Search for the Crucial Extracellular Nucleotide Receptor: Kinase Receptor DORN1's Role in the eATP and eADP Stomatal Signaling Pathways in *Arabidopsis thaliana*

by

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Extracellular adenosine triphosphate (eATP) functions as a signaling molecule in plants, regulating processes such as root hair growth, wound response, and stomata behavior. Although the eATP receptors in animals have been studied extensively, the equivalent receptors in plants are just beginning to be investigated. Recently, a transmembrane receptor kinase called "Does Not Respond to Nucleotides" (DORN1) was identified as the first eATP receptor in plants. This study was aimed at testing the hypothesis that the DORN1 receptor is the receptor for the eATP signaling pathway regulating stomatal opening and closing. Previous results in *Arabidopsis thaliana* wild-type plants indicate a bi-phasic response to applied eATP and eADP, with low concentrations inducing stomatal opening and high concentrations inducing stomatal closing. The effects of applied ATP were tested on leaves of *Arabidopsis thaliana* wild type (Col-0) and the loss-of-function mutant *dorn1-3*. In addition, ATPγS, the poorly hydrolyzable version of ATP, was tested on wild type and mutant plants. If the *dorn1-3* mutant lacks the wild type's responses to applied eATP, then DORN1 is the likely receptor for the guard cells' responses to eATP. Another hypothesis tested in this study is whether extracellular adenosine diphosphate (eADP), the hydrolysis product of eATP, controls stomata behavior via DORN1. If *dorn1-3* responds like the wild type to eADP, then DORN1 may not be the receptor for the guard cells' responses to eADP. Using scanning electron microscopy, differences were documented in *dorn1-3* and wild type *Arabidopsis thaliana* leaf epidermal morphology. Altered guard cell patterning with slight clustering of guard cells was observed in the *dorn1-3* mutant leaves. Preliminary results support the hypothesis that DORN1 is part of the eATP–induced changes in stomatal aperture, suggesting that DORN1 is the receptor for the eATP signaling pathway in guard cells. In contrast, the preliminary results support the hypothesis that DORN1 is not involved in the eADP signaling pathway in guard cells.

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Introduction

1.1 The Importance of Extracellular Nucleotides in Cell Signaling

Cell signaling is a complex communication system coordinating cell activities and actions, making it vital to all organisms. In order to respond appropriately, cells must be able to perceive signals from their environment. Multicellular organisms such as animals and plants depend on networks of signal molecules transmitting chemical messages to cells. In plants, cell signaling is essential to processes including flowering, fruit ripening, germination, photosynthetic development, growth of shoots and roots, as well as responses to pathogens and herbivores (Rudd and Franklin-Tong 2001). As sessile organisms that survive by adapting to their surroundings, plants are especially reliant on physical and physiological responses to their environment through stimuli such as light and dark, temperature, mechanical damage, pest attack, and drought.

Although adenosine triphosphate (ATP) is well known as the "energy currency of the cell", this molecule has a secondary function as a signaling molecule outside the cell. The role of extracellular ATP (eATP) as a signaling molecule was first documented in animals as a neurotransmitter (Khakh and Burnstock 2009). In plants, eATP regulates a diverse range of processes including root hair growth, wound response, and stomatal behavior (Clark et al. 2010; Clark et al. 2014). Intracellular ATP moves into the extracellular matrix (ECM) due to wounding, hypotonic and hypertonic stress, pathogen attack, and mechanical stimulation of cells. The eATP can also be released into the ECM through vesicle secretion and protein transporters. Luciferase-based methods have revealed that eATP accumulates in the extracellular matrix during root hair growth as well as during stomata opening and closing, providing further evidence of eATP's presence outside plant cells (Kim et al. 2006; Clark et al. 2010). Studies on ectoapyrases have revealed that these enzymes, which break down eATP into extracellular adenosine diphosphate (eADP) and eADP to extracellular adenosine monophosphate (eAMP), are expressed in cellular regions that are actively growing (Wu et al. 2007). Since actively growing cells release ATP via vesicle transport, this action could indicate a method to control levels of the eATP signal in the ECM during growth.

Although the eATP receptors in animals have been studied extensively over the last 20 years, the equivalent receptors in plants are still being investigated. The downstream steps in the extracellular nucleotide signaling pathway, such as increases in intracellular Ca^{2+} and nitric oxide (NO), have been identified in plants. However, the initial signaling step in plant cells remained unknown. In mammals, ligand gated ion channel P2X receptors, which respond to purines, and G-protein coupled P2Y receptors, which respond to both pyrimidines and purines, are the main extracellular nucleotide receptors. For example, at least 14 different eATP receptors have been characterized in humans (Knowles 2011; Burnstock 2012). Based on knowledge of the mammalian receptors, researchers looked for equivalent purine receptors in plants. Although the eATP responses in plants can be blocked using mammalian receptor antagonists such as pyridoxalphosphate-6-azophenyl-2',4'-disulfonic acid (PPADS), surveys of plant genome sequence data yielded no similar receptors in plants.

1.2 Discovery of the Plant eATP Receptor

In 2014, a screening for eATP-insensitive mutants of *Arabidopsis thaliana* identified the first plant eATP receptor (Choi et al 2014). This mutant was found to be defective in the lectin receptor kinase-I.9 (LecRK-I.9) encoded by the gene At5g60300. Known as "Does Not Respond to Nucleotides" (DORN1), this receptor has an extracellular binding domain with a strong affinity for eATP as well as its extracellular hydrolysis product eADP. Research indicates that the binding domain is associated with the extracellular lectin domain while the intracellular kinase domain is responsible for activating the signaling pathways inside the cell. DORN1 loss-of-function mutants (*dorn1*) were found to lack the intracellular Ca^{2+} increase in response to biotic and abiotic elicitors. These mutants also showed altered gene expression associated with eATP controlled defense responses to wounding.

There are still numerous questions to be resolved regarding DORN1's role in the extracellular nucleotide signaling pathways of plants. As previous research has shown, eATP and other extracellular nucleotides such as eADP control a variety of responses in plants. Whether DORN1 is the receptor for all extracellular nucleotide controlled plant responses is currently undetermined. In addition, LecRK is a large gene family with at

least 45 members described in *Arabidopsis thaliana* (Bouwmeester et al 2014). Therefore, it remains unknown whether DORN1 is the only extracellular nucleotide receptor in plants or if DORN1 is one of multiple extracellular nucleotide receptors. Although previous studies have shown that *dorn1* does not have differential growth compared with the wild type, *dorn1* has been shown to be more susceptible to oomycete attack than the wild type (Bouwmeester et al 2014). Other studies have pointed to the importance of LeRK-I.9 in processes such as cell wall and plasma membrane adhesion (Bouwmeester et al 2011). Whether DORN1 loss-of-function results in other side effects related to cell morphology is another question worth investigating.

1.3 Research Objectives

The first research question in this study addresses whether DORN1 is involved in additional eATP controlled signaling pathways. This study specifically tests the role of DORN1 in the extracellular nucleotide controlled signaling pathway that regulates stomatal behavior in *Arabidopsis thaliana*. Wild-type plants have a biphasic response to eATP, in which high concentrations cause stomatal closing and low concentrations stimulate stomatal opening. This treatment is applied to both wild type and DORN1 loss-of-function plants to find out if the mutant has a different response than the wild type. To control for the hydrolysis of the third phosphate group, the experiment is repeated with the poorly hydrolysable analogue of ATP known as ATPγS. If the DORN1 loss-of-function plants lack the wild type responses, then DORN1 may be the receptor for eATP-induced changes in stomatal aperture. However, if the DORN1 loss-of-function mutant and the wild type plant exhibit similar responses, this could indicate that DORN1 is not the critical eATP receptor for the signaling pathway controlling stomatal opening and closing.

The second research question is whether DORN1 interacts with both eATP and eADP in order to regulate stomatal behavior. Previous studies indicate a biphasic response to eADP similar to the stomatal responses to eATP. Therefore, this extracellular nucleotide and its poorly hydrolysable analogue, ADPβS, will be tested in wild type and DORN1 loss-of-function plants. Research has indicated eADP and eATP may work through different signaling pathways, with eADP inducing a more rapid influx

of Ca^{2+} across the plasma membrane (Demidchik et al. 2009, 2011). Testing the importance of DORN1 in both eATP and eADP signaling pathways could indicate whether both pathways rely on the same receptor. If there is a difference in wild type and loss-of-function DORN1 mutant plants, this may suggest DORN1 is also an eADP receptor. If the wild type and the loss-of-function mutant have the same response to eADP, then a receptor other than DORN1 may be responsible for eADP stimulated stomatal opening and closing.

The third research question is whether the DORN1 loss-of-function mutant exhibits differences in its epidermal morphology. Observation of *Arabidopsis thaliana* leaf epidermis hints that reduction of DORN1 gene expression may produce phenotype changes in the guard cells and the surrounding epidermal cells. Although light microscopy images have given indication that morphological differences between mutant *dorn1* and wild type may be present, scanning electron microscopy is used to collect more detailed images for comparison.

The purpose of this project is to better understand the importance of DORN1 in extracellular nucleotide controlled signaling pathways in plants. Determining whether DORN1 is a key initial step in the extracellular nucleotide controlled signaling pathways regulating stomatal opening and closing would be the initial test of whether DORN1 is essential to pathways other than the defense pathway previously documented (Choi et al. 2014). If experimental evidence indicates that DORN1 is a crucial step in other plant signaling pathways controlled by extracellular nucleotides, the hypothesis that DORN1 is the primary extracellular nucleotide receptor in plants would gain further support. In a broader view, stomata play an important role in plants' gas exchange and water loss. Enhanced knowledge of the physiological and genetic factors involved in regulating stomatal responses may eventually be applied to create drought-resistance plants that conserve water by reducing stomata opening. The morphology study may also provide insight into the other roles DORN1 plays in plant cells.

Materials and Methods

2.1 Stomata Response Experiments

The *Arabidopsis thaliana* varieties used in the experiments were the Columbia wild type (Col-0) and a particular allele of the loss-of-function mutant (*dorn1-3*). The plants were grown in Growth Chamber 5 at Welch Greenhouse under 16 hour light and 8 hour dark cycle conditions at a constant temperature of 23°C. The plants were between 3 and 6 weeks old at the time of the experiments. The plants' stomata responses were typically measured between 1:00 pm and 3:00 pm.

Leaves from Col-0 and *dorn1-3* plants were placed in 3 mL Falcon dishes filled with *Arabidopsis* leaf buffer (ALB). The ALB contained 0.03275 g KCl and 0.244 g MES per 50 mL of distilled water. Control groups contained only ALB while treatment groups contained specific concentrations of ATP, ATPγS, or ADPβS diluted in ALB. Nucleotide stock concentrations of 2 mM were used for low treatment concentrations. Stock concentrations of 50 mM were used for high treatment concentrations. Positive control groups for closing experiments contained 10 μM abscisic acid (ABA) to induce a closing response. The ABA was prepared from a 10 mM stock solution in ethanol. Sigma Aldrich supplied all chemicals.

