Genetic and biochemical characterization of a "lost" unstable flower color phenotype in interspecific crosses of *Nicotiana*

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ABSTRACT

In the 1930's a genetic instability was observed in tobacco plants derived from an interspecific cross between *N. langsdorffii* and *N. sanderae*. In the intervening half century, the plants showing this variegated phenotype have been lost. The work described in this manuscript was an effort to reconstruct plants that showed a genetic instability in a flower color locus, and to determine the site of the lesion of the instability. The first purpose of this work was, therefore, to recreate the genetic crosses which gave rise to the original genetic instability. These crosses yielded progeny which showed instabilities in flower coloration. The pigment responsible for the flower coloration was isolated and its structure determined to be delphinidin. Because flowers blocked in the production of delphinidin showed the accumulation of the flavonoid precursor myricetin, the site of the lesion can be narrowed to one of a few genetic loci: dehydroflavonol reductase, leucodelphinidin dehydratase, or a regulatory locus controlling the expression of one of these two loci.

INTRODUCTION

It is almost 60 years since the initial observations of a genetic instability affecting flower coloration in the interspecific cross, *Nicotiana langsdorffii* X *N. sanderae* (Smith and Sand, 1957; Sand, 1957). In the decades following its discovery, a considerable effort was made to understand this unstable flower color phenotype, but in the ensuing years lines bearing this phenotype have all been lost. This manuscript describes our efforts to reconstruct similar genetic material harboring this variegated flower color phenotype.

Both of the tobacco species, *N. langsdorffii* and *N. sanderae* are diploid species belonging to the Alatae section of the genus *Nicotiana* and each has nine chromosomes. Interspecific crosses are readily performed between the members of the Alatae (Goodspeed and Thompson, 1945) Members of these species have proven useful in the study of genetics of tobacco (East, 1916a; Smith, 1937a; Smith, 1937b) the production of genetic tumors (Kehr, 1954; Ahuja 1962), and the identification of genetic instabilities (Smith, 1937b; Smith and Sand, 1957).

The earliest studies on the genetics of *N. langsdorffii* and *N. sanderae* allowed an identification of the several genes involved in corolla coloration (Smith, 1937a). Continuation of these early studies identified a genetic instability in the flower color genes in progeny derived from these crosses. This instability was first observed in 1939 and was widely studied. The early papers described the appearance of the variegated phenotype in the standard *N. langsdorffii* X *N. sanderae* crosses. At least six different variegated lines were isolated from this material. These different lines expressed variegated sectors of differing sizes which have been interpreted as differences in the timing of the sectoring event (Smith and Sand, 1957). Subsequently, clones of one variegated plant, variegated-1, were tested for temperature sensitivity during development (Sand, 1957). The sectoring frequency was highest at 80°F (the highest temperature tested) and these studies also demonstrated a positive correlation between the culture temperature of the parental clone and the frequency of sectorial events in the progeny. Thus variegation in the *N. langsdorffii* X *N sanderae* crosses result from reversible changes in the functional

condition of a gene that results in alteration of flower color from red to white. This alteration is also influenced by environmental factors.

Due to the length of time since these early studies, the characterized genetic stocks containing these instabilities have been lost (H. H. Smith and S. A. Sand, personal communication). The parent stocks, however, were maintained through the intervening years and were kindly provided to us by Dr. H. H. Smith, Brookhaven, New York. We have grown them and constructed crosses similar to those produced in the first half of the century and have reconstructed plants which have a similar phenotype as those previously reported. An analysis of the progeny indicates that the genetic instability may be caused by a transposable element which appears to reside in either the gene encoding flavonone 3' hydroxylase, dihydroflavonoid reductase, leucoanthocyanin dehydratase or a regulatory factor affecting these loci.

MATERIALS AND METHODS

Plant Materials

Inbred lines of *Nicotiana langsdorffii* Weinm. (n=9) and *N. sanderae* Hort. var. Sutton's Scarlet (n=9) were used as parents. *N. langsdorffii* is a valid taxonomic species native to southern Brazil. *N. sanderae* is a commercial adaptation of the flower color genes from *N. forgetiana* ex. Hemsl. (n=9) in *N. alata* Lk. and Otto (n=9) (East, 1916b; Smith, 1937a; Goodspeed, 1945; Sand, 1957).

