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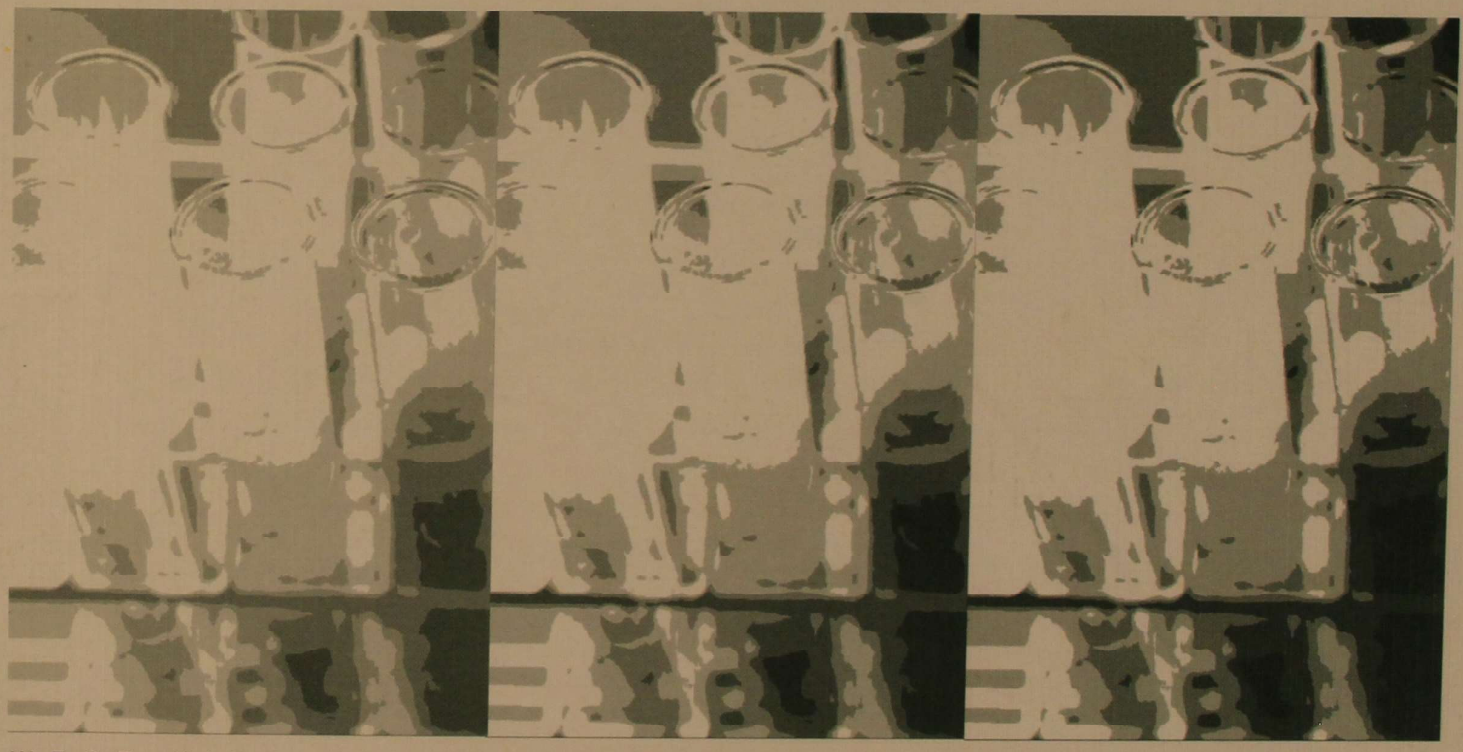

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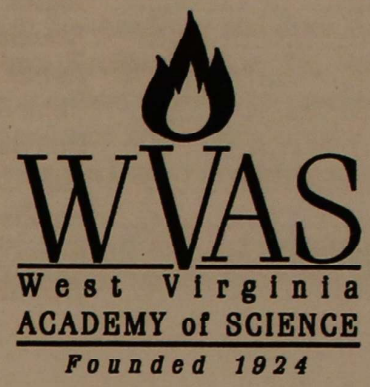

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GROWTH AND GRAVITROPIC CURVATURE IN ETHYLENE MUTANTS OF *ARABIDOPSIS THALIANA*

Dora D. Gonzales, Jeremy Traylor, Ann Hubbard, Benjamin Lowman, Azam Mohiuddin, and Marcia A. Harrison

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ABSTRACT

Gravitropism, the physiological and cytological events associated with a change in orientation to gravity, is regulated by light and plant hormones. The gaseous plant hormone, ethylene, inhibits cellular growth and alters the gravitropic response in plant organs. In this investigation, growth and gravitropic curvature were measured in mutant strains of *Arabidopsis* (common wall cress) with altered ethylene response or biosynthesis. Growth, diagravitropism (horizontal growth determined by angle from vertical), and gravitropic curvature (angle after gravistimulation, a 90° change in orientation) were measured from 3-day-old light grown *Arabidopsis* seedlings. Roots of the *eto1-1* (ethylene overproducer) mutant exhibited significantly less growth, diagravitropism, and less gravitropic curvature. However, *eto1-1* hypocotyl measurements were similar to wild type, indicating lower ethylene production compared to other *eto* strains. The *ctr1-1* (constitutive triple response; short, swollen stems with horizontal orientation in dark-grown plants) mutant seedlings exhibited both root and hypocotyl inhibition. Hypocotyl curvature was reduced in *ctr1-1* roots only. The *hls1-1* (hookless; low ethylene levels and altered differential cellular apical growth in dark-grown seedlings) mutant exhibited somewhat reduced gravitropic curvature proportional to reduced growth in both roots and hypocotyls. The mutant's altered differential growth along the stem does not appear to affect the plant's ability to curve in response to gravity. In conclusion, *eto1-1* and *hls1-1* mutants differ from the wild type and each other in their orientation to the vertical and their responses to changes in orientation. Further studies are planned to investigate ethylene's effect on gravitropic pattern and curvature kinetics in *Arabidopsis* mutants.

INTRODUCTION

Arabidopsis thaliana, the common wall cress, has been selected as a genetic model for the plant kingdom (Alberts et al. 1998). It is a convenient model because it is small, easy to cultivate, fast growing, and can be grown indoors in large numbers; producing thousands of offspring per plant within 8 to 10 weeks. In addition, numerous mutant strains are available for

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Arabidopsis (http://arabidopsis.info/, and http://www.arabidopsis.com/). Several of these strains exhibit altered responses to the gaseous plant hormone, ethylene (Arteca 1996). Ethylene, the chemically simplest plant hormone, is produced in virtually all plant tissue. Its biosynthesis is controlled by a large number of environmental and physiological factors. Increased ethylene production accompanies such morphological events as fruit ripening, senescence, pollination, and abscission (Ecker 1995). In dark-grown seedlings, ethylene produces changes called the 'triple response' characterized by 1) shortened, 2) swollen stems with 3) horizontal orientation or diagravitropism (Arteca 1996).

Arabidopsis mutants that exhibit a constitutive triple response phenotype have been central in the study of ethylene biosynthesis, perception, and response (Kieber 1997). In the constitutive ethylene response mutant, *eto1* (ethylene overproducer), ethylene biosynthesis inhibitors and antagonists can reverse its phenotype. The *ctr1* (constitutive triple response) mutant is defined in dark-grown plants by having a very short root, a short thickened hypocotyl (seedling stem below the seed leaves) and an exaggerated apical hook (Kieber et al. 1993). The *CTR1* gene encodes a putative serine/threonine protein kinase that is most closely related to the *Raf* protein kinase family (Kieber et al. 1993). *Raf*, an animal oncogene product, is associated with hormonal signal transduction cascades, and is activated upon hormone-receptor binding via an intermediate molecule (Kieber 1997). In contrast to the *ctr1* mutant, the *hls1* (hookless) mutant lacks an apical hook in dark-grown plants (Lehman et al. 1996). *Hls1* mutant seedlings produce less ethylene than the wild type (Guzman and Ecker 1990). The *HSL1* gene encodes a protein similar to a class of N-acetyltransferases found in animal tissues and not previously identified in plants. The HSL1 protein is required for the regulation of differential growth in hypocotyl bending (Lehman et al. 1996). In addition, Lehman et al. (1996) reported that the *HLS1* gene alters the expression pattern of the plant hormone auxin, and proposed that coordination of ethylene and auxin in differential cellular growth is critical to processes such as formation of the apical hook of dark grown dicot seedlings. All ethylene response mutants exhibit altered apical hook formation, indicating changed growth pattern along the hypocotyl.

