A HISTOCHEMICAL AND MORPHOLOGICAL STUDY OF FLOWER INITIATION IN THE CHABAUD TYPE CARNATION (DIANTHUS CARYOPHYLLUS L.)

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EVERETT RAYMOND EMINO
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This is to certify that the

thesis entitled

A HISTOCHEMICAL AND MORPHOLOGICAL STUDY OF FLOWER INITIATION IN THE CHABAUD TYPE CARNATION (DIANTHUS CARYOPHYLLUS L.)

presented by

Everett Raymond Emino

has been accepted towards fulfillment
of the requirements for

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ABSTRACT

A HISTOCHEMICAL AND MORPHOLOGICAL STUDY OF FLOWER INITIATION IN THE CHABAUD TYPE CARNATION (*DIANTHUS CARYOPHYLLUS* L.)

By

Everett Raymond Emino

The transition from vegetative to reproductive organogenesis in the Chabaud carnation (*Dianthus caryophyllus* L.) was studied histochemically and morphologically. A non-flowering teratological pine cone mutant carnation was included for comparative studies with two normal flowering clones.

A new technique of sample preparation for the scanning electron microscope was developed for the study of fresh intact carnation shoot apices. Within five min of separation from the plant a scanning electron micrograph of the apical meristem could be obtained showing the topographic features.

This technique combined with standard histological methods revealed that apices of both the pine cone mutant and normal clones were morphologically similar with leaves arising from a single primordium whorl. At flower initiation in the normal flowering clones the apex broadened and flattened initiating centripetal whorls of primordia which

differentiate alternately. Sepals are initiated first, followed by petals, anthers, carpel and placenta. At flower initiation in the pine cone mutant there was a disruption of the centripetal whorl pattern of initiation to a spiral-like pattern of initiation. Attempts to alter this pattern of initiation by grafting and growth regulator application were not successful. These studies show that the spiral-like pattern is controlled in the shoot. A partial redifferentiation of bracts into carpels by cytokinin PBA indicate the control of initiation and differentiation is under separate mechanisms.

DNA, RNA, protein, and starch were localized in the apex region and protein was separated by disc gel electrophoresis. RNA was localized in initiating primordia. Starch was concentrated in the subapical region. The differences between the pine cone mutant and normal were a greater starch accumulation and different protein banding in the mutant. The results of the differential starch accumulation studies in the mutant were inconclusive. However, labeled ^{14}C glucose-1-phosphate incorporation as determined by microautoradiography was not responsible for the starch distribution pattern in the apex. The mutant in addition to low glucose had two sugar complexes absent in extracts separated by thin layer chromatography. The disruption of carbohydrate metabolism in the pine cone

mutant may be related to the disruption of primordia initiation.

Finally the data was discussed in terms of a model based on the reaction system theory of shoot apex morphogenesis. The data and model are consistent with the theory.

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By

Everett Raymond Emino

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NOTE TO COMMITTEE

This thesis has been prepared in two sections. Section I, 'Scanning Electron Microscope Studies of the Shoot Apex in Dianthus caryophyllus L. cv. Scania', is a paper in journal format that has been published in the Journal of the American Society for Horticultural Science (96:235-256, 1971) and was an integral part of this thesis research.

Section II is in the traditional thesis form.

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A HISTOCHEMICAL AND MORPHOLOGICAL STUDY OF FLOWER INITIATION IN THE CHABAUD TYPE CARNATION (*DIANTHUS CARYOPHYLLUS* L.)

INTRODUCTION

Shoot apex morphogenesis has been studied since the mid-nineteenth century. Hanstein (1868) studied the shoot meristem of higher plants and presented a theory on its organization. Schmidt (1924) proposed the tunica-corpus theory. Interest in shoot apex morphology was renewed in the 1950's and many papers have appeared since that time.

The transition from vegetative growth to a reproductive meristem has received considerably less attention. The physiology of flower induction has been extensively studied and reviewers have attempted to bring these two areas together but the processes and morphology involved in the transition from the vegetative to reproductive phase are still not clear.

The study of carnation shoot apices has received less attention than many plants. More research has been directed to the control of flowering by environmental manipulation due to the economic importance of the carnation.

A tetratological carnation called the wheat ear or pine cone mutant has been reported (Masters, 1869) and

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reference has been made to it several times. A brief description of the pine cone mutant has been made but there has been no detailed anatomical study of the shoot apex.

Jensen (1962) states:

> "Plants and their parts are extremely complex, both morphologically and physiologically. Histochemical procedure permits the recognition of this complexity and provides data which can be interpreted in terms of cells, tissue and tissue systems."

The technique of histochemistry has been applied to flower initiation morphology and the studies reported have contributed significantly to the knowledge of flower initiation.

The primary objective of this research was a comparative study of shoot apex morphogenesis in Chabaud carnations (Dianthus caryophyllus L.) and a non-flowering pine cone mutant of the Chabaud type. The plan of investigation evolved around the hypothesis that the pine cone mutant was in a prefloral condition and by comparative study would provide information on the morphogenic sequences necessary for flower primordia initiation and development. First, a morphological study was made to determine the developmental sequence of events in leaf and flower primordia initiation in the normal Chabaud type carnation and pine cone mutant. Since carnation shoot apex morphology had been studied previously only new data and interpretations will be presented in the results. Where data and interpretations are similar to that presented in the past reference will be made to it.

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Second, attempts to alter the pattern of primordia initiation and differentiation were made in the pine cone mutant by grafting and exogenous growth regulator applications. Third, histochemical investigations were made of vegetative and reproductive organogenesis and the comparative differences in the pine cone mutant, then these differences were pursued to find the relationship with flower primordia initiation and differentiation.

The scope of the study was not restricted to the microscope but included physiological and cytological techniques as well. These plants were selected for their potential in comparative studies to provide further knowledge on shoot apex morphology and the initiation of flowering as an event in the life of a plant. Additionally this research involved working in the overall framework of control of flower initiation in carnation as an economic practice in flower production by better understanding carnation shoot apex morphology.

SECTION I

Scanning Electron Microscope Studies of the Shoot Apex in Dianthus caryophyllus L. cv. Scania[1]

E. R. Emino and H. P. Rasmussen[2]

Michigan State University, East Lansing

Abstract. Intact shoot apex development of the carnation was studied with a scanning electron microscope using fresh tissue. Reproductive meristems were more resistant to desiccation from the hard vacuum and electron beam than vegetative meristems. Vegetative meristems, however, can be studied if the work is done quickly.

Structural changes which occurred in the developing carnation shoot showed that leaf primordia are initiated in a circular whorl. Floral initiation was easily determined by the appearance of a flattened apex and a pentagonal whorl of sepal primordia. Subsequent centripetal initiation of flower parts was easily recognized and identified.

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[1]Received for publication September 18, 1970. Michigan Agricultural Experiment Station Journal Article No. 5230. Recognition is extended to V. E. Shull, Microprobe supervisor, for technical assistance and operation of the SEM.

[2]Instructor and Associate Professor respectively, Department of Horticulture.

Many studies of shoot apex morphology have involved viewing paraffin sections 5 to 20 microns thick with the light microscope, dissection under a binocular dissecting microscope or both (Berg, 3; Godkin and Ballantyne, 7; Rajv, 11). The paraffin method provides cellular detail in median longitudinal sections; however, structural changes of the whole apex are difficult to reconstruct and interpret. Techniques of photographing dissected apices through a microscope as described by Beijer (2), Cutter (4) and Ball (1) partially overcomes this problem, but do not give sufficient detail for critical study. Sattler (12) described a technique for the study of floral development by viewing whole fixed and stained shoot apices in 100% ethanol with an incident light condenser and objectives with dipping cones. Einert et al. (5) has recently described a method of determining floral initiation and differentiation of the shoot apices of Lilium longiflorum Thunb., using the scanning electron microscope (SEM). Results presented by Einert et al. (5) on Lilium and Heslop-Harrison (8) with Elodea show promise for such a technique. The SEM is useful in studying microtopographical features because of the depth of field, magnification, and resolution.

Topographical changes in the carnation shoot apex have been studied by the paraffin method. Shushan and Johnson (13) studied the vegetative shoot apex of lateral branches

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of carnations and presented interpretative drawings. Emino (6) described the shoot apex during the plastochron and the transition from a vegetative to reproductive apex. Additional investigations are necessary for a complete interpretation of the changes that occur in the shoot of carnations during flower initiation and development.

This paper illustrates the structural changes in the developing shoot apex of carnation during vegetative and reproductive organogenesis. In addition a new technique for studying shoot apices using fresh tissue is described.

Materials and Methods

Carnations, Dianthus caryophyllus L. cv. Scania, were grown in the greenhouse using standard cultural practices for carnations (Holley and Baker, 9). Shoots representing different morphological stages of development as described by Emino (6) were selected and placed in beakers of warm water prior to preparation for SEM analysis. Leaves were carefully removed from a selected shoot as close to the apex as possible. The terminal section of the stem containing the apex was cut one-half inch in length and inserted into a block of water saturated Niagara Foam (Niagara Foam Products, Mt. Clemens, Mich.) for support. The Niagara Foam and sample were placed in a petri dish under a binocular dissecting microscope. Using a microdissecting needle the remaining leaves, bracts or sepals were removed exposing

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the apex. A drop of distilled water was applied to the exposed apex to prevent desiccation. A drop of carbon Tube Koat (G. C. Electronics Co., Rockford, Ill.) diluted with acetone 50:1 v/v was placed in the center of a polished 1 1/4 inch carbon disc to act as an adhesive as well as an electrical conductor. The terminal 3-4 mm of the shoot was removed and attached to the polished carbon disc with Tube Koat adhesive. The freshly dissected specimen was immediately placed in the sample chamber of the SEM (Applied Research Laboratories model EMX-SM electron microprobe). The sample chamber was evacuated and the sample positioned with the aid of light optics. The instrument was operated at 10 KV accelerating voltage and 0.003 microamp sample current for all samples. Scanning electron micrographs were obtained by a time lapse photograph of a single scan on the Cathod Ray Tube (CRT). The sample preparation procedure was repeated for each apex studied.

To determine the effect of the hard vacuum (5×10^{-5} mm of Hg) and the electron beam on the fresh tissue time course studies were conducted using vegetative and reproductive carnation apices. The micrographs were made at 5 min intervals. A minimum time of 4 to 5 min was necessary for obtaining the proper vacuum, finding the sample, focusing and adjusting the instrument before the first photograph could be taken.

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Results and Discussion

Micrographs of freshly dissected shoot apices gave good detail for critical study (Figs. 1 and 2). Figure 1 A illustrates a typical vegetative apex in late plastochron while Fig. 1 C illustrates a reproductive apex.

The time sequence of desiccation of the vegetative apex is presented in Figs. 1 A and B. The micrograph taken at 5 min shows a hydrated apex which is similar to observations made on the apex with the binocular dissecting microscope prior to placing the specimen in the sample chamber and to interpretations made from observing paraffin sections (Emino, 6). At 10 min the hard vacuum and electron beam severely wrinkled the specimen rendering it unsatisfactory for this study (Fig. 1 B). The dome and leaf primordia show large areas of desiccation due to the electron beam and the vacuum. The desiccation shown by Einert et al. was due to sample preparation artifacts.

The time sequence of reproductive apex desiccation is presented in Fig. 1 C to F. At 5 min the micrographs show the initiated flower primordia in a hydrated condition. Again, this apex appears similar to observations made prior to placing it in the sample chamber of the SEM. After 10 min the hard vacuum and electron beam had done relatively little damage to the apex (Fig. 1 D). During this same time period the vegetative apex would have been completely desiccated. After 15 and 20 min (Figs. 1 E and 1 F)

wrinkling was noted on the petal and anther primordia. Thus, the reproductive apex withstood the vacuum and the electron beam considerably longer than a vegetative apex.

Free hand sections of carnation vegetative and reproductive apices stained with Sudan III and IV (Jensen, 10) for lipids resulted in a slight increase in staining intensity in the cuticle of the floral apex compared with the vegetative apex indicating the possibility of a thicker cuticle on the reproductive apex. In any event extreme care must be exercised in the dissection of the apex. Mechanical damage from the dissecting needle ruptures the water retention mechanism of the apex and causes rapid desiccation of the sample before the vacuum is hard enough for operation of the SEM.

The vegetative shoot apex of the carnation is dome shaped (Figs. 1 A and 2 A to C). Changes in the vegetative apex which occurred at different stages of the plastochron were the result of a cyclical pattern of initiation of opposite leaf primordia. The dome rises to a maximum height of about 100 microns (Emino, 6) and initiates a single circular primordium whorl from its flank. This primordium expands and the dome is then at a minimal height of 40 microns above the whorl (Fig. 2 A).

The leaf primordia were not initiated individually as they appeared to be from longitudinal median sections or cross sections of the apex, but rather from a circular

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primordium whorl (Fig. 2 A) which surrounded the entire apex. This whorl then differentiated into two opposite leaf primordia (Fig. 2 B). The next primordium whorl in the subsequent plastochron stage will have leaf primordia at 90° to the previously formed primordia which resulted in the observed decussate phyllotaxis.

Floral initiation was easily recognized by a broadening of the apex and the initiation of a pentagonal whorl of primordia which develop into sepals (Fig. 2 D). In longitudinal section this stage of development appears as an enlarged vegetative apex with opposite primordia in the SEM micrographs, however, it is clearly an early floral meristem. The sepals are joined laterally forming an undulating calyx ridge with 5 rounded points (Fig. 2 D). The calyx differentiates and enlarges rapidly covering the other floral organs as they develop (Figs. 2 E and F). The calyx eventually forms an enclosure in which all other floral organs differentiate prior to anthesis.

Early in the development of the floral meristem 5 petal primordia are initiated alternately and centripetally to the 5 sepal primordia (Fig. 2 D) and additional petals develop from differentiating anther primordia (Figs. 2 G and H). At anthesis nearly all of the anthers have differentiated into petals. In Fig. 2 G, with sepals removed, the 5 petal primordia with the additional petal primordia which are differentiated from the anther primordia are

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illustrated. Anther primordia are initiated in a pentagonal whorl alternate to the petal primordia and opposite to the sepal primordia (Figs. 2 D and E). The carpel primordium is also initiated as a circular whorl and then differentiates into an apparent bicarpellate primordium leaving the placenta primordium in the center (Fig. 2 E to G).

The stages described have been selected because they demonstrate the morphogenic events of the shoot apex during differentiation. They do not necessarily represent developmental phases equally spaced in time.

Application of the SEM to the study of fresh shoot apices of carnation provides a rapid means of determining the gross morphology of each stage of floral initiation and development. The leaf primordium of carnation is initiated as a single whorl rather than opposite primordia on the flanks of the dome. Flower primordia are initiated centripetally and flower initiation is easily recognized as a broadened apex with a circular whorl of sepal primordia. Use of such a technique on other horticultural crops should provide a valuable tool for studying developmental changes resulting from environmental, cultural, or chemical treatments.

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Fig. 1. Scanning electron microscope micrographs of fresh, dissected apices of carnation showing the time sequence of desiccation of vegetative and reproductive shoot apices due to the electron beam and hard vacuum. A. Vegetative apex showing leaf primordia (lp) and spherical dome shaped apex (d). Taken at 5 min. B. Same apex after 10 min showing severe wrinkling of the dome (d) and leaf primordia (lp). C. Floral apex showing petal (p), anther (a) and gynoecium primordia (gy) taken at 5 min. D. Same apex at 10 min. E. Same apex at 15 min. F. Same apex at 20 min. Wrinkling is obvious on anther primordia (a). White bar represents approximately 100 microns.

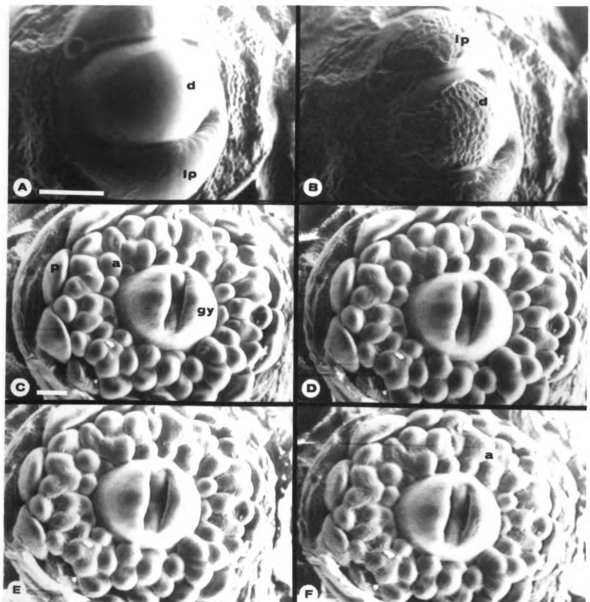
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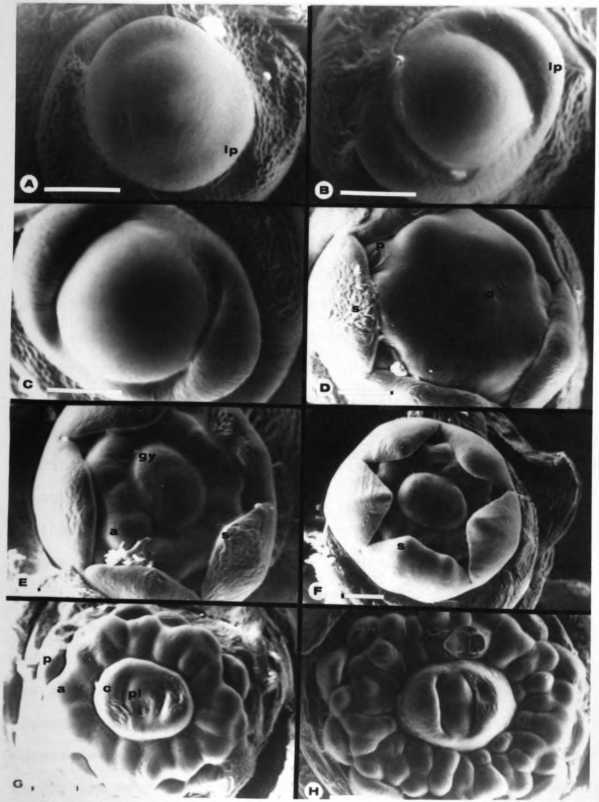
Fig. 2. Scanning electron microscope micrographs of fresh, dissected apices of carnation showing a developmental sequence taken after 5 min in the SEM. A. Vegetative apex in early plastochron showing circular whorl of leaf primordia (lp). B. Vegetative apex in mid-late plastochron with leaf primordia (lp). C. Vegetative apex in late plastochron. D. Reproductive apex with flattened dome (d), sepal (s) and petal (p) primordia. E. Reproductive apex with expanding sepal (s) and anther (a) and gynoecium (gy) primordia. F. Later stage with sepals (s) covering much of the floral apex. G. Similar to F with sepals removed showing petal (p), anther (a), carpel (c) and placenta (pl) primordia. H. Floral apex showing developing floral organs with sepals removed. White bar represents approximately 100 microns.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REVIEW OF LITERATURE

Concepts of Shoot Apices

The shoot apex is defined as the distal region of the shoot comprising both the apical initials (apical meristem) and the subjacent regions of expansion and maturation. This implies a heterogeneous and complex group of tissues (Wardlaw, 1968). The protomeristem is defined as the initiating cells and their most recent derivatives (Jackson, 1953). The partly differentiated tissues are defined as protoderm, which differentiates into the epidermis; procambium or provascular tissue, which becomes the primary vascular system; and the ground meristem, which becomes pith and cortex (Esau, 1965). The promeristem of Clowes (1961) refers only to the apical initials. The term growing point is commonly used as a synonym for shoot apex (Foster, 1939). Since Wolff (Esau, 1965) first recognized the shoot apex in 1759 scientists have attempted to describe the apical meristem region by cell groups or zones to explain its organization and growth. The apical cell theory was the first widely accepted concept of apical growth (Sinnot, 1960). This was superseded by the histogen theory of Hanstein (1868). This theory has been reviewed extensively (Foster, 1939; Gifford, 1954; Romberger, 1963; Cutter, 1965). The theory states

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the body of the plant arises from three histogens. The dermatogen is the primordial epidermis, the periblem is the early cortex, and the plerome is the inner axis. The dermatogen and periblem form a mantle-like layer over the plerome (Esau, 1965). The histogen concept was not generally accepted because of the implication of a predetermined origin of cells and tissues from the apical meristem (Esau, 1965).

