two to four pairs of less-lobed lateral leaflets, which are intermediate in size and one or two pairs of small entire leaflets. Large leaflets are usually arranged in opposite pair or may occur singly or sub-opposite pair. While intermediate and small leaflets are irregularly distributed, the small entire leaflets are petioleless, while large, intermediate and small leaflets are with petiole (Fig.4.13). The venation is also typical pinnate in the tomato leaves (Fig. 4.14). The three classes of *poc* mutant are described hereunder.

Figure 4.12 Phenotype of three and half months old plants of wild-type *(left), poc* A (second from left), *poc* B (third from left), and *poc* C (right) mutants. The *poc* A plant is bushy, leafy and shorter than the wild-type, it also has leaves which are smaller in size. The *poc* B plant is less branched and has less number of leaves compared to *poc* A. The *poc* C plant is also shorter than wild-type, it flowers late from sixteenth leaf onwards compared to wild-type, while *poc* A and B flower from eighth to tenth leaves onward.

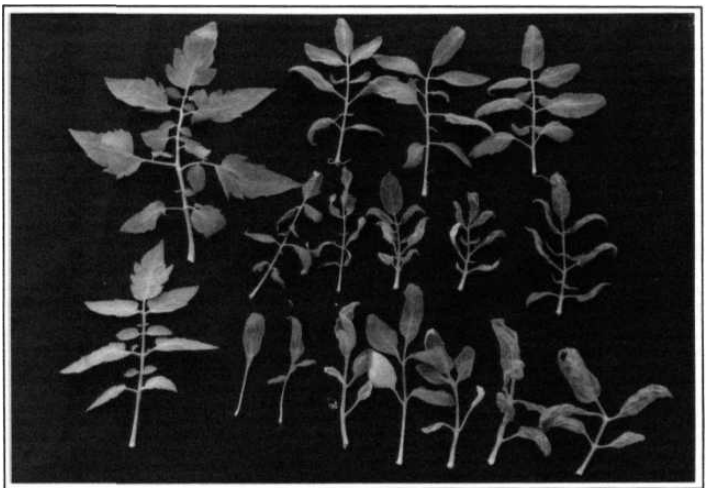


Figure 4.13 Range of variation in morphology of leaf in different classes *of poc* mutant. The leaves were collected from the sixth node onwards of three months old plants of wild-type (upper and lower left), different class of *poc* mutant B (upper right) and A (middle right) and C (lower right). The leaves of *poc* class B show less lobbing as well as the slight curling. The leaves of *poc* A (middle right) show the significant reduction in leaf size and the severe curling. In *poc* C (lower right) leaf complexity varies from the simple lanceolate leaf to leaf with four lateral and one terminal leaflets. In this class *(poc* C) no intermediate and minor leaflets were found and all leaves were with entire margin.

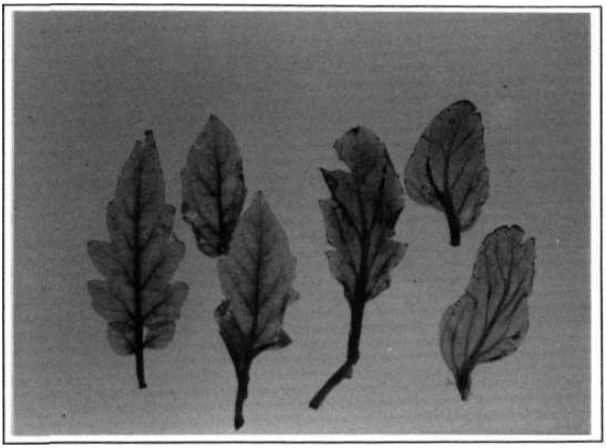


Figure 4.14 Comparison of venation pattern in leaflets of wild-type (three leaflets on left) and *poc* C mutant (three leaflets on right). The leaflets were excised from the seventh node of three months old plants. In the mutant leaflets the veins are thicker and show tendency to run parallel toward the leaflet tip, rather than terminating in leaflets lobes. In addition, minor veins are also eliminated in the mutant.

Class A

Plants, which are extremely shorter than the wild type, characterize the first class. These plants are also very bushy with leafy phenotype. One of the distinctive features of this class is that the mature leaf shows many epiphyllous structures like leaves and shoots on its midrib and leaflet petioles (Fig 4.15). All the individuals of this class show this phenotype of epiphyllous appearance of leaves with nearly 100% frequency. Though in all the three classes curling of leaves is also observed, the class A showed much extreme phenotype of curling. The mutants of this class also showed a maximum reduction in the size of leaf as compared to wild type.

Class B

The class B is slightly shorter than the wild type and shows comparatively less reduction in leaf size than the class A. The plants of class B also have less number of branches, and epiphyllous structures on the leaves and petioles are rarely seen in this class. Compared to class A leaf curling is less drastic in *poc* B mutant. The lobbing of leaves in this class is less compared to wild type (Fig. 4.12).

Class C

The class C is also shorter than the wild type, and shows a longer period of vegetative growth, which continues up to the formation of the sixteenth leaf. On the other hand, in wild type as well as class A and B of *poc* mutant inflorescences are normally formed between the eighth and tenth leaf. In class C the leaf morphology and venation are also drastically altered, and in fact in extreme cases, the venation in leaf tends to be parallel reminiscent of monocot venation (Fig. 4.14). The symmetry of the leaf is also changed and shows wide variation. An individual plant may bear a simple lanceolate leaf; leaf with three leaflets without symmetry, and in many leaves six leaflets and one terminal leaflet (Fig. 4.13). More importantly, in leaves of the class C lobbing is totally

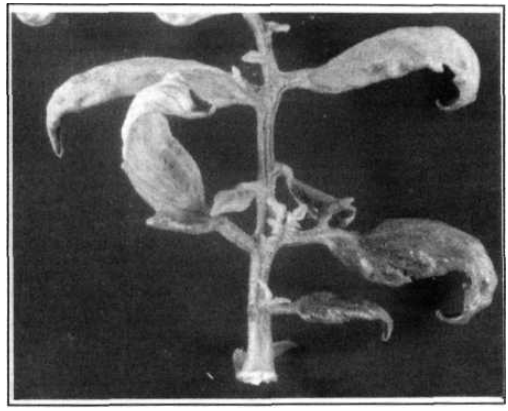


Figure 4.15 The leaf excised from three months old *poc* A mutant of tomato shows epiphyllous structures on the base of the leaflet petiole. Note the epiphyllous structure at the base of the first leaflet and the second (right) leaflet. Leaves in all nodes show this abnormality.

absent, thus, generating leaves with entire margin. Moreover, in this class no small and minor leaflets are found (Fig.4.13).

4.1.11 Reproductive development

In flowering plants, the transition from vegetative to reproductive phase of growth is a critical developmental process, which is marked by a number of changes in the shoot apex at the molecular, physiological and morphological levels. The vegetative meristem is transformed into reproductive meristem, which either directly terminates into flower or remains as inflorescence meristem and produces multiple determinate floral meristems. In contrast to the indeterminate shoot meristem or inflorescence meristem, flowers are determinate structures that produce a defined number of organs, the arrangement and morphology of these organs is species specific. There are many genes, which regulate the development of flower. These genes are classified as flowering time genes that are generally influenced by environment such as day length and vernalization; meristem identity genes, which specify the flower meristem identity; and flower organ identity genes, which determine the fate of organ primordial in the flower (Howell, 1998).

The inflorescence of Ailsa Craig cultivar consists of about 7-14 flowers. In one inflorescence, it is unusual for more than two flowers to open at the same time, so often an inflorescence is found bearing small fruit, open flower and developing floral buds at the same time. Like the leaves, the flowers are also arranged in a regular spiral phyllotaxy. By contrast, the *poc* mutant showed a three-fold increase in the number of flowers per inflorescence with flower numbers ranging from 15-45 (Fig. 4.16). Moreover, most of the flowers bloomed nearly at the same time. The *poc* mutant inflorescence also showed altered phyllotaxy.

At anthesis the normal flower of WT tomato consists of short calyx tube with 6-7 separated sepals and a bright yellow corolla also with a short tube terminating in 5-6

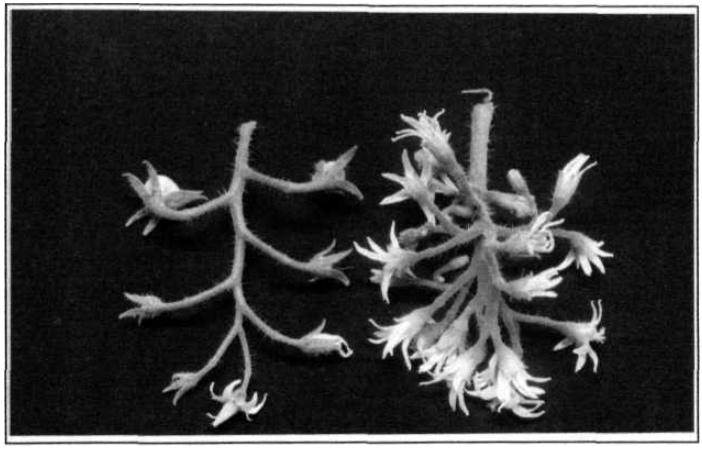


Figure 4.16 Comparison of inflorescences of wild-type and *poc* mutants of tomato. In *poc* mutant the phyllotactic arrangement of flowers is altered. In addition *poc* mutants also produced more number of flowers per inflorescence compared to wild-type. Moreover, all flower in *poc* mutant bloomed almost at the same time.

lobes. The androecium has 6-7 stamens with short filaments and long fully developed bright orange-yellow pubescent anthers, which are laterally joined by lateral hairs to form a hollow cone. Gynoecium consists of two or rarely three fused carpals with a long style terminating in a stigma. The style is contained within the encircling anther cone resulting in perfect determinate flower. The flower shoot ceases growth after formation of last reproductive organs (Fig. 4.17A).

The three classes A, B and C of *poc* mutant classified on the basis of leaf morphology and plant height also show differences in reproductive development. The class A is characterized by more number of sepals in the flower than wild type. About 5% of the flowers of this class show fusion of sepals, whereas, 7.5% of its flowers show sepals with chimeric character of petals in some isolated portions with slightly yellow color resembling petals thereafter termed as petaloid sepals. Class B of *poc* mutant also shows this alteration similar to class A. Interestingly, in the extreme C class 94% of flowers show sepals fusion.

The morphology of petals also shows variation in *poc* mutant. The number of petals increased in all the three classes of *poc* accompanied with decrease in length compared to wild type (Table 4.5, 4.6). The petals of class C are smallest in size compared to other two classes. Additionally, the petals of all the three classes show outgrowth of tissue on the stamen side of the petal and this outgrowth is inserted between stamens resulting in their separation (Fig. 4.17, Table 4.6). However, in some cases the separation of stamens occurs even without this outgrowth, which might perhaps be due to increase in the number of stamens.

In class C petals show sepals like characters with a frequency of 65%. The sepaloid character is manifested by the presence of green tissue in the center of the petals. White green petals were also seen in *poc* C mutant. The above tendency in *poc*-C class of

Figure 4.17 Comparison of morphology of wild-type and *poc* mutant flower.
A- The *poc* mutant shows alteration in the overall morphology of flower compared to wild-type. In the mutant the anther cone is absent and the flower has shorter petals in comparison to wild-type.
B- The sepals of *poc* mutant showing formation of a sector of petal like tissue (petaloid sepals). It can be seen that while the trichomes are present on the wild type sepals they do not form on petal like tissue of the petaloid sepals of the *poc* mutant.
C- The petals of *poc* mutant shows outgrowth on the internal side of the petals towards the stamens.

Table 4.5 Number and length of mature flower organs in WT and *poc* mutant the sample size was 50 flowers from each genotype WT, *poc* A, *poc* B, and *poc* C.

Trait	WT	*poc* **A**	*poc* B	***poc* C**
No of sepals	6.32 + 0.12	7.42 ±0.1	7.26 ±0.14	7.44 ±0.15
No. of petals	5.98 ± 0.099	7.14 ±0.097	6.85 ±0.14	6.92 ±0.14
No. of stamens	6.04 ± 0.09	7.04 ± 0.08	6.84 ±0.13	5.4 ±0.16
No. of carpels	2.12±0.15	2.5 ± 0.08	2.62 ±0.12	Not observed
Length of sepals (mm)	6.84 ± 0.18	5.1 ±0.12	6.06 ±0.15	5.86 ±0.22
Length of petals (mm)	11.32 ±0.21	9.72 ±0.195	10.82 ±0.22	8.48 ± 0.22
Length of stamens (mm)	8.28 ± 0.14	7.38 ± 0.14	7.9 ±0.13	6.08 ± 0.18
Length of carpels (mm)	7.26 ±0.13	6.4 ±0.14	7.56 ±0.12	5.2 ±0.18

Table 4.6 The frequency of abnormalities in flowers of different classes of *poc* mutant.

Phenotypic abnormalities		**Mutant class**		
		poc A	***poc* B**	***poc* C**
Organs fusion	Fusion of sepals	5%	5%	94%
	Lack of fusion of stamens	85%	75%	100%
	Stamens fused to carpels	0%	0%	89.4%
	Petals and stamens fused to carpels	0%	0%	3%
Identity change	Petaloid sepals	7.5 %	7.5%	5.3%
	Out-growth in petals	52.5%	22.5%	60%
	Sepaloid petals	0%	2.5%	65.8%
	White-green petals	0%	2.5%	5.3%

plant to make white green petal is dependent on season. The frequency and intensity of these white green petals increase in summer, and in extreme cases petals appear like green leaves.

The most sensitive floral organ to *poc* mutation is the stamen. In class C of *poc* mutant not only the stamens number is reduced, but also stamen morphology is altered, and they appear as thread like structures, which are totally non-functional. These stamens are fused to the carpels and at the same time no fusion is seen between the stamens (Fig. 4.18).

The *poc* mutant of class A and B also show lack of fusion of stamens to each other. (Fig. 4.18, Table 4.6). In these plants stamens are twisted, distorted, and also variable in size. The plants show male sterility because anthers lack dehiscence. In order to obtain seed setting in these two classes of *poc* mutant female organs are manually pollinated. The highly distorted flower of class C in which all the organs are fused to carpel resulted in fruits with very abnormal external surface, when flowers are manually pollinated by pollen from other source (Fig. 4.19, 4.20).

4.1.12 Fertility estimation

One of the reasons of male sterility in *poc* could be due to loss of viability of pollen. The examination of pollen from mature anthers showed presence of pollen in it, but due to change in morphology of anthers as a result of *poc* mutation, these anthers did not release pollen at anthesis. Therefore we examined *in vitro* pollen viability of *poc* mutant and compared it with wild type pollen. The pollen germination of *poc* mutant was nearly similar to that of the wild type with about 85% germination in wild type and 81% in *poc* mutant. Once the flowers are pollinated manually, the fruit setting was normal in *poc* mutant. On comparing the fruit setting in *poc* mutant and wild type, fruit formation in both A and B classes of *poc* mutant was similar to that of the wild type, while it was 50%

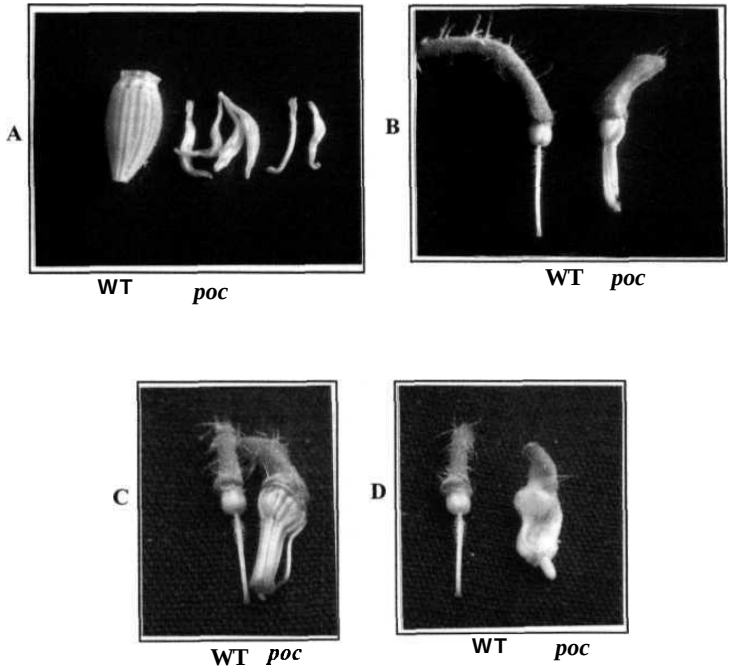


Figure 4.18 Abnormalities in male organs of *poc* mutant flowers

A- The flowers of wild type show a normal anther cone with fused stamens, which are of equal size. On other hand, in *poc* mutant stamens are separated and differ in morphology from wild-type. The length of stamens in *poc* mutant is also different and some of them show twisting.