The treatment and control groups were incubated for 2 hours. Data were collected following incubation. For closing experiments, the groups were placed in bright light. For opening experiments, the groups were placed in a dark room for approximately 24 hours before the treatments were applied to ensure stomatal closure. Treatment groups were kept in the dark while the positive control was placed in the light to induce opening. Peels of leaf epidermis were taken using tweezers from the incubated leaves and placed on wet mounts for light microscopy. Images of each group were taken using a Leica DME microscope at 40X magnification. Approximately 30 images were collected from each group.

Closing or opening responses were measured via analysis of the number of open versus closed stomata and the average width and length of open stomata in each group. The number of open and the number of closed stomata were counted in each group. A selection of open stomata corresponding to a proportion of 50 was measured for width

and length. Measurements were taken using Image J software with the scale set at distance in pixels = 331, the known distance = 100, and the pixel aspect = 1.0. The width and length data were analyzed using Excel. Outliers were removed from each group using the Dixon's Q test. Width and width/length averages from each group were tested for statistical significance using a two-sided Student's T-test.

2.2 Scanning Electron Microscope Imaging

Young leaves from *Arabidopsis thaliana* Col-0 and *dorn1-3* were approximately 1 cm in length when sampled. The selected leaves were treated with a customized aldehyde fixative solution for 2 hours. The customized aldehyde fixative contained 4% glutaraldehyde, 2% paraformaldehyde, 2 mM of $CaCl_2$ and 4 mM of $MgCl_2$. The fixative was prepared using 1/10 strength ALB. The leaves were then washed with full strength ALB and dehydrated using a 50%-70%-95%-100% series of ethanol washes.

The leaf specimens were further dehydrated using the SAMDRI-790 Critical Point Dryer and coated in Pt/Pd metal using the Cressington 208HR Sputter Coater. Images of the undersides of the leaves were taken at 6 x 103 to 1.5 x 103 X magnification using the Zeiss Supra 40V Scanning Electron Microscope (SEM). All equipment and supplies were used courtesy of the Institute for Cellular and Molecular Biology Core Facility.

2.3 Analysis of Stomata Patterning

Images of wild type Col-0 and *dorn1-3* mutant *Arabidopsis thaliana* plants were collected through light microscopy using a Leica DME microscope at 4X magnification. The images were observed in order to note the general appearance of stomata distribution on the abaxial leaf epidermis. Measurements between pairs of closest neighboring stomata were taken using Image J. The stomata patterning was quantified using the "Clark and Evans nearest neighbor method" in R.

2.4 Stomatal Aperture Differences Between Wild Type and Mutant

Measurements of stomatal aperture width in μm and width/length ratio were compiled from the "control" groups of Col-0 and *dorn1-3* in 18 closing experiments. The

control groups were selected because these epidermal peels were kept in bright light and received no treatment. Therefore, the stomata in these control groups are assumed to be closest to the naturally occurring stomatal aperture size. The measurements were analyzed using R to test for statistically significant differences between the average width and average width/length values of Col-0 and *dorn1-3*. The data sets were first checked for normal distribution and equal variance before undergoing an independent two-sided two-sample T Test.

Results

3.1 Results of the Stomata Response Experiments

In order to test opening and closing responses to extracellular nucleotides in *Arabidopsis thaliana* stomata, two series of experiments were conducted using Col-0 wild type and *dorn1-3* loss of function mutants. One group of experiments tested the opening and closing responses caused by eATP and its poorly hydrolysable analogue eATPγS to control for the effects of hydrolysis. The other set of experiments tested the opening and closing responses caused by eADPβS, which is the poorly hydrolysable analogue of eADP. Table 1 summarizes the types of experiments performed, while Table 2 categorizes the overall results of these experiments.

Type	Signal Molecule Tested			Total
	ATP	ATPγS	ADPβS	
Closing	7	9	5	21
Opening	4	9	10	23
Total	11	18	15	44

Table 1. Summary of experiment types conducted over the study.

Result	ATP Closing	ATPγS Closing	ATP Opening	ATPγS Opening	ADPβS Closing	ADPβS Opening
Supported	4	5	3	5	1	5
Not Supported	0	0	0	0	4	3
Inconclusive	3	4	1	4	0	2

Table 2. Summary of results from the experiment types. The conclusion "supported" refers to the majority of experiments supporting the hypothesis that DORN1 is involved in the signaling pathway controlled by the extracellular nucleotide in question. The conclusion "not supported" refers to the majority of experiments failing to support the same hypothesis. Three or more biological repeats determined the overall conclusion in each experiment series.

3.1.1 Effects of eATP Treatment on Stomatal Closing

The closing response of *dorn1-3* loss of function mutant and Col-0 wild type to eATP was tested with the following series of experiments. Concentrations around 2 mM eATP were considered "high" concentrations that stimulate the closing aspect of the bi-phasic response in stomata. The 10 μM abscisic acid (ABA) treatment was the positive control because this plant hormone is known to cause stomatal closing.

In the majority of experiments, treatment with ABA or ATP induced stomatal closing in Col-0 wild type plants. This effect was indicated by statistically significantly smaller average stomatal widths compared to the Col-0 controls. In width, the *dorn1-3* control average stomatal aperture was statistically significantly larger compared to *dorn1-3* treated with 10 μM ABA but not to those treated with high eATP, indicating the *dorn1-3* plants lacked a closing response to high eATP treatment. In fact, the high ATP treatment occasionally appeared to induce statistically significant stomatal opening in the *dorn1-3* plants (Figure 1; Table 3). Comparing stomatal apertures using the ratios of width/length, the Col-0 control group was significantly different from the 10 μM ABA group and to the high eATP group. This indicates the Col-0 plants had the appropriate response to the positive control and also closed in response to high eATP. In width/length, the *dorn1-3* control group was significantly different from the 10 μM ABA group and the high eATP group. This indicates the *dorn1-3* plants had the appropriate response to the positive control, but the high eATP group did not provoke closing, instead inducing slight opening (Figure 2; Table 4).

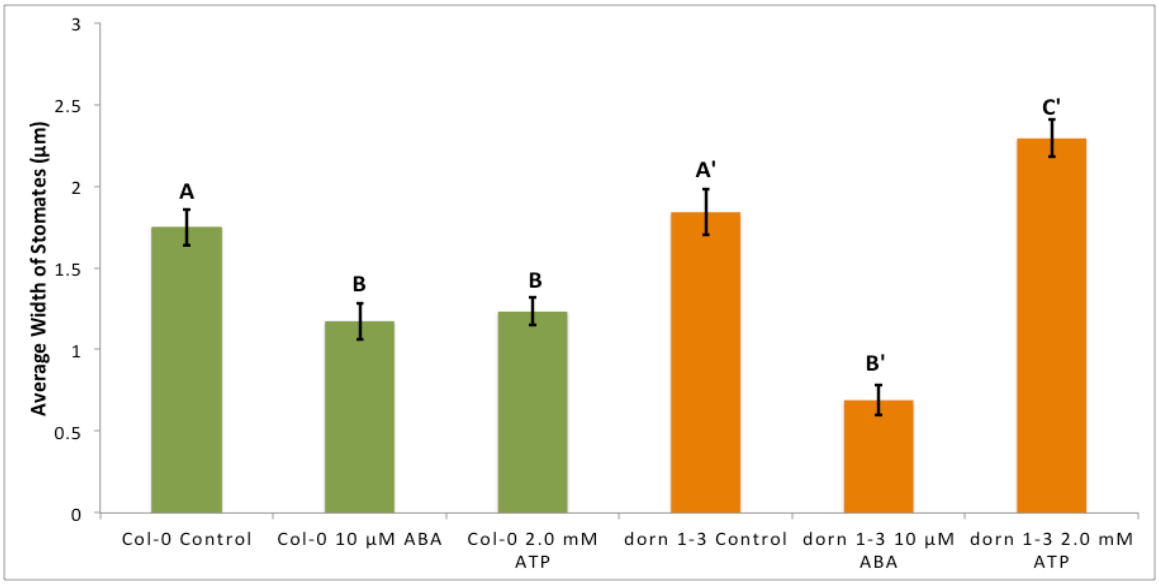

Figure 1. Representative results for average stomata width from the high concentration eATP closing experiment series. The letters above each bar indicate statistical significance.

Width	Col-0 10 µM ABA	Col-0 2.0 mM ATP	*dorn1-3* Control	*dorn1-3* 10 µM ABA	*dorn1-3* 2.0 mM ATP
Col-0 Control	0.0004058	0.000335	0.600	4.436×10^{-11}	0.000880
Col-0 10 µM ABA		0.679	0.000334	0.00115	3.902×10^{-10}
Col-0 2.0 mM ATP			0.000356	3.091×10^{-5}	6.298×10^{-11}
dorn1-3 Control				8.547×10^{-10}	0.0143
dorn1-3 10 µM ABA					2.237×10^{-18}

Table 3. Representative table of statistical significance for high eATP closing responses calculated from average width of stomata. Significance was found using the Student's two-sided T-Test.

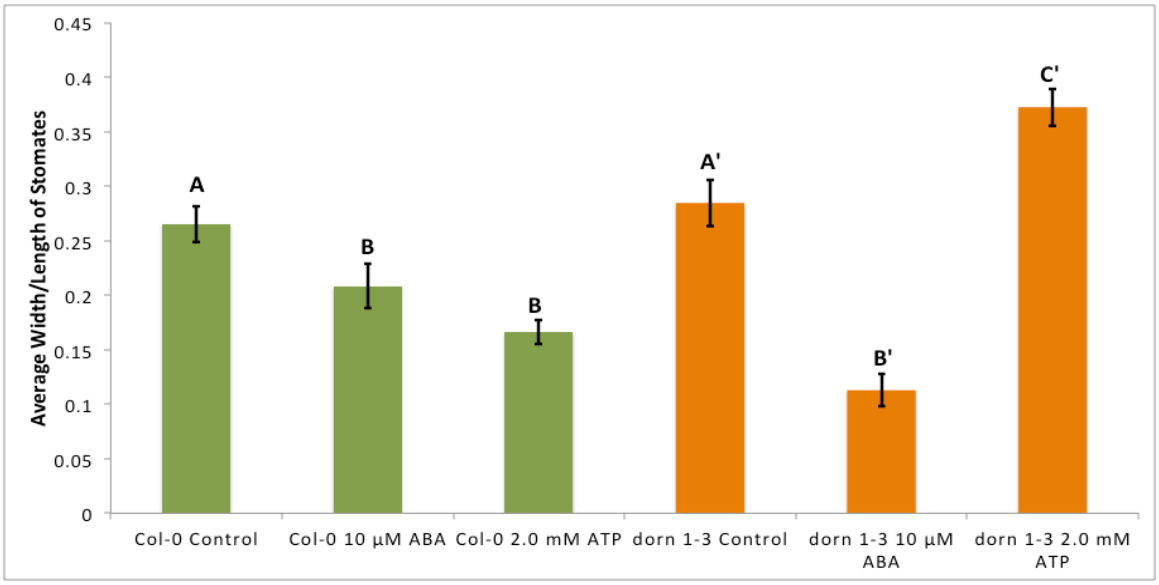

Figure 2. Representative results for average ratio of stomata width divided by stomata length from the high concentration eATP closing experiment series. The letters above each bar indicate statistical significance.

