THE ROLES OF *VANISHING TASSEL2* AND *DEVELOPMENTAL DISASTER1* IN MAIZE VEGETATIVE AND INFLORESCENCE DEVELOPMENT

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Biology

by

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ABSTRACT

Plant hormones control growth and development through the activity of genes that regulate their biosynthesis, transport, and response. Auxin has been found to play a crucial role in many aspects of growth, including cell division and expansion, flower development, adventitious root growth, tropisms, vascular development, and leaf phyllotaxy. Many genes that play a role in auxin-mediated growth and development have been identified in maize. In this thesis, the genetic regulation of maize vegetative and reproductive development will be examined, with a particular focus on the roles of auxin in these processes.

The importance of polar auxin transport has previously been demonstrated in maize through the barren inflorescence2 (bif2) mutant. bif2 is co-orthologous to PINOID (PID) of Arabidopsis, which functions in the subcellular localization of the auxin efflux carrier PINFORMED1 (PIN1). pin, pid, and bif2 mutants all exhibit severe defects in reproductive development, including lack of flowers in pin and pid in Arabidopsis, and lack of branches and production of few or no spikelets in bif2 in maize. Another maize mutant, barren stalk1 (bal), shows a reproductive phenotype similar to bif2, however bal mutants have been shown to transport auxin normally. bal was instead found to play a role in initiating an appropriate response to auxin, as mutants fail to initiate axillary meristems despite having the required levels of auxin at sites of meristem initiation. Finally, the maize mutant spi1 has recently been shown to function in auxin biosynthesis. spi1 mutants exhibit dramatic defects in both vegetative and reproductive development, including reduced height, leaf number, branch number, spikelet number, and kernel number.

In Chapter 2, the characterization and cloning of vanishing tassel2 is presented. vt2 mutants resemble spi1 mutants, with defects in both vegetative and reproductive development. vt2:spi1 double mutants exhibit an additive genetic interaction, suggesting they function
independently in maize development. Conversely, \textit{vt2;bif2} double mutants show a severely synergistic interaction in both vegetative and reproductive growth, indicating the crucial role of both auxin biosynthesis and auxin transport in maize development. Cloning of the \textit{vt2} locus has revealed that it encodes a tryptophan aminotransferase required for auxin biosynthesis.

In Chapter 3, the characterization of the novel maize mutant \textit{Developmental disaster1} is presented. Unlike the other mutants described which are recessive, \textit{Dvd1} mutants are semidominant. Similar to \textit{vt2}, \textit{Dvd1} mutants also show severe defects in vegetative and reproductive development. However, scanning electron microscopy reveals that \textit{Dvd1} inflorescence phenotypes share more similarity with \textit{bal} mutants than with \textit{vt2}. In addition, the genetic interaction of \textit{Dvd1} with \textit{bif2} has revealed that the two genes function independently in maize development, unlike the interaction of \textit{vt2} with \textit{bif2}. Additional results, including current \textit{Dvd1} mapping data and genetic interactions with other maize mutants, are presented in Appendix A. Future positional cloning of the locus will reveal the nature of this mutation and further elucidate the critical role that \textit{Dvd1} plays in maize development.

Characterization of the \textit{vt2} and \textit{Dvd1} phenotypes illustrates that mutants with similar severe defects in vegetative and reproductive development can have different underlying mechanisms. The cloning of \textit{vt2} is a significant contribution to the field since it reveals the importance of one of the Tryptophan-dependent auxin biosynthesis pathways for the first time in monocots. The mapping of \textit{Dvd1} provides the groundwork for its cloning in the future.
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CHAPTER 1

The role of auxin in Arabidopsis and maize vegetative and reproductive development
1.1 Introduction

Hormones are chemical messengers known to regulate growth and development in multicellular organisms. Phytohormones play a crucial role in plant life cycles since plants lack mobility and must quickly adapt to environmental changes in order to survive (Taiz and Zeiger, 2006). Thus, the proper synthesis, transport, and signaling of many different hormonal compounds are vital for proper vegetative and reproductive development in plants. Auxin is one of the most important plant growth hormones and is most commonly found in the form of indole-3-acetic acid (IAA). Throughout development, auxin is known to control such processes as cell division and elongation, apical dominance, shoot growth, leaf initiation, phyllotaxy, fruit development, gravity and light tropisms, and lateral root initiation.

