AN ABSTRACT OF THE THESIS OF

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Title: Genetic Analysis of Interactions Between the Plant Hormones Auxin and Ethylene

Abstract Approved: Terri L. Lomax
Dr. Terri L. Lomax

Auxin and ethylene regulate many important aspects of plant growth and development and are essential to the plants survival. They help regulate such diverse processes as cell elongation, growth of adventitious roots, fruit growth, germination, and a variety of other processes. Few studies have examined how the hormones auxin and ethylene interact. An attempt to understand how these two hormones interact is presented. To try and observe and interaction, two mutants, *diageotropica* (*dgt*) and *Never Ripe* (*Nr*), both near isolines of the tomato (*Lycopersicon esculentum)* parent Alisa Craig (AC). *dgt* is an auxin insensitive, single gene, recessive mutant. *Nr* is a semi-dominant mutation in the AC background as characterized by its fruit phenotype in which some fruit ripens and others remain orange. *Nr* has a mutation in an ethylene receptor (ETR3) but it is not impaired in its ethylene biosynthesis capabilities, only its ability to perceive ethylene. These two mutants (*dgt* and *Nr*) were cross-pollinated using standard pollination techniques to generate an F1 population. The F1 was backcrossed to homozygous *dgt* plants to obtain an F2 population. The four resultant populations (double mutant phenotype, *dgt* phenotype, *Nr* phenotype, and WT phenotype) each have a 25% change for being observed. For each cross completed, a reciprocal was also conducted. To try and characterize an interaction at the seedling level, WT, *Nr*, *dgt*, F1, and F2 seed were gravistimulated under red light and the curvature of growth was measured. In addition the number of lateral roots present was counted. The F2 had a significant proportion of seedlings lacking lateral roots, so the F2 populations were segregated based on the presence or absence of lateral roots. The F1 population, which was expected to have a gravitropic response similar to *Nr*, exhibited an intermediate response, while all F2 populations, regardless of lateral root presence, had responses similar to *dgt*. It was found that *dgt* may have be semi-dominant because the F1 response was lower than that of the expected and the all the F2 populations exhibited a similar response to gravity. *Nr* may also help to partially repair the *dgt* lesion in the F1 as indicated by the intermediate response.

Genetic Analysis of Interactions Between the Plant Hormones Auxin and Ethylene

by
Kristi K. Barckley

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APPROVED:

Terri L. Lomax

Dr. Terri L. Lomax

John G. Fowler Jr.

Dr. John Fowler

Dr. Anita Azarenko

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INTRODUCTION

Arabidopsis has been the model plant for several years, but tomato has several advantages. Tomato genetics and physiology are very well known and the ripening process has been very well researched and is very understood. Other plant processes can therefore be better understood. Tomato, due to its larger size, is more advantageous when studying certain aspects such as stem growth. Furthermore, tomato is a very important economic crop and is consumed around the world. Also, tomatoes have been proven to have significant health benefits, so research on this important crop can help improve the quality of the fruit, increasing consumption and promoting healthier people.

Plants, like humans, have hormones that function to help regulate multiple aspects of the plant's growth and development. Two of the main plant hormones, auxin and ethylene, help regulate such diverse processes as cell elongation, growth of adventitious roots, fruit growth, germination, and a variety of other processes. There have been a number of studies done to understand the pathways influenced by the individual hormones. However, few studies have examined how the hormones auxin and ethylene interact. Hormone interactions and how they affect plant processes is a very challenging but intriguing topic. If hormone interaction can be understood, then not only can these mutants be repaired, but improvements on varieties of various crops can be created. For example, the post-harvest quality of fruit can be improved as well as the ability for the fruit to be stored and the overall firmness and sweetness of the fruit can be improved. Hormones regulate all of these fruit qualities and to be able to understand the intricacies of these processes would greatly benefit our consumer society.

Auxin was the first plant hormone to be discovered and is required for viability of the plant. Because it is essential for maintaining the life of the plant, no true auxin mutants have been found, presumably because such a mutant would be lethal. Auxin is primarily synthesized in the apical meristem and according to the

Cholodny-Went hypothesis, is transported laterally down the shaded side of a plant to induce elongation. It is also known that auxin is responsible for the ability of the plant to sense gravity and lateral and adventitious roots. Auxins are also used in commercial applications. For example, auxins are used as herbicides and to promote root formation on cuttings (Taiz, Zeiger, 1998).

Ethylene is the only hormone that is a gas. The main functions of ethylene in the plant involve fruit ripening, senescence, and protection against wounding and pathogenic attack. The production of ethylene is very well regulated through the 1-aminocyclopropane-1-carboxylate (ACC) gene family. In arabidopsis, a mutation of an ethylene receptor, ETR1, produced a dominant ethylene-insensitive mutant that has reduced affinity for ethylene. In other words, the mutated receptor cannot be turned off by ethylene binding. This mutation is homologous to the tomato mutant *Never Ripe* (*Nr*) (Johnson, et al, 1998).