B- The free wild-type carpels and carpels of *poc* mutant showing fusion of two stamens to carpels.

C- The stamens of *poc* class C showing malformation and separation from each other. The stamen are also fused to the carpels in this mutant. The normal wild type carpels are shown on left for comparison.

D- The *poc* C showing fusion of petals and stamens to carpels. The normal wild-type carpels are shown on left for comparison.

Figure 4.19 The flower of *poc* C mutant showing the fusion of all the four organs sepals, petals, stamens and carpels. The normal wild type flower is shown on right for comparison.

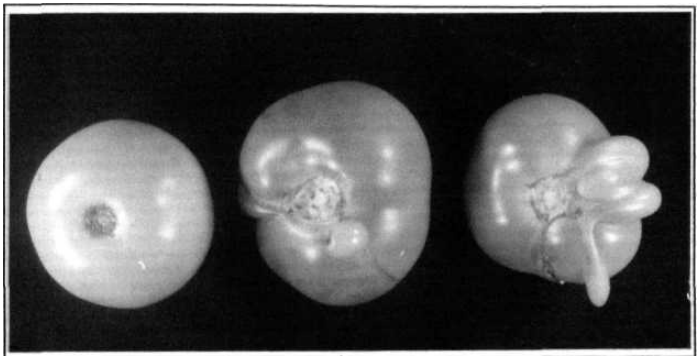


Figure 4.20 The flowers of *poc* C mutant with fusion of all organs to the carpels produce fruits with abnormal lobbing of the external surface. The abnormality in fruit shape arises due to the fusion of tissues of the first three whorls (sepals, petals and stamens) to the carpels. The normal wild-type fruit is shown on left for comparison.

less in class C (Fig.4.21). We observed that the mutants grown in net- and green-houses did not produce any fruits unless they were self-pollinated by hand.

On the other hand, without any manual pollination *poc* mutant grown in the open field produces an average of two fruits compared to 34 fruits in the wild type, the resultant seeds in these fruits are to be tested for cross- pollination. We also compared number of seeds produced per fruit in self-pollinated and manually pollinated fruits. In seed setting test, the female response to *poc* mutation was examined, and it was found that the wild type normally produces more than 116 seeds per fruit compared to selfed mutant which yield only ca. 29 seeds per fruit (Fig.4.22). However, in controlled selfing the wild type produces about 43 seeds per fruit, whereas in the crossed *poc* mutant with wild type as pollen donor the average seed number was 54.84 per fruit (Fig. 4.22).

4.1.13 Transformation of flower into shoot

In *Arabidopsis,* mutants like *leafy,* which determine the fate of the floral meristem, the flowers are replaced by intermediate structures between floral and vegetative shoot. In case of *poc* mutant, the most striking feature is the conversion of fully differentiated open flower into a shoot or an inflorescence (Fig. 4.23, 4.24). The above transformation of flower to shoot or inflorescence was observed in plants grown during March to July at Hyderabad. This conversion is most likely stimulated by the high temperature or may be by the day length in the period of March to June of Hyderabad summer (see appendix for temperature data). However, the above transformation is observed only in *poc* mutant belonging to the class C and rarely in A and B classes.

4.1.14 Embryogenesis

Embryogenesis is very critical stage of the sporophytic life cycle; during this stage the basic body plan of the plant is established. Embryogenesis begins with the process of fertilization. The general embryonic development of tomato has not been described in

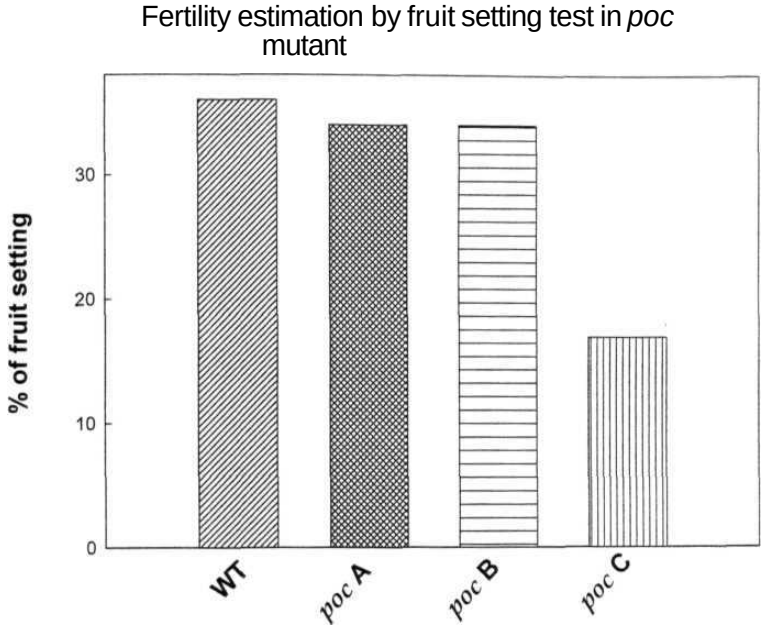


Figure 4.21 The estimation of fertility by comparing fruit setting frequency in wild-type and *poc* mutant after manual pollination. In wild type on manual pollination about 36% flowers produce fruits. In comparison *poc* A and *poc* B mutant flowers produce about 34% fruits showing nearly equal success to wild-type. On other hand, on hand pollination flowers of *poc* C class mutant produce only 17% fruits.

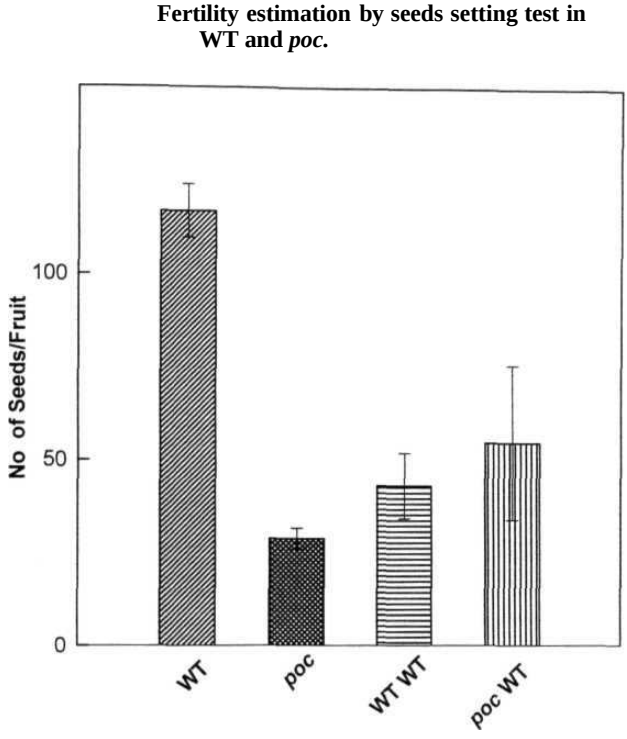


Figure 4.22 Comparison of seed setting in wild type and *poc* mutant after natural and manual pollination. The naturally pollinated wild-type flowers produce large number of seeds in comparison to manually pollinated *poc* mutant. However, manually pollinated wild-type with wild-type pollen and *poc* mutant pollinated with wild-type pollen produces nearly the same number of seeds. This data indicates that mutant showed normal seed setting whether it was selfed or cross pollinated.

Figure 4.23 Flowers showing formation of inflorescence from a fully differentiated flower of *poc* mutant. The fully differentiated flower of *poc* mutant with sepals, petals, malformed stamens and normal carpels produce inflorescence shoot from within the flower. The *poc* phenotype is clearly manifested as evident by large number of flowers in the inflorescence and malformed leaves.

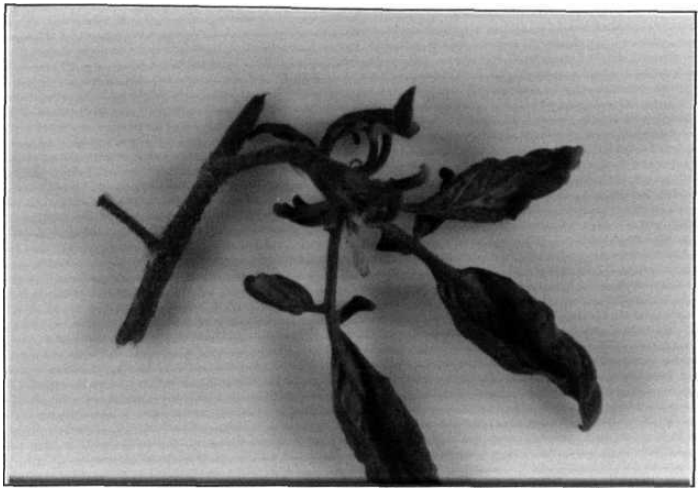


Figure 4.24 A fully differentiated flower of *poc* mutant showing formation of shoot. The shoot shows both flowers and leaves. However, the leaves are larger and number of flowers are less .

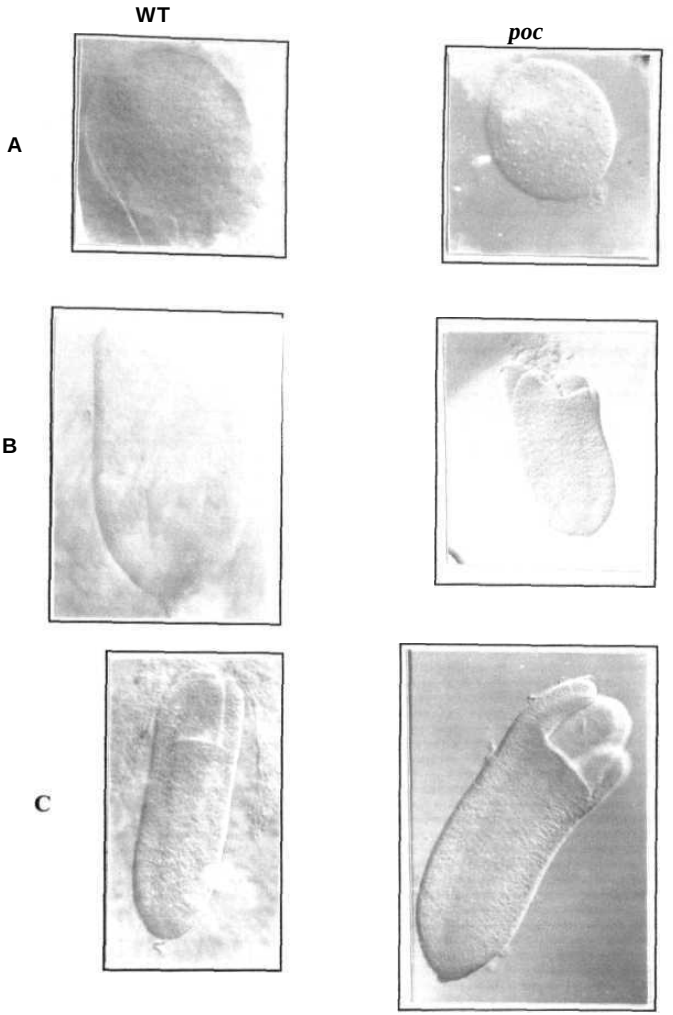


Figure 4.25 Embrogenesis in wild-type and *poc* mutant of tomato. (A) Wild-type and *poc* mutant embryos at globular-stage ten days after pollination, no significant differences were found between wild-type and mutant (magnification 40X). (B) Wild-type embryo fifteen days after pollination at heart stage (magnification 20X), *poc* mutant embryo 16 day after pollination, (note that the magnification is only 10X compared to that of the wild type which is 20X at this stage), the mutant embryo is thicker than the wild type. (C) Wild-type embryo eighteen days after pollination at the torpedo-stage, **the** mutant embryo 21 days after pollination. Note that the thicker radial axis of the mutant and the short cotyledons which is nearly one-third of the embryo in the wild-type, whereas in *poc* mutant they are nearly one-fourth of the embryo (magnification is 10X for both,WT and poc mutant).

literature in details. To investigate the sequence of normal embryogenesis process in tomato, flowers were manually pollinated and fruits were harvested at defined time intervals of different developmental stages. The examination of wild type embryos 10 days after pollination showed the globular stage embryo, which lasted till 14 days after pollination. Thereafter, heart stage was observed at 15 days first as early heart stage and at 16 days or in some cases at 17 days as late heart stage. It was followed by the torpedo stage at 17-19 days after pollination (Fig.4 25). The folding of cotyledons was seen 21 days after pollination in wild type. The embryo reaches maturity approximately 35 days after pollination.

In the *poc* mutant, flowers were manually pollinated and similar to wild-type embryo development was followed from 10 days after pollination. The *poc* mutant also showed globular stage embryo 10 days after pollination, no significant differences were observed in size or morphology of the *poc* embryo from that of the wild type at this stage. However, the *poc* mutant showed abnormality in transition phase from globular to heart stage. Firstly, transition from globular to heart stage was not seen in *poc* mutant due to enlargement of embryo. Secondly, embryo showed initiation of 3-4 cotyledon and clear heart stage was not seen. Rather than heart stage, a pseudo-heart stage was observed 16 days after pollination. On comparative scale *poc* mutant embryo is bigger in size than wild-type and the radial axis of the embryo was 1.8 times thicker than the wild type embryo. The embryo development showed a clear torpedo stage after 21 days after pollination. The cotyledon was one third of the length of the embryo at torpedo stage in wild type while it was much shorter in the *poc* mutant at this stage (Fig. 4.25).

4.2 Isolation and characterization of *npc* mutant

4.2.1 Isolation of *n*arrow *p*etioleless cotyledon mutant *(npc)*

The M_2 population was screened for narrow *p*etioleless cotyledon *(npc)* mutants among seedling grown under continuous light for one week after germination. In a population of 50,000 seedlings seventy-one seedlings showing narrow *p*etioleless cotyledons were isolated. The isolated putative mutant seedlings were grown to maturity in net house. Out of 71, only 31 plants survived till maturity and produced seeds for M_3 generation. In M_3 generation, on examination of phenotype, only three lines showed the *npc* phenotype and these lines were retained. The *npc* phenotype is at present in M_7 generation in our lab.

4.2.2 Genetic characterization

4.2.2.1 Segregation analysis

The reciprocal crosses were made using the *npc* mutant lines as female as well as male parent. In both crosses, the F_1 phenotype was similar to the wild type. The F_2 generation segregated into typical Mendelian 3:1 ratio. However, sometimes the ratio of *npc* mutant is less than 1. The above reduction in ratio of mutant seedling obviously results from a strong reduction in germination rate in *npc* mutant. The seeds of this mutant as mentioned above show delayed germination and also a large percentage of seeds do not germinate in comparison to wild-type (Table 4.7).

In addition, test cross was done to further confirm the single gene inheritance of this mutation. The F_1 generation of test-cross shows typical 1:1 wild-type to mutant phenotype, indicating monogenic recessive nature of this mutation.

4.2.2.2 Complementation analysis

In this study we isolated three lines of *npc* mutants. To determine the number of alleles controlling the *npc* phenotype among these lines, crosses between the three lines

Table 4.7 Genetic segregation in *n*arrow *p*etioleless cotyledon *(npc)* mutants of tomato.

