Isolating Drought Tolerant Wheat Using Germination Screens for Increased ABA Hormone Sensitivity

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Précis

Drought can decrease the production of any crop. Drought means low output for farmers and low input for hungry mouths. The solution is to develop drought tolerant cultivars of the major cereal crops like wheat. Transgenic methods can accomplish this but are not publicly accepted. Mutation breeding may be a solution. Plants isolated by mutation breeding are not genetically engineered and can be marketed immediately.

The purpose of this study was to isolate drought tolerant mutants in two varieties of wheat. Drought tolerant mutants were obtained by screening mutagenized grain for increased sensitivity to the plant hormone abscisic acid (ABA) during germination. In addition to inhibiting seed germination, ABA causes drought tolerance by inducing stomatal (pore) closure to retain water, and by inducing protective genes. A plant that is hypersensitive to ABA should induce these responses more readily. I hypothesize that if hypersensitivity to ABA correlates with drought tolerance, then a plant isolated for ABA hypersensitivity during seed germination should show a drought tolerant phenotype.

After screening 30,000 mutagenized Chinese spring wheat seeds and 15,000 cultivar Zak seeds, we recovered 25 and 4 mutants respectively that failed to germinate in the presence of 5μM ABA, a concentration that is normally too low to inhibit germination. Four tests were used to determine if mutants had improved drought tolerance, including observations of plants under drought stress, measurement of "transpiration rate" or the speed at which a plant looses water, measurement of leaf surface temperatures, and scanning electron microscope (SEM) observations of stomatal/pore opening size.

The behavior of greenhouse-grown wheat plants over the 21 days following the cessation of watering was observed. It was found that all 25 of mutants in the Chinese spring variety showed greener color and less wilting than normal Chinese spring. Also, it was noted that many of the ABA hypersensitive mutants began to flower earlier than normal Chinese spring both when drought stressed, and when well watered.

It was expected that ABA hypersensitive mutant should loose water more slowly than normal plants. Plants wick water out of the ground via a process called transpiration. Pores or stomata in the leaves open allowing water to evaporate. As water evaporates out of the leaves, more water is sucked up from the roots by capillary action. To prevent water loss during drought roots produce ABA, and ABA triggers stomatal closure in the leaves. Closing the stomates allows the plant to retain its water and survive drought. A side effect of this is that the leaves should also become a bit warmer. Transpiration, like perspiration, causes evaporative cooling. Thus, a side effect of ABA hypersensitivity and closed stomates may be higher surface temperatures. Indeed, when surface temperatures of ABA hypersensitive mutants were measured with an infrared thermometer, three very ABA hypersensitive mutants were 0.5 to 1 C warmer than normal Chinese spring. An attempt was also made to measure the transpiration rate. However, we failed to detect a statistically significant difference between normal and mutant Chinese spring because an insufficient number of plants were examined. Stomatal observations using SEM indicated that ABA hypersensitive mutants had a larger fraction of closed stomates than normal. Overall, the data support the notion that ABA hypersensitive plants identified using a germination screen, have increased drought tolerance. Future work will examine the behavior of the mutants in the field.

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Introduction

The world is running out of food. It is estimated that the global population will expand from just over 6.4 billion to 8 billion people by the year 2025 (Conway and Toenniessen, 1999). In order to keep up with this increase in demand for food, grain production of staple crops — rice, wheat, and maize — must increase by 50% (Khush, 2003). Any increase in crop yields for the coming years means a decrease in the number of mouths that go unfed. It is clear that efforts must be made by the scientific community to procure such an increase. The benefits of scientific endeavors to increase crop production have been demonstrated in the past by the, aptly named, "green revolution". Beginning in the 1960s higher yielding crops of rice, wheat, and maize were introduced into the field (Conway and Toenniessen, 1999). Many assert that it is only because of the introduction of these scientifically bred crops that food production was able to support the immense population growth since then (Conway and Toenniessen, 1999; Sakamoto and Matsuoka, 2004).

A twenty percent increase in production was accomplished with the green revolution crops by breeding dwarf or semi-dwarf plants. The process was non-transgenic, meaning, first, that the crops are not considered genetically engineered and, second, that the genes affecting this phenotype were not identified until recently (reviewed by Sakamoto and Matsuoka, 2004). These genes have been characterized as negative regulators of gibberellin (GA) response (reviewed by Sakamoto and Matsuoka,

2004). Knowledge of genes involved simplifies the process of creating new higher-yielding crops.

With this in mind, many researchers have become of the opinion that there must, and will be a second green revolution and that the medium in this situation will be genetic engineering of cereal crops (reviewed by Sakamoto and Matsuoka, 2004). Various ideas for development of genetically modified crops have been developed in order to increase yield to feed the peoples of the world. While these prospects are quite promising from a logistical perspective, they are likely to suffer rejection by the public.

To genetically engineer a crop involves manipulation at the genetic level. Sometimes, genes identified in one plant are inserted into a different variety or even a different species. Other times, a gene is identified in the target cultivar and negative regulators of that gene are knocked out. Genetic engineering has many variations but sometimes — rarely — it even involves gene splicing from animal to plants. This has raised all sorts of concerns among the public. GE (genetically engineered) and GMO (genetically modified organism) have become dirty words in the consumer society, both in developed and underdeveloped countries. Some countries have begun refusing or restricting the entry of GMOs. In response to the US government's World Trade Organization (WTO) case against European Union's policies on GE foods Felix Cohen, head of the Netherlands consumer organisation Consumentenbond, said,

> "[E]ven if the US were successful in its suit, European consumers would not eat the stuff; you cannot force consumers to eat products they do not trust yet." (www.tacd.org).

President of the US consumer organisation Public Citizen, Joan Claybrook, in response to the same suit, pointed out that,

"[It] has been repeatedly said by African governments and academic experts, GE crops are not the answer to hunger in Africa and in fact could worsen existing problems, in part because all GE crops are subject to patent restrictions." (www.tacd.org). This means that seed from one GE crop cannot be reused for the next without paying an annual fee. In general, the public's acceptance of GE crops has been low.