Seeds from *N. langsdorffii* cv. 885 and *N. sanderae* var. Sutton's Scarlet were obtained from Dr. Harold H. Smith, Brookhaven National Laboratory, Long Island, New York. These were the original lines used in the original investigation of this genetic instability. Seeds from *N. langsdorffii* cv. 28A were received from Dr. Vernon Sisson at the USDA Tabacco Seed Repository in Durham, North Carolina. *N. alata* var. Domino White was obtained from Carolina Biological Supply, Burlington NC. All plants were grown in the greenhouse under supplemental high pressure sodium light for long day plants (16 hr days/8 hr nights) or under field conditions during the summers (June to September) of 1988 to 1992 in Ames, Iowa. Since both of these species as well as the F_1 and F_2 progeny flower indeterminately, the flowers were frequently scored for flower color and for the presence of genetic instabilities over a several month period. Flowers were scored when 60 to 70% of the plants from a cross were blooming. To correctly score the phenotype of the flowers, it is advisable to wait until late in the blossoming because full coloration take place midway through a flower's life time.

Anthocyanin isolation

Anthocyanins were isolated according to the methods of Harborne (Asen, 1972; Asen, 1983; Asen, 1984; Harborne, 1976; Harborne, 1988). In a typical isolation, approximately 2 g of fresh flower petals were extracted in an acidified methanolic solution (methanol, water, acetic acid; 19:19:2). After filtering through Whatman No.1 paper, the filtrate was extracted 3 times with equal volumes of ethyl acetate to remove flavonoids from the anthocyanins. Both extracts were taken to dryness *in vacuo* at 30°C, resuspended in methanol and purified by TLC on microcrystalline cellulose plates in BAW (butanol, acetic acid, water; 6:1:2). Each pigmented band was collected separately from the TLC plates. The cellulose containing the pigments was scraped from the plates and extracted into 5% acetic acid in methanol. After separating the pigment from the cellulose by centrifugation,

the solvent was taken to dryness. A second round of purification was performed on silica TLC plates using 15% acetic acid in water as a solvent. Again each pigment was eluted from the TLC plate in 5% acetic acid in methanol, dried under vacuum at 30°C and stored at -70°C.

Preparation of the aglycone

Glycosides were hydrolized by placing 5 g of fresh flowers in 10 to 15 ml of 2N HCl in a 25 ml round bottom flask and boiling for 60 minutes. After cooling, the extract was washed 3 times with an equal volume of ethyl acetate to separate flavonoid aglycones from the anthocyanin aglycones. The upper phase consisting of ethyl acetate with the flavonoid aglycones was removed and further purified according to previously described procedures. The darkly pigmented lower phase was treated with a small amount (2 to 3 ml) of isoamyl alcohol, which extracted the anthocyanin into the organic phase. This phase was taken to dryness and purified using cellulose and silica TLC plates.

HPLC of plant pigments

HPLC of plant pigments was carried out on a reverse phase LiChrosorb RP-18 column with gradient elution. For flavonoids, the gradient system was solvent A (1% triethylamine made to pH 3.0 with phosphoric acid) and solvent B (acetonitrile). The flavonoids were detected at 340 nM at a flow rate of 1 ml/min. The gradient was established by increasing the amount of solvent B from 0% to 20% linearly for the first 20 minutes, then maintained at 20% solvent B in A for the next 15 minutes, when the concentration of solvent B was reduced to 0% over 5 minutes. For anthocyanins, solvent A contained 15% acetic acid, 1.5% phosphoric acid in water and solvent B was 100% acetonitrile. Anthocyanins were detected at 540 nM. The flow rate again was 1 ml/min. The initial solvent mixture was established at 90% solvent A and 10% solvent B. The mixture was increased linearly to 20% solvent B after 40 minutes and then dropped to the initial ratio over 5 minutes.