Although IAA has been the most thoroughly studied auxin in plants, elucidation of its biosynthetic pathways has not yet been completed. It is known that IAA is produced both by release from other conjugates and through de novo synthesis (Bartel, 1997). Biochemical studies have been performed to identify the enzymes and intermediates involved in de novo synthesis, and although not every step has been confirmed, some genes functioning in the different pathways have been identified in bacteria and plants. IAA is produced from the aromatic organic compound indole either with or without utilization of the amino acid tryptophan (Trp) as an intermediate, deemed the Trp-dependent and Trp-independent pathways of auxin biosynthesis. Evidence for Trp-independent synthesis has been found in both Arabidopsis and maize, and is predicted to occur directly from indole or from indole-3-glycerol (Wright et al., 1991; Woodward and Bartel, 2005). Within Trp-dependent biosynthesis, four pathways entailing different enzymes and intermediates have been predicted. The pathways are characterized by the key intermediates they are thought to employ: indole-3-acetaldoxime (IAOx), indole-3-acetamide (IAM),
tryptamine (TAM), and indole-3-pyruvic acid (IPA) (Bartel, 1997; Sugawara et al., 2009). A summary of the proposed Trp-dependent auxin biosynthesis pathways in Arabidopsis, including identified enzymes and the genes that encode them, is illustrated in Figure 1-1 (adapted from Sugawara et al., 2009).

After synthesis, the efficient transport of auxin is crucial for obtaining a long-distance response in other tissues of the plant (Petrasek and Friml, 2009). Unlike other plant hormones, auxin has been shown to move in a specific unidirectional manner throughout the plant, known as the polar auxin transport system. This phenomenon occurs through the action of cellular efflux carriers such as the PINFORMED (PIN) proteins, and their associated regulators such as the PINOID (PID) protein kinase. Following polar transport, auxin signaling can induce or repress auxin-related genes to achieve physiological responses (reviewed in Santner and Estelle, 2009). This signal transduction involves F-box proteins which function in a SCF complex, such as the auxin receptor TRANSPORT INHIBITOR RESPONSE (TIR1), as well as AUXIN/INDOLE-3-ACETIC ACID (Aux/IAA) and AUXIN RESPONSE FACTOR (ARF) transcription factors.

A great deal of work has been completed in recent years to identify the genes that function in auxin biosynthesis, transport and signaling, particularly in the model systems of Arabidopsis and maize. The mutant phenotypes associated with these genes have helped reveal the important roles they play in auxin-mediated growth and development in plants.

1.2 The role of auxin biosynthesis in Arabidopsis vegetative and reproductive development

*Arabidopsis thaliana* serves as a model dicot system for studying the genetic regulation of plant development. Scientists have utilized Arabidopsis for extensive genetic experiments due to its fast life cycle, small genome size, and the ease with which it can be grown and used to produce transgenic plants (reviewed in Pang and Meyerowitz, 1987).
Some of the predicted auxin biosynthesis pathways have been confirmed, and in some cases modified, through recent Arabidopsis genetic studies. In the first step of the indole-3-acetaldoxime (IAOx) pathway, Trp is converted to IAOx. Two genes, \textit{CYP79B2} and \textit{CYP79B3}, have been cloned and found to encode cytochrome P450 enzymes which catalyze this reaction (Zhao et al., 2002). At increased temperatures, \textit{cyp79B2;cyp79B3} double mutants exhibit a phenotype with reduced stature and hypocotyl length similar to what would be expected for a plant that is deficient in IAA, and decreased levels of IAA were found in these mutants (Zhao et al., 2002). After conversion to IAOx, it is predicted that the indole-3-acetonitrile (IAN) intermediate is produced, although the enzymes for this conversion and the genes which encode them have yet to be identified. In the final step of the pathway, IAN is converted to IAA by the enzyme nitrilase, which is encoded by the \textit{NITRILASE1/2/3} genes (Pollmann et al., 2006). \textit{nit1} mutants do not exhibit strong morphological phenotypes (Normanly et al., 1997).

In the indole-3-acetamide (IAM) pathway, it is predicted that Trp is converted to IAM, which in turn is converted to IAA. Genes regulating the initial conversion of Trp were previously detected in bacteria (Comai and Kosuge, 1982) and a recent study by Pollmann et al. (2009) found evidence that this step of the pathway is present in Arabidopsis and other plant species as well. In the final conversion of IAM to IAA, the Arabidopsis \textit{AMIDASE1} (\textit{AMI1}) gene has been shown to encode a protein similar to amidase proteins of bacteria which are known to hydrolyze IAM (Pollmann et al., 2003), suggesting \textit{AMI1} may regulate this step of the pathway in Arabidopsis.

