Characterization and mapping of a wilting *Zea mays* mutant

by

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Abstract

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Understanding water stress responses in maize and the genetic factors controlling these traits has direct application to breeding and genetic study. To understand water stress responses in more detail, this thesis characterizes a novel B73 ethyl methanesulfonate induced wilting maize mutant designated *wi*-NM3219. This mutant is shown to express a wilting phenotype under both field and greenhouse conditions, exhibiting leaf roll, stunted stem growth and reduced fertility. Germination is unaffected in mutant seeds and no abnormal seedling phenotypes are observed. Onset of the phenotype is between V3 and V6 growth stage, and corresponds with a disruption of stem elongation. To characterize the genetic elements underlying the wilting phenotype, *F*₂ and *F*₃ mapping populations were phenotyped and genotyped. The mutant phenotype was found at variable frequencies between populations; ¼ or less of all mapping population members were observed as wilting, implicating a single genetic factor controlling the mutant phenotype. Background effects of the non-mutant parent and residual EMS mutations are hypothesized to be responsible for the unexpected frequency of wilting in *wi*-NM3219 populations. Single nucleotide polymorphism and microsatellite markers localized the *wi*-NM3219 mutant allele to the long arm of chromosome 7, to a 7.4-Megabase region centred on marker umc1301. The phenotype results and rough mapping data provide a base for further study of *wi*-NM3219 and cloning of the mutant allele.
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<tbody>
<tr>
<td>ABA</td>
<td>abscisic acid</td>
</tr>
<tr>
<td>BAC</td>
<td>bacterial artificial chromosome</td>
</tr>
<tr>
<td>bp</td>
<td>base pairs</td>
</tr>
<tr>
<td>cM</td>
<td>centiMorgan (1/10&lt;sup&gt;6&lt;/sup&gt; of a Morgan)</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>EMS</td>
<td>ethyl methanesulfonate</td>
</tr>
<tr>
<td>GA</td>
<td>giberellic acid</td>
</tr>
<tr>
<td>hcf</td>
<td>high chlorophyll fluorescence (mutant phenotype)</td>
</tr>
<tr>
<td>IDP</td>
<td>insertion/deletion polymorphism</td>
</tr>
<tr>
<td>LOD</td>
<td>log of odds</td>
</tr>
<tr>
<td>M</td>
<td>molar concentration (moles per litre)</td>
</tr>
<tr>
<td>Mb</td>
<td>megabase or 1 million bp</td>
</tr>
<tr>
<td>ML</td>
<td>maximum likelihood (mapping)</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger ribonucleic acid</td>
</tr>
<tr>
<td>QTL</td>
<td>quantitative trait locus</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>SNP</td>
<td>single nucleotide polymorphism</td>
</tr>
<tr>
<td>SSR</td>
<td>simple sequence repeat</td>
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<tr>
<td>V stage</td>
<td>leaf collar growth stage</td>
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Introduction

Maize (*Zea mays*) of the grass (*Poaceae*) family is among the most economically important crop plants in the world. Annual production of maize in Canada occupies 1.2 million hectares and produces 11.7 million metric tons of grain, with worldwide production on 162.7 million hectares yielding 820 million metric tons (USDA, 2011). Maize is used primarily in food production; directly consumed, as animal feed or used in industrial processes for its high starch content. Creation of high yielding stress tolerant varieties for future food security requires a significant base of research knowledge and understanding. Maize plays an important part in this research base as both a commercial crop species and model system in many research disciplines. Significant advancements in maize research have been made since the start of this research project, most notably the publishing of a fully assembled B73 reference genome (Schnable et al., 2009). This resource allows for increasingly powerful molecular genetic studies of maize development at all plant levels.

Maize requires significant quantities of water to achieve maximum biomass production (Rochette et al., 1996). Because water is a limiting factor for maize growth both locally during dry spells and on a global scale during dry years (Nielsen et al., 2010), it is important to study how plants make use of the water available in their environment. Breeding for increased drought and stress tolerance has been an important goal of breeding programs worldwide. Research into plant water use mutants will help characterize key components of plant stress pathways, an important research area in which this thesis will contribute.
Understanding how and why mutant phenotypes are expressed in a plant provides knowledge of important plant processes and developmental characteristics relating to water use. This information is vital for improving breeding stock and crop productivity. In this thesis I report my progress in characterizing a wilting mutant found segregating from an EMS mutagenized B73 population. This mutant is further designated \textit{wi*-NM3219}. In this thesis I describe the identification of a locus containing a putative novel wilting gene whose mutation causes a wilting plant phenotype, along with several other phenotypes. The mutant allele is shown to segregate in multiple inbred backgrounds with varying expressivity, though molecular characterization of the mutant allele remains incomplete. To understand the underlying cause of the \textit{wi*-NM3219} wilting phenotype, I measured several plant traits and found growth and developmental changes associated with plant wilting. The measurements used were obtained through whole plant qualitative and quantitative field observations, including controlled greenhouse experiments. I report that the mutant does not respond to available water after the onset of wilting and is impaired in stem elongation compared to wild type controls. Through testing of several plant processes known to be associated with a wilted plant phenotype in maize I hypothesize that mutant \textit{wi*-NM3219} expresses its wilting phenotype through a novel mutation not previously reported in the maize literature. We have assessed the phenotype onset and identified water use trends which merit further investigation.

Overall the study of this wilting mutant will further our understanding of plant water interactions and may have implications for development of drought tolerant maize lines for commercial use. Identification of the genetic element(s) responsible for the \textit{wi*-}
NM219 wilting phenotype remains a key step in understanding both wilting mutant development and this mutant's significance in further plant-water physiological study. Experiments studying the phenotypic characteristics of the wi*-NM3219 mutant, its phenotype expression and penetrance as well as further genetic study are ongoing.

**Research objectives**

This project has two major objectives for understanding the physiology and genetics of the wi*-NM3219 wilting mutant and its phenotype.

**Physiology**

Major Objective: Understand the underlying physiological cause of plant wilting observed in NM3219 mutants.

Approach: Use anatomical study and whole plant physiological techniques to characterize and understand the wilting phenotype observed in wi*-NM3219 wilted mutants.

Hypothesis: A major water use pathway is impaired in the NM3219 wilted mutant, affecting growth and development of the plant.

**Genetics**

Major Objective: Map and identify the genetic element(s) responsible for the wilting phenotype in the NM3219 EMS induced wilting mutant and assess phenotype expression in multiple inbred backgrounds (genotypes).

Approach: Use segregating populations for mapping the genetic elements of the wilted phenotype to a chromosome position. This information can be used to understand the inheritance of the element(s) through crosses in multiple inbred backgrounds and
associate background genotype effects with phenotype expression.

Hypothesis: A single mutation resulting from EMS mutagenesis is responsible for the wilting phenotype in \textit{wi*-NM3219} and can be mapped to a single chromosomal locus.
Literature Review

To introduce the relevant literature reviewed for this thesis I have broken the literature review into two major sections: 1) Plant Structure and Physiology, 2) Genetics and Mapping

1) Plant Structure and Physiology

In the first section of this literature review I describe plant processes and anatomical development which will help to understand any structural or functional impairments found in the characterization of mutant \( wi^*\)-\( NM3219 \). I describe studies which have helped elucidate plant water interactions at several biological levels, from molecular studies to whole plant physiology to field scale studies. I have included a description of important mutants in many anatomical features and plant processes related to this project and describe mutant developmental effects in relation to characterizing a novel wilted mutant. Wilting mutants have been studied in non-maize species ranging from important crops (\( Oryza\ sativa \)) to model systems (\( Arabidopsis\ thaliana \)). The literature contains examples of characterized wilting mutants from other species, especially rice which may prove to be important in maize research due to the close phylogenetic relationship between maize and rice. Due to mutant stocks available in these species, these organisms represent a large knowledge base for studying the mechanisms of wilting in plants. The findings reviewed in this section have helped frame the physiological characterization of mutant \( wi^*\)-\( NM3219 \).

2) Genetics and Mapping
The genetics section covers a range of genetics topics from markers and mapping to multi-locus traits and positional cloning. Linkage mapping and positional cloning are reviewed from a theoretical and molecular techniques standpoint. I describe the marker technologies and the underlying genetics which make gene identification possible. I describe the genetics of previously characterized wilted mutants and review mapping strategies used in previous studies. Also reviewed are mutant creation techniques and the various resources available to geneticists in using available mutants for future characterization.

In both major sections I emphasize the historical context of important techniques and the basic science underlying many of the modern techniques and research to understand how our current knowledge base was created.

**Plant structure and physiology**

**Characteristics of wilting mutants**

There are a number of plant processes and anatomical features whose delayed or improper development may cause wilted plant phenotypes. This section describes the literature of plant wilting phenotypes and provides examples of wilting plant genetic mutants in depth. Characterized mutants with a wilting phenotype are the focus, as these mutants will help define aspects of plant physiology which will be useful in the characterization of *wi*-NM3219.

To understand the wilting phenotype under study in *wi*-NM3219 it is important to
understand how plants normally respond to low water availability. Wilting is a whole plant physiological response to suboptimal plant water status due to low water availability from soil or to transpirational demands greater than its transport ability, or to a combination of these factors. It is visible as an above-ground phenotype characterized by leaf rolling if the stress is mild, eventually leading to plant death if the water deficit is severe or prolonged. The environmental conditions causing wilting in healthy plants have been studied in depth. The term “wilting coefficient” is used to describe the level of water in soil (in a water-saturated atmosphere) at which a plant will begin to wilt and be unable to continue normal physiological functions and growth (Briggs and Shantz, 1912). Wilting can be viewed as a protective mechanism for the plant, as rolled leaves intercept less solar radiation than open leaves, lowering the amount of water lost to evaporation. The cost of wilting is a decreased photosynthetic capability and ultimately reduced plant productivity. Wilting helps to protect photosynthetic proteins from damage that is caused by photosystem excitation (Aro et al., 1993) with no water for heat dissipation. Mutants have been discovered in multiple plant species, which show an increased tendency to wilt under low water conditions. Some mutants display a constitutive wilting phenotype regardless of water availability. Identification and characterization of these diverse mutants will increase the understanding of plant water regulation, a valuable tool for breeding and production systems.

Maize vascular bundle development

Wilting in wi*-NM3219 may be due to a defect in the vascular system moving
water throughout the plant, therefore it is important to review how water is transported in a functional vascular system and how the morphology and anatomy of this system develops during growth. The developmental importance of vasculature is highlighted by the mutant *wilted1*; a metaxylem vessel formation mutant (Postlethwait and Nelson, 1957) which is further discussed below.

Describing vascular development, a landmark study in maize vascular structure and development was published by Esau (1943). Her work describes the anatomy of maize vascular bundle formation and how vascular bundle morphology changes throughout plant development. Lachaud et al. (1999) reported that the first visible differentiated vascular tissue is the vascular cambium, whose main role is to create further differentiated vascular tissue through periclinal divisions. Protophloem are the first developed transport elements. Once two to three protophloem elements are present, the protoxylem begins to differentiate (Bosabalidis et al., 1994). Protoxylem and protophloem are present early in seedling development and provide water and nutrient transport capabilities, respectively, for the developing plant (Heimsch et al., 1950). The physical stretching of stem elongation causes mechanical damage to the protophloem and protoxylem elements, impairing their transport function. These early vessels have some elasticity, but they cannot maintain their transport functions throughout stem elongation. The tearing damage to the protophloem and protoxylem elements leaves behind a characteristic empty cavity in the vascular bundle termed the lacuna (Esau, 1943). As the plant matures the functioning replacement vessel elements are called metaphloem and metaxylem. Comparison of vascular bundles across developmental stages has proved to
be technically challenging because of the range of tissue maturity levels from different parts of the stem (Sharman, 1942). From the reviewed literature there are several important developmental events, such as the switch from protoxylem to metaxylem transport, which could be disrupted and play a role in a wilted plant phenotype, as found in *wilted1*.

Mutant *wilted1* is the best studied wilting maize mutant, with the wilting phenotype is attributed to delayed metaxylem vessel formation as described above. This mutant is a constitutive wilting mutant; available water will not ameliorate plant wilting. Vascular elements in the stem are not capable of moving sufficient amounts of water to the leaves to prevent wilting during normal growth (Postlethwait and Nelson, 1957). The *wilted1* phenotype is characterized by visible leaf roll in the youngest leaf tips approximately 3 weeks after emergence, and is most prominent during daylight hours. The youngest leaves show the strongest wilting phenotype, with recovery of normal leaf water potential during the night. As the plant grows, the most mature leaves at the lowest portion of the stem show recovery of the wild type phenotype while the top leaves remain wilted. *wilted1* plants are also characteristically dwarfed likely due to chronic drought stress during growth. Mutant *wilted1* is able to produce pollen but rarely produces seed. Stomatal number and function were normal, while the mutant was found to transpire less than wild type controls indicating a water transport or uptake problem. For comparison of mutant plants to wild type plants the *wilted1* allele was crossed into a *pigmy* mutant background to eliminate possible plant height effects on phenotype characterization (Postlethwait and Nelson, 1957). Eosin uptake and transport through the vascular system
showed poor dye uptake in the highest vascular bundles of mutant plants, where wild type plants had even uptake of the dye throughout the stem. Postlethwait and Nelson (1957) hypothesized that transport capabilities in the stem were compromised. Stem tissue sections were observed, confirming that a defect in metaxylem vessel formation was impairing water movement. The older basal portions of the stem were found to have normal vascular development in most bundles, while the youngest sections had metaxylem elements that were poorly or improperly developed causing a water shortage in the uppermost leaves of the plant (Postlethwait and Nelson, 1957).