Width/ Length	Col-0 10 µM ABA	Col-0 2.0 mM ATP	*dorn1-3* Control	*dorn1-3* 10µM ABA	*dorn1-3* 2.0 mM ATP
Col-0 Control	0.0319	2.496×10^{-6}	0.472	6.896×10^{-10}	1.717×10^{-5}
Col-0 10 µM ABA		0.0704	0.0108	0.000292	1.594×10^{-8}
Col-0 2.0 mM ATP			4.0524×10^{-6}	0.00511	2.454×10^{-16}
dorn1-3 Control				2.866×10^{-9}	0.00175
dorn1-3 10 µM ABA					1.326×10^{-19}

Table 4. Representative table of statistical significance for high eATP closing responses calculated from average width/length of stomata. Significance was found using the Student's two-sided T-Test.

3.1.2 Effects of eATPγS on Stomatal Closing

The closing response of *dorn1-3* loss-of-function mutant and wild type Col-0 to eATPγS was tested in this experiment series. Concentrations around 250 μM of eATPγS were considered "high" concentrations and are known to stimulate the closing aspect of the biphasic response in stomata. Since eATPγS is relatively non-hydrolysable compared to eATP, concentrations roughly 10 times less than the eATP equivalent were used in the closing experiments. The 10 μM ABA group was the positive control because this plant hormone is known to cause stomatal closing.

In the majority of experiments, treatment with ABA or ATPγS induced stomatal closing in Col-0 as indicated by statistically significantly smaller average stomatal widths compared to Col-0 controls. In width, the *dorn1-3* control group was significantly different from the 10 μM ABA, but statistically similar to the high eATPγS group, indicating the *dorn1-3* plants lacked the closing response to the eATPγS treatment (Figure 3; Table 5). In width/length, the Col-0 control group was significantly different from the 10 μM ABA group and from the high eATPγS group. This indicates the Col-0 plants had the appropriate response to the positive control and closed in response to high eATPγS. In width/length, the *dorn1-3* control group was statistically significantly larger than the 10 μM ABA treatment (Figure 4; Table 6). The *dorn1-3* control group was statistically the same as the group treated with high eATPγS. This indicates the *dorn1-3* plants had the appropriate response to the positive control, but the high eATPγS group did not provoke closing.

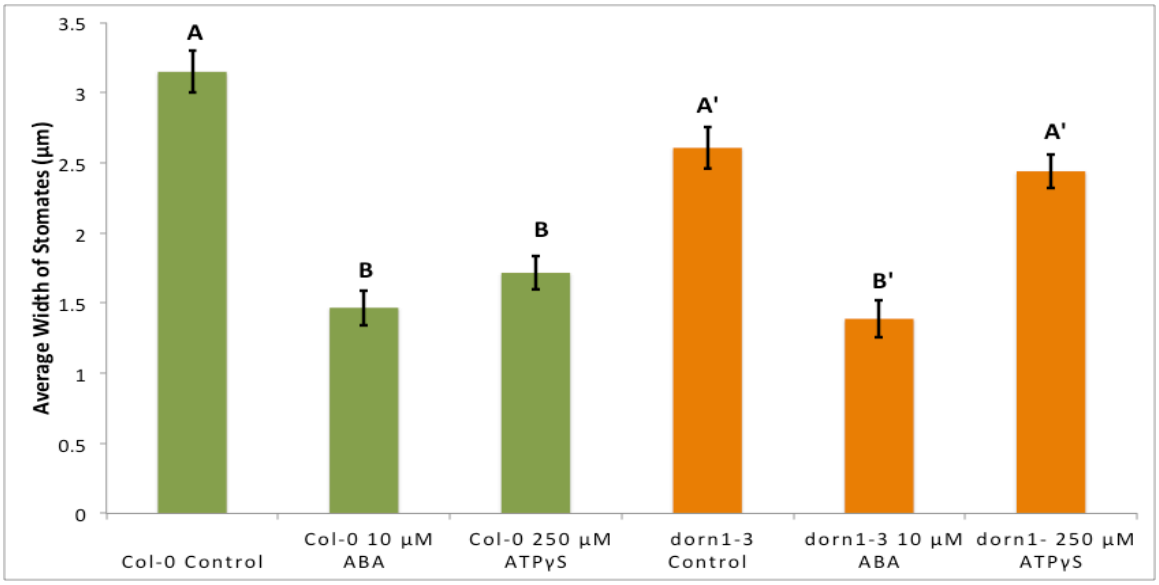

Figure 3. Representative results for average stomata width from the high eATPγS closing experiment series. The letters above each bar indicate statistical significance.

Width	Col-0 10 µM ABA	Col-0 250 µM ATPγS	*dorn1-3* Control	*dorn1-3* 10 µM ABA	*dorn1-3* 250 µM ATPγS
Col-0 Control	4.09874×10^{-12}	7.107×10^{-10}	0.0300	1.263×10^{-12}	0.00177
Col-0 10 µM ABA		0.151	5.521×10^{-8}	0.666	2.061×10^{-7}
Col-0 250 µM ATPγS			7.898×10^{-6}	0.0669	4.145×10^{-5}
dorn1-3 Control				1.608×10^{-8}	0.381
dorn1-3 10 µM ABA					5.843×10^{-8}

Table 5. Representative table of statistical significance for high ATPγS closing responses calculated from average width of stomata. Significance was found using the Student's two-sided T-Test.

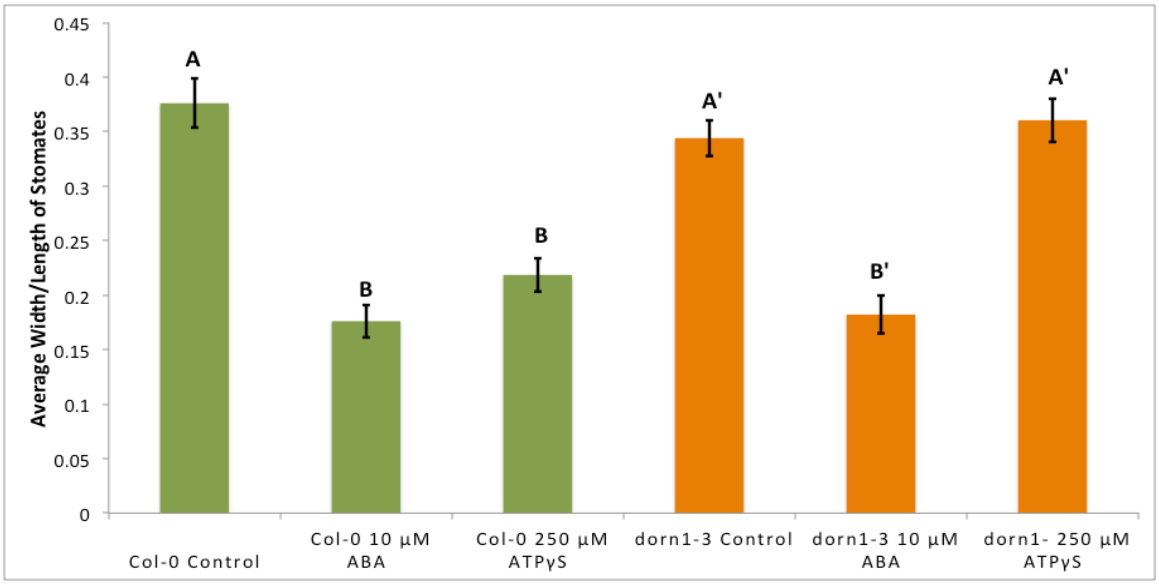

Figure 4. Representative results for average ratio of stomata width divided by stomata length from the high eATPγS closing experiment series. The letters above each bar indicate statistical significance.

Width/ Length	Col-0 10 µM ABA	Col-0 250 µM ATPγS	*dorn1-3* Control	*dorn1-3* 10 µM ABA	*dorn1-3* 250 µM ATPγS
Col-0 Control	1.00869×10^{-10}	1.245×10^{-7}	0.256	9.554×10^{-10}	0.606
Col-0 10 µM ABA		0.0490	1.937×10^{-11}	0.791	5.744×10^{-11}
Col-0 250 µM ATPγS			1.917×10^{-7}	0.116	1.666×10^{-7}
dorn1-3 Control				7.466×10^{-10}	0.526
dorn1-3 10 µM ABA					9.243×10^{-10}

Table 6. Representative table of statistical significance for high ATPγS closing responses calculated from average width/length of stomata. Significance was found using the Student's two-sided T-Test.

3.1.3 Effects of eATP on Stomatal Opening

The opening response to low eATP in the wild type Col-0 and the mutant *dorn1-3* was tested in this series of experiments. Light functioned as the positive control in this series of experiments because stomata are induced to open by exposure to bright light. A "low" concentration of applied ATP, usually 300 μM, was used to stimulate stomatal opening.

In the majority of experiments, the average width of stomatal aperture for the Col-0 dark control was significantly smaller than the Col-0 light control and those treated with low ATP. This indicates that both treatments had an opening effect in the wild type. The average stomatal width for the *dorn1-3* dark control group was statistically significantly smaller than the light control but not significantly different than the low ATP treatment, which indicates that treatment with ATP had no opening effect in the mutant (Figure 5; Table 7).

In width/length, the Col-0 group was significantly different from the light control and from the low ATP group in the majority of experiments. In width/length, the *dorn1-3* mutant control group was significantly different from the positive control but not significantly different from the low ATP group (Figure 6; Table 8). This may indicate the *dorn1-3* mutant lacks an opening response to the eATP treatment.

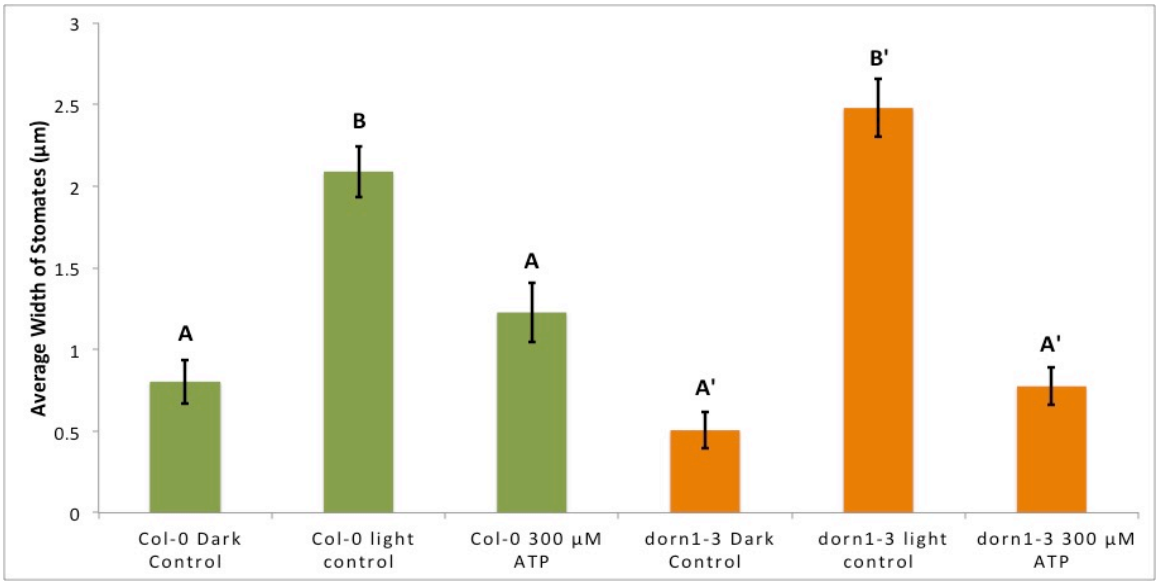

Figure 5. Representative results for average stomata width from the low eATP opening experiment series. The letters above each bar indicate statistical significance. Please note that the statistical significance of the Col-0 dark control and and Col-0 200 µM ATP is P = 0.0616, which is considered "borderline" significance when the critical value is P = 0.05.