The tryptamine (TAM) pathway of auxin biosynthesis is predicted to begin with the conversion of Trp to TAM through tryptamine decarboxylase enzymes (Woodward and Bartel, 2005), however genes encoding these enzymes have not been identified in Arabidopsis. Next, TAM is converted to \textit{N}-hydroxyl tryptamine (HTAM) and identification of the \textit{YUCCA} (\textit{YUC}) genes which encode flavin monooxygenase-like enzymes confirmed this step of the pathway in
Arabidopsis (Zhao et al., 2001). After conversion of TAM to HTAM, an undetermined process converts HTAM to IAA. Previous studies have suggested that an IAOx intermediate may follow the conversion of TAM to HTAM; however, a more recent study by Sugawara et al. (2009) did not find significantly reduced levels of IAOx in Arabidopsis YUC mutants, indicating that the YUC genes are unlikely to play a role in the synthesis of IAOx.

It has been shown that the conversion of TAM to HTAM through action of the YUC genes is a rate-limiting step in the TAM pathway, indicating a critical role of these genes in auxin biosynthesis (Zhao et al., 2001). Further investigation of the YUC family in the Arabidopsis genome has revealed a total of ten YUC-like homologs. Single and double mutant YUC knockouts do not produce a dramatic phenotype in Arabidopsis, indicating that the YUC genes may be functionally redundant (Cheng et al., 2006; Cheng et al., 2007a). However, constructing triple and quadruple mutant combinations revealed that the YUC family plays a role in many aspects of development, as defects in embryogenesis, inflorescence development, and leaf development were observed. Similar to the CYP genes of the IAOx pathway, overexpression of some YUC genes has been shown to produce a phenotype consistent with overproduction of auxin, including increased apical dominance and long, narrow leaves (Zhao et al., 2001).

The potential function of YUCCA genes was investigated in other plant systems as well. When YUCCA was overexpressed in tobacco, transgenic plants displayed significant morphological phenotypes similar to those observed in Arabidopsis, including long and narrow leaves (Zhao et al., 2001). In petunia, the floozy (fzy) mutant was identified as an ortholog of YUC and demonstrated significant morphological phenotypes throughout development, including defects in apical dominance and vasculature (Tobena-Santamaria et al., 2002). fzy mutants also had reductions in floral organ number and defects in floral organ identity, suggesting YUC genes play a key role in reproductive development. Finally, in tomato, ToFZY has been characterized and identified as a YUC ortholog (Exposito-Rodriguez et al., 2007). Preliminary expression
analyses of ToFZY indicated that the gene likely plays a similar role in development to that of its Arabidopsis YUC and petunia FZY orthologs, since the strongest expression signals were found in developing leaves and flowers. These experiments indicate that the YUCCA pathway is utilized for auxin biosynthesis in many plant species; however, the number of genes regulating the pathway and/or the genetic redundancy of those genes may be divergent among species.

The final predicted Trp-dependent pathway of auxin biosynthesis is that which includes the major substrate indole-3-pyruvic acid (IPA), and until very recently this pathway had never been confirmed. It was predicted that Trp is first converted to IPA, after which IPA is converted to indole-3-acetaldehyde (IAAld), then finally IAAld is converted to IAA. The gene regulating the conversion of Trp to IPA in the initial step of the pathway was recently identified. TRYPTOPHAN AMINOTRANSFERASE OF ARABIDOPSIS1 (TAA1) was shown to encode the predicted aminotransferase and belong to a small gene family that also includes the TRYPTOPHAN AMINOTRANSFERASE RELATED1/2/3/4 genes (Stepanova et al., 2008).

Studies showed that TAA1 and TAR1/2 function in many different aspects of plant growth, including embryo patterning, gravitropism, and development of roots, inflorescences, leaves and vasculature (Stepanova et al., 2008; Tao et al., 2008; Yamada et al., 2009). In the next step of the pathway, it is predicted that the activity of an IPA decarboxylase converts IPA to IAAld, however this has not been genetically confirmed in Arabidopsis. Similarly, the final conversion of IAAld to IAA has not been confirmed, but evidence of an aldehyde oxidase that may function in this step has been found through identification of the ARABIDOPSIS ALDEHYDE OXIDASE (AAO) genes (Sekimoto et al., 1998).