In higher plants, shoots show negative gravitropism (upward growth) and roots show positive gravitropism (downward growth). Many physiological and cytological events are associated with gravitropism and are regulated by growth substances and light (Fujisawa et al. 1997). Stem or root curvature is most often attributed to changes in growth on the lower side as a result of increased accumulation of the growth hormone, auxin (Arteca 1996, Taiz and Zeiger 1998). In plant stems, auxin stimulates cell elongation on the lower side of the stem, causing the upward curvature; however, in roots, auxin accumulates to an inhibitory level, causing the lower side to grow more slowly compared with the upper side (Arteca 1996, Taiz and Zeiger 1998). High levels of auxin in the tissue induce the increased biosynthesis of ethylene (Taiz and Zeiger 1998). Many instances of auxin-induced cell inhibition are attributed to auxin-stimulated ethylene biosynthesis (Arteca 1996). Ethylene has also been associated with regulating the kinetics of gravitropic curvature by modifying later stages of bending (Clifford et al. 1983), and altering the plant's sensitivity to auxin (Wheeler et al. 1986). Therefore, auxin and ethylene work together in coordinating cell elongation patterns in a variety of plant growth processes.

In this investigation, measurements of root and hypocotyl growth were compared with gravitropic curvature in the *A. thaliana* ethylene-response mutants *ctr1*, *eto1*, and *hls1*.

MATERIALS AND METHODS

Plant Material and Growth Conditions

Seeds of the wild type (Columbia ecotype) and mutant alleles, *ctr1-1, eto1-1* and *hls1-1*, (Arabidopsis Biological Research Center, Columbus, Ohio) were grown on 0.9% agar plus 0.5 X Murashige and Skoog basal salt medium (Sigma Chemical Co.) in Petri dishes. The dishes were sealed with Parafilm and stored at 4^0 C for an imbibition period of 3 days. After imbibition, seedlings were grown under Sho&Gro Brightsticks™ fluorescent lamps with an average continuous irradiance of 20 :mol m^{-2} s^{-1} at 23 to 24° C.

Growth and Gravitropic Measurements

Growth measurements of hypocotyls and roots were taken periodically after germination. Root and hypocotyl angles from vertically-oriented plants were measured to determine the extent of diagravitropism prior to altering their orientation by 90° (gravistimulation). The extent of gravitropic curvature was determined by the difference between the angle in vertical 3-day-old seedlings and that of plants 24 hrs after gravistimulation. Statistical analyses were performed using single factor Anova (Microsoft Excel™).

Results and Discussion

Roots of *eto1-1* mutants exhibited significantly less growth than the wild types for all time points except 19 days after cold treatment (Figure 1). Hypocotyls of *eto1-1* did not exhibit slower growth compared to wild type (Figure 2). Both hypocotyls and roots tended to display diagravitropic behavior and deviate from the vertical orientation compared with control seedlings but not to a significant level (P=0.055 for hypocotyl; P=0.15 for roots), and only root gravitropic curvature was less than the wild type seedlings (Figures 3 and 4). The *eto1* mutant has been reported to produce 10 to 100 times more ethylene than wild type seedlings (Kieber 1997). Inhibited root growth and diagravitropism support a typical response characteristic of ethylene overproduction by the *eto1-1* mutant (Ecker 1995). However, for these seedlings, under the experimental growth and lighting conditions, hypocotyls did not exhibit the full characteristic phenotype, indicating that the seed lot did not have a high enough ethylene production rate to severely inhibit growth. The *eto1-1* mutant overproduces ethylene, an effect that can be reversed by ethylene biosynthesis inhibitors (Kieber 1997). Thus, this gene encodes a protein involved in the regulation of ethylene biosynthesis. Our data supports the increased ethylene production, but at the lower end of the reported rates, since only the roots exhibited inhibition. Gravitropic curvature was reduced somewhat in the roots, but not in the hypocotyls, of these mutants when compared to wild type seedlings (Figures 3 and 4). When the relative amount of gravitropic curvature (as percent wild type values) was compared with relative growth for 3-day old seedlings, the decrease in root gravitropic curvature was greater than the growth inhibition (Table 1). This suggests that the increased level of ethylene is sufficient to inhibit gravitropic curvature beyond the effect on cellular elongation. However, since these tissues did not maintain a vertical orientation, it should be noted that then the amount of

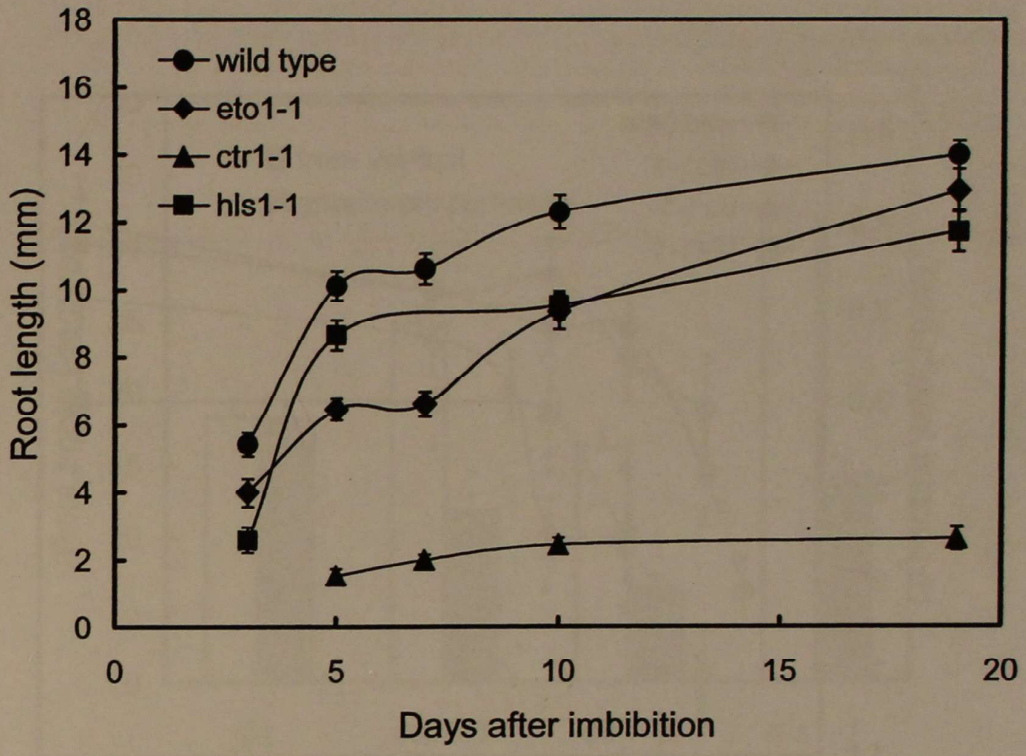

Figure 1. Comparison of root length measurements in ethylene response mutants *eto1-1* (♦), *ctr1-1* (▲), and *hls1-1* (■), compared to wild type (●) seedlings. Means ± SE. N = 12-30 seedlings. All values for the ethylene response mutants are significantly lower ($P<0.05$) than the wild type with the exception of 5- and 19-day-old *hls1-1* and 19-day-old *eto1-1* seedlings.