Width	Col-0 Light Control	Col-0 300 µM ATP	*dorn1-3* Dark Control	*dorn1-3* Light Control	*dorn1-3* 300 µM ATP
Col-0 Dark Control	1.0369×10^{-8}	0.0619	0.0950	2.470×10^{-11}	0.890
Col-0 Light Control		0.000511	1.237×10^{-12}	0.101	1.285×10^{-9}
Col-0 300 µM ATP			0.00111	3.104×10^{-6}	0.0392
dorn1-3 Dark Control				6.718×10^{-15}	0.0968
dorn1-3 Light Control					3.594×10^{-12}

Table 7. Representative table of statistical significance for low eATP opening responses calculated from average width of stomata. Significance was found using the Student's two-sided T-Test.

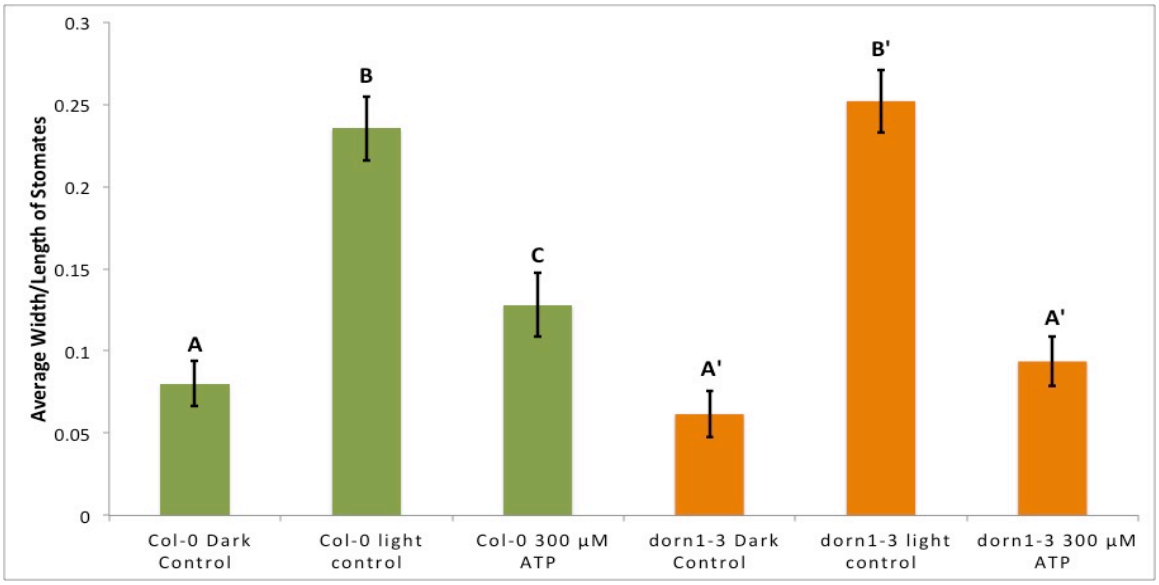

Figure 6. Representative results for average ratio of stomata width divided by stomata length from the low eATP opening experiment series. The letters above each bar indicate statistical significance.

Width/ Length	Col-0 Light Control	Col-0 300 µM ATP	*dorn1-3* Dark Control	*dorn1-3* Light Control	*dorn1-3* 300 µM ATP
Col-0 Dark Control	4.745×10^{-9}	0.0474	0.344	1.0248×10^{-10}	0.505
Col-0 Light Control		0.000189	1.726×10^{-10}	0.545	1.156×10^{-7}
Col-0 300 µM ATP			0.00688	1.590×10^{-5}	0.165
dorn1-3 Dark Control				3.245×10^{-12}	0.120
dorn1-3 Light Control					3.376×10^{-9}

Table 8. Representative table of statistical significance for low eATP opening responses calculated from average width/length of stomata. Significance was found using the Student's two-sided T-Test.

3.1.4 Effects of eATPγS on Stomatal Opening

The opening response to low eATPγS in the wild type Col-0 and the mutant *dorn1-3* was tested with a series of experiments. Light functioned as the positive control in this series of experiments. A "low" concentration of eATPγS, such as 7.5 μM, was used to stimulate stomatal opening.

In the majority of experiments, the Col-0 dark control was significantly different from the Col-0 light control and different from the low ATPγS treatment in width. This indicates the treatment had an opening effect in the wild type. In width, the *dorn1-3* control group was significantly different from the light control but not significantly different from the low ATPγS group, which indicates the treatment had no opening effect in the mutant (Figure 7; Table 9). In width/length, the Col-0 group was significantly different from the light control and from the low ATPγS group in the majority of experiments. In width/length, the *dorn1-3* mutant control group was significantly different from the positive control but not significantly different from the low ATPγS group (Figure 8; Table 10). This seems to indicate the *dorn1-3* group had no opening response to the eATPγS treatment.

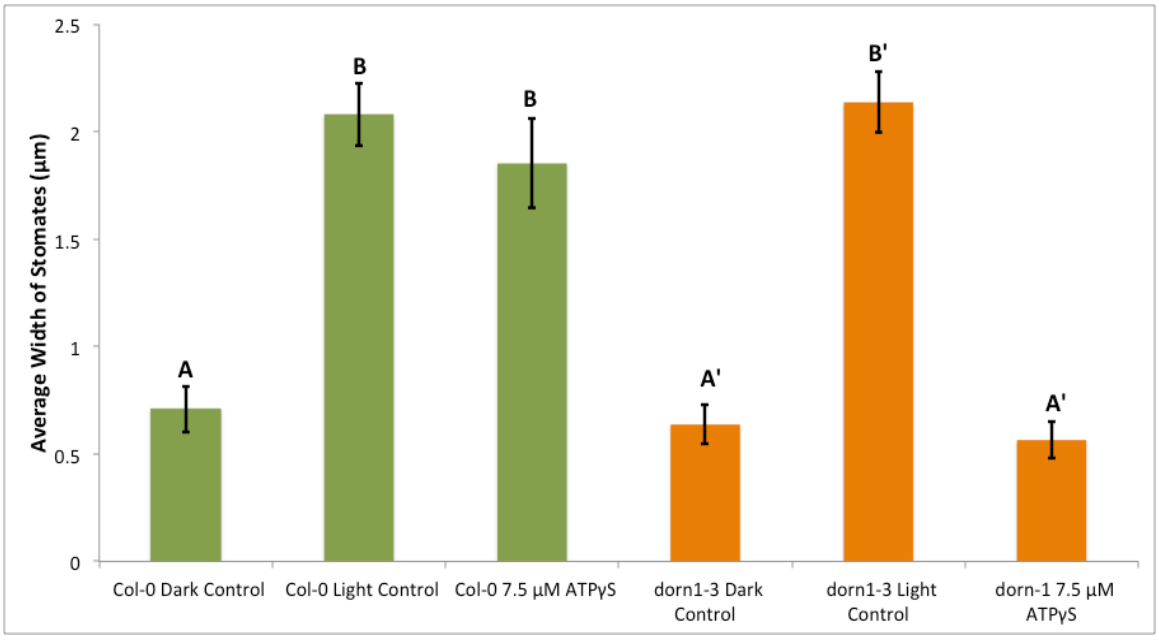

Figure 7. Representative results for average stomata width from the low eATPγS opening experiment series. The letters above each bar indicate statistical significance.

Width	Col-0 Light Control	Col-0 7.5 µM ATPγS	*dorn1-3* Dark Control	*dorn1-3* Light Control	*dorn1-3* 7.5 µM ATPγS
Col-0 Dark Control	3.218×10^{-11}	5.167×10^{-6}	0.610	2.198×10^{-12}	0.293
Col-0 Light Control		0.367	1.239×10^{-12}	0.785	1.085×10^{-13}
Col-0 7.5 µM ATPγS			9.755×10^{-7}	0.256	2.288×10^{-7}
dorn1-3 Dark Control				6.075×10^{-14}	0.562
dorn1-3 Light Control					4.489×10^{-15}

Table 9. Representative table of statistical significance for low eATPγS opening responses calculated from average width of stomata. Significance was found using the Student's two-sided T-Test.

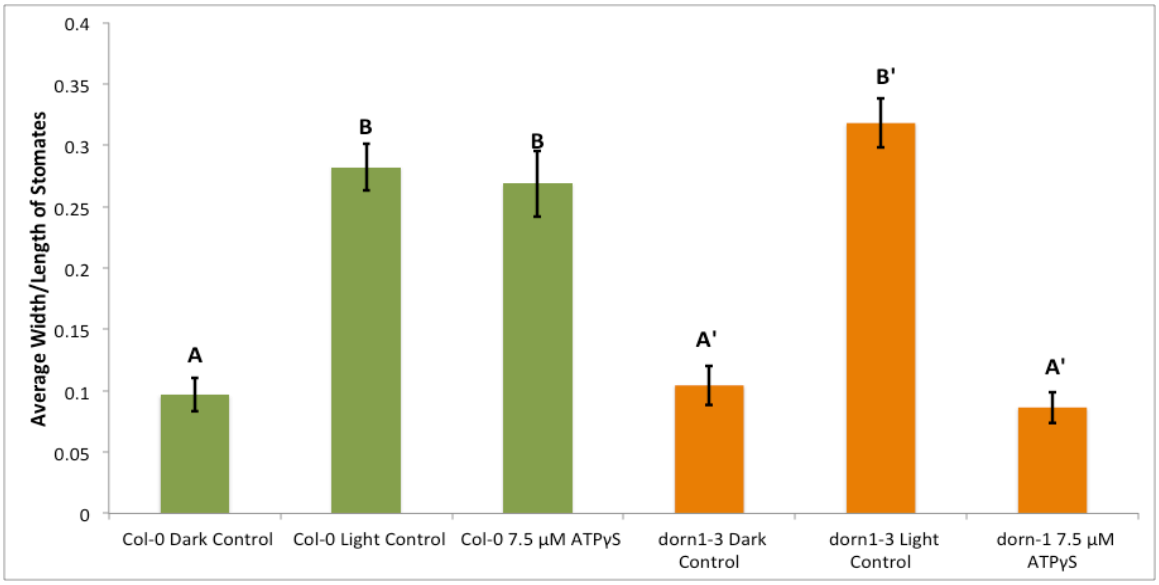

Figure 8. Representative results for average ratio of stomata width divided by stomata length from the low eATPγS opening experiment series. The letters above each bar indicate statistical significance.