A mutant of tomato (*Lycopersicon esculentum*) known as *diageotropica* (*dgt*) is auxin insensitive. This mutant tends to grow horizontal to the gravitational field, has curled dark green leaves, and an absence of lateral roots (Zobel, 1972). While fruit growth is affected, the fruit ripening process is unaffected by the plants reduced sensitivity to auxin (Balbi and Lomax, work in progress). The single gene, recessive *dgt* mutant (Zobel, 1973) has reduced sensitivity to IAA (indole-3-acetic acid) with respect to the induction of ethylene synthesis, but their ethylene synthesizing capacity is normal except for the reduced response to auxin (Kelly and Bradford, 1986). When ethylene was applied to *dgt*, the reduced gravitropic response was somewhat restored indicating low levels of ethylene can somewhat compensate for auxin insensitivity (Madlung, et. al., 1999).

The ethylene-insensitive mutant of tomato known as *Never Ripe* (*Nr*), is a single gene, partially dominant in the AC background which causes some fruit to fully ripen, pleiotropic trait (Lanahan et al., 1994). While the vasculature of *Never ripe* plants looks like the parent line, most of the fruit only ripens to an orange color. The *Never Ripe* mutation in the Alisa Craig (AC) background is somewhat

leaky and some fruits reach a nearly normal red color. The *Nr* mutation is in the tomato homologue of ETR3, an ethylene receptor. This means that *Nr* is not impaired in any step of the biosynthesis of ethylene, nor is it impaired in auxin biosynthesis (Lanahan et al., 1994).Analysis of a double mutant between *dgt* and *Nr* will help us better understand how auxin and ethylene interact. In a previous double mutant study, Andreas Madlung, a Ph.D. student in Dr. Terri Lomax's lab at Oregon State University, generated *dgt/Nr* double mutants. However, for his study, the *Nr* and *dgt* alleles used were in different genetic backgrounds, which could result in background effects that would be difficult to separate from mutant interactions. To control for background effects, seedlings of the F2 generation (*Nr* x *dgt*) were included (Madlung, 2000). Various assays were performed to test for the effects of ethylene and auxin interaction. The most noticeable effects were in flower development where there was a lack of carpel fusion and an increased number of floral organs in the double mutant (Madlung, 2000). However, due to the difference in parental genotype it was difficult to assess the source of this variation. Fortunately, both the *dgt* and *Nr* mutations are available in the same background, Alisa Craig (AC). The purpose of my study was to repeat the *Nr/dgt* crosses within the same genetic background to be certain that the characteristics observed in the previous study result from the interactions between mutant auxin and ethylene response genes and are statistically significant compared to the wild-type parental variety and the single mutants.

According to the observations made by Andreas Madlung, we would hypothesize that the double mutant would exhibit *Nr* phenotypic characteristics, excess floral organs, and abnormal fruit development, as well as a lack of response to ethylene. We expect this because *Nr* is a dominant mutation and therefore should be expressed. However, it is not clear whether the observations made in Madlung's study were really significantly different or the result of maternal effects. From preliminary observations of the single mutants and the wild-type parent, I hypothesize that the double mutant will not be significantly different from the

single mutants or the wild-type parent. I expect that the double mutant will exhibit little abnormality in floral organs, that the time from anthesis to breaker will not be significantly longer because *Nr* is not significantly different from the wildtype, that the gravitropic response will be intermediate between wild type and the slower response normally exhibited by *dgt* seedlings, and that the double mutant will have lateral roots. The presence of both the *Nr* and *dgt* genes is expected to cause a slightly slower growth, but the dominant *Nr*, which has lateral roots, will preside.

MATERIALS AND METHODS

PLANT MATERIAL

Seeds of AC and *dgt* in the AC background were originally obtained from the C.M. Rick Tomato Genetic Resource Center and the University of California at Davis, and were self propagated at the Oregon State University Botany farm in Corvallis, Oregon. Seeds of *Nr* in the AC background were also obtained from the C.M. Rick Tomato Genetic Resource Center. Seeds were sterilized in 20% bleach solution for 20 minutes (15 min for *Nr* seeds) and rinsed with water for 40 minutes. The seeds were then sown onto moistened filter paper placed in a box, and allowed to germinate in complete darkness at 30°C. Seedlings were transplanted into potting soil, then after 3 weeks transplanted once more to half gallon pots filled with a 3:1 mixture of vermiculite and cat litter (to allow for better drainage), and grown under standard greenhouse conditions (14 hours light and 10 hours dark at 25°C day and 15°C night).

CROSS POLLINATIONS

To obtain F1 seeds, reciprocal cross-pollinations between *Nr* and *dgt*, were carried out. Flowers from the female plant were emasculated and pollen from the donor flower was placed on the stigma of the receiving flower (*dgt*♀*Nr*♂, and *Nr*♀*dgt*♂).

F2 GENERATION AND SCREENING

To obtain the F2 generation, the pollen from the F1 generation was back-crossed to *dgt* mutant flowers. The reciprocal cross was also carried out. To locate the predicted F2 populations, the plants were screened for a *Nr* phenotype, a *dgt* phenotype, and a WT phenotype. Characteristics screened for were upright growth, the presence of orange fruit, and horizontal growth. From the backcross, it was expected that 25% of the F2 generation would be *Nr*/+; *dgt*/*dgt*, 25% would be *Nr*/+; *dgt*/+, 25% would be +/+; *dgt*/*dgt*, and 25% would be +/+; *dgt*/+. (+ denotes a wild type allele.)