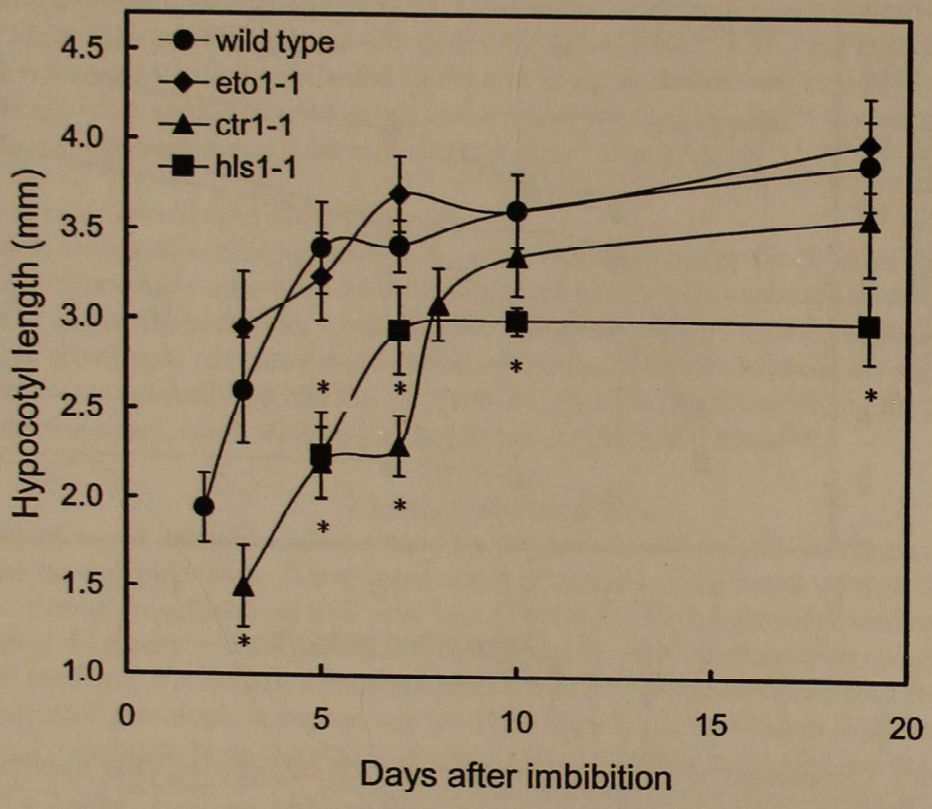

Figure 2. Comparison of hypocotyl length measurements in ethylene response mutants *eto1-1* (♦), *ctr1-1* (▲), and *hls1-1* (■), compared to wild type (●) seedlings. Means ± SE. N = 12-30 seedlings. * indicates values which are significantly ($P<0.05$) different from the wild type.

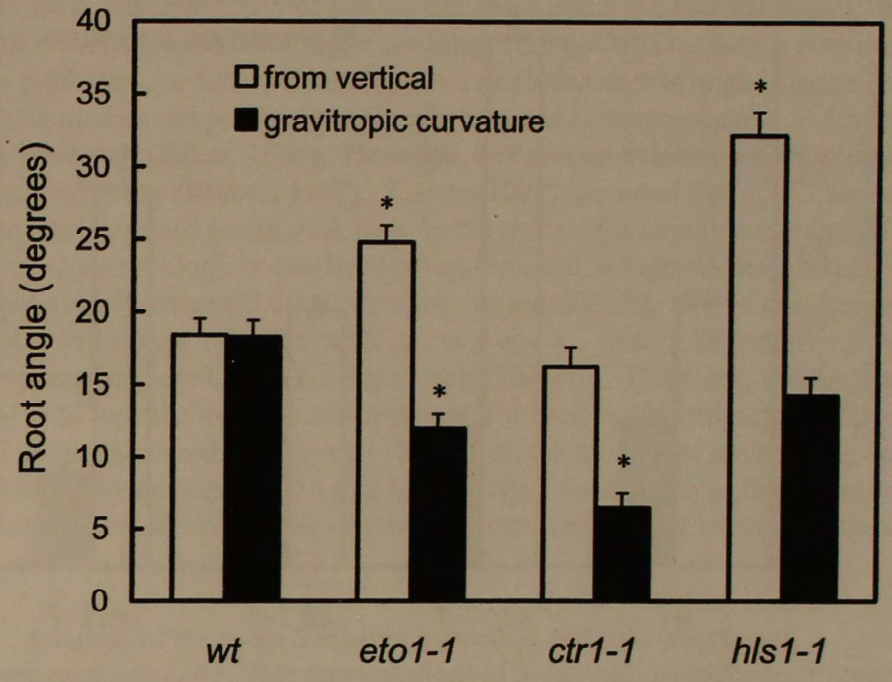

Figure 3. Comparison of root diagravitropism (angle from vertical orientation-open bars) and gravitropic curvature 24 h after gravistimulation (filled bars) in ethylene response mutants *eto1-1*, *ctr1-1*, and *hls1-1*, compared to wild type seedlings. Means $\pm$ SE. N = 12-30 seedlings. * indicates values which are significantly ($P<0.05$) different from the wild type.

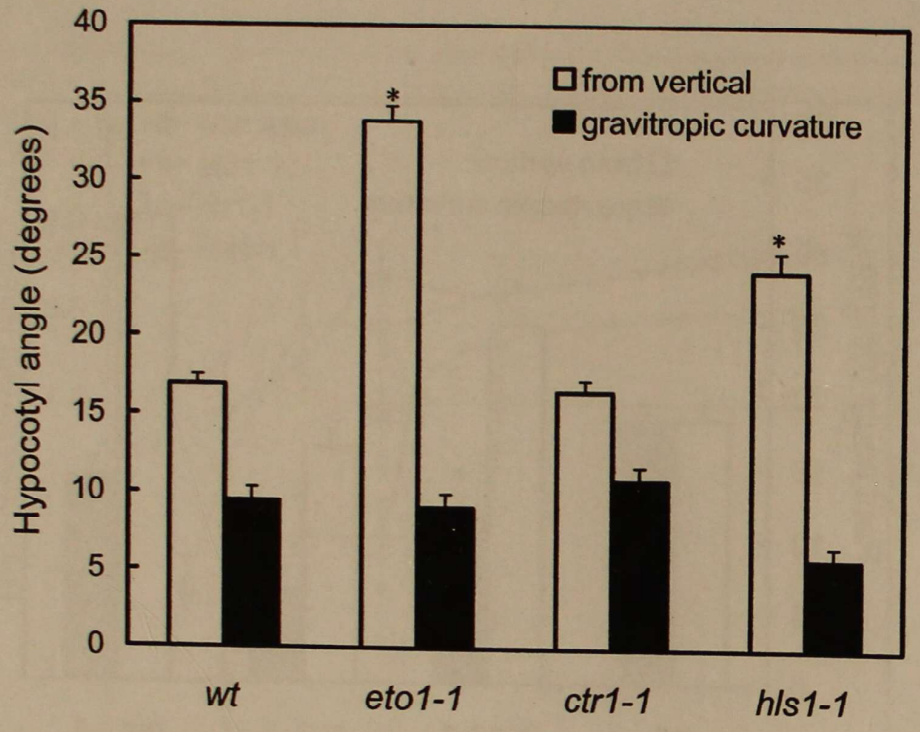

Figure 4. Comparison of hypocotyl diagravitropism (open bars) and gravitropic curvature 24 h after gravistimulation (filled bars) in ethylene response mutants *eto1-1*, *ctr1-1*, and *hls1-1*, compared to wild type seedlings. Means $\pm$ SE. N = 12-30 seedlings. * indicates values which are significantly ($P<0.05$) different from the wild type.

gravistimulation varied when the seedlings were reoriented due to differences in stem diagravitropism.