Width/ Length	Col-0 light control	Col-0 7.5 µM ATPγS	*dorn1-3* Dark Control	*dorn1-3* Light Control	*dorn1-3* 7.5 µM ATPγS
Col-0 Dark Control	6.973×10^{-12}	2.132×10^{-7}	0.710	3.017×10^{-14}	0.577
Col-0 Light Control		0.683	2.151×10^{-10}	0.202	5.548×10^{-13}
Col-0 7.5 µM ATPγS			1.186×10^{-6}	0.145	4.432×10^{-8}
dorn1-3 Dark Control				8.517×10^{-13}	0.378
dorn1-3 Light Control					2.805×10^{-15}

Table 10. Representative table of statistical significance for low eATPγS opening responses calculated from average width/length of stomata. Significance was found using the Student's two-sided T-Test.

3.1.5 Effects of eADPβS on Stomatal Closing

This series of experiments tested the hypothesis that DORN1 is essential to eADP-induced stomata closing. Instead of eADP, the poorly hydrolysable analog to ADP was tested in order to control for the effects of hydrolysis. A high concentration such as 200 μM ADPβS was used to stimulate the closing bi-phasic response, which resembles the biphasic response with eATP.

In the majority of experiments, the Col-0 control was significantly different from the 10 μM ABA and the high eADPβS groups. In Col-0, the high eADPβS group had a significant closing effect comparable to the ABA group. In width, the *dorn1-3* control was significantly different from the 10 μM ABA group and from the high eADPβS group (Figure 9; Table 11). This indicates *dorn1-3* had a closing response similar to the wild type. In width/length, the Col-0 control was significantly different from the 10 μM ABA group and the high eADPβS group. Again, the high eADPβS group was much statistically closer to the positive control. In width/length, the *dorn1-3* control was significantly different from the 10 μM ABA and different from the high eADPβS group, indicating a closing effect (Figure 10; Table 12).

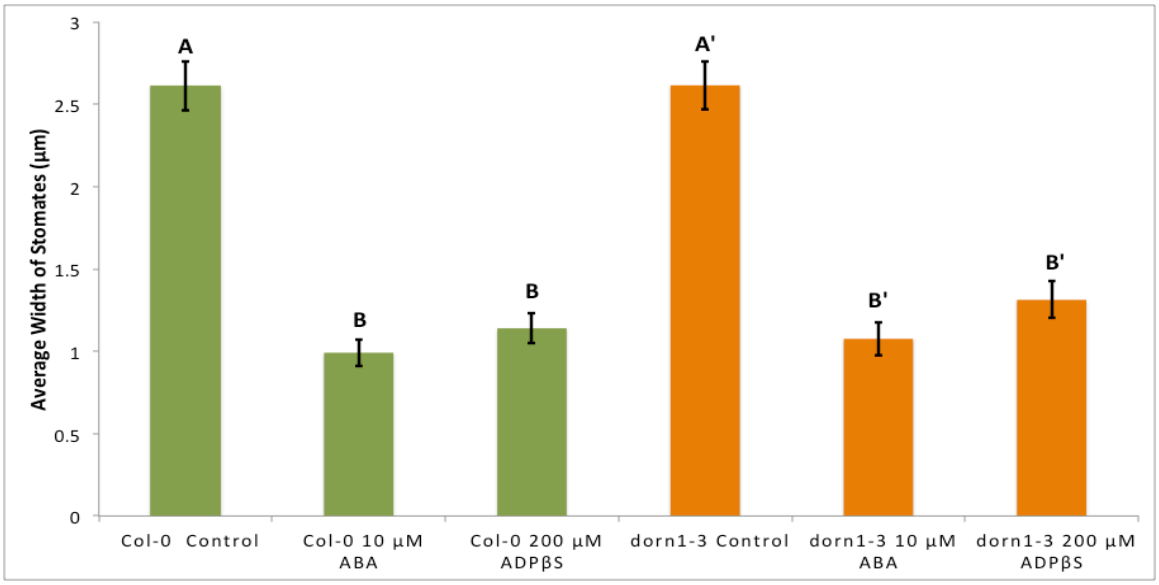

Figure 9. Representative results for average stomata width from the high eADPβS closing experiment series. The letters above each bar indicate statistical significance.

Width	Col-0 10 µM ABA	Col-0 200 µM ADPβS	*dorn1-3* Control	*dorn1-3* 10 µM ABA	*dorn1-3* 200 µM ADPβS
Col-0 Control	8.664×10^{-15}	9.125×10^{-13}	0.983	3.057×10^{-13}	4.021×10^{-10}
Col-0 10 µM ABA		0.231	4.719×10^{-15}	0.513	0.0221
Col-0 200 µM ADPβS			5.394×10^{-13}	0.641	0.237
dorn1-3 Control				1.812×10^{-13}	2.696×10^{-10}
dorn1-3 10 µM ABA					0.118

Table 11. Representative table of statistical significance for high eADPβS closing responses calculated from average width of stomata. Significance was found using the Student's two-sided T-Test.

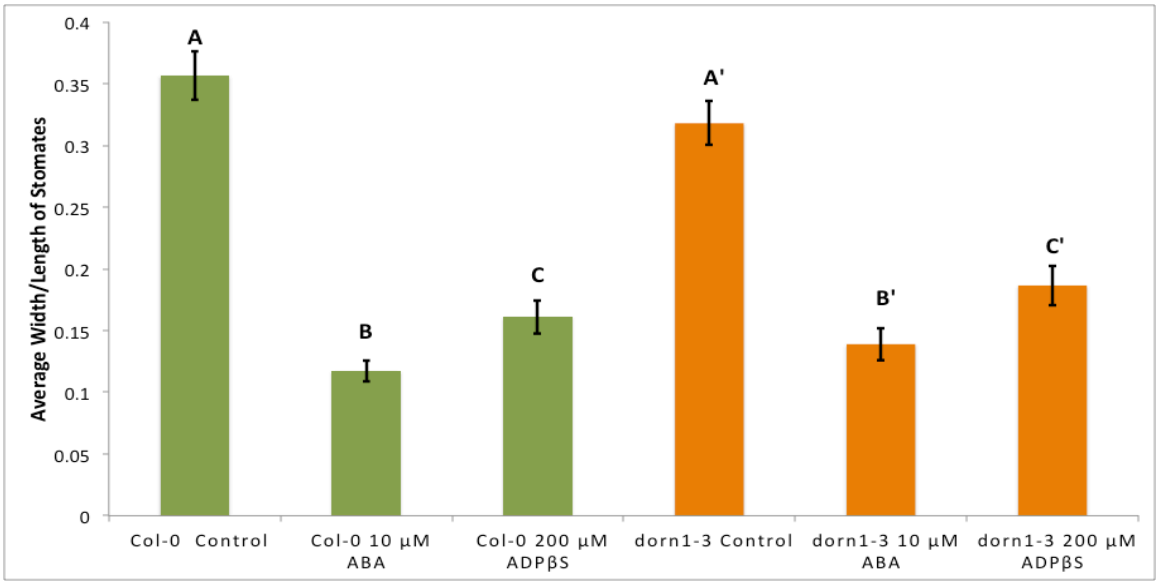

Figure 10. Representative results for average stomata width divided by stomata length from the high eADPβS closing experiment series. The letters above each bar indicate statistical significance.

Width/ Length	Col-0 10 µM ABA	Col-0 200 µM ADPβS	*dorn1-3* Control	*dorn1-3* 10 µM ABA	*dorn1-3* 200 µM ADPβS
Col-0 Control	1.072×10^{-16}	2.934×10^{-12}	0.153	2.886×10^{-14}	2.048×10^{-9}
Col-0 10 µM ABA		0.00692	2.048×10^{-15}	0.159	0.000273
Col-0 200 µM ADPβS			4.08002×10^{-10}	0.241	0.229
dorn1-3 Control				2.584×10^{-12}	3.605E-07
dorn1-3 10 µM ABA					0.0238

Table 12. Representative table of statistical significance for high eADPβS closing responses calculated from average width/length of stomata. Significance was found using the Student's two-sided T-Test.

3.1.6 Effects of eADPβS on Stomatal Opening

This series of experiments tested the hypothesis that DORN1 is essential to the eADP-regulated pathway controlling stomata opening. A low concentration, usually 7.5 μM ADPβS, was used to stimulate the opening response. Exposure to bright light, which induces stomata opening, served as the positive control.

In the majority of experiments, the Col-0 control was significantly different from the light control and the low eADPβS groups. In Col-0, the high eADPβS group had a significant opening effect comparable to the light control group. In width, the *dorn1-3* control was significantly different from the light control group but was not significantly different from the low eADPβS group (Figure 11; Table 13). This indicates *dorn1-3* lacked the opening response seen in the wild type. In width/length, the Col-0 control was significantly different from the light control and the low eADPβS groups. In width/length, the *dorn1-3* control was significantly different from the light control and different from the low eADPβS group, indicating a lack of an opening effect (Figure 12; Table 14).

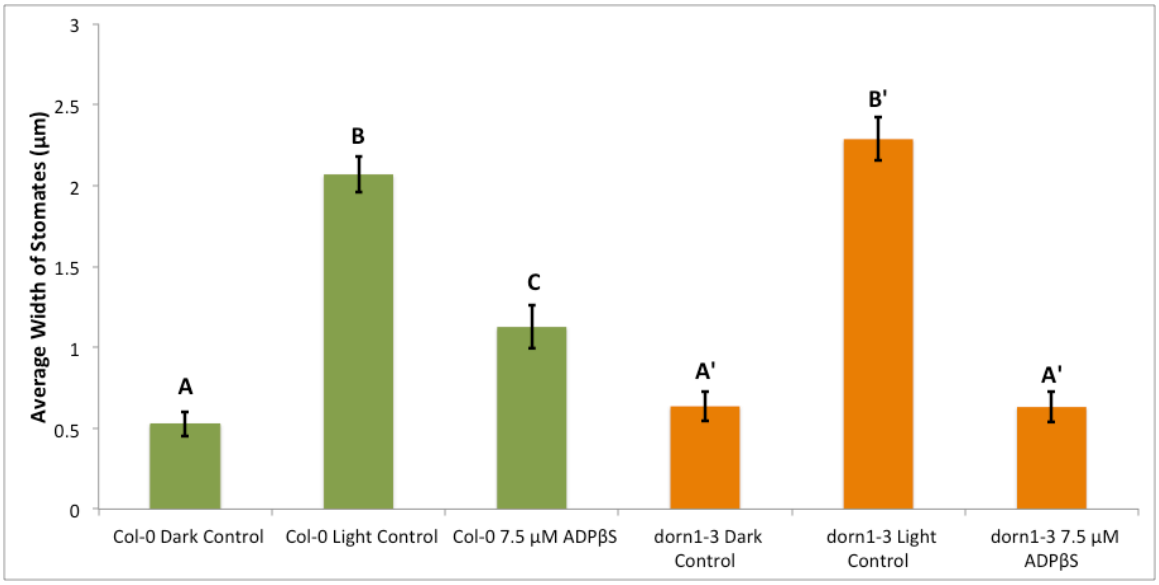

Figure 11. Representative results for average stomata width from the low eADPβS opening experiment series. The letters above each bar indicate statistical significance.