The tunica-corpus theory (Schmidt, 1924) for angiosperm apices described two tissue zones. The tunica is the outer zone of layered cells, one to four cells thick and the corpus, a core of unlayered cells. Anticlinal divisions and surface growth predominate in the tunica while the corpus has planes of divisions in many directions for mass increase. Reeve (1948) stated the theory does not imply that the zones produce specific organs as the histogen theory does but rather descirbes a common type of shoot apex organization for angiosperms. The concept of the tunica is varied and Popham (1951) stated that the tunica should have no periclinal divisions. Reeve (1948) and Gifford (1954) stated that Schmidt's original definition included a small fraction of periclinal divisions. Popham and Chan (1950) suggested the use of mantle for the tunica with periclinal divisions and core for corpus as a less restrictive terminology.

Majumdar (1942) presented a discussion on cytohistological zonation in the angiosperm apex. Later Boke (1941)

documented the frequent occurrence of a zonal structure overlying the tunica-corpus organization in Trichocereus and Opuntia. Romberger (1963) pointed out the tunica-corpus and cytohistological zonation can be complimentary rather than antagonistic. The use of cytohistological terminology does not exclude the tunica-corpus organization.

Popham and Chan (1950) applied the terminology of zonation to the angiosperm apex using Chrysanthemum morifolium as a model. They have described five zones as follows: I. mantle layers; II. central mother cell zone; III. cambium-like zone (not always present); IV. rib meristem; V. peripheral zone. The peripheral zone may be called flank meristem (Esau, 1965).

Buvat (1952, 1953, 1955) suggested an organization of the angiosperm shoot apex where the summit areas of the vegetative apex are inactive and the histogenic activity is subapical. This concept is commonly referred to as the French schools theory and has been interpreted as follows: the summit area is called the waiting meristem (meristeme d'attente); the peripheral zone becomes the initiating ring (anneau initial); and the inner zone the medullary meristem (meristeme medullaire) (Esau, 1965). This concept is not generally accepted and has been severely criticized (Wardlaw, 1957a) mainly because the summit area does have cell division and is the ultimate source of all cells in the shoot.

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Wardlaw (1957b) proposed a five region organization of the shoot apex based on morphogenic activity. The distal region was the summit of the apical meristem; the subdistal region was below containing growth centers or induced areas but no morphological changes; then the organogenic region where primordia initiation was obvious. This area was followed by the subapical region with primordia enlargement, widening of the axis, differentiation of the vascular tissue, and elongation of internodes. Finally the region of maturation where morphogenic patterns initiated and developed were fixed.

Leaf Organogenesis

Turing (1952) proposed a diffusion-reaction theory which attempted to explain growth centers and primordia initiation in the subdistal region on a physical chemistry basis. Wardlaw (1953) interpreted Turing's theory as a localized accumulation of metabolites distributed non-randomly which became growth centers and initiated primordia.

Foster (1939) stated that the leaf primordia were apparent outgrowths from the tunica and outer corpus. In cytohistological zonation the primordia arise from the peripheral zone (Popham and Chan, 1950) and in the morphogenic pattern the growth centers were organized in the subdistal region and became visible in the organogenic region (Wardlaw, 1957b).

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Several theories have been proposed to describe the phyllotaxic pattern developing from these zones or regions. Bunning (1956) proposed the "repulsion theory" which states a mutual incompatibility of growth centers of the same type. Romberger (1963) suggested that in a developing growth center an enzyme system may become very active which would deplete the substrate in the surrounding area. The depletion could prevent additional growth centers from forming nearby. Therefore a new primordia would arise at a point most distant from the last formed primordia. The "first available space theory" (Snow and Snow, 1947) is more specific than the repulsion theory in that the exact position of the primordia depended only on those leaves already formed. All tissue in the apex can form leaf primordia, however, this leaf forming tendency is inhibited by the previously formed primordia. A large enough space must become available for new primordia to form.

The "excessive apical surface growth" theory presented by Priestly (1928) held that the outer surface of the apex grew faster than the interior resulting in folds. Snow and Snow (1947) reasoned that if this theory were true small cuts in the apex would remain closed by compression, however, they found cuts opened suggesting the outer layers of the apex are under tension rather than compression.

The "prior procambial development" theory advanced by Ball (1948) and Snow and Snow (1948) stated that procambial

strands were initiated before the primordia was formed implying some substance was transported by the strands affecting the growth centers and primordia initiation. These workers found that growth centers isolated from the procambium by incisions continued to develop normal primordia.

The theory of "foliar helices" as described by Cutter (1959) suggested that leaves arise along helices of some leaf forming substance or impulse. As primordia are initiated the growth centers move upward in a helical path ending in the peripheral zone.

The Reproductive Shoot Apex

In the change from vegetative growth to reproductive growth the reproductive apex replaces the vegetative apex either directly or by the development of an inflorescence (Esau, 1965).

Philipson (1949) and Gifford (1954) have extensively reviewed this change. The first noticeable difference was a broadening of the apex. In some species the tunica layers increased or decreased in number with flower initiation. The distribution of eumeristematic cells and more highly vacuolated cells often changed with a small dense layer of cells forming a mantle over a vacuolated core. This mantle usually includes the tunica and outer corpus and meristematic activity seemed to be located in that zone.

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In cytohistological zonation the zonation disappears into the inflorescence (Popham and Chan, 1950), however, the reproductive apex up to the stage where floral primordia were recognized may exhibit zonation of the vegetative type (Vaughan, 1955; Esau, 1965).

These observations and interpretations suggest that the change from vegetative to reproductive meristems is a gradual change of the existing meristem (Gifford, 1954).

The French school of thought which proposed a waiting meristem organization in the vegetative apex has been discussed by Esau (1965). This concept stated that the distal region of the apex was inactive during vegetative growth but became active during reproductive growth and in essence grew out and replaced the vegetative meristem. Esau (1965) stated that this concept implied a functional discontinuity between the vegetative and reproductive apex. Wardlaw (1957a) stated that current evidence on the transition to flowering could not support such a concept.

Histochemical Studies of Shoot Apices

Gifford and Tepper (1961, 1962a,b) studied the histochemical changes in the shoot apex of *Chenopodium album* during flower initiation. They observed an increase in RNA in the cells of the summit region about two days after the first long night and a subsequent increase in histones and protein in the same region. Gifford (1964) further

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demonstrated an increase in histones three days after the inductive night with Xanthium.

More recently Corson and Gifford (1969) studying Datura reported changes in histones, RNA and total protein in all zones of the apex during the change from a vegetative to a transitional meristem. The axial cells had a lower concentration of RNA than the peripheral cell in both the vegetative and transitional apices.

In Lilium temulentum, Knox and Evans (1966) found an increase in RNA as the first evidence of flower initiation followed by an increase in size of the nuclei and nucleoli. Histone staining was constant over time and followed the DNA staining pattern. Starch staining with iodine-potassium iodite or the periodic acid Schiff's reaction was negative in the apex and subapical region.

Kinet, et al. (1967) found increased mitotic activity in apices after floral induction and suggested this rapid appearance of mitotic figures was due to sudden release from the G_2 phase, the post-DNA synthesis period in the mitotic cycle. Later Bronchart, et al. (1970) showed a parallel increase in mitotic activity with greater incorporation of ^{3}H-uridine in induced apices indicating RNA synthesis. Electron microscope microautoradiographs showed a differential RNA synthesis pattern with more synthesis occurring in the cytoplasm than in the nucleus of the induced apices.

Watson and Matthews (1966) studying Chenopodium with actinomycin D speculated on the possibility of a "floral messenger RNA" or a new RNA component produced at flower induction. Bronchart, et al. (1970) showed an increase in the chromatin-nucleolus ratio. Furthermore they found that exogenous applications of 2-thiouracil inhibited flower initiation most strongly when the chromatin-nucleolus ratio was highest.

Thorpe and Murashige (1969) noted a heavy accumulation of starch in shoot-forming tissue of tobacco callus culture with no difference in cell wall polysaccharides. Sadik and Ozbun (1967), in addition to finding an increase in nucleolus size with induction, found cauliflower had little starch present in non-induced vegetative apices but after 1 week of cold, starch accumulated especially in the subapical zones. They suggested starch may be important in the flowering process.

Salisbury and Ross (1969) summarized the histochemical data of flowering by stating "The importance of these changes to flowering remains unclear, but an activation of the DNA-RNA-protein system seem to be indicated".

Carnation Shoot Apices

Schnabel (1941) was the first to anatomically describe the shoot apex region of carnations. He noted during the plastochron a change in volume. He described the apex as normally having a two-layered tunica with anticlinal

divisions over a massive corpus with both periclinal and anticlinal divisions. Leaf initiation occurred on the flanks of the dome resulting from divisions in the corpus. Axillary bud primordia occurred at the third leaf pair from the apex.

Shushan and Johnson (1955) studied the lateral vegetative shoot apex of Dianthus caryophyllus. The dome of the apex which changes in volume was characterized as a half ellipsoid with minor and major axis. The average dimensions of lateral branch apices were minor axis, 135 µm, major axis, 155 µm and height 63 µm. The minor and major axis reoriented in line with leaf primordia initiation and a pair of foliar buttresses were produced at the expense of the major axis.

Shushan and Johnson (1955) further described the lateral vegetative apex as having a 0.5 µm cuticle and a biseriate tunica and corpus with no zonation evident in the corpus. Each tunica layer was about seven microns thick. They reported axillary branch primordia initiation at the next to youngest pair of leaf primordia.

Emino (1966) described four stages of shoot apex development in the carnation cultivar 'White Sim' as follows: 1. vegetative; 2. transitional, similar to the vegetative apex but wider; 3. early reproductive, a broadened apex with flower primordia beginning to be initiated; 4. late reproductive, flower primordia distinctly visible and their development leading to anthesis.

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This work was substantiated by Cheng and Langhans (1971) who studied the floral initiation morphology of the carnation cultivar 'Colorado White Pikes Peak'. However, they stated the petal, stamen and carpel primordia were initiated concurrently rather than centripetally.

Carnation Flowering

The effect of light on flower initiation and development in carnations has only within the past ten years been satisfactorily explained yet disagreement still exists on these factors (Laurie, et al., 1967; Holley and Baker, 1963).

Arthur and Harvill (1938) found that carnations flowered better in lighted greenhouses. Post (1942, 1949) reported that additional light hastened flower development, but had no effect on initiation. Hanzel, et al. (1955) reported that flower initiation was not affected by additional light and higher temperature.

Blake and Spencer (1958) demonstrated that up to the time of flower bud initiation daylength is the important environmental factor affecting flower initiation. After flower initiation takes place temperature is the most important environmental factor.

Originally the carnation was a long day plant but breeders have selected varieties with a year-round flowering habit and now is considered a facilitative long day plant that is able to flower under any daylength (Blake,

1955). Carnation plants grown under short days have more nodes than plants grown under long days indicating flower initiation was delayed by short days (Blake, 1955; Harris and Harris, 1962).

Blake and Harris (1960) showed long days promoted flower initiation. Harris and Griffin (1961) demonstrated that two hours of low intensity light during the middle of the night had the same effect on flower initiation as a 16 hour photoperiod. The effect of additional light was too small for commercial application (Blake and Whitehead, 1961). Harris and Asford (1966) found pronounced promotion of flowering when plants were grown in daylengths of up to 24 hours for a period of six weeks. Harris (1967) demonstrated that additional light could be used to increase commercial production. Butterfield, et al. (1968) demonstrated a 75-100% increase in production with artificial light. Harris (1968) showed artificial light high in far-red was most effective in promoting flower initiation.

Emino (unpublished data) found by noting initiation in random samples from an eight hour day, a control normal day and two hour extended day that flower initiation took place about two weeks earlier than normal in the two hour extended day treatment and two weeks behind the control in eight hour day treatment. Peak flower production from each treatment was two weeks early for long days and two weeks behind for the short day compared to the control confirming Blake and Spencer's (1958) work.

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The Chabaud Carnation

The Chabaud carnations, a seed grown garden type, are true breeding doubles (Saunders, 1916). The gene for doubleness is on a different loci than that used for commerical cultivars and provides male fertile doubles.

Cytology

The Chabaud and commercial types of carnation have been investigated cytologically and have a 2n chromosome number of 30 (Andersson-Kotto and Gairdner, 1931; Mehlquist, 1945; Mehlquist and Geissman, 1947; Darlington and Wylie, 1955 and Carolin, 1957).

Grafting and Flower Initiation

Melchers (according to Wardlaw, 1968) first studied the effect of grafting on flowering. Using annual and biennial forms of *Hyoscymus niger* he demonstrated that if scions of the annual type were grafted to plants of biennials in their first year, flower initiation took place in the biennial plants. Also if first year biennial scions were grafted to an annual plant flower induction took place in the scion. These results indicated there was some transmissable substance inducing flower initiation.

Zeevaart (1958, 1962, 1966) and Zeevaart and Lang (1962) using *Perilla*, *Kalanchoe*, *Sedum*, *Bryophyllum*, and *Nicotiana* demonstrated that there was transmission of an inducer of flower initiation across a graft union, that

is, plants that were induced to flower transmitted this induction to a receptor plant, not induced, resulting in flower initiation.

Origin of the Pine Cone Carnation

The Dianthus caryophyllus Chabaud pine cone mutant used in this study was obtained from Franklin J. Campbell, Asst. Prof. of Floriculture, Waltham Field Station, University of Massachusetts in 1964. Campbell (1965) stated that this plant grew as a seedling from a commercially prepared seed mixture of the Chabaud type.

Masters in 1869 described a similar carnation as follows: "where the floral axis is prolonged, and produces from its side a successive series of sepals, as is what is called the wheat-ear carnation".

Of the several carnation abnormalities described by Worsdell (1916), two are of interest to this study. The first is sepalody where all flower parts were absent except for sepals spirally arranged and greatly increased in number. The second abnormality is referred to as bractelody where all the organs of the flower are absent except for the bracts which are concentrated in great numbers on an elongated floral axis. Worsdell (1916) referred to this phenomenon as bracteomania. This author's observations on the pine cone carnation concurs with the description and illustration by Worsdell (1916).

Holley and Baker (1963) mentioned a mutant which does not develop normal flowers but produces bract-like appendages resembling a green pine cone. None of the descriptions of carnation abnormalities mentioned above included anatomical studies. Emino (1966), studying the present mutant, made histological sections and found the apex to be similar to bract initiation in normal development.

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MATERIALS AND METHODS

Plant Material

Dianthus caryophyllus L. Chabaud carnation is the garden type, propagated from seed and adaptable to greenhouse culture. This carnation type was chosen for two reasons. First, it is similar to commercial carnations but its flowering time is shorter, making it a more desirable experimental plant. Second, the non-flowering teratological pine cone mutant is of this type and similar types are desirable for comparative research.

Seeds of the Chabaud carnation were sown in vermiculite, covered with cheesecloth and germinated at 60°F on a greenhouse bench. Seedlings were used in the cytological study and for the shoot apex development study of the normal Chabaud type. For comparative study with the mutant two plants were selected from the seedling population.

These two selected plants and the non-flowering pine cone mutant were propagated vegetatively by stem tip cuttings. Cuttings were made by snapping off terminal growth and placing the cuttings in flats of vermiculite until rooting took place. The cuttings were covered with cheesecloth and syringed with water daily. After rooting the cuttings were placed in sterilized three inch pots and

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pinched leaving two to three nodes. The growing media consisted of one part sterilized soil, one part peat, one part sand, and one part perlite. Plants were fertilized biweekly with 20-20-20 soluble fertilizer at the equivalent rate of 30 ppm nitrates Spurway.

In most studies plants were pruned to remove all lateral branches except the one to be utilized for sampling or flowering studies. In some studies plants were placed in larger pots and several branches allowed to develop.

Experimental Material Characterization

The two flowering clones of the Chabaud carnation and the pine cone mutant are illustrated in Figure 1. Eleven plants of each clone were grown to flowering and the flowers dissected at anthesis and the number of flower parts recorded. The experimental material characterization is presented in Table 1. The normal flowers were typical double Chabaud flowers at anthesis with two bract pairs subtending the flower, five sepals, about 30 petals, 0-3 petaloid structures (intermediate between petals and stamens, but neither), about 30 anthers, quadralocular ovary, and four stigmas and styles (Figure 1, A and C). The non-flowering pine cone mutant is made up entirely of bracts with no flower parts (Figure 1 B). The two normal flowering clones are designated as N-15 and N-30 and the pine cone mutant as C-2.