Crosses	Generation	**No.** of seedlings	**Seedling Phenotype**		**P**
			Wild Type	***npc***	
WT x *npc1-1*	**F1**	87	**87**	**0**	
	F2	888	707	181	0.01
WT x *npc]-2*	**F1**	37	**37**	**0**	
	F2	129	103	**26**	0.02
WT x *npc]-3*	**F1**	37	**37**	**0**	
	F2	330	255	**75**	**0.2 - 0.5**
npc]-] x WT	**F1**	**24**	**24**	**0**	
	F2	146	111	**35**	0.95
npc 1-3 x WT	**F1**	15	**15**	**0**	
	F2	456	356	100	**0.2 - 0.5**
NPC\lnpcl-l x *npcl-1/npcl-]*	**F1**	133	**67**	**66**	0.99

was carried out. The F_1 generation showed only the mutant phenotype indicating that all the three lines likely represent one locus. In view of this finding, the lines were designated as *npc1-1*, *npc1-2 and npc1-3* (Table 4.8). The complementation analysis of *npc* mutant with other narrow cotyledon *(nc)* mutant, which were obtained from TGRC collection (LA, 3178) showed the wild type phenotype in F_1 indicating that these two mutants are non-allelic. Moreover, the narrow cotyledon phenotype *of npc* mutant is more extreme than *nc* mutant. In addition, narrow cotyledon mutant (nc) have normal petiole.

4.2.3 Expression *of npc* phenotype is age dependent

The freshly harvested *npc* seeds on germination generate seedlings, which are phenotypically similar to wild-type. However, if *npc* seeds are germinated three months after harvest the seedlings show distinct phenotype with narrow petioleless cotyledons. Moreover, germination of older *npc* seeds manifest much stronger phenotype accompanied with strong reduction in seed germination. The *npc* seeds older than one year are difficult to germinate and thus, the mutant is maintained by annual multiplication. Thus, it may be concluded that the *npc* phenotype manifested itself in an age dependent manner.

4.2.4 Germination

Germination in *npc* mutant was examined over a period of one year from the harvest of seeds. The results showed that the mutant's germination behavior was similar to the cotyledon expansion phenotype. There was a tendency of slow and reduced germination, which correlated with advancement in the age of the seeds. The three months old *npc* mutant seeds reach 50% of germination by 14^{th} day after sowing and only 62% of seeds germinated even after three weeks. On the other hand, in the one-year-old mutant seeds only 16.35% seeds showed germination, even after three weeks. In comparison, three months and one year old wild type seeds reached 50% germination by

Table 4.8 Complementation analysis of *npc* mutant lines and *nc* mutant.

Line	Generation	No. of seedlings	Seedling Phenotype		Mutant (%)
			Wild Type	Mutant	
nc* x *npcl-1	**F_1**	**63**	**63**	**0**	**0%**
npcl-1* x *npcl-2	**F_1**	**66**	**0**	**66**	100%
npcl-1* x npc1-*3	**F_1**	**47**	**0**	**47**	100%
npcl-2* x *npc1-3	**F_1**	**23**	**0**	**23**	100%

the fourth and fifth day after sowing, respectively. There was slight reduction in germination percentage of the one-year-old seeds of wild-type, which was 82.44%, compared to 87.5% of the three months old wild type seeds. The data on seed germination, which was quantified over a period of three weeks, are presented in Table 4.9.

4.2.5 Interaction with *au* mutant

In epigeally germinating dicot seedlings, light is one of major factor controlling cotyledon expansion (Chory, 1997). Since light is perceived by plant photoreceptor(s), which in turn regulates light mediated cotyledon expansion, it is likely that loss of photoreceptor(s) or one of the signal chain components may cause *npc* phenotype. To test this possibility, the *npc* mutant was crossed with *au* mutant, which is phytochrome chromophore deficient and is deficient in all phytochromes. The double mutant *npc au* unexpectedly showed a reduction in hypocotyl elongation in light, which is usually longer in au mutant indicting that npc mutant, is epistatic to *au* in controlling hypocotyl elongation. At the same time, double mutant showed the cotyledon phenotype of *npc* mutant (Fig. 4.26, 4.27).

4.2.6 Interaction with *poc* mutant

The *npc* homozygous mutant was crossed to the homozygous *poc* mutant. The F_1 progeny showed wild-type phenotype. The F_2 generation segregated to *npc* and *poc* phenotype and no intermediate phenotypes could be detected in F_2 While we expected to detect double mutant with *npc poc* phenotype in F_2, the phenotype could not be easily discerned. Thereafter, each of F_2 population of *npc* and *poc* mutant was screened in F_3 for double mutant phenotype. The double mutant phenotype was obtained in F_3 progeny in population of both *npc* and *poc* mutant. The double mutant showed the polycotyledon phenotype of *poc* and the narrow cotyledon of *npc* in the seedling stage (Fig. 4.28).

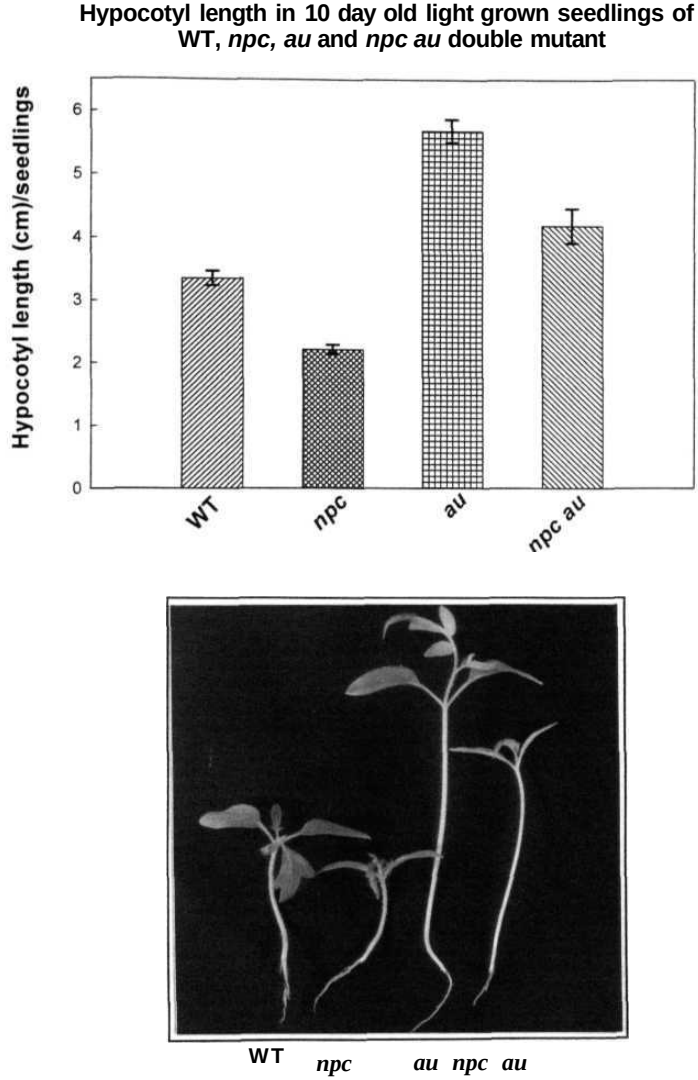


Figure 4.26 Comparison of hypocotyl length of *npc* mutant with wild-type, *au* mutant and *npc au* double mutant. The *npc* mutant shows shorter hypocotyl length than wild type whereas *au* shows elongated hypocotyls. The effect of *au* mutation on hypocotyl elongation was partially suppressed by *npc* mution which shows intermediate length of hypocotyl in double mutant.

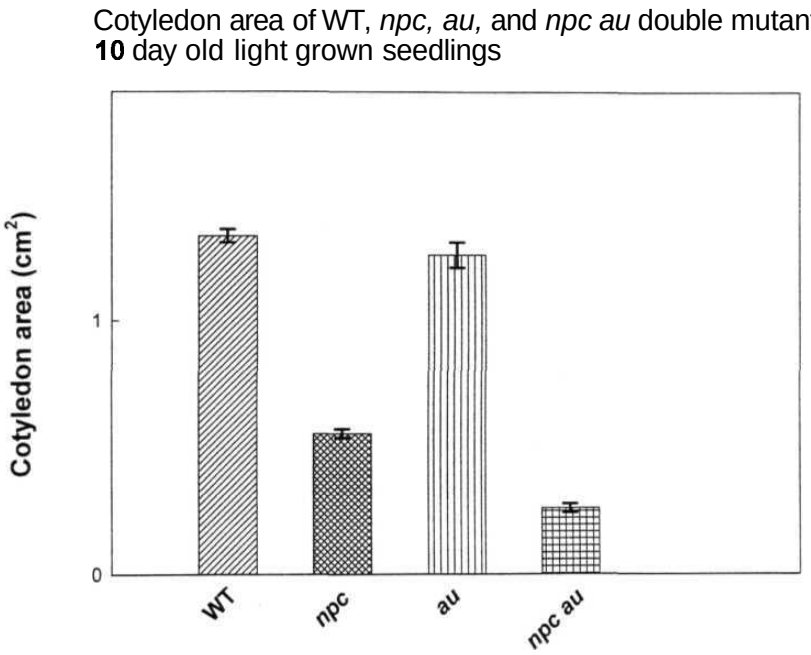


Figure 4.27 Comparison of cotyledon area of wild-type (WT), *npc* mutant, *au* mutant and *npc au* double mutant. The *npc* mutant of tomato shows significant reduction in cotyledon area compared to wild-type. The *npc au* double mutant also showed reduction in cotyledon area similar to *npc*. The values in figure represent area of a single cotyledon.

Figure 4.28 Phenotype of wild-type, *npc*, *poc,* and double mutant of *npc poc*. The photograph show the wild-type (top) with typical two cotyledons, *npc* mutant (left) with two narrow cotyledons, *poc* mutant with four cotyledons(center). The *npc poc* double mutant seedlings (bottom) show the polycotyledon phenotype of *poc* mutant and the narrow cotyledons of *npc* mutant.

Table 4.9 The percentage of germination after three weeks and days to 50% germination was examined in three months and one year old stored seeds batches of wild-type and *npc* mutant. The percentages are an average of three replicates for each batch of seeds.

Line	**Germination (%). After three weeks of sowing in dark.**	**Days needed to reach 50% germination**
Wild type (three months old seeds)	87.5%	**4**
Wild type (one year old seeds)	82.44%	**5**
***npc* mutant (three months after harvest)**	62.76%	**14**
***npc* mutant (one year after harvest)**	16.35%	

During subsequent vegetative and reproductive development the double mutant resembled *poc* in all aspects, and on basis of morphology it could not be distinguished from the single mutant of *poc*.

4.2.7 Background effect

The role of genetic background in eliciting *npc* phenotype was examined. The *npc* mutant was crossed with wild-type cultivar Moneymaker. The F_1 generation showed normal phenotype as that of wild type. In addition, the F_2 segregation showed a normal segregation ratio of 3:1. Moreover, examination of morphology of *npc* mutant showed that mutant in money maker background has the same morphological characters and pattern as observed in Ailsa Craig background. These results indicate that expression of *npc* mutant is background independent. Moreover in the money maker background too, the phenotype appears only after storage of seeds for few months.

4.2.8 Phenotypic Characterization *of* *npc* mutant

Compared to the *poc* mutant, *npc* mutant showed less pleiotropic effect on the plant phenotype. The following morphological parameters were investigated in *npc1-1* mutant, as all the three lines where allelic and no differences were observed between them.

4.2.8.1 Cotyledon

Comparative information about cotyledon area and other cotyledon parameters of both the *npc* mutant and of the wild type was obtained by analyzing scanned images of cotyledon using Image Tool Program. As described earlier the freshly harvested *npc* mutant seeds germinate like the wild type producing seedling with cotyledon shape and area similar to wild type. However, in seedlings grown after three month of harvest of seeds the cotyledon area of the *npc* mutant was reduced by more than 50% compared to the wild- type. The reduction in cotyledon area of *npc* mutant followed an age dependent

pattern reducing cotyledon area to about 0.11 cm^2 after one year of harvest of seeds (Fig. 4.29).

As a result of the reduction in the cotyledon area the perimeter of the cotyledon is also reduced in a similar fashion. While the cotyledon perimeter in the wild type is about 5.339 cm, it is reduced to 4.09 cm in *npc* mutant. This represents about 24% reduction in perimeter compared to wild type. In fact after one year of storage *of npc* seeds, cotyledon perimeter in *npc* seedlings was 1.717±0.04 cm, which was 67.8% less than the wild type (Fig. 4.29, Table 4.10).

Similar to cotyledon area and perimeter, the *npc* mutant also shows reduction in the length of the cotyledon on storage. The length of the *npc* cotyledon after three months of harvest was about 1.786 cm compared to the wild type which was 2.149 cm indicating a 16.89% reduction in length. Interestingly after one year of harvest the length of *npc* cotyledon was only 0.735 cm, showing a reduction by 65%.

The width of cotyledon in *npc* mutant also showed a similar reduction after three months of harvest while it was 0.389 cm in *npc* mutant in the wild type it was 0.8474 cm. After one year of storage it was 0.1598 cm in *npc* mutant (Fig. 4.29,Table 4.10).

4.2.8.2 Epidermal cells of the cotyledons.

The abaxial (lower side) epidermal cells of the *npc* mutant cotyledon showed a different shape compared to the wild type. These cells showed very small lateral protrusions compared to the wild type, which shows long protrusions in all the directions (Fig. 4.30).

4.2.9 Hypocotyl elongation

Seedlings of dicot plants can follow two developmental strategies depending on the surrounding light conditions. In the dark, dicotyledonous seedlings adopt morphology and physiology adapted to subterranean growth conditions called skotomorphogenesis:

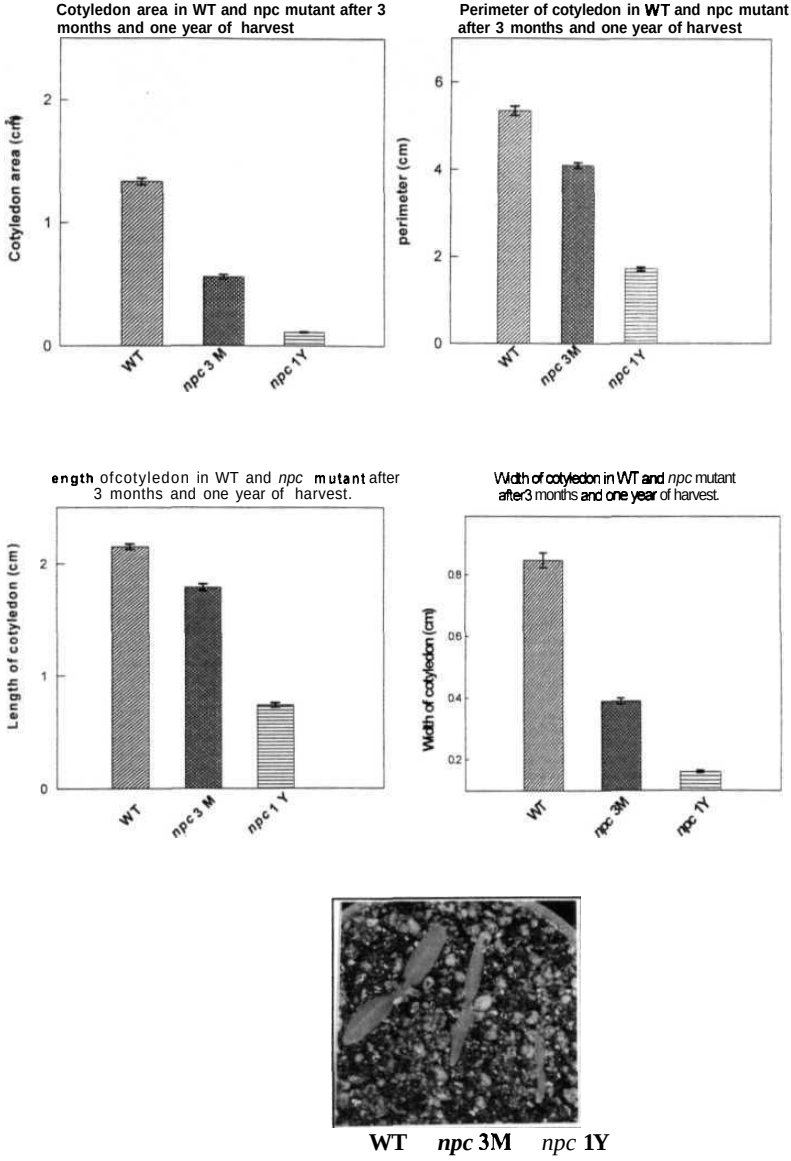
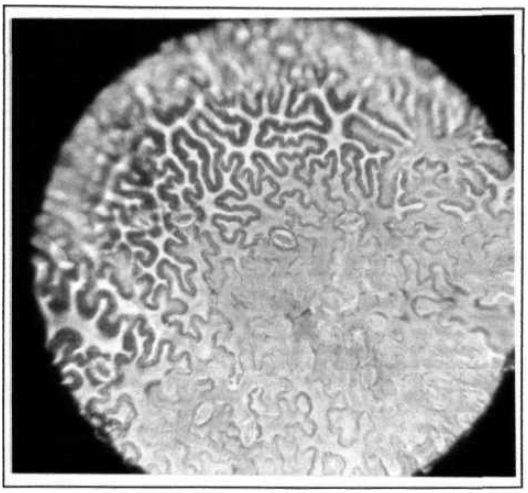


Figure 4.29 Comparison of cotyledon area, perimeter, length and width in wild type and *npc* mutant, seeds were germinated three months (3M) and one year (1Y) after harvest. A total of 30 seedling were analyzed for above parameters. The value in graph represents mean value for a single cotyledon.