GERMINATION ASSAY

Seeds from the wild-type parent line (AC), single homozygous mutant lines (*dgt* and *Nr*), and reciprocals of the F2 population (*dgt*♀(*Nr*♀*dgt*♂)♂ and (*Nr*♀*dgt*♂)♀*dgt*♀) were used. To test for differences in germination and possible maternal effects, seed from all populations were grown in darkness on water-moistened filter paper placed in plastic boxes in five different chambers. Each chamber had a different temperature ranging from 15°C to 35°C at 5° intervals. The number of seeds that germinated (seeds that have a hypocotyl length of 3mm) were counted every other day. Two replications were completed with the number of seeds per replicate ranging from five to eight seeds.

ANALYSIS OF PLANTS

The number of internodes required for flowering was counted for each plant. Once every other week, flowers at the anthesis stage were collected and their morphology analyzed. The number of floral organs were counted, weighed and scored for fusion, lack of fusion, and any other abnormal characteristics. In addition, time

from anthesis (flower opening) to breaker (first stage of color on the bottom of the fruit), the number of seeds per tomato, fruit diameter, and weight were analyzed.

GRAVITROPIC RESPONSE

WT, *Nr*, *dgt*, F1 generation, and the F2 generation were grown in darkness for 4 days. Seedlings from each population were selected and placed on 1% agar plates and placed under red light. Pictures were taken at time 0 hr, 1 hr, 2 hr, 4 hr, 6 hr, 8 hr, 10 hr, 12hr, 24 hr, and 30 hr after exposure to red light. These seedlings were measured for their gravitropic response in light by measuring the angle of curvature with a protractor (Madlung, 2000). The seedlings were kept on agar plates under red light for 3 additional days and the number of lateral roots counted. The seedlings were then classified according to their rooting phenotype.

RESULTS AND DISCUSSION

CROSS POLLINATIONS

Mutants homozygous for the *dgt* and *Nr* mutant genes were crossed to obtain an F1 population. Both the *dgt* and *Nr* alleles used are in the same wild type parental background, Alisa Craig (AC). The F1 population was backcrossed to *dgt* to allow for a greater chance of observing a double mutant phenotype due to the expected 1:1:1:1 ratio. The predicted F2 phenotypes are shown in Figure 1 from left to right: double mutant with an unknown phenotype, dgt mutant phenotype, Nr phenotype, and wild type phenotype. Only five seedlings survived the severe disease infestation. Out of those five plants that reached maturity, two had a *dgt* phenotype and the remaining three had a distinct *Nr* phenotype. No plants with completely wild-type phenotypes were found (Table 1).