Figure 1. The three Chabaud clones of carnation Dianthus caryophyllus L. used in this study. A. N-15, B. C-2 and C. N-30. Both N-15 and N-30 are normal flowering clones with two decussate bract pairs, five sepals, 30 petals, 30 stamens, and a quadralocular ovary. C-2 is a non-flowering teratological mutant called the pine cone mutant and only produces bracts.

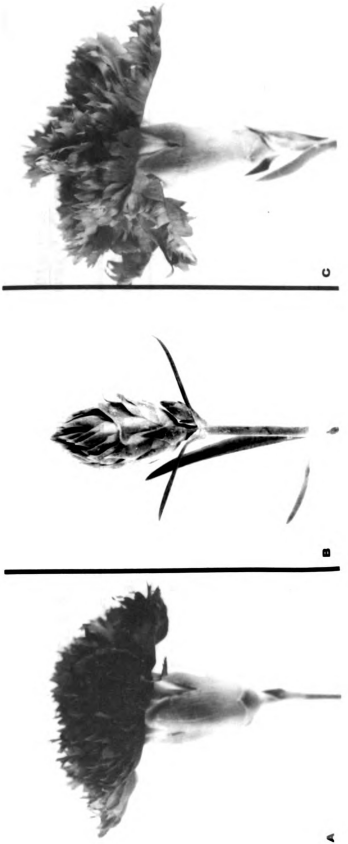
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Table 1.--Characterization of the three clones of Dianthus caryophyllus L. used in this study. Average* flower parts present in each clone.

Clone	Bract Pairs	Sepals	Petals	Petaloid Structures	Anthers	Carpels in Ovary	Stigma Styles
N-15	2	5	35.55	2.73	34.82	4	4.27
C-2	Many	0	0	0	0	0	0
N-30	2	5	29.91	1.82	30.27	4	3.73

*Eleven observations.

Shoot Apex Development in the Chabaud Carnation

Sampling of shoot tips started at seedling emergence and continued daily for five days then weekly for 12 weeks. Six samples were collected from the seedling population such that three could be orientated parallel to the cotyledons and three orientated perpendicular to the cotyledons during sectioning. The samples were killed and fixed in acrolein since it penetrates and fixes rapidly using a modified method of Feder and O'Brien (1968) as follows: The shoot apices were fixed in a freshly prepared 10% solution of acrolein in distilled water cooled to about 0°C in an ice bucket. The cold acrolein was carried into the greenhouse for sample collection.

The fixed specimens were transferred through two changes of 100% Methyl Cellosolve for 6-12 hours each at 0°C. The specimens were then transferred to 100% ethanol for 6-12 hours then to 100% n-propanol, 6-12 hours and finally to 100% n-butanol, 6-12 hours all at 0°C. The samples were brought to room temperature and transferred to 100% t-butyl alcohol (3 changes) and imbedded in Paraplast (Sherwood Medical Industries Inc., St. Louis, Mo.). Longitudinal sections were cut on a rotary microtome at 6 µm.

The sections were affixed to glass slides with Haupt's adhesive (Johansen, 1950), the paraffin was removed and the sections stained with Heidenhains Iron Hematoxylin (Sass,

1958). The sections were dehydrated and permanently mounted with a cover slip. Median sections were determined and identified for future reference. The seedling apex was measured (height and width) for comparison with apices from previous studies. The change in apex height and width over time, and the developmental morphology of the vegetative and reproductive apex was determined.

The developmental morphology of the non-flowering pine cone mutant apex was determined using similar histological techniques.

Scanning Electron Microscope

Plants to be used for the comparative SEM study were grown in the greenhouse and selected plants pinched at weekly intervals. This technique provided specimens at various stages of growth so the topographic features of the apices could be related to apices studied histologically.

Samples were prepared for analysis with the scanning electron microscope by the technique of Einert et al. (1970) and by the technique of Emino and Rasmussen (1971) with minor modifications. Modifications included the replacement of Tube Koat with Silver Conductive Paint (G. C. Electronics, Rockford, Illinois). The silver paint was as effective as an adhesive and electrical conductor as Tube Koat with the additional advantage of making the freshly dissected shoot apex mounted on the carbon disc easier to locate through the light optics. This advantage speeded

the location of the specimen thus a photograph could be taken sooner with less damage due to the vacuum and resulted in a photograph more representative of the actual topographic features.

Recently Falk et al. (1970, 1971) has similarly used the scanning electron microscope in the study of fresh shoot apices of Tropaeolum.

Microscopic Histochemistry

General. The sample preparation procedure used for microscopic histochemistry is the same as that used for the shoot apex development study. Each staining series is given below along with modifications from the general procedure.

The plant material, representing the three clones, was sampled weekly over about a 14 week period or until flower buds were visible macroscopically. This provided a developmental, as well as comparative, sequence in which to interpret the microscopic histochemical data.

Starch. Starch was localized by the IKI procedure (Jensen, 1962). The basis of this reaction is the accumulation of iodine in the center of the helical starch molecule. Short molecules have more red color and long molecules more blue. As a result newly formed starch is generally red while older starch stains dark blue.

Insoluble Polysaccharides. Total insoluble polysaccharides was determined by the Periodic Acid-Schiffs' (PAS) reaction (Jensen, 1962). The basis of this reaction is the

formation of aldehydes by the oxidative action of periodic acid on the polysaccharides which form highly colored complexes with Schiff's reagent. Since the reaction depends upon the production of aldehydes, free aldehydes in other constituents interfere with the reaction (Jensen, 1962). Feder and O'Brien (1968) recommend a 24 hour pre-incubation in a saturated solution of dimedone which would tie up the free aldehyde making them unavailable for reaction with Schiff's reagent. This step was included in the procedure. As a control sections were placed directly in Schiff's bypassing oxidation with periodic acid. These sections did not take on stain.

DNA-Feulgen Method. Deoxyribonucleic acid was localized by use of the procedure of Gomori (1952) with minor modifications. This procedure is based on the Schiff's reaction. The insoluble polysaccharides do not stain because periodic acid has been omitted from the procedure. The tissue is treated with warm HCl which hydrolyzes the purine-deoxyribose linkages giving free aldehyde groups for reaction with Schiff's reagent (Jensen, 1962). Dimedone was employed as a free aldehyde blocking agent (Feder and O'Brien, 1968). This reaction is quantitative, thus the color produced is proportional to the amount of DNA present. As a control for the reaction hydrolysis was bypassed. These sections did not stain.

RNA-Pyronine B Method. Ribonucleic acid was localized by staining with Pyronine B (Jensen, 1962). Because of the phosphates present, nucleic acids have a tendency to bind to basic dyes at acid pH.

As controls, sections were placed in 0.5 N perchloric acid at 70°C for 30 min to remove DNA. Other sections were placed in 1 N perchloric acid for 12 hours at 4°C to remove RNA. This extraction removed most of the pyronine B positive material resulting in a faint staining in the cell wall.

DNA-RNA. The Azure B method as used by Flax and Himes (1952) was combined with differential enzymatic extraction of DNA and RNA (Brachet, 1953). Brachet (1953) described the use of deoxyribonuclease and ribonuclease for removal of these cell components from histological sections. These enzymes are highly specific and provide good controls for histochemical localization of DNA and RNA. Modifications of the general procedure are: acrolein fixed apices were imbedded in paraplast and sectioned at 10 microns. Specially prepared Haupt's without a preservative was used as an adhesive.

As controls sections were placed in distilled water adjusted to pH 6.5 for one hour to measure the effect of pH on nucleic acid removal (Jensen, 1962). Also sections were placed in both enzyme solutions as described and no stain was taken up.

Histones. Protein high in basic amino acids was studied by the Fast Green test of Alfert and Geschwind (1953). Tissue chemically fixed with either acrolein or FFA was sectioned at 10 microns. Nucleic acids, the only substances known to interfere with this otherwise specific reaction, were removed by extraction with 15% trichloracetic acid for 15 min (Jensen, 1962).

Fresh Weight-Dry Weight Changes

Plants grown in three inch pots were arranged randomly on the greenhouse bench. Weekly, three plants were randomly selected of C-2, N-15, and N-30 and total height and fresh weight were recorded. Dry weight was recorded after plants were dried in an oven and three consecutive weighings over a three day period were constant.

Cytology

Chromosome counts were made of Dianthus caryophyllus L. Chabaud type as follows: actively growing shoot tips of mutant and normal plants were harvested and placed in 3:1 mixture of 95% alcohol and glacial acetic acid for 30 min (Jensen, 1962). Shoot tips of the normal Chabaud carnation were randomly collected from a population of 35 seedling plants. The acetocarmine method of Darlington and LaCour (1960) gave very poor preparations. The Feulgen method was tried with greater success. The tissue was removed from the fixative to 70% alcohol, transferred to 1 N HCl at 60°C for

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15 min, washed with distilled water, and stained in Schiff's reagent for 30 min. Then the tissue was placed on a slide with 45% acetic acid, macerated, flattened, heated, and sealed with vaseline. In order to get good resolution of chromosomes the preparations were examined with phase contrast microscopy. Ten mitotic figures were counted for the pine cone mutant and the seedling group. Photomicrographs were taken where chromosomes appeared close to being on the same focal plane. A matt and glossy print were made for each figure, then using the original figure and the glossy print an interpretive drawing was made on the matt.

Grafting Studies

In an attempt to alter the pattern of primordia initiation and differentiation in the pine cone mutant reciprocal grafts were made with the two normal flowering clones. Rooted cuttings of these three selected clones of carnations were taken from the vermiculite rooting media and grown in sterilized three inch clay pots in a medium of one third sterilized soil, one third perlite, one third peat. At the time of potting each plant was pinched to the second node forcing lateral growth. One shoot was selected for grafting studies, the remaining side shoots were removed. When three to four internodes had elongated wedge grafts were made followed by wrapping the graft with masking tape. Stretched floral tape was also used with similar success but was more difficult to apply.

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Reciprocal grafts were made by cutting selected plants in the internodal region of the third or fourth expanded node with a new razor blade. The razor blade was dipped in 95% ethyl alcohol between cuts for sterilization. The stock was cut perpendicular to the stem and a slit about one cm long was made longitudinally in the stem. The scion was cut in an extended v-shape and inserted into the stock. The union was immediately wrapped with a single layer of masking tape and the plant tied to a stake and covered with a plastic bag. The plants were placed pot to pot with two layers of cheesecloth placed over the plastic bags to reduce heat buildup.

The plastic bags were removed after ten days and the plants randomly spaced with four inches between pots and grown at 60°F on a greenhouse bench. Only the terminal shoot apex was allowed to develop by removing all lateral branches from the stock and scion during the course of the experiment.

Reciprocal graft combinations were as follows: C-2/C-2, N-15/C-2, N-30/C-2, N-15/N-15, N-30/N-30, C-2/N-15, C-2/N-30. Each graft combination was replicated six times.

Observations were made on the plants at anthesis for scions N-15 and N-30 and at the anthesis of the last replication of the normal scion for the mutant C-2. Comparisons were made only between reciprocal grafts of one kind, i.e. N-15/C-2, N-15/N-15, and C-2/C-2, etc. Data recorded were

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number of nodes above and below graft, bract pairs, sepals, petals, anthers and general notes on plant development.

Growth Regulator and Environmental Studies

Because of the success of Wittwer and Bukovac (1962) and Pike and Peterson (1968) using gibberellin application to induce maleness in cucumbers and the influence of cytokinin in promoting femaleness in several species (Heslop-Harrison, 1963) a series of exogenous growth regulating compounds were applied to the pine cone mutant carnation in an attempt to alter the pattern of primordia initiation and differentiation. An experiment was designed where GA_3 and BA was applied to the root system of the pine cone mutant carnation. Rooted cuttings growing in vermiculite were transferred to styrofoam supports suspended over a one gallon container containing an aerated one half Hoagland solution (Hoagland and Arnon, 1950) in the greenhouse. Levels of GA_3 used were 10^{-6}, 10^{-7}, and 10^{-8}M and BA 10^{-6} and 10^{-7}M. There were 11 treatments replicated three times arranged in a complete randomized block design with four observations per treatment (four plants per jar). This resulted in a total of 36 treatments including the control. Solutions were changed every 10 days. The staked plants were pruned to allow only one stalk to grow per plant. Treatments were applied for 14 weeks.

Pike and Peterson (1968) found spray applications of 50 ppm gibberellin A_4/A_7 significantly induced a greater number of staminate flowers than GA_3 at 1000 ppm. The experiment was repeated using GA_4/GA_7 mixture in place of GA_3.

Two plants in one of the three replications growing in a mixture of 10^{-6} BA and 10^{-6} GA_4/GA_7 showed an unusual amount of differentiation of stigma-like structures. Because of these results this part of the experiment was repeated twice with no effect.

The remainder of the experiments were conducted by spraying the plant surface with a measured amount of growth regulating compound.

Preliminary observations with PBA (SD-8338) (Shell Development Co., Modesto, California) showed some differentiation of stigmoid structures. This experiment was repeated using SD-8338 in combination with IAA and GA_3. All combinations of 0, 10, 100 and 1000 ppm were used in a completely randomized design with rearrangement. Plants were grown single stem in a 3 inch pot on the greenhouse bench. Temperature was maintained at approximately 55°F. Each treatment was replicated three times. Differentiated buds were compared by viewing free hand sections through a dissecting microscope. Selected buds were chemically fixed in FAA and sectioned at 10 µm on a rotary microtome for further study. Weekly spray application was made using a

Son-Of-A-Gun sprayer (Ampco, Broomfield, Colorado). Five ml of solution containing 0.1% Tween 20 was used per plant. Plants were removed from the growing area to be sprayed to avoid drift. This experiment was repeated once. An additional spray study involving ethephon, IAA, GA_3, and BA was conducted. As before a completely randomized design with rearrangement was used. Solutions of 0, 10, 100, and 1000 ppm were used in combination for each treatment which was replicated twice using a total of 513 plants. Plants were observed as previously described.

Plants of the pine cone clone were grown under varying environmental conditions in several experiments to further attempt to alter the pattern of primordia initiation and differentiation. Plants were grown in four inch pots under 8 hour and 16 hour days for 3 weeks. Ten plants were removed from the 60°F night greenhouse to a cold room at 35°F for 14 hours daily for four weeks. After this time plants were maintained under the 8 hour and 16 hour day-length regime. Shoot apices were free-hand sectioned with a razor blade and viewed through a dissecting microscope.

Disc Gel Electrophoresis

The results of the histochemical studies indicated further work needed to be done on the enzyme systems for starch synthesis and degradation. Selected stems of the three Chabaud clones were brought into the lab and one gram of fresh weight of stem tissue including the apical meristem

with the leaves removed was chopped into discs about one mm in length and placed on ice in 10 ml of cold Hepes buffer (Good et al., 1966) pH 7.1-7.2 containing 0.1 mm dithiothreitol. The protein extraction procedure was modified from McCown, et al. (1968). All protein extraction was carried out at 70°F. The tissue and buffer were placed in a pre-chilled mortar along with one gram of insoluble PVP (Polyclar-AT powder) and one gram grinding sand. The tissue was ground for five min or until the tissue was completely macerated. The resultant slurry was strained through Mirrorcloth (Chicopee Mills Inc., New York, N.Y.) into a centrifuge tube. The sample was centrifuged for 30 min at 20,000 x g and the supernatant was decanted into a test tube and stored at 4°C until used.

The extract containing from 400 to 600 micrograms of protein was applied to each gel and gave acceptable band resolution. The protein in the extract was routinely estimated by the Folin reaction according to Lowry, et al. (1951) using bovine serum albumin as the standard.

The electrophoresis procedure was a modification of Davis (1964), the alternate procedure was used where the sample was layered above the gel rather than using a sample gel. A chilled upper bath buffer was used for layering over the protein. Running time took about three hours and the current (4 ma/tube) was turned off when the bromophenol blue tracking band was within one cm of the bottom of the gel.

Gels were immediately placed in a 0.5% solution of buffalo blue black made up in 7.0% acetic acid for 30 min. The gels were destained electrophoretically with 7.0% acetic acid circulated through a charcoal filter and 12 volts potential applied.

Gels were stored in 7.0% acetic acid, photographed with Polaroid P/N film (type 55) with a green filter to enhance contrast. R_f values were calculated using the tracking band as R_f 100 and measuring to the middle of the various bands with a vernier caliper.

Enzyme Identification of Electrophoretically Separated Protein

Amylase bands were qualitatively identified using a modified procedure of Macko et al. (1967). Hydrolyzed starch 0.1% was included in the water fraction of the gel before electrophoresis. After separation the gels were incubated in a solution of 0.1% iodine in 0.5% KI in a 0.2M acetate buffer at pH 4.8-5.0. Bands were observed by negative staining.

After electrophoretic separation the gels were immersed in a 0.02M solution of guaiacol for 30 min, washed in distilled water and incubated in 0.1% H_2O_2 for band development of perioxidase isoenzymes. No bands were resolved using guaiacol. Gels were immersed in a saturated 80% alcoholic solution of benzidine mixed with an equal volume of 0.1%

H_2O_2 for band development of perioxidase isoenzymes. Banding appeared within 30 min.

Starch phosphorylase was determined using the technique outlined by Jensen (1962). Gels were placed in a 1.0M solution of glucose-1-phosphate in acetate buffer pH 6.0. Gels were incubated for two hours at room temperature then transferred to IKI solution for color development. Where starch was synthesized banding should appear. Gels were stored in 7.0% acetic acid.

Starch Synthesis and Degradative Enzymes-Quantitative Determination

Starch synthesis and degradative enzymes were studied by a modified technique of Machlis and Torrey (1956). Plant material was prepared as previously described for disc gel electrophoresis. Phosphate buffer (0.1M, pH 6.2) was used in place of Hepes buffer which interfered with IKI color reaction. Extracts were kept in an ice bath until use. Potato tissue was employed as a control.

The incubation medium for starch synthesis was as follows: 0.1 ml (10 µg) ATP in 0.1M phosphate buffer at pH 6.2; 0.01 ml starch at 0.2%; 0.3 ml 1.0% Glucose-1-Phosphate, and 0.2 ml of the appropriate extract at 0 time. The reaction was allowed to run for 0, 15, 30, 60, 90, 120, 150, and 180 min in a water bath at 30°C. A standard of 75 mg/ml starch was included. The reaction was stopped by the addition of 1.0 ml of IKI made according to Chrispeels and

Varner (1967) by diluting 1.0 ml of iodine stock solution (6 µg of KI and 600 mg of iodine dissolved in 100 ml water) to 100 ml with 0.05 N HCl. Five ml of water was added to each tube and mixed well. OD was read at 620 mµ against a reagent blank minus starch.