**Maize root development and function**

The root system functions as a physical anchor while allowing for water and nutrient uptake from the environment. A disruption of root growth or alteration of root morphology may also explain the wilting phenotype in *wi*-NM3219. This root development and function section begins by describing historical studies of roots and root/soil interactions to provide a basis for the experimental hypotheses in this thesis. This literature describes water movement through plants and root-soil interactions during normal growth and development.

As a basis for characterizing *wi*-NM3219, historical studies describing plant water interaction are discussed. To understand why plants showed varying wilting phenotype levels under changing soil conditions, Briggs and Shantz (1912) studied the moisture equivalent of soils; defined as the amount of water which soil can hold under a force equal to 1000 times that of gravity (1000g). Using physical science knowledge of
the time, the authors realized that water moves to reach an equilibrium between soil water and atmospheric water content, using the plant as a channel for this water. They found the wilting coefficient of soils, a soil water content level where water is physiologically available to the plant. Anything below this content causes wilting (Briggs and Shantz, 1912). Other work from the early 1900s describes water relations at the agronomic level, looking at uneven plant productivity related to varying field water conditions (Elliott, 1924). This study related root morphology and penetration to tiling characteristics in an experimental field, finding that field water availability had an effect on root development. At the time of these studies, the mechanisms of water movement in plants was poorly understood. There was a significant debate as to whether the process of moving water throughout the plant was an active or passive process.

Kramer conducted several experiments in the 1930s to characterize water movement through maize plants. Kramer (1932) found that osmotic pressure generated in the roots was insufficient to move the quantities of water required for normal plant function. From this it was hypothesized that water tension from plant transpiration would cause the roots to act as water absorption surfaces. This was tested by measuring stem exudate rates under normal atmospheric pressure and under suction, finding that suction always improved exudate flow from the roots. It was further found that live plants with dead roots remained unwilted longer than unwatered, live root control plants, showing that active root processes were not responsible for the majority of water flow during transpiration. Kramer (1933) expanded on this study using several plant species (sunflower, tobacco, tomato, cotton, yellow pine, red oak, loblolly pine) with hot water
treated/killed roots and found that in all cases plants with dead roots maintained above ground water potential longer than unwatered plants with living root systems. Sunflower plants with living roots wilted in three days, but those with dead roots and water remained unwilted for ten days. Above ground tissues of plants with dead roots were shown to have normal physiological responses to ethylene containing lamp gas, showing that above ground plant responses were normal and the plant was alive. These early experiments elucidated the role of the root system in water movement and showed that transpiration pulling force and not root pressure was responsible for moving water to the leaves from the soil.

Further study of root water uptake showed that plants preferentially draw water from dilute solutions compared to concentrated ones, with the solute composition of the concentrated solutions having no effect (Eaton, 1941). This demonstrated that osmotic pressure could affect root development and that roots were responding to their environment. Following this work there was interest in determining what portions of the root were important for water uptake. A capillary device was designed to measure root water absorption, which could be fitted to unbranched root endings (Hayward et al., 1942). This device was used on corn roots to determine that maximal absorption was taking place 10 cm distal to the root tip. Root water availability was shown to be important for root morphology changes, where availability of water at different regions of a root system caused local developmental changes of the root system (Sharp and Davies, 1985). Current research in root systems is heavily genetics-based, with root morphological changes in response to water availability being characterized on a
molecular level (Tuberosa et al., 2002). Root traits can also have large scale morphological defects, as has been recently found in several maize root morphology mutants (Hochholdinger et al., 2004). Major root morphology QTLs have been identified which explain root development traits and highlight inter-relationships between root and above ground traits (Giuliani et al., 2005). As a complex system with many factors influencing development there is much progress to be made in understanding the plant root system. Using this understanding of root function it is reasonable to consider that wi*-NM3219 may be defective in some aspect of its soil water interaction or water uptake, resulting in the water deficient phenotype.

Root development rate and root:shoot ratio are important indicator traits describing plant development and response to osmotic stress conditions. An altered root:shoot ratio provides a clue as to how the plant is responding to stress conditions. Due to the wilted phenotype observed in wi*-NM3219, the literature describing growth under stress is discussed here for identification of traits to help with characterizing wi*-NM3219. The changes in root:shoot ratio and the different developmental changes occurring under water stress are well reviewed by Wu and Cosgrove (2000). An example of a wilted mutant with altered root:shoot ratio is present in the Arabidopsis literature, mutant lew2-1. This mutant is wilted due to collapsed xylem vessels, and was found to have normal root development (Chen et al., 2005), while above ground development is severely impaired.

Root growth and development has been shown to be affected in rice wilting mutants, notably oscow1-1 and oscow1-2 (Woo et al., 2007). Both alleles of this single
gene mutant are associated with decreased overall plant growth. They were discovered via T-DNA insertion mutagenesis. OsCOW1 displays a wilting phenotype from an early developmental stage, with wilting visible 7-10 days after germination and persisting throughout the plant's life cycle. Severity of wilting is dependant on the environmental conditions in which the mutant is grown (Woo et al., 2007). OsCOW1 is a putative indole-acetic acid (auxin) biosynthesis mutant based on sequence homology to the YUCCA subgroup from Arabidopsis. A key finding of this study is the significant decrease in root growth of the OsCOW1 mutant. This leads to a decreased root:shoot ratio, which is hypothesized to be responsible for the wilting phenotype observed. Root growth in OsCOW1 mutants was found to be 50% of the wild type value 34 and 60 days after germination, while shoot growth was 90% of wild type (Woo et al., 2007). Transpiration rates 90 to 100 days after germination were found to be significantly lower in the mutant than non-mutant plants, which can be attributed to a lower total leaf water content and ultimately due to decreased root development. Identification of a root:shoot or root development phenotype may help to explain the wilting phenotype in wi*-NM3219, therefore, these phenotypes merit further investigation.

Only recently have efforts been made to characterize below ground maize phenotypes and understand root traits in terms of molecular genetics. The maize literature contains many examples of systems used to understand and characterize root development. One such system in use by the Lukens Lab and other groups uses a polyethylene glycol solution to place roots under osmotic stress, allowing for study of developmental effects of water availability (Verslues et al., 1998), with current work
aimed at understanding the underlying genetic interactions. Study of roots under aeroponics allows for observation of large scale mature plant root phenotypes (Gaudin et al., 2011). Several maize root mutants have been identified, but the plethora of above ground phenotypic mutants would suggest that there is more to learn about root morphology and development with modern molecular methods (Hochholdinger et al., 2004). Further study of root systems will increase the number of characterized mutants and improve our knowledge of root processes, and the characterization of mutant \( wi^* - NM3219 \) may provide valuable information into the function and development of the root system.

**Leaf maturity and senescence**

With previous field notes of early leaf senescence in \( wi^* - NM3219 \) mutant plants it is important to understand the normal progression of leaf senescence in wild type maize to understand the precocious initiation of the phenotype. Leaf senescence is an actively regulated process in which plants degrade leaf tissue for remobilization of nutrients for use in sink tissues, and may play a response role to stress conditions (Pastori and Trippi, 1993). Senescence in maize is a sequential process, occurring from the oldest leaves to the youngest leaves in controlled progression, and is associated with plant physiological maturity and the grain filling period. The earliest phenotypic characteristic is a visible yellowing of the leaf originating in the chloroplast (Smart, 1994). As cited in Smart (1994), Mothes (1928) found that drought stress could cause initiation of leaf senescence in *Nicotiana* and *Helianthus*, though the mechanisms underlying senescence were not
understood at the time of the study. Early studies such as that of Ingram and Bartels (1996) looked for evidence of dehydration mechanisms through analysis of drought related physiological pathways thought to be linked with senescence. It has been noted that leaf senescence may be a contributing factor to plant survival under drought stress, as plant resources may be allocated to younger parts of the plant for continued growth (Munne-Bosch and Alegre, 2004). Regulation of the molecular processes of senescence has been studied extensively. Lim et al. (2003) describe a number of Arabidopsis mutants with altered senescence phenotypes including rapid onset and late senescence mutants acting through multiple pathways. The authors stated that more work was required to understand the relationship between these phenotypes and applied stress senescence, especially in the area of senescence initiation and signalling. Molecular methods will help to explore and understand senescence signalling pathways and initiation in future studies. Hormones may interact with reactive oxygen species to integrate senescence responses and regulation (Hoeberichts and Woltering, 2003), with studies showing ethylene triggered release of \( \text{H}_2\text{O}_2 \) as a critical step and feedback mechanisms in senescence as well as other hormone interactions (de Jong et al., 2002).

The natural initiation of senescence plays a role in determining maturity date of a crop. Plants which are delayed in senescence initiation and correspondingly maintain green leaves are termed stay green plants. This trait can be studied from physiological and genetic perspectives through mutants, with stay green traits appearing to have complex genetic control (Zheng et al., 2009). In general terms, early senescence is associated with low grain yield due to a short grain filling period; conversely, longer stay
green is associated with higher grain yield (Borrell et al., 2001). Peroxisomes play a key role in the senescence process, with the delay of their activation allowing for longer stay green (del Río et al., 1998). In maize leaves senescence has been linked to higher oxidative stress coupled with decreased antioxidant production (Prochazkova et al., 2001), and regulation of these factors may influence the timing of senescence through integration with other plant signals. Linking the mechanisms of drought stress and senescence under field conditions is complicated by many environmental factors, however field trials may offer the best clues to the underlying molecular mechanisms of senescence. There are many greenhouse development artifacts associated with senescence phenotypes (Munne-Bosch and Alegre, 2004). Stay green is a similarly difficult trait to work with as there may be significant environmental influences affecting the trait. The study of mutants with senescence phenotypes, including wi*-NM3219, may help understand the initiation and regulation of senescence processes and how they are affected by environmental factors and their interactions.

There are several identified and characterized senescence mutants in the maize literature, relating to both early and delayed senescence. Mutant id1, or indeterminate1 is a maize mutant noted for continued vegetative growth with no change to reproductive growth (Colasanti et al., 1998). The gene responsible is a transcriptional regulator of flowering, whose disruption stops reproductive organ formation and initiation of flowering. Early senescing mutant pre1 or premature senescence 1 was identified in maize, and has been shown to initiate leaf senescence 2 weeks before flowering occurs (Neuffer et al., 1997). The molecular basis of this phenotype remains unknown, meriting
further study. Characterization of the leaf senescence phenotype of mutant *wi*-NM3219 may increase the knowledge of how senescence processes are regulated and how these processes are integrated with environmental stresses.

**High chlorophyll fluorescence mutants**

Many pathways in leaf development may be important in determining wilting expression in *wi*-NM3219. This section describes the high chlorophyll fluorescence (*hcf*) class mutants, previously noted to have leaf colour phenotypes similar to *wi*-NM3219. The *hcf* maize mutants are a mutant family deficient in photosynthetic protein synthesis or activity (Miles, 1994). Many of these mutants are seedling lethal due to their inability to undergo normal photosynthesis, while other members of this family have reduced (compared to non-*hcf*) photosynthetic capability (Miles and Daniel, 1974). Typically *hcf* mutants can be observed through application of long wave ultraviolet light (~600 nm wavelength) to the leaves, resulting in red fluorescence (light emission ~680-685 nm). Wild type plants have blue leaf fluorescence under UV exposure. Over 130 mutants with the described *hcf* phenotype have been found (Neuffer et al., 1997), including several mapping to the long arm of chromosome 7. This class of photosynthetic mutants has visible leaf colour phenotypes resembling the early wilting leaf phenotype *wi*-NM3219.

**Gibberellins and plant height**

The *wi*-NM3219 mutant has a stunted growth phenotype. To understand normal plant growth, this section describes gibberellin and gibberellin mutants in terms their
Gibberellins (gibberellic acid, GA) are a group of tetracyclic diterpenoid plant hormones, which play an important role in plant growth and developmental regulation (Richards et al., 2001). This group of hormones has a wide variety of actions in plants, including regulation of stem elongation, maintenance of apical dominance and several seed development roles. The study of stem elongation factors led to the discovery that gibberellin A$_1$ is the main active form of GA influencing stem elongation (Phinney, 1985). Early study of gibberellins linked their function to diterpenoids, and has led to the discovery of many active GAs (Graebe et al., 1965). The best known GA mutants in maize are the dwarf class of mutants. These are GA sensitive GA biosynthetic pathway mutants, which have a reduced but varied plant height phenotype under application of exogenous GA. Mutants $d1$, $d2$, $d3$ and $d5$ are recessive, non-allelic and gibberellin responsive (Phinney, 1985). Several GA insensitive dwarf mutants are known to exist: $d7$, $D8$, $d9$ and $d10$. Mutant $D8$ is of particular interest to the study of GA metabolism as this mutant displays a plant height phenotype similar to the GA sensitive dwarf mutants while remaining completely GA insensitive, with a maximum height that was 20-25% of the wild type control (Phinney, 1985). $D8$ was found to be impaired in perception of GA at a putative GA binding site or at a downstream signal from the GA binding protein. With the range of plant height phenotypes described for GA mutants, the plant height phenotype of $wi^{*-NM3219}$ mutants may be related to GA function.