Width	Col-0 Light Control	Col-0 7.5 µM ADPβS	*dorn1-3* Dark Control	*dorn1-3* Light Control	*dorn1-3* 7.5 µM ADPβS
Col-0 Dark Control	5.347×10^{-19}	0.000156	0.355	1.434×10^{-18}	0.377
Col-0 Light Control		3.629×10^{-7}	2.687×10^{-16}	0.211	2.619×10^{-16}
Col-0 7.5 µM ADPβS			0.00291	1.189×10^{-8}	0.00274
dorn1-3 Dark Control				1.198×10^{-16}	0.973
dorn1-3 Light Control					1.136×10^{-16}

Table 13. Representative table of statistical significance for low ADPβS opening responses calculated from average width of stomata. Significance was found using the Student's two-sided T-Test.

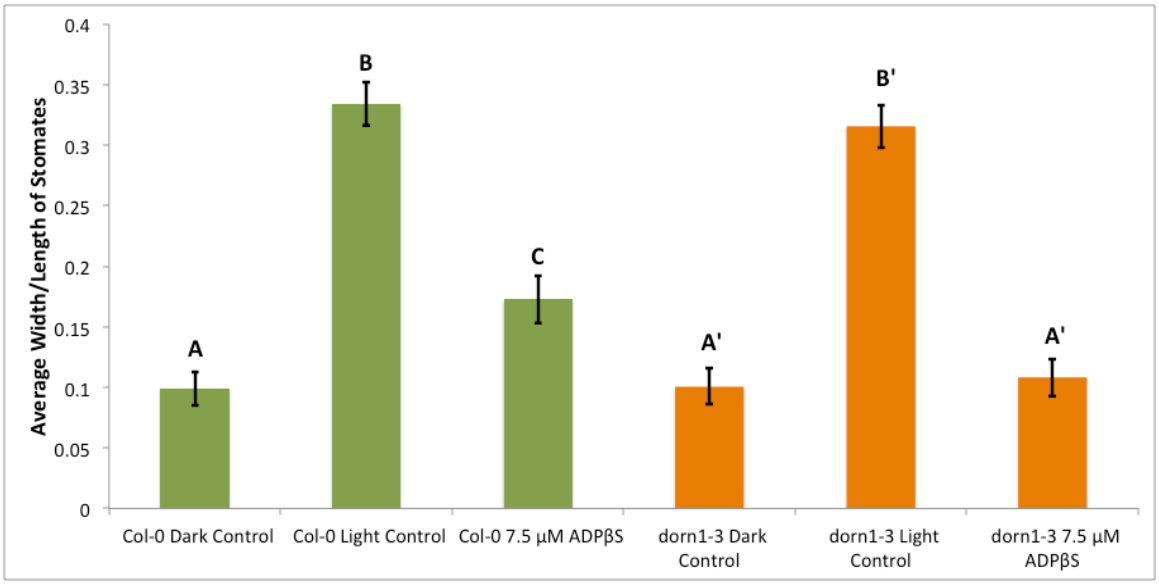

Figure 12. Representative results for average stomata width divided by stomata length from the low eADPβS opening experiment series. The letters above each bar indicate statistical significance.

Width/Length	Col-0 Light Control	Col-0 7.5 µM ADPβS	*dorn1-3* Dark Control	*dorn1-3* Light Control	*dorn1-3* 7.5 µM ADPβS
Col-0 Dark Control	2.914×10^{-17}	0.00283	0.9317	1.951×10^{-15}	0.653
Col-0 Light Control		1.735×10^{-8}	9.218×10^{-17}	0.465	9.890×10^{-16}
Col-0 7.5 µM ADPβS			0.00408	4.158×10^{-7}	0.0111
dorn1-3 Dark Control				5.837×10^{-15}	0.721
dorn1-3 Light Control					5.828×10^{-14}

Table 14. Representative table of statistical significance for low ADPβS opening responses calculated from average width of stomata. Significance was found using the Student's two-sided T-Test.

3.2 Results of the Epidermis Morphology Study

In order to study the possible morphological differences between Col-0 wild type and *dorn1-3* mutant *Arabidopsis thaliana*, a series of scanning electron microscope (SEM) images were taken of the stomata on the abaxial surfaces of young leaves. The study focused on the morphological differences in the epidermis, guard cell morphology, and stomata patterning between the wild type and mutant plants. The SEM images were collected at various magnifications in order for morphological details to be observed.

Measurements of the distances between neighboring stomata were taken and statistically analyzed utilizing epidermal peel images taken via light microscopy.

Previously collected measurements of Col-0 and *dorn1-3* stomatal apertures were compared using statistical methods to test for a significant difference in aperture size between the two groups.

3.2.1 Comparison of Wild Type and Mutant SEM Images

Direct observation of the SEM images yielded qualitative rather than quantitative data. Notable differences were seen in the appearance of the epidermal pavement cells, the size of the stomatal apertures, and the evidence of stomata development.

	Pavement Cells	Stomatal Aperture	Stomata Development
Wild Type (Col-0)	- Sharply defined with deep creases between cells - Overall homogenous appearance	- Longer aperture than in dorn1-3 - Less variation in stomata size than in dorn1-3	- Most stomata fully developed and functional - Few under-developed stomata
Mutant (*dorn1-3*)	- Cells have less distinct definition between them - Pavement cells are hetero-geneous, some large and "inflated", cells around stomata are flat	- Stomatal apertures are shorter than in Col-0 - Aperture size and shape more variable than in Col-0	- Numerous circular cell clusters, likely guard cell initials with arrested development - Underdeveloped stomata found near mature stomata

Table 15. Observed differences in epidermal and stomatal morphology in wild type and mutant *Arabidopsis thaliana*.

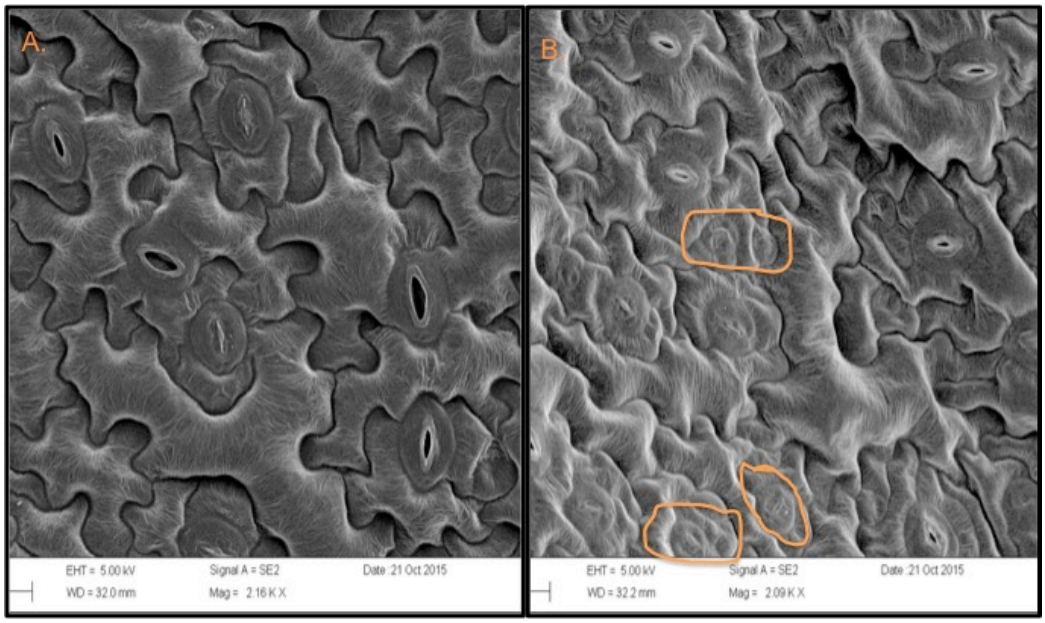

Figure 13. Comparison of Col-0 wild type (Figure 13 - A) and *dorn1-3* mutant (Figure 13 - B). The areas highlighted show the stunted guard cell initials mentioned in the table.

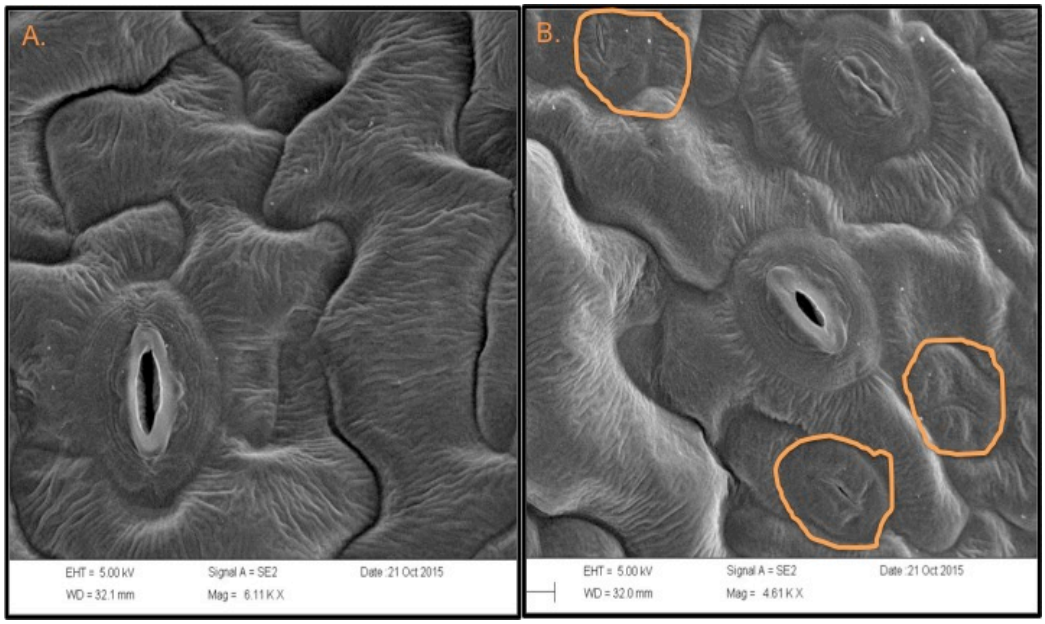

Figure 14. Comparison of Col-0 wild type (Figure 14 - A) and *dorn1-3* mutant (Figure 14 - B) at higher magnification. Highlighted areas show the stunted guard cell initials clustered around mature stomata.