A



B

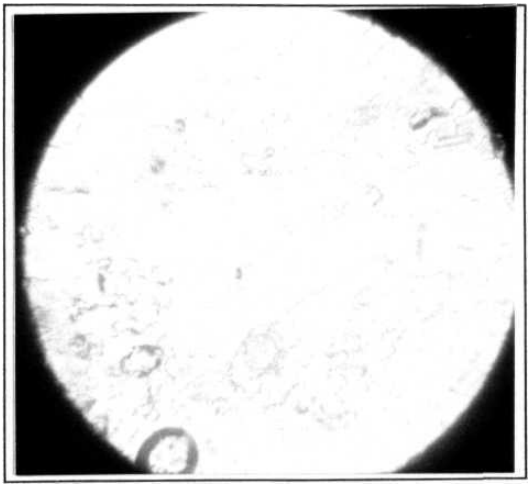


Figure 4.30 The comparison of epidermal cells shape on abaxial (lower) side of WT (A) and *npc* (B) mutant cotyledons. Note the difference in lateral protrusions. The pink color is due to the anthocyanin which is abundant in WT.

Table 4.10 The cotyledon area, perimeter, length and width of wild-type and *npc* mutant three months and one year after harvest (n = 30 seedlings). The values in tables represent mean obtained for single cotyledon.

Parameter	WT	***npc*** **(3 months from harvest)**	*npc* **(one year from harvest)**
Cotyledon area (cm^2)	1.33 ±0.27	0.56 ± 0.02	0.11 ±0.004
Perimeter (cm)	5.339 ± 0.106	4.09 ± 0.666	1.717 ±0.04
Length (cm)	2.149 + 0.024	1.786 ±0.030	0.739 ± 0.020
Width **(cm)**	0.847 ± 0.0245	0.398 ± 0.004	0.1598 ±0.004

cotyledons do not expand, leaf and chloroplast development remain inhibited, whereas the hypocotyl forms an apical hook and undergoes rapid elongation. Upon exposure to light an alternative developmental program, photomorphogenesis is induced. Seedlings adopt a so-called de-etiolated phenotype: the apical hook opens, cotyledons expand, the chloroplast and leaves develop, and hypocotyl growth is inhibited. The photomorphogenic pathways appear to be repressed in the absence of light, which is regulated by negative elements identified in *Arabidopsis* (Howell, 1998). The *npc* mutant departs from the normal development of the wild type, the dark grown seedling of *npc* showed reduction in the hypocotyl elongation in dark in an age dependent manner. After three month of harvest, the hypocotyl of dark grown seedling showed significant reduction in hypocotyl length. In fact, one-year old dark grown *npc* seedling showed hypocotyls length nearly similar to light grown wild-type and has an open hypocotyls hook (Fig. 4.31).

4.2.10 Splitting of shoot

Normally tomato plant possesses only one axis, which give rise to branches and leaves. However, many *npc* plants showed splitting of shoot into two axis after three weeks either at the first or second node. In a population of about 500 one month old *npc* plants, as many as 124 plants showed splitting of axis into two identical shoots and in some rare cases even three identical shoots. The splitted shoots grow normally showing identical morphology and produce flower and fruit normally.

4.2.11 Altered phyllotaxy

The *npc* mutant showed alteration in phyllotaxy in about 30% of the seedlings. However, this alteration was mostly seen in the first internode and was rarely seen in others internodes.

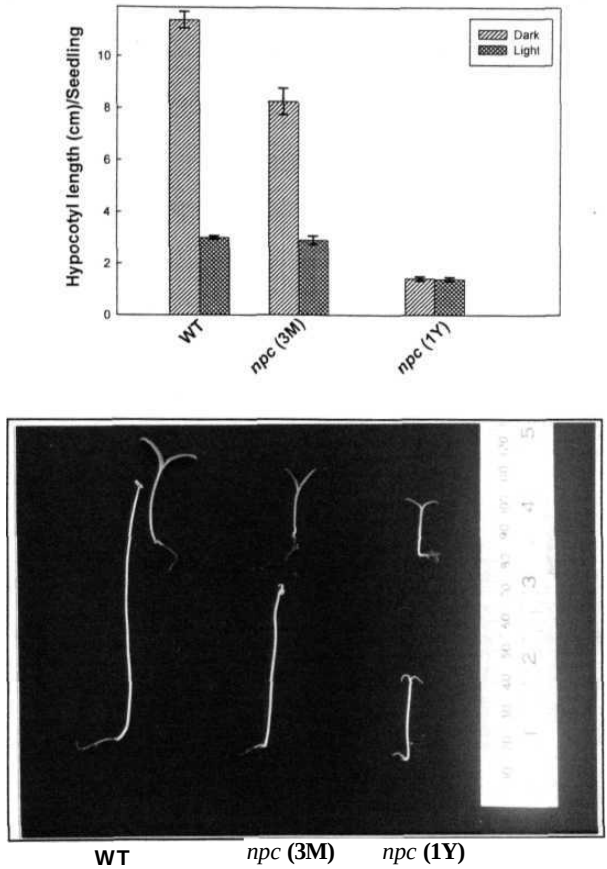


Figure 4.31 Comparison of hypocotyls length of nine days old light grown (upper) and dark grown (lower) wild-type and *npc* mutant seedlings. The dark grown seedling of wild-type show typical elongated hypocotyl compared to the light grown control. On the other hand, *npc* mutant seedlings show reduction in hypocotyl length in both light and dark grown seedlings. The reduction in hypoctyl length of seedling was correlated to the date of harvest of seeds. The *npc* seedlings germinated after one year(l Y) of harvest show a severe reduction in hypocotyl length in dark grown seedlings and also of light grown seedlings.

5. Discussion

The presence of two cotyledons is the basic characteristic of dicots, forming the basis on which this group of plants is named. The information about genetic and biochemical basis of the dicotyledon phenotype is still not fully available. In the wild type population of tomato, both in AC and MM background, no spontaneous mutant showing variation in the cotyledon number was observed during the course of this study. In this study genetic and morphological analysis of the *poc* and *npc* mutant have been carried out. The study indicates that the *poc* loci may represent a new class of gene regulating cotyledon number in the tomato and *npc* mutant may represent new class of mutants controlling germination and cotyledons expansion in age dependent manner.

5.1 Genetic analysis of *poc* mutant

5.1.1 *poc* is a recessive nuclear mutation

The *poc* mutants were crossed with the parental wild type background and the resultant progeny was analyzed for the pattern of inheritance and segregation of the mutant phenotype. The segregation and inheritance analysis of *poc* mutant revealed that each of the nine *poc* lines is defective in a single locus. The mutated gene was functionally recessive to the wild type and followed Mendelian segregation pattern indicating that it is a single nuclear gene. The notion that *poc* is a recessive, single locus gene was also confirmed by data from test-cross analysis.

Complementation analysis of nine lines of *poc* mutant showed that all these lines were allelic to each other, representing only one group. Based on this observation these lines of *poc* were classified in one group and were designated *poc1-1* through *poc1-9*. The isolation of multiple lines of same mutation in population resulted from our screening of *poc* mutant from a M_2 seed stock, which was harvested in bulk. Since we used this bulk pool of M_2 seeds and isolated *poc* mutant, these lines had very similar phenotype, points

to the possibility that the nine *poc* lines used in this study are the progeny of a single M, plant.

5.2 Seedling phenotype of *poc* mutant

5.2.1 A transposon-tagged line of tomato shows seedling phenotype similar to *poc*

There are isolated reports of multiple cotyledons in the tomato. As early as 1952 it was reported as a part of G. Reynard thesis (1952), which reported that the polycot trait was inherited quantitatively, however, the homozygous lines inheriting multiple cotyledons were not derived. It was followed by a report by Rick *et al.* (1992) who characterized TGRC line LA 2896; however, this line is in a determinate stock, a factor that might affect expression of certain morphological features. The mutation is reported to be highly pleiotropic in nature and is located on short arm of chromosome 9. Moreover, the characteristics of above mutant have been reported only in a very short report, which is meant for circulation among the Tomato Genetics Cooperative members.

The occurrence of multiple cotyledons were also observed in *dem* mutant, a transposon tagged line carrying maize *Ds* in tomato. The *dem* mutant is recessive and mutant progeny occurred at a frequency of 10-15%. The *DEM* gene encodes for a novel protein with significant homology to yeast YNV2, a hypothetical protein with unknown function in yeast. In the *dem* mutant, seedlings phenotype ranged from monocots to tetracots. A comparison of *dem* and *poc* phenotype showed much dissimilarity between two mutants. First, in the *poc* population no monocot seedlings were observed, while *dem* showed a minor percent of monocot seedlings. Second, the *dem* mutant had no functional shoot or root meristems (Keddie *et al.*, 1998), therefore the seedling growth was terminated, whereas *poc* has functional meristems and completes its life cycle normally as homozygote. The examination of the vegetative and reproductive morphology of plants

showed that the *poc* mutation is strongly pleiotropic, affecting all the stages of plant development.

The question that *poc* mutant isolated in the present study is allelic to the other mutants reported above needs the complementation tests. We have recently obtained the seeds of both *pct* and *dem* mutants and the complementation analysis would be done this season at Hyderabad.

5.2.2 *poc* mutant shows high penetrance and variable expressivity of multiple cotyledon phenotype

The most prominent character of *poc* mutant is occurrence of multiple cotyledons in the seedlings. Several mutants with multiple cotyledons have been reported in *Arabidopsis.* A comparison between tomato and *Arabidopsis* shows many features, which are novel to the *poc* mutant. The *Arabidopsis* cotyledon mutants show low penetrance, with mutant seedlings ranging between 20-45% even in a homozygous population of mutants, for example, the *amp1* mutant of *Arabidopsis* showed only 20-30% non-dicot seedlings (Chaudhury *et al.*, 1993). In contrast, in case of *poc* the proportion of polycotyledon seedlings was about 98.5% in the successive generations. Similarly, the *pinoid* mutant of *Arabidopsis* shows mutant phenotype only in 20-50% of seedlings and *pin* mutant shows a penetrance of about 30-45% (Bennett *et al.*, 1995). To the best of our information, the *poc* mutant of tomato is the only one that shows high degree of penetrance for the multiple cotyledon phenotype.

The expressivity of *poc* gene varied within the mutant population and mutant phenotypes ranged from dicot to tetracot with intermediates showing partial splitting of the cotyledon into two. The expressivity of gene was in the order of nearly 50% tetracot, followed by about 35% tricot, and the remaining showed dicot or tricot phenotype with fused cotyledons. Similar variations in the expressivity were also found in *Arabidopsis*

mutants. In *pinoid* mutant of *Arabidopsis,* among abnormal seedlings, the majority were tricots (80%), and remaining were dicots and a minor percent were tetracot and even monocot. The auxin polar transport defective *pin-formed (pin1-1)* mutant of *Arabidopsis* on the other hand, showed majority of the mutant seedlings with single cotyledon (60%), and remaining with fused cotyledons or abnormal dicots and also cup-shaped cotyledon were found (Bennett *et al,* 1995). In *amp]* mutant of *Arabidopsis* the single cotyledon, dicotyledons and polycotyledons were detected (Chaudhury *et al.*, 1993). In contrast, the *poc* mutant never showed any monocot seedlings or cotyledons fused to form funnel, which was observed for *amp* and *pin1* mutants *of Arabidopsis.*

5.2.3 The extra cotyledons of *poc* mutants are true cotyledons

In the dicot seedlings, the seedling development is accompanied with expansion of two cotyledons which are formed during embryogenesis, which is followed by expansion of leaf pair, the primordia for which too forms during embryogenesis. However, in few mutants such as *xtc1, xtc2,* and *amp1* the mutation causes the transformation of the first pair of seedling leaves into 'extra cotyledons'. These transformed leaves show few characteristics of embryonic cotyledons, such as reduced trichome numbers and the presence of lipid and protein bodies (Conway and Poethig, 1997). In fact, these mutants bear resemblance to precociously germinated immature *Brassica napus* embryos, which shows conversion of leaf primordia into cotyledon-like organs that lack trichomes and express embryo-specific genes (Fernandez, 1997) Apparently, precocious activation of the shoot apical meristems during embryogenesis causes the observed extra cotyledon phenotypes of the mutants and the cultured embryos. A close observation of *poc* mutant ruled out the possibility of the transformation of leaf into cotyledon. The *poc* mutant in addition to supernumerary cotyledon also shows normal first pair of leaf. All cotyledons have same morphological characters and show no evidence of transformation of leaf in to

cotyledon. Moreover, the analysis of embryo development in mutant (See section 4.1.7) also do not show any precocious activation of the SAM, which is evident by the fact that, all the cotyledons arises at same node and therefore can easily be distinguished from transformed leaf pair which arises at a different point on shoot axis.

5.2.4 The *poc* mutation affects the root and hypocotyl elongation in seedlings

The *poc* mutation has a strong pleiotropic effect on plant development right from seedlings stage, as evident by the observation that the length of the primary root of *poc* mutant grown in vermiculate is significantly less than that of the wild type control. Likewise, the etiolated seedlings of *poc* mutant also show reduction in hypocotyls length compared to the wild type. It is believed that both the root and hypocotyl elongation are controlled by regulation of the relative level of auxin and cytokinins. In tomato, the need for auxin to regulate the root and hypocotyl elongation is illustrated by single-gene, recessive *diageotropica (dgt)* mutant of tomato exhibiting pleiotropic effects including reduction in the root and shoot growth.

The *dgt* phenotype is basically caused by the reduced sensitivity of mutant tissues to auxin (Muday *et al*., 1995). Since the hypocotyls and roots of etiolated *dgt* seedlings are shorter than those of wild-type seedlings (Coenen and Lomax, 1998) due to lack of responsiveness to auxin, the reduction in root and hypocotyl length of *poc* may similarly result from perturbation in auxin action/metabolism and or polar transport. Alternately, the reduction in root length can also result from elevation of auxin concentration, as seen in wild-type tomato where application of auxin to seedlings reduces root elongation. However, in later case auxin-mediated reduction in root elongation was accompanied with induction of lateral roots (Muday and Haworth, 1994).

