1999


Katrina Maria Ramonell

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EFFECTS OF LOW OXYGEN ATMOSPHERE ON THE GROWTH AND DEVELOPMENT OF *ARABIDOPSIS THALIANA* (L.) HEYNH

A Dissertation

Submitted to the Graduate Faculty of the
Louisiana State University and
Agricultural and Mechanical College
in partial fulfillment of the
requirements for the degree of
Doctor of Philosophy

in

The Department of Plant Pathology and Crop Physiology

by

Katrina Maria Ramonell
B.S., Auburn University, 1991
M.A., Columbia University, 1992
May, 1999
Dedication

This dissertation is dedicated to my mother, Sandy Sanford Ramonell. She has supported me throughout my education and has always been confident that I would reach my goals, even when I was not. Without her constant support and belief in my abilities, this work would not have been possible.
Acknowledgments

I am indebted to many people who have assisted me in reaching my goals throughout graduate school. My graduate advisory committee, Drs. Sue Bartlett, Bill Piastuch, Roy Brown, Tom Moore, Nikolaus Fischer and Ray Schneider have consistently supported my research and given me beneficial advice. My graduate advisor, Dr. Mary Musgrave, has constantly challenged and encouraged me throughout my graduate work, providing me with an exceptional and rewarding learning experience. I am grateful to Dr. Musgrave for her advice, friendship and support.

There are others who have helped me complete graduate school by sharing the results of their efforts with me. The carnation ACC synthase and ACC oxidase clones were provided by Dr. William R. Woodson of Purdue University and the Arabidopsis ACS2 cDNA clone was kindly given to me by Dr. Athanasios Theologis. Dr Steven Barker and Dr. Nikolaus Fischer shared their expertise with me in performing the brassinolide detection experiments using GC-MS. I am also grateful to the Louisiana Space Consortium for their support of me through the LaSpace graduate fellowship program.

My lab mates past and present: Hee Jin Kim, Beth Floyd, Michael Bracey, Linda Shaffer, Xiaochun Xi, Anxiu Kuang, Ying Xiao, Marshall Porterfield, Mark Crispi, Stephen Stout and Gloria McClure. I have been fortunate to work with each of them.
Finally I would like to thank my parents and fiancé. Their unfailing support of me during my graduate career has helped me through many tough times. I appreciate all of their patience and love during these years.
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Abstract

Interest in the improvement of crop productivity through repression of photorespiration led to experimental growth of plants in low O₂ and the surprising finding that reproduction is inhibited under these conditions. Using Arabidopsis thaliana (L.) Heynh. cv. Columbia, studies were undertaken to understand the mechanism behind oxygen control of plant development. The hypothesis that developmental changes observed in low O₂ are due to repression of the plant hormones ethylene and brassinolide (which require O₂ for biosynthesis) was tested. Arabidopsis was grown for 35 days in Sun bags in one of five altered O₂ atmospheres (210, 160, 100, 50, 25 mmol/mol) with 0.35 mmol/mol CO₂ in N₂. Molecular analysis of ethylene biosynthesis was accomplished using cDNAs encoding ACC synthase and ACC oxidase in ribonuclease protection assays and in situ hybridizations. To understand if low O₂ inhibition of brassinolide biosynthesis was responsible for developmental changes in low O₂, brassinolide-replacement experiments were performed. Arabidopsis was grown for 10 days on nutrient agar or nutrient agar + 10⁻⁷ M brassinolide in magenta vessels coupled to gas mixtures of 210 or 25 mmol/mol O₂, 0.35 mmol/mol CO₂ in N₂. When grown in 25 mmol/mol O₂, plants exhibited dwarf morphology resembling the brassinolide-deficient mutant det2, and this line was incorporated into the brassinolide studies at low O₂ for comparison. Leaf development under low O₂ resulted in changes in leaf size, stomatal density and stomatal patterning. Stomatal density increased in all low O₂ treatments but was not linked to changes in ethylene or brassinolide. Low
O$_2$ changes in leaf size, however, were mediated through depression of brassinolide biosynthesis. There was a loss of ethylene biosynthesis in siliques from plants grown at 50 and 25 mmol/mol O$_2$, and silique ACC oxidase mRNA increased as O$_2$ was lowered. As O$_2$ decreased, tissue-specific patterning of ACC oxidase and ACC synthase gene expression shifted from the embryo to the silique wall. Loss of ethylene in the silique was tightly correlated with loss of the embryo in seeds produced in low O$_2$. These data show that changes in development under low oxygen can be explained by repression of O$_2$-sensitive plant hormones.
Chapter 1
Oxygen Control of Plant Development

Introduction

Plants must constantly adapt to their environment, including fluctuations in the atmosphere surrounding them. In nature, the components of the atmosphere are constantly in flux due to changes in humidity, altitude, wind speed and temperature. Table 1.1 summarizes fluctuations in oxygen concentrations which significantly affect plants. These fluctuations fall into two general categories; decreasing atmospheric pressure with increasing altitude resulting in lower oxygen (O$_2$) availability and changes in soil oxygen concentration due to flooding or compaction.

The normal oxygen concentration at sea level is 210 mmol/mol. As elevation increases, atmospheric pressure drops resulting in decreased amounts of oxygen available to biological systems. Plants growing in mountainous regions, such as montane forests (2100 m) and at the alpine treeline (3350 m), must contend with decreased oxygen levels as well as extremes in temperature. At the elevation of the montane forest, oxygen levels fall to 199.3 mmol/mol and at alpine treeline to 191 mmol/mol. At an elevation of 6000 m, the oxygen available is half that at sea level (105 mmol/mol) and at extreme elevations such as the summit of Mt. Everest (8848 m), the oxygen concentration is 63 mmol/mol (Fitter and Hay, 1987).

Flooded soils are a second type of environment where oxygen is limited. Wetland soils are under constant oxygen stress. Depending on the degree of
Table 1.1. The distribution of oxygen concentrations found in nature.

<table>
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<th>Oxygen Concentration (mmol/mol)</th>
<th>Environment</th>
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<td>210</td>
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<td>200 - 150</td>
<td>Normal Soils</td>
<td>Fitter and Hay, 1987</td>
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<tr>
<td>150</td>
<td>50% agricultural yield for a C3 crop.</td>
<td>Quebedeaux and Hardy, 1975</td>
</tr>
<tr>
<td>100</td>
<td>50% agricultural yield for a C4 crop.</td>
<td>Quebedeaux and Hardy, 1975</td>
</tr>
<tr>
<td>100</td>
<td>Photorespiration inhibited</td>
<td>Quebedeaux and Hardy, 1975</td>
</tr>
<tr>
<td>63</td>
<td>Summit of Mt. Everest (8848 m)</td>
<td></td>
</tr>
<tr>
<td>42</td>
<td>Respiration inhibited</td>
<td>Gale, 1974</td>
</tr>
<tr>
<td>2 - 0</td>
<td>wetland soils</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>anoxia</td>
<td>Taiz and Zeiger, 1991</td>
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flooding and the time of year, oxygen levels in wetland soils may range from 2 mmol/mol (hypoxic) to 0 mmol/mol (anoxic) (Fitter and Hay, 1987). Wetland plants have adapted to these environments in several ways. Their root systems form large gas spaces (aerenchyma) allowing for greater gas exchange in the root zone. In addition, some research suggests that these plants have a lower critical oxygen pressure (20-40 mmol/mol) than non-wetland adapted species allowing for survival under near anoxic conditions (Jackson and Drew, 1984). Agricultural crops may also be exposed to low oxygen environments in the field due to flooding and soil compaction (Table 1.1, Quebedeaux and Hardy, 1975). This lack of oxygen may result in decreased crop yields.

Clearly, the range of oxygen concentrations present in nature presents constant challenges to plant systems. The sections below will address specific changes in the growth and development of the plant root, shoot and reproductive structures caused or influenced by low oxygen environments.

**Oxygen effects on the plant rootzone**

There is a large body of literature which discusses low oxygen (hypoxic) conditions affecting the root zone of plants. Generally, roots obtain adequate oxygen from the soil surrounding them. However, in flooded or compacted soils the diffusion of oxygen in the soil is restricted resulting in the depression of plant growth, decreased crop yields and injury to the root system. Root damage in soils depleted of oxygen involves several factors. Under these conditions, ATP is produced by fermentation which results in the production of only 2 moles ATP/ mole hexose sugar respired compared to 36 moles ATP/
only 2 moles ATP/ mole hexose sugar respired compared to 36 moles ATP/
mole hexose sugar respired under normal oxygen conditions (Taiz and Zeiger, 1991). This loss of ATP causes an interruption in root metabolic processes including a loss of efficient uptake and transport of soil nutrients to the xylem (and consequently the shoot) and an overall acidification of the root cell caused by lack of ATP to maintain normal pH gradients (Xia and Roberts, 1996). Loss of nutrients to the shoot causes shortages of necessary ions in the developing and expanding tissues above ground. Acidification of the root can also interrupt ion exchange and transport in the root system. In addition, other research has shown that substantial changes occur in mitochondrial structure under hypoxic conditions. The mitochondria show an irregular shape and dilation of the cristae within 1-3 hours of the onset of hypoxia. This initial change is followed by a general degradation of the mitochondrial structure within 24-48 hours (Vartapetian et al., 1987).

Root structure is also altered by oxygen deficiency. Hypoxic roots exhibit the formation of large gas-filled spaces within the cortex called aerenchyma. These gas-filled spaces may then act as areas of gas exchange for the root system and in the stems of aquatic plants (Taiz and Zeiger, 1991).

While much is known about responses of the root to low oxygen conditions, there is little known regarding the response of the shoot to these environments. The focus of the studies in the following chapters is on the responses of the leaf and the reproductive structures of Arabidopsis thaliana to low oxygen environments.
Oxygen effects on foliage

Altered oxygen environments have been shown to effect changes in plant vegetative growth in closed systems (Bowes, 1993, du Cloux et al., 1989, Leadley and Reynolds, 1989, Musgrave and Strain, 1988, Tremmel and Patterson, 1993). The change in the growth of C\textsubscript{3} and C\textsubscript{4} plants under low oxygen (O\textsubscript{2}) conditions has been the focus of several studies. Björkman et al. (1968 & 1969) were the first to observe a large increase in the vegetative matter of C\textsubscript{3} plants when grown under low O\textsubscript{2} tensions. *Mimulus cardinalis* and *Marchantia polymorpha* showed a 130- to 1000- fold increase in dry weight of vegetative matter over a 10 day experiment in 40 mmol/mol O\textsubscript{2} compared with control plants grown in 210 mmol/mol O\textsubscript{2}. *Zea mays*, a C\textsubscript{4} plant, showed no significant response to low O\textsubscript{2} tensions. Quebedeaux and Hardy (1976) repeated these experiments using soybean and sorghum and carried out the low O\textsubscript{2} treatment over the complete life cycle of the plants. The aerial portions of soybean and sorghum were grown in 50 mmol/mol oxygen under controlled conditions. The observations of Björkman et al. were confirmed: there was an increase in vegetative growth of C\textsubscript{3} but not C\textsubscript{4} plants under lowered oxygen tensions. They observed a larger leaf surface area and density, increases in overall lateral growth, diameter of stems and branches, and some delay in senescence of the C\textsubscript{3} plants. Final dry weight of the vegetative portions of the C\textsubscript{3} plants showed a 74\% increase when grown in 50 mmol/mol O\textsubscript{2} compared with plants grown in 210 mmol/mol O\textsubscript{2}. C\textsubscript{4} plants showed no obvious vegetative changes.
C₃ plants showed dramatic increases in growth due to the inhibition, at low oxygen tensions, of photorespiration. In C₃ plants, oxygen concentrations below 100 mmol/mol allow for less competition between oxygen and carbon dioxide for the active site of ribulose-1,5-bisphosphate carboxylase oxygenase (Rubisco). This shift in the CO₂/O₂ ratio favors the carboxylation reaction. C₄ plants are essentially unaffected in this regard by low O₂ tensions due to their naturally low rates of photorespiration. The efficient activity of phosphoenolpyruvate (PEP) carboxylase and the sequestering of Rubisco in the bundle sheath cells ensures that C₄ plants maintain optimal CO₂ concentrations at the site of Rubisco activity, favoring the carboxylation reaction (Campbell et al., 1988; Chollet and Ogren, 1975; Forrester et al., 1966; Scheidegger and Nösberger, 1984, Sharkey, 1985).

C₃ and C₄ species have markedly different leaf anatomy which reflects the differences in their carbon assimilation cycles. The typical C₃ leaf consists of a photosynthetically active mesophyll which is differentiated into palisade parenchyma, with long columnar cells, and spongy parenchyma with cells of irregular shape. In contrast, the C₄ leaf has two distinct chloroplast-containing cell types; the mesophyll and bundle sheath cells. The mesophyll cells and the bundle sheath cells form two concentric layers around the vascular bundles (Raven et al., 1992). Studies by Ku et al. (1983) and Holaday et al. (1984) have characterized species of Flaveria as either C₃, C₄ or C₃-C₄ intermediates by documenting changes in the leaf anatomy and the photosynthetic response.
to CO₂ sensitivity of net photosynthesis to O₂. These studies indicate that O₂ has a pronounced impact on species evolution and leaf anatomy.