For starch degradation 0.2 ml of extract was added to 0.3 ml of 0.2% starch at pH 6.2 in phosphate buffer. The reaction was allowed to run as in the previous experiment. The reaction was stopped as before with IKI and diluted with 5.0 ml water, mixed and read at 620 mµ.

Because of the difficulty with some extract reducing the IKI indicator a modification suggested by Varner (Bienvenido and Varner, 1969) was used. This included the use of 1.5 ml iodine solution and 4.5 ml of H_2O instead of 1.0 ml iodine and 5 ml H_2O.

To determine if inhibitors of starch synthesis were present in the carnation extracts aliquots of the carnation extracts were combined with the potato extracts (or distilled water as a control) and run as described above for starch synthesis.

Analysis of Sugar and Starch

Starch and sugar were quantitatively determined by a modification of the colorimetric technique of McCready et al. (1950) and Clegg (1956) based on the anthrone reaction. Plant material was selected in the greenhouse and placed on ice. One gram fresh weight of stem tissue including the

apical meristem with leaves removed was chopped into discs about one mm in length and ground in a mortar with 4.0 ml of hot 80% ethanol for five min. The mixture was placed in a centrifuge tube and stirred. The mortar was rinsed with 2.0 ml ethanol twice and added to the original extract. After standing 5 min the sample was centrifuged 10 min at 13,300 x g. The supernatant was carefully decanted into a flask. Six ml of 80% hot ethanol was added to the residue, stirred and centrifuged as before. The residue was washed an additional two times with hot 80% ethanol and the washings combined.

The combined sugar extract was washed with 40 ml of petroleum ether in a sepatory funnel to remove the chlorophyll. The sugar remained in the alcoholic fraction. After 20 min the sugar solution was separated from the petroleum ether fraction and brought to 25 ml with H_2O prior to analysis.

Five ml of H_2O was added to the sugar extraction residue in the centrifuge tube followed by 6.5 ml of 52% perchloric acid. The mixture was stirred occasionally for 20 min and then 10.0 ml of H_2O was added and the mixture was centrifuged at 13,300 x g for 10 min. The supernatant was transferred to a 50 ml volume flask and the residue again treated with perchloric acid. After 20 min the sample residue and solution was washed into the 50 ml flask, brought to volume with H_2O and filtered. The first 5.0 ml of filtrate

was discarded. The solubilized starch was placed on ice until assayed.

The determination of starch and sugar was carried out by taking duplicate aliquots of 0.1 ml sugar extract from each sample and 1.0 ml of starch extract from each sample and placed in test tubes. Five ml of anthrone sulfuric acid made according to McCready et al. (1950) was added to each sample aliquot, stirred and heated in a boiling water bath for 7.5 min. The samples were quickly cooled in an ice bath to 21°C and read at 630 μm using a K-64 filter on a colorimeter. A glucose standard (Clegg, 1956) was routinely run with the samples. Glucose or starch in micrograms/gram fresh weight were plotted graphically. Glucose readings were multiplied by 0.9 to convert to starch since 0.9 g starch yields 1.0 g of glucose on hydrolysis (Clegg, 1956).

Thin Layer Chromatography of Sugars

The extraction procedure for sugars used for thin layer chromatography was the same as for the quantitative sugar and starch determination. The combined 80% ethanol extracts obtained from the extraction procedure were evaporated to dryness in a rotary vacuum evaporator and redissolved in 2.0 ml of 80% ethanol. The residue was redissolved first in 1.0 ml than 0.5 ml and a final 0.5 ml for three washings totaling 2.0 ml.

The thin layer chromatographs were made according to Stahl (1969) and Lewis and Smith (1969). Silica gel G, Cellulose, and Avicel (Analtech, Inc., Wilmington, Del.) prepared thin layer plates at 250 microns thickness were tested for separation in several solvent systems. Silica gel G was found to be best for sugar separation with the following solvent systems: Methyl ethyl ketone-glacial acetic acid-methanol (3:1:1; v/v/v) (Shellard and Jolliffe, 1969) and isobutyric acid-ammonium hydroxide-water (66:1:33; v/v/v) (Stahl, 1969).

Extracts (25 µl) were applied to 2.0 cm wide etched strips and the plates placed in a saturated chromatography chamber. For methyl ethyl ketone-acetic acid-methanol the chromatograms were developed without equilibration since the solvent system was not aqueous. With isobutyric acid-ammonium hydroxide-water the chromatograms were equilibrated by leaving them in the closed saturated developing chamber overnight (12 hr) before developing. Developing time was about 30 min and 90 min respectively at 22°C.

After the solvent front reached 10.0 cm the chromatogram was removed from the chamber and air dried. Visualation of sugars was made by spraying with either anisaldehyde-sulfuric acid or benzidine-trichloracetic acid (Stahl, 1969) and heating at 110°C for 15 min. R_f values were calculated by measuring from the center of each band.

Known sugars of 10^{-2}M were applied in an attempt to identify the unknowns in the extracts. The following were used: Mannose, fructose, sucrose, glucose, galactose, xylose, maltose, raffinose, glucose-1-phosphate and glucose-6-phosphate. Additional spray reagents were applied to the unknowns for clues to their identity such as ninhydrin-cupric nitrate for amino sugars.

Incorporation of Labeled ^{14}C Glucose-1-Phosphate Microautoradiography

Since the pine cone mutant had more starch than the normal clones it was hypothesized that glucose-1-phosphate would be differentially incorporated into starch in the mutant, indicating a more active starch synthesis enzyme system. To test this hypothesis, uniformly labeled ^{14}C glucose-1-phosphate, dipotassium salt was placed in a vial at the rate 3.0 microcuries per ml. Three cuttings approximately 5.0 cm long were placed in each vial containing the labeled material and allowed to take up the solution and incorporate it into the apex. Samples were collected at 0, 3, 6, 12, and 24 hours, killed and fixed in acrolein, dehydrated and imbedded as described earlier. Sections were made at 6 µm and affixed to slides with autoradiographic adhesive (Jensen, 1962). The microautoradiographic procedure outlined by Jensen (1962) was used. Kodak nuclear track liquid emulsion type NBT 2 was applied to the slides and exposed for five days.

The emulsion was developed in the dark for 20 min at 14°C in a solution composed of 1.125 gm amidol, 4.5 gm of anhydrous sodium sulfide, 2.1 ml of 10% potassium bromide and 250 ml of water (Jensen, 1962). The slides were passed through a 1.0% acetic acid stop bath and fixed in sodium thiosulfite at one third saturation for 45 min, placed in a running water bath for one hour, 70% ethanol for 30 min, air dried overnight and permanently mounted with a cover slip. The median sections were determined and marked for future reference.

An uptake period of six hours gave excellent glucose incorporation. Therefore this time was selected for the next experiment.

The general procedure was the same as above except one-half of the samples after sectioning were brought down to water and placed in two changes of fresh 52% perchloric acid of 20 min each for starch solubilization (Jensen, 1962). After the perchloric acid treatment the section were dehydrated through 100% ethanol, air dried, liquid emulsion applied, exposed and developed as above. The microautoradiograms were examined by phase contrast and dark field microscopy.

Phosphorus Fractionation

Phosphorus was extracted from carnation meristems using a modification of Olney and Pollock (1960) procedure for high nucleotide phosphate and ortho-phosphate fractionation.

Shoot apices with the associated leaf sheaths and stems were collected in the greenhouse from each clone and immediately frozen on dry ice. One gram fresh weight tissue was ground in a mortar and pestil with 4.0 ml of 10% perchloric acid for five min. The mortar was rinsed with an additional 1.0 ml of 10% perchloric acid. The extract was centrifuged at 2000 x g for 10 min. The residue was washed twice with 5.0 ml of 5% perchloric acid and centrifuged as before. One gram of acid washed Norite A carbon was added to absorb organic phosphates, thoroughly mixed and removed by centrifugation at 6,000 x g for 10 min. The carbon was washed three times with 1.0 ml of glass distilled water and the supernatants combined. This extract contained the orthophosphate present in the sample.

High energy nucleotide phosphate was extracted by hydrolysis of the carbon residue with 7.5 ml of 1 N HCl in a boiling water bath for 12 min. The extract was centrifuged and washed with 2.5 ml of glass distilled water, centrifuged, and the supernatants combined.

Phosphorus was measured colorimetrically (Fisk and Subbarow, 1925) using Elon as a reducing agent (Clark, 1964). Aliquots of 1.0 µm/ml stock solution of potassium phosphate monobasic was used to construct a standard curve. Optical density was read at 660 mµ against a reagent blank. The carbon blank had a sufficient phosphorus background to reduce the reliability of calculating actual phosphorus

values in the extract. Therefore mutant-normal ratios were calculated to gain some ideas of the comparative amounts of phosphorus present.

RESULTS

Shoot Apex Development in the Chabaud Carnation

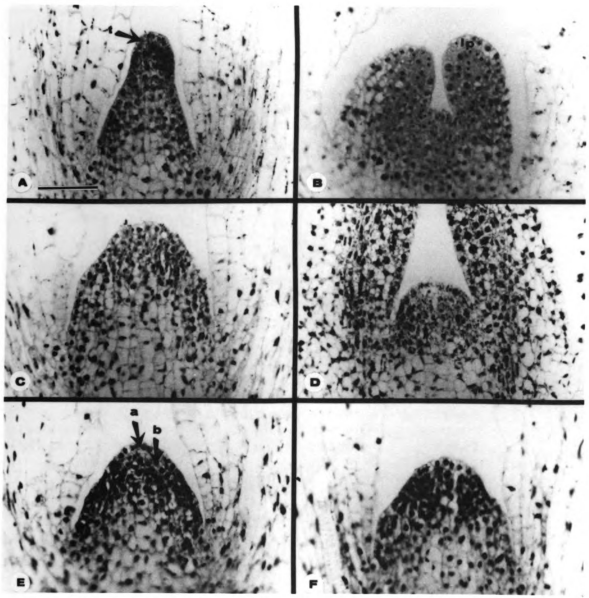
Carnation seedlings have a 4-6 mm hypocotyl with two cotyledons upright and parallel to the hypocotyl at seedling emergence. In two to three days after germination these cotyledons are fully expanded. Median longitudinal sections through the seedling apex oriented at 90° and parallel to the cotyledons show the early development and initiation of the first leaf pair (Figure 2 A, B). The meristem at this stage is about 25 µm wide, when orientated parallel to the cotyledons appears 35-40 µm high, and orientated perpendicular to the cotyledons only 5-7 µm high. This is the result of two leaf primordia enlarging lateral to the meristem and oriented opposite each other and at right angles to the cotyledons, thus setting the pattern for the observed decussate phyllotaxis. Median longitudinal sections of the apex indicate a single tunica layer, although not well defined, overlying a corpus (Figure 2 A, B).

During the ontogeny of the apex as the cotyledons expanded and the first pair of leaf primordia enlarged there was a broadening of the apex relative to the height

(Figure 2 C, D). This results in an apex that begins to appear as the normal apex previously described (Emino, 1968). Also during the second plastochron interval the apex exhibited a two-layered tunica overlying a corpus which had an organized upper layer that could give the appearance of a third tunica layer (Figure 2 E, F). However, both anticlinal and periclinal divisions regularly occurred in this group of cells and thus should be considered part of the corpus. The corpus cells radiate outward and downward from the central apical region to the peripheral zones, however, there was no evident cytohistological organization to the corpus. The shoot apex by the time the second leaf primordium had been initiated was typical of apices initiating leaf primordia at later stages of growth (Figure 2 E, F). From the initiation of the second set of leaves until flower initiation the apex had an average width of 110 μm and a height of 82 μm (Figure 3 and 4). As seen in Figure 3 and 4 width and height did not increase over time (analysis of variance not significant at $P = .05$). The apex exhibits only a minimal and maximal height and width changes during a plastochron interval.

The morphological changes taking place during the transformation from a vegetative to a reproductive apex were similar to the commercial cultivar 'White Sim' investigated earlier. The apex broadened out and centripetally initiated sepal, petal, anther, carpel and placenta

Figure 2. Photomicrographs of the normal flowering Chabaud carnation showing the development of the vegetative shoot apex stained with Heidenhains Iron Hematoxylin. A. A shoot apex orientated parallel to the unexpanded cotyledons at seedling emergence (t, arrow, tunica layer). B. The apex orientated perpendicular to the cotyledons with the leaf primordia (lp) present. At this stage only one tunica layer is present. C. An apex sampled three days later parallel to the expanded cotyledons. D. A similar apex orientated perpendicular to the expanded cotyledons with longer leaf primordia. E,F. Additional samples taken six days later showing an organized apex with a two-layered tunica (a,b) and corpus (c, arrow, organized upper corpus layer). Bar represents approximately 100 µm. A-F are all at the same magnification.


A
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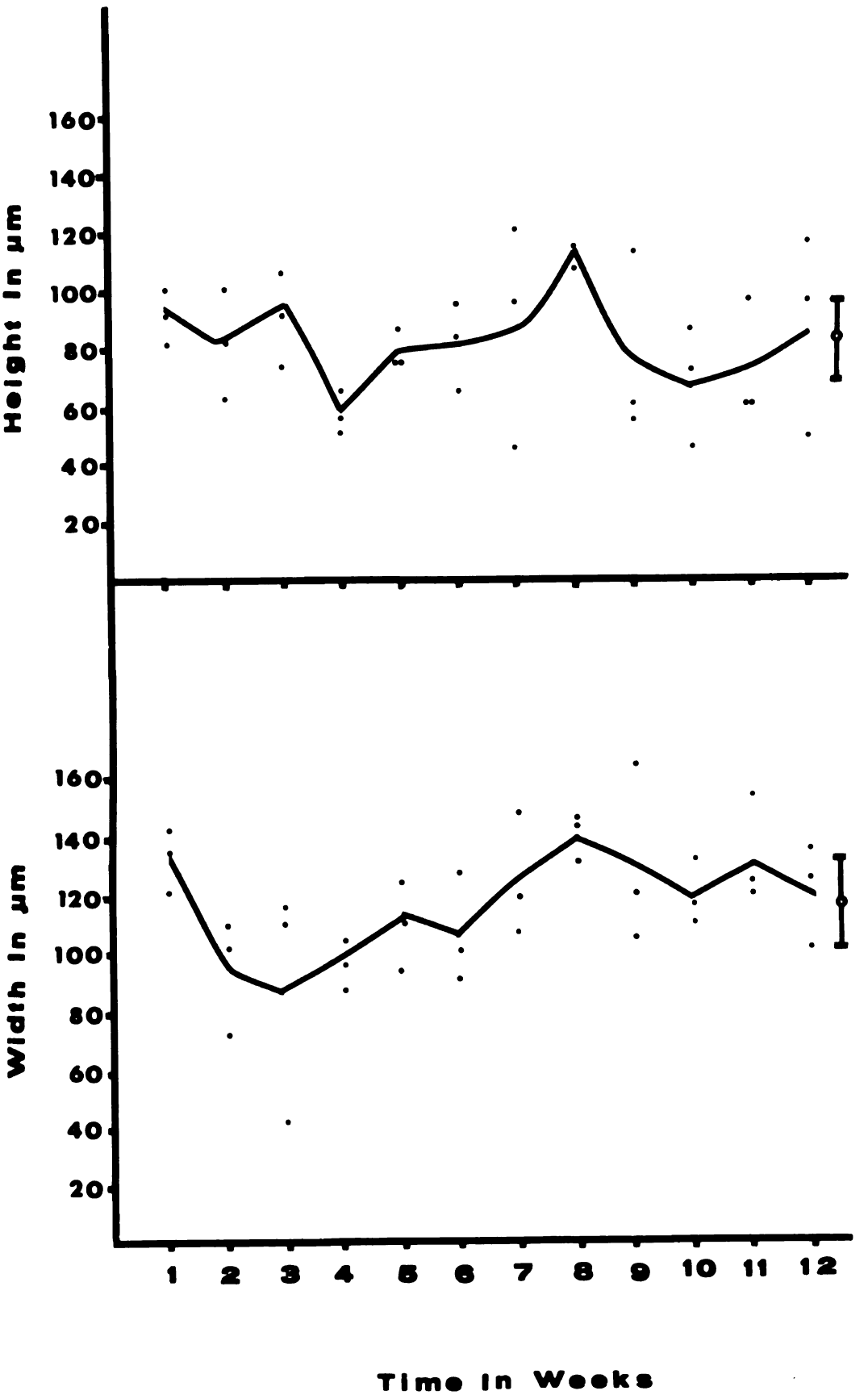
C
D
a
b
E
F

Figure 3. Dome height changes during vegetative growth of seedling Chabaud carnations. Vertical bar represents the variance of the mean. The means for each treatment (time) were not significantly different (P = .05).

Figure 4. Dome width changes during vegetative growth of seedling Chabaud carnations. Vertical bar represents the variance of the mean. The means for each treatment (time) were not significantly different (P = .05).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primordia. At anthesis in commercial cultivars all the anther primordia redifferentiate into petal primordia. In the Chabaud only half of these primordia differentiate into petals. However, this difference was not detected in these histological sections.

Shoot Apex Development in the Pine Cone Mutant

The vegetative shoot apex of the pine cone mutant is presented in Figure 5 A. The dome is hemispherical and has a two-layered tunica overlying a massive corpus. Typical cyclical changes in volume during a plastochron interval were observed and leaf initiation appeared to be opposite. The vegetative apex appeared to be similar to the normal Chabaud carnation.

