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.