Wilting mutants and abscisic acid
Several wilted maize mutants thought to have abscisic acid phenotypes have undergone cursory characterization. \(Wi2\), \(Wi3\) and \(Wi4\) are dominant, non-allelic mutants which wilt under mild stress conditions but do not have a wilted phenotype when well watered (Rock and Ng, 1999). \(Wi2\) was shown to have a dose response curve to exogenous abscisic acid similar to that of a wild type control, indicating that ABA perception was not affected in this mutant. Transpiration rates were measured for all mutants finding a trend with the mutant class transpiring less than the wild type control (though statistical significance of these results is unclear and the results likely not statistically significant). \(Wi2\) shows stress response based on \(Rab17\) expression (ABA reporter gene (Gomez et al., 1988)), assayed on both unstressed and drought stressed leaves. The wilted phenotype of these mutants was suggested to be caused by xylem element defects in the stem as found in \textit{wilted1}. Fushcin acid stain uptake was found to be delayed in these mutants, supporting the hypothesis that \(Wi2\), \(Wi3\) and \(Wi4\) are wilted due to delayed metaxylem formation. Recent work presented via poster at the 2012 Maize Genetics Conference has described \(Wi2\) as encoding a \(\beta\)-tubulin\(6\) gene (likely \(\beta\)-tubulin\(6b\) due to map position) whose disruption affects microtubule dynamics within the cell (Haiming et al., 2012). Further characterization is required to confirm transport vessel disruption in \(Wi3\) and \(Wi4\). Recent mutant screens from the Maize Inflorescence Architecture Project have provided additional EMS-induced recessive wilted mutants available through the Maize Stock Centre for future characterization efforts.

There are examples throughout the maize literature of mutants with a secondary wilting phenotype, which are classified based on their original phenotype of interest. One
class of wilting plants with pleiotropic phenotypes is the viviparous mutants. These are ABA production or sensitivity mutants and were initially divided into two classes, the first that have normal coloured endosperms and the second where seeds have white endosperms with albino seedlings (Robertson, 1955). Further work has since blurred the lines between these classes, however they can still be classified by the pathway location of the mutation (Robichaud et al., 1980). Viviparous mutants can appear wilted under field conditions due to stomatal control problems resulting from various ABA pathway mutations. In addition to the viviparous mutants, other mutants associated with the ABA pathway have been shown to display viviparous and osmotic stress phenotypes. The EMS induced maize mutant rea displays a red embryonic axis in its seed, but has normal ABA biosynthesis (Sturaro et al., 1996). This mutant is less sensitive to exogenous ABA than wild type controls, but does exhibit stronger rab17 transcriptional responses to water stress than vp1. Much of the research published on the various ABA mutants is directed at understanding seed dormancy and embryogenesis without consideration for pleiotropic phenotypes later in development. This may be justified by the seedling lethality of many of these mutants and special care they require for growth. Further study would increase understanding of how many of the early development and later growth drought tolerance mechanisms are integrated.

**Leaf wax composition and wilting**

An interesting rice mutant is wilted dwarf and lethal 1 (wdl1); a recessive seedling lethal wilting mutant whose phenotype results from uncontrolled leaf water loss.
This mutant was discovered in a T-DNA insertion population, and mapped using the Knowledge-based Oryza Molecular Biological Encyclopedia (Kikuchi, 2003). The phenotype was found to be conditional on water status, but the transcript levels of wild type WDL1 could not fully explain the phenotype (Park et al., 2010). Microscopic analysis of the leaves found that stomata and pavement cells were smaller than the wild type, measured by the stomatal index (Gray et al., 2000). Through measurement of water loss in detached leaves, the authors determined that the high transpirational losses of wdl1 could not be attributed to stomatal loss alone, as the stomates closed within 30 minutes of leaf detachment from the plant (Kerstiens et al., 2006). To address the high transpirational losses, leaf wax production composition was analyzed. It was found that mutants had normal wax levels, but wax composition differences could account for the wilting phenotype (Park et al., 2010).

**Genetics and mapping**

**Genetic linkage and recombination**

The concept of genetic linkage is the foundation for undertaking a gene mapping project. The methods used in mapping are easily derived from basic principles. The basic concepts of linkage and recombination are reviewed in terms of their historical background to start this section. Current work and the molecular aspects of linkage mapping and recombination are also described. Genetic linkage is the inheritance of genes/loci at a frequency not expected by independent assortment/independent Mendelian
segregation genes/loci. This process occurs due to close physical proximity of genes on
the chromosome, with closely spaced physical loci often segregating together at a greater
frequency than would be expected if the traits were independently segregating.
Recombination is the physical crossing over of genetic material between sister
chromatids. This occurs during prophase I of meiosis I. The chromosome tetrads line up
alone the metaphase plate of the cell, where there is close physical proximity of the
chromosome arms. Double strand break is the current accepted model for the method of
recombination (Szostak et al., 1983). Recombination frequency between loci (or their
visible associated traits) can be converted into a genetic distance; denoted by the unit
Morgan. Commonly the unit is converted to centiMorgans (cM, 1/100th of a Morgan) for
ease of use and relevance to usable genetic distance. 1 cM represents 1 recombination
event between loci per 100 meioses.

The use of a percentage of crossing over between traits for linkage mapping was
first described by Sturtevant (1913) using Drosophila melaogaster (ampelophila) while
in the lab of Thomas Morgan. Drosophila provided a good model system for this work
because of the large numbers of progeny produced per individual, the easy
characterization of individual traits and large number of described traits. Linkage was
identified when individuals with different sex-linked mutations were crossed together and
the F₂ progeny did not exhibit the expected Mendelian segregation ratio (Morgan, 1911).
Using the ratio of crossing over events as an index of distance between any two genetic
factors led to the formation of the first genetic linkage map. Fewer crossing over events
between traits indicated that two traits were more closely linked than two traits which
recombined more frequently. These early results lead to speculation of double cross over events which might influence the recombination frequency of traits (Haldane, 1919). The current accepted method of calculating genetic distance from recombination information stems from work by Kosambi (1943), who derived a formula for corrected genetic distance, including the chances of a double cross over event.

**Genetic markers**

A genetic marker is a DNA polymorphism which can be probed for information regarding its state. This allows for determination of genotype, or a description of which parent's genetic material is within the region of the genome being queried. Genetic polymorphisms are abundant in maize and have contributed greatly to the initial molecular research of this plant (Tautz et al., 1986). Simple sequence repeat (SSR) markers are series of short repetitive nucleotide elements which are easily probed by PCR amplification. The marker sequence consists of repetitive short nucleotide motifs of 2 to 6 base pairs in length with repeat number determining amplified sequence length. A large list of SSR markers was published for maize by Sharopova et al (2002). At the time of publishing this list was the most comprehensive set of genetic markers available in any plant species. Sequence length polymorphisms are not limited to repetitive elements. There are many insertion/deletion (indel) polymorphisms (IDPs) present in maize which can be used for mapping. These are often located around coding sequences, often mapping to intronic sequence. Sequence length polymorphisms are highly effective genotyping tools but in many cases are being replaced by newer techniques such as single
nucleotide polymorphism (SNP) genotyping. SNPs are single base pair changes present between inbred genotypes, which can be probed using next generation sequencing methods (such as Illumina, 454) or SNP specific detection platforms (Sequenom MassArray, KASP, TaqMan). SNPs are the ultimate marker based genotyping tool in maize as they occur at a high frequency, estimated to be 1 SNP per 104 base pairs of sequence between inbred lines (Tenaillon et al., 2001). SNPs provide high resolution data for mapping in maize. Though SSRs are an older technology, there is value to length polymorphisms, resulting from the data resolvable based on their fragment length (Senior et al., 1998). While a SNP can only be 1 of the 4 nucleotide bases, an IDP or SSR marker can be any length. This can give clues to the evolutionary links between population groups (Haasl and Payseur, 2011), however as a general genotyping tool SNPs are the most efficient way to gather genetic data on a large scale without sequencing. Markers may be anchored to genomic sequence, meaning they have a defined physical position based on sequencing projects, but this is not required for their effectiveness in the construction of linkage maps. This thesis makes use of several of the techniques above for gathering genetic data, making extensive use of SSR/IDP markers and SNP genotyping for the characterization of \textit{wi*-NM3219}.

**Positional cloning and gene mapping**

Identification of the genomic region responsible for the wilting phenotype in \textit{wi*-NM3219} is a major focus of this thesis. This section describes the literature behind positional cloning, as well as the first attempts at cloning using this method. Positional
cloning is the use of recombination and genetic markers for determining the location of a specific gene within a genome. This is achieved by analyzing the phenotypes and genotypes of a segregating population to discover markers linked with the trait of interest. Positional cloning basics are well reviewed by Collins (1992). The positional cloning method is somewhat different from the older functional genetics approaches to gene mapping. In functional cloning biological function of the mutant is characterized, with sequence data generated from proteins of interest used to amplify the gene of interest.

The first example of positional cloning in any organism is from Royer-Pokora et al. (1986), a study conducted in humans for mapping the gene responsible for chronic granulomatous disease based solely on its map position. This work was possible through creation of a partial linkage map of the human X chromosome by Davies (1983). In comparison with plant genetics, human genetics relies on pedigree information for mutation study where plant studies can make use of specifically created mapping populations for gene identification (Lukowitz et al., 2000). Positional cloning in plants has been aided greatly by the wealth of sequence data (including reference genomes) being produced and published for many crop and model plant species. Early examples of cloning in plants made use of chromosome walking, a technique that relies on screening bacterial artificial chromosome (BAC) based genomic segments for markers co-segregating with a phenotype of interest. One such example of chromosome walking in plants was the mapping of Arabidopsis mutant SUPERMAN (Sakai et al., 1995).

With high throughput genotyping and sequencing platforms becoming cost efficient there are new and powerful ways to associate phenotypes to genomic regions.
One such technique is bulk segregant analysis using SNPs for genotyping. In this technique genome wide SNPs are evaluated on bulks of mutant and non mutant individuals from a segregating population for comparison of mutant to non-mutant ratios for each SNP (Xia et al., 2010). This technique is viable in organisms with curated published genome sequence along with high SNP density allowing for efficient candidate SNP validation. SNPs can then be associated with traits of interest with or without linkage maps. SNPs linked to the mutation of interest will reflect the mutant parent allele at a greater frequency than expected from a non-linked allele. This technique allows for mapping with a bulk sample representing a small number of individuals, but the phenotyping must be accurate or the mapping will not be effective. Similar to bulk segregant analysis is the use of bulk extracted mutant DNA for high throughput sequencing. This technique was demonstrated in Arabidopsis for identifying the location of a single nucleotide mutation in the AT4G35090 gene using SHOREmap software (Schneeberger et al., 2009). As a technology demonstration this study showed that a range of sequencing depth and SNP coverage allowed the mutation to be easily mapped. Though it is still prohibitively expensive to get sufficient sequencing depth in maize for full genome sequencing of a mutant bulk, technology advances are bringing the cost down rapidly for wide scale use of these mapping techniques.

With the publication and release of a fully assembled B73 genome (Schnable et al., 2009) we can expect many advances in gene mapping in the near future. Each new genome revision contains a more precise picture of the functional elements of the genome. Complete sequence data allows for faster generation of markers and testing of
candidate genes. High throughput SNP mapping of maize mutants has been demonstrated by Liu et al (2009), used in rough mapping dozens of recessive mutants in a short period of time using Sequenom SNPs.

The rapid technology advances behind positional cloning techniques have allowed for many genes to be discovered in recent years which would not have previously been possible (Bortiri et al., 2006). As a result of perseverance with older techniques combined with new methods of gene identification being developed, the literature contains many examples of genes isolated using positional cloning in maize. An example of a maize locus isolated using positional cloning is the tga1 locus (Dorweiler et al., 1993), a major kernel architecture trait hypothesized to be a key trait in domestication of teosinte (Zea mays spp. parviglumis) This locus was fully sequenced 12 years after its initial isolation (Wang et al., 2005). As summarized by Bortiri et al. (2006) there is a wide range of population sizes which may be used for effective positional cloning of a gene, from 600 to 6000 individuals. Local recombination frequency occurring at the locus of interest will affect the population size required for mapping, however the local level of recombination is generally unknown when starting an experiment. Population size is also dependent on the mapping resolution desired and gene density (for selection of candidate genes) in the area of interest.

This thesis makes use of the previously described studies in determination of the genetic element(s) linked to the wilting phenotype in wi*-NM3219. Locating the genetic basis of the trait will require populations of sufficient size to ensure sufficient crossing over events at a putative genetic region of interest. Due to technological limitations it was
not possible to use the described bulk mapping techniques on next generation platforms in this project, however future studies such as this may use such techniques as they become cost efficient.

**Mapped wilting mutants in maize**

Genetic studies have mapped several of the recessive maize wilting mutants to chromosome segments. The above described *wilted1* mutant is inherited through a single recessive nuclear gene responsible for the wilted phenotype and is located on the long arm of chromosome 6, identified through co-segregation with the visible phenotype marker locus *Y1* (Neuffer et al., 1997). *Wi2* (mapped to the long arm of chromosome 3, near CAPS047), *Wi3* (unmapped) and *Wi4* (mapped to the short arm of chromosome 5) are dominant, non-allelic wilted mutants which do not have a wilted phenotype when well watered (Rock and Ng, 1999), possibly associated with metaxylem defects as described above. *Wi2* is thought to encode a *β*-tubulin6 gene (likely *β*-tubulin6b which maps to the long arm of chromosome 3), as described recently (Haiming et al., 2012). Further genetic mutant resources are available for characterization through the Maize Genetics Stock Centre and will help elucidate further large effect single gene wilted mutants.