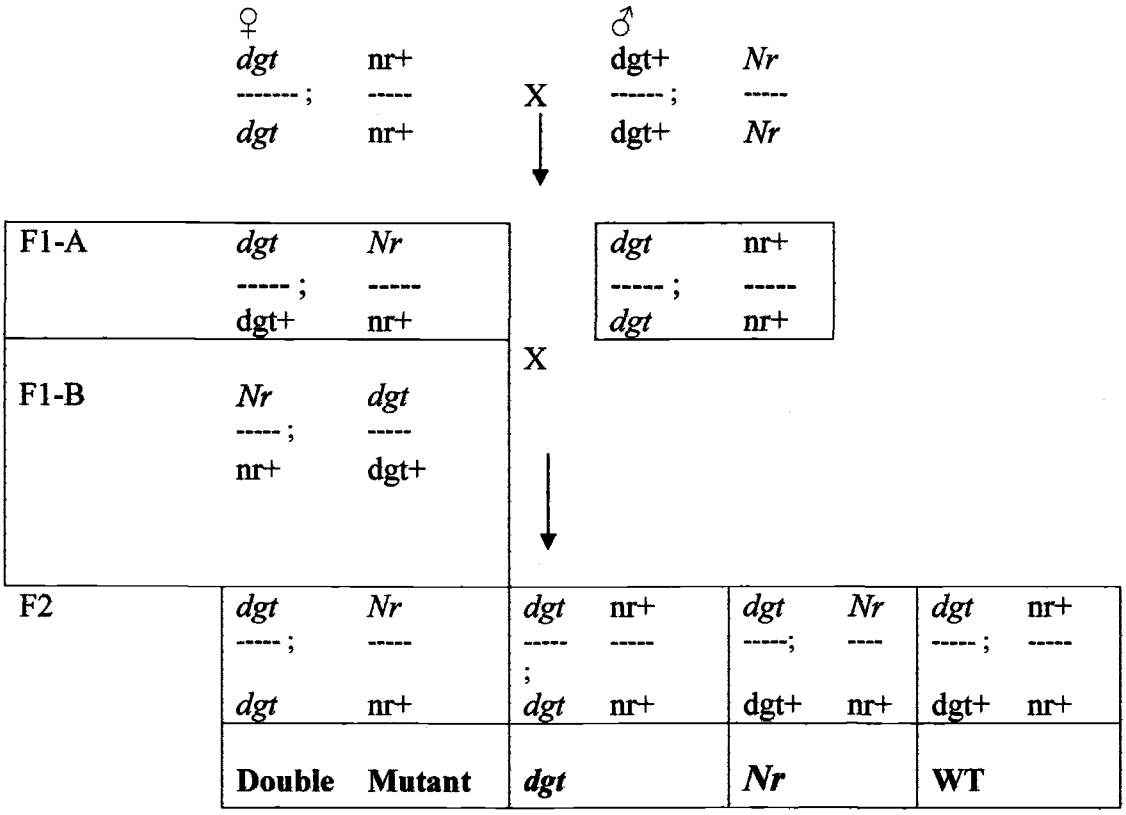

Table 1: cross-pollinations. pollen from the donating parent (either *dgt* or *Nr*) was placed on the stigma of the emasculated flower which resulted in an F1 population. The F1 was backcrossed with homozygous dgt via the same transfer of pollen technique and the resultant F2 population was obtained. There is a 25% chance of observing all four populations. The chromosome listed on the left is always the mother. For each cross, a reciprocal was also completed to account for the possibility of a maternal effect.

GERMINATION ASSAY

When F2 seeds were germinated in the usual conditions of 30°C, F2 from reciprocal crosses germinated at different rates ((*Nr*♀*dgt*♂)♀ *dgt*♂ began germinating slightly sooner than its reciprocal *dgt*♀ (*Nr*♀*dgt*♂)♂). To see if there was a maternal effect, seeds were incubated at different temperatures and analyzed

for the total number of seeds that germinated. Two replications were completed with reciprocal F2 populations with each replication having between five to eight seeds. The total seed germination determines seed germination success. Differences in rate of germination are very difficult to determine and cannot be used to determine maternal differences due to the sensitivity of the seeds to their conditions. It is very difficult to maintain exact conditions, and as a result, the only way to establish a difference is if the percentage of total germinated seed is significantly different (Hiro Nonogaki, personal interview). Therefore, no conclusive results could be drawn from this experiment since there was no significant difference of percent germination for the various genotypes. The seed coat, which is derived from the ovary and is genetically maternal, delivers nutrients to the growing hypocotyls. Therefore, it is still possible to have a maternal effect, although it causes only a slight delay in germination and is probably not significant.

Plant Analysis

Plants were grown under greenhouse conditions with 16-hour days and 8-hour nights. The number of internodes produced before the first flower appeared was about the same for the F2 population as for the wild-type and F1 parents (Fig. 1). This number is probably slightly high because of poor survival. Only five plants out of 20 F2 plants survived. The lack of survival was due to damping off, mite and virus infestations. The infected plants were removed so as not to falsify the data as well as infect subsequent plants. Analysis of the ovary weight indicated that there was no significant difference between wild-type plants and the double mutant. Once again, low numbers make the accuracy of these values difficult to assess (Fig. 2). The F2 was very similar to *Nr* with respect to fruit characteristics. However, the *dgt* values measured, which included weight, polar and equatorial diameters, and the number of seeds, were higher than expected. This is most likely due to a low number of fruit analyzed. The time from anthesis, or flower opening,

to breaker, which is appearance of color at the calyx end of the fruit, showed that in general, those fruit that derived from crosses in which *dgt* was the mother (*dgt*♀(*Nr*♀*dgt*♂)♂ and *dgt*♀*Nr*♂), had values similar to *dgt*. Those fruit that were derived from platns where *Nr* was the mother ((*Nr*♀*dgt*♂)♀*dgt*♂) had values similar to *Nr*, with the exception of the *Nr*♀*dgt*♂ F1 population. The data for this population had significantly less fruit analyzed, and therefore cannot accurately be compared with the other values. However, this trend does not follow for any of the other characteristics measured (Fig. 3). Lack of seedling survival, pest problems, and lack of flower to fruit set were the causes of the low fruit number. Standard error values of the mean were calculated in Excel. Overall, these data are inconclusive. To be sure that a double mutant was obtained the sampling size needs to be increased. If more samples were analyzed and the survival rate increased, a slight maternal effect may be observed in the F2 populations in terms of time from anthesis to breaker. In the data that was collected, it appears that most of the F2 values are very similar, but when looking closely at the data for anthesis to breaker, those fruit that were from an F2 cross in which *dgt* was the mother (*dgt*♀(*Nr*♀*dgt*♂)♂ and *dgt*♀*Nr*♂), take slightly longer to mature than those coming from *Nr* mother ((*Nr*♀*dgt*♂)♀*dgt*♂) plants. The difference is not significant, but a larger sample size may indicate that the difference is slightly larger than observed here.