Similar to the YUC family, characterization of mutant phenotypes has revealed important roles for the TAA/TAR genes in Arabidopsis development. In fact, the groups that simultaneously identified the TAA1 gene were screening for three mutants with virtually unrelated phenotypes. In one case, mutants were identified based on their inability to exhibit shade avoidance responses
typical for Arabidopsis (Tao et al., 2008). The shade avoidance syndrome (SAS) is initiated by a decrease in the ratio of red:far-red (R:FR) light perceived by the plant. A lower R:FR ratio is an indicator of weaker light, which typically arises due to crowding from neighboring plants. After perception, the plant induces a rapid response to stimulate growth, which ultimately results in increased plant height to avoid the shade. When grown in a shaded environment, shade avoidance3 (sav3) mutants exhibited an overall failure to initiate SAS, including reduced length of hypocotyls and petioles compared to wild-type.

In another screen, weak ethylene insensitive8 (wei8) mutants were identified based on root-specific insensitivity to ethylene, another important phytohormone in plants (Taiz and Zeiger, 2006; Stepanova et al., 2008). No defects in auxin transport or signaling were observed in wei8 mutants, nor were any morphological phenotypes besides the weak insensitivity to ethylene identified by increased root length compared to wild-type. To further study the role that the TAA1 and TAR genes play in development, wei8 double and triple mutants were constructed with alleles of TAR1 and TAR2 (Stepanova et al., 2008). The ethylene insensitive mutant phenotypes were found to be significantly more severe in double and triple mutants compared to wei8 single mutants, indicating an overlap in function of the TAA1 and TAR genes. In addition, other auxin-related phenotypes were observed, including decreased apical dominance, reduced height, and defects in flower and vasculature development.

Most recently, a screen for mutants exhibiting resistance to auxin transport inhibitors identified the TRANSPORT INHIBITOR RESPONSE2 (TIR2) gene of Arabidopsis (Yamada et al., 2009). tir2 mutants transported auxin normally and showed resistance to the auxin transport inhibitor N-1-naphthylphthalamic (NPA) but did not show resistance to exogenous auxin, indicating that TIR2 functions in auxin biosynthesis rather than transport or signaling. tir2 mutants exhibited multiple auxin phenotypes including reduced hypocotyl length, defects in vasculature, decreased lateral root growth, and altered root gravitropism. It was also observed that TIR2
expression is induced by increasing temperatures and functions in certain aspects of temperature-dependent development, such as hypocotyl elongation.

Studies to observe the impact of over-expressing TAA1 were also performed. Tao et al. (2008) did not find evidence of auxin over-producing phenotypes or enhancement of the shade-induced hypocotyl elongation phenotype when TAA1 was over-expressed. Stepanova et al. (2008) similarly did not find evidence of auxin overproduction when TAA1 was over-expressed. These results indicate that the conversion of Trp to IPA by TAA1 is not likely to be the rate-limiting step in the IPA pathway in Arabidopsis.

Based on the phenotypes of sav3, wei8, and tir2, it is clear that defects in the TAA1 and TAR genes have the potential to dramatically affect many aspects of growth and development in Arabidopsis. In addition, similar to the YUC family of genes, the double and triple mutant phenotypes observed between TAA1 and TARs suggest that the genes are functionally redundant in Arabidopsis.

1.3 The role of auxin transport and signal transduction in Arabidopsis vegetative and reproductive development

Efficient transport of auxin is also crucial for maintaining proper levels of the hormone in various tissues. Extensive studies in Arabidopsis have demonstrated the importance of auxin transport and have identified many of the genes which regulate transport.

The cell-to-cell flux of auxin in a specific directional pattern is known as polar auxin transport, and is accomplished through the action of auxin influx and efflux carriers (reviewed in Zazimalova et al., 2007). Auxin movement into the cell is facilitated by the action of influx carriers such as AUXIN RESISTANT1 (AUX1) (Bennett et al., 1996), and loss of AUX1 function results in auxin-related phenotypes such as reduced lateral root formation (Marchant et al., 2002).
Auxin is transported out of the cell through the function of *PINFORMED (PIN)* efflux carriers such as *PINFORMED1 (PIN1)* (Galweiler et al., 1998). The localization of *PIN1* must be directed to one end of the cell in order for polar auxin transport to occur in a unidirectional manner, and this localization has been found to be regulated by the serine/threonine protein kinase *PINOID (PID)* (Friml et al., 2004). Arabidopsis mutants defective in *PIN1* or *PID* display similar phenotypes including defects in organogenesis; most notably, mutants are defective in the initiation of floral meristems which results in a pin-shaped inflorescence (Bennett et al., 1995). Although other factors contribute to polar auxin transport, it is clear that disrupting genes which play a major role in either influx or efflux to the cell can sufficiently alter growth and development in Arabidopsis.