The question whether or not the *poc* mutant is defective in auxin level/action was examined by studying the induction of adventitious roots after excising primary root from

the seedling hypocotyl. It was observed that both wild type and *poc* generated the adventitious roots on excised hypocotyl grown on agar, indicating that *poc* mutant retains the normal pathway for wound induced root generation. Interestingly, *poc* mutant formed root on the agar media containing kinetin, whereas the root formation was inhibited by kinetin in the wild type. These results indicate that the *poc* mutation causes reduction in the cytokinin sensitivity at least for formation of roots after wounding of tissue. It is likely that initiation of root in *poc* mutant is because the cytokinin is unable to antagonize endogenous auxin, whereas in wild type it does so leading to formation of callus at cut end of hypocotyls instead of roots.

The interaction between cytokinin and auxin is a complex phenomenon. The investigations on the interdependency of auxin and cytokinin signaling using tomato *dgt* mutant showed that etiolated *dgt* seedlings display cross-resistance to cytokinin with respect to root elongation (Coenen and Lomax, 1998). At the same time, cytokinin effects on hypocotyl growth and ethylene synthesis in *dgt* seedlings were not impaired indicating that auxin and cytokinin may regulate plant growth through both shared and separate signaling pathways (Coenen and Lomax, 1998).

The observed reduction in hypocotyl elongation in *poc* seedlings similarly could result from the defect in auxin action/metabolism. The reduction in the hypocotyl elongation in darkness has been seen in several photomorphogenic mutants of *Arabidopsis* belonging to *COP/DET/FUS* gene family. A link between auxin signaling and hypocotyl elongation is provided by cloning of *SHY2* gene. The *shy2* mutation suppresses the long-hypocotyl phenotype of *hy2* mutant of *Arabidopsis* and this dominant mutation resides in the auxin-induced gene IAA3, indicating a role link between hypocotyl elongation and auxin (Tian and Reed, 1999). The reduction in hypocotyl elongation is also seen in *ampl* mutant of *Arabidopsis* showing a multiple cotyledon

phenotype (Chaudhury *et al.,* 1993) and *dumpy* (*dpy*) mutant of tomato, which is brassinosteroid deficient (Koka *et al.,* 2000). The phenotype of etiolated *poc* seedling is similar to wild type showing a prominent apical hook and closed cotyledons except reduction in hypocotyl length, which was not as severe as in *dumpy*. Moreover, the phenotype of the mature *poc* plants and *dumpy* are very different, discounting the possibility of brassinosteroid deficiency causing hypocotyl reduction in *poc* mutant. One of the attractive possibilities is that the reduction in hypocotyls elongation of dark grown *poc* mutant could be due to defect in cell elongation, which in turn is mediated by levels of plant hormones.

5.2.5 Effect of *au* and *hp-1* gene on *poc* phenotype

The double mutants are very valuable to study interaction, hierarchy and relationship between different genes. The tomato *aurea* mutant, deficient in phytochrome shows elongated hypocotyls in the light-grown seedlings, whereas *hp-1* mutant shows exaggerated phytochrome responses. (Sharma and Kendrick, 1999). The *poc* mutant was crossed with these mutants to examine the phenotype of the double mutants particularly at the seedling stage. The double mutant of *poc au* showed the phenotype of both single mutants, long hypocotyls of *au* and polycotyledon of *poc*. In case of mature plant the double mutant shows the phenotype of *poc* but retains golden color of leaf, characteristic of *au*. However, in case of *amp]* mutant of *Arabidopsis* the double mutant with phytochrome deficient *hy2* mutation showed intermediate phenotype between *amp]* and *hy2* indicating that *hy2* is perhaps needed for *amp1* expression (Chaudhury *et al.,* 1993). In *poc au* double mutant it is evident that *au* and *poc* genes are independent from each other in their expression. Similarly, the *poc hp-1* double mutant showed the phenotype of respective single mutants indicating no interaction. The analysis of these double mutants

indicated that *poc* phenotype results from endogenous changes and the environmental factor light plays no role in regulation of *poc* phenotype.

5.3 Vegetative development in *poc* mutant

In tomato, the shoot development is sympodial and consists of two distinct phases; in the first phase, which represents vegetative development, the SAM forms metamers consisting of an elongated internode, a leaf, and a bud. Subsequently, after the formation of 7-11 metamers, the SAM is transformed into an inflorescence meristem (IM). In second phase of shoot development, while the IM give rise to inflorescence, the bud in the axil of the youngest leaf primordium grows vigorously gives SAM. This bud displaces the developing inflorescence to a lateral position and transfers its subtending leaf to an elevated position above the inflorescence (Schmitz and Theres, 1999). However, the SAM of the sympodial shoot is transformed to an IM after the formation of three leaves, while main axis is again continued by growth of bud in the axil of the youngest leaf primordium. The *poc* mutant shows strong pleiotropic effect on several aspects of vegetative and reproductive development through out the life cycle of plant.

5.3.1 *poc* mutation alters phyllotaxy with low penetrance

The arrangement of leaves on tomato follows spiral pattern with only one leaf at each node and each successive leaf is positioned at 137.5 degrees from the last leaf. The *poc* mutant showed altered phyllotaxy in the first internode, but this character appeared with low penetrance with only 30% of plants showing this phenotype. The genetic basis of the phyllotaxy particularly the mechanism that triggers leaf initiation and pre-determined positions or angle remains to be established. In most mutants, changes in phyllotaxy results from pleiotropic effect of mutation meaning that mutation regulates phyllotaxy indirectly. Only in case of maize *abphyl1* mutant, a specific phyllotactic alteration is observed. In this mutant leaf position changes from wild type pattern, i.e.

initiation of leaves singly, alternating from one side to the other, to a different pattern where mutant plant initiates leaves in opposite pairs, a pattern called as decussate phyllotaxy. Morphologically the mutant shows a SAM larger in size and this enlargement of SAM occurs during embryogenesis, prior to true leaf initiation. The enlargement of SAM is associated with larger expression of the homeobox gene *KNOTTED1* (Jackson and Hake, 1999).

Most information on regulation of phyllotaxy has been gathered from surgical and inhibitor experiments (Steeves and Sussex, 1989; Lyndon, 1998). These experiments suggested that either a chemical or a physical stimulus determines the leaf position and the angle of leaf initiation. First hypothesis assumes that the biophysical forces and tissue mechanism together promote morphogenesis through bulking of the tissue in predictable pattern (Jackson and Hake, 1999). While there is no direct proof in favor of biophysical mechanism, it can be assumed that enlargement of *poc* embryo may enlarge the SAM, which in turn may affect phyllotaxy by changing the homeostasis between balancing meristem size and leaf initiation. Alternatively, the chemical hypothesis assumes that the leaf positioning is determined by inhibitory field presumably biochemical in nature emanating from existing primordia and from the apex of the shoot meristem itself (Jackson and Hake 1999; Lyndon, 1998). According to this chemical hypothesis, the initiation of a different phyllotactic pattern may arise due to increase in size of the SAM, which in turn may affect the inhibitor gradients that determine the position of the leaf.

In tomato, most likely the chemical stimuli is responsible for the generation of phyllotactic patterns. Using excised tomato shoot apical menstems, which were cultured on defined media, Reinhardt *et al*., (2000) showed that inclusion of auxin transport inhibitors in media specifically inhibits leaf initiation, leading to menstems devoid of leaf primordia, though meristem continues to grow and forms a *pin* like stem. The application

of the natural auxin IAA to the apex of such *pins* in culture restores leaf formation. The above effect was specific for auxin and other hormones were unable to trigger primordium formation. Moreover, site of leaf formation strictly coincided with the site of IAA application the radial dimension. Apparently in regulating phyllotaxy the auxin determines the radial position and the size of lateral organs. The operation of an IAA based mechanism regulating initiation of leaves on tomato indicates that alteration of phyllotaxy in *poc* could result from interference with auxin formation, signaling or transport in the mutant. The low penetrance of the phenotype may therefore relate to ectopic expression of *poc* mutation in the SAM during formation of the leaf pair.

5.3.2 Leaf morphology of *poc* mutant

Tomato shows heteroblastic leaf development (Coleman and Greyson, 1976; Dengler, 1984) the first true leaves produced by the shoot are larger and can be morphologically and anatomically distinguished from cotyledon. As the tomato shoot grows it produces bigger and more lobbed leaf, a final adult leaf type is achieved by sixth node. The tomato plant has a unipinnately compound leaf made up of leaflets distributed along the leaf rachis. The tomato leaf exhibits reticulate venation typical for dicot leaves with two types of trichomes, multicellular hairs and glandular trichomes. The *poc* mutation affected the leaf morphology with variable penetrance and expressivity. However, based on the phenotype of leaf three distinct classes could be distinguished. Within each class leaf morphology though showed variability also had many common characters.

5.3.2.1 *poc* class A leaves show epiphyllous structure

The class A of *poc* mutant showed very severe altered leaf phenotype as evident by reduction in leaf size and extreme curling of leaf. One of the distinct features of this class was the formation of epiphyllous structure on the leaf. The factors responsible for

epiphylly are not known, however, in plants such as *Bryophyllum*, the leaf supports development of a tiny shoot bud, which is the mode of vegetative propagation as these buds break off from the leaf and they fall onto the ground and produce roots. The formation of epiphyllous structures is also seen in transgenic plant over-expressing different members of *KN1* class of plant homeobox genes and cytokinin-synthesizing genes (Kerstetter and Hake, 1997). Transgenic tobacco and *Arabidopsis* that over-express the maize *KN1* cDNA or related genes show retarded growth, reduced apical dominance and perturbed leaf development (Kerstetter and Hake, 1997). However, a class *of KN1* group of genes was able to induce epiphyllous structures in both tobacco (Sinha *et al.*, 1993) and *Arabidopsis* (Lincoln *et al.*, 1994). In addition to *KN1*, the *ISOPENTYL TRANSFEREASE* (*IPT*) gene over-expressed in tobacco also lead to epiphyllosy, though it was not expressed in all the tissues (Estruch *et al.*, 1991). Moreover, it indicates that these buds form from movement of cytokinin generated at a different site. In fact, this finding is analogous to *Bryophyllum*, where it is believed that the cytokinins accumulating at the leaf margins stimulate cell division in the notches to produce epiphyllous buds.

Though this evidence suggests a role for ectopic expression of gene like *KN1*, the transgenic tomato overexpressing *KN1* showed a strikingly different phenotype in the leaves. The expression of *KN1* gene in young leaf primordia of tomato leads to severe leaf dissection and produced a super-compound leaf (Hareven *et al.*, 1996). Moreover, all *KNOTTED*-type genes described in tomato till now are ubiquitously expressed in the meristem (Hareven *et al.*, 1996) and the comparable tomato gene(s) is yet to be discovered. This finding may discount that *KN1* family of gene is the cause of altered morphology and epiphylly in *poc* mutant, but it is also possible that altered expression of another gene may determine epiphylly in the tomato. Such a possibility is indicated by the

recent finding that over expression of *KNOTTED*-like genes is correlated with overproduction of cytokinin (Frugis *et al.,* 1999). In that case an interference with cytokinin action, production or mobilization by *poc* mutation can be considered as one of the likely cause for the epiphylly. Nevertheless, information on cytokinin action in plants is limited since so far no plant *IPT* gene is isolated. The above observation need further work to determine if or not the causal factor of *poc* phenotype is cytokinin, and also if any of the homeobox genes are involved in to regulate epiphylly in class A group of *poc*. In the class B, the phenotype is weak but the leaf curling and leaf size is different from that of wild type and rarely the leaf shows the epiphyllous structure. This may be due to leakiness of this gene.

One of the proteins whose expression is correlated with formation of incipient leaf primordia is expansins, which are extracellular proteins present in plant cell wall. The exogenous application of expansin protein can trigger the initiation of leaf-like structures on the SAM of tomato. Out of two tomato expansin genes, *LeExp2 (Lycopersicon esculentum EXPANSIN)* and *LeExpl8,* the *LeExpl8L* expresses preferentially in cell layer called II, which is the site of incipient leaf primordium initiation (Reinhardt *et al.,* 1998). The ectopic expression of regulatory genes by *poc* mutation may induce the expression of gene such as *LeExp18* leading to epiphyllous leaf formation.

5.3.2.2 *poc* class C mutant shows altered leaf symmetry

In class C of *poc* mutant the leaf morphology showed striking alteration in the shape and size. The leaf showed a range of variation right from being simple leaf to compound leaf. However, the leaflet had smooth margin and there was loss of small and intermediate leaflets. One prominent feature was change in venation pattern from reticulate to parallel. The cause for above leaf phenotype in this class, as seen for class A, may result due to variable degree of interaction between genes regulating leaf

development and *poc* mutation. Alternatively, the *poc* mutation through its epistatic interaction causes a kind of hormonal imbalance, which in turn results in this phenotype.

In tomato plants treated with GA, leaflet showed smooth margin coupled with loss of small and intermediate leaflets and a similar phenotype was observed in mutants presumed to be overproducers of GA (Jones, 1987). However, these plants did not show any alteration of symmetry or altered venation, which is distinctly manifested by the leaflets of class C of *poc* mutant. The *solanifolia* mutant of tomato to some extent resembles class C of *poc* in showing no lobbing in leaflets. Moreover, application of GA to wild type can phenocopy *solanifolia* mutation (Chandra Sekhar and Sawhney, 1991). These observations indicate the possibility of GA involvement in *poc* phenotype. On the other hand, recently Ross *et al*. (2000) provided evidence that auxin promotes gibberellin A_1 biosynthesis in pea shoot, if such a situation exists in tomato, then the causative factor *of poc* mutant phenotype could be increase in auxin level which may then increase the GA level.

Another possibility is that the *poc* mutation has epistatic interaction with other genes regulating leaf development in tomato. One of feature of class C of *poc* is the reduction in complexity of leaf. In tomato several mutations affect leaf development, which either reduce or increase the complexity of leaf such as *wiry*, *lanceolate*, *solanifolia, mouse ear* etc. These mutants can be classified in four different classes based on the phenotypes produced (Sinha, 1999). Type 1 mutants are defective in expansion of leaf blade such as *wiry*, the type 2 mutants reduce leaf dissection and change leaf into a simple leaf such as *lanceolate* and type 3 mutant alter the degree of leaflet lobbing and produce leaflets with marginal or no lobing such as *solanifolia.* In contrast, type 4 mutant show increased dissection of leaf such as *mouse ear* or *curl.* The leaf phenotype of class C of *poc* to some extent resembles type 2 and type 3 of tomato mutants, however, in the

absence of information on the molecular nature of the genes it is difficult to speculate on the reason or cause of resemblance in the phenotype. In the case of *mouse ear* and *curly* mutants, the mutation may cause unchecked meristematic activity by expression of a *KN1* like gene in the tomato (Parnis *et al.,* 1997), whereas *wiry* appears to be defective in expression of a gene *PHAT* involved in leaf expansion, which is an ortholog *of PHAN* gene of *Arabidopsis* (Kim *et al,* 2000). The alteration in *PHA T* expression causes loss of both radial symmetry and compound nature of leaf.

Recent investigations on formation of compound leaf by Sinha's group have revealed few interesting features of leaf development, which has bearing on variation in leaf morphology in *poc* mutant. They showed that the tomato leaf has a distal and proximal domain and sequential expression of genes in leaf primordia specify the leaf development. The leaf development is regulated by genes similar to maize homeobox gene *KN1*. The investigation on *LeT6* gene in tomato showed that plants underproducing *LeT6* have very simple leaves and no laminar expansion. In contrast, the plants overexpressing *LeT6* gene show drastic changes in leaf morphology (Janssen *et al,* 1998a). The expression patterns of *LeT6* indicate an important role for *LeT6* in leaf morphogenesis in tomato. One of the interesting observations is that the morphological states generated by overexpression of *LeT6* are variable and unstable. These variations in the phenotypes produced in transgenic plants show existence of inherent level of indeterminacy in the expression of tomato leaf phenotype. It is likely that extreme variation in leaf phenotype in different classes of *poc* mutation is the manifestation of above indeterminacy in leaf phenotype observed in *LeT6* transgenic. The interaction between *poc* mutation and perhaps a member of gene family of *LeT6* may be the cause resulting in different leaf morphology in *poc* mutant. Interestingly, the expression study

of *LeT6* showed that it is expressed in meristems and developing ovaries and perhaps may have a role in ovule and embryo morphogenesis (Janssen *et al.*, 1998b).