**Mutagenesis in maize**

The use of mutants to study phenotype is a powerful tool in modern genetics. One such technique was used to create the *wi*-*NM3219* mutant used in this thesis and is
described in detail in this section. Other techniques are also described, as well as specific advantages and disadvantages of each method. There are several techniques available for creating maize mutants for genetic study; most commonly used for non-targeted mutagenesis is ethyl methanesulfonate (EMS) treatment. EMS is a chemical compound which induces point mutations throughout a genome non-specifically (at random). The mechanism of function for EMS mutagenesis is guanine alkylation, which results in G/C to A/T transitions (Bautz and Freese, 1960). In the early use of EMS as a mutagen it was found to produce mutant phenotypes without the deleterious chromosomal aberrations often observed with the use of ionizing radiation for mutagenesis (Amano and Smith, 1965). Due to the relatively non-lethal effects of EMS on cells at high concentration, it can be employed for high density generation of heritable genetic mutations in an organism (Neuffer and Ficsor, 1963). A method for EMS mutagenesis in corn is well explain by Neuffer (1997). Soaking pollen in an EMS solution is the most effective way to generate large numbers of mutant seed for study. In some cases a direct seed treatment with EMS may be the most effective way to generate specific mutants in certain pathways of interest, such as seedling and seed germination phenotypes. In genome wide studies using Caenorhabditis elegans and a 50 mM EMS solution, a transition event was found (from the homozygous state) at a rate of 1 mutation per 100,000 nucleotides (Sarin et al., 2010). This study points out the importance of backcrossing the genome to a non-mutant for several generations before looking at a phenotype of interest due to the heavy mutational load present in genes which are not being studied, yet potentially affecting the phenotype being observed.
A large number of mutants resulting from transposon tagging are becoming available for genetic study, and while many of these mutants were not available at the initiation of this study, they may represent a wealth of data for locating independent knockout alleles in future genetic study of \textit{wi*-NM3219}. Transposon tagging is the process of inserting a transposon to a new location in the genome, fixing its position and observing if a gene has been disrupted by the insertion. This allows for disruption of a single gene and easy cloning of the disrupted gene, provided the mutation produces a viable plant. The first reports of transposable elements in any organism are from maize, and originate from observations of mutable loci which could not be explained by classic Mendelian genetics (McClintock, 1950). These loci were found to map to different genomic regions when mapped in different seed stock. The functional mechanism of tranposons are well reviewed by Gierl et al. (1989). Transposons can be very useful in mutant studies because the location of a phenotype-causing transposon can be determined quickly by PCR based techniques. Use of transposon tagging for gene isolation is on the decline with the increased throughput and power of modern genotyping and sequencing techniques (Bortiri et al., 2006). Several major projects are underway to create transposon insertion mutants in all maize genes for genetic study; the RescueMu and UniformMu projects. RescueMu uses transgenic driven transposons for creation of mutants, making testing phenotypes difficult under field conditions. UniformMu has the same project goals but uses Robertson's mutator, a non-transgenic system allowing for easier transfer of seed across boarders (Settles et al., 2007). Projects such as these provide a valuable resource of knockout mutants for finding additional alleles of a mutant phenotype in gene
mapping projects and discovering new null allele mutant effects.

With the range of mutagenesis techniques available for use and variety of genomic effects, mutant screens and databases can provide valuable information. Several of the large scale mutant generation projects are just beginning to produce data for research community use through the application of high throughput screening and genetic approaches, which should significantly expand the use of mutants for many fields of study. While mutants can be a powerful tool for genetic study, the importance of genome purification cannot be overstated. In the case of EMS mutations it is extremely important to ensure that few point mutations remain in mutant lines so as not to affect the final phenotype under study. Final characterization will determine the exact genetic mutation underlying the \textit{wi*-NM3219} wilting phenotype, but it is expected to be a single point transition in a protein coding gene given the mechanism of EMS mutagenesis previously described.
Materials and Methods

Plant Materials

EMS (ethyl methanesulfonate) mutagenized B73 seeds were obtained from Dr. Nathan Springer (Associate Professor, Department of Plant Biology, University of Minnesota) for use in this research. From these mutant seeds, population NM3219 was found to segregate for a wilted plant phenotype in the field and noted for further study. Wilted NM3219 plants were crossed to A619 and Mo17 plants for creation of F\textsubscript{1} seeds (Appendix Figure 1). These F\textsubscript{1} plants were observed and selfed for the creation of F\textsubscript{2} mapping populations in both A619 and Mo17 genetic backgrounds. In F\textsubscript{2} populations where the wilting phenotype segregated, phenotypically wild type plants were selfed for to create F\textsubscript{3} seeds for further mapping and characterization.

Crosses were made in the field according to standard maize breeding methods (Neuffer et al., 1997). Ear shoots were covered early in development before silks were visible and cut back 1 day before pollination. Tassels were cleaned of old pollen and debris, bagged, then labelled the night before being used for crossing.

Ears from the summer nursery were transported to the Elora Research Station for drying at harvest. Once dry, ears were shelled individually to avoid seed stock contamination. Over winter, seeds were stored in the Crop Science Building (University of Guelph) seed storage area at low temperature and humidity.

Mutant seed stock for phenotypic observation and crossing was ordered from the Maize Genetics Cooperation Stock Centre (Operated by the United States Department of
Agriculture: Agriculture Research Service, located at the University of Illinois, Urbana/Champaign). The following wilted mutant stocks were ordered for this study: 301D (Wi2-N1540), 512E (Wi4-N2445A), 605A (wi1y1), 605E (wi1Y1Pi1), U840G (Wi3-N1614), 4209E (wi*-N811A), 4209F (wi*-N986B), 4209G (wi*-NA1190), 4209J (wi*-N2419). The following high chlorophyll fluorescence mutant stocks were also ordered: 502H (hcf21-N1259A), U940D (hcf11-N1250A), 125D (hcf41-N1275C), 125E (hcf50-N1481), 111B (hcf3-N846B).

Tissue sampling and field phenotyping

All mapping populations and field work was conducted in the corn nursery located near Guelph, Ontario, Canada at Terry Daynard's farm (Paisley Road, east of Guelph, 43.49.91N, 80.31.98W).

F$_2$ populations from 2008 and 2009 (B73 mutant x A619) were hand sampled, leaf tissue was cut into 2-cm$^2$ sized sections and placed in 2-mL centrifuge tubes. These samples were lyophilized for 48 hours before DNA extraction. Plant phenotype, wilted or unwilted, was recorded directly onto the collection tube at the time of tissue sampling.

F$_2$ mapping populations from 2010 were sampled before flowering time into 2-mL centrifuge tubes and frozen at -80º C, then lyophilized (minimum 48 hours) for storage until DNA extraction. Sampling commenced on July 12th, 2010. Each plant in both Mo17 and A619 mapping populations was individually tagged and labelled for phenotyping. Wilting status was assayed on the 2010 populations on the following days: July 21, July 27, Aug 6. A final confirmation check was completed on October 8th 2010 during harvest. Plants were identified as unwilted wild type, of medium height and wilted or extremely
wilted in this population. Phenotypic data from all recording days was compiled together to make a final list of wilted plants for summer 2010. Plants called wilted on more than 50% of sampling dates were considered mutant.

\[ F_2 \text{ and } F_3 \text{ populations from 2011 were sampled on July 12}\text{th}, 2011. \] Sampling procedures were simplified based on previous field experience. Each row of interest was scouted and counted prior to sampling, with 96-well templates made up for the sampling procedure. Tissue was harvested from these rows using tweezers to place thumb sized leaf pieces into the 2-mL well of a 96-well deep collection plate. After collection, these plates were placed at -80\(^\circ\) C prior to DNA extraction. Trait data were recorded for segregating rows 8 times: July: 25 and 30, August: 2, 3, 4, 5, 8 and 10. Plant characteristics other than wilting were noted based on visual observation and plant appearance. Data from all plants was compiled together to reduce phenotyping errors, with a final list of phenotype scores prepared for downstream use. Scoring was performed early in the day when possible, as July, 2011 corresponded with an extremely dry period during which all B73-based plants in the field had extremely rolled leaves by mid-afternoon.

**DNA extraction procedures**

Three methods of DNA extraction were used for this project, depending on the volume of samples to be extracted and end use quality requirements. A single tube Sigma-Aldrich kit was used where high molecular weight and high purity DNA was required. A Two Tube method (adapted from Dr. Joe Colasanti, Molecular and Cellular
Biology, University of Guelph) was used for fast extraction of DNA from large populations. For genotyping in summer 2011 and onwards, a 96-well plate-based method was used for increased extraction throughput.

**Sigma-Aldrich kit extractions**

The Sigma-Aldrich GenElute Plant Genomic DNA Miniprep Kit was used for extraction of DNA where high purity and high molecular weight DNA was required. This extraction method yields of 20-80 ng/uL DNA in 100 uL MBG water, with A260/280 around 1.8. This DNA is acceptable for all end use techniques including Sequenom MassARRAY iPLEX Gold SNP genotyping. Extraction throughput limits the use of this technique to 24 samples extracted per run.

Procedure for the extractions followed manufacturer's instructions except for plant tissue collection, storage and grinding. Tissue for high molecular weight DNA extraction was harvested and frozen at -80°C or processed fresh directly after harvest. Tissue for high molecular weight DNA was hand ground with a mortar and pestle in liquid nitrogen. High molecular weight DNA was used for verification of the Sequenom SNP platform. Samples not requiring high molecular weight DNA were lyophilized and ground using the Fast Prep machine and stainless steel or ceramic beads in a 2-mL screw top tube. After grinding, the extraction followed the manufacturer's procedure until elution. Final DNA was eluted from the binding column only once using 100 uL of 60°C molecular biology grade water. A second elution was not performed in order to maintain the higher concentration of DNA from the first elution.
Two tube DNA extraction method

The two tube DNA extraction procedure was received from Dr. Joe Colasanti, originating from University of California, Berkley. It was modified and optimized by the author for compatibility with available lab equipment and sampling procedures (Appendix Procedure 1). This method uses 2 tubes per sample during the course of the extraction, requiring less pipetting compared to the Sigma-Aldrich kit method, minimizing errors during extraction. The procedure is faster than the Sigma-Aldrich kit method, allowing higher sample throughput. DNA yields are higher per sample as there is no binding step (100-1500 ng/uL in 100 uL MBG water, A260/280 ~1.6-2.1). A consequence of not having a binding step is a reduction in final DNA quality. Often, contaminants (both protein and RNA) are co-purified with the final DNA. The co-purified contaminants do not affect the function of this DNA in lab PCR applications, as samples are heavily diluted for end use applications. Limited testing of this DNA with Sequenom MassARRAY iPLEX Gold SNP genotyping and TaqMan SNP genotyping has produced positive results (Luis Avila Bolivar, personal communication). Mapping population tissue samples were processed using this method by summer students working in the Lukens Lab in the summer of 2010.

96-well plate-based extractions

A 96-well plate-based extraction method developed in the Rajcan lab by Chris Grainger for high throughput extraction of high quality DNA from large populations was
used. The sampling and extraction procedures are geared toward the analysis of many samples with the final eluted DNA ready for direct use in PCR and other 96-well applications. A full procedure, reagent and equipment list is provided (Appendix Procedure 2). Tissue was collected directly into a deep 96-well plate for direct grinding. Tissue for extraction can be of any source: fresh, frozen or lyophilized. The method functions similarly to the Sigma-Aldrich GenElute Plant Genomic DNA Miniprep kit method, scaled for high sample throughput in 96-well-plate format. Extractions are performed 2 plates at a time, both for equipment optimization and extraction speed. DNA quality is similar to the Sigma-Aldrich kit method in terms of co-purified contaminants and end use applications of the DNA.

**DNA quality test**

Inbred DNA was tested for purity and shearedness before initial Sequenom MassARRAY iPLEX Gold SNP genotyping. To test for high molecular weight DNA, samples were run on agarose gels. 140 mL of 1% agarose tris-acetic acid (TAE) buffer gels were used for visualization of quality. 250 ug of DNA in 10 uL of MBG water was loaded per well (with 1.5 uL of glycerol blue dye). DNA was confirmed to be high quality if a band of slowly migrating DNA was found at the top of the gel. Low molecular weight sheared DNA appeared as a streak along the gel. Any samples showing sheared DNA were re-extracted to ensure that the SNP validation genotyping would not fail on account of poor quality DNA.
Sequenom SNP genotyping

SNP genotyping was carried out by the Analytical Genetics Technology Centre (AGTC) at the University Health Network (Princess Margaret Hospital, Toronto, Ontario, Canada). Sequenom MassARRAY iPLEX Gold genotyping uses MALDI-TOF (matrix-assisted laser desorption/ionization - time of flight) mass spectrometry to obtain the final weight of each single nucleotide addition primer. Time of flight correlates with primer weight, allowing for a call of the nucleotide base present in the genomic DNA. Putative SNP locations were discovered in silico by Greg Downs and validated on the MassARRAY platform. Primers were designed by the AGTC using Sequenom software and placed in multiplex arrangement for population genotyping. Sixty-nine validated SNPs were used for genotyping in this research. SNP genotypes were called at the AGTC using Sequenom software and manually called when software would not produce a call. Final SNP allele calls were returned to the lab for analysis.

Simple sequence repeat and insertion deletion polymorphism markers

Simple sequence repeat (SSR) and insertion deletion polymorphism (IDP) markers are polymerase chain reaction (PCR) based markers allowing for genotype calling based on amplified fragment length. The final fragment length is visualized on 3% agarose gels stained with the intercalating agent ethidium bromide (EtBr). Markers were selected from the Maize Genetics and Genomics Database in genomic regions identified for characterization. Marker physical position in B73 genome (B73_revision_1 and B73_revision_2) and centiMorgan position (from the Genetics 2008 recombination map)
were recorded. Primer sequences obtained from MaizeGDB were synthesized by the University of Guelph Laboratory Services group. Primers were diluted to working concentration with molecular biology grade (MBG) water using standard lab protocol. Markers were screened with parental (B73, Mo17 and A619) inbred DNA to verify the polymorphism before population screening. Fifteen microsatellite markers were used for mapping in this research. Parental polymorphisms were compared to reference figures available for each marker on MaizeGDB before population genotyping use.

**SSR and IDP genotyping**

Mapping populations selected for SSR and IDP genotyping were subjected to PCR for amplification of the markers being queried. Procedures for PCR are provided in the Appendix, Procedure 3. Two variants of the PCR procedure are listed due to the varying extraction methods used for obtaining DNA. PCR reactions were visualized on 3% agarose gels at <150V to observe the size polymorphism. Bands were scored by visual gel screening of fragment length, and results were manually entered into a spreadsheet for analysis. Genotypes were called unknown if a sample did not produce a clean band, or a pair of bands, or if no bands were present. Failed markers were re-run where possible to fill gaps in data, depending on tissue sample availability and sample phenotype data.

