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Characterization and Genetic Analysis of a Pigmentation Mutant of *Neurospora crassa*

Craig West Banks

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By

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Characterization and Genetic Analysis of a Pigmentation Mutant of
<u>Neurospora</u> <u>crassa</u>

(Under the direction of Dr. Sara Neville Bennett and Dr. Wayne A.
Krissinger)

The mutant overaccumulator of carotenoids, <u>ovc,</u> was obtained from
the Fungal Genetics Stock Center in Kansas City, Kansas. This mutant
had been isolated following UV irradiation of the colonial mutant <u>col-4</u>
and mapped to Linkage Group IV (R. Harding, 1984 Neurospora
Newsletter <u>34</u>: 23-25). Examination of <u>ovc</u> in our laboratory revealed
that in addition to having increased pigmentation, the mutant was
osmotic sensitive, failing to grow on 6% NaCl. Morphology of <u>ovc</u> closely
resembles that of <u>cut</u>, another osmotic sensitive mutant in LG IV. A
cross of <u>ovc</u> to the osmotic sensitive mutant <u>cut</u> failed to produce
recombinant progeny, supporting allelism of the two mutants. Reported
location of <u>ovc</u> placed it between <u>col-4</u> and <u>met-5</u> in LG IV; however, <u>cut</u>
is located to the far left of <u>col-4</u>. Examination of <u>ovc</u> gave no evidence of
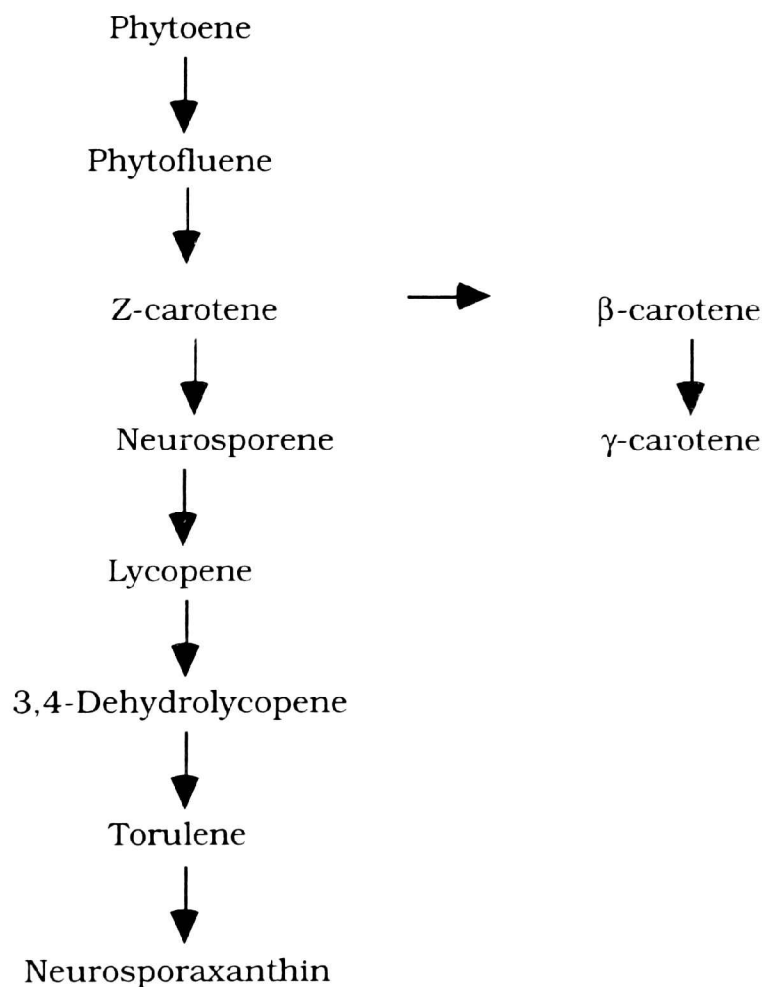a translocation in the mutant.

INTRODUCTION

Neurospora crassa is a filamentous fungus of the class Ascomyctes. The wild type strain grows as a spreading mycelium composed of branching hyphae. Described as the red bread mold, wild type N. crassa actually exhibits a reddish-orange coloration. The morphology of Neurospora crassa is a result of the structure of the hyphal wall. The wall is structually composed of three layers: an inner chitin layer, a middle layer of peptides, and an outer glucan layer (Mahadevan and Tatum, 1965).. The components of the cell wall of wild type are 80-90% polysaccharides and 10-20% lipids and proteins. These components are divided into 4 fractions which were chemically separated by Mahadevan and Tatum (1965). Fraction I consists of peptide polysaccharide complexes, Fraction II consists of glucan, Fraction III consists of β-1,3-Glucan, and Fraction IV consists of chitin. Changes in the concentrations of these four fractions, particularly Fraction I and III, affects the morphology of N. crassa. This is supported by studies of morphological mutants (e.g. colonials) which have shown differences in these fractions of the hyphal walls in comparison to the wild type strain (Brody and Tatum, 1966; Brody 1973).

Pigmentation in N. crassa is due to the presence of carotenoids which are believed to act as a stabilizers for cells and cell wall components against damages caused by photo-illumination (Ruddat and Gerber,

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1983; Lyudnikova, et al., 1990). These bright red and yellow pigments are synthesized in all photosynthetic organisms and in some nonphotosynthetic organisms such as bacteria and fungi (Ruddat and Gerber, 1983). In the mycelium, production of these pigments is photoinduced by exposure to blue-light (Russo, 1986). In contrast to the mycelium, conidia undergo constitutive carotenogenesis (Fincham, et. al., 1979) .

The following biochemical pathway for the synthesis of carotenoids in N. crassa was proposed by Harding et al. (1969).