After flower initiation the apex broadened typical of early flower initiation (Figure 5 B). Primordia were evident on the broadened dome flanks resembling sepal initiation in normal development. All mutant buds exhibited the flattened dome-shaped apex (Figure 5 C). This morphological characteristic persisted for the life of the bud. It was difficult studying serial sections to determine the pattern of initiation of the primordia on the flanks of the dome due to the difficulty of constructing a three dimensional mental image from histological sections. The apex continued to lay down bracts until it was 2.5-4 mm long at which time it began to senesce and die starting at

Figure 5. Photomicrographs of near median sections of the pine cone mutant stained with Chlorozol Black E. A. A vegetative apex exhibiting typical carnation vegetative growth. The apex has a two-layered (a,b) tunica overlying a massive corpus (c). B. A broadened apex at flower initiation similar to sepal initiation in normal flowering carnations with primordia initiated on the flanks of the dome that will differentiate into bract-like structures (bp). C. A more mature pine cone mutant bud showing the persistent transitional shaped apex and differentiated bract-like structures. D. The apex of a very large pine cone mutant bud showing death of the apex and surrounding tissue. Each bar represents 100 µm.

a
b
c
A
bp
B
C
D

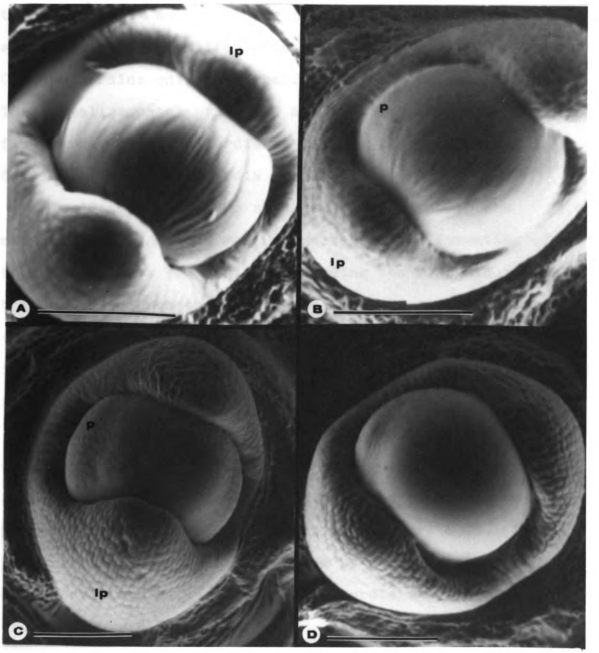
the tip and progressing basipetally toward the receptacle. A typical apex exhibiting this phenomena is depicted in Figure 5 D.

Scanning Electron Microscope Observations

Viewing fresh shoot apices with a scanning electron microscope provided a major breakthrough in this research by providing data which could be more accurately and completely interpreted. The morphogenic events especially at flower initiation and during early development clearly show and pinpoint morphologically the differences between the normal and pine cone mutant Chabaud carnation.

The normal flowering Chabaud clones (N-15 and N-30) exhibited a pattern of shoot apex differentiation essentially the same as the commercial cultivar 'Scania' with minor exceptions. The apex was dome shaped (Figure 6 A-D) and leaf primordia arose from a single primordium whorl which differentiated into two opposite leaf primordia. Figure 6 A and C are vegetative apices of N-30 in different stages of leaf initiation and Figure 6 B and D are vegetative apices of N-15. The first visible signs of flower initiation were more obvious in the Chabaud type than in the commercial cultivar 'Scania'. This apex is similar to a vegetative apex producing foliage leaves except for its greater width and differentiation of less pointed bract primordia which stand more erect away from the broadening apex

Figure 6. Scanning electron micrograph of fresh vegetative apices showing different examples of leaf primordia (lp) organogenesis in the normal flowering clones. A. N-30 and B. N-15 show apices in mid-plastochron with a pair of opposite leaf primordia (lp) and the primordium whorl (p) for the next pair of primordia. C. An N-30 apex in late plastochron showing younger decussate primordia (lp). D. A vegetative apex of N-15 with younger leaf primordia. White bar represents 100 µm.


lp
A
p
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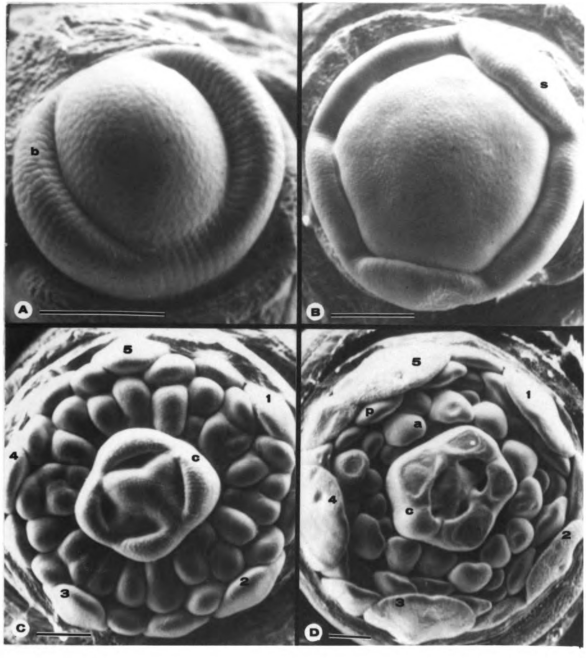
(Figure 7 A). Sepal initiation (Figure 7 B) and petal initiation (Figure 7 C) are very similar to 'Scania' however, the five petal primordia were larger in early development than 'Scania'. As previously mentioned in 'Scania' all anther primordia redifferentiate into petals while in the Chabaud strains only about half differentiate into petals. These smaller petal primordia are evident along with the five larger petal primordia as well as the centripetally developing anther primordia (Figure 7 D).

In 'Scania' only two stigmas were present in the mature flower and this was recognized in the formation of the bicarpellate primordia surrounding the placenta. With the Chabaud clones there were usually four carpel primordia (Figure 7 C) and occasionally five (Figure 7 D) resulting in the observed four and five stigmas present in the mature flower.

The pine cone mutant (C-2) exhibited a pattern of vegetative growth similar to normal development (Figure 8 A, B). The leaf primordia arise from the single circular primordium whorl previously described (Figure 8 C). Bract primordia initiation is recognized by a broader apex and differentiation of less pointed primordia which are more erect (Figure 8 D).

At flower initiation the apex broadens and bracts are initiated. Figure 9 A shows an apex in the transitional stage but rather than the normal symmetrical pattern of

Figure 7. Scanning electron micrographs of fresh apices of the normal flowering clones showing reproductive organogenesis. A. An apex of N-30 that is initiating bract (b) primordia prior to sepal initiation. The primordia stand more erect and at a wider angle compared to regular leaf primordia. B. An apex of N-15 showing five sepal primordia (s) in a circular undulating whorl surrounding the flattened dome. C. A reproductive apex of N-15 showing five (1-5) larger petal primordia that were first initiated centripetal and alternate to the sepal primordia. The carpel (c) primordia normally appears in fours. D. A reproductive apex of N-30 showing the five first formed petal primordia (1-5) and additional petal primordia (p) and anther primordia (a). In this example five carpels (c) were initiated rather than four. White bar represents 100 μm.


A
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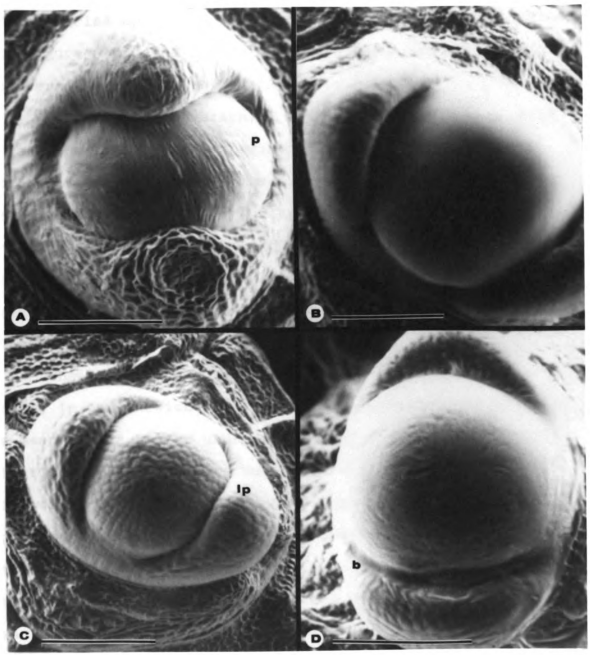
2
3

Figure 8. Scanning electron micrographs of fresh vegetative apices of C-2 showing a similar pattern of leaf primordia (lp) organogenesis to the normal flowering clones. A. Mid-late plastochron with the primordium whorl (p) easily seen. B. Similar to A only slightly earlier. C. Picture taken at a lower magnification showing good cellular detail on the apex and leaf primordia (lp). D. An apex that is in an early transitional stage of bract initiation (b). Note the smaller primordia and the wider angle between the primordia and dome. The wrinkling on the surface of the dome and leaf primordia was caused by the hard vacuum. White bar represents 100 µm.


p
A
B
lp
C
b
D

initiation there is a spiral-like initiation of individual primordia. Figure 9 B, C shows this type of initiation continuing as the pine cone enlarges. Figure 9 D is a freeze dried apex which can be compared to the fresh tissue in Figure 9 C. The freeze dried specimen shows a similar pattern of spiral-like initiation.

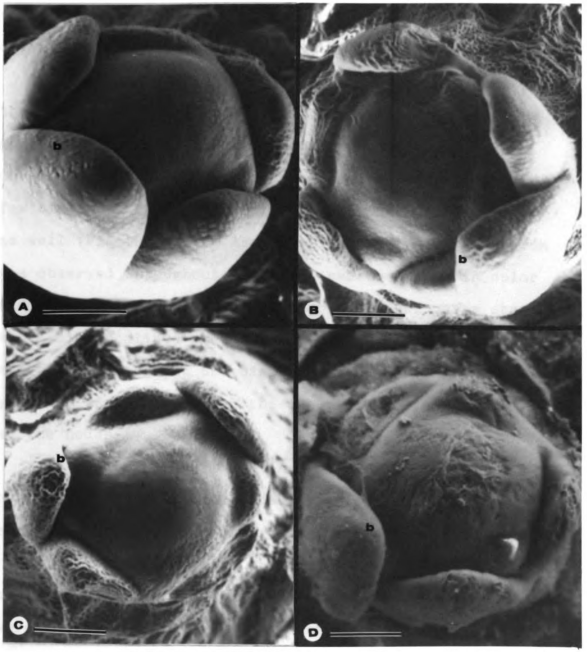
By studying the scanning electron micrographs of both fresh and freeze dried specimens it appears the sample preparation technique of fresh tissue was superior in this study to freeze drying. In vegetative and reproductive organogenesis of both the normal and mutant clones fresh tissue had fewer artifacts from the hard vacuum of the scanning electron microscope than from the freezing and drying procedure (Figure 9 C, D).

The use of the scanning electron microscope in this study in conjunction with the light microscope has provided a means for a better understanding of the pattern of initiation in the pine cone mutant as well as the normal flowering carnation. Reconstructing a three dimensional picture from serial longitudinal sections is difficult and therefore interpretational errors are increased. The SEM has made this important part of the research straight forward.

Microscopic Histochemistry

Starch. Starch grains in the pine cone mutant were relatively large, numerous and blue-black in color. In

Figure 9. Scanning electron micrographs of fresh and freeze dried shoot apices of C-2. This sequence shows the disruption of initiation of flower primordia from centripetal whorls to a spiral-like arrangement of only bract primordia (b). A. An early stage of spiral-like bract initiation showing the first formed bracts. Only the leaves were removed in dissection. B. A later stage where there were numerous bracts dissected away which shows the spiral-like pattern of initiation. C. The apex of a bud that is just macroscopically visible with many bracts dissected away showing the persisting pattern of spiral-like initiation. D. A freeze dried apex comparable to B. White bar represents 100 µm.


A
b
B
b
C
b
D
b

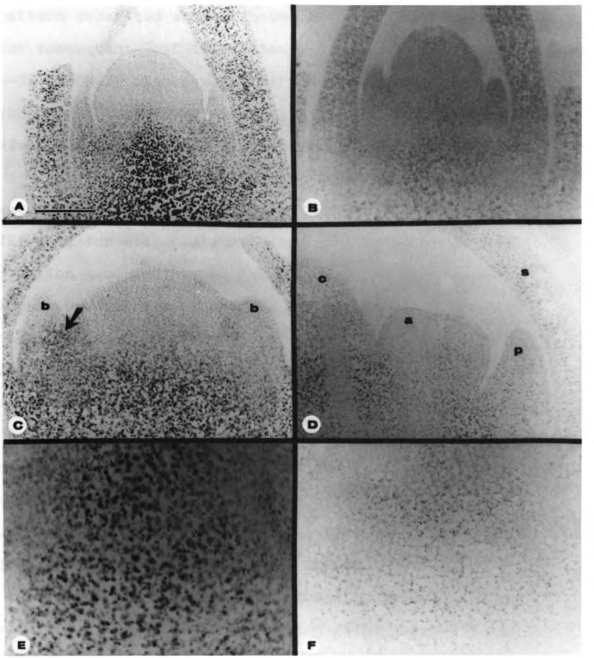
vegetative meristems starch was localized in the subapical region with very little present in the apex proper (Figure 10 A). As bract initiation took place the large amount of starch persisted in the subapical region and appeared in the differentiating bracts (Figure 10 A).

Starch stained red in the normal flowering clones, the grains were small and less prevalent. In vegetative meristems starch appeared in greatest amounts in the developing leaves and in the subapical region, however, a few small starch grains could be seen in the apex proper as well (Figure 10 B). As flower initiation began starch was observed throughout the apex, red to purple in color and small grained. In the developing flower parts starch was again localized as small red-purple staining grains scattered throughout the tissue (Figure 10 D) with the greatest concentration in the subapical region especially at the nodes.

In the third nodal region from the apex starch was critically observed and illustrated (Figure 10 E, F). The starch in the mutant was very dark staining and stained so heavily it appeared to fill the cell cavity and occasionally obscured the cell walls. In the normal apex starch grains were small, red and appeared to be located around the inside of the cell wall.

In addition to differential starch staining between clones the distribution of starch during the developmental

Figure 10. Photomicrographs of the histochemical localization of starch by IKI in the shoot apex and subapical region of the Dianthus clones. A. A vegetative apex of C-2 in early plastochron with a large amount of black staining starch in the subapical region. B. A vegetative apex of N-30 with red staining starch. C. A transitional apex of C-2 with large dark-staining starch. There is also starch localization below the initiated bracts (b, arrow). D. A reproductive apex of N-15. Starch grains are small and red: s, sepal; p, petal; a, anther; and c, carpel primordia. E. Starch localization about 500 µm below the tip of the apex in C-2 showing numerous large dark staining starch. F. Starch localization in N-30 about 500 µm below the tip of the apex showing smaller lighter staining starch. White bar represents 100 µm. All figures A-F are the same magnification.


A
B
b
b
C
c
s
a
p
D
E
F

sequence from early vegetative growth through flower initiation and early development was uniform. In C-2 starch was observed in the earliest sections in the distribution pattern described and exhibited this distribution throughout subsequent leaf initiation and bract initiation. In N-30 and N-15 starch was observed early and persisted throughout vegetative growth. As flower primordia became distinguishable starch accumulated in the primordia.

Insoluble Polysaccharides. The localization of polysaccharides with periodic-acid-Schiffs (PAS) supported the IKI data for starch distribution within the apex. The reaction gave an intense purple-red color with starch appearing dark red as well as PAS staining cellulose, hemicellulose, and other cell constituents. This additional staining interfered with the starch localization since all polysaccharides stained similarly.

In the pine cone mutant (Figure 11 A, C) and both normal flowering lines (Figure 11 B, D) starch stained the same color and appeared the same size due to the nature of the reaction, however, the mutant had considerable more grains evident than the normal clones.

In the third nodal region from the apex starch was compared between normal and mutant clones with the PAS reaction. The starch appeared around the inside of the cell wall in all cases and stained similarly, however, there were more grains evident in the mutant (Figure 11 E)

when compared to the normal (Figure 11 F). During the ontogeny of the shoot apex starch distribution and accumulation was similar to IKI observations.

Additional observations on the PAS stained sections indicated no difference in the staining pattern or intensity of the cell wall constituents. Figure 12 shows the cell wall polysaccharides in the apex as well as lack of starch in the promeristem region of both vegetative and early reproductive meristems of both the mutant and normal clones. The vegetative apex of C-2 exhibits a two-layered tunica and corpus (Figure 12 A). The upper layers of the corpus are stratified giving the appearance of a third tunica layer. The first tunica layer is about 6-7 μm and the second layer 8-9 μm. The layer thicknesses between clones were not significantly different ($P = .05$).

Similar structural features are evident in the normal vegetative apex (Figure 12 B) and the transitional and early vegetative apex of the mutant (Figure 12 C) and normal (Figure 12 D).

DNA. Deoxyribonucleic acid was localized using either Feulgen based on the Schiff's reaction or Azure B after ribonuclease extraction. The nucleus stained a light pink in the case of Feulgen and a light blue with Azure B. In order to increase contrast sufficiently for photography it was necessary to use filters for both stains.

Figure 11. Photomicrographs of the localization of polysaccharides with the periodic-acid-Schiffs reaction in median sections of the shoot apex and subapical region of Dianthus clones. The dark inclusions within the cells are predominantly starch grains. A. A vegetative apex of C-2 showing a similar pattern of starch distribution as with IKI in the subapical region. B. A vegetative apex of N-30 showing less starch. C. An early reproductive C-2 meristem. D. An early reproductive N-15 meristem. E. The subapical region of C-2 vegetative meristem with polysaccharide localization. F. The subapical region of a N-30 vegetative meristem showing polysaccharide localization. The bar represents 100 µm A-D and 10 µm E-F.