5.4 Reproductive development of *poc* mutant

The tomato, which is a day neutral plant, shows sympodial pattern of shoot growth, where after formation of inflorescence meristem the vegetative and reproductive shoot formation alternate regularly. In tomato after the formation of about 8-10 leaves the SAM changes from vegetative to reproductive growth and is converted into an inflorescence meristem (IM), which gives rise to scorpioidial cymose inflorescence. However, the vegetative growth continues by formation of a side shoot growing from the axil of the last leaf, which forms a small number of leaves (2-4) and then differentiates to form the second IM. The vegetative growth also continues from axil of the leaf a pattern called as sympodial, or indeterminate growth. Finally, the stem appears continuously and the inflorescence seems to arise at internodes. Most tomato cultivars produce a minimum of seven leaves before the first flowering branch and thereafter usually three leaves between new inflorescence.

5.4.1 *poc* C class mutant show delayed transition to inflorescence meristem

In contrast to wild type, the *poc* mutant shows alteration of timing and position in the development of IM. In case of *poc* class C mutant, the transition from vegetative development to reproductive development is delayed. The *poc* class C mutant starts flowering on the sixteenth leaf onwards, whereas wild-type, *poc* class A, and *poc* class B mutant flower normally after forming eight to ten leaves. The transition to flowering is a complicated developmental process controlled by both internal and external factors, which in turn regulates an extensive network of flowering-time genes (Ruiz-Garcia *et al*, 1997). One of the major internal factors may be the plant growth hormones, which play a crucial role in flower induction (Weigel, 1995). The major external factor is the

photoperiod or vernalization, which control flowering via regulation of specific set of flowering genes (Irish, 1999). Since tomato is a day neutral plant, the delay of flowering in *poc* class C mutant may be due to alteration in an internal process, which perhaps could be due to alteration in the hormone signaling. Alternately, the *poc* mutation can affect expression of one of the gene, which is involved in transition from SAM to IM.

Since information about tomato is not available, the information on genetic regulation of flowering in *Arabidopsis* can provide some clues for the possible reasons for the delay. However, this genetic regulation of flowering is quite complicated and even in *Arabidopsis* more than twenty genes are involved in it (Theiban and Saedler 1999). The studies using flowering mutants have shown that flowering in *Arabidopsis* is regulated by three pathways, a daylength-dependent pathway that promotes flowering in long days, a daylength-independent pathway that ensures flowering in the absence of inductive photoperiod, and a third autonomous pathway, that probably acts by modulating the other two pathways. These pathways are genetically separable, which is evident by the fact that there are mutations that delay flowering in long day but not in short days. On the other hand, mutants with block in gibberellin synthesis do not flower in short days, but flower normally in long days.

In *Arabidopsis* formation of IM is controlled by regulation of floral meristem-identity genes such as *LEAFY (LFY), APETALA1 (AP1), CAULIFLOWER (CAL), APETALA2 (AP2) and UNUSUAL FLORAL ORGANS (UFO)* (Theiban and Saedler, 1999). Out of these *LFY* and *AP1* are considered as primary genes to regulate flowering, and both genes encode putative transcription factors, which are strongly expressed in floral primordia. On the other hand, sympodial growth of tomato requires continued expression of the vegetative SAM too. In tomato the lateral meristem initiated in the leaf axil continues the shoot growth. In *Arabidopsis* recessive mutations in *TERMINAL*

FLOWER 1 (TFL1) gene result in the conversion of all apical meristems into floral meristems upon floral evocation indicating that this gene maintains the vegetative identity in inflorescence meristems. It is thought that *TFL1* is a negative regulator of the *LEAFY (LFY)* genes.

In tomato *SELF-PRUNING* gene is the functional ortholog of the *TERMINAL FL0WER1 (TFL1)* gene of *Arabidopsis*. The mutation in *SP* gene or reduction in its level by antisense RNA causes premature conversion of the sympodial vegetative apex into a terminal determinate inflorescence shoot. However, the mutation has no effect on the inflorescence architecture or flower morphology. On the other hand, overexpression of *SP* results in an extended vegetative phase of sympodial shoots and replacement of flowers by leaves in the inflorescence. The *sp* mutant phenotype indicates that *SP* probably acts to prevent early flowering in newly developing sympodial shoot meristems. Based on analogy with *Arabidopsis* it can be assumed that the extended vegetative growth in *poc* class C mutant may result from prolonged functioning of *SP* gene or another gene of similar function, which maintains vegetative meristems in tomato, thus delaying formation of reproductive meristem (Pnueli *et al*., 1998).

5.4.2 The flowers of *poc* mutants show defects in morphological development

The flowers of the cultivated tomato are bright yellow in color and normally have six sepals (calyx) and six petals (corolla), however, some time seven appendages are seen. The stamens have short filaments and enlarged anthers, which coalesce together to form a narrow-necked anther cone. The style is shorter than the anther cone, and therefore the stigma is enclosed within the anther cone. This fusion of anthers ensures self-pollination because the pollen is released inside the anther cone close to stigma. The development of flowers in *poc* mutant showed several abnormalities with low degree of penetrance and variable expressivity. The abnormalities in *poc* flower development can be basically

classified in four categories *viz.* fusion or lack fusion of organs, aberrant development of organs, changes in the organ number and the change in organ identity. The *poc* flowers in particular showed the abnormalities such as fusion of sepals, partial transformation of sepal showing tissue patches like petals (petaloid sepals), partial transformation of petal showing tissue patches like sepals (sepaloid petals). In addition there was reduction in length of these organs, the number of sepals and petals were also more and petals showed outgrowth towards the side facing stamens. Similarly, the stamens showed the lack of fusion of stamens as well as malformed stamens. The flowers of *poc* class C mutant also showed the fusion of the stamens to the carpals.

Similar to abnormalities in the flower development of *poc* mutant, the low temperature treatment of tomato plant induced severe abnormalities in flower development, the flowers showed extra stamens and nearly double number of carpels and sometimes flower splits into two (Lozano *et al*., 1998). The low temperature also caused lack of anther fusion, and carpel non-fusion, instead it also leads to fusion between stamens and carpels etc. Similar to *poc*, the low temperature also gave rise to chimeric organs showing tissue sectors of neighboring organ, giving rise to petaloid sepals and staminoid petals. The examination of floral meristem showed that the low temperature enlarges the floral meristems, which leads to increase in the number of organs. Likewise, the separation of stamens in *solanifolia* mutant of tomato was attributed to smaller primordial widths, greater distance between the primordia and larger flower apex diameter prior to the initiation of stamens primordia (Chandra Sekhar and Sawhney, 1989). Such a possibility may also exist in *poc* mutant too where mutations can lead to a large floral meristem. Such an enlargement of the meristem is implied by the fact that the *poc* shoot shows splitting which could be due to large SAM, and in such a case few of the *poc* floral meristems may be enlarged. It is therefore possible that in *poc* mutant the

increase in number of stamens and their separation could arise due formation of a lager floral primordia. The larger flora] meristem may also lead to increase in the number of calyx and corolla. Though in this study we did not examine SAM or FM size, it remains a possibility that needs examination.

Interestingly, treatment of tomato flower buds with GA3 also causes floral transformations similar to low-temperature induced changes (Sawhney, 1983). Since quantification of GA shows higher level in low temperature treated flower, the defect in *poc* could be also due to interference with GA metabolism. On the other hand, the GA treatment of male sterile mutant of tomato, in which stamens and pollens development are arrested results in production of normal viable pollen that is capable of inducing fruits and seed set (Sawhney and Greyson, 1973). In *Arabidopsis* the cytokinin (BAP) treatment to wild-type flowers at three developmental stages results in increase in floral organ number and formation of abnormal floral organs (Venglat and Sawhney, 1996) indicating that exogenous BAP suppresses expression of genes regulating floral meristem identity affecting flower development and organ differentiation. It is therefore likely that one of the possibility is alteration in cytokinin metabolism in *poc* mutant may cause abnormalities in the *poc* flowers.

It is believed that these changes result from abnormal expression of genes regulating floral organ number, or organ initiation and differentiation, or organ identity. A wealth of information has accumulated in *Arabidopsis* about the genetic regulation of flower development, and it is reasonable to assume that similar genes may also regulate tomato flower development. In tomato several genes, which are similar to MADS-box genes controlling flowering in *Arabidopsis* have been characterized (Pnueli *et al.*, 1994). The gene such as *TM4* shows sequence similarities with *AP1* and *SQUAMOSA* (*SQUA*) and is probably an "early" gene as its transcripts are below detection level in mature

flowers. Whereas transcripts of genes such as *TM5* and *TM6* are more abundant in mature flowers than in floral meristems (Pnueli *et al.,* 1991, 1994), and between these *TM6* shows the sequence similarity to class B *DEFICIENS* (*DEF*) gene of snapdragon. The *TAG1* gene controlling stamen and carpel development in tomato flowers is homologous to the *AGAMOUS* (*AG*) a class C gene of *Arabidopsis.*

The changes like formation of petaloid sepals or sepaloid petals indicate some kind of homeotic changes in these organs giving rise to chimeric organs. In case of low temperature treatment, the homeotic and meristic alterations in flowers show an increase in the level of steady-state mRNA of tomato genes *TM4, TM5, TM6,* and *TAG1.* It is also possible that altered expression of another gene, which controls floral organ number, may cause the increase in organ numbers in *poc* flowers. Such a gene has been reported for *Arabidopsis* where *F0N1 (FLORAL ORGAN NUMBER 1),* a novel gene regulates floral meristem activity and controls floral organ number (Huang and Ma, 1997). Similarly, the fusion of organs may result from mis-expression of genes responsible for the organ separation, for example mutations in any one of three *FUSED FLORAL ORGANS* genes in *Arabidopsis* cause the fusion of adjacent floral organs within and/or between whorls (Levin ***et al.,*** 1998).

Interestingly, the *cuc1* and *cuc2* double mutants of *Arabidopsis,* which have fused cotyledons and without SAM show fused sepals and stamens in flowers on adventitious shoots (Aida *et al.*, 1997). The *CUC2* mRNA is shown to express at the boundaries between meristems and organ primordia during both vegetative and reproductive phases indicating that *CUC2* gene may be generally involved in organ separation in shoot and floral meristems (Ishida *et al.,* 2000). Similarly, the petunia *nam* mutants with fused cotyledons and absence of SAM occasionally produced shoots and form flowers with increased petal number and deformed floral organs (Souer *et al.* 1996). It is possible that

the floral organ boundary functions are encoded by a different set of genes, whereas the floral organ identity is mainly encoded by MADS box genes.

5.4.3 The male sterility of *poc* flowers results from mechanical barrier to pollen release

The flowers *of poc* mutants rarely set the fruits and were therefore male sterile. However, an examination of the anther cones of the *poc* flowers showed that some anthers contained the normal pollen. The *poc* pollen also showed normal germination in *vitro*. Moreover, hand pollination of the *poc* flowers with homozygous *poc* pollen resulted in normal setting of fruits. Apparently, the male sterility in the *poc* mutant was due to mechanical defect in anthers preventing release of pollen from anthers. The anther dehiscence, which is needed to release the pollen grains for pollination, involves the breakage of the anther wall at a specific site. This site forms a notch between the locules of each theca and has two special cell types, the stomium and septum present within the notch region, in tomato the septum is the intersporangial type (Bonner and Dickinson 1989). The anther dehiscence requires a temporally regulated cell-degeneration program involving degeneration of the septum and the stomium leading to final release of pollen, which has to be co-ordinated with pollen maturation.

These studies on male sterility in relation to plant hormones have indicated that the relative ratio of plant hormones plays a critical role in the normal stamen and pollen development (Sawhney and Shukla, 1994). It has been suggested that the gene-regulated male sterility may also be mediated through alteration in endogenous levels of plant hormones in developing flowers and stamens. The *Arabidopsis delayed dehiscence1* mutant shows male sterility as the mutant releases pollen grains too late for pollination, even though the pollen grains within the mutant anthers are capable of fertilization. This defect is caused by the delay in the timing of stomium degeneration in the anthers. The

DELAYED DEHISCENCE1 gene encodes 12-oxophytodienoate reductase, which is an enzyme involved in jasmonic acid biosynthesis (Sanders *et al.*, 2000). The exogenous application of jasmonic acid rescues the mutant phenotype leading to seed set in otherwise previously male-sterile plants. These experiments imply that time of anther dehiscence in *Arabidopsis* may be regulated by formation of jasmonic acid. In contrast, *non-dehiscence1* mutant of *Arabidopsis* anthers fail to dehisce due to degeneration of the anther wall layer and the connective cells as result of which the stomium fails to break (Sanders *et al.*, 1999). The absence of the connective cells and the anther wall blocks the mechanical "springing" required for wall opening for pollen release (Sanders *et al.*, 1999). It is likely that in tomato *poc* mutant, the male sterility may result from similar mechanical defect as the *poc* plant makes viable pollen but cannot release them. The application of GA to the *poc* flowers does not rescue the phenotype. The role of jasmonic acid in regulation of male sterility in tomato *poc* mutants is yet to be examined, however, tomato *def1* mutant defective in jasmonic acid response is fertile (Howe *et al.*, 1996).

5.4.4 The *poc* class C mutant shows reduced fertility

In spite *of poc* mutant being male sterile, the homozygous plants can be obtained by mechanical self-pollination of flower. The success of the pollination for the mutants and wild type is nearly the same as monitored by frequency of fruit setting. In *poc* mutant class A and B, the fertility as estimated by fruit setting showed no significant differences from the wild type. However, the *poc* class C flowers showed a 50% reduction in fertility compared to the wild type. Since the flowers of *poc* class C show severe abnormality in the development (see section 5.4.1.) it is likely that defective development of carpel leads to observed reduction in the fertility. The reduction in female fertility of *poc* class C mutant needs further study to find the causal factor, as this class show severe alteration in leaf and floral morphology.

5.4.5 The flowers of *poc* class C mutant show formation of inflorescence

The tomato SAM converts to an IM after making at least 8-10 leaves and thereafter growth is sympodial. The IM produces flower meristems not as lateral primordia, but by a series of nearly equal divisions, each time yielding a flower meristem and an inflorescence meristem (Allen, 1996). In few of the *poc* class C flowers, a new inflorescence develops from a full-differentiated flower, giving rise to fresh set of flowers. In some species reversion from floral to vegetative growth is under environmental control (Battey and Lyndon, 1990), however, such a reversion has not been observed for tomato and other members of Solanaceae family.

Little is known about the signals that govern the network of meristem and organ identity genes that control flower development. In *Arabidopsis* under short day (SD) photoperiod heterozygous *lfy-6* and homozygous *ag-1* flowers display a heterochronic transformation from flower to inflorescence shoot meristem showing floral meristem reversion (Okamuro *et al*., 1996). Interestingly, this transformation from flower to shoot meristem is suppressed by *hy1* mutation, which produces inactive phytochrome protein, or by *spindly* mutation, which exaggerates the basal gibberellin signal transduction, or by GA application. Unlike *Arabidopsis,* tomato is a day neutral plant therefore the mechanism other than the photoperiod is likely to be responsible for floral reversion.

A good example of floral reversion is species *Impatiens balsamina,* which flowers in short days and remain vegetative in long days. In this species the interruptions of the short day induction by transfer into long day result in flower reversion (Krishnamoorthy and Nanda,1968). The reverted meristem produces whorls of leaves lacking axillary meristems and separated by long internodes, however, it behaves differently from a vegetative meristem and resumes flower development without a lag period when transferred back to inductive conditions.