Auxin signal transduction has also been extensively studied in Arabidopsis (reviewed in Santner and Estelle, 2009). AUXIN SIGNALING F-BOX (AFB) proteins and the TRANSPORT INHIBITOR RESPONSE1 (TIR1) F-box protein act as principal auxin receptors and function as a subunit of a SKP1/CULLIN/F-box (SCF) ubiquitin E3 ligase complex. Similar to other ubiquitin ligases, the SCF<sup>TIR1/AFB</sup> complex functions to tag transcriptional regulators with ubiquitin for proteolytic degradation. The transcriptional regulators targeted by the SCF<sup>TIR1/AFB</sup> complex are known as AUXIN/INDOLE-3-ACETIC ACID (Aux/IAA) proteins. Aux/IAA proteins dimerize with proteins of another major family of transcription factors involved in auxin signaling, known as the AUXIN RESPONSE FACTOR (ARF) family. ARFs bind to the promoters of certain auxin-responsive genes and can either inhibit or activate their transcription. In the presence of auxin, the SCF<sup>TIR1/AFB</sup> complex is activated and triggers the degradation of Aux/IAAs. In turn, ARFs are liberated and can activate or repress transcription of certain auxin-responsive genes.

Such auxin signal transduction genes were identified in Arabidopsis through screens for auxin-related phenotypes, which demonstrates their important roles in plant growth and
development. For instance, \textit{tir1} mutants exhibit phenotypes representative of auxin resistance including decreases in hypocotyl elongation and lateral root development (Ruegger et al., 1998). Dominant \textit{Aux/IAA} mutants show significant defects in primary root length and root gravitropism while loss of function mutants do not exhibit significant defects, which is probably due to redundancy in the genome (Reed, 2001). Finally, ARF mutant phenotypes have been found to vary greatly depending on which gene function is lost. ARF single and double mutants can exhibit defects in hypocotyl elongation, vasculature and root development, phototropism, and gravitropism (reviewed in Wei et al., 2006). Specific ARF and Aux/IAA protein combinations could impact development in different ways, and the result of each pair of interactions has not yet been determined (Benjamins and Scheres, 2008).

The importance of both auxin transport and signal transduction in plant development has been demonstrated through the phenotypes of mutants disrupted in these processes. Further characterization of gene families and protein interactions will likely reveal additional roles in plant development in the future.

1.4 Overview of vegetative and reproductive development in maize

The monocot maize (\textit{Zea mays}) also serves as a model system for studying plant development. As in all plants, small groups of undifferentiated cells known as meristems initiate the growth of new structures, and these meristems are directly responsible for the vegetative and reproductive architectures of maize (McSteen et al., 2000).

Maize vegetative development is characterized by the production of a single main stalk with leaves initiated in alternate phyllotaxy by the shoot apical meristem (SAM) (Steeves and Sussex, 1989; McSteen and Leyser, 2005). The growth of the shoot is separated into units called phytomers, with each phytomer consisting of four parts: a leaf, a node to which the leaf is
attached, an internode composed of stem section, and an axillary meristem which forms in the axils of each leaf node. Some maize mutants such as *vanishing tassel2* (*vt2*) are reduced in height at maturity due to the production of fewer phytomers (Chapter 2). In other cases, defects in internode elongation can be attributed to the reduction in height, as in the case of *Developmental disaster1* (*Dvd1*) mutants (Chapter 3). After all leaves have been initiated in vegetative development, the SAM transitions to an inflorescence meristem and proceeds with reproductive development.

Vegetative architecture in maize is controlled in part by the *teosinte branched1* (*tb1*) locus, which functions to repress the outgrowth of the axillary meristems that develop in each phytomer (Doebley et al., 1997; Hubbard et al., 2002). Loss of function of *tb1* results in mutant plants that have a highly tillered (branched) phenotype since all vegetative axillary meristems are allowed to grow out. This phenotype resembles that of the bushy teosinte plant, which was identified as the wild ancestor from which maize was domesticated.