**Oxygen effects on reproductive development**

The above experiments show low oxygen effects on photosynthesis; however, low oxygen has been shown to effect changes in seed development as well. In contrast to the effects of low O₂ on vegetative growth, Quebedeaux and Hardy (1973, 1974, 1975, 1976; and Quebedeaux et al., 1975) observed an almost complete repression of seed formation in both C₃ and C₄ species tested. C₃ and C₄ plants subjected to 50 mmol-mol⁻¹ O₂ had no completely developed seeds at time of normal maturation. The ratio of reproductive to vegetative growth (harvest index) showed a controlling role of O₂ with respect to the balance between vegetative and reproductive growth. Plants grown at 50 mmol/mol O₂ had one third the reproductive growth (in dry weight/ plant) compared to those grown in ambient O₂ concentrations. As O₂ concentrations were increased, vegetative dry weight decreased to normal levels and reproductive dry weight increased to normal levels. At superambient O₂ concentrations (400 mmol/mol), a reduction in both total vegetative and reproductive mass was observed. This was attributed to higher rates of oxidation relative to carboxylation of RuBP by Rubisco. However, at 400 mmol/mol, the partitioning of the photosynthate between vegetative and reproductive structures was not altered in contrast to partitioning at subambient (50 or 100 mmol/mol) oxygen concentrations. Quebedeaux and Hardy's experiments demonstrate that O₂ is a factor in controlling seed and
reproductive development. They hypothesized that an oxygen-mediated process is a major factor in the translocation and accumulation of photosynthate in reproductive structures.

Subsequent studies attempted to show that this $O_2$ effect on reproduction was simply mediated by a lowering of dark respiration by the low $O_2$ tensions under which the plants were grown (Quebedeaux and Hardy, 1976; Gale, 1974). Gale observed an inhibition of respiration by 42 mmol/mol $O_2$ treatment in excised soybean tissues. This effect may not be significant in intact tissues. The $K_m$ for the fixation of $O_2$ by the dark respiration apparatus is quite low and should be saturated at 50 mmol/mol $O_2$. Levels of 10 mmol/mol $O_2$ have been shown to provide adequate $O_2$ for dark respiration in intact plants (Armstrong and Gaynard, 1976; Quebedeaux and Hardy, 1976). Therefore, the effects of low $O_2$ on reproductive structures are not due to a decrease in dark respiration.

Thome (1982) conducted experiments to test whether the low $O_2$ effect on reproduction was a consequence of prevention of phloem unloading of sucrose from the seed coat phloem and uptake by the embryo. Import of $^{14}C$-photosynthate into the intact soybean fruit was found to be $O_2$-dependent, in soybeans grown at 0, 210, and 1000 mmol/mol oxygen. However, in excised embryos, $^{14}C$-sucrose uptake in vitro was depressed by only 30% under anaerobic conditions (0 mmol/mol $O_2$). Thome hypothesized that anoxic conditions within the intact fruit prevented sucrose efflux from the seed coat phloem and subsequent uptake by the embryo. Under anoxic conditions in the
developing seed, the high energy demands of phloem unloading could not be met due to shifts in dark respiration. This short-term experiment implied that the local $O_2$ environment around the developing pods influenced seed set. However, Sinclair et al. (1987) exposed developing soybean pods to 100 mmol/mol $O_2$ in a two-year field experiment and found only a 20% decrease in seed dry weight, compared to an 81% decrease observed by Quebedeaux and Hardy (1976) when the whole aerial portion of the plant was exposed to the same $O_2$ concentration. These results support the argument that seed set is indirectly influenced by low $O_2$ on the vegetative portion of the plant in addition to any localized effects which may occur in the pod.

Recent studies in our laboratory have indicated that at oxygen tensions below 160 mmol-mol$^{-1}$, embryo development in *Arabidopsis thaliana* is depressed and at ultra low oxygen levels (50 mmol/mol, 25 mmol/mol) the embryos arrest during early stages of development. Storage substances (protein bodies) within the seeds themselves were shown to be greatly reduced under subambient (<210 mmol/mol) oxygen conditions (Kuang et al., 1998). These results clearly demonstrate that oxygen concentration around the developing embryo plays a crucial role.

**Role of oxygen in the biosynthesis of plant growth regulators**

From the above experiments it is clear that $O_2$ plays a significant role in plant growth and development. Extensive work by Quebedeaux and Hardy (1975) as well as by Gale (1974) has shown that the inhibition of reproductive growth observed in low oxygen environments is unrelated to the effects
observed on photosynthesis and respiration. Therefore, another process integral to seed and embryo development and influenced by $O_2$ must be affected. A hormonal imbalance may be responsible for the repression of reproductive structures and seed formation. The five classical plant hormones are auxin, cytokinins, gibberellins, ethylene and abscissic acid. A new class of plant hormones, the brassinosteroids, has recently been characterized. Plant growth regulators, especially auxin and cytokinin, are known to be important in the regulation of plant cell division and differentiation in plant tissue culture. Ethylene and the plant steroid brassinolide require oxygen in their biosynthesis (Kende, 1993; Lizada and Yang, 1979; Mandava, 1988; Fujioka et al., 1995). It is well established that plant hormones are involved in an intricate interplay in the regulation of their various syntheses and activities (Taiz and Zeiger, 1991). Therefore, the repression of synthesis of one or more of these hormones due to changes in the oxygen environment could result in serious abnormalities in overall plant growth and development.

**Auxin.** Auxin was the first plant hormone discovered and is probably the best characterized of all the plant growth regulators. Auxin plays a major role in the development of vascular tissues, the formation of lateral and adventitious roots, responses to tropisms and the control of apical dominance in plants (Went and Thimann, 1937). Though there are several naturally occurring auxins in plant tissues, indole-3-acetic acid (IAA) is by far the most active in regulating plant growth and development.
IAA is synthesized from the amino acid tryptophan (Last and Fink, 1988). In most plant species, tryptophan is converted to indole-3-pyruvic acid by tryptophan transaminase. Indole-3-pyruvic acid is then converted to indole-3-acetaldehyde by indolepyruvate decarboxylase and finally to active IAA by indoleacetaldehyde dehydrogenase. These reactions require a single molecule of oxygen. However, due to the presence of other naturally occurring auxin compounds (4-chloroindole-3-acetic acid and phenylacetic acid) it seems likely that any depression in IAA biosynthesis due to low oxygen would be compensated by these compounds (Taiz and Zeiger, 1991).

Gibberellins. Gibberellins were first discovered in the 1930's by Japanese scientists working with rice. However, they were unknown in the West until the 1950's when two research groups, one in Britain and the other in the United States, isolated a compound from fungal cultures which they termed gibberellic acid (Taiz and Zeiger, 1991). The gibberellins are a large group of related compounds which are defined by their chemical structure. Not all gibberellins are biologically active. Their main biological effect is that they are responsible for plant height. Dwarf plants have a decreased concentration of gibberellins as compared to plants of normal height.

Gibberellins are terpenoid compounds comprised of 20 carbons derived from four isoprene (five-carbon) units. The starting compound for gibberellin biosynthesis is mevalonic acid, which is synthesized from acetyl-CoA. Isoprenoid units are consecutively added until geranylgeranyl pyrophosphate has been formed. Geranylgeranyl pyrophosphate is then cyclized to form ent-
kaurene, the first compound specific to gibberellin biosynthesis. *Ent*-kaurene is then oxidized to carboxylic acid and the B-ring contracts to a five-carbon ring to form GA$_{12}$-aldehyde, the precursor of all other gibberellins (Sponsel, 1987). Other gibberellins are formed by successive oxidation at carbon 20 followed by a loss of carbon 20 in combination with hydroxylation at carbon 13 or carbon 3 (possibly both). In this way, a broad range of gibberellins are synthesized with over 84 known to date. These oxidation steps would require some molecular oxygen but the effects of lowered oxygen environments on gibberellin biosynthesis are unclear.

**Cytokinins.** Cytokinins were first discovered in attempts to isolate compounds which would promote plant cell division. Since that time, cytokinins have been shown to affect many processes in plant development, including delay of senescence, mobilization of nutrients, cotyledon expansion, chloroplast maturation and the control of morphogenesis (Taiz and Zeiger, 1991). The principal free cytokinin in plants is *trans*-zeatin discovered by D.S. Letham in 1973 (Letham, 1973). Besides *trans*-zeatin, there are several other naturally occurring cytokinins in plants. These include dihydrozeatin and isopentenyladenine. Naturally occurring cytokinins are found as free molecules in plant tissue but they may also occur in bound form as modified bases of transfer RNA molecules in all organisms.

In cytokinin biosynthesis, the key enzyme, cytokinin synthase, catalyzes the transfer of an isopentyl group from $\Delta^2$-isopentenyl pyrophosphate to the $N^6$ nitrogen of adenosine monophosphate. The product, $N^6$-(\(\Delta^2\)-isopentenyl)}
adenosine monophosphate (i6 AdoMP), may then be readily converted to zeatin or isopentenyladenine. The enzymes catalyzing the intervening steps between i6 AdoMP and zeatin have not been fully characterized (Letham and Palni, 1983). Plant tissues also contain the enzyme cytokinin oxidase which converts active cytokinins to an inactive form. It has been postulated that this enzyme may regulate cytokinin, preventing its accumulation to toxic levels (Letham and Palni, 1983). Though oxygen is not involved in the biosynthesis of cytokinins, the oxidation by cytokinin oxidase requires oxygen and lowered oxygen environments may inhibit its activity allowing abnormal levels of the hormone to accumulate.

**Abscissic Acid.** Abscissic acid (ABA) is a ubiquitous plant hormone which has been detected in all vascular plants and in mosses (Taiz and Zeiger, 1991). It has been isolated from every major plant organ and tissue system and is synthesized in almost all cells containing chloroplasts and amyloplasts (Taiz and Zeiger, 1991). ABA is involved in bud and seed dormancy, inhibition of auxin-induced cell growth, stress responses, stomatal closure, response to drought and tissue senescence. ABA was initially isolated as a factor involved in the formation and development of abscission zones. However, it is now known that ethylene is the major hormone responsible for abscission.

The complete pathway for ABA biosynthesis is not known. There are two alternative metabolic routes which are currently under study (Zeevaart and Creelman, 1988). The first pathway involves the conversion of mevalonic acid to farnesyl pyrophosphate with a final conversion to ABA. This is known as the
direct pathway. The second or indirect pathway involves the conversion of violaxanthin, a 40-carbon compound, to xanthoxin and finally to ABA. Interestingly, xanthoxin is itself a plant growth inhibitor with properties similar to ABA. Abscisic acid concentration in various plant tissues seems to involve a balance between biosynthesis and catabolism. ABA can be catabolized in an oxidation reaction to phaseic acid or 4'-dihydrophaseic acid. This oxidation of ABA may be inhibited by low oxygen environments causing abnormal levels of the hormone to remain active in various plant tissues.

**Ethylene.** Ethylene (C$_2$H$_4$) is the smallest plant hormone and among the best characterized of the plant growth regulators (Taiz and Zeiger, 1991, Theologis et al., 1990). It can produce a myriad of effects on plant growth, development and physiology, most notably the ripening of fruits, inhibition of stem and root elongation, promotion of seed germination and flowering, senescence of leaves and flowers, and sex determination (Ecker, 1995). Ethylene has been implicated in shifting the allocation of photosynthate from vegetative to reproductive structures in the initiation of fruit ripening; thus, alteration in the translocation and accumulation of photosynthate in the reproductive structures grown under low O$_2$ tensions could indicate a change in concentration or lack of ethylene.

Ethylene is synthesized via the Yang pathway (Fig.1.1) which was elucidated by Shang Fa Yang and co-workers in 1979 (Yang and Hoffman, 1984, Boller et al., 1979). In higher plants, methionine is the precursor of
ethylene and is converted to ethylene via several steps. The rate limiting step in ethylene biosynthesis is the conversion of s-adenosyl methionine (SAM) to 1-aminocyclopropane-1-carboxylic acid (ACC). This conversion is catalyzed by the enzyme ACC synthase which is present in very low amounts in plant tissue (0.0001% in ripe tomato) (Bleecker et al., 1986). ACC synthase was first identified in tomato pericarp extracts by Boller et al. in 1979. ACC synthase is a pyridoxal enzyme which catalyzes the elimination of 5'-methylythioadenosine (MTA) from SAM to form ACC (Abeles et al., 1992, Adams and Yang, 1979). SAM was shown to be the substrate of ACC synthase with a K_m of between 13-60 μM and aminoethoxyvinylglycine (AVG) was shown to inhibit ACC synthase activity with a K_i of 0.2 μM (Boller et al., 1979). Two-dimensional gel electrophoresis identified ACC synthase as a protein of ~ 45-50 kD with a pH optimum of 8.5 and a specific activity of 4.5 x 10^5 units per mg protein (one unit = one nmol ACC produced per hr at 30°C) (Boller et al., 1979; Bleecker, 1986; Bleecker et al., 1987; Abeles et al., 1992). ACC synthase is encoded by multiple genes which appear to be environmentally and developmentally regulated (Abeles et al., 1992).