Phytoene
↓
Phytofluene
↓
Z-carotene → β-carotene
↓   ↓
Neurosporene   γ-carotene
↓
Lycopene
↓
3,4-Dehydrolycopene
↓
Torulene
↓
Neurosporaxanthin

In this pathway, phytoene, the first of the carotenoids, is produced by a series of reactions which began with mevalonic acid. The production of phytoene can occur in the dark, but is greatly increased by the presence of light (Davies, 1979; Spurgen et. al., 1979; Mitzka-Schnabel and Rau, 1981; Russo, 1986) which activates the enzyme phytoene dehydrogenase. This enzyme converts phytoene to phytofluene, the next compound of the pathway. The production of the other carotenoids follows in sequence (Harding et. al., 1969) to produce the red acidic neurosporaxanthin and the yellow neutral β-carotene and γ-carotene. In the wild type strain the completion of the pathway to both neurosporaxanthin and γ-carotene is accomplished, and a mixture of the pigments produces the observed reddish-orange color of the fungus (Harding et. al., 1969; Goldie and Suben, 1973).

In N. crassa the photoinduced carotenogenesis involves three phases: (1) a light reaction (2) a period of protein synthesis, and (3) an accumulation of the carotenoid pigment (Harding and Shropshire, 1980). Enzymes which were absent in dark grown cultures were produced de novo following light induction. These enzymes were found within the mycelium and triggered carotenoid production which was detectable in the mycelium within about 30 minutes of the light pulse (Rau, 1976). A requirement for protein synthesis following illumination was documented in studies utilizing protein inhibitors. These studies showed that protein synthesis had to occur before carotenoids could be synthesized from the colorless precursors which had accumulated in the dark (Harding, et. al., 1969; Harding and Turner, 1982; Chambers, et. al., 1985). These

carotenoids are located in cell fractions which are enriched in membranes of the endoplasmic reticulum and in a supernatant lipid layer (Ursula and Rau, 1981).

Mutants which differ in the color and/or the degree of pigmentation can be useful for investing the pathways of pigment biosynthesis. Harding et. al. (1984) reported the isolation and characterization of a mutant which exhibited an over-accumulation of carotenoids with an increased pigmentation above that of the wild type strain. It was also found (Harding et. al., 1984) that when dark grown mycelia were harvested, exposed to light for two minutes, and returned to the dark at 25C, those of the mutant accumulated more carotenoid pigment than those of the wild type strain. When the mycelia were incubated in the dark at 6C following the light exposure, both ovc and wild type accumulated more pigment than they had at 25C. The mutant, ovc, was isolated following UV irradiation of the colonial mutant, col-4 (allele 70007c) and was mapped to the right arm of linkage group IV (LG IV) between col-4 (10% recombination) and met-5 (14% recombination).

Our laboratory, which has long been interested in osmotic-sensitive mutants, became interested in ovc when, during the routine use of the mutant in a study of pigmentation, it was found that in addition to being a pigmentation mutant, ovc was also osmotic-sensitive. Osmotic-sensitive mutants are those that fail to grow on medium supplemented with 4% NaCl (Perkins 1959), 6% NaCl (Mishra 1975; 1977b), elevated concentrations of sucrose (Kuwana 1953), or with various other osmotica (Livingston, 1969).

Seven osmotic-sensitive mutants have been reported and mapped in three of the seven linkage groups of N. crassa. In LG I are os-1, os-4, and os-5. The report of another mutant in this linkage group, os-3, could not be confirmed (Perkins, et. al., 1982) due to loss of the strain (Mays and Barratt, 1974). In LG III is os-8, and in LG IV are os-2 and cut. Two other osmotic mutants, os-6 and os-7 are not used to determine loci (Perkins, et al., 1982) since they were obtained by transformation experiments (Mishra 1977).

The typical osmotic mutants show an altered morphology together with osmotic sensitivity. This connection between the two traits has led to the practice of identifying osmotic-sensitive cultures by scoring morphology. In these mutants, the cell wall composition was shown to be altered when compared to wild type and exhibited a higher ratio of glactosamine to glucosamine. The walls were found to be weaker in comparison to those of wild type. They were also shown by physiological studies, rather than by visualization to have abnormally large pores (Trevithick and Metzenberg, 1966).

The altered morphology of the typical, osmotic-sensitive mutants, os-1, os-2, os-4, and os-5, includes close-cropped aerial hyphae that tend to rupture and bleed, and intense pigmentation of aggregated hyphae (Perkins, et. al. 1982). The mutant os-8 (isolated in the Neurospora Genetics Laboratory at Georgia Southern University) differs from the typical osmotic mutants, resembling the wild type strain in morphology. The other osmotic mutant, cut, which does resemble the typical osmotics, was first isolated by Kuwana (1953). The name 'cut'

came from its form of growth on a slant in a culture tube. Under usual conditions of culture the hyphal tips of the mutant all end at the same height, giving the culture a "crew cut" appearance. When the mutant is grown under conditions of high humidity, its morphology resembles that of wild type. Kuwana's allele of cut, HK53 was mapped to LG I because of the presence of a translocation in the strain. However, another allele of cut (LLM1) which did not contain a translocation was mapped to LG IV (Perkins and Barry, 1977). Thus the locus of cut is now known to be in the left arm of LG IV.

Since osmotic sensitivity had not previously been reported for the pigmentation mutant ovc which had been characterized only in terms of overaccumulation of carotenoids, this study was undertaken to investigate the trait of osmotic sensitivity of ovc and the relationship of this mutant to the other osmotic-sensitive mutants.

METHODS AND MATERIALS

Strains

Wild-type strains 74-OR23-1A (74A) and 74-OR8-1a (74a) of
Neurospora crassa were obtained from Dr. H. Branch Howe, Jr.,
University of Georgia, Athens, Georgia and from the Fungal Genetics
Stock Center, University of Kansas Medical School, Kansas City, Kansas.