Mass spectrometry analysis

Chemical ionization desorption mass spectrometry was performed on a Finnigan 4000 Quadrupole mass spectrometer in the negative ion mode using ammonia as reagent gas. The ion source had a temperature of 110°C and the desorption ramp time was 30 sec. with 1 scan every 0.75 sec. Scans covered the range from 150 to 600 dalton.

NMR analysis

Purified and dessicated pigment sample (ca. 10 mg.) was dissolved in 450 uL d4-methanol for NMR analysis. Spectra were obtained on a Varian Instruments Unity 500 spectrometer at a proton resonance frequency of 500 MHz. The sweepwidth was set at 6000 Hz to cover a range of 0 to 12 ppm. The peak due to residual protiated solvent was suppressed by low-power presaturation of the resonance. Typically, 64- 128 scans were collected with a recycle time of 1.8 s using a 10 us 90-degree pulse and 4096 data points. The sample temperature was regulated at 21°C by cooled air flow. Chemical shift values were referenced to the methyl group resonance of d4-methanol centered at 3.30 ppm.

RESULTS

***N. langsdorffii* is self-compatible, however, *N. sanderae* var. Sutton's Scarlet has both**

self- sterile and self-fertile alleles at the *S* locus. The *N. sanderae* plants that we have used are self-incompatible as are many members of the Alatae (Brieger, 1935; East, 1916b). Both of these lines grew readily, however, the *N. sanderae* had not been grown in many years and germinated very poorly. Of approximately 100 seeds, only 2 germinated and only a single plant survived. Because this *N. sanderae* plant is self- incompatible and Sutton's and Son's no longer market this variety, we have propagated it asexually.

When the single surviving *N. sanderae* cv. Sutton's Scarlet plant was grown and examined, it was found to have large, deep red flowers as opposed to *N. langsdorffii* which has small green flowers (Figure 1). The original description of the tobacco flower color instability stated that this instability was activated by making an interspecific cross between *N. langsdorffii* and *N. sanderae* (Sand, 1957; Smith and Sand, 1957). Therefore, we also prepared crosses between these two species. The crosses using *N. sanderae* as the female parent with *N. langsdorffii* as the male parent were unproductive, presumably because pollen of *N. langsdorffii* cannot grow the full length of the *N. sanderae* style to fertilize the *N. sanderae* ova. Thus, all interspecific crosses described in this manuscript were made using *N. sanderae* as the male parent and *N. langsdorffii* as the female parent. Flowers of the F1 progeny were intermediate in size to both of the parents (Figure 1). Several generations of crosses and backcrosses were made to determine that the genotypes controlling flower color of *N. sanderae* and *N. langsdorffii* were as previously described (Smith, 1937a), and that the flower color genes behaved in a Mendelian fashion.

In addition to verifying the earlier work of Smith and Sand, these genetic studies permitted us to establish several standard lines bearing well characterized genetic traits. When F1 flowers from the interspecific cross were closely examined, the pink flowers demonstrated a high degree of genetic instability as evidenced by the appearance of sectoring events on the pink background . The initial crosses were made with two independent lines of *N. langsdorffii* crossed with the sole surviving *N. sanderae* (Sutton's Scarlet) plant. The frequency with which sectors occurred in each of the crosses varied as did the size of the observed sectors.