**Chi square goodness of fit test**

The Chi Square Test was used to analyze both observed genotype and phenotype
results for ratios of best fit. The equation for calculating a Chi Square test statistic ($x^2$) is as follows:

$$x^2 = \sum \frac{(\text{observed} - \text{expected})^2}{\text{expected}}$$

Each entry in the ratio has this calculation applied and the final $x^2$ stat is the sum of these values. Chi Square significance charts were used to determine significance thresholds with appropriate degrees of freedom based on the ratio tested.

**Student's t-test**

Physiological data used Student's t-test for pairwise statistical testing. Unpaired 2-tailed tests were used in all experiments unless otherwise stated. Null hypotheses were rejected if the resulting test statistic was less than the threshold significance value of $p=0.05$. Data tested using this statistic is graphed with error bars representing one standard deviation around the mean.

**Linkage map creation**

Linkage maps were created from genotyping data using JoinMap 4 (Kyazma Software). The interval mapping algorithm was used for construction of linkage maps. For creation of genome wide linkage maps the complete data set of SNP calls were entered into JoinMap. Manual grouping was performed for each chromosome based on known anchored SNP positions (from B73 reference genome revision_1). Each set of individual chromosome SNPs were grouped within JoinMap. SNPs found to be linked with a LOD score greater than three were subgrouped together and a map was created to
identify their relative centiMorgan distances. Markers which remained unlinked were analyzed independently. SNP markers were also tested with forced linkage, where SNPs appearing physically close but genetically unlinked were forced into a single linkage group. This did not produce usable linkage maps and was not further used.

Linkage maps from SSR and IDP marker data were created one chromosome at a time. The same grouping procedures were used for single chromosome SSR and IDP markers. Anchored marker physical position was used to check the correctness of the final linkage map. Relative cM distances were checked against the Genetics 2008 maize composite recombination map.

**Greenhouse wilting phenotype expression experiment**

This experiment was designed to determine the onset of expression of the wilting phenotype. A wi*-NM3219 F1 B73_mutant x Mo17 (F2 plant heterozygote at umc1301, selfed for F3) seed population was used in this experiment from summer 2010, row S10 LL 564-8. This population was confirmed to contain wilting individuals under field conditions in the summer of 2011. Plants were grown in 3-gallon plastic pots containing a 50/50 volume mixture of Promix (general purpose potting soil from Premier Horticulture Inc.) and Turface MVP (course baked clay substrate from Profile Products LLC.) as growing media. The experiment was carried out in the Crop Science Greenhouse facility (University of Guelph). Pots were watered to saturation 1 day prior to the start of the experiment. Three seeds per pot were sown. Media was manually kept moist through germination and early development, until installation of an automatic watering system.
Upon installation, automatic watering kept plants well watered to ensure no wilting from lack of water. Fertilizer was injected into the watering system at a 5% dosing rate. Fertilizer composition is listed in Appendix Table 1. The final fertilizer and water mixture had a pH of 6.0. Biological pest control was used when required; no chemical pesticides were applied.

Tissue for genotyping at marker umc1301 (linked to wilting phenotype) and two flanking markers was collected when the plants reached V1 stage. After genotyping, the 120-plant population was thinned to 40 plants, one plant per pot. The population was enriched for the B73 (\textit{wi*-NM3219}) allele at umc1301, ensuring that 20 pots had B73 homozygous plants at this allele. Heterozygotes and Mo17 homozygotes were randomly assigned to remaining pots where possible.

The seeds were first screened for soil emergence timing. One hundred and twenty segregating F_{3} seeds from S10 LL 564-8 were germinated in the greenhouse. Seeds were checked for germination by surveying for above ground tissue at 24 hour intervals post planting. A plant was counted as emerged when the plane of the soil surface was broken by green tissue. Germination and seedling emergence were recorded for each plant.

Stem diameter and top ligule height of each plant were recorded for calculation of stem volume. Measurements were taken every fourth day starting at the V1 stage. Leaf collar stage of each plant was recorded at the time of stem measurement. The timing of wilting expression was recorded for all plants daily. Visual observation of the plants took place daily during growth to search for characteristics that may influence the expression of the wilting phenotype. Pictures of all plants were taken to compare leaf morphology,
leaf colour and leaf development. Notes on anomalies visible during growth were made.

Stem volume (SV) was calculated during the greenhouse experiment using the following formula:

\[
SV = LH \pi ((0.5)(0.1)(SD))^2
\]

SV is expressed in cm\(^2\). The 0.5 is a factor to convert measured diameter to radius. The 0.1 converts millimetres to centimetres. Stem diameter (SD) was measured in millimetres with an analog vernier calliper approximately 2 cm below the lower leaf collar.

**Long wave ultraviolet light testing for the hcf phenotype**

The 120 segregating F\(_3\) seedlings grown for the greenhouse experiment were checked for an hcf phenotype when one fully developed leaf was present. Visual observation of the phenotype occurred under long wave ultraviolet light (Spectroline UV-4B, 365 nm light). The hcf phenotype was noted as expressed if the observed leaves had a red glow under long wave UV light (Miles, 1994). Normal leaves appear blue under long wave UV light. Other signs of the hcf phenotype which were checked for included seedling lethality and early developmental defects of the leaves, including albinism.

**Tissue sections and staining**

Stem sections were made for observation under a light microscope to determine if vascular bundles were normally developed. Both wilted and unwilted stems from field
grown plants were brought to the lab for sectioning. Thin sections were cut from the stem and placed in water for a two-minute soaking. Sections were then transferred to a dry Petri dish for application of toluidine blue stain. The stain was allowed to soak for 60 seconds or less, depending on section thickness. More than 60 seconds in the stain resulted in a loss of detail from overstaining. Sections were rinsed with water and wet mounted for immediate observation with a compound light microscope.

Toluidine blue stain was prepared from dry powder (toluidine blue O, certified – Sigma-Aldrich Inc.) and distilled water (Parker et al., 1982). The final mixture was 0.05% dry powder (50 mg dry powder per 100 mL distilled water), giving a pH 6.5 final solution for staining. Images were visualized with a Zeiss Axiophot microscope (Crop Science imaging equipment) and captured digitally at 250x magnification. Metaxylem vessels were visually assessed for signs of developmental irregularity and collapse observed according to Postlethwait and Nelson (1957).

**Photographs**

All field and greenhouse pictures were taken with a Nikon D60 digital camera. No image colour manipulations were made unless otherwise stated with the exception of white balance on greenhouse and stem section pictures. Pictures were cropped for best representation of the material being observed.
Results

Genetic study of the $wi^*\text{-NM3219}$ wilted mutant

Genetics experiment 1. wilting frequency in $wi^*\text{-NM3219}$ mapping populations

The first major objective for the genetic study of $wi^*\text{-NM3219}$ was to determine the frequency of wilting plants in mapping populations from $wi^*\text{-NM32319}$ plants. In determining wilting frequency, the effects of environment (year effect) and genetic background were considered. Because the wilting phenotype in $wi^*\text{-NM3219}$ is proposed to be caused by a Mendelian, recessive mutation, wilting plants are expected at a 3:1 unwilted:wilted ratio in mapping populations. In addition, the wilted phenotype is expected to have high penetrance and expressivity between field seasons and across genetic backgrounds.

Populations were grown in summer nurseries to determine wilting plant ratios of $wi^*\text{-NM3219}$ populations. Figure 1 shows that $wi^*\text{-NM3219}$ x A619 $F_2$ populations segregated approximately 3:1 for wilting plants in 2008 and 2009. Mo17 $F_2$ populations from summer 2010 segregated about 3:1. In all cases, $F_2$ populations observed during this study had a wilting plant frequency of 25% or less (Figure 1). Observing that no population has more wilted plants than expected indicates that the responsible mutation may be recessive within a single Mendelian gene, but with poor penetrance or poor expressivity.

Observing that Mo17 $F_2$ populations (Figure 1) had 3:1 unwilted:wilted plant ratios, unwilted individuals were selfed for further genetic study. Seed derived from two
of three unwilted individuals in an F₂ population are expected to have a 3:1 unwilted:wilted ratio. Seed from three of four F₃ populations displayed the expected mutant ratio (3:1, Chi square statistic p<0.05) in summer 2011, while one population had a lower mutant count than expected (Figure 1, F₃ population 1). The data from F₃ plants is consistent with previous field observations of F₂ populations.

A single A619 F₂ population was grown in three field seasons to test for year-to-year frequency of wilting plants. It was hypothesized that the wilting plant phenotype would have stable expression across the tested field seasons. Figure 1 shows that A619 F₂ populations had 3:1 unwilted:wilted ratios in 2008 and 2009, but showed a decreased frequency of plants wilting in 2010. The wilting phenotype was expressed at differing frequencies between years within a single population. The environment or year appears to affect wilting plant frequency in wi*-NM3219 populations.

Plants from a wi*-NM3219 Mo17 F₃ population were observed in the greenhouse under well-watered conditions. The whole population had 1:2:1 genotypic segregation (Chi Square, 1:2:1, p<0.05, df=2) at umc1301 (Figure 2). Interestingly, plotting marker data by date of emergence revealed that for the 3 day emergence period, only day 6 showed 1:2:1 segregation. Day 7 did not show this ratio, and day 8 had insufficient data for computation of a Chi Square test statistic. Day 7 showed an increase in the Mo17 allele at umc1301 compared to the B73 (wi*-NM3219) allele. Overall the F₃ population used in the greenhouse experiment segregated 1:2:1, consistent with a Mendelian segregation ratio.
Genetics experiment 2. mapping the \( wi^{*-NM3219} \) locus

The first step in mapping the causative mutation is to apply genome-wide markers to a segregating population, allowing for determination of markers which show an enrichment for the \( wi^{*-NM3219} \) (B73) allele within mutant individuals. The populations chosen for single nucleotide polymorphisms (SNP) genotyping were A619 F\(_2\) populations grown in 2008 and 2009, as both were found to have 3:1 unwilted:wilted ratios (Figure 1). Seventy three individuals were genotyped with 69 genome-wide SNPs. SNP marker data were filtered for expected segregation (1:2:1) at each marker, then the subset of wilted individuals was tested for enrichment of the \( wi^{*-NM3219} \) (B73) allele (Table 1). The marker with the highest B73 allele count was found to be located near the tip of the short arm of chromosome 2. Several chromosome 4 markers were found to segregate differently than 1:2:1 (Chi square, 1:2:1 \( p<0.05 \)), however these markers also show an enrichment for the A619 allele, which does not confer the \( wi^{*-NM3219} \) wilting plant phenotype. Two markers on the long arm of chromosome 7 show an enrichment for the B73 allele, and are 25 Mb apart. These two markers could not be linked together using linkage mapping software, either because of significant recombination between markers or insufficient population size for linkage calculation. The C2 and C7 markers with B73 alleles that were over-represented in the mutant class are further explored below.

As a result of identifying two genomic regions putatively containing the \( wi^{*-NM3219} \) mutation, the short arm of chromosome 2 and the long arm of chromosome 7, further genotyping was required to rule out a false positive result from the SNP data. To test if the short arm of chromosome 2 contributed a causative mutation, the region
surrounding 006964|C2|27686619 was genotyped with SSR markers in a Mo17 F₂ population (88 individuals, 19 wilted). The null hypothesis was that marker alleles would segregate 1:2:1 both in the mutant class and across the entire population. Table 2 shows the SSR marker data for the entire population, and the mutant plant subset. Marker umc1542 was the only marker found to have segregation at a ratio different from 1:2:1 within the entire population. All markers tested in the wilted plant sub-population segregated 1:2:1, evidence supporting that the short arm of chromosome 2 does not contain the mutation conferring the wi*-NM3219 wilting plant phenotype.

Having found that the short arm of chromosome 2 is not linked to wi*-NM3219 wilting, the region of chromosome 7 identified through SNP analysis was genotyped with SSR markers. The genotyped population contained 274 Mo17 F₂ individuals, of which 54 were wilted plants. Eleven markers spanning the entire length of the long arm of chromosome 7 were assayed and a linkage map was constructed in JoinMap (Figure 3A). The total physical distance covered by the markers was 60.9 Mb, while the genetic distance was calculated to be 103.2 cM (Figure 3B). The genetic distances correlate well with the published Genetics 2008 Composite Recombination Map. Only the distal portion of the map is inflated in genetic distance, which may be attributed to the decreased marker density applied to this region coupled with the large genetic (vs physical) distance around this region of the chromosome. The plant phenotype data for this population was collected quantitatively, with two classes of wilting plants recorded. The wilting phenotype data and linkage map were run in MapQTL 5 to determine if there was a QTL present on the long arm of chromosome 7. QTL analysis determined the marker with the
The highest logarithm of odds (LOD) score was umc1301, at LOD 33.7 (Figure 3A). Given the high LOD score, this finding supports a single gene mutation as responsible for the wilting phenotype. The wilting plant phenotype in \textit{wi*-NM3219} segregating populations is associated with a genetic element linked to marker umc1301, mapping to the long arm of chromosome 7.

The marker data described was assessed directly in terms of marker segregation in both the whole population and wilted classes of individuals. Figure 4 shows the genotypic segregation of a Mo17 F$_2$ population with 274 individuals genotyped at 11 markers. All markers had the expected 1:2:1 genotypic segregation within the whole population. The mutant class does not display 1:2:1 segregation from markers umc2092 to phi328175. Within these markers, in the mutant population there is an enrichment of the B73 (\textit{wi*-NM3219}) parental allele. Marker umc1301 previously identified as being strongly associated with the phenotype does not have any Mo17 alleles within the wilted population and also has the highest frequency of B73 parental allele. Due to the small population size and marker limitations no further refinement of map position was possible using the summer 2010 Mo17 F$_2$ plants.