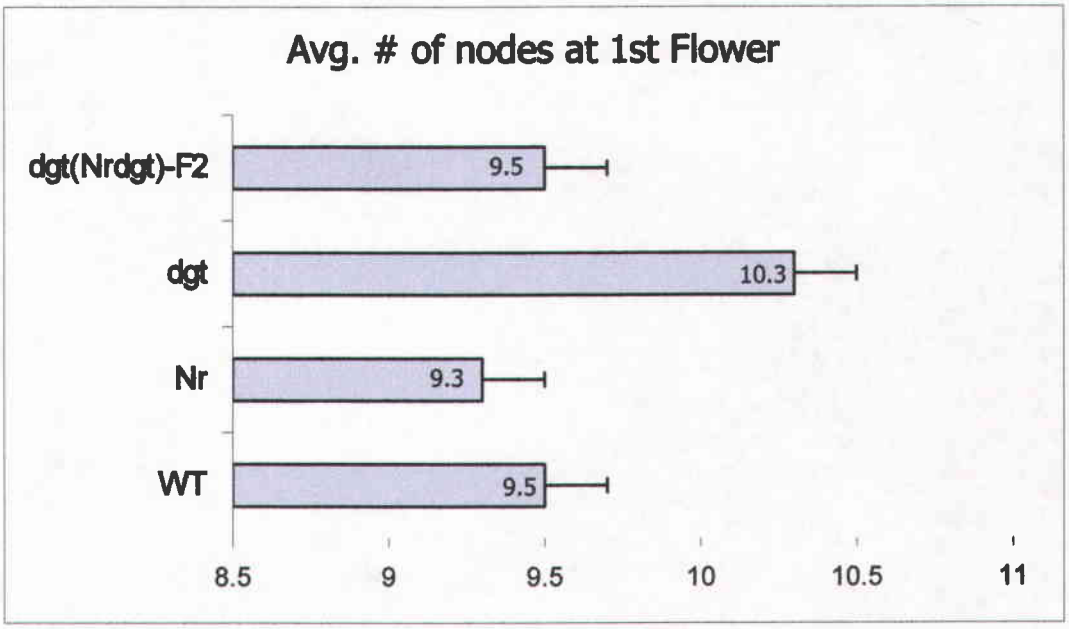

Figure 1: The number of internodes was counted at first anthesis for each plant. Error bars represent S.E. of mean, n= 2 for F2, n=4 for *dgt*, n=6 for *Nr*, n=5 for WT.

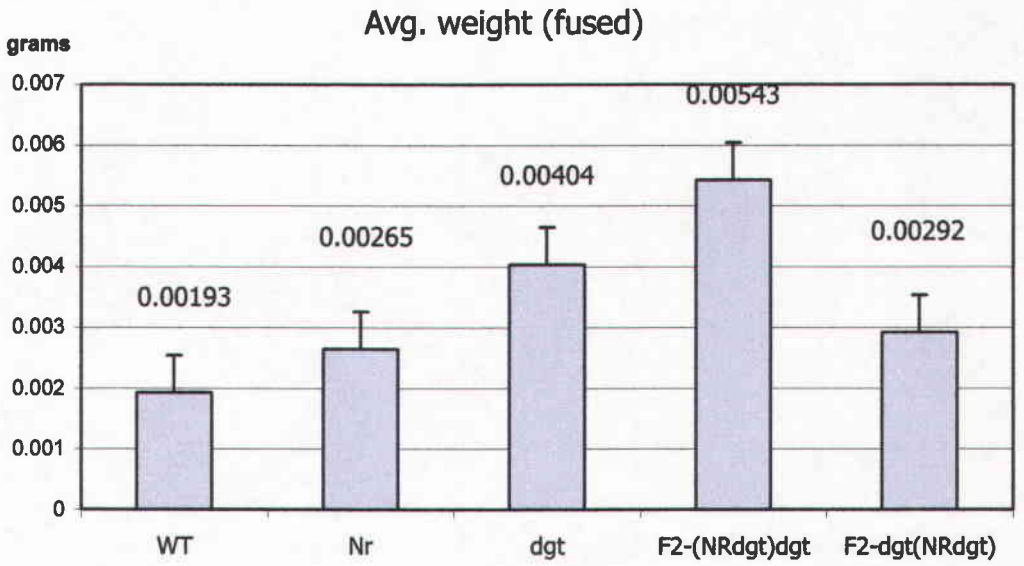

Figure 2: Flowers at anthesis from each genotype were collected and segregated based on ovary fusion. The total weight of the fused and unfused ovaries was weighed. The weight/ovary was then calculated. Error bars represent S.E. of the mean; n = 38 for WT, n= 32 for *Nr*, n= 14 for *dgt*, n= 12 for *dgt*♀(*Nr*♀*dgt*♂)♂-F2, n= 7 for (*Nr*♀*dgt*♂)♀*dgt*♂-F2.