For a crop to naturally develop a desired phenotype it takes millions of years of random mutations with a strong selection for desired phenotype. A million years is a long time, but there exists a shortcut. To increase the speed of this process mutagenesis can be performed on the seeds of a crop to increase the rate of randomly occurring mutations. Then selection can be applied by human intervention for the desired phenotype. Mutagenesis can be achieved by soaking the seeds in a mutagenic, or mutation causing, chemical, or by exposure to mutation causing radiation. Ethyl methane sulfonate (EMS) is a commonly used mutagenic chemical and a well-known mutagenesis technique using radiation employs fast neutron emissions to increase mutation rate. Every seed contains a multicellular plant embryo. When you mutagenize a seed, some cells of the embryo will contain mutations and others will not. The resulting M1 plant is called a genetic chimera, because it contains a mixture of mutations in different cells. The M1 plant is allowed to self-pollinate, and the cells of the resulting progeny will be of uniform genetic composition. Thus the M2 generation is screened for the trait of interest. The screen is repeated on the subsequent M3 generations to confirm that the desired trait is reproducible. Cases of natural mutations include errors in DNA replication, UV

radiation, and cosmic radiation. Mutagens simply increase the speed of this natural process.

Since the 1920s and the discovery of mutagenic chemicals, or mutagens, for use in increasing mutation rate, the process of mutagenesis has been responsible for development of numerous advantageous new varieties of our staple crops, including mutant varieties resistance to powdery mildew, stem rust, leaf rust, and various other stressors (Strader et al, 2003). The process that led to the first green revolution can be used to aid in the second, but we cannot perform the same selection. The same selection that led to the green revolution mutants would yield the same mutants again, with little or no gain, so it is apparent that selection for an exclusive yield increasing trait be formulated.

In order to discover a trait needed to increase production of these crops, we must consider the limiting factors in growth. Since the previous selection was based on an increase in overall production, selection for mutants that can survive and produce in non-ideal conditions — for instance, during a late frost, a drought, or an early rain — would increase annual production by eliminating yield lost to random climatic effects. I'm proposing that, instead of trying to directly increase production, try to indirectly increase production by decreasing loss in production. We must especially consider any stresses that, if imposed on the plants, can reduce grain fill or grain production and, thus, crop yield.

One major cause of decreased yield is drought. Drought can affect any crop in any part of the world and the consequences can be disastrous. If uncontrolled water stress occurs during grain filling it generally reduces final grain fill (Yang et al, 2004).

Especially in places like India, with a higher average temperature, heat stress (Rane and Nagarajan, 2002) and water deficit (Reddy et al, 2004) due to drought is a significant factor in grain production. Drought tolerance would be a benefit to any crop, and would increase the overall production of those crops which are likely to be subjected to drought. In similar studies maize selection for drought tolerance has increased grain yield (Bänziger et al, 1999).

Exposing a field of mutagenized wheat to drought conditions and trying to "see who survives" will, likely, yield very scant results. In order to screen for resistance to drought we must understand the mechanisms of drought stress. During a drought, the main stressor is water deficit. Not only are plants not getting as much water during this period, but also they are losing water more quickly due to increased heat. Water loss in plants occurs mainly via evaporation of water escaping through open pores in the leaves called stomates.

How does a plant respond to this? Studies of maize have shown that soil moisture deficit causes an increase in the concentration of abscisic acid in the plant (Bahrun et al, 2002). Abscisic acid (ABA) is a plant hormone that inhibits seed germination, triggers stomatal closure, and induces genes that protect the plant from drought, cold, and salt damage. ABA is produced in the roots and sent to the leaves causing the stomates to close. One function of open stomates is to create capillary action to draw water out of the ground like a siphon. The other function is to allow gas exchange of carbon dioxide and oxygen. During a drought there is little water to be drawn up, so closing stomates to avoid evaporative water loss is a good strategy. Complete stomatal closure for any significant period of time would prevent gas exchange altogether, so plants have a partial

response to ABA. ABA concentration present directly correlates with stomatal conductance (Borel and Simonneau, 2002). As more evidence for this idea, mutants were created that were unable to function ABA properly and these were discovered to be unable to close their stomates and therefore wilt easily (Finkelsten and Rock, 2002). Sensitivity to ABA also determines how much stomatal closure occurs. Thus, an isolated ABA hypersensitive (ABH) mutant (a mutant that has a high sensitivity to ABA) should keep more of its stomates closed more of the time. This would prevent water loss more readily during a drought. ABA also triggers other processes, mostly involving gene transcription, which aid in survival under water deficit (Chrispeels and Sadava, 2003; Ingram and Bartels, 1996). Therefore, if we screen for ABA hypersensitivity, we can produce mutants with a drought tolerant phenotype.

The trick is in how to screen for ABA hypersensitivity. Some ABA regulating genes are known in wheat (Marcotte et al, 1989), but this is of little use for random mutagenesis. To explain the solution, knowledge of what ABA is and how it functions is necessary. As mentioned before, ABA is a hormone. It has many functions that may seem unrelated to drought, but may prove useful for identifying ABH mutants. It induces seed dormancy during embryo maturation. ABA inhibits seed germination, studies with coffee suggest that this is due to ABA preventing the endosperm cap from weakening (Silva et al, 2004). It also stimulates stomatal closure, and induces genes that give response to stresses imposed by drought, cold, and salinity (Chrispeels and Sadava, 2003; Mäntylä et al, 1995). It controls senescence, carbon remobilization (important for grain filling), and accelerated grain fill during water deficit (Yang et al, 2003). Seed dormancy is one of the most well known results of ABA. When a seed falls off a plant it does not

begin to grow immediately. There is a signal that tells it to wait for a while. This period of waiting, during which no growth occurs is the dormancy period. It protects the seed until the time comes for it to germinate, since the unborn seed is unaffected by drought, cold, or lack of nutrients. ABA is the signal that tells the seed to wait. So ABA determines the length of the dormancy period. A dormant seed will not grow even if you give it water. A seed that has passed through its period of dormancy is known as afterripened and will germinate if given water. People who don't think of ABA as a stress hormone, usually think of it as the dormancy hormone.