The reversion of the flower in *poc* class C mutant appears to be different than the above cases. In case of *poc* the flower gives rise to not a new flower bud or vegetative leaves, rather a full inflorescence appears with many flowers and sometimes large leaves. In tomato, *falsiflora (fa)* and *anantha* mutants block the acquisition of floral meristem identity, in both mutants the SAM is converted to IM, but determinate flower meristems are replaced by indeterminate shoots. The *FALSIFLORA* gene is cloned and it likely encodes a protein having about 80% and 70% identity with either FLO or LFY proteins of *Arabidopsis,* supporting the view that *FA* is the tomato ortholog to *FLO* and *LFY* (Molinero-Rosale *et al.*, 1999). The formation of IM in the *poc* flowers may results from ectopic expression of genes controlling IM formation while the flower is undergoing differentiation or after it differentiates. In such a case, few tissue sectors in flower may suppress the *FLORICULA* gene or other floral meristem identity genes to form new flowers. Alternatively, since in tomato the IM produces flower meristems not as lateral primordia, but by a series of nearly equal divisions, each time yielding a flower meristem and an inflorescence meristem, a loss of separation between these meristems and/or loss of tissue determination for floral meristem may lead to formation of IM. It is also likely that though photoperiod may not contribute in tomato, but the temperature may play some role in causing this phenotype.

5.4.6 The *poc* phenotype results from defective embryo development

The expression of multiple-cotyledon phenotype obviously is a manifestation of altered embryo development of tomato. The embryo development in tomato wild type and *poc* mutant is nearly identical till the globular stage, thereafter the difference between them becomes apparent. While the wild type embryo undergoes a normal transition from globular to heart stage, in *poc* mutant this transition is associated with increased thickness of the radial axis. The radial expansion of *poc* embryo is followed by simultaneous

formation of 3 or 4 cotyledon primordia on it. It is evident from this observation that first manifestation *of poc* mutation is at the transition from globular to heart stage of embryo development.

The genetic regulation of embryo development has been examined in detail for *Arabidopsis*, which also has several mutants defective in cotyledon development and number of cotyledons. One possibility is that the multiple cotyledons in *poc* can form by transformation of leaves into cotyledons, like in the case of *xtc1, xtc2* and *pt* (primordia riming) mutants of *Arabidopsis*. The transformation of first leaf pair into cotyledon in *xtcl* and *xtc2* mutant is associated with change in timing of events in embryogenesis. In *xtcl* and *xtc2* mutants, the transition form globular-to-heart stage embryo is delayed, and the development of shoot apex is advanced. Unlike the *xtc* mutants, *poc* mutant shows initiation of multiple cotyledons at the same position in the embryo, which can also be seen in developing *poc* seedlings. At no stage during seedling growth, the *poc* cotyledons show any character of transformed leaf, like in case of *xtc* mutants. It is evident that the mechanism of extra-cotyledon(s) initiation in *poc* mutant is different from that of the *xtc1*, *xtc2* and *pt* mutants of *Arabidopsis*. It is evident from the foregoing that *poc* phenotype results from some other mechanism rather by transformation of first pair of leaf into cotyledon.

The mutation such as *sin1* in *Arabidopsis* though gives rise to few seedlings with multiple cotyledons, the seedlings lack functional SAM. Multiple cotyledons have also been observed in *hydra* mutant of *Arabidopsis*, which shows abnormal embryo development at globular stage. At this stage the *hydra* embryo lacks the characteristic cell arrangement both in upper and lower tiers of embryo. One of the primary defects in the *hydra* mutant is in regulation of cell shape, where cell is unable to expand in correct orientation. Topping *et al.* (1997) proposed that multiple cotyledons in *hydra* mutant arise

as a secondary effect of the wide hypocotyl and broad shoot apex. Moreover, they also proposed that an inhibitor might be involved in cotyledon initiation, and depletion of its level due to widening of shoot apical meristem leads to the formation of multiple cotyledons.

The embryo development in *poc* mutant of tomato to some extent resembles that *of hydra* in *Arabidopsis*. Similar to *hydra* that has wide hypocotyl, the *poc* embryo shows radial expansion after globular stage, where the radius of *poc* embryo is nearly 1.8 times larger than the wild type. The wider *poc* embryo in turn would have a larger surface area available to initiate cotyledons at the apical end of embryo, compared to the wild type. The enlargement of area at apical end of embryo may cause formation of a larger SAM, and like *hydra* mutant leads to reduction in the level of an inhibitor blocking cotyledon initiation, which in turn would induce formation of multiple cotyledons in *poc* mutant.

One of the recurrent regulator for cotyledon initiation and separation appears to be plant hormone - auxin. The loss of auxin polar transport in the mutants such as *pin1* leads to formation of embryos with fused collar shape cotyledon. Conversely, the defect in auxin signaling leads to formation of multiple cotyledons in embryo, such as in *pid* mutants of *Arabidopsis*. In several features, *poc* mutant resembles the *pid* mutant of *Arabidopsis*, for example the *pid* mutant also shows strong pleiotropic effect on structure and development of inflorescence, floral organs and leaves. It is possible that *poc* mutant may have a defect in level or signaling of hormone auxin, which leads to radial expansion of embryo during development. The enlargement of embryo in turn allows the embryo to initiate multiple cotyledons at the apical end leading to polycotyledony. However, the exact cause for the formation of multiple cotyledons can be determined only after identification of the *POC* gene product and its interaction with other genes during tomato embryo development.

5.5 Genetic analysis of *npc* mutant

5.5.1 *npc* is a recessive mutant

The *npc* mutants were crossed with the parental wild-type and resultant progeny was analyzed for the pattern of inheritance and segregation of the mutant phenotype. The segregation and inheritance analysis of *npc* mutant revealed that similar to *poc,* each of the three *npc* lines is defective in a single locus. The mutated *npc* gene was functionally recessive to the wild type and followed Mendelian segregation pattern indicating that the *npc* is a single nuclear gene. The idea that *npc* is a recessive, single locus nuclear gene was also confirmed from data of test-cross analysis.

Complementation analysis of three lines of *npc* mutant showed that all these lines were allelic to each other, and cannot be genetically distinguished, therefore represent only one group. In view of this finding, the three lines of *npc* are considered as one group and were designated *npc1-1* through *npc1-3*. Since we used bulk pool M_2 seeds (see section 5.1.1) to isolate *npc* mutant lines and they were allelic it is likely that the three *npc* lines may have descended from a single M_1 plant. The *npc* mutation is a loss of function mutation and the predicted role of *NPC* gene is to regulate the germination, cotyledon expansion and hypocotyl elongation. To our knowledge no other mutant with a phenotype similar to *npc* has been reported. It is likely that *npc* mutation may represent a new class of loci regulating plant development in seedling stage in age dependent fashion.

5.5.2 *npc* mutant phenotype is age dependent

The freshly harvested seeds of the *npc* mutant on germination showed seedling phenotype identical to wild type with broad cotyledon and petiole. However, the *npc* seeds after storage of about three months progressively lost the vigor for germination. The loss of vigor was accompanied by appearance of *npc* phenotype with narrow petioleless cotyledon. Since the wild type seeds even after storage for several years do not show this

phenotype at the seedling stage and also the above trait shows Mendelian segregation, it may be assumed that this phenotype has a genetic basis. However, expression *of npc* traits needs decline of yet an unknown factor during storage of seeds, which once drops below a threshold level leads to appearance of *npc* phenotype.

The nature of above factor/mechanism determining the regulation of seed vigor and the *npc* phenotype is not known. One of the potential candidates could be GA, the loss of which may cause decline in the seed germination. However, the application of the GA to *npc* seed neither improved germination nor rescued the phenotype of mutant. One of the possibilities is that the *npc* mutation causes a change in metabolism of dry seeds, which accelerates the aging and loss of cotyledon expansion. It was shown that in mung bean the non-enzymatic modifications of proteins through Amadori and Maillard reactions plays an important role in the loss of seed viability during storage (Narayana Murthy *et al.*,2000). In these seeds the level of glucose and lipid peroxidation products in seed axis increased significantly during storage. The changes in the sugar level may cause the *npc* phenotype on storage. However, the role of sugar in regulation of *npc* phenotype is yet to be examined. The *Arabidopsis petit1* (*pet1*) mutant shows reduction in cell elongation in various organs such as the hypocotyl, root, flower stalk, leaf, petal etc (Kurata and Yamamoto, 1998). The growth defect of *the petl* mutant was only obvious on medium containing sucrose, which promoted hypocotyl elongation in *Arabidopsis* but had no effect on elongation in *petl,* therefore, the *petl* phenotype is conditional, depending on the presence of sucrose.

Several studies in tomato have indicated that the germination needs build up of the water potential thresholds for radicle emergence and enzymatic weakening of tissues surrounding radicle. There is some evidence that endo-P-mannanase is involved in the process of endosperm weakening in tomato (Bewely, 1997). Genetic studies have

confirmed that in tomato the endosperm, not the embryo, is the primary determinant of the time to germination at reduced water potential (Foolad and Jones, 1991). Both the development of endo-P-mannanase activity and germination are strictly dependent upon exogenous gibberellin in GA-deficient mutant tomato seeds (Still and Bradford, 1997). The possibility of the loss of endo-P-mannanase or another enzyme as cause for decline in vigor of germination in the *npc* mutant needs examination.

5.5.3 Interaction of *npc* mutation with *aurea (an)* mutant

The light has strong influence on the cotyledon expansion and hypocotyl elongation in tomato. Since *npc* mutant has reduced hypocotyl length, we examined the interaction between *npc* and *aurea* mutant of tomato on the seedling phenotype. The examination of double mutant *npc au* phenotype showed that in the seedling stage the *npc* mutant is epistatic to *aurea (au),* only for elongated hypocotyl phenotype, as it partially suppresses the elongated hypocotyls of *aurea*. The double mutant shows intermediate hypocotyl length between that of *aurea* and *npc*. This may be due to effect of *npc* on cell elongation in tomato independently of photoreceptor action, because the action of *aurea* mutation is due to defect in phytochrome signaling pathway. On the other hand, the *npc au* double mutant showed the narrow petioleless cotyledon rather than *au* cotyledon indicating the epistatic action of *npc* mutant on *aurea*. At the same time the double mutant retains the golden yellow color of *aurea*.

5.5.4 Interaction of *npc* mutation with *poc* mutation

The interaction of *npc* mutation with *poc* mutation of tomato was studied by examining phenotype of *npc poc* double mutant, which showed that *npc* phenotype is restricted only to the seedling stage. The double mutant showed the narrow petioleless phenotype of *npc* mutant and the polycotyledon of *poc* mutant in the seedling stage. During the subsequent vegetative and reproductive growth plants showed characteristic

phenotype of *poc* mutant. These results also indicate that these two mutations are in different pathways of development and there is no interaction between them. In double mutants too, the phenotype of *npc* was age dependent and no *npc* phenotype could be seen on germinating the freshly harvested seeds. Nevertheless, the double mutant phenotype clearly manifested when the seeds were germinated after storage for three months from harvest.

5.5.5 Background effect on *npc* mutation

When the *npc* mutation was transferred to Moneymaker *(Lycopersicon esculentum)* background it displayed similar phenotype as observed in the original Ailsa Craig background. This indicates the stability of the mutation as well as supports its monogenic and recessive nature. Moreover, it is evident that this gene is background independent.

5.6 Seedling phenotype of *npc* mutant

5.6.1 *npc* mutant shows attenuated cotyledon development and expansion

The phenotype of the *npc* is unique in the sense that it shows age dependent expression of the phenotype. The other tomato mutant *nc* is not allelic to this gene and shows petioles on the cotyledon in the seedling stage. Moreover, in our laboratory conditions the *nc* cotyledons are much wider than the *npc* mutant. Since the *npc* cotyledon though show reduction in the cotyledon length, the major effect is seen in cotyledon width, it can be assumed that the *npc* mutation specifically affects the lateral expansion of the cotyledons.

The factors regulating lateral expansion of cotyledons are yet to be discovered. There are many internal and external factors, which regulate the cotyledon and leaf expansion such as light and hormones, for example; auxin and gibberellins are likely involved in regulating elongation of cells in the direction of length (Kende and Zeevaart

1997). Similarly, it was reported that brassinolides are involved in polar elongation of cells in the length direction (Creelman and Mullet 1997). There are few reports for the loss of lateral expansion in the leaf development in *Arabidopsis* and maize. Though leaf and cotyledon are not identical entities in terms of their ontogeny and genetic regulation, information about the defect in lateral development of leaf mutants may provide some clue about possible causes of *npc* phenotype. In maize *LEAFBLADELESS1* (Timmermans *et al.,* 1998) and *NARROW SHEATH* genes (Scanlon *et al,* 1996) are likely involved in the process of lateral expansion of leaf and may play a role in the down regulation of the homeobox gene *KNOTTED 1*. The microsurgery of leaf primordia and blade in *Solarium* provided the evidence that lateral expansion of the primordium and blade formation are related events with a connection to dorsoventrality (Sussex, 1955). In *phantastica* mutants in *Antirrhinum* (Waites and Hudson, 1995), the leaf primordia show a defect in dorsoventrality that is related to the failure in lateral growth and blade formation. *PHAN* is a MYB-related transcription factor that acts together with a temperature-dependent pathway (Waites *et al,* 1998). All *phan* mutants develop normally at 25°C temperature, but show mutant phenotypes at a lower temperature of 15°C. In case of *npc* the expression of mutant phenotype is conditional similar to *phan* mutant in the sense that the expression of *npc* phenotype needs three month storage of seeds.

The narrow-leaf mutant *angustifolia (an)* in *Arabidopsis* was isolated from irradiated seeds. It shows narrow cotyledons, narrow rosette leaves, and slightly twisted seed pods compared to wild type (Redei, 1962). In contrast to *npc* mutant, the *an* mutant shows the petiole and the leaf-blade length in the leaves similar to that of the wild type (Tsuge *et al.,* 1996), whereas in *npc* cotyledons the petiole is absent. In the leaves of the *an* mutant, the total number of cells was nearly similar to wild type, but at the cellular level compared to wild type the cells were smaller in the leaf-width direction and larger in

the leaf-thickness direction. It has been suggested that the *ANGUSTIFOLIA* gene controls leaf morphology by regulating polarity-specific cell elongation. In contrast to *an* mutant, *rotundifolia3* mutant of *Arabidopsis* also had the same number of cells as the wild type in the leaf but showed reduced cell elongation in the direction of leaf-length. The analysis of double mutants of *angustifolia rotundifolia3* mutant indicated that these two genes independently regulate the leaf expansion. In contrast to these mutants, *npc* mutant showed the reduction in both the length and width of only cotyledons, whereas the leaf morphology in the *npc* mutant was unaffected.

The molecular nature of the product of *ANGUSTIFOLIA* is not known, however, the molecular cloning of *R0TUNDIF0LIA3* (ROT3) gene indicated that it encodes a cytochrome P450 that might be involved in steroid biosynthesis as it has domains homologous to regions of steroid hydroxylases of animals and plants. (Kim *et al.*, 1998). Even though the *ROT3* transcript is ubiquitously present in the cells, the gene appears to specifically function to regulate the polar elongation of leaf cells. The transgenic plants overexpressing a wild-type *ROT3* gene showed longer leaves without any changes in leaf width from the parent plants (Kim et al 1999). Interestingly the transgenic plants overexpressing the *ROT3* gene had longer leaf blades but leaf petioles were of normal length. In this the cotyledon phenotype was not reported therefore it is not know whether or not *ROT3* overexpressing plants had longer cotyledons.