Ethylene biosynthesis is directly affected by O_2. The final step in ethylene biosynthesis is the conversion of ACC to ethylene via the enzyme ACC oxidase (formerly Ethylene Forming Enzyme, [EFE]), a reaction which requires O_2. ACC oxidase is a bisubstrate enzyme requiring O_2 and ACC (Abeles et al., 1992). The kinetics of the reaction between ACC, O_2 and ACC oxidase indicate that the reaction follows an ordered binding mechanism where
Figure 1.1. The Yang Pathway. Adapted from Yang and Hoffman, 1984.
the enzyme binds $O_2$ first and then ACC (Yip et al., 1988). ACC oxidase has been classified as a protein related to the 2-oxoglutarate-dependent dioxygenases which require $Fe^{2+}$ and ascorbate for *in vitro* activity (Kende, 1993). It was shown to be a 41 kD protein with a $K_m$ of 60 $\mu$M and a pH optimum of $\sim$ 7.5 (Kende, 1993). Further characterization proved that the enzyme did not require 2-oxoglutarate as a co-factor (Smith et al., 1992). ACC oxidase is inhibited to varying extents by $Co^{2+}$, SH-reagents, free-radical quenchers and EDTA (Kende, 1993). The most potent inhibitor of ACC oxidase is 1,2-dihydroxynaphthalene, possibly due to its iron-binding ability (Smith et al., 1992). Numerous studies have attempted to localize ACC oxidase within the cell with various results (Kende, 1993). It has been consistently found in the particulate fraction of homogenates (Dong et al., 1992; Fernández-Maculet and Yang, 1992; Peck et al., 1992; Kende, 1993) and has been shown to be sequestered in the vacuole (Abeles, 1992) and this has led to the proposal that the enzyme is associated via protein-protein interactions with an integral membrane protein. The region between amino acids 113-134 of the tomato ACC oxidase shows strong probability for the formation of an amphipathic $\alpha$-helix containing multiple leucine residues on the hydrophobic side (Kende, 1993). This putative leucine zipper is conserved in all known ACC oxidases and may provide a site for ACC oxidase interaction with the plant membrane (Kende, 1993).
Brassinolides. Plant steroids have been studied as plant growth regulators since the 1930's and 1940's, when USDA scientists recognized that pollen extracts could promote growth. Mitchell and co-workers (1970) found that this substance from pollen extracts, especially from the pollen of *Brassica napus*, produced a response in the bean-internode assay which combined elongation (typical of gibberellins) with swelling and curvature (Mandava, 1988). Mitchell proposed that the active component was a new group of lipoidal hormones which they termed brassins. In 1979, the steroidal lactone brassinolide was identified as the growth promoting component of *Brassica napus* pollen (Mandava, 1988). Brassinolide is present in low amounts in plant tissue with its highest concentration in the pollen and seeds (1-1000 ng/kg tissue). It is found in varying lower amounts in shoots, leaves and fruit. Data from bioassay and immunoassay experiments have identified several brassinosteroids (BR) present in higher plants and suggest that BR's are widely distributed throughout the plant kingdom (Mandava, 1988).

Brassinosteroids all have similar structural features. They all contain a steroid nucleus and some have their oxygen function in the B ring, and a side chain at C-17 similar to that found in cholesterol. Other conserved features include: β-oriented angular C-18 and C-19 methyl groups, α-orientation at the C-5 A/B ring junction, α-oriented hydroxyl groups at C-22 and C-23 and α-oriented hydroxyl groups with cis-geometry at C-2 and C-3 in ring A of the steroid nucleus (Mandava, 1988). Brassinosteroids generally differ in their functional group at C-24 of the steroid side chain, although BR's have been

The brassinolide biosynthetic pathway was elucidated by Shozo Fujioka et al. in 1995 in *Catharanthus roseus* crown gall cells using feeding experiments. The pathway from campesterol to brassinolide is shown in Figure 1.2 (Fujioka et al., 1995) and has 5 oxygen-dependent steps. The proposed pathway is: campesterol → campanestol → 6α-hydroxycampestanol → 6-oxocampestanol → cathasterone → teasterone → 3-dehydroteasterone → typhasterol → castasterone → brassinolide. The step from 6-oxocampestanol to cathasterone is tentative but there is experimental evidence in support of this hypothesis. From studies with synthetic brassinolide analogs, several structural requirements for brassinolide biological activity were determined (Mandava, 1988):

- *cis*-vicinal glycol function at C-2 and C-3 in ring A.
- oxygen function at C-6 in the form of either a ketone or lactone.
- cholesterol (steroid) side chain with vicinyl group at C-22 and C-23.
- *trans*-A/B ring junction.
- substitution at C-24.
Figure 1.2. Brassinolide biosynthetic pathway. Adapted from Fujioka et al., 1995. Asterisks indicate steps which require oxygen.
Brassinosteroids have received much recent attention as a possible new class of plant growth hormone (Li et al., 1996; Szekeres et al., 1996; Clouse, et al., 1996). Li et al. characterized an Arabidopsis mutant defective in light-regulated development, DET 2 (de-etiolated 2). It was shown to have a defect in a protein which shared significant sequence homology with the mammalian steroid 5α-reductases. Two det2 alleles showed a substitution of lysine at glutamate 204, a residue absolutely required for activity in human steroid reductases. This substitution abolished the activity of the DET2 gene product causing defects in light-regulated development indicating a similar critical activity at this site. Another Arabidopsis mutant, CYP90, a cytochrome P450 mutant defective in cell elongation and etiolation, was shown by Szekeres et al. (1996) to be rescued by the addition of brassinolides. The cytochrome P450 encoded by CYP90 was further shown to have sequence homology to conserved domains of P450 monoxygenases which include the steroid hydroxylases (Szekeres et al., 1996; Nelson et al., 1993). The authors speculate that the CYP90 gene product is involved in the biosynthesis of active brassinosteroids and that they are required for normal cell elongation in plant development. Clouse et al. (1996) have recently identified a brassinosteroid-insensitive mutant, bri1, in Arabidopsis thaliana. Although the bri1 mutant retained normal physiologic responses to auxins, cytokinins, ethylene, abscisic acid and gibberellins, it displayed abnormal development. As exemplified by dwarfed stature, dark green, thickened leaves, male sterility, reduced apical
dominance and de-etiolation of dark grown seedlings. Taken together, these data indicate that plant steroid compounds may represent a novel class of plant growth hormones.

**Hypotheses and goals for this research**

Changes in the biosynthesis of ethylene and brassinolide in low oxygen environments may play an important role in the control of reproductive development under low O₂ conditions. Previous hypotheses have focused on the perturbations in photosynthesis and respiration caused by low O₂ as explanations for the reproductive inhibition. However, studies have shown that the oxygen-controlled process is not directly related to photorespiration and involves an unknown aspect of metabolism that requires a higher O₂ concentration than respiration (Quebedeaux and Hardy, 1976). The O₂-controlled process seems to occur and be mediated in reproductive tissue. We propose that a hormonal imbalance involving the inhibition of ethylene and brassinolide biosynthesis may be responsible for the repression of reproductive structures and seed formation.

This dissertation discusses the effect of low oxygen atmospheres on the growth and development of *Arabidopsis thaliana*. Initial discussion describes the morphological changes which occur in the rosette and reproductive structures of *Arabidopsis thaliana* over a range of subambient oxygen concentrations. The dissertation then describes the similarity between plants grown in extreme low oxygen environments and *Arabidopsis* mutants which are defective in brassinolide biosynthesis and discusses attempts to alleviate these
low oxygen effects by supplementation with brassinolide. Finally, a molecular analysis of changes occurring in ethylene biosynthesis under low oxygen conditions is presented. Herein is presented the first discussion of reasons for the reproductive inhibition observed by Quebedeaux and Hardy some twenty-five years ago.

References Cited


Chapter 2

Morphological changes in the Leaf Structure of *Arabidopsis thaliana* Grown Under Low Oxygen Conditions

Introduction

Changes in the growth of C₃ and C₄ plants under low oxygen (O₂) conditions have been the focus of several studies. Björkman et al. (1968 & 1969) were the first to observe a large increase in the vegetative matter of C₃ plants when grown under low O₂ tensions. *Mimulus cardinalis* and *Marchantia polymorpha* showed a 130- to 1000- fold increase in dry weight of vegetative matter over a 10 day experiment in 40 mmol/mol O₂ compared with control plants grown in 210 mmol/mol O₂. *Zea mays*, a C₄ plant, showed no significant response to low O₂ tensions. In experiments by Quebedeaux and Hardy (1973, 1975, 1976; Quebedeaux et al., 1975) soybean and sorghum were grown at 50 mmol/mol over their complete life cycle. As expected, there was an increase in vegetative growth of C₃, but not C₄, plants under lowered oxygen tensions. They observed a larger leaf surface area and density, increases in overall lateral growth and diameter of stems and branches, and some delay in senescence of the C₃ plants. Final dry weight of the vegetative portions of the C₃ plants showed a 74% increase when grown in 50 mmol-mol⁻¹ O₂ compared with plants grown in 210 mmol-mol⁻¹ O₂. C₄ plants showed no obvious vegetative changes.

C₃ plants show dramatic increases in growth due to the inhibition, at low oxygen tensions or elevated CO₂ concentrations, of photorespiration. These
conditions allow for less competition between oxygen and carbon dioxide for the active site of ribulose-1,5-bisphosphate carboxylase oxygenase (Rubisco). This shift in the CO₂/O₂ ratio favors the carboxylation reaction. C₄ plants are essentially unaffected in this regard by low O₂ or elevated CO₂ tensions due to their naturally low rates of photorespiration. The gas exchange responses to O₂ and CO₂ levels have been well characterized, however there is little information on anatomical changes which occur due to long-term exposure to altered O₂ and CO₂ levels. Ferris et al. (1996) found that in response to elevated CO₂ or elevated CO₂ and elevated temperature, leaf anatomy was significantly affected in Lolium perenne L. The leaves exhibited increases in stomatal density, epidermal cell density and stomatal index. These studies indicate that atmospheric gas composition has a pronounced effect on plant development.

Here we present data detailing morphological changes which occur in Arabidopsis grown under low oxygen conditions including detailed analysis of changes in leaf development. These experiments investigated long-term effects of low oxygen on overall plant stature, stomatal density and patterning, and epidermal cell shape over a range of low oxygen concentrations (210 - 25 mmol/mol O₂). We discuss the similarities of leaf anatomy changes to those caused by CO₂ enrichment, and propose that some of the changes caused by very low oxygen may be due to decreased or blocked biosynthesis of brassinosteroids, a new class of plant hormone.
Materials and Methods

Growth Conditions. *Arabidopsis thaliana* (L.) Heynh. var. 'Columbia' seeds were seeded in 10 mesh sieved Redi-earth (Scotts-Sierra Horticultural Products Co., Marysville, OH), amended with 5-6 granules of Osmocote 14-14-14 per cell (Grace Sierra Horticultural Products Co., Milpitas, CA), in six-cell seedling flats (45 ml per cell) and placed under a 300 µmol/m²/s, PAR light source. The growth medium was kept moist using a wick and reservoir watering system. Temperature was approximately 25°C.

Six days after seeding, seedlings were thinned to two per cell. Seven days after seeding, each six-cell seedling flat was placed into modified, transparent Sun bags (polypropylene film, No. B-7026, Sigma Chemical Co., St. Louis, MO) that were closed with an impulse sealer (Model: AIE-300, American International Electric) 1 cm from the open end of the bag (Figure 2.1). The plants were then exposed to one of five oxygen atmospheres (25, 50, 100, 160, 210 mmol/mol). Gas mixtures contained either 25, 50, 100, 160, or 210 mmol/mol O₂ plus 0.35 mmol/mol CO₂ in N₂. Relative humidity (90 ± 5% RH) and temperature (25°C ± 4°C) were monitored continuously in the controlled atmospheres with a Vaisala HMP 114Y T/RH sensor (Vaisala, Inc., Helsinki, Finland). Irradiance at soil level and at the top of each bag were 200 and 280 µmol/m²/s PAR, respectively. Gas composition in the premixed tanks and in the bags was confirmed by periodic sampling and gas chromatography.
Figure 2.1. *Arabidopsis thaliana* after growth for 30 days under normal oxygen A: (210 mmol/mol) or B: 25 mmol/mol oxygen. Plants grown at 25 mmol/mol O\(_2\) show a dwarf phenotype compared with plants grown under normal O\(_2\) conditions. Plants grown at 160, 100 and 50 mmol/mol O\(_2\) showed no obvious changes from the control plants.
Fixation of Tissue for Microscopy. Tissues were harvested 35 days after planting. Leaves were cut from mature rosettes and were immediately fixed in 1% formaldehyde and 2.5% glutaraldehyde in 0.1 mM phosphate buffer, pH 7.0, and postfixed in 1% osmium tetraoxide. For SEM analysis, tissue was dehydrated in ethanol and dried in CO₂ using a Denton DCP-1 critical-point drying apparatus (Denton Vacuum, Inc., Cherry Hill, NJ, USA). Tissue was observed and photographed with a Cambridge S-260 scanning electron microscope (Cambridge Instruments, Ltd., Cambridge, UK). Fresh material was observed and photographed using a Nikon Camera.

Morphometric analysis. SEM photographs of the adaxial (upper) and abaxial (lower) leaf surface were analyzed using grids of 200 μm² area. Only points occurring within the grid were counted, with edge points being counted half the time. A total of ten individual fields were counted for each treatment. Data were then analyzed by analysis of variance (ANOVA) using Number Cruncher Statistical Software (Hintze, 1987).

Results

Changes in the overall morphology of Arabidopsis in low oxygen environments. Arabidopsis thaliana grown under 25 mmol/mol oxygen exhibits a dwarf phenotype compared with plants grown at higher oxygen concentrations (Figure 2.2). Plants grown at 50, 100, and 160 mmol/mol oxygen show no obvious morphological changes from the control plants grown under 210
mmol/mol. In 25 mmol/mol oxygen, the rosette is smaller and the abaxial (lower) surface of the leaf is purple due to the accumulation of anthocyanins

**Changes in leaf epidermal cell structure.** Examination of rosette leaves by scanning electron microscopy revealed significant changes in leaf epidermal cell shape, stomatal density and stomatal distribution in low oxygen environments. Under normal conditions, leaf epidermal cells have a characteristic irregular shape on both the adaxial and abaxial surfaces (Figures 2.3a, 2.4a). However, as oxygen concentration was lowered, epidermal cell shape became more regular in appearance, shifting to elongated rectangular cells in 160, 100 and 50 mmol/mol oxygen treatments and finally to a smaller, round cell shape in 25 mmol/mol oxygen (Figures 2.3b-e, 2.4b-e).