Dormancy is much easier to measure than drought tolerance. A screen for drought might leave you with a field of dead plants; there is no significant mortality in a dormancy screen. Selection is based on whether a seed is germinated or not. As such, ABA hypersensitivity can be measured — since ABA inhibits germination — by selecting only those mature seeds, which do not germinate or germinate very poorly.

One might ask how an "ungerminated" seed is different from a dead seed. How do you plant a seed that won't grow? The answer lies in another hormone; gibberellic acid (GA, also gibberellin). GA is a hormone with almost opposite effects to that of ABA. GA is the signal that stimulates a seed to break dormancy. It has other functions, such as, stem elongation, leaf expansion, development of leaf hairs called trichomes, and flower and fruit production (Steber et al, 1998). The plants which caused the green revolution were GA insensitive (GAI) mutants (Sakamoto and Matsuoka, 2004). That is the reason they are dwarfs, reduced sensitivity to GA means reduced stem elongation, and reduced leaf expansion. It is interesting to note that since ABA and GA have an

antagonistic relationship, an ABH mutant will share many traits with a GAI mutant. The major difference is that an ABH mutant will not be a dwarf.

There are three cereal crops that are of the greatest importance for feeding the world. They are rice, wheat and maize (Conway and Toenniessen, 1999). The average annual increase in yield of wheat both from 1975-84 and from 1985-94 was less than the average annual increase of rice or maize during these years (Conway and Toenniessen, 1999). Because of this, wheat is the ideal candidate for a study to create drought resistant mutants by screening for ABA hypersensitivity at the time of germination. Similar experiments have been performed in different plants to see if ABA insensitivity in germination correlates with ABA insensitivity in adulthood, and have found this to be the case (Finkelstein, 1994). Genes responsible for enhanced response to ABA have been identified, abh1 (Hugouvieux et al, 2001) and era1 (Cutler et al, 1996) However, this is the first study with the proposal of showing that wheat plants screened for ABA hypersensitivity by germination tests will isolate ABH plants and that these plants will show a drought tolerant phenotype. Using wheat, this research examined the hypothesis that: if hypersensitivity to ABA correlates with drought tolerance, then a plant isolated for ABA hypersensitivity during seed germination should show a drought tolerant phenotype.

Materials and Methods

Examining this hypothesis consisted of three major steps. First, third generation ABH mutants were isolated using mutagenesis and selection via germination screens. Second, drought tolerance of these mutants was tested. Finally, it was determined whether ABH and observed drought tolerance are associated with closed stomates.

Two cultivars of wheat were used to isolate ABA hypersensitive (ABH) mutants from, Chinese spring and Zak wheat. Approximately 30,000 Chinese spring seed were mutagenized by fast neutron mutagenesis. Approximately 15,000 Zak seeds were mutagenized by EMS mutagenesis. This was accomplished by soaking the grains in EMS for 16 hours before neutralizing the mutagen. Isolation from Chinese spring was done in conjunction with PhD candidate (and subsequent recipient) Lucia Strader in the department of plant physiology. Protocols for isolating ABH mutants during germination were based on protocols which she designed.

Grain from mutagenized wheat was grown to maturity in a field by members of K. Kidwell's laboratory. The harvested heads contained M2 (second generation after mutagenesis) seed. The seeds were afterripened which involves storing them at room temperature for about 6 months (time depends on variety) until the natural period of dormancy has passed. Once grain afterripened a wild type (wt; non-mutagenized) seed will germinate. These heads were screened for ABA hypersensitivity by placing 4 grains from each head on a petri plate, with only 10 heads per plate. The petri plate contains an absorptive growth disk which is soaked with a solution of MES (as a buffer) plus 5μM ABA. (Concentration determined by L. Strader.) Grains were lined up into rows for easier identification and to prevent interaction between them. The plates were sealed with parafilm, wrapped in aluminum foil (to prevent light interference in germination), and incubated at 26°C. Germination data was recorded every 24 hours for four days. Those grain which did not germinate, or which poorly germinated, were moved to new plates containing GA_3 to stimulate germination. These plants were planted in a greenhouse and grown to maturity. Each plant passing this screen was considered one

mutant line and given a number to identify it. The afterripened grain of these lines were retested by placing 30 grain of one line per petri plate with 5μM ABA plus MES. Plates were sealed with parafilm, wrapped with aluminum foil, and stored at 26°C. Germination data (germinated/ungerminated) was taken every 24 hour for 4 days and those seed which germinate poorly or not at all were considered to retest. These plants are M3 generation and will produce M4 seed. (For similar procedure see: Strader et al, 2003)

QUANTIFYING DROUGHT TOLERANCE

To test drought tolerance of isolated mutants two experiments were performed. First, water to the plants was cut off and the response was compared to that of wild type for both Zak and Chinese spring mutants. Second, an experiment to measure transpiration was conducted with Chinese spring mutants, based on inconsistent results from this procedure, the experiment was not performed with Zak mutants. (for related experiment see: Finkelstein, 1994) Isolated ABH mutants were grown to maturity in growth chambers alongside wild type in large pots with equal weights of soil, equalized after the first week of growth. Once adulthood was reached water was stopped and the pots were covered by saran wrap in order to prevent water loss due to evaporation. Individual plant weights were measured each day to quantify water loss. Once all plants were dead a final weight was measured and the results were graphed.

DROUGHT TOLERANCE ASSOCIATION WITH STOMATAL CLOSURE

Two methods were employed to measure stomatal closure of the mutants of both Zak and Chinese spring cultivars. First, the average leaf temperature was measured of adult mutants and compared to that of wild type using an infrared thermometer. Second, observations of the leaves were performed using scanning electron microscope (SEM).

In order to capture the stomates in their natural position, since cutting the leaf will stimulate stomatal closure. Imprints of ABH leaves were obtained using transparent fingernail polish. These imprints were then observed under the SEM. Since open stomates were difficult to observe, steam was applied to the leaves of wild type and mutants in order to stimulate stomatal opening. Response of mutants were compared to that of wild type.

Results

ABA hypersensitive mutants were successfully isolated in both Zak and Chinese spring wheat varieties. Drought tolerance of the Chinese spring mutants was sufficiently demonstrated, while further work is needed to examine drought tolerance of Zak wheat mutants. Both cultivars of wheat did, however, show heightened stomatal closure activity in at least one ABH mutant.