Genotypically heterozygous (at umc1301) Mo17 F$_2$ plants were selfed to create \textit{wi*-NM3219} F$_3$ populations for summer 2011. A total of 226 F$_3$ individuals were genotyped and phenotyped from these populations. Figure 5 shows the distribution of alleles at umc1301 and two flanking markers, idp8199 (152.4 Mb) and idp6850 (159.8 Mb). The whole population was found to segregate for allele frequencies at 1:2:1, as expected, while the wilted class did not segregate 1:2:1. Within the wilted class marker
umc1301 shows the highest frequency of the B73 (\textit{wi*-NM3219}) allele, and is the closest linked marker currently identified to the \textit{wi*-NM3219} mutation. The \textit{wi*-NM3219} mutation lies within a 7.4 Mb region between umc1301 (155 562 009 – 155 562 882 bp, genome rev_2) flanking markers: idp8199 (152.4 Mb) and idp6850 (159.8 Mb).

**Physiological study of wilted mutant \textit{wi*-NM3219}**

**Physiology experiment 1. Study of wilting expression and plant trait characterization**

In determining wilting onset and characterizing traits associated with wilting in \textit{wi*-NM3219} plants, it was found that field grown plants showed stunted growth (Figure 6D), and were unable to set seed, with a strong wilted phenotype even in wet field conditions. The \textit{wi*-NM3219} wilted plant phenotype was observed to be expressed several weeks before flowering and not until several fully developed leaves were present. Onset of wilting expression was hypothesized to occur after emergence and the seedling stage, before flowering. It was expected that wilted plants would have a decreased growth rate compared to unwilted plants; with a corresponding lower plant water content. To test these hypotheses, a greenhouse experiment with 120 seedlings and 40 fully grown plants was undertaken to study the developmental timing of wilting onset, and to characterize wilting plant traits.

A soil emergence phenotype was tested for with 120 F\textsubscript{3} seeds. No impairment in ability to emerge from soil was found, and eight days after planting 100\% of sown seeds
had emerged (Figure 2). As previously described, this population was genotyped at umc1301 (linked to \textit{wi*-NM3219} locus), demonstrating expected 1:2:1 genotypic population segregation emergence. Plants with the B73 (\textit{wi*-NM3219}) allele at umc1301 appeared to emerge earlier than those with the Mo17 allele. Plants which became wilted during development emerged earlier (Student's t-test, \textit{p}<0.05), with an average emergence time of 6.3 days, while plants which did not wilt emerged 6.8 DAP (Figure 7). This early emergence may be conferred by linkage of the emergence trait to the B73 (\textit{wi*-NM3219}) allele at the umc1301 marker, and may not represent a cause or effect of the wilting phenotype.

Ligule height of the seedlings grown to maturity (\textit{n}=40) was recorded at 15 and 21 days after planting to test if plants which wilted later in development had a decreased growth rate compared to unwilted plants. Seedlings which became wilted plants were significantly taller (Student's t-test, \textit{p}<0.05) at 15 days after planting (5.0±0.9 cm nonwilted, 6.1±1.2 cm wilted) and 21 days after planting (9.5±1.4 cm nonwilted, 10.6 ±1.3 cm wilted) compared to unwilted plants (Figure 8). As with emergence timing, the B73 (\textit{wi*-NM3219}) allele at umc1301 may confer earlier emergence than the Mo17 allele, and this earliness would confer the ligule height increase present in the future wilted plants.

Wilting in the greenhouse was defined as visible rolling of at least one fully emerged leaf. Plants (\textit{n}=40) were scored for leaf collar stage (V Stage) and plant wilting/leaf rolling every three days in order to determine the onset of plant wilting. Figure 9 shows the onset of wilting in the population by leaf collar stage. Wilting onset
ranged from V3 to V7, with four plants expressing wilting at the V6 stage. Thus, the onset of wilting occurs later than the seedling stage but before flowering occurred in the greenhouse, consistent with field observations. Interestingly some other characteristics were observed while measuring leaf collar stage of the plants, shown in Figure 6A-C. Several leaves on wilted plants were found to have light green stripes running along the vasculature (Figure 6A, 6C). The earliest wilting seedling in the greenhouse displayed wilting in leaf 5 as this leaf emerged from the whorl, with a light green colour (Figure 6B).

The effect of wilting on ligule height and stem diameter in greenhouse plants was measured throughout the greenhouse experiment. Figure 10 shows ligule height of the greenhouse population over 70 days of growth. Stem volume is shown in Appendix Figure 2, with a similar graph appearance to ligule height; variance in stem diameter measurements make the data noisier than ligule height measurements. Plants were found to develop normally until the onset of wilting, at which point leaf number and ligule height stagnate for the duration of development, as seen for ligule height in wilted plants (Figure 10). The two unwilted plants had growth curves representative of the remaining unwilted plants (not shown on graph). Wilted plants were not found to be shorter than unwilted plants until the onset of wilting, where they were found to stagnate developmentally. After the cessation of growth, wilted plants did not regain normal growth and remained shorter than unwilted plants. They did not proceed to flowering in the greenhouse experiment.

Wilting plants, which imply insufficient water to maintain normal turgor pressure,
were identified from among well-watered greenhouse plants. Student's t-test was used to compare unwilted and wilted samples for significant differences in water content between wilted and unwilted plants. Samples were categorized into three classes for comparison: above ground biomass, leaf tissue and shoot (Figure 11). Each wilted to unwilted comparison uncovered lower water content in wilting plants than unwilted plants. Leaf water content of wilted plants had the highest variability (44.8±22.7% water content) among the tested plant tissues, possibly due to tissue necrosis or early senescence of several wilted leaves at the time of sampling. All unwilted plants had consistent water content, demonstrating a high level of regulation for this trait. These results support the hypothesis that wilting plants have decreased water content compared to unwilted plants in all above ground tissues, demonstrating that wi*-NM3219 wilted plants are unable to maintain normal above ground water content under well-watered conditions.

**Physiology Experiment 2. Mechanisms of plant wilting**

Literature review of known wilting mutants has identified several mechanisms which may explain wilting visible wi*-NM3219. The hypothesis of this experiment was that wilting and abnormal leaf traits in wi*-NM3219 are caused by previously characterized mechanisms. This is tested by assessing two mechanisms known to function in pathways whose disruption results in plants that look similar to wi*-NM3219 mutant individuals, the wilted1 mutation and the high chlorophyll fluorescence (hcf) seedling phenotype.

The wilted1 metaxylem formation and development mechanism was studied in
plants; characterized by (Postlethwait and Nelson, 1957). Plants with the wilted1 mutation have characteristically stunted growth and upper leaf wilting due to retarded metaxylem development. To examine metaxylem structure and function in wi*-NM3219, stem sections were made of wilting and unwilted field grown plants. Stems were hand sectioned and stained with toluidine blue, then observed for characteristically deformed metaxylem elements, expected if plant wilting was due metaxylem formation defects as similar to wilted1. Sections from n=3 wilted B73 (wi*-NM3219), and n=3 unwilted siblings were examined for abnormal appearance of vascular bundles. No evidence of a delayed metaxylem development phenotype was visible in stem sections of wilted plants, with all examined vasculature appearing the same between wilting and unwilted plants (Figure 12A-C). From the stem sections observed, the mechanism of wilting in wi*-NM3219 does not appear to be the same as described in wilted1.

The second characteristic studied in wi*-NM3219 plants was chlorophyll fluorescence, described in terms of the characterized maize hcf mutants. Mutant hcf plants have small and yellowed leaves, a trait seen in wi*-NM3219 mutant plants. To test if wi*-NM3219 plants display an hcf mutant phenotype, 120 greenhouse F3 seedlings were subjected to long wave ultraviolet light and observed for colour of fluorescence emission. A red leaf emission is expected if plants are hcf mutant, as mutant plants are unable to quench fluorescence in photosystem II. Blue leaves are expected for non-mutant plants. Only blue leaf emission was visible in the greenhouse, with no evidence of a red leaf phenotype observed during this test. This evidence supports that the leaf traits observed in wi*-NM3219 are not due to an hcf type mutation.
Figure 1. Mutant frequency data for all field grown F$_2$ and F$_3$ populations with a wi*-NM3219 wilted plant as an F$_1$ parent. Significance calculated using Chi Square test for 3:1 unwilted:wilted plant ratio, data displayed as a percentage (p<0.05, df=1, * denotes significant deviation from expected 3:1 ratio). Populations from a single year are differentiated based on the selfed F$_1$ or F$_2$ individual used to produce the observed seed. Combined populations are the sum of all plants for the indicated cross, within the summer of observation.
**Figure 2.** Emergence timing (days after planting) of 120 seeds from an F$_3$ population genotyped at marker umc1301. 100% of sown seeds had emerged 8 days after planting. The results displayed represent 107 individual allele calls. Thirteen individuals could not be accurately genotyped. The whole population was found to segregate with a 1:2:1 allele ratio. Day 6 emerged seedlings segregated 1:2:1, while day 7 did not and 8 had insufficient data for calculation of a ratio. * denotes non-1:2:1 allele segregation, Chi Square test, p<0.05, df=2.
Table 1. Allele frequencies of SNP markers in an A619 F$_2$ mapping population

Sixty nine SNPs were tested for 1:2:1 allelic frequency in the entire population (Chi Square statistic, df=2, p<0.05, statistic over 5.99 is significant deviation from expected segregation), any SNPs which did not show 1:2:1 allele frequency were not considered for this analysis. SNPs were then arranged by Chi Square statistic within the class of wilted individuals, identifying individuals above the 5.99 significance cutoff, ie those individuals that did not show 1:2:1 allelic frequency in the wilted plant class. SNPs with an over-representation of the $wi^*-NM3219$ (B73) allele amongst wilted plants may be linked to the wilted plant phenotype. Marker name is in the form markerID|Chromosome|PhysicalPosition_rev1. Physical position is from maize genome sequence revision 1 (4a.53, released October 2009).

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Table 2. Marker data for Chromosome 2 short arm SSRs from 2010 Mo17 F₂ individuals

19 mutant and 69 wild type individuals were genotyped from 4.7-33.4 Mb on the short arm of chromosome 2. Chi square statistic (p<0.05, df=2, populations with * are significantly different from 1:2:1). Since no markers in the wilted sub-population were found to show an enrichment in the B73 (wi*-NM3219) allele, the region does not appear to be associated with the wilting plant phenotype.

<table>
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**Figure 3.** A) Linkage map of chromosome 7 long arm constructed from 11 SSR markers in a 2010 F2 mapping population (n=274 individuals). MapQTL graph of LOD score for markers listed, from quantitative trait data. B) Marker physical and genetic positions based on mapping data compared Genetics 2008 Composite Map, zeroed at umc2092.
Figure 4. Genotype data for the Mo17 F₂ population (n=274 total, n=54 wilted individuals), at 11 genotyped markers the long arm of chromosome 7. Bars with * are significantly different from 1:2:1 segregation (Chi Square test, df=2, p<0.05). Markers are linked together as shown in Figure 3A, but kept separate here to show allelic frequency at each marker.
Figure 5. Genotype data for Mo17 F₃ plants (n=226 total population, n=60 wilted) grown in summer 2011, genotyped at umc1301 and two flanking markers (idp8199 and idp6850). In the whole population the three tested markers show 1:2:1 allelic frequency as expected, while the mutant class markers do not display 1:2:1 allelic frequency (Chi Square Statistic, df=2, p<0.05, * denotes different ratio from 1:2:1). Marker umc1301 has the highest frequency of B73 (wi*-NM3219) alleles and the lower frequency of Mo17.
**Figure 6.** Pictures of wilting plant traits observed during greenhouse and field experiments with *wi*-NM3219 populations.

Figure 7. Emergence time (days after planting) of 40 plants, 10 which became wilted during development. Nonwilted plants had an average emergence time of 6.8 DAP while future wilted plants emerged earlier, 6.3 DAP (Student's t-test, p<0.05). Error bars represent standard error of the mean. Plants which wilted had earlier seedling emergence, a trait possibly conferred by the B73 (wi*-NM3219) allele at the umc1301 locus.
Figure 8. Top ligule height of 15 and 21 day old seedlings, comparing seedlings which later wilted to unwilted plants (n=40 seedlings total, n=10 wilted). Error bars represent standard error of the mean. At both 15 and 21 days after planting seedlings that will grow into wilted plants are significantly taller than plants that will not become wilted (Student's t-test, p<0.05). 15 DAP (5.0 cm nonwilted, 6.1 cm wilted), 21 DAP (9.5 cm nonwilted, 10.6 cm wilted).
Figure 9. Leaf collar stage (V stage) at onset of leaf rolling in 10 wilted F₃ plants which under well-watered greenhouse conditions. Four plants were found to express the wilting phenotype at V stage 6, with the range of expression stages from V3 to V7.
Figure 10. Top ligule height of F$_3$ greenhouse plants (filled circles) that wilted before day 69, compared to two unwilted plants (representative of the 30 unwilted plants measured, based on observations for plant wilting). Wilted plants stopped growing at the onset of the wilting and did not regain normal growth during the experiment. The unwilted plants are representative of all control unwilted plants observed in the experiment.
Figure 11. Water content (measured as % of fresh weight) of greenhouse grown F$_3$ plants. Samples were air dried to determine water content for comparison. All tissue types had significantly less water content in the wilted plants compared to unwilted (Student's t-test, 1 way, p<0.05, * denotes significance). Error bars show standard error of the mean. Above ground water content (90.6% unwilted, 82.7% wilted), shoot (94.2% unwilted, 88.8% wilted), leaf (78.8% unwilted, 44.8% wilted).
Figure 12. Stem sections of two wilted individuals, (A) and (B) showing normal metaxylem vessel morphology. An unwilted sibling (C) shows normal metaxylem vessel development as well. The sections are representative of n=3 wilted plants and n=3 unwilted siblings observed.
Discussion

Genetic study of the \( wi^*\)-NM3219 mutant

Identification of mutant plants in the field proved difficult in mapping populations for genetic study of the \( wi^*\)-NM3219 mutant. The frequency of wilting plants within mapping populations from \( wi^*\)-NM3219 parents was found to be variable across inbred backgrounds and environmental conditions. Year-to-year variation was found in replicated mapping populations. These results were somewhat surprising given the initial expectation of a strongly expressed 3:1 unwilted:wilted ratio of the wilted phenotype in 2008 and 2009 populations.