In addition to changes in leaf epidermal cell shape, the patterning of stomata on both sides of the leaf was significantly affected by changes in oxygen concentration. At 210 mmol/mol oxygen, stomata are evenly distributed across the surface of the leaf with at least a single epidermal cell spaced between individual stomata. As oxygen concentration is lowered, stomata begin to appear in linear arrays (Figure 2.3b-c, 2.4b-c) without intervening epidermal cells in the 160 and 100 mmol/mol treatments. In extremely low oxygen conditions, 50 and 25 mmol/mol, stomata are clustered in groups of two or three so that guard cell walls are directly adjacent to one another (Figure 2.3d-e, 2.4d-e).
Figure 2.2. Comparison of rosette size in Arabidopsis grown under 210 or 25 mmol/mol O₂. Growth under low oxygen conditions results in an overall decrease in rosette size as compared to control plants.
Figure 2.3. Scanning electron micrographs of the adaxial leaf surface. (A) 210 mmol/mol O₂; (B) 160 mmol/mol O₂; (C) 100 mmol/mol O₂; (D) 50 mmol/mol O₂; (E) 25 mmol/mol O₂. Arrowheads indicate aberrant stomatal pairs and patterning across the leaf surface. Asterisks indicate changes in the epidermal cell shape as O₂ is lowered. 250X.
Figure 2.4. Scanning electron micrographs of the abaxial leaf surface. (A) 210 mmol/mol O₂; (B) 160 mmol/mol O₂; (C) 100 mmol/mol O₂; (D) 50 mmol/mol O₂; (E) 25 mmol/mol O₂. Arrowheads indicate aberrant stomatal pairs and patterning across the leaf surface. Asterisks indicate changes in the epidermal cell shape as O₂ is lowered. 250X.
Changes in stomatal density in low oxygen environments. Stomatal density per 200 μm² area was compared in wild-type and low oxygen treated rosette leaves. Stomatal density on both the adaxial and abaxial surfaces of the leaf increased as oxygen concentration decreased (Figure 2.5). In both experiments, there was a gradual increase in stomatal number over wild-type levels in the 100 and 50 mmol/mol oxygen treatments on both the adaxial and abaxial surfaces (Figure 2.5a, 2.5b). At 160 mmol/mol O₂, stomatal density fluctuated depending on the leaf surface and experiment. Data analysis from the first experiment showed a decrease in stomatal density on the adaxial surface under 160 mmol/mol oxygen (Figure 2.5a, 2.5b). In contrast, data from the second experiment showed stomatal density gradually increasing from wild-type levels in the 160, 100, and 50 mmol/mol oxygen treatments on both adaxial and abaxial surfaces. In the lowest (25 mmol/mol) oxygen treatment, stomatal density on the adaxial surface sharply increased while only a slight increase was observed on the abaxial surface in both experiments (Figure 2.5a, 2.5b).

Discussion

Morphological changes under low oxygen - parallels with elevated CO₂ studies

Elevated carbon dioxide levels significantly affect leaf anatomy on both the cellular and molecular level. Ferris et al. (1996), in studies tracking the response of Lolium perenne to increasing CO₂ and temperature in the atmosphere, have noted increases in leaf thickness, changes in epidermal cell size and an increase in the stomatal density of leaves grown under elevated...
Figure 2.5. The effects of low oxygen on stomatal density in *Arabidopsis thaliana*. Panel A shows stomatal density increasing as $O_2$ is lowered on the adaxial leaf surface. Panel B shows the increase in stomatal density as $O_2$ concentration decreases on the abaxial surface.
CO₂ conditions. The gradual increase in the stomatal density and leaf thickness of *Arabidopsis* leaves with decreasing oxygen concentration (Figure 2.5; Table 2.1) resembles increases in stomatal density observed in *Lolium perenne* grown under elevated carbon dioxide and elevated CO₂ and temperature conditions (Ferris et al., 1996). Epidermal cell shape in *Arabidopsis* also changed with decreasing oxygen compared to normal conditions. In 160, 100, and 50 mmol/mol O₂ treatments, cells became more rectangular in shape with straight cell walls (Figure 2.3a-d, 2.4a-d). Leaf epidermal cells in the 25 mmol/mol treatment became smaller, exhibiting a rounded cell shape with uniform straight cell walls (Figure 2.3e, 2.4e). *Lolium perenne* leaves under elevated CO₂ conditions were shown to have an increase in leaf epidermal cell density compared with normal conditions (Ferris et al., 1996). Anatomical traits such as increases in plant size, stomatal density and leaf thickness were affected in similar ways by both low oxygen and elevated carbon dioxide. Therefore, these effects are most likely mediated by changes in photosynthesis since both elevated CO₂ and low O₂ promote increased carboxylation of Rubisco. Clearly, atmospheric gases exert a strong influence on overall leaf development. However, changes in patterning of stomata across the leaf surface, leaf epidermal cell shape and the dwarf phenotype of plants grown at 25 mmol/mol O₂ are unique to growth under low oxygen (Table 2.2). These features closely resemble anatomical changes which have been documented in several recently characterized brassinolide-deficient mutants (Table 2.2), as will be discussed below.
Table 2.1. Measurements of leaf cross-sections indicating changes in leaf thickness and sizes of epidermal and mesophyll cells as oxygen concentration is decreased. Mean values followed by the same letter were not significantly different at the 0.05 probability level based on Fisher's LSD following ANOVA. Adapted from Kuang et al., in review.

<table>
<thead>
<tr>
<th>O₂ (mmol/mol)</th>
<th>Leaf Thickness (µm)</th>
<th>Cell Size (µm²) Epidermis</th>
<th>Cell Size (µm²) Mesophyll</th>
</tr>
</thead>
<tbody>
<tr>
<td>25</td>
<td>370.7c</td>
<td>1170ab</td>
<td>1082a</td>
</tr>
<tr>
<td>50</td>
<td>262.0ab</td>
<td>1209ab</td>
<td>1893b</td>
</tr>
<tr>
<td>100</td>
<td>261.7ab</td>
<td>1325bc</td>
<td>1916b</td>
</tr>
<tr>
<td>160</td>
<td>284.7b</td>
<td>1654c</td>
<td>2623c</td>
</tr>
<tr>
<td>210</td>
<td>236.0a</td>
<td>851a</td>
<td>1335a</td>
</tr>
</tbody>
</table>
Dwarfism, stomatal patterning and changes in epidermal leaf shape- a loss of brassinolide at low oxygen tensions? Plants grown under 160, 100, and 50 mmol/mol oxygen show no obvious differences from plants grown at normal (210 mmol/mol) oxygen concentrations. However, plants grown at ultra-low oxygen concentrations (25 mmol/mol) are dwarves. These low-oxygen dwarves resemble two recently described brassinosteroid-deficient mutants, *det2* and *cpd* (Li *et al.*, 1996, Szekeres *et al.*, 1996). Brassinosteroids have received much recent attention as a possible new class of plant growth regulators (Clouse *et al.*, 1996; Hooley, 1996). They are implicated in the normal development and elongation of cells and require molecular oxygen for their biosynthesis (Szekeres *et al.*, 1996; Fujioka *et al.*, 1995; Mandava, 1988). Li *et al.* (1996) characterized an *Arabidopsis* mutant defective in light regulated development, *det2* (de-etiolated 2), whose gene product was shown to encode a protein which shared significant sequence homology with mammalian 5-α-reductases. Another *Arabidopsis* mutant, *cpd*, defective in both cell elongation and de-etiolation, was shown by Szekeres *et al.* (1996) to be rescued by the addition of brassinolides, which restored normal cell elongation in the hypocotyl, leaves and petioles. The CPD gene product, CYP90, was shown to encode a cytochrome P450 which shared homology to conserved domains of steroid hydroxylases (Szekeres *et al.*, 1996). The authors speculate that CYP90 is involved in the biosynthesis of active brassinolides and that they are required for normal cell elongation in plant development.
Table 2.2. Changes in leaf anatomical traits mediated by low O$_2$, by elevated CO$_2$ and by loss of brassinolide biosynthesis. Citations: a: Szekeres et al., 1996 b: Ferris et al., 1996 c: this study.

<table>
<thead>
<tr>
<th>Anatomical Trait</th>
<th>Brassinolide-deficient mutants</th>
<th>Elevated CO$_2$</th>
<th>Low O$_2$</th>
<th>All conditions</th>
<th>Citations</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plant size and leaf length</td>
<td>↓</td>
<td>↑</td>
<td>↓</td>
<td>-</td>
<td>a, b, c</td>
</tr>
<tr>
<td>Stomatal Density</td>
<td>- , ↑</td>
<td>↑</td>
<td>↑</td>
<td></td>
<td>b, c</td>
</tr>
<tr>
<td>Stomatal Patterning</td>
<td>Δ, loss of epidermal cells between stomata</td>
<td>?</td>
<td>Δ, loss of epidermal cells between stomata</td>
<td></td>
<td>a, c</td>
</tr>
<tr>
<td>Leaf Thickness</td>
<td></td>
<td></td>
<td></td>
<td>↑</td>
<td>b, c</td>
</tr>
<tr>
<td>Epidermal Cell Shape</td>
<td>Δ, cells have straight cell walls</td>
<td>?</td>
<td>Δ, cells have straight cell walls</td>
<td></td>
<td>a, c</td>
</tr>
</tbody>
</table>
Both det2 and cpd mutants display overall dwarfism, male sterility and the activation of stress regulated genes (Szekeres et al., 1996; Li et al., 1996). Plants grown under 25 mmol/mol oxygen also exhibit overall dwarfism, loss of fertility and high anthocyanin production. In addition, the cpd mutant was shown to have aberrant stomatal patterning and leaf epidermal cell shape on the adaxial surface of the leaf (Szekeres et al., 1996). Rosette leaves from plants grown under low oxygen conditions also show altered patterning of stomata across the leaf surface and an altered epidermal cell shape (Figure 2.3a-e, 2.4a-e, Table 2.1). The inhibition or complete repression of brassinolide biosynthesis as the oxygen concentration is lowered from ambient levels could be responsible for changes in the anatomy of plants grown under low oxygen which are not reflected in studies involving elevated CO₂. The precise role of brassinolide in the developmental changes observed under low oxygen conditions will be investigated in the following chapter.

References Cited


The Effects of Low Oxygen Environments on Brassinolide Production in Arabidopsis thaliana

Introduction

Brassinosteroids are a class of plant growth regulators which are known to elicit numerous biological responses in plants. Brassinolide, the most biologically active brassinosteroid, was first isolated from rape pollen in 1979 by Grove et al. First thought to be most active in pollen and reproductive development, brassinosteroids are now known to be important in stem elongation, leaf bending, leaf unrolling, root inhibition, proton pump activation, the promotion of 1-aminocyclopropane-1-carboxylic acid (ACC) and xylogenesis (Yokota, 1997). Furthermore, DNA, RNA and protein synthesis have been reported to be affected by brassinosteroids (Xu, 1995; Zurek, 1994). Brassinosteroids have now been identified in a broad range of species. The large body of knowledge accumulated indicates that steroids are as important to plants as they are to animals, insects, crustaceans and microorganisms (Yokota, 1997).

In brassinolide biosynthesis, it is the addition of hydroxyl groups to the cholesterol backbone which confers biological activity on the compound. As subsequent hydroxyl groups are added, the steroid becomes more and more active as a plant growth regulator. For this reason, the three most active brassinosteroids are typhasterol, castasterone and brassinolide, the final three compounds in the brassinosteroid biosynthetic pathway (Figure 1.2; Yokota, 1997).
1997; Fujioka et al., 1995). The addition of hydroxyl groups occurs through hydroxylation reactions which require molecular oxygen.

Plants grown under 25 mmol/mol oxygen exhibit a dwarf phenotype, changes in leaf epidermal cell shape and stomatal patterning which resemble anatomical changes reported in brassinolide-deficient Arabidopsis mutants. We propose that ultra-low oxygen environments may inhibit or repress the biosynthesis of biologically active brassinosteroids in plants. Therefore, we have used the det2 mutant, which is deficient in brassinolide biosynthesis, in the following experiments. If low oxygen dwarfism is caused by a block in brassinolide biosynthesis then 1) Columbia wild-type grown under low oxygen and supplemented with brassinolide should return to its normal size and 2) the det2 mutant should a) exhibit no further size changes under low oxygen conditions and b) when grown under low oxygen supplemented with brassinolide should return to normal size (Table 3.1). Here we present data detailing the role of brassinolide in developmental changes observed under low oxygen. Supplementation of plants growing under 25 mmol/mol oxygen with 10^{-7} M brassinolide partially rescued the dwarf phenotype and resulted in more normal leaf thicknesses and leaf mesophyll development.

**Materials and Methods**

**Experimental System.** The experimental system consisted of eight modified coupled magenta units arranged in groups of four with each group receiving a different oxygen treatment (Figure 3.1). The two oxygen treatments used
Table 3.1. Diagram of predicted and known experimental results which would follow from the hypothesis that low oxygen dwarfism is due to the inhibition or a block in brassinolide biosynthesis. Highlighted blocks indicate predicted observations.
consisted of: 210 mmol/mol O\textsubscript{2} and 350 ppm CO\textsubscript{2} in N\textsubscript{2} and 25 mmol/mol O\textsubscript{2} and 350 ppm CO\textsubscript{2} in N\textsubscript{2} with four magenta vessels receiving 210 mmol/mol O\textsubscript{2} and four receiving 25 mmol/mol O\textsubscript{2}. Gases were filtered prior to entry into each coupled unit with an autoclaved, Nalgene PTFE membrane syringe filter (#199-2020, Nalge Co., Rochester, NY). Teflon PFA tubing (# H-06375-02, Cole-Parmer Instrument Co., Niles, IL) and brass fittings (Swagelok Co., Solon, OH) were used to connect the gas sources to each magenta chamber. Gas flow into each magenta unit was set to 35 ml/min, creating a slight positive pressure inside each unit. Flows were set by adjusting brass Nupro JNR valves (Nupro Co., Willoughby, OH).