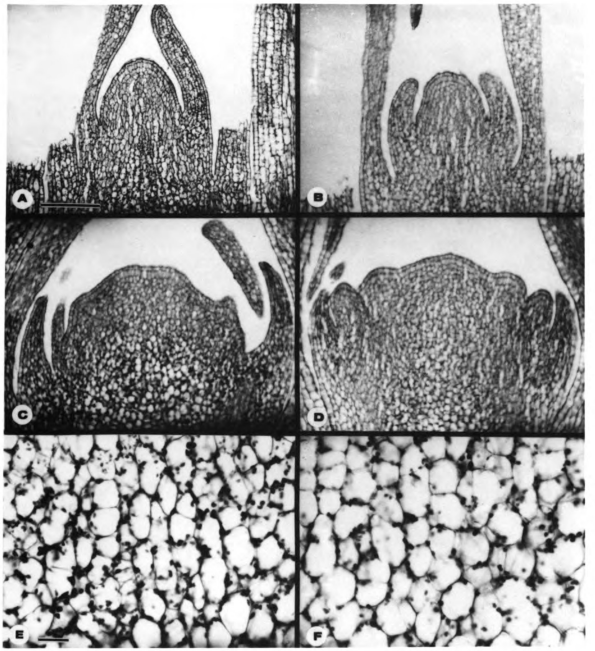
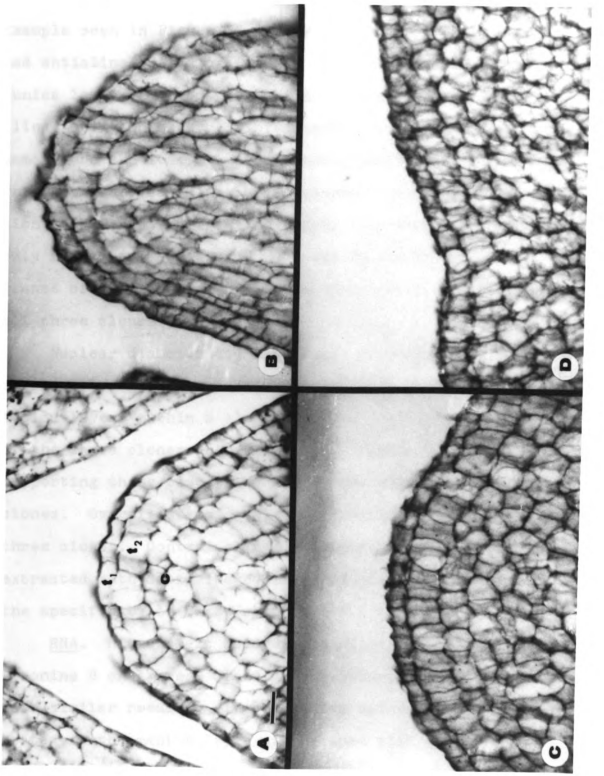

A
B
C
D
E
F

Figure 12. Photomicrographs of the localization of insoluble polysaccharides with the periodic-acid-Schiffs reaction in vegetative and transitional apices of Dianthus clones. A. The vegetative apex of C-2 showing the two-layered tunica (t_1-t_2) overlying the corpus (c). The corpus upper layers appear stratified. B. The vegetative apex of N-15 showing similar organization. C. The transitional apex of C-2 with obvious two-layered tunica and thicker cells making up the second tunica layer. D. The early reproductive apex of N-30 showing similar interval structural features. Bar represents 10 µm.

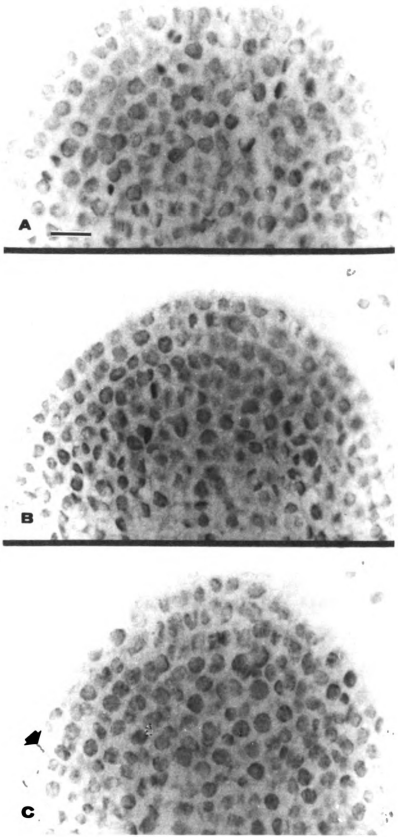

A
B
C
D
t_1
t_2
c

Since metaphase chromosomes formed a nice line across the cell, planes of cell division could be determined. Example seen in Figure 13 (arrow). The outer tunica only had anticlinal division for all three clones. The second tunica layer had predominantly anticlinal division. Periclinal division occurred infrequently on the flanks of the dome where the leaf primordium whorl would be originating. This was observed in all three clones. During the transition to a floral meristem and early reproductive growth only anticlinal division were noted in all three clones. Planes of division in the corpus occurred in all planes in all three clones.

Nuclear diameter did not change between vegetative and reproductive growth nor did it vary between clones or tunica layers within a clone ($P = .05$). Vegetative apices of the three clones are presented in Figure 13 visually supporting these results by noting the similarity between clones. Overall staining distribution was the same in all three clones. Control slides not hydrolized or additionally extracted with deoxyribonuclease did not stain indicating the specificity of these reactions for DNA.

RNA. Ribonucleic acid was localized with either Pyronine B or Axure B after deoxyribonuclease extraction with similar results. In vegetative apices RNA was concentrated in the mantle layer of the apex with a greater

Figure 13. Photomicrographs of Feulgen localized DNA in the vegetative meristems of Dianthus clones sectioned at 6 µm. A. N-15. B. C-2. C. N-30. Arrow is an example of an anticlinal division in the outer tunica layer. Bar represents 10 µm.


A
B
C

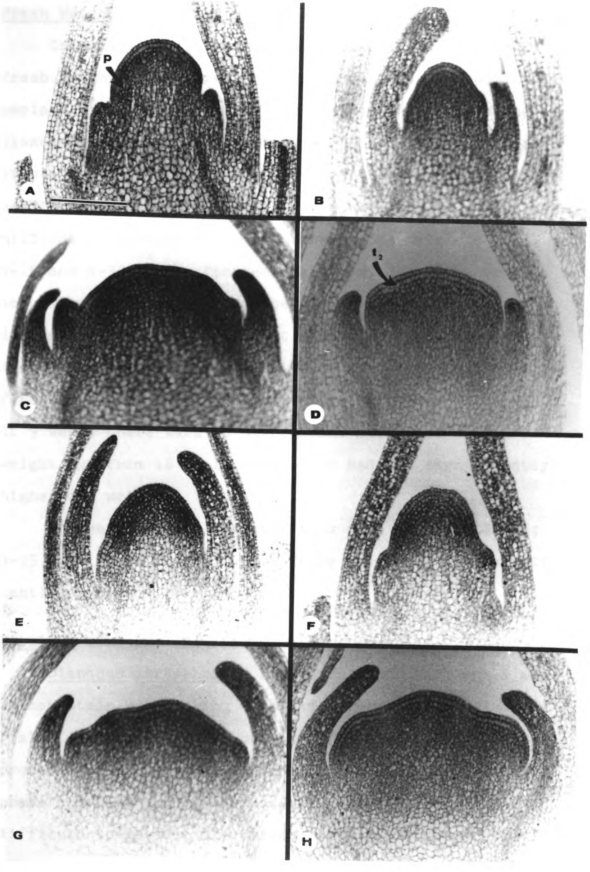
concentration at sites in the peripheral zones where leaf primordia originated. This pattern persisted during vegetative growth in all three clones (Figure 14 A, B, E, F).

At flower initiation in the normal clones during the transitional phase the RNA distribution was fairly uniform in the mantle layer but as the petal and anther primordia become visible a greater staining intensity occurred in these areas and progressed centripetally (Figure 14 C, G). In the mutant exhibiting an early transitional apex the RNA distribution was fairly uniform across the mantle layer. As the apex continued to broaden and initiate bract primordia the pattern continued but with a slightly greater staining under the primordia buttresses (Figure 14 D, H). In all three clones the second tunica layer exhibited less RNA staining (example, Figure 14 A).

The controls where the sections were placed in 1.0 N perchloric acid for 12 hours at 4°C or treated with ribonuclease did not stain RNA indicating the specificity of the reaction for RNA.

Histones. Sections placed in basic Fast Green after TCA extraction for nucleic acids exhibited no dye binding. This result may indicate there is no stainable concentration of basic protein present in the meristem or the sample preparation techniques used was not working. No controls were used. All clones at all sampling times stained similarly.

Figure 14. Photomicrographs of RNA localization with Pyronine B or Azure B. A. A vegetative apex of C-2 showing RNA in the peripheral zone (p). B. A vegetative apex of N-30. C. A transitional apex of C-2. D. A reproductive apex of N-15 with the example of the lighter staining second tunica layer (t_2). A-D stained with Pyronine B. E. Vegetative apex of C-2. F. Vegetative apex of N-15. G. Transitional apex of C-2. H. Transitional apex of N-30. E-H stained with Azure B. Bar represents 100 µm.


P
A
B
C
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G
H

Fresh Weight-Dry Weight Changes

Changes in height, fresh weight, dry weight and dry/fresh weight ratio were recorded throughout the sampling periods to be able to relate to the growth of the carnation clones to the histochemical observations. Measurements started at the third week after pinching and continued until buds were macroscopically visible. There was no significant difference ($P = .05$) between the height of C-2, N-15 and N-30 over a fourteen week period. Fresh weight accumulations over the same period were not significantly different for C-2 and N-15 but were significantly higher for N-30. Dry weight similarly was not significantly different for C-2 and N-15 but significantly higher for N-30. At 9 weeks there were no significant differences in dry weight but from 10 to 14 weeks N-30 had the significantly higher dry weight.

Dry weight/fresh weight ratios were for C-2, 12.0%; N-15, 12.1% and N-30, 11.3%. These values are not significantly different ($P = .05$).

Cytology

Dianthus caryophyllus chromosomes are very small and do not stain well making them difficult cytological material. Acetocarmine and conventional light microscopy proved inadequate but with Feulgen staining combined with phase contrast a chromosome count could be made. It was difficult to get all the chromosomes in the same focal

plane but with selective focusing and making an interpretive drawing the chromosome number of the Chabaud carnation was determined to be 2n = 30 for both the pine cone mutant and normal flowering types. The original photograph of the 2n = 30 chromosomes of C-2 and the interpretive drawing are presented in Figure 15 A, B respectively. Similarly Figure 15 C and D show 2n = 30 for the normal flowering types.

Grafting Studies

The reciprocal grafts between the two normal clones and the pine cone mutant revealed that by grafting the pattern of primordia initiation and differentiation cannot be changed either in the normal flowering clones or the pine cone mutant. The pine cone mutant scions and the scion of the two normal clones in all cases had their characteristic development (Table 2). A functional graft union was formed (Figure 16 A) and the scions allowed to grow and differentiate (Figure 16 B). The pine cone mutant on either N-15 or N-30 root systems exhibited only bract proliferation. Either N-15 or N-30 on the pine cone mutant root system flowered normally (Figure 16 B).

Greenhouse Growth Regulator and Environmental Studies

It is not my intention to dwell on the negative results of these experiments except to record that this work was carried out as an attempt to chemically induce additional

Figure 15. Photomicrographs and interpretive drawings of Dianthus caryophyllus chromosomes showing a 2n = 30 number. A. The pine cone mutant showing prophase chromosomes of 2n = 30. B. An interpretive drawing of the same photograph. C. The normal flowering type showing prophase chromosomes of 2n = 30. D. An interpretive drawing of the same photograph. Magnification 1,250x.

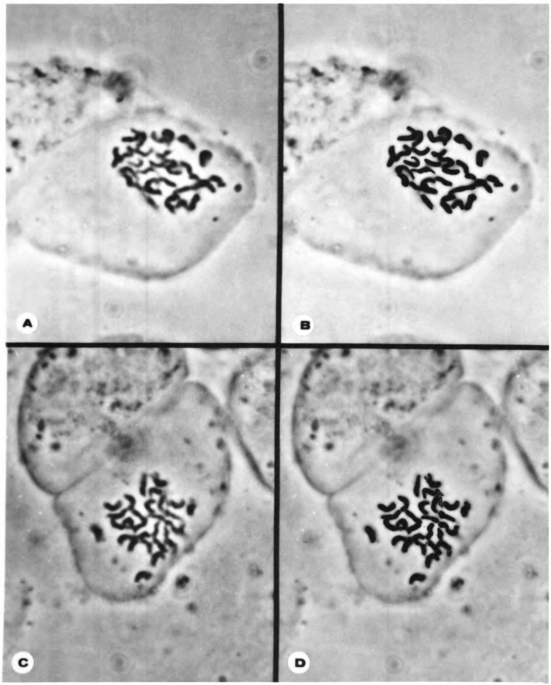

A
B
C
D

Table 2.--Morphological characteristics of reciprocal grafts of normal (N-15 and N-30) Chabaud carnations and the pine cone mutant (C-2).

Description	*Average Nodes Below Graft	Average Nodes Above Graft	Bract Pairs	Sepals	Petals	Anthers	Petaloid Structures
C2/C2	3	6.2	Many	0	0	0	0
N15/C2	2.6	5.8	2	5	35.4	33.2	2.6
N30/C2	3	7.2	2	5	29.8	29.8	1.2
N15/N15	3	6.5	2	5	35.6	36.1	3.4
N30/N30	3.1	7.5	2	5	30.1	31.1	1.6
C2/N15	3	6.8	Many	0	0	0	0
C2/N30	3.6	6.6	Many	0	0	0	0

*Average of 6 grafts except C2/C2, C2/N15, N15/C2 where 5 grafts were analyzed.

Figure 16. A reciprocal wedge graft between the selected normal flowering clones and the pine cone mutant. A. The functional graft union. B. The typical results of the grafting experiment showing reciprocal grafts of C-2 and N-30. The N-30 scion flowered as the N-30 plant normally does and the C-2 scion had its usual proliferation of bracts.

N-30
C-2
C-2
N-30
B
A

initiation of flower parts and change the direction of differentiation of structures already initiated.

The experiments involving the use of GA_4/GA_7 and BA had at concentrations of 10^{-6}M GA and BA on two observations in only one of the three replications, a pronounced effect on the differentiation of a distorted stigma-style like structure and a distorted carpel and placenta with ovules. There was no evidence of staminate or perianth structures, just the usual large number of bracts subtending the differentiated area. The other two plants in the replication as well as the other replications of the same treatment showed no such differentiation. Attempts to repeat these results were not successful.

All other experiments described using growth regulators (other than PBA) only showed bract initiation and development.

Daylength studies and a cold period prior to initiation also gave negative results. There was no alteration in the pattern of development by viewing free-hand sections under the dissecting microscope.

It can be concluded that GA_3, GA_4/GA_7, BA, IAA, ethephon, and others under the timing of application, concentrations used, and the environment under which the plants were grown were not the factors to induce flower part initiation and development in the pine cone mutant.

The effects of PBA on differentiation of the pine cone mutant are presented in Figure 17 A-B. The control showed no differentiation of flower parts while at 1,000 ppm PBA there were distorted carpels, placenta, ovules, and stigma-style evident.

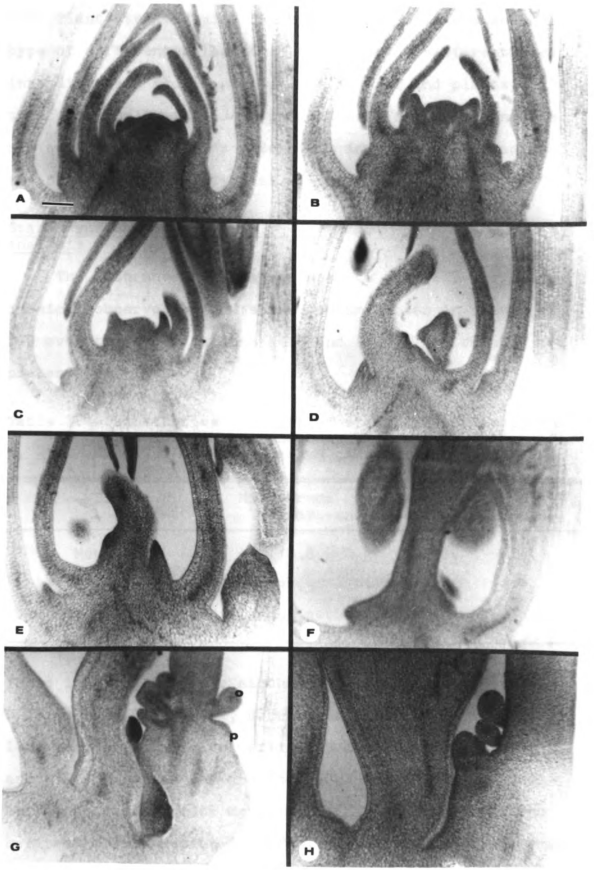
In the pine cone mutant plants treated with high concentrations of the PBA, the buds, after attaining a diameter of about 4 mm or greater, exhibited this type of bract differentiation compared to that previously described for the mutant. For example, at 1,000 ppm on a bud 9.8 mm in diameter two areas of differentiation were noted to occur. First, the apex itself continued to form bract primordia which then differentiated into a distorted carpel and placenta with ovules but persisted as a transitional meristem. The differentiation of these primordia from the apex was only evident at the higher concentration of PBA and only after a large number of bracts had been initiated (Figure 18, A-D). Secondly, a change in the pattern of differentiation took place only on the older bracts. These bracts redifferentiated into a distorted carpel and placenta with ovules (Figure 18 E-H). Plants receiving no treatment and younger buds on treated plants differentiated only bract-like appendages, however, these bracts appeared to redifferentiate in the higher concentration treated plants into a distorted carpel, stigma and a placenta with ovules. Such a differentiation was not enhanced by GA_3 or IAA.

Figure 17. Free-hand sections through the pine cone mutant's bract proliferation. A. The control with no induced differentiation. Other growth regulator applications resulted in only this type of bract proliferation. B. The effect of cytokinin PBA on bract differentiation at 1000 ppm with weekly applications.

B

A

Figure 18. Photomicrographs of serial longitudinal sections through a bud of the pine cone mutant treated with 1000 ppm cytokinin PBA. These sections are from a bud similar to that illustrated in Figure 17 demonstrating the two areas of differentiation. A. A near-median section of the transitional meristem. B. The same bud 60 µm from section A. C. 120 µm from section A. D. 180 µm from section A. E. 210 µm from section A showing distorted placenta (p) and ovules (o). H. 540 µm from section A. Bar equals 100 µm.


A
B
C
D
E
F
G
P
H

Plants treated with 100 ppm PBA had only the second type of differentiation where the bracts redifferentiated into distorted flower parts. The control and plants treated with 10 ppm had no induced redifferentiation. In all treatments the buds under 4 mm in diameter had apices and bracts that were typical of the pine cone mutant.

Starch and Sugar Quantitative Analysis

The anthrone reaction was used to quantify the histochemical observations on starch accumulation in the mutant C-2 over the normal clones N-15 and N-30. The results are presented in Table 3.

Table 3.--Quantitative analysis of starch and sugar in the pine cone mutant and normal flowering clones of the Chabaud carnation.

	Micrograms Sugar	Micrograms Starch
Mutant	1178.8	4325.6
Normal	2137.5	3127.5
(H.S.D. .05)	690.8	1056.3

There is about 45% more alcohol soluble sugar in N-30 and N-15 as compared to C-2, correspondingly there is about 28% less perchloric acid solubilized starch present in N-30 and N-15 clones as compared to C-2. The above data indicate that carbohydrate metabolism in the upper shoot and meristem of the mutant is definitely different from normal

flowering types. This further verifies the histochemical procedures which utilize IKI and Schiff's reagent.