Mutant strains used in this study were obtained from the Fungal
Genetics Stock Center now located at the Department of Microbiology,
University of Kansas Medical Center Kansas City, Kansas. These strains
and their allelic designations were: cut (LLM1); os-2 (UCLA80);
ovc (S20-16); fl (P); int (ALS8); os-1 (M16). Detailed descriptions and
genetic maps of the strains may be found in Perkins et al. (1982) and in
Fungal Genetics Newsletter, Number 34 (1987)

Media

The medium used for crossing, growth, and storage of all mutant
strains was that of Westergaard and Mitchell (1947) and is designated
W-M. W-M consisted of a carbon source (sucrose); the inorganic salts:
potassium nitrate, potassium phosphate monobasic, magnesium sulfate,
calcium chloride, and sodium chloride; trace elements; and the vitamin

biotin. When solidification was needed, 1.5% Difco Bacto-agar was added.

Some mutants were crossed and stored on complete medium. Complete medium consisted of W-M with the addition of 0.25% yeast extract and 0.50% malt extract and is designated W-M (complete). To test for osmotic-sensitivity, media were altered with the addition of 6% NaCl. If restricted growth was needed, W-M (complete) or W-M media was altered by the reduction of sucrose to 0.1% and the addition of 1.0% sorbose. To restrict conidation Tween 80 (0.8%, v/v) was added to the medium. This medium was designated WM(TW80) (Spurgen et. al. 1979).

Media were adjusted to pH 6.5. For sterilization media were autoclaved for 20 minutes at 121C in a Castle T-60 Sterilizer.

Chemicals

All inorganic salts were obtained from J. T. Baker Chemical Company, Phillipsburg, N.J. or from Fisher Scientific Co., Fair Lawn, New Jersey.

Tween 80, polyoxyethylene (20) sorbitan mono-oleate, practical grade, was obtained from Fisher Scientific Co., Fair Lawn, N.J.

Sucrose was from Dixie Crystals brand sugar obtained at a local grocery store.

Light Source

In pigmentation experiments in which the effect of light was being examined, the light source used was a 25 in. (63.5 cm), GE, 33 watt, fluorescent bulb, no. #FBS25/WX. In experiments requiring a red safe light, a GE Delta 1 Universal Bright Lab Jumbo Safelight CAT. #12-0025, CPM Inc., Dallas, Texas 75238, was used.

Petri Plates

All 100 mm X 15 mm petri plates were Fisher Polystyrene/VMR scientific brand obtained from, VMR Scientific Inc., Miles Laboratories Inc., Naperville, IL 60540, cat. no. 25384-070.

Culturing Conditions

All cultures were maintained at constant light and at ambient temperature (23C-25C) unless experimental methods directed otherwise.

Complementation Tests

The complementation tests were performed on W-M (6% NaCl) medium. Neither member of the pairs of mutants being tested for complementation could grow alone on this medium. A 100 x 15 mm petri plate was marked on the underside with 3 circles located equidistant from each other and labeled #1, #2, and #3. Inocula from the two

mutants, were placed separately in circles circle #1 and circle #2 to serve as controls. Inocula of both mutants were placed in circle #3 so as to touch one another. The mutants were of the same mating type to allow for heterokaryon formation and to avoid fertilization. The petri plate was placed in constant light at 25C for 3 days and scored for growth of the strains. Evidence for the formation of a heterokaryon and occurrence of complementation was obtained by the presence of wild type growth of the two mutants placed together (circle #3) accompanied by no growth of either of the mutants alone (circle #1 and #2). Complementation is an indication of non-allelism of the two mutants. Although failure to show wild type growth is consistent with allelism of the two mutants being tested, in the absence of forcing markers heterokaryon formation can not be assured therefore non-allelism of the mutants can not be ruled out by the negative complementation test.

Crosses for Genetic Analysis

Crosses of mutant strains to wild type were made by fertilizing 5-7 day old lawns with 5 drops of conidial/hyphal suspensions made by placing conidia/hyphae in tubes of water which were then vortexed to insure separation and an equal suspension of the conidia/hyphae. In designation of crosses, the female parent is indicated first and precedes the "X" (e.g. ovc a X 74 A). Ripe ascospores which had been shot from perithecia were removed from the sides of the culture tubes with an inoculating loop and placed onto a 4% agar slab on a microscope slide.

Using a Bausch and Lomb dissecting stereoscope Cat. No. 31-35-30 at 3x power using 20x eyepieces, a flattened, sharpened platinum iridium needle was used to cut out individual ascospores which were placed into 10 x 75 mm tubes, each containing 1.0 ml of medium. The tubes containing the individual ascospores were heat shocked for 30-45 minutes in a 60C water bath in order to induce germination of the ascospores and to kill parental hyphae and conidia.

Mass Plating of Ascospores

When large numbers of progeny were needed for genetic analysis, mass plating of ascospores was used. Ascospores were collected with an inoculating loop, placed in 0.1% agar, and the average number of ascospores per drop of the suspension was determined. The suspension was then heat-shocked at 60C for 45 minutes and aliquots were spread in petri plates which contained W-M (complete) medium with sorbose and/or 6% NaCl. The total number of ascospores placed on each plate was calculated.

The petri plates were then placed in an incubator at 25C under constant light. After 72 hours pieces of growing colonies were cut out with a flattened transfer needle and placed into individual tubes containing 1.0 ml of the appropriate medium. The tubes were then put back into the incubator, allowed to grow, and scored at times indicated by the experiment.