Roots of *ctr1-1* were severely inhibited compared with wild type ($P < 0.001$ for all data points; Figure 1). Hypocotyls of *ctr1-1* seedlings exhibited significantly lower elongation during the first week after imbibition ($P = 0.006$ for day three and $P = 0.003$ for day 7), indicating a slow initial growth rate compared to wild type seedlings (Figure 2). Our data is consistent with the growth rates published for 5-day-old dark grown *Arabidopsis* seedlings (Roman et al., 1995). The *ctr1-1* mutant does not produce excess ethylene and is not responsive to ethylene biosynthesis inhibitors (Ecker, 1995). However, this mutant exhibits a high expression of ethylene-regulated genes (Kieber, 1997). Kieber (1997) proposed that CTR1 protein negatively regulates ethylene response genes, and, thus, in the mutant the negative regulation is removed. Therefore, *ctr1-1* morphology is similar to plants exposed to high ethylene levels. The *ctr1-1* mutant did not exhibit increased diagravitropism or significantly altered gravitropic curvature, although root gravitropism was reduced (Figures 3 and 4). In fact, the relative gravitropic curvature response increased in *ctr1-1* hypocotyls (Table 1). Therefore, seedling response based on percent of wild type showed that the observed decrease in gravitropic curvature in roots was proportional to the decreased root growth. However, the kinetics of the bending was not evaluated. Since ethylene may play a role in modifying the kinetics of the response, the *ctr1-1* mutant may exhibit an altered kinetic or growth pattern in terms of location of the cellular response (Evans and Ishikawa, 1997).

Table 1. Analysis of the overall relative hypocotyl and root growth and gravitropic response (24 h after reorientation) in 5-day-old *Arabidopsis* mutants compared to wild type seedlings. Values are percent measurements in mutant seedlings compared to wild type for a given set of seedlings. N = 12-25 seedlings.

HYPOCOTYLS	***eto1-1***	***ctr1-1***	***hls1-1***
Length	109%	64%	72%
Gravitropic curvature	96%	115%	62%
ROOTS			
Length	84%	35%	57%
Gravitropic curvature	65%	35%	78%

Growth rates for *hls1-1* mutants were generally slower compared to wild type seedlings (Figures 1 and 2). This supports the Lehman et al. (1996) investigation noting decreased

hypocotyl length 3 to 12 days after germination and inhibited root length 4 to 6 days after germination. Since dark-grown *hls1-1* seedlings had an 86% reduction in ethylene production (Guzman and Ecker, 1990), increased growth might be anticipated. Thus, the morphological changes are not attributed to an ethylene response. The *HLS1* gene causes altered differential growth rate in the apical hook and is thought to change the growth response in cells along the plant stem (Kieber, 1997). The *HLS1* gene encodes an N-acetyltransferase, a proposed target of an ethylene response pathway that regulates differential cell growth along plant organs in bending processes such as the apical hook or gravitropism. In the *hls1-1* mutant, increased diagravitropism was found in both hypocotyls and roots, along with a reduction in gravitropic bending but without statistical significance (Figures 3 and 4). As noted for the *eto1-1* mutant, increased diagravitropism alters the initial angle when the seedling orientation is changed to induce gravistimulation. This may affect the gravitropic response. Comparison of the relative growth and gravitropic responses shows that the gravitropic response in hypocotyls was slightly lower than would be anticipated from the overall growth rate. This indicates a possible alteration in differential growth in the hypocotyl *hls1-1* seedlings, which would also affect the gravitropic response.

In conclusion, the ethylene response mutants of *Arabidopsis* can be valuable tools for the evaluation of ethylene's role in root and hypocotyl growth and gravitropic curvature. Mutants such as *eto1-1* and *hls1-1* clearly differ from the wild type and each other in their orientation to the vertical and their response to changes in orientation. The *ctr1-1* mutant does not exhibit a difference in relative gravitropic curvature compared with its overall growth rate, but may have an altered pattern of response. Further studies on the kinetics of the gravitropic bending and the cellular growth patterns will elucidate the effect of ethylene on these processes in *Arabidopsis* seedlings.

ACKNOWLEDGMENTS

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Effects of Cytosolic Calcium Regulators on Ethylene Biosynthesis in Etiolated Pea Stems

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ABSTRACT

Physical wounding, temperature extremes, chemical signals, and plant hormones all stimulate the production of the gaseous hormone, ethylene. Ethylene level is regulated by the production of the ethylene precursor, 1-aminocyclopropane-1-carboxylic acid (ACC) by ACC synthase and turnover of ACC to ethylene by ACC oxidase. Transduction of environmental, hormonal, and chemical signals resulting in altered ethylene biosynthesis involves a network of biochemical steps connecting signal-to-receptor binding with a cellular response. Numerous second messengers, including Ca^{2+}, have been identfied in transduction mechanisms in plants. The objective of this research is to determine the relationship between cytosolic Ca^{2+} and ethylene biosynthesis in dark-grown pea stems through exogenous application of various cytosolic Ca^{2+} mediators. Ethylene level and ACC oxidase activity were determined in excised stem segments treated with calcium or signal transduction inhibitor solutions. Short-term $CaCl_2$ treatment significantly enhanced basal ethylene production. However, ACC oxidase activity was not significantly altered by changes in the endogenous levels of Ca^{2+}. Wound-induced ethylene production that begins, in peas, 26-min after excision was not significantly affected by exogenous Ca^{2+} treatment or by application of Ca^{2+} signal transduction inhibitors. Our results suggest that the signal transduction associated with wounding does not appear to follow the Ca^{2+} pathway in the subsequent stimulation of ethylene biosynthesis. In conclusion, these results support a model of basal ethylene regulation via altered cytosolic Ca^{2+} released from the vacuole or endoplasmic reticulum, its binding to calmodulin, and subsequent activation of a protein kinase that directly activates ACC synthase.

INTRODUCTION

Ethylene, the chemically simplest known plant hormone, is produced in all plant tissues at low levels. Ethylene production increases in response to many endogenous and external stimuli, such

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as hormone levels, pathogen attack, wounding, or temperature extremes. Changes in ethylene concentration modify many plant growth and developmental processes. Increased ethylene production inhibits such responses as stem and root elongation, and alters the stem's orientation to gravity (Abeles et al. 1992). The enzyme, 1-aminocyclopropane-1-carboxylic acid (ACC) synthase regulates the primary rate-limiting step in the ethylene biosynthetic pathway, the conversion of S-adenosyl-L-methionine (SAM) to ACC (Yang and Hoffman 1984, Kende and Zeevaart 1997). ACC is converted to ethylene by ACC oxidase (Kende and Zeevaart 1997).

Signal transduction in plants is a network of biochemical steps that connect signal binding, usually on the plasma membrane, to a cellular response associated with growth and development (Trewavas and Malhó 1997). Intermediates in this network include numerous second messengers such as Ca^{2+}; inositol 1,4,5-trisphosphate (IP_3, a product of the breakdown of a phospholipid); cAMP (cyclic adenosine monophosphate); peroxide; and free radičals (Trewavas and Malhó 1997). Second messengers are involved in the transduction of plant signals such as hormones or fungal cell wall elicitors, thus altering ion transport across membranes, enzyme activity, and/or gene expression. An example of a proposed signal transduction pathway for the activation of ethylene biosynthesis includes the following steps. (1) A signal in the plant cell wall space binds to a plasma membrane receptor, (2) signal-receptor binding actives a GTP (guanosine 5'-triphosphate)-binding protein (G-protein) associated with the cytoplasmic face of the plasma membrane, which in turn activates phospholipase, (3) phospholipase cleaves the membrane phospholipid, phosphatidylinositol 4,5-bisphosphate, releasing inositol IP_3 and 1,2-diacylglycerol, (4) IP_3 activates membrane-bound Ca^{2+} channels (in the plasma membrane, ER, or vacuole), causing increased cytoplasmic Ca^{2+}, (5) Ca^{2+} binds to a small cytoplasmic protein, calmodulin, (6) the Ca^{2+}-calmodulin complex activates protein kinases that in turn activate target proteins (such as ethylene biosynthetic enzymes) or genes within the cell (Taiz and Zeiger 1991, Trewavas and Malhó 1997).