The most important step to create drought tolerant mutants it isolating the initial mutants. The number of mutants that will screen positive for any desired phenotype is relative to the amount of seed mutagenized, the amount of mutagen used, and — in large part — due to dumb luck.

ISOLATING ABH MUTANTS IN CHINESE SPRING

Screening for ABA hypersensitivity is not absolute. There are many ways that an apparent ABA hypersensitivity phenotype can be observed when the cause is not hypersensitivity to ABA. We are looking for those seed which do not germinate in the presence of exogenous ABA. GA insensitive (GAI) mutants would fail to germinate due to their lack of response to GA, the "wake-up" signal. GAI mutants can be identified in adulthood, because they will be dwarfs, as GA stimulates a range of growth processes including stem elongation and leaf extension. If seeds are not allowed to afterripen,

which involved being stored in dry conditions until the dormancy period has passed, then they will appear ABA hypersensitive because they will all have poor germination or no germination. Dormancy can be broken by cutting the seed in half. A true ABH mutant will have 100% germination when cut in half but have poor germination when ABA is applied exogenously to a cut grain. If there are mutants that do not afterripen as quickly as wild type does, then they will appear ABA hypersensitive when screened, because they are not past their period of dormancy and this period has been prolonged past the length of wild type. Normally, when the grain is cut dormancy is broken; however, some mutants will show reduced germination even when cut. With some mutants a longer period of afterripening normal germination will be restored, In other mutants the phenotype cannot be rescued with any amount of afterripening. This information must be kept in mind when isolating ABH mutants.

We screen the second generation mutant grains (M2). M1 seed was mutagenized, planted, and the seed that these plants produced is the M2 generation. All seeds from one plant are considered to be of the same mutant line. Seeds from one plant are put in one petri dish, usually a quantity that is sufficient for statistical evaluation, in this case 30. A small amount of ABA is mixed with a buffer to a concentration of 5μM. This is added each plate. If a plant is normal, then it will germinate even though there is 5 μM ABA present. If a plant is ABA hypersensitive this means that it has a heightened reaction to ABA, so an ABH mutant will have a big reaction to ABA which will outweigh the GA signal that tells it to germinate, thus it will not germinate.

Of the 30,000 mutagenized Chinese spring seed, 89 were isolated from the first selection screening of M2 grain by L. Strader. These were grown to maturity. From the

original 89, 44 mutant lines either died or were infertile. The remaining 45 were allowed to self-pollinate and set seed. Their progeny, the seed produced by these plants, are the M3 generation and these were screened once again for inability to germinate on 5μM ABA. This is the retest. Twenty five mutant lines retested with greater than 60% ungerminated seed, many with 100% ungerminated seed. Five of these mutants have characteristics of a GA insentitive (GAI) mutant and must be considered as such. Characteristics include dark green appearance and dwarfism (See figure 1.1). Each mutant line has a unique numerical identity. The shown GAI-suspect is of the 1314-45 mutant line. The other four probable dwarfs are 910-22, 11314-76, 910-69, and 78-69.

Figure 1.1: Suspected GAI mutant (left) appears to be a dwarf compared to Chinese spring wild type (right).

The 25 mutants were separated into 5 classes based on their degree of sensitivity to ABA in germination experiments performed on cut afterripened grain as shown in figure 1.2. Class 5 produced a phenotype suggesting that mutants in class 5 do not

Figure 1.2: Five classes of sensitivity to ABA of cut dormant and afterripened grain.

Mutant Group	# isolated	Mutant line	Dormant ABA Dose-response Phenotype	Afterripened ABA Dose-response Phenotype
1	8	1314-1, 1314-26A, 1314-26B, 1314-35, 1314-115C, 46-17, 78-112, 910-55A	Wild-type	Hypersensitive
2	5	1314-34, 1314-42, 1314-93, 78-7, 78-39	Hypersensitive early, germination recovers later	Hypersensitive
3	9	1314-45, 1314-46, 1314-64, 1314-76, 1314-82A, 1314-130, 78-15, 910-13, 910-22	Hypersensitive	Hypersensitive
4	2	1314-28A, 78-68	Reduced germination in absence of ABA	Hypersensitive; Germinates in absence of ABA
5	1	1314-16	Reduced germination in absence of ABA	Reduced germination in absence of ABA with 3 years afterripening!

achieve afterripening. Class 4 mutants took 2 years to achieve afterripening. Class one produced germination data from cut afterripened grain that looked the same as the data produced by wild type plants, it is possible that those mutants in class 1 are afterripening mutants without an altered ABA response.

ISOLATING ABH MUTANTS IN ZAK

There were 15,000 Zak grains mutagenized and the M2 seeds were screened by germination on 5μM ABA. Twenty-eight isolates of these were identified. Of these only 12 mutant lines survived or were able to produce seed. Four out of these twelve lines retested at the M3 generation. The isolates are: ZakERA26A, ZakERA33A, ZakERA19A, and EMS-ZakERA0. (Naming note: ERA stands for enhanced response to ABA and is identical in meaning to ABH.) There were also three mutants that all had a shriveled grain appearance and sprouted out of the wrong end of the grain: ZakERA15A, ZakERA26A, and ZakERA35A. They were not considered ABH, but were planted for observation.

PRELIMINARY DROUGHT TOLERANCE TESTING

All 25 of the isolated ABH mutants of Chinese spring wheat appeared to show some degree of drought tolerance compared to wild type when artificial drought was imposed. This is expected because ABA stimulates stomatal closure, so a mutant that responds more sensitively to ABA will tend to keep its stomates closed more often which can be beneficial in a drought by preventing water loss. Artificial drought, created by stopping water to the plants, was recreated 3 times with similar results. Wild type expressed a wilty phenotype, while the fourth generation mutants (M4) remained green and erect. There were 5 plants with the appearance of more promising drought tolerance phenotypes than the rest. These plants were 910-55A, 1314-26A, 1314-26B, 1314-16,

and 1314-28A. The best looking of these was 910-55A (see figure 2.1). Time required to transition to flowering (heading date) was also recorded; heading is when the wheat plant produces the flowers as a head, or spike, where the grain will be formed. It was found that some of the mutant lines appeared to have a faster maturation rate. To determine if this was either due to drought imposed stress, or, occurring even in unstressed plants the same mutants were grown to maturity without inducing water stress. Many of these mutant lines showed an earlier heading date relative to wild type, even without drought stress. The fastest maturing mutant was 910-55A. Three other mutant lines were exceptionally fast maturing; these are 1314-26A, 1314-45, and 1314-130. Further study of these results is needed.