Protein Changes Between Clones and During Vegetative Growth and Development

Disc gel electrophoretic protein patterns were determined for the C-2, N-15 and N-30 clones. Twelve bands were detected from vegetative and reproductive terminal stem and meristematic tissue. The banding pattern and band intensity were similar between N-30 and N-15 and somewhat different for C-2 (Figure 19 A). With the twelve bands identified in the gels of N-30, N-15, and C-2. There was some variability in R_f values between the normals and C-2 and differences in the intensity of banding. For example, R_f 27-28 was very dark and conspicuous in the normal where as R_f 27-28 in C-2 was blurred and less distinguishable.

Comparison of protein separated on gels within clones made from vegetative tissue to those made at biweekly intervals until macroscopic flower buds appeared showed no gross difference in banding pattern or band intensity. Neither qualitative or quantitative differences in protein could be observed within clones from vegetative samples compared to samples with macroscopically visible flower buds.

Perioxidase isoenzyme banding was observed using benzadine as a substrate since guaiacol did not resolve bands. Perioxidase isoenzyme banding is presented in

Figure 19. At R_f 54-55 there was a very faint staining present in C-2 and was absent in N-30 and N-15. Vegetative and reproductive samples gave similar perioxidase isoenzyme patterns with no qualitative or quantitative differences evident on gross examination of the gels.

Because of the differential histochemical and quantitative differences in starch between the mutant and normal clones starch synthesis and degradative enzymes were studied to try to explain the differential starch accumulation. Amylase and starch phosphorylase localization by disc gel electrophoresis always gave negative results.

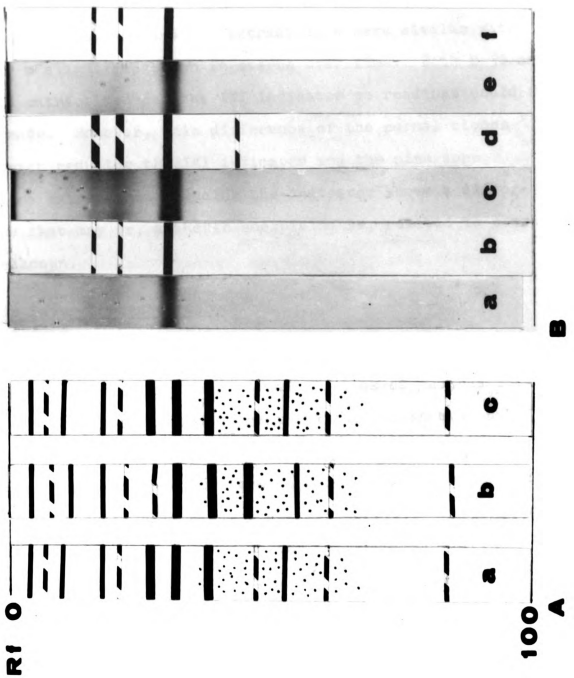
Starch Synthesis and Degradative Enzymes

Since the protein separated by disc gel electrophoresis did not show differences in starch synthesis and degradative enzymes another approach was attempted. The working hypothesis stated that there is a greater starch synthesis in the mutant therefore a more active enzyme system. Similarly there could be less starch breakdown in the mutant therefore an absent or inhibited enzyme system or both.

The potato which is known to synthesize starch was used as a control.

The N-30, N-15, and C-2 extracts did not synthesize starch in the same manner as the potato control. Combined potato and carnation extracts did not synthesize starch as

Figure 19. Zymogram pattern of the basic extractable protein from the three Dianthus clones. A. Total banding visualized with buffalo blue black. a. N-30. b. C-2. c. N-15. Narrow solid line represents clear band, dashed line represents faint and blurred bands, and broad solid line represents dominant dark staining bands. o represents origin. B. Perioxidase isoenzyme pattern a-b, N-30; c-d, C-2; and e-f, N-15. b, d, and f are actual photographs.


Rf 0
100
A
a
b
c
B
a
b
c
d
e
f

did the potato extract alone indicating inhibitors of starch synthesis were present in the experimental extracts.

Starch breakdown experiments provided inconclusive data. The potato and C-2 extract data were similar with only a slight reduction in starch over time. Both N-30 and N-15 extract reduced the IKI indicator so readings could not be made. However, this difference of the normal clones extract reducing the IKI indicator and the pine cone mutant extract not reducing the indicator shows a difference that may be, although unexplainable, related to starch breakdown.

Thin Layer Chromatography of Sugars

Because of the quantitative difference in sugar between the mutant and normal clones and the lack of success looking at starch synthesis and degradative enzymes an approach using thin layer chromatography was used. The hypothesis stated that the sugar difference would be detected qualitatively, specifically glucose and its derivatives would be different and thus related to the differential starch accumulation observed in the mutant.

The results revealed a qualitative difference between the alcohol extractable sugars chromatogrammed on silica Gel G thin layer plates, as well as the quantitative differences in carbohydrate between normal and mutant clones. Using a methyl ethyl ketone-acetic acid-methanol solvent

system the extracts of C-2 had two conspicuous bands absent when chromatographed against the N-30 and N-15 extracts. These are seen in Figure 20 A and B chromatogram f and h for C-2, e for N-30, and g for N-15. Sugars were visualized with anisaldehyde-sulfuric acid and with benzadine-trichloroacetic acid for Figure 20 A and B respectively. R_f values for the two sugars present in N-30 and N-15 were about 67 and 76 respectively. Known sugar solution of 10^{-2}M were applied and chromatographed in an attempt to identify the sugars which were absent in the mutant, C-2. Mannose, fructose, sucrose, glucose, galactose, xylose, maltose, raffinose, glucose-1-P and glucose-6-P were used. Sugars tentatively identified in all extracts were sucrose, glucose, and fructose. Sucrose had an average R_f value of 41; glucose, 57; fructose, 56; and xylose, 67. Xylose in this solvent system co-chromatogrammed with the R_f 67 band in the normal and stained similarly with anisaldehyde-sulfuric acid but differently with benzadine-trichloroacetic acid giving a dark brown reaction rather than light grey. However, this band was tentatively identified as xylose or a close derivative of xylose. Anisaldehyde-sulfuric acid also stained glucose dark grey and fructose light grey. Benzadine-trichloroacetic acid stained glucose light brown and fructose dark brown. Since glucose and fructose had close R_f values it was difficult to observe these bands but by this differential staining glucose was further identified

with a weaker staining reaction in the mutant (Figure 20 A and B chromatograms b and c). This result showed quantitatively less glucose in the mutant and similarly showed fructose present in all clones.

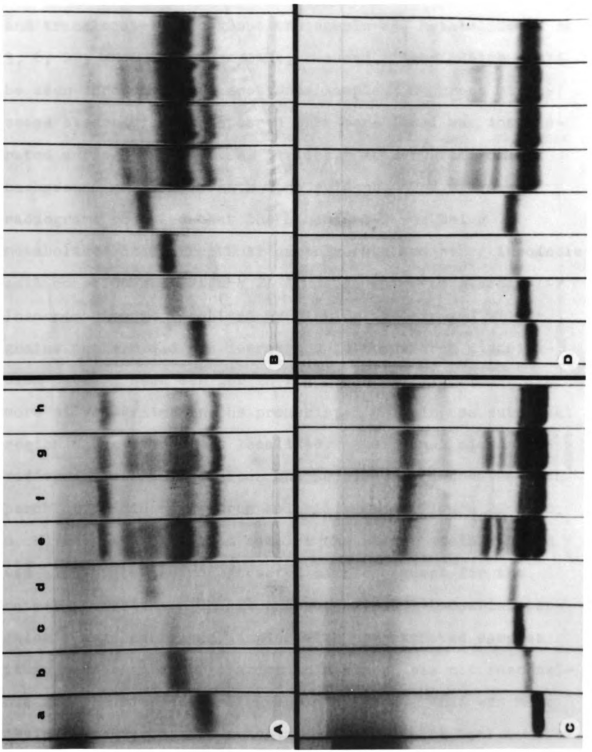
By using the solvent system isobutyric acid-ammonium hydroxide-water with the same developing techniques it was possible to determine that xylose was not one of the missing bands. Xylose had an R_f value of 24 while the band in question had a value of 30 (Figure 20 C and D). Chromatogram d was xylose. The observation on weaker glucose staining in the mutant was consistent in this solvent system.

Thus the above data reveals a qualitative difference in the soluble carbohydrates between the mutant and the normal with two sugar complexes absent in the mutant and a considerably weaker staining reaction for glucose in the mutant, however, the identity of the missing sugars remains unknown.

Microautoradiography of ^{14}C Labeled Glucose-1-Phosphate

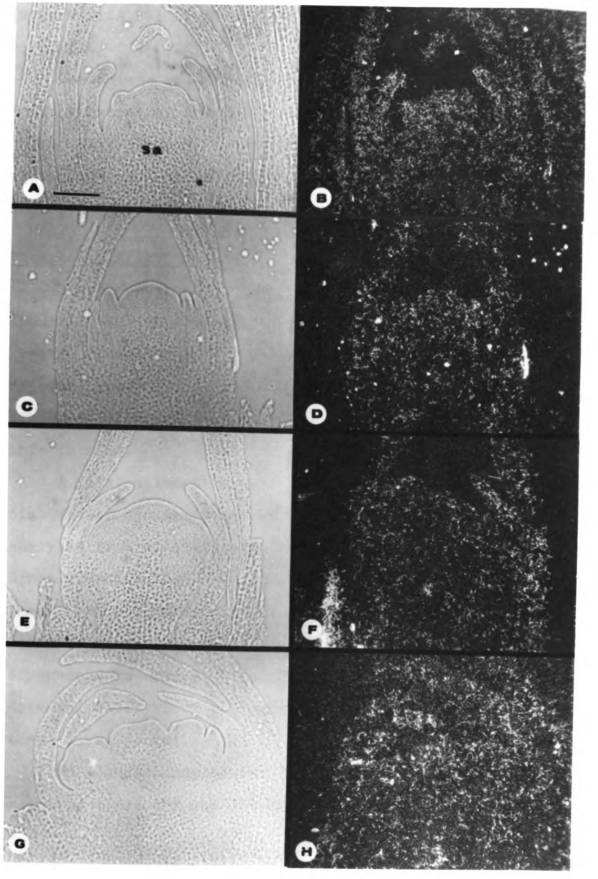
Because of the difference in soluble sugars and the amount of sugar present between the mutant and normals and the lack of success working with cell free systems an intact plant approach using microautoradiographs was attempted. The hypothesis stated that glucose-1-phosphate would be differentially incorporated into starch in the

Figure 20. Thin layer chromatographs of sugar extracts from the mutant C-2 and normal flowering clones N-15 and N-30. Solvent system used in A and B was methyl ethyl ketone-acetic acid-methanol and in C and D was isobutyric acid-ammonium hydroxide water. Sugar development on the chromatogram was with anisaldehyde-sulfuric acid in A and C and benzadine-trichloroacetic acid in B and D. Chromatogram strips for A and B are as follows: a. glucose, b. sucrose, c. fructose, d. xylose, e. N-30, f. C-2-1, g. N-15, h. C-2-2. Chromatogram strips for C and D are similar except b. fructose, and c. sucrose. Chromatogram C-2 (f and h) have two bands missing in both solvent systems and a weaker staining reaction for glucose in the anisaldehyde-sulfuric acid developing system.


a
b
c
d
e
f
g
h
A
B
C
D

mutant indicating diversion of energy into storage rather than other pathways. The labeled glucose-1-P was taken up and translocated throughout the sample and metabolized. At 3, 6, 12, and 24 hours uptake, reduced silver grains could be seen throughout the sectioned sample. On gross microscope observation it appeared that more label was incorporated during longer uptake periods. At zero time only background silver deposits were evident. The microautoradiograms revealed that the glucose-1-P was being metabolized into structural carbohydrate and other insoluble cell constituents (Figure 21 A, B, E, F). The glucose-1-P incorporation as localized by viewing the exposed silver grains pattern did not correspond to the starch distribution pattern seen earlier in Figure 10 and 11. There are more silver grains in the promeristem than in the subapical region where starch was localized. Additional microautoradiographs were made after the sections were treated with perchloric acid for starch solubilization (Figure 21 C, D, G, H). In the extracted samples the silver grain pattern was similar to the nonextracted samples except for the normal variation in uptake between apices. Comparing perchloric acid extracted samples with nonextracted samples it appears that incorporation into starch was not responsible for the microautoradiographic pattern. This was most obvious looking at the subapical region of the nonextracted

Figure 21. Photomicrographs of phase contrast and dark field images of labeled ^{14}C glucose-1-phosphate microaudiographs. A. A phase contrast image of a C-2 apex with a six hour incorporation time, (sa) is the subapical region of starch localization. B. A dark field image of the same apex showing the distribution of silver grain deposits. C. A phase contrast image of a C-2 apex after perchloric acid treatment for starch solubilization. D. A dark field image of the same apex. E. A phase contrast of N-15. F. A dark field image of the same apex. G. A phase contrast image of N-15 apex after perchloric acid treatment. H. A dark field image of the same apex. Bar represents 100 µm.


A
SA
B
C
D
E
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G
H

(Figure 21 A, B, E, F) and extracted samples (Figure 21 C, D, E, H).

The mutant and normals had similar exposure patterns in both extracted and nonextracted samples indicating little difference between them concerning the uptake and metabolism of labeled glucose-1-P into insoluble carbohydrate.

Phosphorus Fractionation

Since phosphorus is an end product of starch biosynthesis it was hypothesized that the mutant would have a greater phosphate accumulation than the normal clones. However, phosphate is the end product of many plant biosynthetic pathways so the data must be interpreted with this in mind.

A final attempt to gain further evidence of differential starch synthesis was made where orthophosphate was analyzed from extracts made from the normals and mutant. Since contamination from carbon used to absorb nucleotide phosphates occurred only relative amounts of phosphorus could be accurately determined. The mutant/normal ratios of phosphorus were 1.44 (significant at P = .01). This data indicates about 68% more orthophosphate in the mutant than in the normal.

In addition nucleotide di- and triphosphate was measured since ADP and ATP are involved in starch

biosynthesis and the mutant/normal ratio of 1.19 was found (not significant at P = .05).

The greater phosphate in the mutant allows us to accept the hypothesis. However, the major hypothesis of a more active starch synthetic enzyme in the mutant cannot be accepted or rejected from this data because of the many other sources of phosphate but provides some evidence in support of the hypothesis.

DISCUSSION

General

The shoot apex transition from vegetative to reproductive growth of the Chabaud type carnation (Dianthus caryophyllus L.) was investigated morphogenically. The Chabaud carnation has not been investigated in this respect, however, the shoot apices of commercially available cultivars of the carnation have been investigated morphologically. The non-flowering teratological mutant or pine cone mutant has not been studied morphologically or been subjected to a comparative morphological investigation with normal flowering types.

Ideally isogenic lines would be desirable for this type of comparative research. Since the origin of the pine cone mutant was from a commercially prepared seed mixture a sister line could not be obtained. However, plants were selected from commercially available seed mixtures of the Chabaud type. It was probable that the morphological characteristics of the apices of the selected flowering clones would be similar to sister lines.

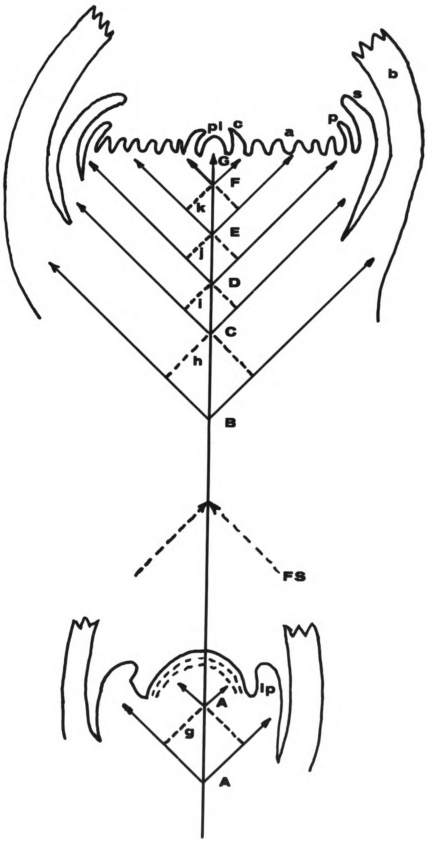
The transition of an apex from producing leaf primordia (vegetative organogenesis) to producing flower part primordia (reproductive organogenesis) is the morphological

stage of flower initiation. It can be described as a sequence of events eventually leading to the complete development of a flower. This concept is presented schematically in Figure 22. This figure is a result of the applications of the theories of differentiation of flower primordia advanced by Wardlaw (1957b) and Heslop-Harrison (1963) of a sequentially controlled reaction system to the carnation.

Carnation Shoot Apex Morphology

The observation that the pine cone mutant only produced bracts led to the hypothesis that the morphology of the apex was typical of an apex producing bract primordia. In developing the plan of investigation it was apparent that the pine cone mutant would have to be propagated vegetatively as well as the comparative normal flowering types. After studying the literature it was obvious that all previous studies on carnation shoot apex morphology were made on vegetatively propagated commercial cultivars, thus plants that were induced to flower. This induction could be carried over in the cutting. Therefore, prior to the main comparative study, it was decided to study the ontogeny of the normal flowering seedling shoot and see if it was typical of previously described shoot apices. It was found that the normal flowering seedling shoot apex attains the typical form of vegetative shoot apices (Schnabel, 1941;

Figure 22. A model for the sequence of events leading to flower primordia initiation and development in the carnation. A is the gene complex responsible for leaf initiation (lp). As the leaf differentiates factor g allows gene complex A to initiate another set of leaf primordia, and so on. After a variable number of leaves are initiated the floral stimulus (FS) is received and activates the flowering gene complex (B-G). The first is B which controls bract (b) initiation. As the bracts differentiate a similar system to that of leaf initiation allows for two bract pairs. The last bract pair differentiates factor h allowing gene complex C to initiate sepals (s). The differentiating sepals produce factor i which allows gene complex D to initiate petals (p). The petals differentiate producing factor j allowing E, the anther (a) gene complex to operate. The differentiating anthers produce factor k which allows gene complex F and G to initiate carpels (c) and placenta (pl). At each new step in the sequence the newly activated gene complex would produce factors which deactivate the previously operative gene complex. Modified from the reaction system theory of shoot apex morphogenesis after Heslop-Harrison (1963).


b
e
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A

Shushan and Johnson, 1955; Emino, 1966). Therefore there is morphologically no difference between a true vegetative carnation apex or one that has received floral stimulus but is still producing leaves. In the model flower induction is placed prior to bract initiation and after leaf initiation since there is no morphological evidence to place it earlier.