**Growth Conditions.** *Arabidopsis thaliana* (L.) Heynh var. ‘Columbia’ and *det2* seed were surface sterilized, sown on nutrient agar plates (0.6% Hoagland’s amended with 0.5% sucrose) and vernalized for two days. Seeds of the *det2* mutant were obtained from the Arabidopsis Biological Resource Center (ABRC, Ohio State University, Columbus, OH). The plants were then grown under continuous fluorescent light (250 μmol/m\textsuperscript{2}/sec) for ten days until the first leaves had emerged and expanded. The plants were next aseptically transferred to autoclaved, coupled magenta vessels containing nine 7-ml polycarbonate centrifuge tubes. Plants in each experiment were grown for ten days for comparison of leaf development, gas and brassinolide response of *det2* and Columbia wild-type plants. Plants were then harvested for analysis.

**Brassinolide Concentration Series.** In experiments to determine the optimal
Low Oxygen plus BR Experiment

Concentration Series Experiment

Figure 3.1. Diagram of experimental system for brassinolide experiments. Squares represent magenta vessels, each containing 9 plants growing in individual test tubes.
concentration of brassinolide for use in this system, the test tubes contained either nutrient agar or nutrient agar amended with $10^6$ M, $10^7$ M, or $10^8$ M brassinolide in ethanol (Cidtech Research, Ontario, Canada) (Figure 3.1). The coupled magenta vessels were then grown for ten days under the illumination conditions discussed above and harvested for analysis. The brassinolide concentration used in the following low oxygen experiments ($10^7$ M brassinolide) was determined based on the optimal response of the det2 mutant (in terms of increased leaf length) to brassinolide supplementation in the concentration series.

**Oxygen Effects On Mutant × Brassinolide Interaction.** For comparison of leaf development, gas and brassinolide response of det2 with var. Columbia under low oxygen conditions, plants were grown on agar following vernalization as illustrated in Figure 3.1. Test tubes contained either nutrient agar or nutrient agar amended with $10^7$ M brassinolide in ethanol (Cidtech Research, Ontario, Canada). The coupled magenta vessels were then connected to compressed gas mixtures as described in the experimental system and grown for ten days. Multiple experiments were conducted (n=3) to verify results and obtain statistically complete data.

**Analysis.** In both the brassinolide concentration series and the low oxygen × brassinolide supplementation experiments, plants were harvested after ten days. Four leaves from each treatment were harvested for scanning electron microscopy (SEM) analysis, four leaves for leaf thickness analysis and ten leaves were measured for leaf length analysis. Leaves for SEM and leaf
thickness analysis were cut from the rosette and placed in fixative (1% formaldehyde, 2% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4) for three hours at room temperature. Tissue was then washed three times in 0.1 M phosphate buffer and postfixed overnight in buffered 1% osmium tetraoxide (OsO₄). For leaf thickness measurements, leaf tissues were washed in 0.1 M phosphate buffer, pH 7.0, dehydrated in an ethanol series and then infiltrated and embedded in Spurr’s resin (Spurr, 1969). Embedded leaf tissues were sectioned with a Dupont Sorvall microtome (Dupont, Newton, CN). Sections 1 μm thick were stained with toluidine blue O (TBO) in 2% sodium borate for general tissue staining. Sections were observed and photographed using a Nikon light microscope. Leaf blade thickness was determined by measuring the thickness of leaf cross sections at four locations midway between the minor veins immediately flanking the midvein of the lamina. For scanning electron microscopy, leaf tissue after fixation in 1% osmium tetraoxide was washed in 0.1 M phosphate buffer, pH 7.0, taken through a dehydration series, critical point-dried, coated in gold-palladium (2Å) using a Hummer II sputter coater and examined on a Cambridge S-260 scanning electron microscope.

Ten leaf measurements from each gas x brassinolide treatment were taken. Ten 200 μm² fields from the SEM leaves were counted to determine stomatal density on both the adaxial (upper) and abaxial (lower) surfaces. These results were analyzed by analysis of variance (ANOVA) and t-test analysis using Number Cruncher Statistical Software (Hintze, 1987).
Results

**Determination of Optimal Brassinolide Concentration - Leaf length.** Initial experiments were undertaken to determine the optimal brassinolide concentration for use under low oxygen conditions. Plants were grown as described above in agar supplemented with 10^{-6} M, 10^{-7} M or 10^{-8} M brassinolide under 210 mmol/mol oxygen. Response of the *det2* mutant was used to determine the optimal brassinolide concentration for subsequent experiments. Visually, Columbia and *det2* most closely resembled one another when treated with 10^{-7} M brassinolide (Figure 3.2). In response to brassinolide supplementation, *det2* leaf length most closely matched wild-type leaf length at 10^{-7} M brassinolide (Figure 3.3). Supplementation with 10^{-6} or 10^{-8} M brassinolide resulted in significant differences in *det2* leaf length (Figure 3.3) when compared with Columbia based on ANOVA. For these reasons, 10^{-7} M brassinolide was determined as the optimal concentration for use in the low oxygen experiments.

**Leaf thickness.** Leaf thickness of Columbia and *det2* was also measured (Figure 3.4) over a range of brassinolide concentrations. Previous experiments (Kuang *et al.*, unpublished) have shown that the leaves of the *det2* mutant are thicker than wild-type when grown under normal oxygen conditions (Figure 3.4). Plants grown under 25 mmol/mol oxygen also show increased leaf thickness (Kuang *et al.*, in review). For this reason, changes in the leaf thickness of *det2* in response to supplementation with brassinolide was investigated.
Figure 3.2. Photograph of Columbia wild-type and det2 plants grown in agar supplemented with various brassinolide concentrations. All plants are 19 days old.
Figure 3.3. Graph comparing Columbia wild-type and det2 leaf lengths when grown in agar supplemented with various brassinolide concentrations. A-Z indicate significant differences between brassinolide treatments. Asterisks indicate significant differences between Columbia and det2 plants at a given brassinolide concentration based on ANOVA analysis.
Supplementation with all brassinolide concentrations (10^{-6}, 10^{-7}, 10^{-8} M) resulted in a decrease in leaf thickness in \textit{det2} though leaves still remained slightly thicker than those of wild-type plants (Figure 3.4). In wild-type plants supplemented with 10^{-6} M brassinolide, leaf thickness increased (Figure 3.4). Columbia supplemented with 10^{-8} M brassinolide had leaves which were slightly thinner than wild-type grown on agar alone (Figure 3.4). Columbia plants were most similar to normal leaf thickness when supplemented with 10^{-7} M brassinolide.

Changes in the structure of the palisade and spongy mesophyll were also observed in both \textit{det2} and Columbia supplemented with brassinolides (Figure 3.5). In Columbia, at 10^{-7} M brassinolide there was a loss of palisade organization and an increase in the amount of spongy mesophyll compared with wild-type leaves (Figure 3.5). At 10^{-6} M brassinolide, there was an increase in leaf thickness compared with the other treatments. As in the 10^{-7} M brassinolide treatment, the palisade layer was less organized and there was an increase in the amount of spongy mesophyll (Figure 3.5).

In the \textit{det2} mutant, leaves fluctuate in thickness with increasing amounts of brassinolide (Figure 3.5). \textit{Det2} leaves grown under ambient conditions are much thicker than those of wild-type plants (Figure 3.5). In the 10^{-8} M brassinolide treatment, \textit{det2} leaves showed a decrease in thickness and more organized palisade and spongy mesophyll arrangement more closely resembling wild-type plants. At 10^{-7} M brassinolide, \textit{det2} leaves increased in thickness and had palisade mesophyll cells which were smaller and more
Figure 3.4. Changes in leaf thicknesses in Columbia and det2 plants grown at various brassinolide concentrations. A-Z indicates significant differences between brassinolide treatments as determined by ANOVA analysis.
Figure 3.5. Light micrographs of cross-sections from Columbia and det2 leaves grown in agar or agar supplemented with brassinolide. Magnification 33.3 X.
random in arrangement than in the $10^{-8}$ or agar alone treatments (Figure 3.5). Leaves in the $10^{-6}$ M treatment were significantly smaller than those from any other brassinolide treatment (Figure 3.5). Leaves from the $10^{-6}$ M brassinolide treatment had tightly packed palisade mesophyll cells which accounted for an abnormally high percentage of the total leaf area (Figure 3.5). Though spongy mesophyll was present, it accounted for much less of the leaf volume than under normal conditions.

**Stomatal Density.** Changes in the stomatal density of both det2 and Columbia were observed when supplemented with brassinolide (Figure 3.6). Det2 stomatal density increased on both the adaxial and abaxial leaf surfaces in all brassinolide concentrations tested (Figure 3.6). The most significant increases were observed in the $10^{-7}$ M and $10^{-6}$ M treatments on the abaxial leaf surface and in the $10^{-7}$ and $10^{-8}$ M treatments on the adaxial surface (Figure 3.6). On both leaf surfaces, the det2 mutant showed more substantial increases in stomatal density with brassinolide supplementation compared with wild-type plants. Interestingly, the differences in stomatal density between det2 and Columbia grown on agar were more pronounced on the abaxial leaf surface (Figure 3.6). There was no difference under normal growth conditions between wild-type and det2 stomatal density on the adaxial leaf surface when grown on agar alone or on agar supplemented with $10^{-6}$ M brassinolide (Figure 3.6). On the adaxial surface, Columbia exhibited no significant change in stomatal density, except at $10^{-8}$ M brassinolide, where a decrease in stomatal density
Figure 3.6. Changes in stomatal density on the adaxial and abaxial leaf surfaces of Columbia and det2 grown in agar or agar supplemented with brassinolide. A-Z indicates significant differences between brassinolide treatments. Asterisks indicate significant differences between varieties at a given brassinolide concentration. Panel A: Adaxial (upper) surface; Panel B: Abaxial (lower) surface.
was observed. On the abaxial surface, wild-type plants showed an increase in stomatal density at all brassinolide concentrations tested.

**Changes in leaf length under low oxygen with brassinolide supplementation.**

*Arabidopsis thaliana* plants grown under 25 mmol/mol oxygen exhibit a dwarf phenotype when compared with plants grown at higher oxygen concentrations (Kuang *et al.*, in review; Figure 2.1 & 2.2). The *det2* mutant, which is blocked in brassinolide biosynthesis, exhibits a similar dwarf phenotype (Li *et al.*, 1996). In an attempt to see if brassinolide supplementation could overcome the low oxygen dwarf phenotype, leaf lengths of Columbia and *det2* grown under 210 and 25 mmol/mol oxygen and supplemented with $10^{-7}$ M brassinolide were compared. When supplemented with brassinolide in 25 mmol/mol O$_2$, Columbia leaf lengths increased to an intermediate length between the dwarf and wild-type (Figure 3.7, 3.8). The *det2* mutant under the same conditions increased its leaf length to near wild-type levels (Figure 3.8). *Det2* showed no response to the decreased oxygen environment (Figure 3.7), exhibiting the same growth habit when grown on agar in 210 mmol/mol O$_2$ and in 25 mmol/mol O$_2$. This indicates that the *det2* mutation renders the plant's leaf length insensitive to changes in atmospheric oxygen.

**Changes in leaf thickness in low oxygen with $10^{-7}$ M brassinolide.** The *det2* mutant has significantly thicker leaves than wild-type *Arabidopsis* when grown under normal conditions (Figure 3.4, 3.5). Previous studies have shown that wild-type plants grown at 25 mmol/mol oxygen also exhibit an increase in leaf thickness (Kuang *et al.*, unpublished and Table 2.1). Since *det2* is deficient in
brassinolide biosynthesis it was surmised that addition of brassinolide to plants growing in low oxygen environments might alleviate the increase in leaf thickness. However, when supplemented with $10^{-7}$ M brassinolide, Columbia and det2 plants grown in 25 mmol/mol showed no significant change in leaf thickness (Figure 3.9). Both Columbia and det2 leaves supplemented with $10^{-7}$ M brassinolide whether grown in ambient or low O$_2$ showed no change in leaf thicknesses when analyzed by ANOVA (Figure 3.9).