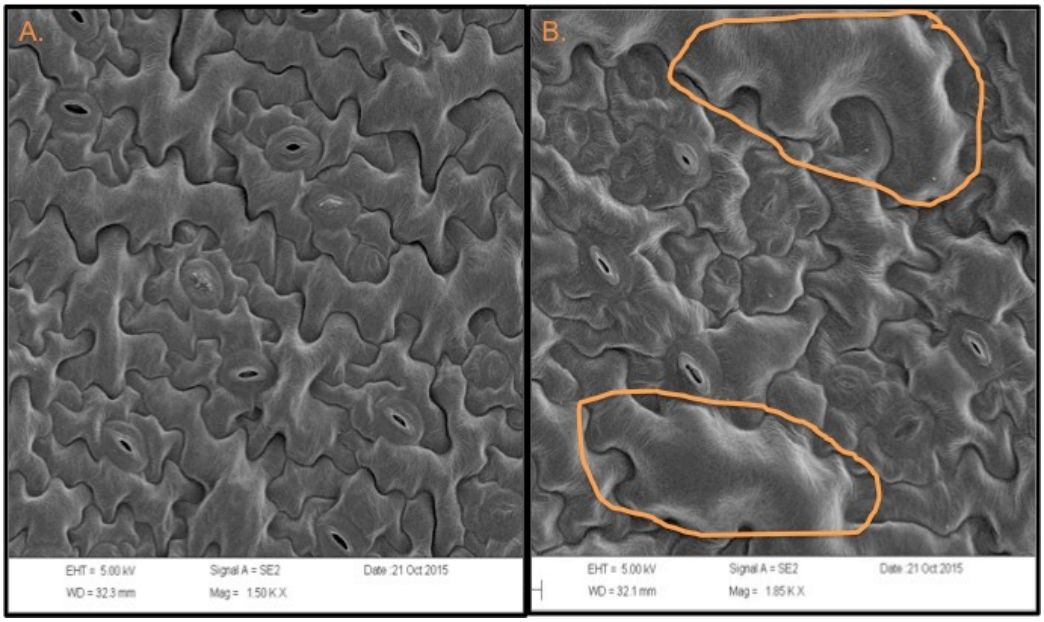

Figure 15. Comparison of Col-0 wild type (Figure 15 - A) and *dorn1-3* mutant (Figure 15 - B) pavement cells. Highlighted areas indicate the heterogeneous pavement cells observed in *dorn1-3*.

3.2.2 Comparison of Wild Type and Mutant Stomata Patterning

Observation of the distribution of stomata indicated that the Col-0 ecotype may have stomata scattered relatively evenly over the abaxial surface while *dorn1-3* loss of function mutants typically have stomata occurring in a more clustered distribution. This difference is not obvious and required a statistical test to try the hypothesis that the mutant has a more clustered stomata pattern than the wild type. The Clark-Evans nearest neighbor test was selected as the appropriate statistical test. The results of the Clark-Evans nearest neighbor method indicated that both the mutant and the wild type have R values less than 1, which indicates both phenotypes show a "clustered" stomata pattern. However, the R value was lower in the mutant, signifying that the stomata were relatively more clustered than in the wild type.

Both wild type and mutant differed significantly from an entirely random stomata distribution pattern, as shown by the Z value obtained in the second part of the Clark-Evans test. The Z value also indicated that the mutant group departs more from a random distribution than the wild type because the Z value of *dorn1-3* group was greater than the Col-0 group.

Finally, an ANOVA test was performed to check if the difference between nearest neighbor distances was greater within the two groups or between the two groups. The results of the ANOVA indicated that the variation between the two groups was greater than the variation inside the two groups because the P value calculated from the F test statistic is less than the significance value of 0.05.

In conclusion, the statistical analysis indicates that the nearest neighbor distance is significantly different in the mutant and wild type. The tests also suggest the mutant's stomata pattern may be more clustered than the wild type.

	R value	Z value	F test statistic	P value
Wild Type (Col-0)	0.668	6.593	11.68	0.00834
Mutant (*dorn1-3*)	0.533	9.538		

Table 16. Summary of the R values and Z values related to the Clark-Evans test. The results of the ANOVA test are represented by the F test statistic and the P value.

3.2.3 Comparison of Average Stomatal Aperture Widths and Width/Length Ratios in the Wild Type and the Mutant

This study tested the hypothesis that there is a consistent difference in average stomatal aperture size through a statistical comparison of Col-0 wild type and *dorn1-3* mutant stomatal aperture measurements. Based on observations of the SEM and light microscopy images, wild type plants appeared to have typically larger stomatal apertures than the mutant plants. Data from the control groups of stomata response experiments were used to calculate the average width and width/length values for the wild type and the mutant. A two-sided independent two sample T-Test was selected as the appropriate statistical test and the analysis performed in R.

The results indicated that wild type plants have significantly wider stomatal apertures than mutant plants. However, there was no significant difference between the stomatal aperture width/length ratios of the wild type and the mutant. This may indicate that the mutant's stomatal apertures are proportionally smaller than the wild type's stomatal apertures.

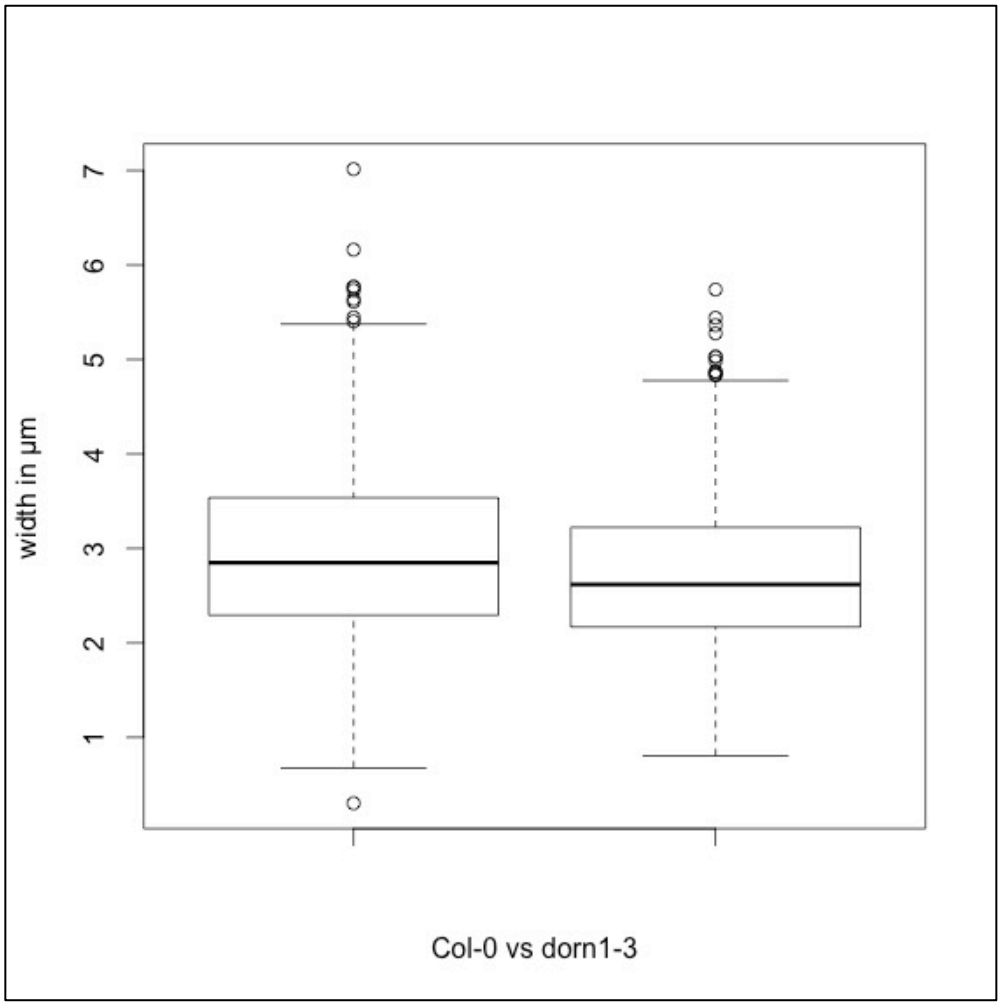

Figure 16. Boxplot presenting the distribution of wild type and mutant stomatal aperture width measurements. Of the wild type (left) and mutant (right) groups, the wild type has a visibly greater mean value and a greater spread of values than the mutant.

	Mean Width (µm)	N Value
Wild Type (Col-0)	2.948	788
Mutant (*dorn1-3*)	2.699	777
Welch Two – Sample T-Test	P-value = 2.621×10^{-8}	

Table 17. Results of Welch's T Test on average stomatal aperture width. The P-value was less than the critical value of P = 0.05, which indicates there is a statistically significant difference between the two groups.

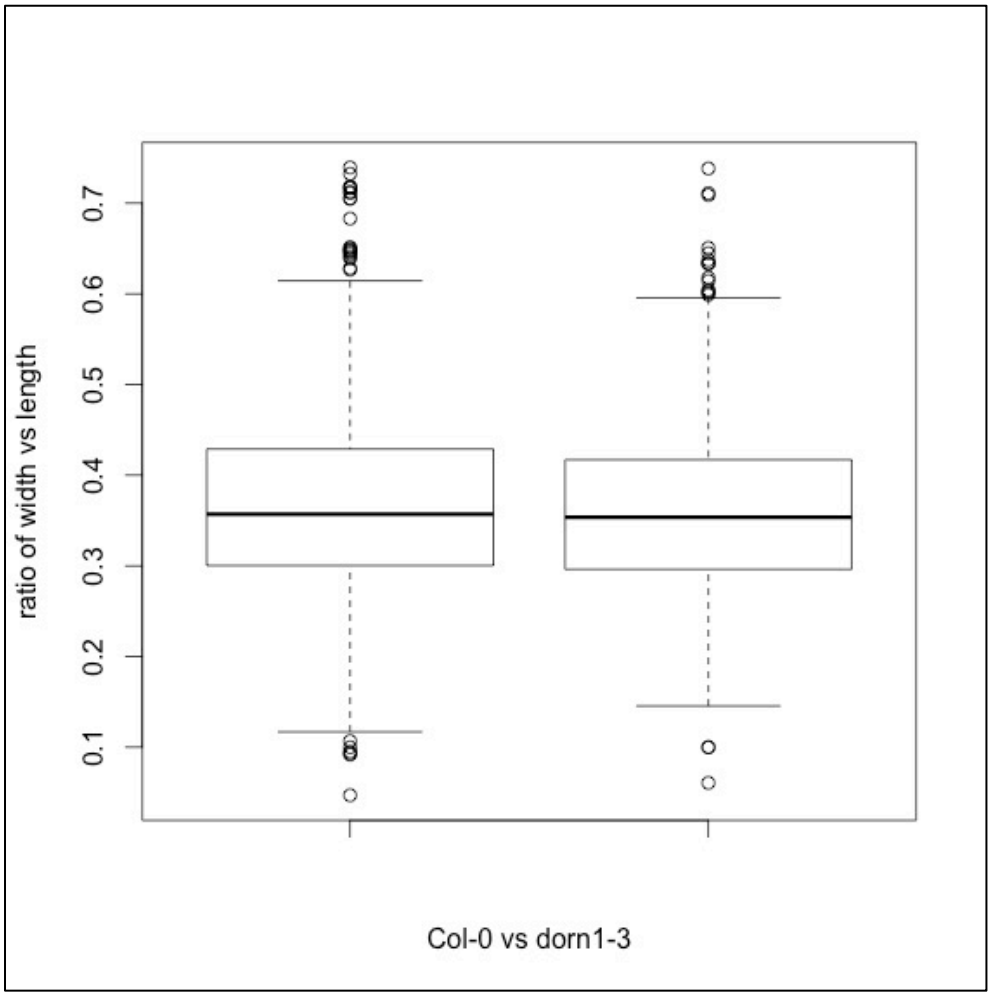

Figure 17. Boxplot presenting the distribution of wild type and mutant stomatal aperture width/length ratios. Of the wild type (left) and mutant (right) groups, the wild type has a visibly greater spread of values than the mutant although their means appear to be close.