To obtain further support for this the apex size was studied over time. In some plants, like hops (Humulus), the apex increases in size over time (Thomas and Schwabe, 1970). This provides a means of determining the stage of development the apex is in prior to flower initiation. The diameter of the apices in the carnation fluctuated between 80 and 150 µm and averaged 116 µm and was about 82 microns high but did not increase in size over time. These measurements are in agreement with Schnabel (1941); Shushan and Johnson (1955); and Emino (1966) and in disagreement with Cheng and Langhans (1971) whose apex measurement ranged from 220 to 300 µm wide and 100 to 200 µm high. Thus apex size does not provide morphological evidence for the earlier placement of flower induction in the model.

The observed transition from the vegetative apex to reproductive apex was in agreement with earlier work (Emino, 1966). At anthesis the Chabaud type carnation has half the outer initiated anther primordia differentiate into petals while in commercial cultivars all anther primordia differentiate into petals.

It appeared that the proximity to the petals was the determining factor for the observed differentiation; that is, those near the petals became petals and those more centrally located became stamens. In addition, observations on the mature flower substantiate this by having antheroid-petaloid structures in the transition zone midway between the inner anthers and outer petals. Similar conclusions have been reached with double flowering Petunia (Natarella et al., 1971).

In the model, the initiation of anthers is controlled by a gene complex (E) which continues to operate. We could think of a factor (k) not being produced in sufficient concentration to start the next gene complex (F). Thus many anther primordia are laid down before enough activator (k) is produced. Finally the initiation of carpel primordia produces a factor which could suppress further anther primordia initiation. The model can be further developed to account for the difference in the pattern of anther differentiation between the Chabaud and commercial types. Each primordia would be under different initiation and differentiation mechanisms, thus primordia that are initiated can differentiate into different organs. For example, in the commercial carnation, differentiating petals produce a factor which suppresses the anther differentiating gene complex. This results in the petal differentiating gene complex taking over and redifferentiating all

anther primordia into petals. In the Chabaud type a different gene is known to be responsible for doubleness. The anther differentiating gene complex might not be completely suppressed so the further away from the petals the primordia are the less effect this factor has. Finally the anther differentiating gene complex takes over. Alternately the differentiating petals could produce less of the suppressing factor and lose control of the gene complex after about half of the anthers were redifferentiated into petals.

In studying the longitudinal sections of both the pine cone mutant and the normal flowering clones, it became evident that different observations were necessary for a more comprehensive understanding of the shoot apex morphology. The scanning electron microscope provided the additional parameter. With the SEM technique evidence for the single primordium whorl for vegetative growth, centripetal primordium initiation and alternate differentiation of flower part primordia was obtained for the normal flowering clones. Similarly, the result of the histological observation on the pine cone mutant vegetative apex being similar morphologically to the normal flowering clones was confirmed. However, the first morphological difference in the comparative study between the pine cone mutant and normal flowering clones was observed at sepal initiation with the normal clone initiating primordium whorls

centripetally and the pine cone mutant initiating bract primordia in a more primitive spiral-like fashion. These data partially support the hypothesis that the apex of the pine cone mutant is typical of an apex producing bract primordia, however, the pattern of primordia initiation was changed. This transformation of the apex of the pine cone mutant from initiating whorls of primordia to a spiral-like pattern of initiation seems to involve a disruption of the shoot apical meristem at an unknown level. This transformation to the spiral-like pattern could be thought of in terms of the model as the first visible factor and may be related to the disruption of the sequentially controlled system.

The model in terms of the normal flowering clones is complete, that is the apex completely initiates and differentiates a flower. The model in terms of the pine cone mutant is incomplete. Either gene complexes C-D-E-F are all or partially missing disrupting the sequence of events or factor h is not produced or is inactive. In any event the pine cone mutant only reacts to the floral stimulus at bract initiation and differentiation and related to this phenomenon is the change to the spiral-like pattern of initiation.

The scanning electron microscope technique used to reach these conclusions has provided a major breakthrough in shoot apex morphology studies. Now available to the

researcher in addition to gross observation of the flower at anthesis, microscopic dissection, and histological sections is an additional parameter which allows for a topographical interpretation of shoot apex development.

Preparative techniques for the SEM are important but with the carnation fresh shoot apices can be used and they provide a rapid means of determining gross morphology at each stage of development. Additional techniques developed by Einert et al., (1970) using freeze drying; Heslop-Harrison (1969) using chemical fixation; and Falk et al. (1970, 1971) using fresh tissue indicate that with each species studied with the SEM, techniques have to be developed specifically for the plant material in question. For example, the technique used for Lilium does not provide the best result for carnation. Whichever technique is used it should provide the most accurate picture of the topographical pattern of the living apex.

With the results of the morphological study two working hypotheses were developed to gain further insight into flower primordia initiation and differentiation in the carnation. First, because prior research indicated an activation of a RNA-DNA-Protein system (Salisbury and Ross, 1969) and the implication of starch build up with flowering (Sadik and Ozbun, 1967) it was hypothesized that histochemical techniques used to study RNA, DNA, Protein and starch would show a difference between the pine cone mutant and

the normal flowering clones. The differences, if present, could be developed into further studies. Second, it was hypothesized that spiral-like pattern of initiation of bract primordia in the pine cone mutant could be altered by the exogenous application of growth regulators or by grafting to normal flowering types.

Histochemical Observations

Comparative and time course histochemical studies on apices of the pine cone mutant and normal flowering clones have shown important similarities and differences in staining patterns. Specifically, DNA localization was similar with the techniques used. For RNA, localization was observed in the organogenic region in all clones. During vegetative growth RNA was localized in and below the developing leaf primordia and at flower initiation RNA was observed sequentially in the organogenic region of sepal, petal, anther, carpel and placenta primordia of the normal clones. In the transitional apex of the pine cone mutant RNA was observed in the organogenic area of bract primordia. These observations are important for two reasons. First, my interpretation based on the study of histological sections and the scanning electron micrographs indicate that in normal flowering clones flcwer primordia are initiated centripetally. This is not in agreement with Cheng and Langhan's (1971) interpretation. They stated that the flower parts arise at the same time. Secondly, the model

fits the observations. As the next gene complex is activated RNA is localized in the area where the next primordia arise. In the pine cone mutant the model states no new gene complex will be activated but the already operating gene complex which initiates additional bract primordia continues to operate allowing RNA to be localized in these areas.

Basic proteins or histones usually associated with genetic material did not stain, indicating possibly a low concentration of these nuclear proteins, the reaction did not take place, or inductive factors have temporarily denatured them. Additional protein differences were determined by separation of protein by disc gel electrophoresis. Protein banding was different between the pine cone mutant and normal flowering clones. This different banding pattern seen in the pine cone mutant could be enzyme related to the change from centripetal whorls of primordia to the spiral-like pattern observed in the pine cone mutant. However, McCown et al. (1968), looking at carnation stem protein in relation to winter hardiness, reported zymogram patterns considerably different than reported here using different clones of carnation. This would indicate there is considerable variability in protein banding among diverse clones.

Morphogenetic changes in the carnation such as flower initiation in terms of the model suggest that activation or

deactivation of specific gene complexes which ultimately change protein are involved. Nitsan (1962) showed small changes in leaf protein by electrophoresis from photoperiodically induced cocklebur plants over non-induced plants in support of this hypothesis. In spite of the resolution offered by disc gel electrophoresis no differences between protein banding of shoot apices from vegetative and reproductive meristems of carnations could be observed. Recently Sherwood et al. (1971) found no difference in protein banding with photoperiodically induced cocklebur plants. They concluded protein synthesized specifically for flower initiation, if it exists, must represent an extremely minor fraction of the total protein and that its detection by electrophoresis is impossible under the present preparatory scheme.

Additionally, perioxidase isoenzymes were differentially localized on the gels with benzadine. No quantitative or qualitative differences were noted within a clone but the pine cone mutant had one additional band and a more intense staining.

The histochemical localization of starch in the subapical region of carnation meristems was significant in that the pine cone mutant had considerably more starch than the normal flowering clones. In the mutant starch accumulated in the subapical region to a great extent and at flower initiation continued to accumulate starch. Although

not conclusive it indicates a possible role in the continued differentiation of the apical meristem. Quantitative data supported the histochemical observation that there was a differential accumulation of starch in the mutant.

Starch may play an important role in flower initiation and differentiation as a source of energy. Sadik and Ozbun (1967) have noted a build up of starch in the subapical region of cauliflower during the cold treatment prior to bolting. In tissue culture work Thorpe and Murashige (1969) noted that before the mass of undifferentiated tissue showed some organization starch accumulated in the region that would differentiate into an apex suggesting a relationship with primordia initiation and differentiation.

Assuming this suggestion to be true the differential accumulation of starch in the pine cone mutant could be related to the mechanism preventing additional flower part primordia from being initiated and to the change from centripetal whorls to the spiral-like pattern of initiation. This idea could be developed to fit the model by thinking in terms of a threshold level of carbohydrate necessary for flower initiation and development. Blake (1955) speculated that flower initiation in the carnation was controlled by a "primary metabolite" reaching a threshold level for flower primordia to be initiated; below that level leaves would be produced. If a given sugar or one of its derivatives was

the metabolite and was tied up in starch synthesis or starch was not being broken down, then it would result in low concentrations of sugar which could keep the critical level below that necessary for further flower primordia initiation. In the model then the pine cone mutant reaches the critical level for bract initiation but not sepals, etc., in the sequential system.

Primordia Initiation and Differentiation as Influenced by Grafting and Growth Regulators

Attempts to change the spiral-like pattern of bract initiation and to initiate flower primordia in the mutant followed two courses. First, experiments were conducted by grafting the pine cone mutant to the normal flowering clones. Second, growth regulators were applied to the pine cone mutant through the root system as well as the leaves.

The results of the grafting experiments indicate that by grafting additional flower primordia and differentiation could not be induced in the pine cone mutant. Conversely the pine cone mutant's characteristics could not be induced on the normal pattern of centripetal whorls. This would suggest floral primordia initiating or inhibitory substances are not transmitted acropetally across a graft union. Preliminary studies where the grafted plant was allowed to develop two flowering shoots, one from the stock and the other from the scion indicate a lack of basipetal movement

also. These results may also indicate there was no stimulus to be translocated.

However, the negative response of these plants to form additional primordia or change their pattern of differentiation to the normal strongly indicates the control for this morphogenic phenomenon lies in the scion and more probably the shoot and apex region and is not transmitted from lower plant parts as the flower inducing factors are in some species.

Growth regulators have been used to alter sex expression in plants, thus controlling primordia initiation and differentiation. The lack of response by the pine cone mutant in changing the pattern of primordia initiation and differentiation by application of GA, IAA, Ethephon, and some cytokinins indicate these materials under the conditions applied (concentrations, combinations, timing) are not factors which alter the pattern of primordia initiation and differentiation in the pine cone mutant.

The cytokinin PBA is known to promote the differentiation of carpel primordia in Vitis (Weaver, et al., 1966; Negi and Olmo, 1966; and Moore, 1970). Moore (1970) proposed a model for the effect of cytokinin PBA on differentiation of carpel primordia in Vitis. PBA would reduce the level of an inhibitor of the female differentiating gene complex or hormone below a threshold level. A similar system could be present in the pine cone mutant for a

partial explanation of the observed differentiation. The effect of PBA was seen only after many bracts have been produced and then only distorted carpel and placenta tissue were differentiated. Since the applications of PBA did influence the pattern of differentiation in the pine cone mutant this suggests in terms of the model that gene complex for sepals is inoperative, missing or replaced by bract initiating gene complexes. The effect of PBA bypasses these gene complexes and partially activates the carpel then placenta differentiating gene complexes by reducing the level of an inhibitor. Correspondingly, the other compounds used did not influence the gene complexes or factors to levels at which changes in the pattern of initiation and differentiation could take place in the pine cone mutant.

In any event the results of this section provide evidence for a concept of initiation and differentiation of flower primordia being under separate control mechanisms; that is distorted carpels can be differentiated from primordia that without exogenous PBA application would differentiate into bract-like organs.

Carbohydrate Studies of the Carnation Shoot

The mechanism of starch biosynthesis found in higher plants (Ghosh and Preiss, 1965, 1966) is as follows:

ATP + glucose-1-phosphate → ADP-glucose + PPi (1)
ADP-glucose + 1,4-glucan → 1,4-glucosyl-glucan + ADP (2)

The regulation of starch biosynthesis is in reaction 1 with the enzyme ADP-glucose pyrophosphorylase, commonly referred to as starch phosphorylase. It seemed logical that this enzyme might account for differential starch accumulation in the pine cone mutant. Several experiments were designed to test this hypothesis. Histochemical staining of protein separated by disc gel electrophoresis for starch phosphorylase did not resolve any bands that incorporated glucose-1-phosphate as a substrate into starch.

Therefore an approach using a control, the potato, with an active starch phosphorylase was used. While the potato actively synthesized starch the carnation extracts alone or combined with the potato extracts did not. This indicated an inhibitor of the starch phosphorylase present in both carnation extracts. An indirect approach using the intact shoot, glucose ^{14}C-1-phosphate, and microautoradiography indicated starch phosphorylase was not responsible for the microautoradiographic pattern. In addition to starch biosynthesis, glucose-1-phosphate is the precursor of many metabolic pathways and apparently was incorporated in these other pathways. This would indicate starch is being synthesized by some other pathway.

Two experiments provided data that partially supported the hypothesis. Inorganic phosphate was found to be higher in the pine cone mutant implying a greater starch synthesis. Additionally there is quantitatively less alcohol soluble

sugar present in the pine cone mutant. Qualitative evidence from thin layer chromatograms indicates the low sugar is principally due to two sugars being absent from the pine cone mutant and more importantly less glucose. This would suggest glucose is being differentially metabolized into starch.

All these data taken collectively indicate that there is a greater disruption of carbohydrate metabolism in the shoot of the pine cone mutant other than just differential starch synthesis. This study along with other evidence shows that stored carbohydrate may be associated with primordia initiation since it accumulates in organogenic regions. Therefore it is reasonable to suggest further that the disruption of carbohydrate metabolism may be related to the morphogenic disruption of primordia initiation and differentiation characterized in the pine cone mutant.

In Retrospect

In retrospect the study of carnation shoot meristems was an exciting area of plant study. Most investigations reported on flower initiation and development have dealt with photoperiodic or cold requiring plants. Little work had been done in relation to control of shoot apex development on day-neutral plants or plants such as *Dianthus caryophyllus*, a facultative long day plant that flowers year round but faster under long days. In terms of the

model long days and high light intensities would cause the floral stimulus to be activated earlier. Similarly the carbohydrate threshold level would be reached earlier under these conditions. The use of the carnation was justified economically since it is the second most important cut flower crop grown in the United States. One of the biggest difficulties in carnation production is control of cropping and thus an understanding of the carnation flowering mechanism is necessary before more control can be gained.

The non-flowering teratological pine cone mutant used in comparative study provided a means of gaining further information on the problem as well as explaining the mutant morphologically. Since little information was available about the mutant at the start of this research except that it had been known since 1869, the approach could not be based on prior research. Thus a plan to investigate this plant was based on defining the disruption producing the abnormality morphologically, then attempting to alter the morphology of the pine cone mutant by external means (growth regulators and grafting), investigating the comparative histochemistry then pursuing the difference to find a relationship with the disruption of flowering. This approach did not account for the complexity of the mutant but did provide a new means of approaching the study of flower primordia initiation, of gaining a greater

appreciation of the complex control of morphogenic events and extending the knowledge of carnation shoot apex morphology.

SUMMARY

The transition from vegetative and reproductive organogenesis in the Chabaud carnation (Dianthus caryophyllus L.) was studied morphologically and histochemically with respect to comparative differences between a non-flowering teratological pine cone mutant carnation and two normal flowering clones.

Technique

A new technique was developed and adapted to the carnation using the scanning electron microscope to study fresh intact shoot apices. Within five min of separation from the plant the meristem could be photographed on a scanning electron microscope showing the topographic features of the apical meristem.

Morphological Observations

Using both standard histological and the scanning electron microscope technique the morphology of the normal flowering carnation was determined. The leaf arises from a single primordium whorl which differentiates into opposite leaves. At flower initiation the apex broadens and flattens initiating centripetal whorls of primordia which

differentiate alternately. Sepals are initiated first, followed by petals, anthers, carpel, and placenta.

The pine cone mutant is similar morphologically during vegetative growth to the normal clones but at flower initiation a pattern of spiral-like initiation of bract primordia was observed which persisted for the life of the apex.

Histochemical Observations

DNA, RNA, protein and starch distribution within the shoot apex was studied. RNA was localized in the initiating primordia. Starch distribution was in the subapical region and in the developing primordia. Differences noted between the pine cone mutant and normal clones were a greater starch accumulation in the pine cone mutant and a differential protein banding by using disc gel electrophoresis. By using this technique no differences were observed between vegetative and reproductive meristems of the clones studied.

Grafting and Growth Regulator Studies

The morphological pattern of spiral-like initiation in the pine cone mutant was not changed by grafting showing the control of this phenomenon lies in the shoot. Growth regulators under the conditions applied did not change the spiral-like pattern, however, the cytokinin PBA did influence the pattern of differentiation of the bracts into distorted carpel and placenta indicating the control of

initiation and differentiation of primordia are under separate mechanisms.

Carbohydrate Metabolism

The histochemical observation that more starch accumulated in the pine cone mutant than in normal clones was further studied. Quantitative data revealed there was more starch and less sugar in the pine cone mutant than in the normal flowering clones. The enzyme starch phosphorylase was not active when separated by disc gel electrophoresis and inhibited in cell free extracts. Glucose ^{14}C-1-phosphate microautoradiograms revealed glucose-1-phosphate was incorporated into other compounds besides starch. However, low glucose content and high phosphate were consistent with the starch data. In addition two sugar complexes were missing from the pine cone mutant when compared to the normal revealing the disruption of carbohydrate metabolism in the pine cone mutant was more complex than just difference in starch phosphorylase.

Model

A model for flower initiation was adapted (Heslop-Harrison, 1963) to the carnation and the morphological and histochemical data explained in terms of the model. The model shows a sequence of events leading to flower initiation and development in the carnation. Although the

pattern of morphogenesis was not changed in the mutant the data obtained is consistent with the model.

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