Figure 2.1: Following drought wild type (left) shows a wilty phenotype, 910-55A (right) remains erect.

Zak mutants were not subjected to drought since significant grain increase must first be procured before risking loss of a sample population to drought. Such an increase has yet to be obtained.

PRELIMINARY TRANSPIRATION TESTING

In order to better quantify drought tolerance, water loss versus time measurement was attempted. This is the transpiration experiment; it attempts to measure the speed at which water is lost through the leaves by measuring the decrease in mass over time. The

transpiration experiment began spanning two Conviron growth chambers with 4 plants per mutant line each planted individually. Conviron 2 contracted a catastrophic aphid infection and all plants had to be destroyed. (Insecticide will affect transpiration rate, so there was no other option.) Because of this measurements were only possible for 1-2 plants per mutant line. This must be repeated with a larger sample size to achieve statistically significant data. To measure the transpiration rate total plant weight (including the pot with soil) was measured on a daily basis. Results of the transpiration rate were inconclusive. Data collected conflicted even within one mutant line. Error analysis will be included in the discussion section of this paper. This experiment can neither verify or deny any drought tolerance.

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Drought tolerance cannot be assumed to be the result of closed stomata. Thus, two experiments were performed to see if the stomata of these mutants were actually more closed, more of the time.

INFRARED MEASUREMENT OF CHINESE SPRING

In emulation of work performed with an infrared camera to see the difference between the average leaf temperature an ABA insensitive mutant and wild type (see

Figure3.1: ABA insensitive (ABI) plant (on the right in both images) showed a cooler leaf temperature than wild type (wt) (on the left in both images).

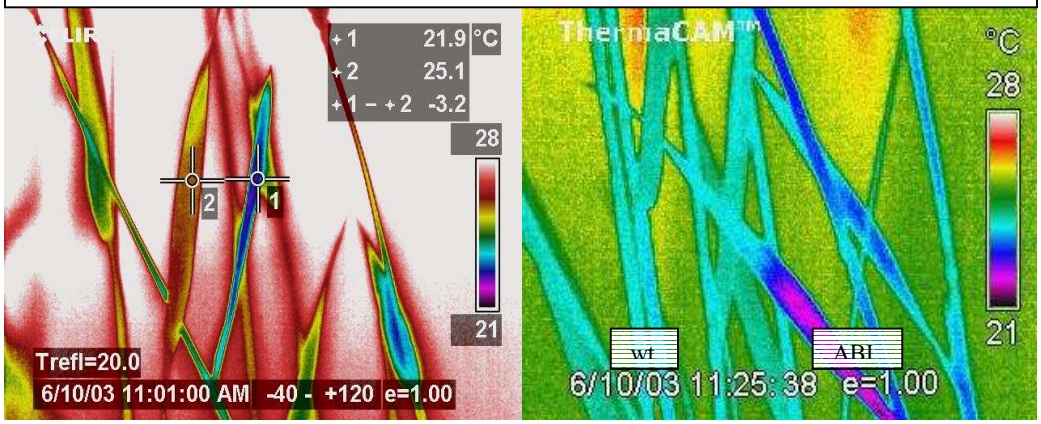


figure 3.1), an infrared (IR) thermometer was used to measure leaf temperatures of ABH mutants and compare them to wild type. This procedure was only performed on the five most promising Chinese spring mutants, as isolated from the drought tests. As can be seen from figure 3.2, 1314-16 showed a significantly warmer average leaf temperature while most of the others did not. The temperature of 1314-16 had an average of being 0.84 degrees Celsius warmer than wild type. Another mutant, 1314-28A shows an average leaf temperature that is above wild type and below 1314-16. It is an average of 0.57ºC higher than wild type. This data is consistent with the dormancy classes chart (figure 1.2), as 1314-16 was the only class 5 and 1314-28A is the only class 4 that made the testing pool.

Figure 3.2: Average temperature differences of Chinese spring mutants difference from wild type

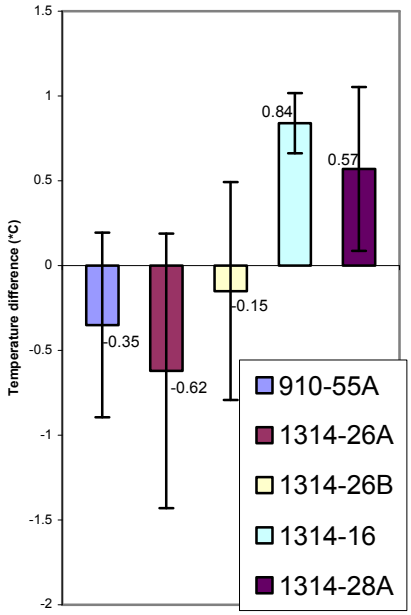
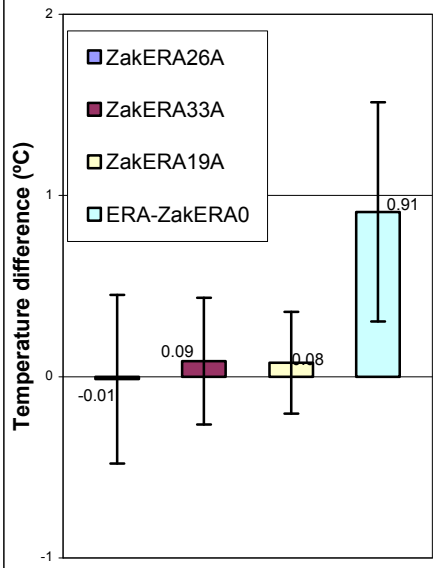


Figure 3.3: Average temperature differences of Zak mutants from wild type



INFRARED MEASUREMENT OF ZAK

Infrared temperature measurement of Zak leaves isolated one mutant as having an increased average leaf temperature when compared to wild type. EMS-ZakERA0 showed a leaf temperature averaging 0.91ºC higher than wild type (see figure 3.3). This mutant could share characteristics with the Chinese spring

mutant 1314-16. The remaining three mutants of Zak showed no noticeable average difference in temperature from wild type.