As shown in Table 1, both crosses demonstrated a high frequency of sectored flowers. However, there was also an apparent genotype variation in the frequency of sectoring depending upon the maternal parent. Those plants that had the maternal parent, *N. langsdorffii* cv. 28A demonstrated a higher frequency of sectoring (72.3% vs. 57.5%) then did the *N. langsdorffii* cv. 885 (the parent used in the original study). In addition, the size of the sectors varied with the different parents. Although flowers from those F1 plants derived from the 885 parent showed a lower frequency of sectoring, the sectors on these flowers were usually larger than the sectors on the F1 plants derived from the 28A parent. The sizes of the sectors gives a rough clue to the timing of the initiation of the genetic instability. The smaller the sector, the later in the development of the flower that the genetic instability occurs. As observed in these plants, sectors may involve only a few petal cells (Figure 2), large floral sectors, half a flower (Figure 3), or even a whole branch. By far the most common are small sectors as shown in Figure 2. The genetic instability in the F1 generation is characterized by several different phenotypes including white, red, or twin spots on the pink background. We, therefore, examined the numbers of each type of phenotype on plants from each of the primary crosses. As shown in Table 1, white sectors were 10 to 30 times more frequent than red sectors. Twin sectors of the type shown in Figure 2, Panel C were relatively rare events occuring in about 2% of all observed events. Even more infrequently, these twin sectors appeared as mottled sectors (Figure 2, Panel D). While the origin of this type of sectoring is not clear, it seems likely that this arises as a twin sector in which the cell lines have undergone a plastic rearrangement to give the mottled phenotype. The observed sectors on the petals of those flowers are reminiscent of the spotting of corn kernels and the flowers of other species by transposable elements (Bianchi, 1978; Carpenter, 1987; Coen, 1986; Coen and Carpenter,

1988; Luo, 1991; Peterson, 1987; Wijsman, 1986).

All of the observed flower coloration patterns can be explained if we consider the possibility of a transposable element residing in a flower color gene. This is shown in Figure 4. The genotype of the *N. sanderae* flower is shown as an undefined locus that affects flower coloration and also harbors a transposable element. The genotype of *N. langsdorffii* is homozygous null at this locus. Homozygosity at this locus results in a full red color as observed in the *N. sanderae* flowers. In many cases of transposable elements, the insertion of an element into a gene results in complete inactivation of the target gene (Federoff, 1989; Coen, 1986). However this is not always the case. In some examples, when transposons have been found in promoter elements or in introns (Sommer, 1988), the presence of the transposon results in low levels of expression giving a phenotype of incomplete dominance (Sommer, 1985). Apparently two copies of this gene in the *N. sanderae* parent produce sufficient mRNA from this locus to result in a deep red coloration.

Heterozygosity (or hemizygosity) at this locus as found in the F1 flowers results in a pink coloration due to decreased expression of the undefined flower color gene. If the *N. sanderae* flowers are examined carefully, we very infrequently observe pink spots against the red background. We interpret these spots as arising from imprecise excision events in one of the two homozygous genes to give a hemizygous genotype. The F_1 flowers most readily show the spotted phenotype. As illustrated above, we observe three different types of spots. The white sectors are the most prevalent pattern observed. These types of spots can be explained by the imprecise excision of a transposable element from the unidentified flower color gene (Figure 4). In this case, imprecise excision produces a non-functional gene that results in cells showing a complete lack of coloration. The red sectors can be explained by the precise excision of the putative transposable element from the undefined flower color gene. When the element precisely excises out of the flower color gene, the gene is restored to a fully functional state which results in the expression of full flower cell coloration. The twin sectors can be explained by a somatic crossover event that occurs during cell division. As shown in Figure 4, such an event would result in daughter cell lineages in which one daughter contains two copies of the flower color gene and the other daughter containing no copies of the flower color gene.

While most of the observed sectors on these plants are small, occasionally, large sectors occur that involve whole flowers, or whole plants. On several occasions we have observed plants that showed an early sectored event. Some plants even produce one branch bearing pink flowers and another branch bearing all red flowers. Thus, from these genetic studies we can say that the basic color genes segregated as previously observed by other workers and there is evidence that these genes are represented by single loci. The phenotypic variegation which was observed in the first half of this century, but subsequently lost has been reconstructed by performing the same crosses as earlier described. We have also determined that the genotypic background of the *N. langsdorffii* parent results in altered frequency of expression of the spotted phenotype in these crosses. While the exact nature of the reconstructed genetic instability has not been elucidated, the observed phenotype is reminiscent of transposable elements, and all observations can be explained by the presence of a transposable element in an uncharacterized flower color locus.