Changes in the distribution and size of leaf palisade and spongy mesophyll were also observed in both varieties under low oxygen conditions (Figure 3.10). In Columbia, plants grown on agar under 25 mmol/mol O$_2$ showed an increase in the amount of starch stored on the leaf, an increase in wax deposition, a more tightly organized palisade layer and a loss of air space in the leaf (Figure 3.10 C & D). Columbia grown under ambient oxygen, both with and without $10^{-7}$ M brassinolide supplementation, showed few visible differences (Figure 3.10). Det2 leaves grown at 25 mmol/mol O$_2$ and at 210 mmol/mol O$_2$ on agar showed an almost complete loss of spongy mesophyll (Figure 3.10). In both oxygen conditions, when supplemented with $10^{-7}$ M brassinolide det2 leaves showed a more normal morphology with both spongy and palisade mesophyll represented (Figure 3.10). However, in the 25 mmol/mol O$_2$- $10^{-7}$ M brassinolide treatment an increase in starch storage in the leaves was observed in det2 plants (Figure 3.10 H). No increases in wax deposition on the surface of det2 leaves was observed (Figure 3.10).
Figure 3.7. Photograph of Columbia wild-type and det2 plants grown under either 210 mmol/mol or 25 mmol/mol oxygen, on nutrient agar or nutrient agar supplemented with $10^{-7}$ M brassinolide. Plants are 19 days old.
Figure 3.8. Graphs of Columbia and det2 leaf length when grown at either 210 mmol/mol or 25 mmol/mol oxygen on agar or agar supplemented with $10^{-7}$ M brassinolide. Asterisks indicate significant differences between treatments as determined by ANOVA ($n = 2$).
Figure 3.9. Graphs of Columbia and det2 leaf thicknesses when grown in either 210 mmol/mol or 25 mmol/mol oxygen on agar or agar supplemented with $10^{-7}$ M brassinolide.
Figure 3.10. Light micrographs of cross-sections from Columbia and det2 leaves grown at either 210 mmol/mol or 25 mmol/mol oxygen on agar or agar supplemented with $10^{-7}$ M brassinolide. A: wild-type, 210 mmol/mol $O_2$; B: wild-type, 210 mmol/mol $O_2$; C: wild-type, 25 mmol/mol $O_2$; D: wildtype, 25 mmol/mol $O_2$; E: det2, 210 mmol/mol $O_2$; F: det2 210 mmol/mol $O_2$; G: det2, 25 mmol/mol $O_2$; H: det2, 25 mmol/mol $O_2$. Magnification 33.3 X.
Changes in stomatal density under low oxygen with brassinolide supplementation. Leaf tissue was analyzed by SEM to observe changes in stomatal density in plants grown under low oxygen with brassinolide supplementation. Plants grown under low oxygen exhibit an increase in stomatal density as oxygen concentration is decreased (Figure 2.3). Det2 also has an increased stomatal density compared with wild-type plants when grown under normal conditions (Figure 3.11). When supplemented with brassinolide under low oxygen conditions, both det2 and Columbia wild-type plants showed a slight increase in stomatal density on the adaxial surface. On the abaxial surface, stomatal density in det2 increased at 25 mmol/mol O₂ with brassinolide supplementation while Columbia stomatal density decreased under the same conditions (Figure 3.11). Under 210 mmol/mol O₂, stomatal density remained constant in both varieties on the abaxial surface but stomatal density increased on the adaxial surface in both Columbia and det2 supplemented with brassinolide under ambient oxygen conditions (Figure 3.11). Interestingly, both Columbia and det2 grown on agar at 210 and 25 mmol/mol O₂ showed no significant differences in stomatal density on the adaxial surface (Figure 3.11). However, on the abaxial surface, Columbia and det2 grown on agar exhibited significantly different stomatal densities from one another in each oxygen treatment. When two independent experiments were analyzed by ANOVA, the only significant factor affecting stomatal density was the change in oxygen.
Figure 3.11. Graph of changes in adaxial and abaxial stomatal density in Columbia and det2 leaves grown under 210 mmol/mol or 25 mmol/mol oxygen on agar or agar supplemented with $10^{-7}$ M brassinolide. A-Z indicate significant differences between treatments within a given variety. Asterisks indicate significant differences between varieties within a given treatment as determined by ANOVA.
concentration. Neither variety nor brassinolide treatment had a significant effect. Therefore, the change in stomatal density across the leaf surface is related to changes in atmospheric composition.

**Discussion**

**Role of brassinolides in low oxygen responses.** Growth of *Arabidopsis* under 25 mmol/mol oxygen results in dwarf plants (Figure 3.2). The appearance of these plants resembles the *det2* mutant which is deficient in brassinolide biosynthesis. To date most dwarf mutations have been associated with deficiencies in gibberellin biosynthesis. However, when tested, several *Arabidopsis* mutants (*det2* and *cpd*) had normal levels of and responses to gibberellins. Both mutants exhibited dwarfism and de-etiolation which were rescued only by the application of brassinolides and not by gibberellins or auxin (Li *et al.*, 1996). Brassinolides are known to elicit divergent biological activities and have been proposed as a new class of plant growth regulator (Hooley, 1996; Yokota, 1997). Their functions include stem elongation, pollen tube growth, leaf bending, leaf unrolling, root inhibition, proton pump activation, promotion of 1-aminocyclopropane-1-carboxylic acid production and xylogenesis (Yokota, 1997). They also require molecular oxygen at several steps in their biosynthesis (Figure 1.2). Li *et al.* (1996) characterized the *det2* mutant, which is defective in light-regulated development. The *det2* gene product was shown to encode a protein which shares significant homology to mammalian steroid 5-α-reductases. Another *Arabidopsis* mutant, *cpd*,
defective in cell elongation and de-etioliation, was shown by Szekeres et al. (1996) to be rescued by the addition of brassinolides which restored normal cell elongation in the hypocotyl, leaves and petioles. Limited anatomical data available on cpd indicate that it has altered leaf structure in its epidermal arrangement and in the internal arrangement of the palisade and spongy mesophyll. The cpd gene product was shown to encode a cytochrome P450 which shared sequence homology to conserved domains of steroid hydroxylases (Szekeres et al., 1996). The authors speculate that the cpd gene product is involved in the biosynthesis of active brassinolides and that they are required for normal cell elongation in plant development.

Figure 3.12 summarizes the results of the low oxygen/brassinolide supplementation studies and compares observed results with those expected if low oxygen inhibition of brassinolide biosynthesis were responsible for the traits observed. In several cases, the observed results were not consistent with the predicted outcomes (Figure 3.12). In both Columbia and det2 supplemented with 10^{-7} M brassinolide and grown under 25 mmol/mol oxygen, the predicted result was a return to normal leaf length. In both varieties at 210 mmol/mol O_2, leaf length only recovered to near wild-type levels (Figure 3.8). Similar results were reported for brassinolide supplementation of the cpd mutant (Szekeres et al., 1996). In Columbia, these changes under ambient oxygen conditions may be explained by a toxic effect of brassinolide being present throughout the life cycle of the plants. Wild-type plants are capable of synthesizing endogenous brassinolide and so excess brassinolide in the system may be responsible for
the depression in leaf length (Figure 3.8). Though not capable of brassinolide biosynthesis, *det2* may also exhibit aberrant development with brassinolide supplementation under 210 mmol/mol O₂ due to the presence of the growth regulator in the medium at all times. Under low oxygen conditions (25 mmol/mol), neither Columbia nor *det2* supplemented with brassinolide achieved the predicted normal leaf length. Low O₂-grown, brassinolide-supplemented Columbia leaf length increased to an intermediate length between normal and dwarf morphology. *Det2* leaf lengths under low O₂ and 10⁻⁷ M brassinolide increased but not to wild-type levels. *Det2* plants reached an intermediate stage between the dwarf and wild-type phenotypes (Figure 3.7, 3.8 & 3.12). These discrepancies may be also be the result of toxic effects on plant growth and development arising from the growth regulator being present at all times or a result of impurities in the commercially available brassinolides. GC-MS spectra indicated that there were impurities present in the commercially available compound although the major component was brassinolide (Appendix). Byproducts of the steroid isolation process may also be present and could be interfering with the experimental results.

In the case of leaf thickness, data did not agree with expected results when *det2* was grown at ambient oxygen with and without brassinolide supplementation (Figure 3.9, 3.12). It is expected that the brassinolide-deficient mutant when supplemented with the missing growth regulator at ambient O₂ would show a decrease in leaf thickness to wild-type levels if the mutant was the result of a single mutation. These data may indicate that the
Figure 3.12. Summary of results from low oxygen x brassinolide supplementation experiments. Highlighted blocks indicate instances where observed results do not equal expected results. N → Dwf: intermediate leaf length. n.s.: not significant when analyzed by ANOVA.
det2 mutant is the result of mutations at more than one locus. This may explain some of the inconsistencies within the data. In addition, Both det2 and Columbia grown at 25 mmol/mol O₂ did not show a significant increase in leaf thickness over ambient O₂ as in previous experiments (Kuang et al., in review). Increases in leaf thickness are also observed in plants grown under elevated CO₂ conditions (Ferris et al., 1996) (Table 3.2). Therefore, changes in leaf thickness may be mediated partially by a process linked to photosynthesis and not completely mediated by loss of brassinolide biosynthesis under low oxygen conditions.

Stomatal density data for both Columbia and det2 showed several instances where observed data did not agree with expected results. Columbia supplemented with brassinolide under ambient O₂ conditions and det2 supplemented with brassinolide under ambient and low oxygen showed increases in stomatal density from the wild-type (Figure 3.13). When several experiments were analyzed using ANOVA, the only significant factor affecting stomatal density was shown to be oxygen concentration. Therefore the changes in stomatal density are not linked with the loss of brassinolide biosynthesis in low oxygen environments. Since increases in stomatal density are also observed in plants growing under elevated CO₂ conditions (Ferris et al., 1996), changes in stomatal density may be related to a process regulated through photosynthesis (Table 3.2). Clearly, changes in leaf development under low oxygen (25 mmol/mol) are influenced by a variety of causes including interruption of brassinolide biosynthesis and changes in photosynthesis.
Table 3.2. Changes in leaf anatomical traits mediated by low O₂, elevated CO₂ or by a loss of brassinolide biosynthesis. Citations: aSzekeres et al., 1996; bFerris et al., 1996; cKuang et al., 1999 (in review); dthis study.

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resulting from increased carboxylation of Rubisco under lowered oxygen conditions (Table 3.2). However, low O\textsubscript{2} also affects other developmental processes in addition to changes in the leaf. Low oxygen environments have been shown to have a profound effect on the development of reproductive structures in plants (Quebedeaux and Hardy, 1973, 1975; Quebedeaux \textit{et al.}, 1975; Kuang \textit{et al.}, 1998). Extensive work by Quebedeaux and Hardy (1975) as well as by Gale (1974) has shown that the inhibition of reproductive growth observed in low oxygen environments is unrelated to the effects observed on photosynthesis and respiration. Therefore, another process integral to seed and embryo development and influenced by O\textsubscript{2} must be affected. In the next chapter, the role of ethylene, another plant growth regulator controlled by oxygen will be examined.

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Chapter 4

Effects of Low Oxygen Environments on Ethylene Production in *Arabidopsis thaliana* Siliques

Introduction

The possibility of increasing crop productivity through the repression of photorespiration has led to interest in the growth of plants under low oxygen environments. In studies of plants grown at 50 mmol/mol O$_2$, C$_3$ species showed a strong induction of vegetative growth while C$_4$ species showed no major changes. Surprisingly, in both C$_3$ and C$_4$ species grown under 50 mmol/mol O$_2$ there was an almost complete repression of seed development (Quebedeaux and Hardy, 1973). These findings led to the speculation that an O$_2$-sensitive process which controlled partitioning between vegetative and reproductive structures was responsible for the inhibition of seed development observed (Quebedeaux and Hardy, 1976). Subsequent studies by Gale (1974) and Sinclair (1987) have argued that the inhibition of reproductive growth observed under low oxygen conditions cannot be completely explained by the obvious effects of low O$_2$ on photosynthesis and respiration. Therefore other processes integral to seed development and influenced by O$_2$ must be affected. We propose that a hormonal imbalance may be responsible for the repression of reproductive structures and seed development observed in plants grown at lower oxygen tensions.

A plant hormone whose biosynthesis is directly influenced by oxygen and which is known to intimately influence seed and fruit development is
ethylene. Ethylene is a small, gaseous plant hormone whose final biosynthetic step, the conversion of ACC to ethylene by the enzyme ACC oxidase, requires molecular oxygen. A lowering of the oxygen concentration around the plant might result in an inhibition or block of ethylene biosynthesis.

Ethylene is known to be important in seed and fruit development. In 1990, Meakin and Roberts showed that ethylene production in the silique of *Brassica napus* increased during seed development particularly during seed reserve accumulation. The high levels of ethylene in the developing siliques declined rapidly when seeds were in the final stages of maturation (Meakin and Roberts, 1990). Further studies by Johnson-Flanagan and Spencer (1994) on two *Brassica* species, *Brassica juncea* and *Brassica napus*, showed that ethylene evolution was particularly high during early embryo development and late seed maturation. In addition, Bertrano *et al.* (1994) in studies with wheat showed that there was a strong correlation between increases in ethylene production and the maturation of the developing grain. Ethylene production increased progressively from pre-anthesis to a peak at the hard dough stage of the grains (Bertrano *et al.*, 1994). In contrast, ethylene levels fell as the grains reached maturation and senescence (Bertrano *et al.*, 1994). These data indicate that ethylene may be integral to normal embryo and seed development in diverse species.

Here we present data detailing the effect of low oxygen environments on ethylene production and biosynthesis in the reproductive structures of *Arabidopsis thaliana*. We investigate the effects of low oxygen on the
expression and patterning of the ethylene biosynthetic enzymes, ACC synthase and ACC oxidase in *Arabidopsis* tissue as well as the ability of siliques produced under a range of low oxygen concentrations (210 - 25 mmol/mol O₂) to produce ethylene. We suggest that the repression of reproductive structures in low oxygen environments is related to the inhibition of ethylene biosynthesis, particularly at 50 and 25 mmol/mol oxygen.

**Materials and Methods**

**Cloning of the genes for the ethylene biosynthetic enzymes.** Homologous clones of ACC synthase and ACC oxidase from Carnation were obtained from Dr. William R. Woodson, Purdue University. ACC synthase (CARACC 3, 1.2 kb EcoRI/Hind III insert in pGEM 72f+ [Promega]) and ACC oxidase (pSR120, EcoRI 1.2 kb insert in pGEM 72f+) clones were restriction digested (ACC synthase: Asp718/PstI, 641 bp fragment and ACC oxidase: Kpnl/PstI, 597 bp fragment) to generate probes for use in a cDNA library screening. An *Arabidopsis thaliana* cDNA library (Promega Corp.) was screened with random primed probes (Ambion) and 30 positive phage plaques were chosen for further analysis. Secondary and tertiary screens indicated that only the ACC oxidase gene was present. Secondary and tertiary screens of the ACC synthase gene indicated that all plaques chosen were false positives. Positive ACC oxidase phage were subjected to PCR amplification between the SP6 and T7 promoters flanking the insert cDNA in the phage arms. The resulting PCR products were restriction digested with EcoRI/PstI and subcloned into pBluescript™ KS+.