Inhibitors of signal transduction steps are commonly used to elucidate the reaction sequence between signal binding to a receptor and a target response. For example, $LaCl_3$ is a Ca^{2+} channel blocker that effectively reduces Ca^{2+}-mediated transduction events (Knight et al. 1996). EGTA (ethylene glycol-bis (2-aminoethyl ether) tetraacetic acid) inhibits Ca^{2+} influx into cells by chelating the ion in the cell wall. Ca^{2+} channel ionophores, such as A23187, increase cytosolic Ca^{2+} concentrations by opening ion channels and allowing intracellular Ca^{2+} concentrations to be balanced across the membranes (Rasmussen and Goodman 1977).

The kinetics of basal and wound ethylene biosynthesis have been well documented for dark-grown pea stems (Saltveit and Dilley, 1978). The objective of this research is to determine the relationship between cytosolic Ca^{2+} and ethylene biosynthesis in dark-grown pea epicotyls through exogenous application of various cytosolic Ca^{2+} mediators.

MATERIALS AND METHODS

Plant Material and Growth Conditions

Seeds of *Pisum sativum* L. cv. Alaska were surface sterilized in 0.525% NaOCl (10% commercial bleach) for 10 min, then rinsed thoroughly in tap water. Seeds were planted in vermiculite and grown in an environmental chamber (Revco Lindberg, Asheville, NC) at 25° C in darkness for 5-7 days. After germination, seedlings were watered daily under a dim green light (490-575 nm) with an average irradiance of 0.5 W m^{-2}.

Treatments and Ethylene Measurements

All chemicals were purchased from Sigma Chemical Co. (St. Louis, MO) and used at concentrations reported to be effective in eliciting a calcium response. Calcium treatments included 20 mM $CaCl_2$ (Kwak and Lee 1997); 20 mM $CaCl_2$ + 4 µM A23187 (an ionophore that increases cytosolic Ca^{2+}) (Schwacke and Hager 1992); or 4 µM A23187 alone. Signal transduction inhibitors included 20 mM LiCl which inhibits the phosphatidylinositol cycle by preventing Ca^{2+} release from intracellular compartments (Knight et al.1996); 10 mM EGTA, a Ca^{2+} chelator (Knight et al.1996); and the Ca^{2+} channel blockers, 10 mM $LaCl_3$ (Knight et al. 1996), and 500 µM nifedipine (DeNisi and Zocchi 1996). Deionized water was used as a control treatment. Preliminary studies were conducted to assess the effective incubation interval in the treatment solution. These studies included incubation of epicotyl segments from 1 hour to 1 day in a test solution in a Petri dish or spraying intact seedlings with a test solution 2-4 hours prior to epicotyl excision and enclosure into vials.

In dark-grown pea seedlings, excision of subapical stem sections induces an increase in ethylene production after a lag of 26 min, with peak production occurring at 60 min after excision (Saltveit and Dilley 1978). Excision of stem segments underwater, followed by a brief incubation in distilled water significantly reduces the wound ethylene response (Harrison 1991). Therefore, subapical stem sections (1.5 cm long) were cut underwater, then incubated with a test solution in a Petri dish for 10 min. Segments were then sandwiched between filter paper disks moistened with the same test solution, and sealed into a 2 mL vial. After 15-min incubation, a 1 mL headspace sample was removed. To determine ethylene levels, samples were injected onto an Alumina F column in a Varian 3700 gas chromatograph equipped with a flame ionization detector. All measurements of ethylene taken within 25 min of excision were considered representative of basal ethylene in the tissue; measurements after 25 min were categorized as wound-induced ethylene. Septa were removed from the sample vials between incubation intervals.

To measure ACC oxidase activity, the filter paper discs were moistened with 70 µL test solution containing 100 µM ACC, then incubated an additional 10 min before sealing the vial for ethylene accumulation. The rate of conversion of ACC to ethylene was used as an indicator of *in vivo* ACC oxidation (Harrison 1997).

Statistical analyses were performed using Microsoft Excel™ and SigmaStat™.

RESULTS AND DISCUSSION

Short-term $CaCl_2$ treatment with or without ionophore significantly enhanced basal ethylene production (P=0.001 and P=0.01, respectively) (Figure 1). These results suggest the rapid activation of an ethylene biosynthetic enzyme. Morgan and Drew (1997) propose that post-translational activation of ACC synthase may occur in rapid ethylene responses to stress. Also, Ca^{2+} has been reported to act as a second messenger in ethylene stimulation by fungal cell wall elicitors (Schwacke and Hager 1992) and applied ethylene (Kwak and Lee, 1997). Therefore, an influx of Ca^{2+} into the cytoplasm induced by an ionophore plus exogenous Ca^{2+} may initiate Ca^{2+}-calmodulin complex formation and subsequent protein kinase activation (Trewavas and Malhó, 1997). A protein kinase may directly phosphorylate and activate an ethylene biosynthetic enzyme such as ACC oxidase in response to increased endogenous ethylene levels (Kwak and Lee, 1997). Instances of both positive and negative feedback regulation mechanisms via ACC oxidase or ACC synthase regulation by applied ethylene have been reported (Abeles et al., 1992). For example, Kwak and Lee (1997) reported ethylene-induced transcription of ACC oxidase as a target of the ethylene response pathway. High exogenous levels of ethylene also inhibit its biosynthesis by reducing the level of ACC by inhibition of ACC synthase or stimulation of ACC conjugation to a bound and inactive form (Abeles et al., 1992).

Ca^{2+}-induced ethylene increase was not observed in studies using longer incubation times in the test solutions, whether applied to excised segments or as a spray prior to excision or enclosure in vials. This also supports a rapid, short-term role for altered cytosolic Ca^{2+} in regulating ethylene production. However, treatment with ionophore alone did not affect ethylene production, suggesting that endogenous Ca^{2+} stores are not sufficient to produce a substantial response. Ca^{2+} signal transduction inhibitors did not significantly affect basal ethylene levels (Figure 2). Since the results using the ionophore treatment indicated low endogenous Ca^{2+} concentrations, it follows that further reduction of the ion by chelation or channel blocking would not elicit an effect.

Wound-induced ethylene production was not significantly different between control seedlings and those treated with exogenous Ca^{2+} (Figure 1) or Ca^{2+} signal transduction inhibitors (Figure 2). However, $LiCl_3$-treated plants exhibited a slight increase (P=0.11) in wound ethylene compared to controls (Figure 2). If Ca^{2+} contributed to the wound ethylene response, then an increase in ethylene should have been observed with exogenous Ca^{2+} treatment. In addition, longer incubation of segments in $CaCl_2$ or inhibitors prior to tissue excision did not affect wound ethylene production, indicating incubation time in the treatment solution was not a factor.

Both ACC oxidase and ACC synthase regulate stress-induced ethylene (Morgan and Drew, 1997). However, ACC synthase and its control of ACC levels are usually attributed to the regulation of ethylene production in plant tissue (Abeles et al. 1992, Morgan and Drew, 1997). Each gene of the ACC synthase multigene family is regulated independently by different stress conditions (as reviewed by Morgan and Drew, 1997). Our results indicate that the signal transduction associated with wounding does not appear to follow the Ca^{2+}-calmodulin pathway in the subsequent stimulation of ethylene biosynthesis.