Effect of Humidity on Growth

The mutant <u>cut</u> was described as having wild type morphology when grown at high humidity (Kuwana 1953). To examine the effect of humidity on the form of growth of <u>ovc</u>, <u>cut</u>, and wild type 74, an experiment modified from that of Kuwana was conducted. Culture tubes containing W-M medium were inoculated separately with <u>ovc</u>, <u>cut</u>, and wild type 74. The cultures tubes were left unplugged and were placed for support in 25 ml flasks. The flasks, in turn, were placed into 600 ml beakers which had been partially filled with water. A single piece of aluminum foil was used to cover each beaker which extended above the level of the flask and culture tube. This allowed for the development of an equilibrium of moisture and the maintenance of a high level of humidity within the culture tubes. The cultures in these moist chambers were placed in an incubator at 25C in constant light and were examined for morphology after three days growth.

Observation for Translocation

Following the procedure of Perkins (1974), crosses of <u>fluffy</u> (<u>fl</u>) and <u>ovc</u> were observed for black to white ascospore ratios. Presence of more than 10% white ascospores is indicative of the presence of a translocation in a parental strain. Four day old lawns of <u>fl</u> <u>A</u> growing in petri plates were fertilized with conidia of <u>ovc</u> <u>a</u>, inverted and kept at 25C in the dark for ten days to allow the perithecia to develop. After ten days of incubation the inverted plates were placed in constant light to induce shooting of

ascospores. To obtain groups of eight ascospores (unordered tetrads), the lids of the petri plates were removed and the plates were inverted over microscope slides containing 4% agar slabs. Ascospores were allowed to shoot onto the slabs and then were viewed under a dissecting stereo microscope to determine the number of white and black ascospores in each tetrad.

Pigmentation Identification

Mycelial pigmentation colors were matched as closely as possible to Sherwin-Williams Colors Collection II Paint Swatches (SWPS): cards [#]84-[#]96 with SW#'s for specific colors [#]1582-[#]1672, the Sherwin-Williams Co., Cleveland, OH. Each culture was given a SWPS color name and number (#) which allowed a qualitative reference for the degree and hue of pigmentation exhibited by each strain.

Effect of Temperature on Pigmentation of Liquid Grown Mycelia

An experiment modified from Harding et. al. (1984) was carried out to examine the effect of temperature on pigmentation in several strains of Neurospora crassa.

Conidial suspensions of each of the five mutant strains, ovc, int, os-2, os-1, and cut, and wild type 74A were used to inoculate nine 125 ml flasks for each strain. Each flask contained 20 ml liquid WM(TW80). The inoculated flasks were wrapped in aluminum foil to exclude light and

divided into three groups with three flasks of each strain per group. Group I and Group II were incubated at 25C for six days. Group III was incubated at 37C for six days. After the six days of incubation the mycelial pads were removed under a safe light and placed in petri plates which were then put into covered dark green plastic boxes, one group per box, to prevent any light from reaching the cultures. The mycelial pads were not filtered and only enough medium to prevent drying was allowed to remain with them. Of the mycelial pads which had grown at 25C, Group I was placed at 6C and Group II at 25C for two hours to allow for equilibration. Group III composed of those mycelial pads which had grown at 37C was returned to that temperature for two hours for equilibration. After the two hour period of equilibration, the plates in each group, Group I (25C —> 6C), Group II (25C —> 25C), Group III (37C —> 37C), were exposed to light for two minutes and then returned to their equilibration temperatures and incubated for 24 hours. The plates were then removed from the boxes and the mycelial pads were scored for color of pigmentation using paint swatches (SWPS).

Statistical Analysis

The Chi-Square Test was used to obtain probability values for tests of alternate classes.

RESULTS

The mutant overaccumulator of carotenoids, _ovc_, was obtained to furnish a comparison to other mutants in a pigmentation study. During a routine analysis of the traits being examined, _ovc_, was growth-tested on W-M (6% NaCl), a medium used to identify osmotic sensitivity in _N. crassa_. The mutant failed to grow on this medium indicating that the _ovc_ strain, in addition to being a pigmentation mutant, was also osmotic-sensitive. With this discovery it became important to further investigate the characteristics of the mutant.

Although _ovc_ was a pigmentation mutant and was a somewhat brighter orange, when grown on W-M medium its color was not dramatically different from that of the wild type strain 74, which was being utilized in these studies. However, it was noticed that in culture tubes the form of growth of _ovc_ could readily be distinguished from that of the wild type strain 74.

Genetic analysis of the _ovc_ strain began with a cross to wild type (74A). The purpose of the cross was twofold: first to determine if the trait of osmotic sensitivity was a heritable trait, and if so its mode of inheritance. second, to determine if the putative osmotic-sensitive trait was due to a second mutation or was a pleiotropic, but unrecognized , effect of the _ovc_ locus.

Because it was somewhat difficult to score mutant vs wild type progeny on the basis of color, the distinguishable morphology of the two parental strains served as the basis for determining <u>ovc</u> mutant vs wild type progeny. In a cross, of <u>ovc</u> <u>a</u> X 74 <u>A</u>, 42 progeny were recovered. Of these 24 exhibited the wild type morphology and grew on W-M (6% NaCl) and 18 exhibited altered morphology and failed to grow on W-M (6% NaCl). Thus, all of the progeny were parentals with no recombinants being recovered. Chi-square analysis of the two parental classes showed 1:1 segregation which indicated one gene under Mendelian control. Co-segregation of altered morphology and osmotic sensitivity is consistent with these traits being pleiotropic effects of the gene.