	Mean Width (μm)	N value
Wild Type (Col-0)	0.3705913	788
Mutant (*dorn1-3*)	0.3603883	777
Welch Two-Sample t-test	P-value = 0.05197	

Table 18. Results of Welch's T Test on average stomatal aperture width/length ratio. The P-value was more than the critical value of P = 0.05, which indicates there is no statistically significant difference between the two groups.

Discussion

4.1 Discussion of the Stomata Response Experiments

In this research study, the hypothesis of whether DORN1 is the only extracellular nucleotide receptor in *Arabidopsis thaliana* was investigated. The first research question was whether DORN1 is the main receptor for eATP signaling pathways regulating stomatal closing and opening. The second research question focused on whether hydrolysis of eATP is involved in the eATP signaling pathway by testing equivalent concentrations of ATPγS, the poorly hydrolysable version of ATP. The third research question asked whether DORN1 additionally functions as the main eADP receptor for stomatal closing and opening. Based on the overall results of the study, DORN1 appears to be the main receptor in both eATP signaling pathways regulating closing and opening of stomata. Comparable results were found with ATPγS, indicating that hydrolysis is not involved in the interaction between ATP and the receptor. While the experimental results indicate DORN1 is a key part of the eADP stomatal opening pathway, the eADP stomatal closing pathway does not seem to depend on the presence of DORN1.

4.1.1 Discussion of Stomata Responses to eATP and eATPγS

Considered holistically, the results of the experiments testing closing effects of high eATP concentrations on wild type and *dorn1-3* loss-of-function mutant plants of *Arabidopsis thaliana* suggest that DORN1 is crucial for stomatal closing in response to this signaling molecule. In the majority of the experiments, the wild type exhibited a closing response to high concentrations of eATP while the mutant did not show a closing response to the same stimulus. This indicates the response is present only when DORN1 is expressed in the plant. Likewise, the closing response is absent when DORN1 is not expressed.

In addition to the high concentration eATP experiments, the poorly hydrolyzable analog eATPγS was tested. Equivalent concentrations were used to induce stomatal closing in wild type and mutant plants to ascertain whether hydrolysis is involved in the eATP response. As in the eATP closing experiments, the wild type plants showed the expected closing response to high concentrations of eATPγS while the *dorn1-3* mutants

did not response with stomatal closing. As well as providing further evidence that DORN1 is crucial to the eATP stomata closing response, the results of this experiment series indicated that hydrolysis of the third phosphate group in eATP is not involved in the signaling response.

To test if eATP acts via the same receptor in both stomatal closing and opening responses, the opening response to low concentrations of eATP was tested on wild type and *dorn1-3* plants. As in the experiments testing the closing response, the wild type plants expressing DORN1 exhibited the typical stomatal opening response to low concentrations of eATP. In contrast, the mutant plants with reduced DORN1 expression lacked the closing responses to the same stimulus. This indicates DORN1 is the required receptor for the signaling pathway regulating eATP stomatal opening as well as eATP induced stomatal closing. The two pathways may share a common receptor and initial step since DORN1 is apparently necessary for both opening and closing responses.

Along with the low concentration eATP experiments, the poorly hydrolysable analogue eATPγS was tested in equivalent concentrations to induce stomatal opening in order to find out whether hydrolysis is involved in the eATP response. As in the eATP opening experiments, the wild type plants showed the expected opening response to high concentrations of eATPγS while the *dorn1-3* mutants did not respond with stomatal opening. These results lend additional support to the hypothesis that DORN1 is involved in the signaling pathways controlled by eATP in stomata by indicating that DORN1 is a main receptor in the stomatal opening response. In addition, results of this series of experiments imply that hydrolysis of the third phosphate group in eATP is not part of the signaling response leading to stomatal opening. This is another similarity shared with the eATP closing pathway.

4.1.2 Discussion of Stomata Responses to eADPβS

Based on the experimental results, DORN1 is the receptor for eADP in the eADP signaling pathway regulating stomatal opening. However, the results imply that DORN1 may not be involved in the eADP signaling pathway leading to stomatal closing. To investigate whether DORN1 is the main receptor for eADP induced stomatal closing responses, high concentrations of eADPβS were tested on both wild type and *dorn1-3*

mutant plants. As in the experiments using high concentrations of eATP, the wild type showed the appropriate closing response. In contrast to the results of the eATP closing experiments, the closing response to high eADPβS was also observed in the *dorn1-3* mutant plants. This unexpected result was repeated in the majority of the experiments, indicating the receptor DORN1 is not required in the stomatal closing signaling pathway induced by eADP. This finding does not support the hypothesis that DORN1 is the main receptor for both eATP and eADP signaling pathways controlling stomatal closing. Instead, the finding suggests that a different receptor is involved in this particular pathway.

The results of the experiments testing low concentrations of eADPβS support the hypothesis that DORN1 is the main receptor in the eADP stomatal opening pathway. As in the eATP opening response experiments, low concentrations of eADPβS caused stomatal opening in the wild type but did not induce opening in the mutant. Since the opening response was present when DORN1 was expressed, the results of this experiment series indicate that DORN1 is the receptor for the signaling pathway controlling stomatal opening via eADPβS. When considered alongside the results of the eADPβS closing experiments, the results suggest eADP may work through different receptors for stomatal opening and closing. This is an interesting difference from the findings on eATP, which appear to indicate that DORN1 is the primary receptor for both stomatal opening and closing.

4.1.3 Overall Conclusions of Stomata Response Experiments

The cumulative findings uphold the hypothesis that DORN1 is one of the important extracellular nucleotide receptors in plants. The outcomes of the stomata response experiments denote that DORN1 is the extracellular nucleotide receptor for some, but not all, signaling pathways controlling stomata closing and opening in *Arabidopsis thaliana*. The results indicate that DORN1 is the extracellular nucleotide receptor in both closing and opening signaling pathways controlled by eATP and in the opening signaling pathway controlled by eADP. However, the findings suggest that DORN1 is not involved in the pathway controlling the stomatal opening response to eADP. This supports the alternative hypothesis that DORN1 is one of a number of

extracellular nucleotide receptors, since this pathway does not depend upon the receptor. In addition, the results indicating that eATP and eATPγS produce similar responses suggest that hydrolysis of the third phosphate group in ATP is not involved in the initiation of the signal. This finding provides more information on the interaction between the extracellular lectin receptor domain and the signaling molecule.

4.2 Discussion of the Epidermis Morphology Study

Although the data collected from the SEM images was qualitative rather than quantitative, differences were noted between epidermis morphology in the wild type and in the mutant. The wild type displayed well-defined homogeneous pavement cells and mature stomata with elongated apertures. In contrast, heterogeneous pavement cells along with numerous underdeveloped stomatal complexes were observed in the mutant. In dorn1-3, the many immature stomatal complexes apparently in a state of arrested development may be a significant difference. This observation could indicate that reducing DORN1 expression interferes with the cell signaling pathways regulating stomatal complex formation and maturation. The inflated or flattened pavement cells may also suggest DORN1 is involved in wall expansion or another developmental pathway related to pavement cell growth.

4.2.1 Discussion of Stomatal Patterning Analysis

The results of the stomata patterning study indicate that both wild type and mutant have a clustered distribution of stomata on the abaxial leaf surfaces. However, the mutant has a more clustered distribution of stomata than the wild type. This finding is based on the distances between nearest neighbor guard cells of stomatal complexes in the epidermis of wild type and mutant plants. Since the SEM image study hinted that the reduction of DORN1 expression interferes with normal stomata development, this result lends support to the hypothesis that DORN1 impacts stomata development and maturation in *Arabidopsis thaliana*. It is possible that decreasing DORN1 expression leads to stomata developing in a more clustered pattern compared to the relatively less clustered stomata development when DORN1 is present.

4.2.2 Discussion of Stomatal Aperture Width and Width/Length Comparison

The study investigating possible differences between wild type and *dorn1-3* plants yielded results suggesting aperture size may depend on the presence of DORN1. A T-Test analysis of the wild type and mutant's stomatal aperture width measurement revealed a statistically significant difference between the two groups. This finding supports the hypothesis that Col-0 and *dorn1-3* have intrinsic morphological differences because the wild type has significantly wider stomata than the mutant under control conditions. According to the Student's t-test results, the width/length ratio of the wild type and the mutant were not significantly different, implying the stomata apertures are proportionally smaller in *dorn1-3* and not simply narrower than in Col-0. This difference in stomatal aperture size is possibly related to the stunted stomata guard cell complexes documented in the SEM images. Based on this study, reducing DORN1 expression may contribute to the formation of guard cells with overall smaller apertures than those found when DORN1 is present. When the results of the epidermis morphology investigation are considered together, normal stomata development could be impacted by the absence of DORN1.

4.3 Summary of Findings

The outcomes of this study should still be considered as preliminary data. Further repeats of the experiments performed in this study will help confirm the responses recorded are repeatable. The eADP signaling pathways have only been tested using the poorly hydrolysable version of eADPβS. Therefore, the next series of experiments should focus on opening and closing responses to eADP in wild type and *dorn1-3* mutant plants to test whether nucleotide hydrolysis is involved in the response. Determining whether *dorn1-3* has a closing response to high concentrations of eADP would clarify whether DORN1 is involved in this signaling pathway. Research has indicated a time difference in the intracellular calcium spikes triggered by eATP and eADP stimuli, implying that eATP works via ion channels while eADP utilizes a hetero- trimeric G protein system (Sun et al. 2012; Clark et al. 2013). The differences in responses to eADP may indicate that the signaling pathways for eATP and eADP diverge more than previously known.

The epidermis morphology studies yielded intriguing preliminary results hinting at DORN1's role in stomata patterning and development. Future directions for this topic could include more SEM image collection to observe the amount of underdeveloped stomata in more mature leaves to find out whether these unusual cell clusters are present only in young leaves. In addition, the stomata clustering study could be enhanced with other, more rigorous, statistical tests along with a larger sample size.

4.4 Future Directions

Further studies could focus on techniques verifying DORN1 expression in the guard cells regulating the stomatal aperture. Proving that DORN1 is localized in the cells controlling the stomata responses would add support to the hypothesis that this receptor is acting on the guard cells. Tagging DORN1 with GFP and observing the protein expression via fluorescence microscopy is a potential strategy. The next field of inquiry could focus on one of the other diverse plant signaling pathways controlled by extracellular nucleotides. A series of experiments testing the differences in root hair growth responses to applied eATP and eADP could demonstrate whether this extracellular nucleotide signaling pathway relies on DORN1 as its main receptor.

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