Many previous single gene studies have worked with highly penetrant mutations which are easily phenotyped across environmental conditions. The \( tdy1 \) mutant is an excellent example (Braun et al., 2006). Background genotype effects on gene and trait expression (Kaeppler et al., 2000) are also well documented in maize, and given the wide range of plant stress response pathways, it is reasonable to conclude that a mutation in a stress response gene could have varying effects between inbred backgrounds.

The results reported for \( wi^*\)-NM3219 characterization remain consistent with a single recessive Mendelian gene hypothesis, if the mutation is considered to be incompletely penetrant or have incomplete expressivity in homozygous \( wi^*\)-NM3219 plants. An incompletely penetrant gene does not always express a phenotype, even if a mutant allele may be present, where expressivity is a measure of the degree of phenotype expression. A wilted mutant with low expressivity may be indistinguishable from non-mutant plants under favourable environmental conditions. Evidence supporting a single
Mendelian gene comes from the observation of 3:1 unwilted:wilted segregating F₃ plants, given that these plants were offspring of an F₂ plant genotyped as heterozygous at a locus linked to wi*-NM3219. The difficulty in observing the expected segregation ratios may be due to the water stressed phenotype of mutant plants. Normal plants may express wilting under dry field conditions, and conversely it may be possible for a mutant plant to appear unwilted if the environmental conditions allow the plant enough water for normal growth. Wilting was also visible in greenhouse grown well-watered plants, though not at the frequency expected from field observations.

Observation and genotyping of the greenhouse seedlings for emergence timing found that the F₃ population segregated 1:2:1 at umc1301 as a whole population, but interestingly seedlings emerging on different days had skewed genotypic make-up. Seedlings with the B73 (wi*-NM3219) allele in the greenhouse tended to have earlier emergence than Mo17. Given that seedling health was thought to be negatively affected by the wi*-NM3219 allele, it is surprising that this allele did not negatively affect emergence and no negative seedling phenotypes were observed. Genotype specific emergence timing is common in maize (Eagles and Brooking, 1981), and through selectively considering the population by an individual allele at a given marker, there may be inadvertent selection for an emergence or growth trait. In general Eagles and Brooks concluded (1981) that maize germplasm from northern environments with earlier maturity showed earlier emergence. In the context of this experiment there appears to be no influence of early emergence in B73 (wi*-NM3219) individuals on the expression of plant wilting, but it is an important note to make when considering future trait
measurement such as plant height and stem volume.

**Future segregation study of **\textit{wi*-NM3219} **mutants**

Because the frequency of the wilting trait was found to be 25% or less in all mapping populations, future segregation studies require larger and more genetically pure populations for study. The mutant individuals originally used for crossing in this project were not back-crossed for several generations which would have been desirable for isolating direct mutant effects from background ethyl methanesulfonate (EMS) effects. Though initial populations showed 3:1 phenotype segregation, once larger populations were used it was found that 3:1 unwilted:wilted ratio was not stable across environments and genetic backgrounds. Back-crossing of the \textit{wi*-NM3219} mutant has been started in B73, A619 and Mo17 inbreds, but these lines will not reach desired homozygosity for several field seasons. Segregation should be re-assessed once fully back-crossed populations are available for study.

**Mapping the **\textit{wi*-NM3219} **mutant**

Mapping of the \textit{wi*-NM3219} mutant locus made use of genome-wide single nucleotide polymorphism (SNP) genotyping to identify markers co-segregating with the mutant phenotype. This initial process uncovered two putative locations for the locus, further tested with microsatellite markers for confirmation of association. As the SNP density used for mapping \textit{wi*-NM3219} left large genomic regions between markers, it was fortunate to discover markers associated with the phenotype. Future mapping using
this technique would benefit from increased marker density and working with a more favourable genetic background. A B73xMo17 cross would allow for leveraging the vast maize genetics resources available to researchers. The rough mapping shown here in A619 populations was successful however, allowing for further fine mapping of the \( wi^-\)NM3219 locus.

Microsatellite marker confirmation of the SNP loci found to be associated with the wilting plant phenotype occurred in a B73 (\( wi^-\)NM3219) x Mo17 F\(_2\) population with 274 individuals. The short arm of chromosome 2 was ruled out as a false positive in the SNP data, which may be a result of not using a multiple testing procedure for the Chi Square results from SNP genotyping. The long arm of chromosome 7 was found to have a strong association with the wilting plant trait. Both QTL mapping and independent marker association highlighted the strong association of SSR marker umc1301 with the wilting phenotype in \( wi^-\)NM3219 plants. Marker evidence from mapping supports the hypothesis that the wilting plant phenotype is conferred by a single Mendelian recessive mutation. Mapping the mutation in \( wi^-\)NM3219 has proven difficult due to the incomplete penetrance or incomplete expression of the wilting phenotype. Hypothesizing incomplete expressivity of the wilting phenotype, it may be possible to identify other traits associated with wilting which would allow for accurate phenotyping of segregating populations. Future mapping may rely solely on mutant individuals for narrowing the interval of the mutant locus, eliminating \( wi^-\)NM3219 mutant individuals which do not wilt and develop normally.

Genetic maps created during the mapping process were found to correlate well
with known linkage maps established in maize (MaizeGDB Genetics 2008 Composite Map). The linkage map created for the long arm of chromosome 7 had expansion towards the tip (Figure 3A), possibly due to the increased recombination in this region of the genome combined with the low marker density used in this study. The physical distance between markers in this region is short (IDP6850 to umc1408 is 11 Mb, but 56 cM), but establishing linkage proved difficult given the large genetic distance. The population size and marker density was sufficient for completion of the linkage map, linking the wilting phenotype to the umc1301 marker.

**Future mapping considerations**

Given that the \textit{wi*-NM3219} locus has been localized to the long arm of chromosome 7, further mapping efforts will focus on refining the identified map position. To complete the fine mapping of \textit{wi*-NM3219}, it is important to increase the number of mutant plants available for genotyping. Larger F\textsubscript{2} populations will be required for genotyping, given the described issues with penetrance or expressivity resulting in lower wilting plant frequency than expected. Currently F\textsubscript{4} populations segregating for the wilting trait are in the field and will be useful in fine mapping \textit{wi*-NM3219}. In order for positional cloning to make best use of the large populations being genotyped, future marker work must decrease the possibility of false positive allele calls. False positive allele calls occur when an allele within an individual is called a genotype which it is not, a major issue when using mutant individuals to rule out genomic segments via markers. One method of going about this would be to move away from gel based markers and use
dye-tagged microsatellite markers or SNPs. An incorrectly called gel based marker may lead to ruling out an important genetic interval, setting back mapping progress (Hackett and Broadfoot, 2003). With SNPs or dye-tagged microsatellites the possibility of a miscall is much lower, as there is no ambiguity in terms of fragment length or amplified allele colour. The worst case scenario using a newer technology is that an individual will be a no call, and not contribute to mapping progress.

**Physiological characterization of wi*-NM3219 traits**

Identification of traits related to wilting plant development in wi*-NM3219 was a key objective in this thesis. The first trait characterized was seedling emergence. Plants that later became wilted emerged earlier than plants that were normal throughout development. From the initial field experiments it was hypothesized that the decreased frequency of wilted plants could be due to a germination or seedling trait affected in wi*-NM3219 plants. Interestingly it was found that plants which wilted emerged earlier than unwilted plants. Several possibilities may explain this earliness, including the over-representation of B73 (wi*-NM3219) allele at umc1301 compared to Mo17 allele. This early emergence in B73 (wi*-NM3219) plants may play a key role in determining future plant wilting status. It is hypothesized that mutant plants which wilt during development may be expending their energy to grow quickly as seedlings, causing developmental issues later during vegetative growth such as decreased root biomass.

Plants that became wilted later in development were also not impaired in early seedling growth. Instead they were taller than their unwilted counterparts. This was an
unexpected result, given that wilted plants are stunted and short later in development. Given that the ligule height measurements were recorded in terms of days after planting (DAP) instead of days after emergence (DAE), earlier emerging mutant individuals appear taller than expected. Future work in segregating populations should choose to focus on DAE as a baseline for plant comparison, as a single day of extra growth can be significant when observing plants less than two weeks after planting. If data had been collected every day for the greenhouse populations, a days after emergence comparison would have been possible. However, as data was collected with off days, it is difficult to model the ligule height for the unmeasured days for comparison.

Wilting onset was found to occur between the V3 and V7 stage of plant development (Figure 4). The V6 stage had four plants express the wilting phenotype, the highest frequency of any stage observed. These results fit with the expectation that wilting would occur after several fully emerged leaves were present and before flowering. The range of wilting onset is fairly large, occurring before and after the V5 stage. Onset is unlikely linked to the vegetative to floral transition (Irish and Nelson, 1991). Wilting in wi*-NM3219 mutants may be linked to plant size or an external stimulus. Because these observations have only been made in greenhouse conditions the onset times may differ in field grown populations given the more extreme light and temperature conditions.

At the onset of wilting, wi*-NM3219 plants stopped increasing in ligule height and leaf number with no growth recovery. This finding is contrary to the growth data described for wilted1 mutant plants (Postlethwait and Nelson, 1957), where metaxylem development problems impair vegetative plant growth, but plants are able to continue
slow growth. Eventually metaxylem elements mature, allowing mutant plants to proceed to flowering. Interestingly under field conditions wilted plants are sometimes able to produce a tassel, which was not observed in the greenhouse. Future work should focus on measuring developmental traits in field grown plants, as the field may provide the stimuli needed to elicit the full range of mutant phenotypes.

Measurement of plant water content showed that wilted wi*-NM3219 plants had lower water content than unwilted plants. Other research has observed lower water content in wilted mutant plant leaves (Rock and Ng, 1999), but the mutants observed in that study are not thought to be allelic to wi*-NM3219 mutants due to differing map positions, or have a similar mechanism of wilting due to differing wilting expression and development in the field. Leaf tissue of wilting plants was found to have the largest range of measured water content, likely a result of leaf necrosis or senescence in wi*-NM3219 mutants. Unfortunately not enough mutant leaf tissue was available to make additional water content measurements which would have allowed for differentiating water content of necrotic or senescenting tissue from wilting green leaf tissue. Stem water content was found to be lower in mutant plants. It is not possible to determine whether the stem water content is lower as a result of the leaf water loss or an unaccounted for factor, such as a root development defect. Further work is required to assess the root development and root water content of wi*-NM3219 mutants, which will help to determine if roots are causing the above ground water content decrease or if a leaf defect is responsible for wilting. As well, other methods of determining visible water status may prove helpful in the study of wilting mutants (Engelbrecht et al., 2007) for large scale wilting characterization. Work is
underway in summer 2012 to quantify leaf water loss in mutant plants.

The metaxylem formation mutation described in *wilted1* plants was assessed in *wi*-NM3219 plants. Stem sections were assessed visually for metaxylem element defects. One area of improvement for future comparisons of *wilted1* and *wi*-NM3219 would be to use *wilted1* mutant plants as controls in stem section observations. No *wilted1* plants were available at the time of sectioning for comparison. No evidence of the vessel defects reported by Postlethwait and Nelson (1957) were found in *wi*-NM3219 wilting plants. Additionally *wi*-NM3219 mutants do not appear to have similar field growth characteristics to *wilted1* mutants, and both occupy different genetic map positions. Further tissue sectioning could be used in *wi*-NM3219 plants to characterize other growth characteristics including cell length in transverse sections, vascular bundle density and cellular root morphology.

The high chlorophyll fluorescence (*hcf*) trait was assessed in greenhouse *wi*-NM3219 populations. Wilted plants were found to have normal fluorescence. The prevalence of *hcf* mutants with genome-wide map positions, (over 130 identified (Miles, 1994)) and leaf phenotypes similar to *wi*-NM3219 made *hcf* a logical trait to test. Unfortunately at the time of *hcf* phenotype testing no *hcf* mutant plants were available as positive controls. No cause for the light coloured leaf sectors in *wi*-NM3219 which led to the *hcf* characterization has yet been identified. Further characterization of mutant phenotypes in *wi*-NM3219 plants could analyze the light yellow leaf striping observed in mutant plants.
**Future physiological work**

Several avenues of future research are available for the characterization of *wi*-NM3219 mutant plants. This project did not assess any root characteristics of mutant plants, which may help in understanding why wilting occurs in well watered plants. Given the difficulty in studying root traits few root mutants have been identified in maize (Hochholdinger et al., 2004). Root weight was a trait for which quantification was attempted in the F$_3$ greenhouse experiment, however due to long term growth in the three gallon pots, it was not possible to separate roots from growth media for measurement. Given the extreme wilting visible under well-watered conditions, it is unlikely that a morphological or root penetration phenotype would be responsible for the above-ground wilting. A cellular or internal root feature may be responsible for plant wilting in light of this.