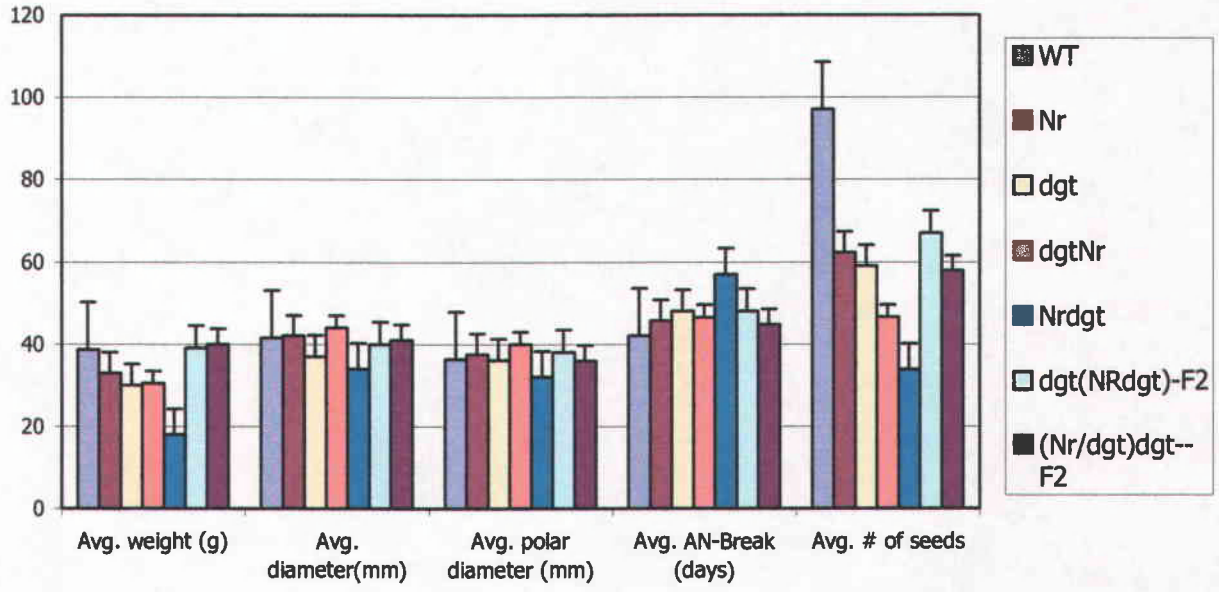

Figure 3: Flowers at anthesis, or flower opening, were tagged and marked with the appropriate data. The fruit derived from the previously tagged flowers were further tagged at the breaker stage, where color first begins to appear on the calyx end of the fruit, of development. Once these fruit ripened, they were removed, weighed and measured for their polar and equatorial diameters. The fruit were subsequently sliced in half and the number of seeds were counted. Error bars represent S.E. of the mean, n= 13 for WT, n= 22 for *Nr*, n = 9 for *dgt*, n= 17 for *dgt*♀*Nr*♂-F1, n= 10 for *Nr*♀*dgt*♂-F1, n= 5 for *dgt*♀(*Nr*♀*dgt*♂)♂-F2, n= 15 for (*Nr*♀*dgt*♂)♀*dgt*♂-F2.

GRAVITROPIC RESPONSE

Wild type shoots exhibit a negative gravitropic response. In other words, they reorient so that they continue to grow up opposite to the gravity vector. (Negative gravitropism is growth away from gravity, positive gravitropism is growth toward gravity) *dgt* mutants exhibit a reduced positive gravitational response (Fig. 4) (Madlung, 2000). Most of the F2 seedlings exhibited a negative gravitational response. However, there was a high percentage ((*Nr*♀*dgt*♂)♀*dgt*♂:75% and

dgt♀(*Nr*♀*dgt*♂)♂: 50%) of seedlings that lacked lateral roots, which is a phenotype associated with *dgt*. Therefore, the only way to determine the phenotype of the plants is to grow them in the greenhouse and characterize their phenotype. Since lateral root formation is the only way to determine a *dgt* phenotype, seedlings for the gravitropic experiment were separated based on the presence or absence of lateral roots. In this way, a difference in gravitropic response due to the presence or absence of lateral roots could be analyzed. (Table 2, Fig. 5) Based on the gravitropic data, the F1 generation appears to have an intermediate response to gravity. Since the F1 has a *Nr* phenotype, we would expect the F1 to exhibit a response similar to *Nr*. The F2 population, as figure 6 illustrates, has a similar response to gravity as *dgt*. There was no significant difference between the seedling populations that had lateral roots and those that did not. A significantly high proportion (29 out of 47 seedlings) had no lateral root formation, which could indicate a semi-dominant *dgt* phenotype. It is also possible that the *Nr* lesion helps repair the dgt lesion since the F1 population had a depressed gravitropic response in respect to *Nr*, but a much larger response with respect to *dgt*. However, one homozygous *Nr* seedling as well as a WT seedling, which normally have wild type phenotype in terms of lateral root formation, had an absence of lateral roots. In addition, abnormal phenotypes such as hypocotyls without roots or leaves were initially observed in the F1 population. (Fig. 8) A screening of the WT, F1, F2, *dgt*, and *Nr* concluded that the abnormal phenotypes were also present in these populations as well. Several of the abnormalities observed included leafless hypocotyls, rootless hypocotyls, hypocotyls with a severely shortened and thickened root, hypocotyls that were fused to the seed at the position where the hypocotyls emerge, seeds without hypocotyls but had a root, and very curled hypocotyls. All of these irregularities were observed in wild type (AC), *Nr*, *dgt*, the F1 populations, and the F2 populations. Significantly more abnormalities (approximately three times as many) were found in the *dgt* and F1 populations that in the AC populations. However, these aberrations were also seen

in the other genotypes as well. Since seedlings respond to their surrounding environment, and it is the hormones that are responsible for sending the signals, these abnormal seedlings could be the result of a response to something in the environment. In addition, the higher frequency that was found in the mutant populations could be an indication that hormone insensitivity generates seedlings that are more sensitive to its environment.