Positive subclones were confirmed by miniprep (Maniatis *et al.*, 1982) and the
DNA was pooled for purification via polyethylene glycol precipitation. Cloned *Arabidopsis thaliana* ACC oxidase was confirmed by standard dideoxy sequencing reactions (United States Biochemicals, Sequenase Version 2.0™ sequencing kit). Alignment of the DNA sequence was accomplished utilizing the National Center for Biotechnology Information’s Basic Local Alignment Search Tool (BLAST) (Altschul *et al*., 1990) network service.

The *Arabidopsis thaliana* ACC synthase cDNA clone (Liang *et al*., 1992), ACS2, was obtained from the laboratory of Dr. Athanasios Theologis at the Plant Gene Expression Center, UC Berkeley. The ACS2 clone was restriction digested with BamHI and subcloned into the pGEM 3Z vector (Promega).

Ribonuclease protection assays of ACC synthase and ACC oxidase mRNA levels in low-oxygen grown *Arabidopsis thaliana*. Total RNA was isolated from the rosette, floral and silique tissue of *A. thaliana* grown under low-oxygen conditions (Maniatis *et al*., 1982). For ribonuclease protection assays, 10 μg total RNA was loaded into each lane to be probed on an RNA gel (Ambion). Antisense and sense RNA probes were generated using the Maxiscript™ *in vitro* transcription kit (Ambion). Briefly, 350 base pair fragments of the ACC oxidase and ACC synthase genes were cloned into the pGEM vector (Promega). Antisense transcripts were generated from the T7 end and sense transcripts from the SP6 end.

*In situ* analysis of ACC synthase and ACC oxidase in the developing siliques of *Arabidopsis thaliana*. *In situ* hybridizations were used to determine the tissue-
specific patterning of ACC synthase and ACC oxidase in Arabidopsis siliques which developed under low oxygen. ACC synthase and ACC oxidase clones from A. thaliana were subcloned as previously described into the pGEM vector (Promega) with the T7 and SP6 promoters flanking each gene. Sense and antisense in situ probes were generated using the Boehringer Mannheim Genius™ non-radioactive RNA labeling kit. Ten-micron sections of A. thaliana silique tissue were hybridized and genes were localized using the Genius™ detection kit (Boehringer Mannheim) using the protocol developed by Gary Drews (1995).

**GC analysis of O₂, CO₂, and ethylene Levels.** Gas composition (O₂ and CO₂ levels) within each Sun bag was sampled by removing 1 ml samples from each Sun bag's exhaust port. The gas samples were analyzed using an HP 5890 series gas chromatograph (GC) (Hewlett Packard co., Wilmington, DE) equipped with a 4.6 m Carboxen 1000 column (Supelco, Inc., Bellefonte, PA), a thermal conductivity detector and an HP 3396 series II Integrator (Hewlett Packard Co., Wilmington, DE).

Ethylene levels were also monitored by gas chromatography. After harvest, ten siliques were placed into a 2.3 ml gas tight vial equipped with a septum for sampling. Siliques were heated at 80°C for ten minutes to drive ethylene from the internal portion of the silique. Vials were then cooled for 3 minutes and 1 ml samples were drawn and analyzed using an HP 5890 series gas chromatograph (Hewlett Packard Co., Wilmington, DE) equipped with an
Altech 1.83 m stainless steel column packed with unibeads A 80/100 (Altech Associates, Inc., Deerfield, IL), a flame-ionization detector and an HP 3396 series II Integrator (Hewlett Packard Co., Wilmington, DE). Results analysis were compared statistically by analysis of variance (ANOVA) using Number Cruncher Statistical Software (Hintze, 1987).

Silique length analysis. Thirty siliques from each oxygen treatment were measured using a Mitutoyo dial caliper (Mitutoyo Corporation, Japan). Individual measurements were then entered into the Number Cruncher Statistical Software (Hintze, 1987) and analyzed using ANOVA.

Results

Effects of low oxygen on the expression of the ethylene biosynthetic enzymes. Ribonuclease protection assays of *Arabidopsis* leaf and floral tissue showed that the expression of ACC oxidase and ACC synthase was not changed by growth under low oxygen conditions over a series of experiments (Figure 4.1, 4.2). ACC synthase expression in silique tissue also remained unchanged regardless of oxygen concentration (Figure 4.3). However, the expression of ACC oxidase in *Arabidopsis* siliques did increase as oxygen concentration was lowered (Figure 4.1, 4.3). In the 100 mmol/mol treatment, ACC oxidase expression increased slightly over wild-type levels. Significant increases (three-fold) were seen in ACC oxidase expression in the 25 mmol/mol oxygen treatment when compared to wild-type (Figure 4.3).

Effects of low oxygen on the tissue-specific expression patterns of the ethylene biosynthetic enzymes in *Arabidopsis* siliques. Though no changes were seen
in the level of expression of ACC synthase in siliques (Figure 4.3), significant changes were seen in the pattern of expression at the tissue level. In Arabidopsis siliques grown under normal oxygen conditions, ACC synthase is expressed by the developing embryo and in the endothelial layer of the seed (Figure 4.4a). This pattern of expression is also seen in the 160 and 100 mmol/mol treatments (Figure 4.4b, c). However, upon lowering the oxygen concentration to 50 or 25 mmol/mol oxygen, changes in the expression pattern of ACC synthase are evident (Figure 4.4d,e). Instead of expression by the developing embryo, most staining is seen in the silique wall in the 50 and 25 mmol/mol treatments (Figure 4.4d,e). Expression also appears to be high in the funiculus of seeds developing at 50 mmol/mol oxygen (Figure 4.4d).

Significant changes in the expression levels of ACC oxidase were observed using ribonuclease protection assays (Figure 4.3). Changes in the pattern of ACC oxidase expression, similar to those in ACC synthase, were also seen in Arabidopsis siliques grown at low oxygen tensions (Figure 4.5). As with ACC synthase, in 210 mmol/mol O₂, the developing embryo and the endothelial layer of the seed show dark staining indicating high levels of expression (Figure 4.5a). This pattern of expression is again reflected in the other high oxygen treatments, 160 and 100 mmol/mol O₂ (Figure 4.5b,c). In the 50 mmol/mol O₂ treatment, the darkest staining is seen in the funiculus of the developing seed and in the silique wall though not to the same extent as with ACC synthase. However, at 25 mmol/mol oxygen, the expression pattern shifts to the silique wall in the same manner as in the in situ with ACC synthase.
Figure 4.1. Ribonuclease protection assay of *Arabidopsis* leaf, floral and silique tissue showing the expression of ACC oxidase and ACC synthase mRNA. Ten micrograms of total RNA from each tissue type was run in each lane. Antisense RNA probes were synthesized as described in Materials and Methods. Representative gels are shown.
Figure 4.2. Graph showing the mRNA expression of ACC oxidase and ACC synthase represented by mean pixel density (n = 3) in scans of ribonuclease protection assays of *Arabidopsis* rosette and floral tissue grown under low oxygen environments.
Figure 4.3. Graph showing mRNA expression of ACC oxidase and ACC synthase represented by mean pixel density (n = 3) in *Arabidopsis* silique tissue grown under low oxygen environments. Letters represent significant differences between treatments at P ≤ 0.5.
Figure 4.4. *In situ* hybridization of *Arabidopsis* silique tissue grown at low oxygen tensions with ACC synthase antisense probe. Hybridization with ACC synthase sense probe is shown in panel F. Panel a: 210 mmol/mol O₂; b: 160 mmol/mol O₂; c: 100 mmol/mol O₂; d: 50 mmol/mol O₂ and e: 25 mmol/mol O₂. Arrows indicate sites of reaction in sections. Magnification 33X.
Figure 4.5. *In situ* hybridization of *Arabidopsis* silique tissue grown at low oxygen tensions with ACC oxidase antisense probe. Hybridization with ACC oxidase sense probe is shown in panel F. Panel a: 210 mmol/mol O₂; b: 160 mmol/mol O₂; c: 100 mmol/mol O₂; d: 50 mmol/mol O₂ and e: 25 mmol/mol O₂. Arrows indicate sites of reaction within sections. Magnification 33X.
(Figure 4.4d,e; 4.5d,e). Taken together, these results show that when oxygen is lowered to 50 or 25 mmol/mol O₂, the change in the atmospheric composition significantly alters the pattern of gene expression.

**Effect of low oxygen on ethylene production in Arabidopsis siliques.** Siliques from each low oxygen treatment were harvested and tested for ethylene production. As oxygen concentration is lowered, the length of siliques decreases at 50 and 25 mmol/mol O₂ (Figure 4.6). A slight decrease in size is also seen at 160 mmol/mol O₂ (Figure 4.6). Gas chromatography of evolved gases from siliques showed that ethylene was produced at normal levels in the 210, 160 and 100 mmol/mol oxygen treatments (Figure 4.7). In the 50 and 25 mmol/mol oxygen treatments, ethylene was either not produced or was below the level of detection (1 ppb) (Figure 4.7). The loss of ethylene production in the lowest oxygen concentrations is consistent with the loss or premature abortion of embryos in the 50 and 25 mmol/mol oxygen treatments (Figure 4.4, 4.5, 4.7).

**Discussion**

Previous work has shown that plants grown under 50 and 25 mmol/mol O₂ are the most affected, as far as embryo development, by growth under low oxygen conditions (Kuang *et al.*, 1998). Kuang *et al.* (1998) showed that seed germination was depressed by oxygen treatments below 160 mmol/mol O₂ and that seeds from plants at 25 mmol/mol oxygen would not germinate at all. Fewer than 25% of the seeds taken from plants grown at 50 mmol/mol O₂
Figure 4.6. Graph showing mean length in millimeters (n = 30) of Arabidopsis siliques grown at low oxygen tensions. Letters indicate significant differences between treatments.
germinated and most of the seedlings were abnormal in appearance (Kuang et al., 1998). Electron microscopy of embryos at 50 and 25 mmol/mol oxygen revealed that embryos arrested at very early stages of development and spontaneously aborted (Kuang et al., 1998). Work by Quebedeaux and Hardy (1973, 1975, 1976) showed only that seed production was inhibited by 50 mmol/mol O₂ in soybean and sorghum. These experiments gave no detail on which stages of embryo development were most affected by growth in low oxygen environments. The explanation given by Quebedeaux and Hardy, that an O₂-sensitive process which controlled photosynthate partitioning between vegetative and reproductive structures was impaired in low oxygen environments, has been shown by subsequent studies to be inadequate (Gale, 1974; Thorne, 1982; Sinclair, 1987). We propose that biosynthesis of the plant hormone ethylene is repressed in low O₂ and is partially responsible for the repression of reproductive structures and seed development observed under these conditions.

Ethylene is known to produce a myriad of effects on plant growth and development most notably on the ripening of fruits (Ecker, 1995). Ethylene has been implicated in the shifting of photosynthate from vegetative to reproductive structures in the initiation of fruit ripening; thus, alteration in the translocation and accumulation of photosynthate in reproductive structures grown under low oxygen could indicate a change in the concentration or loss of ethylene in the plant. In the lowest oxygen treatments, 50 and 25 mmol/mol O₂, there was a
Figure 4.7. Graph showing production of ethylene on a per silique basis under low oxygen conditions.
loss of ethylene production in the developing siliques (Figure 4.7). However, siliques were produced at these oxygen concentrations and contained seeds, though the siliques were significantly smaller than those at higher oxygen concentrations (Figure 4.6).

Though there was no detectable ethylene production at 50 and 25 mmol/mol oxygen, RPA and *in situ* analysis clearly demonstrate that the enzymes involved in ethylene biosynthesis, ACC synthase and ACC oxidase, are being transcribed and are present in the developing silique (Figure 4.1, 4.2, 4.3, 4.4 and 4.5). The transcription of ACC synthase was not affected by growth under low oxygen conditions (Figure 4.2). ACC synthase is known to be a tightly regulated enzyme so it is not surprising that environmental stresses do not override endogenous regulatory mechanisms. However, the expression of ACC oxidase was shown to increase as oxygen concentration decreased (Figure 4.1, 4.3). ACC oxidase catalyzes the final step in the ethylene biosynthetic pathway, the conversion of ACC to ethylene, which requires molecular oxygen. The increased expression of ACC oxidase mRNA may indicate an attempt to drive the ethylene biosynthetic reaction to completion under near anoxic conditions.