Because it was difficult to distinguish the color of <u>ovc</u> with certainty from the color of wild type when the cultures were growing on W-M medium, each progeny of the cross was subcultured onto W-M (complete) since this medium enhanced the degree of pigmentation making it possible to distinguish the two colors. Examination of the progeny for pigmentation showed that 20 had the wild type orange color and 15 had the somewhat more vivid orange coloration of <u>ovc</u>. In each case color of pigmentation co-segregated with the other two parental traits, morphology and osmotic sensitivity. Unexpectedly, the other seven progeny had yellow pigmentation and differed from both of the parental strains. Four of these had wild type morphology and grew on W-M (6% NaCl) and the other three had altered morphology and did not grow on this medium. Thus, the yellow pigmentation did not co-segregate with

the other traits. The reason for the observed yellow pigmentation is unknown.

The next step taken was to more precisely map the ovc locus in its reported position between col-4 and met-5 (Figure 1) in right arm of LG IV (Harding et. al., 1984). The locus of another pigmentation mutant intense, int, is in that same area of LG IV. However, ovc was shown not to be allelic to int (Perkins 1992). Since the ovc strain had been shown to be osmotic-sensitive, it was necessary to determine if it might be allelic to the osmotic-sensitive mutant, os-2 which was also located between col-4 and met-5 (Perkins, et. al.,1982). A complementation test between ovc and os-2 was performed on W-M (6% NaCl). In addition, as a control a similar complementation test was also conducted with cut, the other osmotic-sensitive mutant located in LG IV (Figure 1) but in the left arm and away from the reported location of ovc and therefore not expected to be allelic to ovc.

In the first complementation test, the mutants ovc and os-2 inoculated separately on W-M (6% NaCl) failed to grow, whereas the inoculation of the two mutants side-by-side resulted in growth similar to that of the wild type strain. This indicated non-allelism of ovc and os-2. However, when the ovc a + os-2 a heterokaryon was subsequently grown on W-M and then subcultured onto W-M (6% NaCl), it failed to grow. This failure to grow was possibly due to unequal ratios of the two types of nuclei within the growing hyphae.

To further confirm non-allelism and to determine the linkage relationship, a cross was made between ovc and os-2. Of the 28 progeny

recovered from this cross of the two osmotic-sensitive mutants, eight progeny were identified as wild type recombinants, growing on W-M (6% NaCl). The recovery of wild type recombinant progeny indicated that the osmotic-sensitive trait of the ovc strain was not allelic to os-2 and that the two loci were at least 28 or more map units apart.

In the complementation test of ovc and cut, the two mutants placed side-by-side on W-M (6% NaCl) failed to grow. Although formation of a heterokaryon could not be assured since forcing markers were not available, the results of the complementation test were consistent with allelism of the two mutants. Additionally, in comparing ovc to cut, which has close cropped aerial hyphae giving it an even 'cut' appearance, it was observed that there was little or no difference in the gross morphology of the two mutants. Moreover, there was no difference in response of ovc and cut when grown under conditions of high humidity which resulted in development of wild type morphology of both mutants. Therefore, although cut was not within the area of the linkage group reported for ovc, the morphology and the change in morphology in high humidity along with osmotic sensitivity and the failure of complementation suggested the possibility of allelism of the two mutants.

To further test for allelism, progeny recovered from two crosses of ovc and cut were subcultured onto W-M (6% NaCl). None of the 54 progeny from the first cross or the 117 progeny from the second cross grew on this medium (Table 2). Thus all of the 171 progeny from the two crosses (Table 2) were osmotic-sensitive, as were the parents. To assure that the subcultures of the progeny were not just retarted in growth on the salt

medium, the cultures were checked again after ten days and still no growth was observed, thus no wild type recombinants were recovered, further supporting allelism of <u>ovc</u> and <u>cut</u>.

Although, the crosses supported allelism of <u>ovc</u> and <u>cut</u>, the relatively small number of 171 progeny recovered would not allow for ruling out closely linked, but separate, loci. Therefore, in order to obtain larger numbers of progeny for further confirmation of allelism, a mass plating of ascospores from the two crosses of <u>ovc</u> and <u>cut</u> was carried out. Additionally, a third cross of the two mutants was made and ascospores from that cross were also mass plated.

In the first analysis, approximately 124 ascospores were placed on each of 22 petri plates containing complete medium with sorbose and 6% NaCl. This restrictive medium would allow only wild type recombinant progeny to grow and any such progeny would indicate non-allelism. After a ten day period of incubation, there was no evidence of growth of any of the approximately 2728 ascospores plated (Table 3). For the mass plating of ascospores from the second cross (Table 3) approximately 5760 ascospores were placed on W-M (6% NaCl) in ten plates. Again, after the ten day incubation period there was no evidence of growth and therefore no wild type recombinants were found. Mass plating of the third cross (Table 3) again produced no wild type recombinants from the 16224 ascospores plated onto W-M (6% NaCl) in twelve plates. For the second and third crosses, controls in which plates containing complete medium with sorbose but with no NaCl were plated with the same ascospore suspensions used in each of the experiments such that for each

experiment the number of ascospores on a control plate was equal to the number on an experimental plate. Within a three day incubation period the control plates were filled with confluent growth indicating the ability of progeny of the cross to grow when the medium did not contain NaCl. Therefore, the failure to recover wild type progeny from the 24712 ascospores from the three crosses would not appear to be due to a lack of viability of the ascospores. These results both from individually isolated ascospores and from mass plating strongly support allelism of ovc and cut.

Because ovc had been reported to be in the right arm of LG IV between col-4 and met-5, and as a result of the present crosses, ovc was also found to be allelic to cut in the left arm of LG IV; it was necessary to determine if a translocation might be present in ovc. In N. crassa, presence of more than 5-10% white ascospores from a cross is indicative of a translocation in a parent (Perkins 1974). To check for such a translocation in ovc, a modification of the technique described by Perkins (1974) was followed and unordered ascospore tetrads from a cross of ovc and fluffy (fl) were obtained. A total of 462 tetrads were examined. Of these, one tetrad with a 6:2, black:white ratio was found. The other 461 tetrads were composed of eight black ascospores each. Therefore, there was no evidence that a translocation might be present in the mutant ovc. Additionally, examination of a cross of cut, failed to reveal white ascospores. Thus there was also no evidence of translocation in this strain.