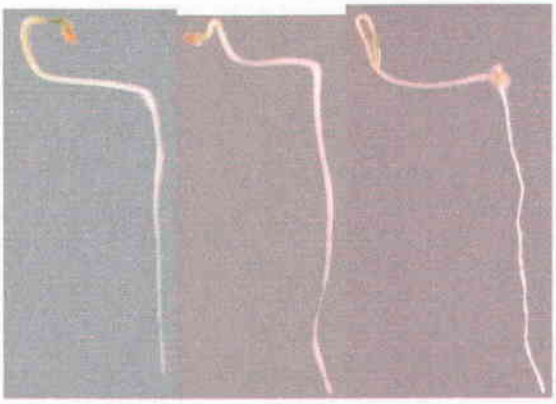

Figure 4: Illustration of three seedlings (From left to right, *dgt*, F2, *Nr*) after 12 hours of exposure to red light. To measure their response to gravity, a line would be drawn though the flat part of the hypocotyl. Another line would be drawn at the point of curvature and the smaller of the two resulting angles would be measured.

Genotype	Total # of seedlings	# with lateral roots	# without lateral roots
WT	59	58	1
Nr	26	25	1
dgt	35	0	35
dgt♀*Nr*♂-F1	17	17	0
Nr♀*dgt*♂-F1	20	20	0
dgt♀(*Nr*♀*dgt*♂)♂-F2	17	8	9
(*Nr*♀*dgt*♂)♀*dgt*♂-F2	32	8	24

Table 2: The number of lateral roots present and absent for each genotype and the reciprocal crosses. The WT and *Nr* seedling without lateral roots was treated as an outlier and not considered in the data.

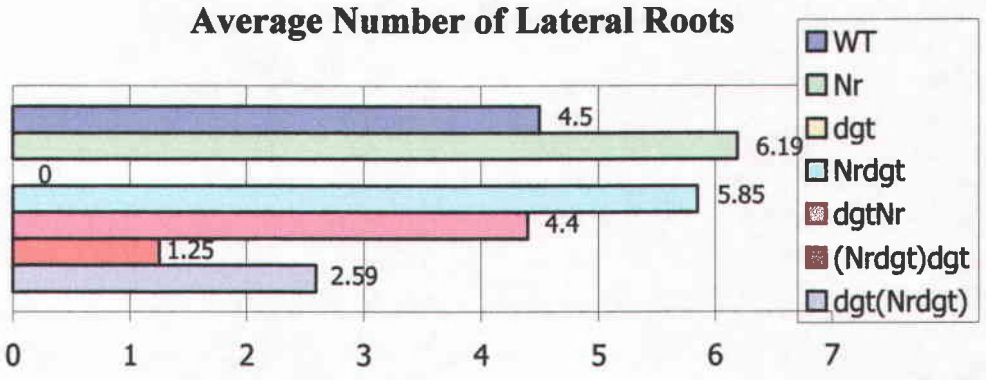

Figure 5: The number of lateral roots were counted five days after seedlings were transplanted onto agar plates. The seedlings were then separated depending on the presence or absence of lateral roots.