SEM OBSERVATION OF STOMATAL APERTURE

Final observation of stomatal closure was conducted with very literal interpretation of the word observation. Leaves of Chinese spring and promising mutant lines were sampled, fixed with osmium tetraoxide, and observed under scanning electron microscope (SEM) (see figue 4.1). Due to the method of fixation in conjunction with the natural state of wheat stomates few open stomates were observed. Fixation with liquid nitrogen and lyopholization (freeze-drying) was also performed to attain open stomates, but failed to do so. The successful method of obtaining images of open wheat stomates was actually an imprint with fingernail polish. No open stomates were initially observed, but application of steam to the leaves prior to fingernail polish caused the stomata to open and then begin to close again. The degree of opening observed by the shape of the nail polish mold may suggest the speed at which the stomates closed and the original degree of opening. Large round white globs in the center of the inverted stomate imprint indicate that the stomate opened very wide and allowed much nail polish to enter before it closed or the nail polish solidified. Thin white slats between the closed lips of a stomate imprint suggest that the stomate did not open very wide and closed almost immediately causing this small amount of nail polish to be compressed to a straight line. Other degrees of open and closed can be most easily

Figure 4.1: Stomate of Chinese spring wild type observed under SEM.

determined by looking at the outermost edge of either lip. These "lips" are called guard cells and stomatal closure is mechanically controlled by them (see figure 4.1). If either or both outermost edges of the lip make a "V" or bend towards the central opening, then the stomate is closed. If the lips seem flat or even bend slightly away from the central opening then the stomate is more open. The results of close stomatal observation with the SEM were consistent with the IR temperature results. The mutant 1314-16 tended to have a more tightly closed stomates. An area containing 8 stomates in figure 4.2 has 5 with the thin white slats and the remaining three appearing tightly closed. From both this and the IR temperature data 910-55A appears to have the opposite phenotype than expected. It shows an average cooler temperature than wild type and has the most open looking stomates of those mutants tested. The large round white globs can be observed numerously on the imprint of 910-55A (see figure 4.3). Figure 4.4 shows wild-type stomates, of which the majority appear to be more open, but not as widely open as 910-55A.

Figure 4.2: Image of wheat stomate showing well defined guard cells.

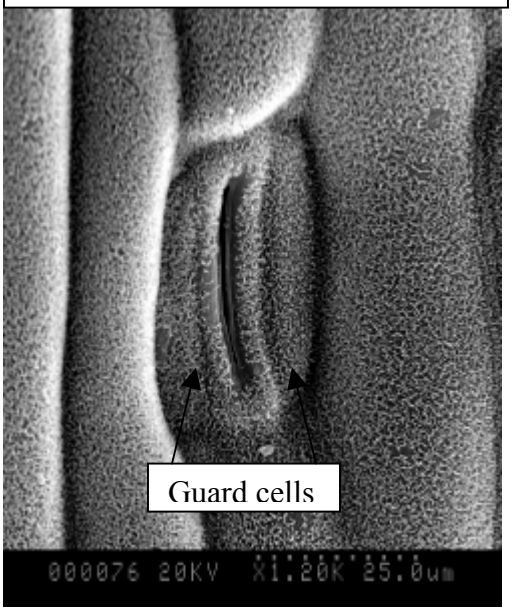
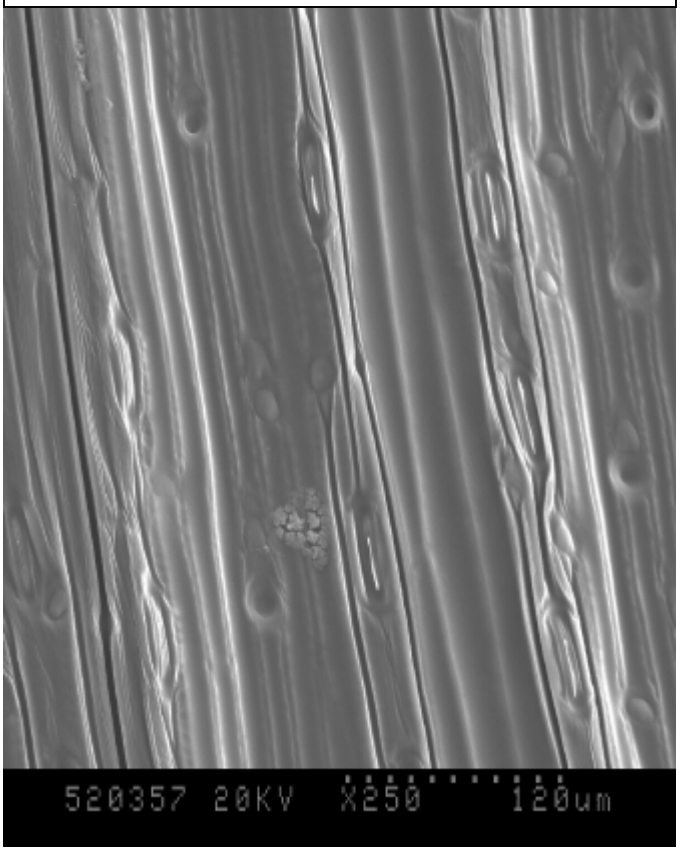
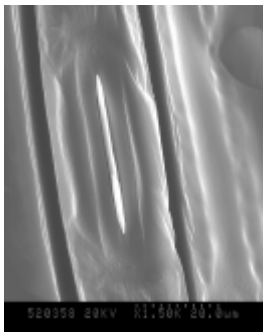
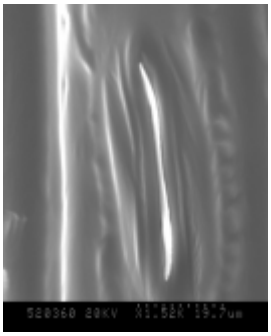


Figure 4.3: 1314-16 leaf impression with nail polish containing stomates.