Structure Determination of the Anthocyanin Aglycone in Red Flowers

To determine the molecular step that is interrupted by the genetic instability, we decided to first determine the structure of the pigment that is produced in these flowers. The general pathway of anthocyanin biosynthesis is known in flowers Figure 5. Anthocyanins are synthesized from malonyl-CoA and 4-coumaryl CoA which are condensed to form a two ringed compound termed chalcone (Forkmann, 1991). The basic chalcone is isomerized into naringenin by the action of chalcone isomerase. Flavones are produced by the

hydroxylation at the 3 position. From the flavonol, a branch can occur. Flavonols are produced by the action of flavonol synthase (Step D in Figure 5) and colorless Lucoanthocyanins are produced by the action of dihydroflavonol reductase (Step E in Figure 5). Removal of a water results in the production of the colored anthocyanins. These compounds are frequently glycosylated in sometimes quite complex patterns.

As stated in the figure, this is a simplified anthocyanin biosynthetic pathway. In reality, hydroxylation of the B-ring frequently occurs early in the synthesis of most flavonoids. Therefore, a more realistic view of the biosynthesis of anthocyanins is shown in Figure 6. Depending upon the expression pattern of the various genes in the plant, different levels of B-ring hydroxylation may occurs from one plant to another. Prior to these studies, the exact anthocyanins produced in these plants were unknown.

We reasoned that, by identifying the pigments that accumulate in the flower petals of the wild type and mutant plants, the site of the blockage could be discovered. For the pigment isolation, flowers from the F_2 generation were used because *N. sanderae* is self-incompatible and it was difficult to obtain large numbers of flowers.

The procedures that we used to isolate the pigment yielded a single anthocyanin band, visible on microcrystalline cellulose TLC plates developed in either BAW (Butanol : HOAc : Water; 6:1:2), or HFW (HCl : formic acid : water; 7:50:40). Once we had purified the anthocyanin, the next step was to identify the aglycone, which formed the core of the anthocyanin. The pigment was therefore isolated and the aglycone, prepared by as described in the Materials and Methods section. When the aglycone was in hand, several methods were used confirm its structure. First, we used TLC analysis on microcrystalline cellulose plates. Regardless of whether the TLC plates were developed using BAW or HFW, we obtained a single band with Rf values corresponding to delphinidin (Table 2). To further verify the identity of the aglycone, this compound was eluted from the TLC plate, dissolved in 30 mM HCl in methanol, and UV and visible spectra were measured. Delphinidin contains vicinal hydroxyls on the B ring. One test for the presence of these vicinal hydroxyls is to examine its hyperchromic shift in the presence of 5% $AlCl_3$ that results from chelation of the metal by the vicinal hydroxyl groups (Harborne and Grayer 1988). Several anthocyanin aglycones including cyanidin, delphinidin, and petunidin contain vicinal hydroxyls and give hyperchromatic shifts. As shown in Table 2, both the absorption maxima of the native aglycone as well as the $AlCl_3$ shifted aglycone match the literature values for delphinidin (Harborne and Grayer 1988).

Chemical ionization mass spectrometric analysis was conducted with the aglycone. Aglycones of cyanidin, delphinidin and petunidin have masses of 287, 303 and 317 respectively. The mass spectral analysis of negative ions (aglycone minus one hydrogen) showed a parent peak at 302 dalton, corresponding to the mass of delphinidin.

Finally, NMR analysis of the aromatic region of the anthocyanin glycoside showed the presence of five protons in the aromatic region. The interpretation of these peaks vis-a-vis the structure of delphinidin is shown in Figure 7. Because of Rf values in two different solvents, the absorption maxima and the hyperchromic shift in the presence of $AlCl_3$, the determined molecular mass of 303 daltons and the presence of five aromatic protons by NMR analysis, we conclude that the pigment accumulating in the red flowers is a derivative of delphinidin.