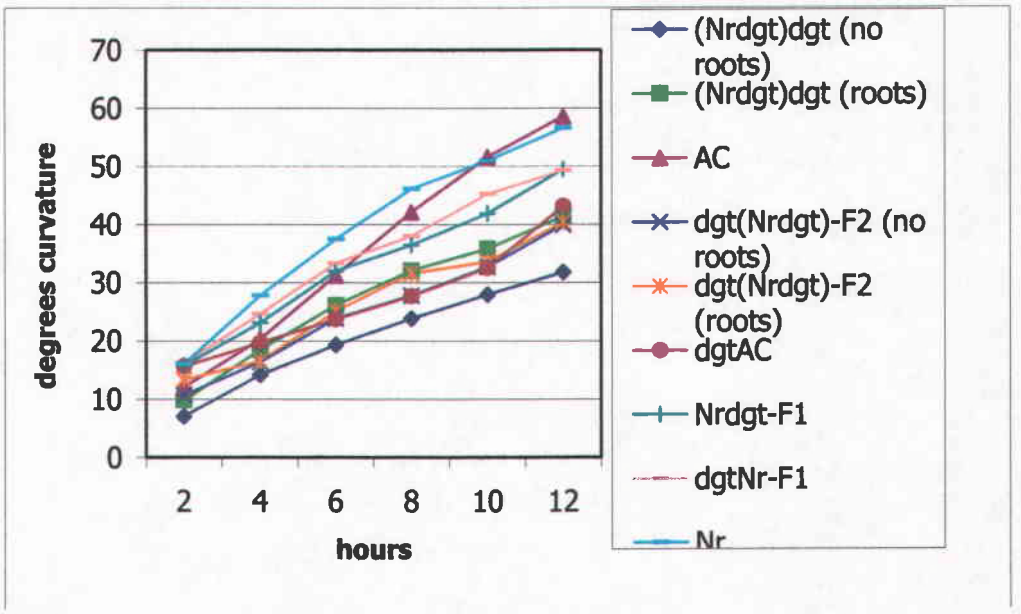

Figure 6: Response to gravity. Seedlings were grown in darkness at 28 °C for four days. They were then placed on 1% agar plates, which were inserted vertically into plastic baskets so that the seedlings would be perpendicular to the ground. Pictures were taken from 0-30 hours and the degree of curvature was measured with a protractor. The values showed here represent the mean of the degrees curvature.

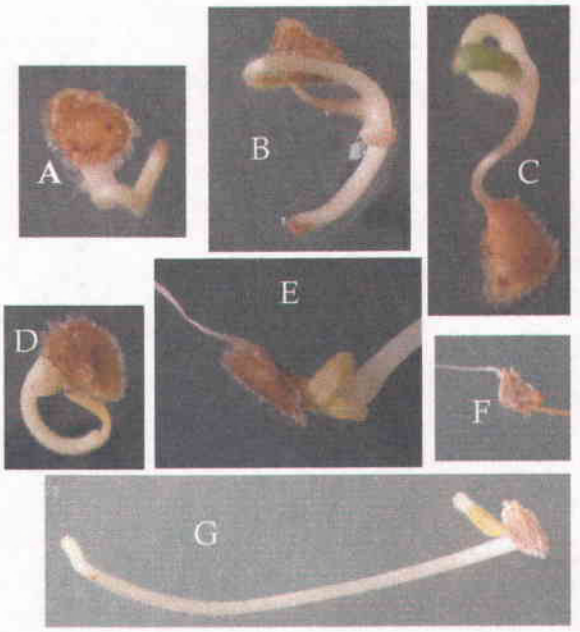

Figure 7: The original abnormalities observed in the F1 population. A: short, thick hypocotyl with no leaves B: thick hypocotyl with no leaves that is fused to the seed C: curled hypocotyl with no root D: short, curled hypocotyl that is fused to the seed, has another leaf protruding form the seed, and no root E: seed with a thin root and leaves emerging from the seed F: very thin root and hypocotyl with seed degenerating G: hypocotyl with only one leaf primordia and another emerging from the seed with no root.

CONCLUSIONS

Auxin and ethylene combine to regulate many processes that occur throughout development of a plant. However, the mechanism by which auxin and ethylene interact is not well known. Since very little data was obtained form the fruit analysis, the conclusions have been drawn from the lateral root and gravitropic data. The F2 populations, regardless of lateral root presence had a gravitropic response similar to dgt and because the F1 generations had an intermediate gravitropic response, it can be concluded that *dgt* is semi-dominant. The presence of *Nr*, did not increase the gravitropic response, even in the populations were lateral root formation was present. In addition, the presence of *dgt* depresses the gravitropic response in the F1 generation so that it has a slower response than *Nr*. In addition, it can also be concluded that the presence of Nr partially repairs the dgt lesion. The gravitropic response of the F1 generation was elevated from the response of dgt. Therefore, because in the F1 there is only one copy of the dgt gene, Nr is able to increase the gravitropic response and allow for the formation of lateral roots. The F2 could be tested for insensitivity to ethylene by observing its triple response. The F2 populations could also be tested for auxin insensitivity by applying auxin. If the F2 exhibits no triple response and does not respond to auxin applications, then it is likely both genes are present. However, if there is a slight response, it could also indicate that one gene is helping to repair the other. Genetic screening would have to be conducted in order to obtain conclusive evidence on the status of the presence of the double mutant.

From this research, no concrete conclusions can be made at this time. From all of the observations made, genetic analysis, increased sampling size, and observations of adult F2 plants are required in order to characterize the hormonal interactions. Perhaps new techniques, such as gene traps should be employed to see if the *Nr* gene is partially masking the effects of the *dgt* gene. There are three types of gene traps: enhancer traps, promoter traps, and gene traps. The enhancer trap involves a

reporter gene that is fused to a minimal promoter that usually is composed of a TATA box and a transcription start site. The promoter and gene traps both contain a promoterless reporter gene, which requires them to be in the correct orientation within a transcriptional unit. Gene traps could prove to be very useful because a mutant phenotype is not required. In addition, it allows for identification of "functionally redundant genes and genes that have functions at multiple developmental stages" (Springer, 2000). If gene traps were used in this research, it would be difficult to predict what the outcome would be, especially if *dgt* is semi-dominant, but it is probable that *Nr* helps to partially repair the *dgt* gene to some degree. Before using such techniques, however, more observations at the adult plant stage would have to be collected.

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