Three 1314-16 stomates exhibiting the "white slat" phenotype described.





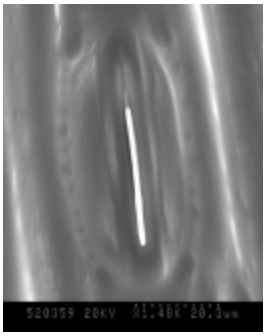
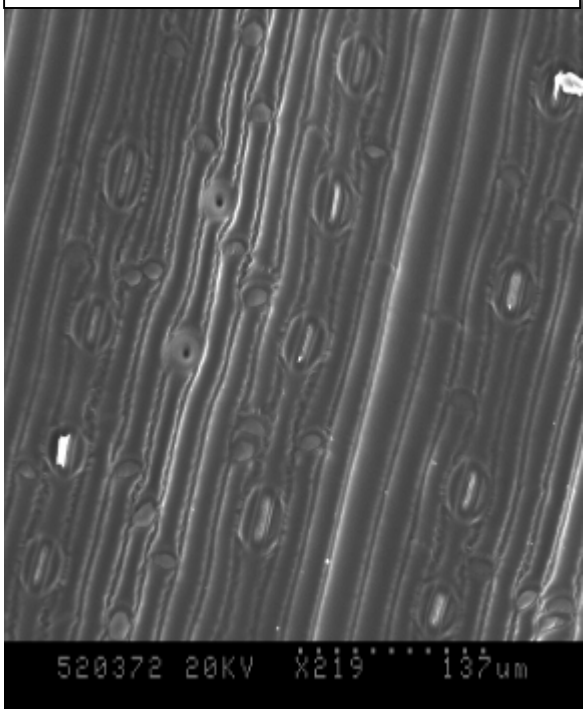
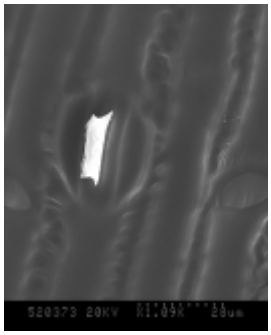


Figure 4.4: 910-55A leaf impression.



910-55A stomates



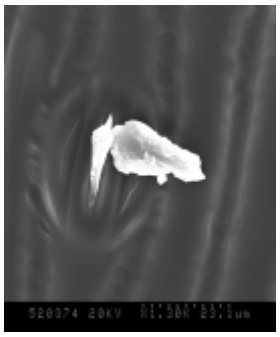
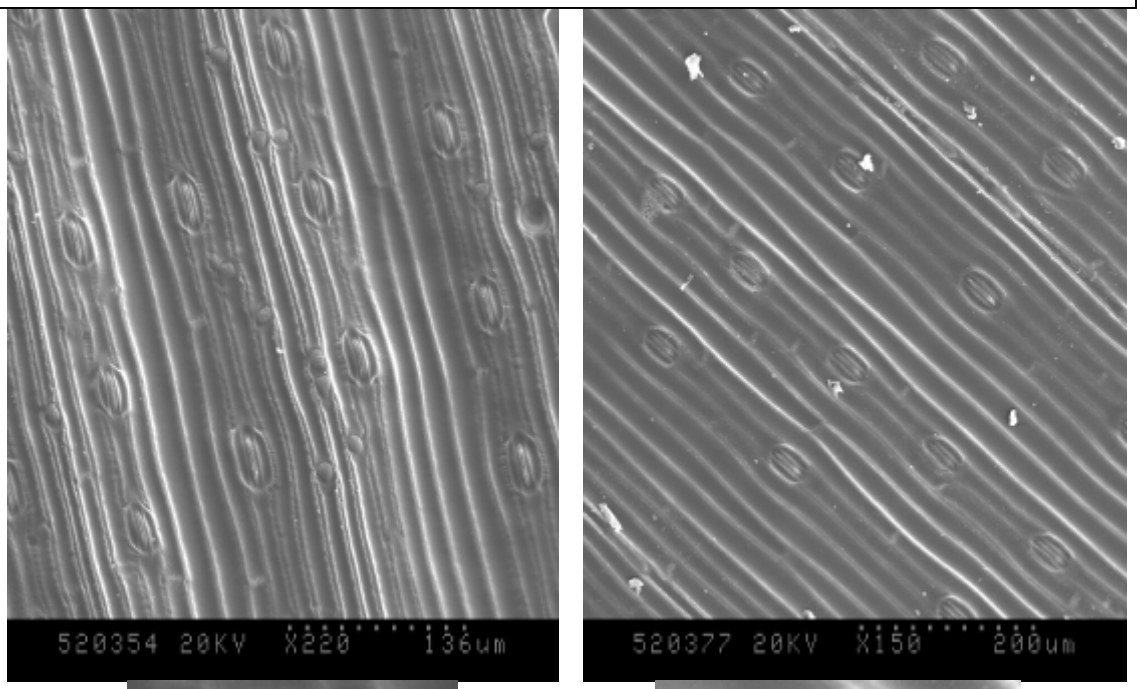
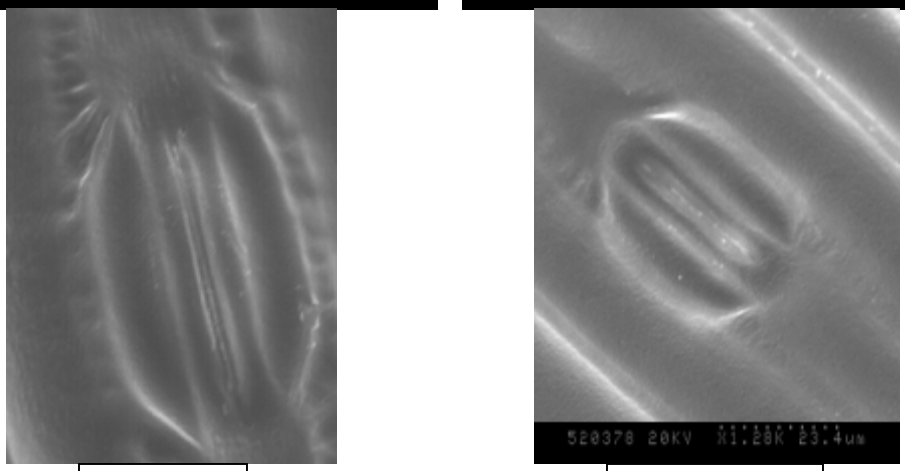


Figure 4.5: Image on the left is a Chinese spring wild type impression collected after the application of steam, right is the same without the application of steam. Pinching of the guard cells can plainly be seen in the right-hand image; whereas, in the left hand image the increased water vapor concentration outside of the guard cells has stimulated opening.