Because most anthocyanins are glycosides, we determined the carbohydrate composition of the anthocyanin pigment. The anthocyanin pigment was subjected to alditol acetate analysis to identify the carbohydrate portion of the pigment. This analysis established an equal molar ratio of glucose and rhamnose present in the pigment. Preliminary two dimensional NMR analyses indicates that this pigment is substituted at both the 3 and 7 positions by carbohydrates. Thus we concluded that the major colored pigment present in these tobacco

flowers is delphinidin derivitized with equal molar amounts of rhamnose and glucose.

Characterization of Accumulating Flavonoids in Pink Flowers

To identify the biochemical lesion responsible for this unstable phenotype, we also determined the nature of the flavonoid precursor which accumulated in plants showing the pigment instability. The plant LxS 552 #11-7 is an F_3 progeny of *N. langsdorffii* X *N. sanderae* . The pedigree of this plant is shown in Figure 8. This plant line has colorless to light pink flowers but shows darkly pigmented spots. Flavonoids were isolated from the flowers of this plant as well as the flowers of the parental *N. sanderae* and *N. langsdorffii* plants as described in Materials and Methods. The flavonoids were identified by elution times on an HPLC calibrated with known Petunia flavonoids (Asen, 1983). As shown in Table 3, in all cases between 45% and 90% of all flavonoids could be identified. All flavonoids found in these flowers were derivatives of quercetin. We did not observe any flavonoids related to other anthocyanins.

Because quercetin is synthesized from dihydroquercetin we can be sure that the biosynthetic pathway is complete up through the synthesis of dihydroquercetin in the pink flowers. In fully red flowers the pathway is complete through delphinidin. Therefore, as shown in Figure 9, we concluded that the block must occur following the step that produces dihydroquercetin (flavone-3- hydroxylase). Likewise, the block must occur before the production of delphinidin. The possible biochemical steps that could be disrupted in these flowers are dihydroflavonol reductase and leucoanthocyanin dehydratase. In addition another possibility is that a regulatory protein controlling the expression of one or both of these genes could also be disrupted.

DISCUSSION

This work was an effort to reconstruct plants that showed genetic instability in a flower color locus, and to determine the site of the lesion of the instability. This genetic instability was first observed in the first half of the twentieth century (Smith and Sand, 1957). However, in the intervening years, the lines with variegated phenotype were lost (Smith, personal communication). The first purpose of this work was, therefore, to recreate the genetic crosses which gave rise to the original genetic instability. Therefore, seeds of the original parents were obtained and interspecific crosses similar to those conducted in the 1930s were performed. Analyses of the progeny of these interspecific crosses demonstrated that the genes involved in the basic pigment production behaved in these crosses as they did in the earlier experiments (Smith, 1937a; Smith, 1937b; Smith, 1937c; Smith, 1943). Further, these crosses yielded progeny which showed instabilities in the flower color similar to those produced in the earlier work.

The pigment responsible for the flower coloration was isolated and its structure determined to be a delphinidin glycoside. Because flowers blocked in the production of delphinidin showed the presence of the flavonoid precursor, quercetin, the site of the lesion was narrowed to one of a few genetic loci: flavonoid-3'- hydroxylase, dehydroflavonol reductase, leucodelphinidin dehydratase, or a regulatory locus controlling the expression of one of these loci.

CONCLUSIONS

- **We have successfully reconstructed genetically unstable germplasm that was originally prepared in 1937 and "lost" in the intervening years.**
- **This genetic instability appears to be caused by a blockage in the anthocyanin pathway prior to delphinidin formation.**
- **Similar genetic instabilities are known to be caused by transposable elements. All phenotypic observations made herein can be explained by the presence of a transposable element.**
- **The frequency of this genetic instability is much higher than has been observed with the other known tobacco transposon (*Tnt* 1)**
- **The anthocyanin produced in these flowers was isolated and characterized as a delphinidin glycoside**
- **The site of interruption of the flavonoid biosynthetic pathway is in one of only a few possible biochemical steps.**
    - **flavonoid-3'-hydroxylase**
    - **dihydroflavonoid reductase (DFR)**
    - **leucoanthocyanin dehydratase or**
    - **a regulatory locus controlling the expression of these loci**

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