Of the leaf phenotypes observed during this project, leaf rolling appeared to be associated with the pale leaf sectors in some mutant plants. The relationship of these pale sectors to wilting is unclear, but the light leaf colour may indicate damage to the photosynthetic machinery of the plant (Covshoff et al., 2008). This damage could be in the form of photochemical bleaching due to exposure to higher levels of solar radiation than can be dissipated by regular coping mechanisms. One consideration is that the *wi*-NM3219 mutant has no early growth phenotype, so it is unlikely a constitutively expressed mutation early in development. The wilting phenotype may be activated through developmental cues as the plant matures.

Other leaf mechanisms are available for study, including the wax composition and
development pathways. Wax structure is implicated in wilting in several wilted rice mutants and has not been identified in maize (Park et al., 2010). Future work could study the wax composition and development in \textit{wi*-NM3219} plants.
Summary

This project has generated interesting results during the study of the \textit{wi*-NM3219} wilting mutant. Through genetic study of populations with the \textit{wi*-NM3219} allele, the frequency of mutant plants was found to be consistent with a single gene mutation conferring the wilted plant phenotype. The mutant locus has been mapped to a 7.4 Mb region on the long arm of chromosome 7, with the most tightly linked marker identified being umc1301 (155 562 009 – 155 562 882 bp, genome rev_2).

The wilting plant phenotype was found to be expressed between the V3 and V7 stages of growth. Ligule height increase stopped at the onset of wilting, even under well-watered growth conditions. Water content in wilted plants was lower than unwilted plants. No evidence of a metaxylem development phenotype was identified in \textit{wi*-NM3219} mutant plants.

Directions for future work include fine mapping the mutant locus to a candidate gene, then studying gene expression levels and tissue specificity. To further study the physiology of \textit{wi*-NM3219} mutants, root growth traits may prove useful to characterize. Additionally there are several wilting mutants in non-maize species which may reveal novel wilting mechanisms for the study of wilting maize plants.


defective mutant. Plant Physiology 146, 1469–1481.


Eaton, F.M. (1941). Water uptake and root growth as influenced by inequalities in the concentration of the substrate. Plant Physiology 16, 545.


Appendix. Supplemental tables and figures from mapping and characterization of

\textit{wi*-NM3219} wilted plants
Figure 1. Schematic of initial NM3219 population and major populations created for mapping experiments. A) Initial NM3219 mutagenized seed and seed pack segregation results. B) A619 F₂ mapping population parents and field seasons tested. C) Mo17 F₂ mapping population and founder lines for F₃ and F₄ populations under current study.
**Procedure 1. DNA Extraction Protocol – Two Tube Method**

**Chemicals:**

Extraction Buffer  
100 mM Tris-HCl pH 8.0  
50 mM EDTA  
100 mM NaCl  

Potassium Acetate Solution  
5 M KAC  

Isopropanol  

70% Ethanol  

**Procedure:**  

1. In a screw cap 2 mL tube add plant tissue (thumbnail size piece), beads for fast prep machine and 500 uL of extraction buffer  

2. Fast prep for 2 rounds of 40 seconds. Ensure tissue is completely broken up, add more time if required.  

3. Add 130 uL of ice cold KAC to tubes, mix samples by inversion. Place on ice for ~25 minutes.  

4. Centrifuge tubes at max speed (~16 000 g's) for 10 minutes. Transfer 500 uL of supernatant to a new tube, discard old tube.  

5. Add 350 uL of isopropanol to new tube with supernatant. Centrifuge at max speed for 10 minutes. A pellet should be visible in these tubes.  

6. Discard supernatant, leaving the pellet. Add 350 uL of 70% ethanol, swirl tube to wash pellet. Discard ethanol after wash. Place tubes with pellet in vacuum bell for 10 minutes to evaporate any remaining ethanol.  

7. Suspend dried pellet in 100 uL of 65 C MBG water. If pellet is slow dissolving leave tubes in fridge overnight to dissolve pellet before freezing tubes.  

Dilute or use 1 uL for PCR applications.
Procedure 2. Modified Plate-Based DNA Extraction

Procedure:

1. Place ~10mm² leaf tissue in 2mL 96-well plate. Cover with cap mat.
2. Add 400 uL lysis buffer to each well
3. Add 1 steel grinding bead to each well, seal with mat and tape down
4. Homogenize plate at 20Hz, run twice for 40 seconds, rotating plates between runs
5. Centrifuge at 5000g's for one minute
6. Incubate at 65C for one hour
7. Centrifuge 1 minute at 5000gs
8. Transfer 100uL lysate to 96 -well DNA plate
9. Add 200uL binding buffer
10. Mix with pipette, transfer 300uL lysate to binding plate
11. Vacuum for 2 minutes at 23 In Hg using vacuum manifold
12. Add 400uL first wash buffer to binding plate, vacuum for 3 minutes as above
13. Add 750uL second wash buffer to binding plate, vacuum for 10 minutes
14. Centrifuge at 5000g for 1 minute
15. Incubate at 56C for 30 minutes
16. Add 100uL 56C MBG water to binding plate
17. Centrifuge for 5 minutes at 5000g to elute DNA

Wide bore pipette tips can be used to prevent clogging during lysate transfer steps.

Reagents:

A) 6M GuSCN Stock (100mL)
- 70.9g GuSCN
- 4mL 0.5M EDTA
- 2mL of 0.5M Tris-HCl (pH 6.4)
- 4mL Triton X-100
- 90mL MBG water

B) Lysis Buffer
- 10g CTAB
- 100mL 0.5M Tris-HCl (pH 8.0)
- 140mL 5M NaCl
- 240mL MBG water

C) Binding Buffer
- 80mL GuSCN stock
• 16mL MBG water
D) First Wash Buffer
• 50mL GuSCN stock
• 50mL 100% ethanol

E) Second Wash Buffer
• 300mL 100% ethanol
• 10mL 0.5M Tris-HCl (pH 7.4)
• 500uL 0.5M EDTA
• 189.5mL MBG water
Procedure 3. PCR microsatellite recipe

**Mastermix volumes** (per individual 15 uL reaction):

<table>
<thead>
<tr>
<th>component</th>
<th>volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCR buffer</td>
<td>1.5 uL</td>
</tr>
<tr>
<td>MgCl₂</td>
<td>1.5 uL</td>
</tr>
<tr>
<td>10 mM dNTP</td>
<td>0.4 uL</td>
</tr>
<tr>
<td>H₂O</td>
<td>8.2 uL</td>
</tr>
<tr>
<td>primer</td>
<td>2 uL</td>
</tr>
<tr>
<td>taq polymerase</td>
<td>0.4 uL</td>
</tr>
<tr>
<td>template DNA</td>
<td>1 uL</td>
</tr>
<tr>
<td><strong>total</strong></td>
<td><strong>15 uL</strong></td>
</tr>
</tbody>
</table>

JumpStart Taq DNA Polymerase (Sigma-Aldrich, Inc.) and Sigma-Aldrich PCR component used in all reactions.

**Cycling Steps:**
Initial Melt: 94C (5 minutes)
Touchdown: 95C (60s) melt, 65C-56C (60s) anneal in 1C increments, 72C (90s) amplification
Amplification: 95C (30s) melt, 55C (30s) anneal, 72C (40s) amplification
Final Extension: 72C (5 minutes)

Visualized on 3% agarose gels, stained with ethidium bromide.
Table 1. Greenhouse corn fertilizer for automatic irrigation

Fertilizer solution was mixed in 100L batches. The component weights given are for 100L of concentrated solution. The concentrated solution described below was fed directly into the automatic irrigation system at a 5% dosing rate (20:1 water:nutrient solution). Plants were watered exclusively with this nutrient solution once irrigation was initiated.

<table>
<thead>
<tr>
<th>component</th>
<th>weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>28-14-14</td>
<td>800g</td>
</tr>
<tr>
<td>15-15-30</td>
<td>800g</td>
</tr>
<tr>
<td>NH₄NO₃</td>
<td>400g</td>
</tr>
<tr>
<td>Plant Prod Micro</td>
<td>60g</td>
</tr>
<tr>
<td>MgSO₄·7H₂O</td>
<td>80g</td>
</tr>
</tbody>
</table>
Table 2. Wilting plant frequency data for multiple environments and years (* denotes significant deviation from 3:1 ratio @ p=0.05 tested by Chi Square with df=1). Populations have all pedigree data and seed IDs listed for comparison to pedigree information.

<table>
<thead>
<tr>
<th>Year</th>
<th>Population</th>
<th>F1 Parents</th>
<th>F1 Seed Pack</th>
<th>F2 self (seed id)</th>
<th># mutant</th>
<th># total</th>
<th>% mutant</th>
<th>Chi Square</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>2008</td>
<td>A619</td>
<td>S07 LL-279-1 (mutant)</td>
<td>W08 LEE 352-1</td>
<td>-</td>
<td>7</td>
<td>28</td>
<td>25.00%</td>
<td>0</td>
<td>F2 mapping population in A619 background</td>
</tr>
<tr>
<td>2009</td>
<td>A619</td>
<td>S07 LL-279-1 (mutant)</td>
<td>W08 LEE 352-1</td>
<td>-</td>
<td>8</td>
<td>45</td>
<td>17.78%</td>
<td>1.25</td>
<td>F2 mapping population in A619 background</td>
</tr>
<tr>
<td>2008 + 2009</td>
<td>A619</td>
<td>S07 LL-279-1 (mutant)</td>
<td>W08 LEE 352-1</td>
<td>-</td>
<td>15</td>
<td>73</td>
<td>20.55%</td>
<td>0.77</td>
<td>Combined F2 population for SNP genotyping</td>
</tr>
<tr>
<td>2010</td>
<td>Mo17</td>
<td>S08 LL-522 (mutant)</td>
<td>S09 1163</td>
<td>-</td>
<td>54</td>
<td>242</td>
<td>22.31%</td>
<td>0.93</td>
<td>F2 mapping population in Mo17 background</td>
</tr>
<tr>
<td>2010</td>
<td>A619</td>
<td>S07 LL-279-1 (mutant)</td>
<td>W08 LEE 352-1</td>
<td>-</td>
<td>18</td>
<td>233</td>
<td>7.73%</td>
<td>37.08*</td>
<td>F2 mapping population in A619 background</td>
</tr>
<tr>
<td>2010</td>
<td>A619</td>
<td>S07 LL-279-1 (mutant)</td>
<td>W08 LEE 352-2</td>
<td>-</td>
<td>8</td>
<td>137</td>
<td>5.84%</td>
<td>26.82*</td>
<td>F2 mapping population in A619 background</td>
</tr>
<tr>
<td>2010</td>
<td>A619</td>
<td>S07 LL-279-1 (mutant)</td>
<td>W08 LEE 352-3</td>
<td>-</td>
<td>25</td>
<td>230</td>
<td>10.87%</td>
<td>24.49*</td>
<td>F2 mapping population in A619 background</td>
</tr>
<tr>
<td>2010</td>
<td>A619</td>
<td>S07 LL-279-1 (mutant)</td>
<td>W08 LEE 352-7</td>
<td>-</td>
<td>4</td>
<td>51</td>
<td>7.84%</td>
<td>8.01*</td>
<td>F2 mapping population in A619 background</td>
</tr>
<tr>
<td>2010</td>
<td>A619</td>
<td>S07 LL-279-1 (mutant)</td>
<td>combined A619</td>
<td>-</td>
<td>55</td>
<td>651</td>
<td>8.45%</td>
<td>95.12*</td>
<td>combined F2 A619 population</td>
</tr>
<tr>
<td>2011</td>
<td>Mo17</td>
<td>S08 LL-522 (mutant)</td>
<td>S09 1163</td>
<td>S10 LL-564-8 (S10 519)</td>
<td>20</td>
<td>81</td>
<td>24.60%</td>
<td>0.84</td>
<td>F3 mapping seed from F2 Mo17 non-mutant</td>
</tr>
<tr>
<td>2011</td>
<td>Mo17</td>
<td>S08 LL-522 (mutant)</td>
<td>S09 1163</td>
<td>S10 LL-074-5 (S10 520)</td>
<td>15</td>
<td>70</td>
<td>21.43%</td>
<td>0.48</td>
<td>F3 mapping seed from F2 Mo17 non-mutant</td>
</tr>
<tr>
<td>2011</td>
<td>Mo17</td>
<td>S08 LL-522 (mutant)</td>
<td>S09 1163</td>
<td>S10 LL-078-4 (S10 524)</td>
<td>18</td>
<td>83</td>
<td>21.69%</td>
<td>0.49</td>
<td>F3 mapping seed from F2 Mo17 non-mutant</td>
</tr>
<tr>
<td>2011</td>
<td>Mo17</td>
<td>S08 LL-522 (mutant)</td>
<td>S10 LL-079-2 (S10 525)</td>
<td>7</td>
<td>66</td>
<td>10.61%</td>
<td>7.29*</td>
<td>F3 mapping seed from F2 Mo17 non-mutant</td>
<td></td>
</tr>
<tr>
<td>2011</td>
<td>Mo17</td>
<td>S08 LL-522 (mutant)</td>
<td>S09 1163</td>
<td>combined Mo17 F3</td>
<td>60</td>
<td>286</td>
<td>20.98%</td>
<td>2.47</td>
<td>combined F3 mapping population</td>
</tr>
<tr>
<td>Greenhouse</td>
<td>Mo17</td>
<td>S08 LL-522 (mutant)</td>
<td>S09 1163</td>
<td>S10 LL-564-8 (S10 519)</td>
<td>10</td>
<td>40</td>
<td>-</td>
<td>-</td>
<td>F3 mapping seed in greenhouse</td>
</tr>
</tbody>
</table>
Figure 2. Stem volume of greenhouse F₃ plants. The trend in this figure is similar to the ligule height graph. Variability in stem diameter measurements is the cause of the noise in stem volume calculations. 10 wilted plants and 2 unwilted plants (representative of 30 unwilted plants) are shown.