STEAM NO STEAM

Discussion

The purpose of this project was to confirm that plants isolated for ABA hypersensitivity during germination will show a drought tolerant phenotype in adulthood. In two cultivars of wheat, screens for ABA hypersensitive germination identified mutants that were also drought tolerant. The results obtained provide strong support for the hypothesis: if hypersensitivity to ABA correlates with drought tolerance, then a plant isolated for ABA hypersensitivity during seed germination should show a drought tolerant phenotype.

In the process of testing this hypothesis, the transgenic method was overlooked in favor of a method that would produce crops which will be more accepted by the public. Because of this, the majority of 2 years was spent isolating the mutant lines. Thus drought tolerance screening and stomatal closure measurements have produced only preliminary results for further investigation. Drought tolerance was observed in all of the Chinese spring ABH mutants to varying degrees; however, those that showed the strongest drought tolerance were either the plants in which a greater degree of stomatal closure had been observed (this study), or the plants which were shown to produce seed faster by flowering earlier (J. Abellera and C. Steber unpublished).

Of the early flowering mutants, 910-55A had the shortest time from planting to seed production. Increasing their rate of transition to flowering is a natural mechanism of a plant under drought stress. The idea is to produce seeds as fast as you can before you die. Thus, a plant that flowers quickly, even when not in the presence of drought stress, is likely to be drought tolerant. These mutants, however, do not appear to be true ABA hypersensitive mutants based on germination tests (Class 1 and 2 Figure 1.2).

Those mutants, such as 1314-16 and — to a lesser extent — 1314-28A, which showed an increased temperature and other evidence of stomates being more closed, more often do appear to be true ABH mutants. Especially, in 1314-16, where the rate of transition to flowering was not increased in relation to wild type, yet drought tolerance was nonetheless observed, we must attribute drought tolerance to stomatal regulation. Therefore, by screening mutants during germination for ABA hypersensitivity, it was possible to create at least one drought tolerant ABH mutant.

In Zak mutants it was possible to observe stomatal closure by leaf temperature measurement; however, I was unable to obtain any drought tolerance data. Based on the correlation between leaf temperatureand stomatal closure in Chinese spring mutants, Zak mutant EMS-ZakERA0 is likely to be drought tolerant based on its slightly warmer than wild type average leaf surface temperature. The use of Zak as an origin cultivar was employed because Zak wheat is currently a cultivar grown by wheat farmers, while Chinese spring is not. A drought tolerant mutant of Zak would be of special interest to the farming community.

With this in mind, further research to confirm the results of the preliminary data presented in this paper must be performed. It is evident that a better method for accurately measuring drought tolerance must be made. The attempted transpiration rate experiment had too many factors to be controlled with a plant as large as wheat. Individual plant size affects results, since a larger plant with more leaf surface area would show an apparent higher transpiration rate due to faster water loss. The crowding of plants in growth chambers may have had effects resulting from leaves of one plant resting on another plant. Furthermore, error can be introduced easily due to soil density, root

mass differences, and other factors. A new protocol involving hydroponicly grown wheat (wheat grown in water with no soil) allowing measures of change in actual water volume would alleviate some root and soil related errors. Another possibility is trying to measure water loss of a group of plants from the same mutant line grown together in one giant container. This would magnify any actual differences in transpiration rate, thereby, increasing accuracy, and take into account that different plants grow to different sizes.

Further study must be conducted to determine whether these mutants will be successful under drought conditions in the farmers' field. Future field experiments will be conducted in dry regions of eastern WA (less than 10cm rainfall/year) to test the agronomic potential of these mutants. Promising mutants can be crossed into other useful wheat cultivars by wheat breeding programs.

Although mutant screening takes significantly longer than most transgenic methods of engineering new crops, the overall process is not extensively drawn out. In only two years I was able to isolate 29 mutant lines from two cultivars of wheat and perform preliminary testing to confirm their drought tolerance. If only one of these lines goes on to produce a successful drought tolerant crop in the field, it may increase wheat production worldwide just like the famous green revolution plants. It is interesting to note that, though the green revolution plants were not developed using mutagenesis, they did have an origin right here at Washington State University. Perhaps, one of the origins of the second green revolution will, also, begin here.

Conclusion

Since the time of the green revolution the world's population has been increasing rapidly. The need for another wave of increased production crops is apparent, and creation of such crops will likely be achieved by reducing the loss that regularly occurs to current crops. Although the abscisic acid hypersensitive mutants isolated in this study are not the solution to this problem, they do serve as a step towards the answer. With a population of soon to be 8 million, any small increase in yield will decrease the number of people who go hungry.

In order to make this step count, further investigation of this research must continue as proposed. Better testing methods, faster screens, and improved facilities would all benefit the research for a drought tolerant crop. Possibilities of crossing these mutant lines with other cultivars that have desired traits are very interesting and warrant consideration. With these proposals fulfilled the mutants discussed in this study and further mutants can be prepared for use in the field. If that happens, it could cause a needed increase in global food production.

With this study I have shown one possible route for creating non-genetically engineered crops with a property that will increase crop production. Mutagenesis followed by selection at the M2 and M3 generation will take more time than genetic engineering, but not so much time as to be impractical. The isolated mutants in this study are, by no means, a miracle solution that will end world hunger, but they are a step in the right direction. With the continued research proposed, these plants may begin field testing and eventually reduce at least a small part of the world's food deficit. The world

is still running out of food, but now at least there are some steps being made to slow this process.

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