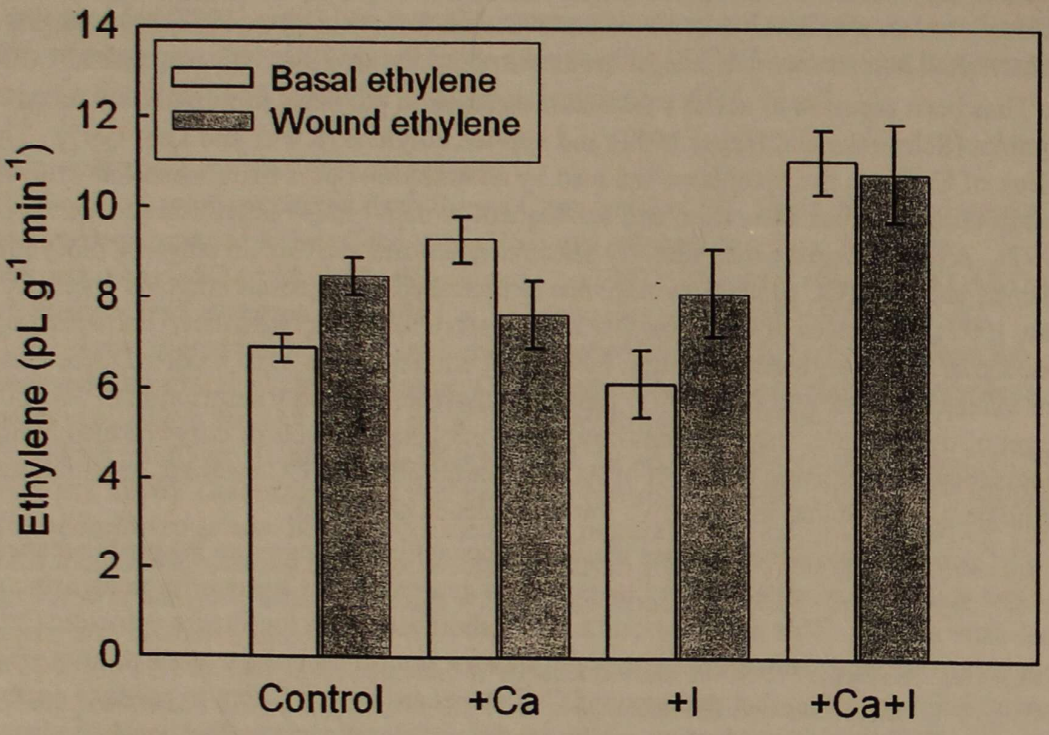

Figure 1. Effects of signal transduction activators on basal (open) and wound-induced (shaded) ethylene production in etiolated pea epicotyls. Treatments: deionized water (Control), 20 mM $CaCl_2$ (+Ca), 4 µM A23187 ionophore (+I), or 20 mM $CaCl_2$ plus 4 µM A23187 ionophore (+Ca+I). Means ± SE. N= 8-22 replicates per treatment.

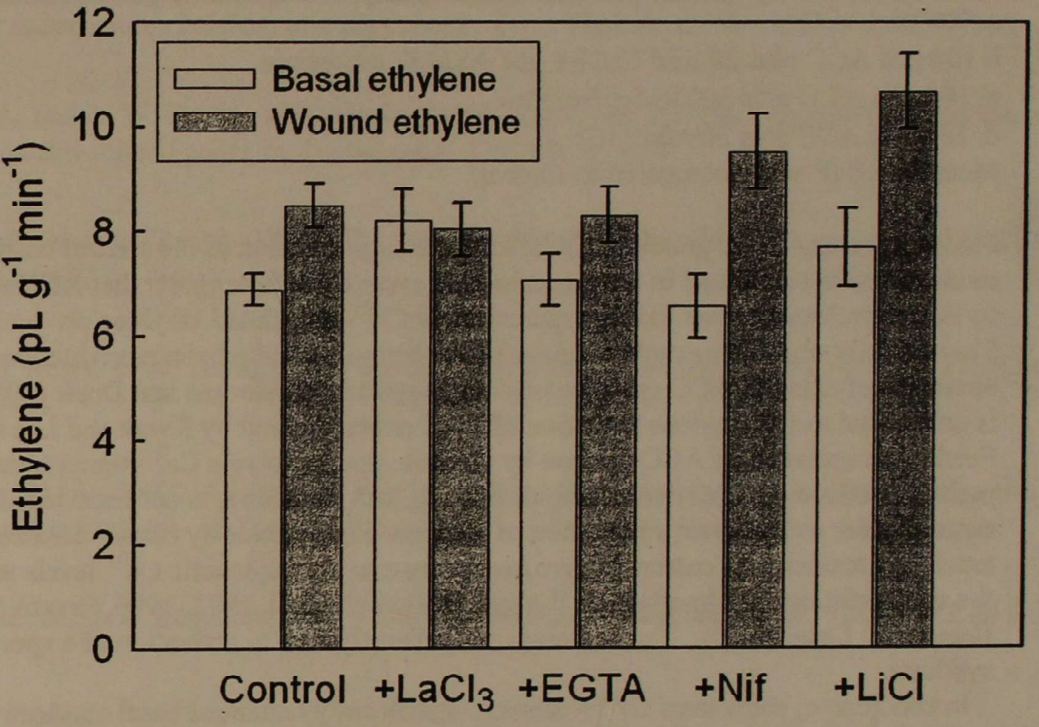

Figure 2. Effects of signal transduction inhibitors on basal (open) and wound-induced (shaded) ethylene production in etiolated pea epicotyls. Treatments: deionized water (Control), 20 mM $LaCl_3$, 10 mM EGTA, 500 µM nifedipine (+Nif), or 20 mM $LiCl_3$. Means $\pm$ SE. N= 5-12 replicates per treatment.

Table 1. Effect of signal transduction inhibitors on *in vivo* ACC oxidase activity in etiolated pea epicotyls.

Treatment	ACC oxidase activity (Ethylene pL g^{-1} min^{-1})
Control[a]	23.8 ± 1.2
Calcium and ionophore[b]	27.6 ± 1.2 (P=0.48)
Nifedipine[c]	33.9 ± 1.9 (P=0.26)
LiCl[d]	24.5 ± 0.9 (P=.96)

a. 100 µM ACC
b 100 µM ACC plus 20 mM $CaCl_2$ 4 µM A23187 ionophore
c. 100 µM ACC plus 500 µM nifedipine
d. 100 µM ACC plus 20 mM LiCl
Means ± SE (P-value compared to control).

Since exogenous ACC greatly stimulated ethylene production in the control tissue, ACC oxidase was not saturated in this experimental system. This suggests that ACC regulation by ACC synthase is more likely responsible for Ca^{2+}-stimulated ethylene production. These results support the rapid stimulation of stress-induced ethylene production by alteration of existing ACC synthase activity suggested by Morgan and Drew (1997), and is not related to the ethylene induction of ACC oxidase found by Kwak and Lee (1997). Feedback regulation of ACC oxidase by ethylene may involve a Ca^{2+}-signal transduction pathway activated by ethylene-receptor binding, and, therefore, is not transduced in the same manner as the direct stimulation of ethylene biosynthesis by stress. Stresses such as touch and temperature extremes have been shown to alter cytosolic Ca^{2+} levels and cause the accumulation of calmodulin in the cytosol (Knight et al. 1992, 1996, Braam 1992, Braam and Davis 1990). These changes may directly lead to activation of a specific ACC synthase.

In conclusion, short-term Ca^{2+} treatment significantly enhanced basal ethylene production, but ACC oxidase activity was not altered by changes in cytosolic Ca^{2+}. The proposed model includes the release of Ca^{2+} from vacuole or endoplasmic reticulum, and its binding to calmodulin producing an important activator for a protein kinase that may directly phosphorylate ACC synthase. Since ACC synthase activity is dependent on different members of a multigene family, the direct study of specific stress-induced ACC synthases will be of importance in future research.