Since these experiments furnished overwhelming evidence that the mutant ovc was allelic to cut, it was decided to examine cut to determine if evidence of pigmentation in that mutant was enhanced by a low temperature (6C) as had been shown for ovc (Harding et. al., 1984). Therefore, an experiment modified from that of Harding et. al. (1984) was carried out to qualitatively examine pigmentation in cultures of cut and ovc. For comparison, another pigmentation mutant, intense, int, as well as two other osmotic-sensitive mutant, os-1 and os-2 were included in the experiment. Wild type 74 served as the control.

An examination of the mycelia of Group I (25C —> 6C) after 24 hours incubation at 6C (Table 4) showed a light pink pigmentation in the wild type 74 strain. The mycelium of the cut strain was a dark orange rose color and showed more pigmentation than that of the ovc strain which was a light peach color. This was the first difference noted in the comparison of ovc and cut. Cultures of int, and os-1 showed more pigmentation than ovc but less than cut. The least pigmented strain in this group was os-2, which had very little pigment.

The next group examined was Group II (25C —> 25C). The amount of pigmentation displayed by strains in Group II was less than that observed for Group I, however as in Group I, the cut strain had the greatest amount of pigmentation followed by int, os-1, ovc, wild type 74, and os-2 in that order (Table 4).

In Group III (37C —> 37C) the only mutant to show any degree of pigmentation was cut (Table 4). The hue of pigmentation was equal for the three strains, ovc, int, and os-1. These three strains exhibited only

slightly more pigmentation than wild type 74 and os-2, both of which were very pale.

When examining all three groups based on overall pigmentation, Group I (25C —> 6C) showed the most pigmentation followed by Group II (25C —> 25C) and then Group III (37C —> 37C). When comparing the ovc strain to the cut strain and wild type 74, ovc showed more pigmentation than wild type 74 in all three groups but it was not equal in pigmentation to cut, which in all three groups was found to be very vividly colored (Table 4). Therefore, cut exhibited more pigmentation than any of the strains (including int and ovc) to which it was compared and in addition exhibited the same response of increased pigmentation at low temperature (6C) as had been reported for ovc. Therefore, cut could, indeed, also be described as a pigmentation mutant.

Since it had been observed that growth on W-M (complete) medium greatly enhanced the pigmentation of ovc, both ovc and cut were grown on this medium to determine if differences observed in the color of their mycelia when incubated in the cold (6C) would again be apparent. After six days growth at ambient temperature on 6 ml of medium in 20 x 150 mm culture tubes, there was little difference in the degree of pigmentation of the two mutants, although ovc may have had a slightly pinker pigmentation than cut.

DISCUSSION

Characterization of <u>ovc</u> (Harding <u>et</u>. <u>al</u>. 1984) identified the mutant as an overaccumulator of carotenoids with an increased pigmentation above that of the wild type strain. The mutant <u>ovc</u> was mapped to an area, but not to a specific locus, in LG IV (Harding <u>et</u>. <u>al</u>., 1984). Since in the present study <u>ovc</u> was shown to be osmotic-sensitive, it was thought that it might be an allele of the osmotic-sensitive mutant <u>os-2</u> which is located in the same area of the linkage group. However, a positive complementation test of <u>ovc</u> and <u>os-2</u> and the recovery of wild type recombinant progeny from a cross of the two mutants ruled out allelism. The only other known osmotic-sensitive mutant in LG IV is the mutant <u>cut</u> which is in the left arm and not within the area mapped for <u>ovc</u>.

In macroscopic examination it was observed that <u>ovc</u> had aerial hyphae, all of which were the same length and thus <u>ovc</u> had the same appearance as did the mutant <u>cut</u>. These hyphae which were so even and so closely packed together may have enhanced the appearence of increase pigmentation observed when <u>ovc</u> was compared to the wild type strain. When a complementation test between <u>ovc</u> and <u>cut</u> failed, it suggested possible allelism between these two mutants which were morphologically the same. Furthermore, when the two mutants were grown in conditions of high humidity, <u>ovc</u> exhibited wild type morphology in the same way that <u>cut</u> did. Additionally no recombinant progeny were

recovered (Table 2) from crosses between ovc and cut, in which large numbers (24712) of ascospores (Table 3) were mass plated. These characteristics: (1) the morphology on slants identical to that of cut, (2) the response of development of wild type morphology in high humidity identical to that of cut, (3) the negative complementation tests with cut, and (4) the failure to recover wild type recombinants in crosses to cut, all gave overwhelming support of allelism of the mutants ovc and cut.

The support of allelism of ovc and cut was convincing in spite of the report of the locus of ovc in the right arm of LG IV, some distance from that of cut in the left arm. It was therefore necessary to determine if a translocation could account for these contradictory results. Although the non-translocated allele of cut, LLM1, was being used in this study, for further proof a cross of cut was analyzed for the presence of white ascospores. Results of the analysis gave no evidence that a translocation was present in that mutant. A similar analysis of a cross of ovc also failed to give any evidence of a translocation. Therefore, it appeared that neither of the mutants, ovc or cut, contained a translocation and that differences from mapping data must be reconciled in some other way.

Although ovc is allelic to cut, it appears not to be an identical allele since some difference was observed between the two mutants. Evidence of this difference was shown by the degree of pigmentation exhibited when harvested mycelia of ovc and cut were incubated at 6C. Although ovc and cut both exhibited more pigmentation than the wild type 74 strain (Table 4) there was an observable difference between the two mutants with cut showing a dramatic increase in pigmentation over that

of _ovc_ (Table 4). Thus the _ovc_ strain used in this study appears to be a new allele of the _cut_ locus.