In *in situ* hybridization experiments, both enzymes showed a shift in expression patterns when grown under low oxygen. At higher oxygen levels, 210, 160 and 100 mmol/mol oxygen, siliques showed expression of both ACC synthase and ACC oxidase by the developing embryo and the inner layer of the seed coat, the endothelium (Figure 4.4, 4.5). The endothelium is known to be a
layer which accumulates tannins during development (Schultz and Jensen, 1971, 1974). The dark staining observed in the \textit{in situ} hybridizations with both the ACC oxidase and ACC synthase antisense probes is most likely due to non-specific binding of the antibody to the accumulated tannins in this layer. Studies in \textit{Arabidopsis} suggest that nutrients enter the developing seed via the chalazal and micropylar pole (Scott \textit{et al.}, 1998; Mansfield and Briarty, 1990a, 1990b). These results agree with the previous findings of Schultz and Jensen (1971, 1974) on the embryogenesis of the related crucifer \textit{Capsella bursa-pastoris}. In the highest oxygen treatments (210, 160 and 100 mmol/mol O$_2$) the silique wall and replum showed only background levels of gene expression. These patterns of expression in the developing \textit{Arabidopsis} siliques agree with the findings of several groups. Johnson-Flanagan and Spencer (1994) showed in \textit{Brassica juncea} and \textit{Brassica napus} siliques that the maturing seed (including the embryo) is the structure which produces ethylene. When tested, the separated silique walls produced only negligible amounts of ethylene while the isolated mustard seed evolved high amounts of ethylene especially in the early stages of development and during the final stages of seed maturation (Johnson-Flanagan and Spencer, 1994). Meakin and Roberts (1990), again working with \textit{Brassica napus}, also determined that it was the developing seed, not the intact pod (including seed) or the pod wall alone which was responsible for the bulk of ethylene production. Beltrano \textit{et al.} (1994) showed a tight correlation between ethylene evolution and the stage of grain development in wheat heads. Ethylene production in wheat gradually increases in wheat ears.
from early milk stages through the hard dough 1 stage (Bertrano et al., 1994). Ethylene production then falls to almost undetectable levels in the late stages of wheat grain development (Bertrano et al., 1994). In sunflower seeds (Corbineau et al., 1989) and cocklebur seeds (Satoh and Esashi, 1984) increases in ethylene production during seed maturation have also been documented. Interestingly, Satoh and Esashi (1984) found that the increase in ethylene production paralleled similar increases in ACC synthase activity though no data is given documenting protein or mRNA levels.

In contrast to the expression patterns in the higher oxygen concentrations, there was a shift in expression of ACC synthase and ACC oxidase from the embryo of the developing seed, to the silique wall and funiculus in 50 mmol/mol O₂ and to the silique wall and replum in the 25 mmol/mol oxygen treatment (Figure 4.4, 4.5). In both 50 and 25 mmol/mol treatments the endothelium of the seed coat is still showing expression of the two enzymes though not at the same levels as in the higher oxygen treatments (Figure 4.4, 4.5).

A number of investigators have found ethylene concentrations around developing seeds to change during maturation. Work by Johnson-Flanagan and Spencer (1994) and by Meakin and Roberts (1990) showed that ethylene production was highest in siliques during early embryo development and at the later stages of seed maturation. Bertrano et al. (1994) observed that ethylene production gradually increased from early stages in wheat grain development through very late stages of development in the ear. Other studies in sunflower
(Corbineau et al., 1989) and in cocklebur (Satoh and Esashi, 1984) have also documented increases in ethylene production paralleling seed development and maturation. These data further suggest that ethylene production may be necessary for normal seed development. Bertrano et al. (1994) found some support for this hypothesis when they showed that upon addition of aminoethoxyvinylglycine (AVG) or silver thiosulfate (STS) to developing wheat ears there was a decrease in ethylene emission, delayed grain maturation (characterized by delayed grain dehydration and chlorophyll degradation in glumes and awns), and a prolonged period of metabolism in the florets resulting in an increase in grain dry weight. The current study is important because it uses low oxygen as a further means to test the hypothesis that ethylene is essential for seed development. If this hypothesis is correct then at low oxygen concentrations there would be a critical point below which no seeds or ethylene would be produced. As shown in Figures 4.4, 4.5 and 4.7, at oxygen concentrations below 50 mmol/mol O$_2$ no seeds are present in the developing siliques, the same O$_2$ concentration below which there is no ethylene production. These findings provide more evidence of a correlation between ethylene production and seed development in plants and demonstrate a plausible explanation for the reproductive inhibition first observed in low oxygen environments twenty-five years ago.

References Cited


Chapter 5
Conclusions and Future Directions

The pioneering work of Quebedeaux and Hardy (1973, 1975, 1976; Quebedeaux et al., 1975) provided little detail describing anatomical changes which occurred during long-term growth of plants in low oxygen. This dissertation presents the first study noting the detailed changes which occur in the leaf under low oxygen conditions. We also present the first explanations of how observed effects of low oxygen on plant reproductive and leaf development are correlated with low oxygen inhibition of the plant growth regulators brassinolide and ethylene.

As shown in Table 5.1, anatomical changes which occur in low oxygen environments may be divided into two categories: 1) changes which occur incrementally as oxygen concentration is lowered and 2) threshold effects which occur only at ultra-low oxygen concentrations. The changes seen only at ultra-low oxygen concentrations (50 and 25 mmol/mol O\textsubscript{2}) include a decrease in overall plant size (leaf length) and a decrease in silique length (Table 5.1). All of the afore-mentioned morphological changes seem to be related to low oxygen effects on the biosynthesis of the plant hormone brassinolide. In addition, increase in leaf thickness, which occurs incrementally as oxygen concentration is lowered, is also related to changes in brassinolide (Table 5.1). In the experiments discussed in chapter 3, the addition of $10^{-7}$ M brassinolide to plants grown at 25 mmol/mol O\textsubscript{2} resulted in an increase in leaf length to an
Table 5.1. Summary of anatomical changes which occur during plant growth in low oxygen conditions. Abbreviations; PS: Photosynthesis; Δ: Changes.

<table>
<thead>
<tr>
<th>Anatomical Trait</th>
<th>Incremental Change</th>
<th>Threshold Effect</th>
<th>Brassinolide Related</th>
<th>Ethylene Related</th>
<th>PS Related</th>
</tr>
</thead>
<tbody>
<tr>
<td>Major Anatomical Effects</td>
<td>↓</td>
<td>25 mmol/mol O&lt;sub&gt;2&lt;/sub&gt;</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Plant Size</td>
<td></td>
<td></td>
<td>X</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Reproductive Inhibition</td>
<td>↓</td>
<td>160 - 25 mmol/mol O&lt;sub&gt;2&lt;/sub&gt;</td>
<td></td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>Effects on Leaf Development</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stomatal Density</td>
<td>↑</td>
<td>100 - 25 mmol/mol O&lt;sub&gt;2&lt;/sub&gt;</td>
<td></td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>Stomatal Patterning</td>
<td>Δ</td>
<td>160 - 25 mmol/mol O&lt;sub&gt;2&lt;/sub&gt;</td>
<td></td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>Leaf Thickness</td>
<td>↑</td>
<td></td>
<td>X</td>
<td>X</td>
<td></td>
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<tr>
<td>Effects on Reproductive Development</td>
<td></td>
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<tr>
<td>Silique Length</td>
<td>↓</td>
<td>50 &amp; 25 mmol/mol O&lt;sub&gt;2&lt;/sub&gt;</td>
<td></td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>Shift in ACC Oxidase or ACC synthase Expression Patterns</td>
<td>Δ</td>
<td>50 &amp; 25 mmol/mol O&lt;sub&gt;2&lt;/sub&gt;</td>
<td></td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>Change in ACC Oxidase Expression Level</td>
<td>Δ</td>
<td>100 &amp; 25 mmol/mol O&lt;sub&gt;2&lt;/sub&gt;</td>
<td></td>
<td>X</td>
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intermediate level closer to normal conditions. Though long-term experiments studying the length of siliques grown under ultra-low oxygen with brassinolide were not performed, the \textit{det2} mutant, which is deficient in brassinolide biosynthesis, has siliques which are shorter in length than Columbia wild-type. Our studies show that \textit{det2} leaves are thicker than those of wild-type \textit{Arabidopsis} (Figure 3.4). Brassinolide is known to control cell elongation and expansion in developing tissues. All of these threshold changes in low oxygen environments are related to changes in the size of plant structures. Clearly, the interruption of brassinolide biosynthesis in low oxygen environments is partially responsible for these alterations in plant anatomy.

Anatomical changes also occur in an incremental manner when plant growth in decreasing oxygen environments is compared. These changes may be grouped into two classes; a) changes in stomatal density, patterning and leaf thickness and b) changes in the reproductive structures under low O$_2$. Changes in leaf thickness have been previously discussed as mediated by interruption of brassinolide biosynthesis in low oxygen environments. However, the changes observed in stomatal density and patterning across the leaf surface seem to be most closely linked to direct effects of low oxygen on photosynthesis. ANOVA results from Chapter 3 clearly showed that the increase in stomatal density was only related to changes in the oxygen concentration implying this effect was mediated through photosynthesis. Stomatal patterning also changed under low oxygen conditions yet was not compensated in plants supplemented with 10$^{-7}$ M brassinolide and plants at
intermediate oxygen levels (160 and 100 mmol/mol O₂) which were capable of normal ethylene production still exhibited aberrant stomatal patterning (Figure 4.7). Therefore these anatomical changes must also be mediated through changes in photosynthesis which occur under low oxygen conditions.

Changes in the reproductive structures of plants also occur incrementally as oxygen is lowered, and this is tightly correlated with a loss of ethylene production in the developing silique (Figure 4.7). We speculate that the shift in the expression patterns of the two ethylene biosynthetic genes, ACC synthase and ACC oxidase, from the developing embryo and endothelium of the developing seed under ambient, 160 and 100 mmol/mol O₂ to the walls of siliques which develop at 50 and 25 mmol/mol O₂ may be an effort to increase the ethylene concentration around the developing seeds promoting further silique development (Figure 4.4, 4.5). The increase in ACC oxidase expression seen as oxygen concentration is lowered from 210 mmol/mol O₂ to 100 mmol/mol and finally to 25 mmol/mol oxygen could be an attempt to drive the ethylene biosynthetic reaction to completion in these siliques. Ethylene plays a key role in embryo and fruit development and the loss of ethylene in the lowest oxygen treatments clearly correlates to the loss of reproductive ability shown in the experiments of Quebedeaux and Hardy (1976).

**Future Directions**

Currently, studies are underway to determine if brassinolide or its precursors can be detected in whole plant extracts from low oxygen tissue. This would shed light on whether or not plants which develop in low O₂ can
produce brassinolide or in its absence can produce brassinosteroid-precursors which may have some limited plant growth regulator activity. Under low oxygen conditions, the *det2* mutant did not decrease in size. This provides strong evidence that brassinolides may be blocked in low oxygen. However, plants grown in low oxygen environments and supplemented with $10^{-7}$ M brassinolide did not return to wild-type leaf lengths. In the brassinolide experiments, plants were grown in agar supplemented with $10^{-7}$ M brassinolide and transport of the steroid to the aerial portion of the plant was dependent on uptake from the media by the root. The failure of Columbia wild-type and *det2* leaves grown at 25 mmol/mol O$_2$ to return to normal leaf lengths may be due to inhibition or inefficient transport of the brassinolide by the root to the expanding leaves. The problem of uptake and transport of brassinolides by the root may be overcome by direct application of brassinolide to the leaf. Experiments comparing the root mass of the *det2* mutant to wild-type plants, documentation of changes in root anatomy upon continuous exposure to brassinolides and application of the steroid to individual leaves of both Columbia and *det2* under ambient and low oxygen conditions would provide data on differences caused by brassinolide in the root and whether direct application of the steroid to the leaves more closely mimics physiological conditions.

Other experiments may focus on the precise mechanism behind the increases in stomatal density observed under low oxygen conditions, particularly focusing on which stage of stomatal precursor is most affected by the low oxygen environment. Biochemical studies to determine the activity of
ACC oxidase and ACC synthase under sub-ambient oxygen environments will also be important to determine the exact point when ethylene production is repressed. Studies examining the turnover and accumulation of ACC in plants grown under low oxygen may also assist in ascertaining the point at which oxygen concentration limits ethylene production. Experiments examining the production of ethylene at specific times during seed development would assist in our understanding of which stages of embryogenesis are most influenced by ethylene and what concentrations of ethylene exhibit the most pronounced effects on fruit development.

**Significance**

This dissertation represents the first detailed anatomical studies of plant development over a broad range of sub-ambient oxygen concentrations. NASA is interested in designing altered atmospheres which would promote plant productivity for use in the proposed controlled ecological life support system (CELSS). CELSS will be utilized on future lunar and Mars bases. This work is beneficial in the design of CELSS since it identifies specific anatomical changes through a range of low oxygen concentrations which impact plant productivity. This work will allow CELSS to be developed at oxygen concentrations which will improve crop yield and avoid repression of essential plant growth regulators.

Using *Arabidopsis* as a model system, this work gives a basic demonstration of oxygen control of plant development. Oxygen partial pressure strongly affects basic processes on the gross anatomical, cellular and
molecular levels. This work provides a foundation for future studies of environmental influence on plant systems and demonstrates the important role of atmospheric gases on plant development.

References Cited


GC-MS spectrum of purified brassinolide obtained from Cidtech Corporation, Ontario, Canada. Major peak (△) at 24.71 minutes corresponds to brassinolide. Minor peaks (*) at 24.97 and 26.13 are isomers of brassinolide or residual contaminating compounds from the purification process.
GC-MS spectrum of purified castasterone obtained from Cidtech Corporation, Ontario, Canada. Major peak (↓) at 20.64 minutes corresponds to castasterone. Minor peaks (*) at 17.91 and 21.76 represent related compounds or residual contaminating compounds from the purification process.
Katrina Maria Ramonell attended Auburn University in Auburn, Alabama, and graduated in June, 1991, with a bachelor's degree in microbiology. She then attended Columbia University in New York, New York, and graduated in December, 1992, with a master of arts degree in microbiology. She worked in neurobiology at Cornell Medical Center in New York, New York, for several years before returning to graduate school at Louisiana State University in Baton Rouge, Louisiana, in 1994. Ms. Ramonell will graduate with the degree of Doctor of Philosophy in plant health from Louisiana State University in May, 1999.
DOCTORAL EXAMINATION AND DISSERTATION REPORT

Candidate: Katrina Maria Ramonell

Major Field: Plant Health

Title of Dissertation: Effects of Low Oxygen Atmosphere on the Growth and Development of Arabidopsis thaliana (L.) Heynh

Approved:

Major Professor and Chairman

Dean of the Graduate School

EXAMINING COMMITTEE:

[Signatures]

Date of Examination:

March 23, 1999