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Records of Black-throated Blue Warbler in Southern West Virginia: Habitat Selection and Confirmed Breeding

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ABSTRACT

We report confirmed breeding of the Black-throated Blue Warbler (*Dendroica caerulenscens*) outside the Allegheny Mountains of West Virginia in both lowland and upland habitats. Although Hall (1983) considered this warbler to breed exclusively in the Allegheny Mountains in this state, we found breeding pairs along streams with rhododendron thickets and along ridgetops dominated by mountain laurel. In southern West Virginia, we first noted breeding records in 1989, and found 32 pairs along Guyandotte (Bolt) Mountain in Raleigh and Wyoming counties in 1995. Although at much lower density than local populations in the Allegheny Mountains, these data indicate a breeding, viable population in southern West Virginia. Habitat variables measured disclosed that shrub density and elevation are both important determinants of Black-throated Blue Warbler density, but breeding in the lowlands along coves and rhododendron thickets merits further investigation.

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INTRODUCTION

Many passerine species show geographical variation in song dialects (Baker and Thompson 1985, Tubaro et al. 1993, Byers 1996), habitat selection (Cody 1978, Noon 1981, Collins 1983, Grzybowski et al. 1994), foraging preference (Morse 1973), morphology (James 1970, Blem 1975, Twedt et al. 1994), reproduction (Young 1994, Baker 1995) and growth (Burns 1993). Some studies have relied on research of single-species and family-level comparisons to elucidate the ecological and evolutionary adaptations of geographical variation among populations (e.g., Lowther 1975). Likewise, geographical variation of subspecies has been of much interest. For example, work on the Black-throated Blue Warbler, *Dendroica caerulenscens*, has shown that there are possibly two subspecies (Holmes 1994). However, the validity of recognizing two subspecies based on geographical variation has been questioned using biochemical and morphological techniques. Studies are needed on the ecology and geographical variation of the two described subspecies, as well as on populations at the limit of their range and disjunct populations, to better understand the ecology of the Black-throated Blue Warbler.

The Black-throated Blue Warbler breeds from the higher elevations in the southern Appalachians from northern Georgia to Pennsylvania, and from New York to southern Nova Scotia and west to Quebec, southern Ontario, and northern Michigan (Holmes 1994). Possible geographical differences in population demography exist between northern or boreal populations and birds that breed in the extreme southern limits of their range. Steele (1993) found that Black-throated Blue Warblers in New Hampshire select nest-sites based on habitat availability (presence of dense shrub), and that foraging had no influence on habitat selection. Recent studies on the Black-throated Blue Warbler have also shown that it has multiple broods and has increased in numbers in the Hubbard Brook Experimental Forest, New Hampshire (Holmes et al. 1992). Holmes et al. (1992) reported 18-32 singing males on his 70-ha study site from 1986-1989. Like other perching songbirds, male Black-throated Blue Warblers must select territories that optimize their chances of breeding, i.e., areas with dense shrub in this case. The selection of territories may depend upon (1) previous reproductive effort, (2) intraspecific competition with other male Black-throated Blue Warblers, (3) interspecific competition with other species for similar habitat and resources, (4) population density; and (5) habitat availability. Population pressure from the north may force more birds to try to breed at southern limits of their range.

In the present study, we report on habitat selection of Black-throated Blue Warblers in an area of West Virginia where it has not been reported to nest (or reported as a probable breeder or nesting at extreme eastern limit within the Western Hill Physiographic Province in West Virginia, see Buckelew and Hall 1994). In addition, we compare habitat selection of Black-throated Blue Warblers near the southern limit of their range with northern populations, and with the population found in the High Alleghenies of the West Virginia (sites with similar habitat to the Canadian boreal forest, Strausbaugh and Core 1981). We test the model that southern populations of Black-throated Blue Warblers also select, like northern populations, dense habitats for their breeding territories.

MATERIALS AND METHODS

We surveyed sites in southern West Virginia where previous breeding bird surveys (BBS) had shown occurrences of Black-throated Blue Warblers, or where we had found birds during atlasing (see Buckelew and Hall 1994) and biodiversity studies for the National Park Service. Sites surveyed within the Western Hill Region were scattered throughout Fayette, Mercer, Raleigh, and Wyoming counties. These sites included Babcock State Park, Beauty Mountain, Chestnut Knob, Egeria, Flat Top Mountain, Glade Creek, Grandview State Park, Guyandotte (Bolt) Mountain (and Ivy Knob), and Saxon. In the Allegheny Mountains Region, we surveyed Bell Knob within the Dollie Sods Wilderness, Beartown State Park, Droop Mountain, and Cheat Mountain (including the Uppers Shavers Fork Watershed). The forest types in the Western Hill (Allegheny Plateau) were mainly oak-hickory-maple and mixed-deciduous forests (hemlock in valley floors and Virginia and pitch pines on ridgetops, and white pine along the forested slopes and permeating throughout the forested areas). On the other hand, the forest types of the Allegheny Region were spruce-northern hardwoods and northern hardwoods. Therefore, the Appalachian or mixed-deciduous forest of the Western Hill Physiographic Province is notably different than the spruce-northern hardwoods of the Allegheny Mountains, but with similarities in some of the shrub layers, especially areas with mountain laurel and rhododendron in both areas.

We conducted singing male censuses from 1989-1995 (see Hall 1983). We mapped the territories of each singing male by observing the position of each male for 1 hour during 10 trips to each site spanning a two week period (Steele 1993). We quantified the habitat for all males whose territorial boundaries were exactly mapped. We placed four 30-m transects in sites occupied by territorial males to sample the vegetation. To make sure transects were placed randomly within territories, we took particular care to record the center of the singing males' territory and noted where most singing bouts took place. Along each transect we counted the number of shrubs > 0.5 m tall and < 2 cm diameter at breast height (Steele 1993). Elevation at the center of each male's territory was determined with GIS mapping. Statistical analyses were performed on the average shrub density of the four transects. We recorded the number of singing males in various forest habitats and regressed these numbers against shrub density and elevation using least-squares regression analysis. Nests were found by searching for nest building or observing parents feeding young. All data were analyzed with SPSS and graphed with SigmaPlot for Windows.

RESULTS

Table 1 shows data on singing male censuses and habitat types. The highest number of territorial males was noted on the peaks of the highest mountains in southern West Virginia, such as Guyandotte (Bolt) and Flat Top Mountains. Pairs of Black-throated Blue Warblers were found at Glade Creek (Raleigh County) and Beauty Mountain (Fayette County), in the New River Gorge National River (NRGNR) during all years of the survey. In addition, Black-throated Blue Warblers were also found each year at Ivy Knob fire tower on the border of Raleigh and Wyoming counties, and at Babcock State Park (Fayette County). The preferred habitat at Glade Creek was bottomland

hardwood forest with rhododendron (*Rhododendron* spp.) thickets and Eastern hemlocks (*Tsuga canadensis*). The Beauty Mountain site was also characterized by similar dense habitat, but also contained open, dry mixed forest. The Ivy Knob site at Guyandotte Mountain harbored territorial males mainly in dry slopes with mountain laurel, but the species occurred throughout this mountain range. A similar trend was noted for the Black-throated Blue Warbler population at Babcock State Park.

Table 1. Average number of singing males (# per 100 ha.) per habitat type in southern West Virginia (Western Hill or Allegheny Plateau Region). Mean number taken from all years (1989-1995) pooled.

Average Number of Males	Main Habitat Type (> 75% of shrub cover)
15	Cove rhododendron
25	Ridgetop Mt. laurel
19	Sloped, clear-cut

Table 2 shows the number of singing males at one of the Allegheny Mountains study sites. The species appears to be declining (negative trend data for all seven years of the study; $r = -0.44$, $p < 0.02$) in the Allegheny Mountains of West Virginia, especially in areas with spruce forest and may be correlated with the disappearance of the boreal-spruce forest from the West Virginia highlands.

Table 2. Singing-Male Censuses (# birds per 100 ha.) in the Uppers Shavers Fork Watershed (Mower Tract) during summers of 1990 and 1995.