Assuming the original identification of _ovc_ as an overaccumulator of carotenoids with its locus in the right arm of LG IV was correct, how did this stock of _ovc_, which is in fact a new allele of _cut_ originate? The simplest explanation is that a second mutation, either spontaneous or induced at the same time as the _ovc_ mutation, had occurred such that the _ovc_ strain was, in fact, a double mutant strain containing both the _ovc_ and the _cut_ loci. (Table 5). Crosses of this putative double _ovc_, _cut_ mutant to wild type would result in both parental and recombinant progeny. The recombinant progeny would express either the _ovc_ or the _cut_ phenotype (Table 5). Since mutant _ovc_ progeny were likely identified by their increased pigmentation and their differences in appearance from wild type, and since the _cut_ locus results in both these characteristics, it is possible that a recombinant progeny, now containing only the _cut_ locus, was selected and misidentified as an _ovc_ mutant. Under this scenario, mapping results placing _ovc_ between _col-4_ and _met-5_ would require that these crosses were carried out using a recombinant progeny containing only the _ovc_ locus, since it would be expected that crosses of a double mutant, where the two mutations could not be phenotypically differentiated, would give skewed results.

Unless a laboratory is interested in osmotic-sensitive mutants, it would be unlikely for isolates to be tested routinely for ability to grow on medium containing elevated NaCl. Thus it is not surprising that nothing was known about osmotic sensitivity of the originally isolated _ovc_ strain.

On the other hand, if there should have been an error, for what ever reason, in mapping ovc to the right arm of LG IV, the strain ovc might be the result of a single mutation which occurred in the cut⁺ locus. Thus the strains separately identified and characterized as ovc and as cut might, in fact be the same mutants.

Names assigned to new loci are usually chosen to reflect the characteristic(s) being studied in the particular mutant. It is therefore understandable that an osmotic-sensitive mutant such as an allele of cut, might be identified and named as a pigmentation mutant, since one of the characteristics of the osmotic-sensitive mutants is possession of intense pigmentation (Perkins et. al., 1982). Precedence for such identification of osmotic mutants was set when the name flame, based on this intense pigmentation along with observed altered morphology, was originally assigned to some of the osmotic-sensitive mutants, i.e. flm-1 {flame-1} allelic to os-1 and flm-2 {flame-2} allelic to os-4. At any rate, results of this study support the present strain of ovc (S20-16) being an allele of the cut locus in the left arm of LG IV.

Studies of mutants, such as the ovc and cut strains, which have osmotic sensitivity and altered morphology can be utilized to gain more information about the fungal cell wall. In addition since these mutant strains also have altered pigmentation they might furnish a means of determining the identity of the putative blue-light photoreceptor and help to elucidate any relationship between the cell wall, altered physiology e.g. osmotic-sensitivity, and carotenogenesis in Neurospora crassa.

Figure 1

Linkage Group IV: Loci of mutants of _Neurospora crassa_ (not to scale)

a New position of the _ovc_ locus.
b Previously reported position of the _ovc_ locus (Harding, _et. al._ 1984).

Table 1

Parental (P): Recombinant (R) ratio in progeny of
a cross of the mutant <u>ovc</u> and Wild type 74A <u>N. crassa</u>

Cross	No. Ascospores Isolated	No. Ascospores Germination	Growth on W-M (6% NaCl)	Morphology Wild Type (+)	Altered (-)	P	R
<u>ovca</u> x 74A	80	42	+	(P)24	(R)0	42	0
			-	(R)0	(P)18		

Table 2

Analysis of osmotic sensitivity in progeny of
a cross of the mutants ovc and cut of N. crassa

Crosses of ovc x cut	No. Ascospores Isolated	No. Ascospores Germinated	Viability (%)	Growth on W-M (6% NaCl)	
				+	-
1	100	54	54	0	54
2	200	117	59	0	117
Total	300	171	57	0	171

Table 3

Analysis of crosses to determine allelism
of the mutants ovc and cut of N. crassa

Crosses of ovc X cut	Approximate Number Ascospores plated [a]	No. Progeny Growing on W-M (6% NaCl) [b]
1	2728	0
2	5760	0
3	16224	0
Total:	24712	0

[a] Ascospores were heat-shocked and plated en masse
[b] Only wild type recombinants would grow on W-M (6% NaCl)

Table 4

Color of pigmentation[a] in five mutant strains and wild type 74 of N. crassa following six days growth in constant dark followed by two minutes of light exposure and then 24 hours of dark at three different temperatures

Strains	Group I 25C for 6 days & 6C for 24 hours	Group II 25C for 6 days & 25C for 24 hours	Group III 37C for 6 days & 37C for 24 hours
74	1626 Shell Flower	1662 Natural Silk	1662 Natural Silk
ovc	1618 Peach Mimosa	1634 Medici Ivory	1641 White Organdy
cut	1614 Duchess Rose	1625 Golden Peach	1639 Dainty Apricot
os-1	1621 Indian Summer	1640 Ivory Memento	1641 White Organdy
int	1603 Sea Coral	1633 Sheer Apricot	1641 White Organdy
os-2	1647 Moon Slice	1662 Natural Silk	1662 Natural Silk

[a] standardized using Sherwin-Williams Paint Swatches.