The *Arabidopsis AINTEGUMENTA (ANT)* gene, which encodes a transcription factor of the AP2-domain family, is localized at the growing zone of immature organs. The recent studies showed that the *ANT* is an intrinsic organ size regulator that likely controls cell number and growth of lateral organs in the shoot development. The *ant* mutant though showed no difference in the timing of leaf primordia initiation or the number of leaf primordia, the width and length of mature *ant-1* leaves were reduced in

comparison with wild-type leaves (Mizukami and Fischer, 2000). The reduction in size of *ant-1* organs was associated with a decrease in cell number rather than the decrease in cell size. The reduced cell divisions observed in *pointed first leaf (pfl)2* mutant of *Arabidopsis* also showed that the reduction in leaf width could also result from less cell divisions (Ito *et al*, 2000). The *pfl2* mutation resulted from disruption of cytoplasmic ribosomal protein. It is apparent from the foregoing discussion that while there are several genes known, which regulate the organ size, the likely candidate for *npc* phenotype is difficult to predict as the *npc* phenotype is dependent on the age of the seeds.

One of the distinctive features of *npc* mutant is that the cotyledon lacks petiole. Apparently the formation of petiole on the cotyledon in tomato is a postembryonic event, which is suppressed in the *npc* mutant. However, the loss of petiole is a part of general effect of *npc* mutation on the cotyledon phenotype. There is little information available if any specific gene regulates the petiole development in plants, particularly cotyledons. In a study on expression of expansins gene *{AtEXPlO)* in transgenic *Arabidopsis,* it was found that the leaf size was substantially reduced in antisense lines with suppressed *AtEXP10 {Arabidopsis thaliana EXPANSIN 10)* expression, whereas overexpression *of AtEXP10* resulted in plants with somewhat larger leaves. Interestingly changes in leaf size were correlated with changes in the petiole length of leaf (Cho and Cosgrove, 2000). In the wild type plants the *AtEXPlO* preferentially expressed in the petiole and the midrib, but its expression in the cotyledons could not be detected. Since petioles appear only in light-grown cotyledons of wild type, obviously light has a distinct effect in regulating petiole development. Using *Arabidopsis* mutants which were deficient in phytochrome E in phytochrome A- and phytochrome B-deficient background, phytochrome E deficiency led to reduced petiole elongation in leaves (Devlin *et al,* 1998). On the other hand, a

phytochrome D mutation in *Arabidopsis* in Ws genetic background showed an increase in petiole length of leaf (Aukermann *et al.,* 1997).

In case of *npc* mutants the abaxial (lower side) epidermal cells of the cotyledons showed round shape and fewer protrusions. This modification in the phenotype of the epidermal cell shows that the *npc* mutation in some way affects the cell shape in the cotyledon. However, a detailed study of the cells shape, number and size in *npc* cotyledon is needed to draw a definite conclusion.

5.6.2 Etiolated seedlings of *npc* mutant shows partial constitutive photomorphogenesis

One of the distinct effects of *npc* mutation is on the phenotype of dark-grown seedlings, which shows inhibition of hypocotyl elongation, absence of apical hook and open unexpanded cotyledons. In a fashion similar to the narrow cotyledons of light-grown seedlings, the above alteration in etiolated seedlings phenotype is also observed after storage of seeds. The observed phenotype of *npc* seedling in darkness is somewhat reminiscent of *Arabidopsis* mutants belonging to *det/cop/fus* family which shows the constitutive photomorphogenesis in dark, such as *de-etiolated (det)* (Chory *et al,* 1989), *constitute photomorphogenic {cop)* (Deng *et al,* 1991), and *plumular hook open (pho)* (Khurana *et al,* 1996). Genetic and physiological evidences have shown that light represses hypocotyl elongation through activation of photoreceptors such as phytochromes, a blue light receptor and one or more UV-B receptors (Kendrick and Kronenberg, 1994). Similar evidences have also been obtained for the role of growth hormones in hypocotyls elongation, where gibberellins and auxins act as stimulatory factors and ethylene, abscisic acid and cytokinins have inhibitory effects (Davies, 1995).

Now it is believed that phenotype of many of the *det/cop/fus* mutants rather results from mutations in the genes encoding nuclear proteins, few of which may form a part of a

nuclear signalosome complex (Deng *et al.,* 2000). On the other hand, in mutant such as *det2,* it results from a block in the biosynthesis of brassinosteroids. Since *npc* mutant shows altered phenotype only in seedling stage, and does not show defect in other light-dependent responses, it suggests that *npc* mutation causes defect in pathway, which specifically acts during the seedlings development.

6. Summary

Though cotyledon number has been a major criterion to classify the flowering plants into monocot and dicot, only during last decade the information about genetic regulation of cotyledon development being obtained. The application of molecular and genetic techniques to study embryo development has provided a wealth of information about the role of different genes in embryo formation in *Arabidopsis.* However, in other plants such as tomato the information about embryo development and regulation of cotyledon number is limited.

In this study, we isolated and characterized two groups of cotyledon development mutants of tomato (*Lycopersicon esculentum* L.). The first group of mutant showed multiple cotyledons in the seedling and were named as *po*lycotyledon (*poc*) mutants. The genetic analysis of *poc* mutant revealed that *poc* is a monogenic recessive nuclear gene. In this group, nine mutant lines were isolated, but all these lines belonged to same complementation group, designated as *poc]-]* through *poc 1-9.* The *poc* mutation has a strong pleiotropic effect at all the stages of plant development right from embryogenesis till fruit setting. The studies on tomato embryogenesis revealed that the primary effect of *poc* mutation on cotyledon number is likely at the transition from globular to heart stage. In *poc* embryo this transition is accompanied with the thickening of radial axis of the embryo, consequently, *poc* embryo is 1.8 times wider than the wild type embryo. The increase in diameter of the embryo in lateral direction is followed by simultaneous initiation of 3-4 cotyledons on shoot end of the embryo.

The seedlings of homozygous *poc* mutants on germination showed cotyledon number ranging from 2-4. Among these tetracotyledons accounted for nearly 50% of seedlings followed by 35% tricots, others were with splitted cotyledons and only 1.5% of seedlings were dicots. All cotyledons in *poc* mutant arose from the same node on the

seedling hypocotyls ruling out the possibility of transformation of leaf in to cotyledons. The *poc* mutation affected the dark phenotype of the seedlings and etiolated seedlings showed shorter hypocotyls than the wild-type control. The roots of *poc* mutant grown in vermiculate were also shorter in length compared to the wild type. The role of phytochrome and/or component of the signal transduction chain was ruled out as *poc* double mutants with either of the phytochrome deficient mutant *au* or *hp* retained characteristic features of *poc* at all the stage of development.

The most prominent feature of *poc* mutant was alteration of vegetative and reproductive developments compared to wild type. The mutant showed altered phyllotaxy with a frequency of about 30% resulting in whorl phyllotaxy at the first node instead of normal spiral phyllotaxy. On the basis of variable expression of leaf morphology, the *poc* mutants were classified in to three major classes.

A. The class A mutant were bushy, leafy and showed formation of epiphyllous leaves with almost 100% frequency. The leaves of this class were strongly curled and had reduced leaf area. The fully mature plant was shorter than the wild type.

B. In class B, the plants were slightly shorter than the wild type. The plants were sparingly branched and have slightly reduced leaf area as compared to the wild type. In this group formation of epiphyllous leaves was rarely seen.

C. The class C mutant showed the extreme changes in leaf morphology with leaves ranging from simple lanceolate to compound leaf with six leaflets and one terminal leaflet. However, in all these leaves, the symmetry of organization was altered and small and minor leaflets were totally absent. The lobbing of leaf margin was also absent in this class.

Similarly, the reproductive development of *poc* mutant was also drastically altered. While the plants of A and B classes flowered at the same time as the wild type,

the onset of flowering was delayed in class C. In *poc* mutant, the reproductive development was severely affected and in most plants the inflorescence shows altered phyllotaxy. Moreover, the number of flowers per inflorescence was much higher than the wild type and all the flowers bloomed at the same time.

The floral organs in the *poc* mutant showed increase in number but a decrease in length of organs. The number of stamens was reduced in the class C of *poc* mutant. In A and B class the sepals showed some homeotic changes to petals at a frequency of 7.5%. The petals showed proliferation of tissue resulting in out growth on the inner side of flower, which invaded the fused stamen tube leading to the separation of stamens. In all the three classes the stamens showed absence of fusion and in class C the stamens were fused to carpels. Interesting, from the fully mature flowers of class C new inflorescence originated leading to formation of many flowers and in some instances big leaves.

In tomato, the expansion of the cotyledons is stimulated by light. We have also isolated a mutant, which showed defect in the cotyledon expansion named Narrow petioleless cotyledons *(npc)*. Interestingly, the expression of the mutant phenotype was age dependent. The freshly harvested seeds of *npc* mutant were phenotypically similar to wild type in all aspects of development; germination, hypocotyl elongation in light and dark, and cotyledon expansion. However, after storage of seeds for one year the *npc* mutant seedlings showed delayed germination, reduction in hypocotyl elongation and narrow petioleless cotyledons. This age dependent effect on these processes appeared only after three months of storage from harvest. The cotyledon area in *npc* mutant was reduced by more than 50% and the petiole was totally absent. In comparison to *poc*, the *npc* mutant showed less pleiotropic effect on vegetative and reproductive development, however plant height was slightly reduced than the wild type.

Interestingly, the one year old *npc* mutant shows partial constitutive photomorphogenesis in dark manifested by short hypocotyls, unfolded cotyledons and open hook. The double mutant *npc au* shows some inhibition of hypocotyl elongation and reduction of the cotyledon area similar to *npc*. In contrast, the *npc poc* double mutant shows the narrow cotyledons of *npc* mutant and all the other features of *poc*.

Finally, it is evident that these two mutants are very unique, in comparison with the mutants of *Arabidopsis*. The highest frequency of polycot seedlings in *poc* mutant and the age dependency of *npc* mutant show the necessity to investigate the plant development in other systems too.

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Appendix

MEAN MONTHLY METEOROLOGICAL DATA AT A. R. I., RAJENDRANAGAR (2000)

Months	Temperature MAX.	MIN	R.H (%) I	II	RAIN FALL (mm)	RAINY DAYS	SUN SHINE (Hrs)	WIND SPEED (Km/hr)	EVAP (mm)	MEAN TEMP.
Jan	30.2	12.9	84	30	25.2	2	9.7	1.9	3.6	21.5
Feb	31.5	18	84	44	Nil	Nil	8.5	3.0	4.4	24.7
Mar	35.4	18.3	70	41	12.4	2	10.4	2.4	6.7	26.9
Apr	39.4	23.1	61	36	12.4	2	9.9	3.1	8.2	31.2
May	36.9	24.9	62	37	66.8	4	10.1	4.7	8.0	30.9
Jun	31.2	23.6	83	63	257.5	12	2.4	4.7	4.1	27.4
Jul	29.8	23.6	82	65	97.3	6	4.7	6.9	4.1	26.7
Aug	29.4	23.1	87	69	Nil	Nil	4.0	5.2	3.7	26.3

MEAN MONTHLY METEOROLOGICAL DATA AT A.R.I., RAJENDRANAGAR (1999)

Months	Temperature (C)		R.H.(%)		Rain fall (mm)	Rainy days	Sun shine (hrs)	Wind Speed (km/hr	Evap. (mm)	Mean Temp.
	Max	Min	I	II						
JAN	28.8	11	87	33	0	0	9.9	2	3.1	19.9
FEB	32	15.8	82	29	2.7	1	10.1	3.6	5.1	23.9
MAR	36.4	19	69	24	0	0	9.8	2.6	6	27.7
APR	39.7	23	57	19	0	0	9.6	2.8	8.2	31.4
MAY	36.8	25.3	70	38	141.7	8	8.2	4.9	6.7	31
JUN	32.7	24.1	80	52	69.4	6	6.8	8	6.3	28.4
JUL	30.7	23.3	86	63	183.5	13	5.4	18.6	4.8	27
AUG	29.3	22.9	88	69	157	10	4.3	5.2	3.1	26.1
SEP	30.2	22.8	87	63	55.5	6	5.4	4.5	4	26.5
OCT	31.1	20.8	87	51	68.6	4	7.9	2.4	3.7	26
NOV	30.4	15.1	81	35	0	0	9.3	2.1	3.7	22.8
DEC	28.3	11.3	84	34	0	0	9.4	1.5	3.1	19.1
TOTAL	386.4	234.4	958	510	678.4	48	96.1	58.2	57.8	309.8
MEAN	32.2	19.5	79.8	42.5	56.5	4.0	8.0	4.9	4.8	25.8

MEAN MONTHLY METEOROLOGICAL DATA AT A.R.I., RAJENDRANAGAR (1998)

MONTHS	TEMPERATURE (C)		R.H.(%)		RAIN FALL (mm)	RAINY DAYS	SUN SHINE (Hrs)	WIND SPEED (Km/hr)	EVAP. (mm)	MEAN TEMP.
	MAX	MIN	I	II						
JAN	30.4	16.3	88.0	42.0	0.0	0.0	9.1	2.7	4.0	23.4
FEB	32.8	17.4	78.0	31.0	0.8	0.0	10.2	2.3	5.7	25.1
MAR	36.1	21.3	70.0	31.0	1.2	0.0	9.3	3.3	7.5	28.7
APR	39.1	24.0	62.0	27.0	3.5	1.0	9.4	2.7	9.1	31.6
MAY	39.8	26.0	63.0	31.0	52.2	3.0	9.3	3.1	9.2	32.9
JUN	36.4	25.1	78.0	54.0	50.5	5.0	7.6	6.2	10.3	30.8
JUL	32.0	23.4	87.0	65.0	198.1	9.0	5.2	6.1	5.1	27.7
AUG	30.0	22.7	91.0	73.0	283.4	13.0	5.2	4.0	2.7	26.4
SEP	29.8	22.6	91.9	72.2	197.3	11.0	4.4	3.8	3.2	26.2
OCT	29.8	20.7	92.0	64.0	241.2	9.0	6.4	2.7	2.5	25.3
NOV	29.0	17.2	91.0	49.0	21.9	4.0	7.7	1.5	2.8	23.1
DEC	28.2	10.1	86.0	34.0	0.0	0.0	9.2	1.9	2.6	19.2
TOTAL	393.4	246.8	977.9	573.2	1050.1	55	93	40.3	64.7	320.1
MEAN	32.8	20.6	81.5	47.8	87.5	4.6	7.8	3.4	5.4	26.7

MEAN MONTHLY METEOROLOGICAL DATA AT A.R.I., RAJENDRANAGAR (1997)

MONTHS	TEMPERATURE (C)		R.H.(%)		RAIN FALL (mm)	RAINY DAYS	SUN SHINE (Hrs)	WIND SPEED (Km/hr)	EVAP (mm)	MEAN TEMP.
	MAX	MIN	I	II						
JAN	27.8	13.6	83.0	48.0	38.0	1.0	8.4	2.6	2.9	20.7
FEB	32.3	13.3	80.6	26.5	0.0	0.0	10.2	1.9	4.2	22.8
MAR	36.2	18.2	67.0	25.0	52.4	2.0	10.1	3.1	4.9	27.2
APR	36.0	21.2	71.0	31.0	45.6	5.0	10.0	3.3	4.9	28.6
MAY	39.5	24.8	54.0	25.0	2.8	1.0	10.1	4.3	8.0	32.2
JUN	36.2	23.8	75.0	40.0	71.0	4.0	7.7	8.3	5.8	30.0
JUL	31.9	23.1	82.0	58.0	131.0	12.0	4.7	9.4	4.8	27.5
AUG	31.4	22.6	86.0	65.0	116.6	5.0	5.3	10.0	4.7	27.0
SEP	31.3	22.6	89.1	66.8	125.1	6.0	5.7	3.2	4.0	27.0
OCT	31.5	20.0	85.0	49.0	73.1	4.0	8.2	2.5	4.0	25.8
NOV	29.9	20.2	91.0	58.0	49.1	2.0	7.5	2.6	2.8	25.1
DEC	29.0	18.4	92.0	56.0	36.4	2.0	7.1	3.3	2.7	23.7
TOTAL	393.0	241.8	955.7	548.3	741.1	44.0	95.0	54.5	53.7	317.4
MEAN	32.8	20.2	79.6	45.7	61.8	3.7	7.9	4.5	4.5	26.5