1990	1995	Habitat
94	85	Cut-over mature hardwoods
81	62	Spruce-northern hardwoods
60	59	Young deciduous forests
72	61	Young spruce stands

Confirmed Breeding

On July 21, 1989 we found a female Black-throated Blue Warbler feeding two fledglings at Glade Creek, Raleigh Co. We observed the female carrying green caterpillars to the young birds. The male was singing nearby and also appeared, making alarm calls. On June 10, 1992, we found an occupied nest with four eggs in a rhododendron at Ivy Knob, Wyoming County, and a nest with three young were observed at Peachtree Ridge of Guyandotte (Bolt) Mountain in a red maple sapling on June 13, 1992. Breeding was also confirmed with eight additional pairs from 1993-1995. Confirmed breeding observed in the Mower tract in 1990 included: Female nest building along Shavers Fork in rhododendron thicket; pair feeding young at Ward Knob on June 28, 1990; pair feeding fledglings on June 30, 1990.

Habitat and Elevation

We found a significant correlation between number of singing males and shrub density in the high Alleghenies ($r = 0.96$, $p < 0.005$, $n = 23$). Figure 1 shows linear regression relationship between number of singing males and habitat quantification (presence of dense shrubs). As noted in Figure 1, the patterns are different for the two regions studied, where highland mountain populations appear to select denser habitats (as noted by the steeper slope in the equations). However, both shrub density and elevation (both regions - Western Hill and Allegheny Mountains - combined) were important predictors ($r \geq 0.41$, $p < 0.03$) of Black-throated Blue Warbler density.

DISCUSSION

The Black-throated Blue Warbler appears to nests in many ravine bottomland hardwoods in southern West Virginia. For example, singing, territorial males have been observed on several occasions at Camp Creek State Forests, Mercer Co. (see Ward 1988). Dense rhododendron and hemlock stands appear to be a preferred nesting site. However, most of the singing males in southern West Virginia were found on the higher, drier knobs of Western Hill Region, such as Beauty and Guyandotte Mountains where the habitat is more open. Thus, habitat selection appears to differ somewhat between high elevation populations and marginal populations at the southern edge of their breeding range. The significance of using high, dry (xeric) habitats as compared to hydric areas along streams and moist rhododendron thickets remains unclear.

Hall (1983) states that the Black-throated Blue Warbler is primarily limited to the Allegheny Mountains of West Virginia as a breeding bird. During the WV breeding bird atlas, probable nesting pairs were found in several localized areas in southern West Virginia, and confirmed breeding was found in only one county (Monroe) outside the Allegheny Mountains (Buckelew and Hall 1994). Thus, scattered records exist for southern West Virginia, but most lack confirmed breeding. For example, Black-throated Blue Warblers were found at Babcock State Park, Fayette County (Phillips 1987). They were found in Kanawha County in 1966 during the first Kanawha Forest sortie, but were absence in 1975 during a second sortie (Koch 1977). Four singing males were reported in Camp Creek State Forest on June 5, 1975 and one at the north overlook in Grandview State Park, Raleigh County (Bell 1976). In addition, one singing male was observed at Camp Creek State Forest

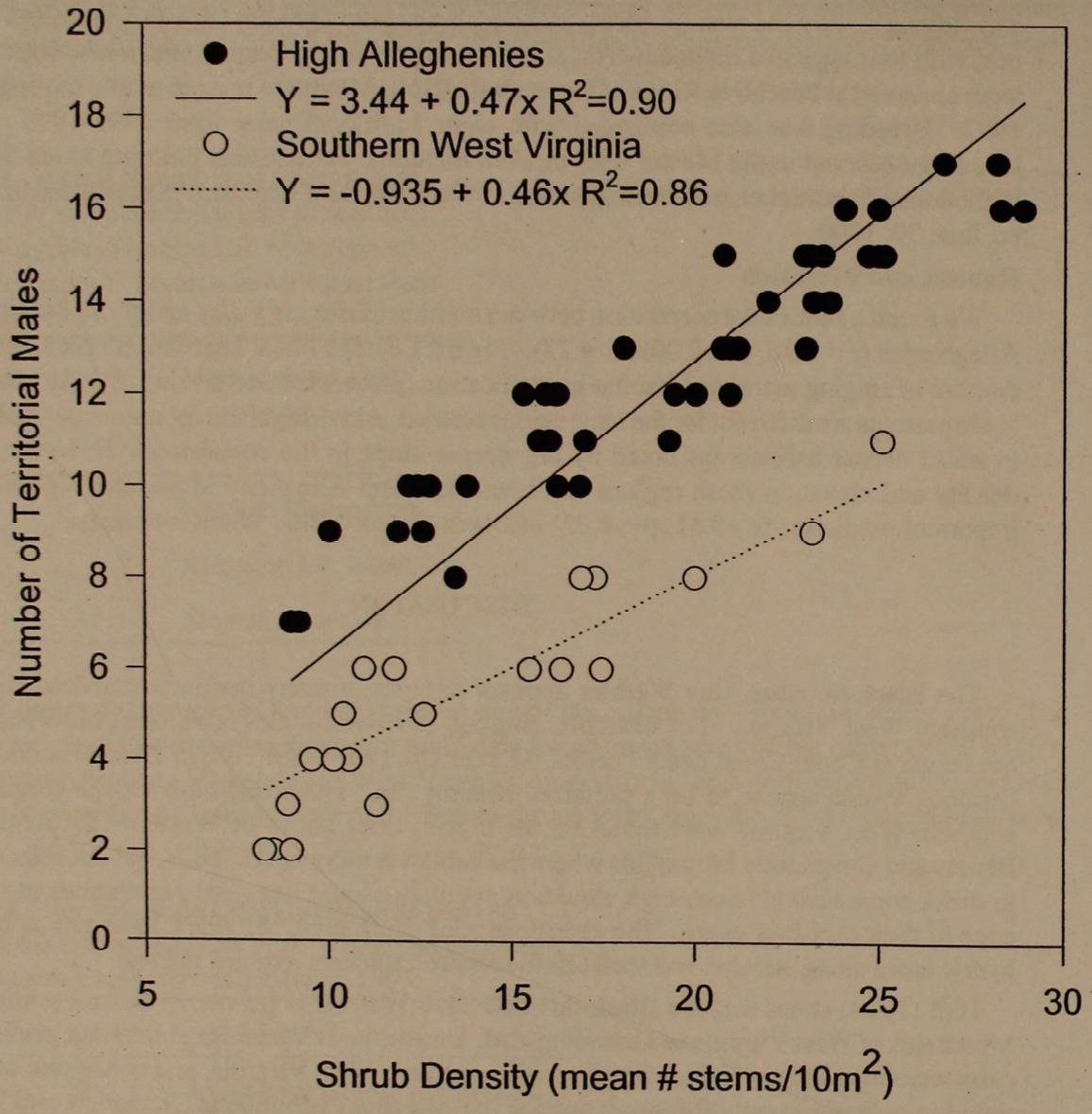

Figure 1. Number of singing male Black-throated Blue Warblers as a function of shrub density.

during the 1987 foray (Ward 1988). They were listed as rare at Ivy Knob fire tower, Wyoming County during the 1984 foray, where only a single female was noted (Temple 1985), but we found as many as 32 males in 1995 along Guyandotte (Bolt) Mountain, including seven at Ivy Knob.

Population declines have been noted in the Allegheny Mountains. For example, only 22 birds were recorded on the BBS routes at the Durbin, Pocahontas County foray as compared to 52 in 1968 (Bell 1974), and 11 in 1983 (Laitsch 1984). A negative trend in the High Mountains was noted for this species during the present study. On the other hand, it must be pointed out that these may be yearly variations and little is known about long term population trends. Despite local declines in the High Alleghenies, Black-throated Blue Warbler populations in West Virginia have been increasing (Buckelew and Hall 1994; Holmes 1994). Range expansion will probably be evident in the lowlands due to population pressures (higher density) in the north. This may explain why more birds are seen in southern West Virginia as opposed to past censuses. Their success in lowlands and throughout the Western Hill Region may depend upon habitat management and shrub density and conservation of the mountain ridgetops in these areas.

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