Table 5

Possible origin of allelism of the <u>ovc</u> strain to the <u>cut</u> locus in the left arm
of LG IV of <u>N. crassa</u>

Orginally isolated <u>ovc</u> mutant: Proposed spontaneousor induced
                                     2nd mutation from <u>cut</u>$^+$ to <u>cut:</u>

<u>cut+_____()_____ovc</u>   <u>cut_____()_____ovc</u>

A Genetic Cross Between unrecognized <u>cut</u> <u>ovc</u> double mutant and wild
type 74:

<u>cut_____()_____ovc</u>   <— <u>ovc</u> strain containing a
                                        double mutation
         (X)

<u>cut+_____()_____ovc+</u>   <— wild type strain

Progeny resulting from the above cross:

<u>cut_____()_____ovc</u>   <–- parental double mutant

<u>cut+_____()_____ovc+</u>   <— parental wild type

<u>cut+_____()_____ovc</u>   <— recombinant <u>ovc</u> mutant

<u>cut_____()_____ovc+</u>   <— recombinant <u>cut</u> mutant

LITERATURE CITED

Brody, S and E.L. Tatum. 1966. The primary biochemical effect of a morphological mutation in Neurospora crassa. PNAS. **56**:1290-1297.

Brody, S. 1973. Metabolism, cell walls, and morphogenesis. Developmental regulation aspects of cell differentiation. Edited by Stuart J. Coward. Academic Press. New York and London.

Chambers, J.A.A., K. Hinkelammert, and V.E.A. Russo. 1985. Light-regulated protein and poly(A)$^+$ mRNA synthesis in Neurospora crassa. EMBO Journal **4** (13B):3649-3653.

Davies, B.H. 1979. Solved and unsolved problems of carotenoid formation. Pure and Applied Chemistry **51**:623-630.

Fincham, J.R.S., P.R. Day, and A. Radford. 1979. Fungal Genetics fourth ed. Botanical Monographs. J.H. Barnett, ed. Berkley and Los Angeles, CA. University of California Press.

Goldie, A. H., and R.E. Suben. 1973. The neutral carotenoids of wild type and mutant strains of Neurospora crassa. Biochemical Genetics **10**(3):275-284.

Harding, R. W., P.C. Huang, and H. K. Mitchell. 1969. Photochemical studies of the carotenoid biosynthesis pathway in Neurospora crassa. Archives of Biochemistry and Biophysics **129**:696-707.

Harding, R.W., and W. Shropshire, Jr. 1980. Photocontrol of carotenoid biosynthesis. Ann. Rev. Plant Physiol **31**:217-238.

Harding, R. W., and R. V. Turner. 1982. Photoregulation of carotenoid biosynthetic pathway in albino and white collar mutants of Neurospora crassa. Plant Physiology **68**:745-749.

Harding, R.W., D.Q. Philip, B.Z. Drozdowicz, and N.P. Williams. 1984. A Neurospora crassa mutant which overaccumulates carotenoid pigments. Neurospora Newsletter **31**:23-25.

Kuwana, H. 1953. Studies on the morphological mutant 'cut' in Neurospora crassa. Cytologia **18**:235-239.

Livingston, L.R. 1969. Locus-specific changes in cell wall composition characteristic of osmotic mutants of Neurospora crassa. Journal of Bacteriology **99**:85-90.

Lyudnikova, T.A., E.A. Chernysheva, A.A. Bezzubou, and M.S. Kritskii. 1990. Interrelationship of desaturation of fatty acids of phospholipids and the synthesis of carotenoids during adaptation of Neurospora crassa. Biokhimiya **55**(12):2211-2220.

Mahadevan, P.R. and E.L. Tatum. 1965. Relationship of the major constituents of Neurospora crassa cell wall to the wild type and colonial morphology. Journal of Bacteriology **99**(4):1073-1081.

Mays, L.L. and R.W. Barratt. 1974. Note on Yale osmotic stocks. Neurospora Newsletter **21**:25.

Mishra, N.C. 1975. Surface architecture of the different strains of Neurospora crassa as revealed by scanning electron microscopy and their relation to morphology. J. Microscopie Biol. Cell **26**:151-160.

Mishra, N.C. 1977b. Characterization of the new osmotic mutants (os) which originated during genetic transformation in N. crassa. Genetic. Res., Camb **29**:9-19.

Mitzka-Schnabel U. and W. Rau. 1981. Subcellular site of Carotenoid Biosynthesis in Neurospora crassa. Phytochemistry **20**:63-69.

Perkins, D.D. 1959. New Markers and multiple point Linkage Data in Neurospora. Genetics **44**:1185-1207.

Perkins, D.D. 1974. The manifestion of chromosome rearrangements in unordered asci of Neurospora. Genetics **77**:459-489.

Perkins, D.D., and E. G. Barry. 1977. The Cytogenetics of Neurospora. Advances in Genetics. **19**:133-285.

Perkins, D.D., A. Radford, D. Newmeyer, and M. Bjorkman. 1982. Chromosomal loci of Neurospora crassa. Microbiol. Rev. **46**:426-570.

Rau, W. 1976. Photoregulation of carotenoid biosynthesis in plants. Pure & Appl. Chem **47**:237-243.

Ruddat, M. and E. D. Gerber. 1983. Biochemistry phycology and genetics of carotenoids in fungi. Secondary metabolism and differention in fungi. ed. J.W. Bennett and Alexander Ciegler. New York, N.Y.:95-151.

Russo, V.E.A. 1986. Are carotenoids the blue-light photoreceptor in the photoinduction of properithecium in Neurospora crassa? Planta 167.

Spurgeon, S. L., R. V. Turner, and R. W. Harding. 1979. Biosynthesis of phytoene from isopentenyl pyrophosphate by a Neurospora enzyme system. Archives of Biochemistry and Biophysics **195**(1):23-29.

Trevithick, J.R. and R.L. Metzenberg. 1966. Genetic alteration of pore size and other properties of the Neurospora cell wall. J. Bacteriol. **92**:1016-1020.

Westergaard, M. and H.K. Mitchell. 1947. A synthetic medium favoring sexual reproduction. Am. J. Bot. **34**:573-577.