Maize plants produce separate male and female inflorescences during reproductive development (Kiesselbach, 1949). The male inflorescence, known as the tassel, is located at the tip of the shoot and produces several long branches, and these branches and the main tassel spike are covered in spikelets which house the pollen. The female inflorescence, known as the ear, is initiated from an axillary meristem a few nodes below the tassel and produces the kernels. The architectures of the tassel and ear are regulated by multiple types of axillary meristems that arise on both inflorescences (Cheng et al., 1983; Irish, 1997; McSteen et al., 2000; McSteen and Leyser, 2005). The inflorescence apical meristem initiates several branch meristems (BMs) at the base of the tassel inflorescence, leading to the long lateral branches observed at maturity. Spikelet pair meristems (SPMs) are also initiated in regular rows on the inflorescence and produce short spikelet branches. Next, spikelet meristems (SMs) are initiated to produce the spikelets which each house two florets. Finally, floral meristems (FMs) are responsible for
producing the floral organs. This inflorescence development is considered to be the result of highly modified phytomers since leaf primordia develop (but are suppressed) in the form of bracts, and the BMs and SPMs develop in the axils of these bracts.

Initiation of these reproductive meristems in maize is controlled by the barren stalk1 (ba1) locus (Ritter et al., 2002; Gallavotti et al., 2004). BA1 encodes a basic helix-loop-helix transcription factor that has been found to function in the initiation of axillary meristems. As a result, ba1 mutants never produce ears and ba1 tassels exhibit completely barren phenotypes, although the location where axillary meristems should develop can be still be observed by the presence of the suppressed bracts in regular rows along the tassel rachis. In addition, ba1 functions in vegetative growth in maize since all vegetative axillary meristems similarly fail to initiate in ba1 mutants. Recent analysis found that ZmPIN1 localization and expression is not disrupted in ba1 mutants, suggesting that ba1 does not play a role in auxin transport (Gallavotti et al., 2008a).

Inflorescence architecture in maize is also regulated in part by ramosa1 (ra1), which encodes a zinc finger transcription factor and acts as a positive regulator of SPM determinacy (Vollbrecht et al., 2005; McSteen, 2006; Kellogg, 2007). Without the function of ra1, SPMs show indeterminate growth and thus result in a highly branched phenotype in both the tassel and ear. ra1 function is thus critical for producing the unbranched ear inflorescence that has allowed for easy grain harvest and helped maize become such an important food crop.

It is clear that normal maize development is dependent on the growth of different meristems, which shape the complex architectures observed in both vegetative and reproductive growth. Many genes that regulate the formation and initiation of these meristems have been identified, and normal growth can be severely impacted if any of these genes is disrupted.
1.5 The role of auxin biosynthesis in maize vegetative and reproductive development

A summary of the Trp-dependent auxin biosynthesis pathways in maize, including identified enzymes and the genes that encode them, is illustrated in Figure 1-2 (Kriechbaumer et al., 2006; Sugawara et al., 2009).

The indole-3-acetaldoxime (IAOx) pathway of auxin biosynthesis has received both skepticism and support in maize. In one case, the pathway has been predicted to be absent in maize since no orthologs of the Arabidopsis cytochrome P450 genes (*CYP79B2* and *CYP79B3*) have been identified for the conversion of Trp to IAOx. In addition, no detectable IAOx could be found in maize tissues (Sugawara et al., 2009). The same study was also unable to detect indole-3-acetonitrile (IAN), the next predicted intermediate, however previous studies have detected IAN in coleoptiles and kernels of maize (Park et al., 2003). Additional support of a maize IAOx-like pathway has been found through identification of nitrilase genes similar to *NITRILASE1/2/3* of Arabidopsis which function to convert IAN to IAA in the final step of the pathway. In particular, *Zea mays Nitrilase2* (*ZmNIT2*) was found to be capable of converting IAN to IAA at very efficient rates (Park et al., 2003). Expression of *ZmNIT2* was identified in many tissues, such as kernels, coleoptiles, and roots and was also found to be light-induced (Kriechbaumer et al., 2007). *ZmNIT2* mutants were found to exhibit reduced primary root growth during early development, however no additional morphological phenotypes were found through maturity. Hence, evidence suggests that an IAOx-like pathway exists in maize however further identification and characterization of genes involved in this pathway are necessary to completely understand its role in development.

Little is known about the indole-3-acetamide (IAM) pathway in maize. Although the recent work of Pollmann et al. (2009) has provided support for the initial conversion of Trp to IAM in Arabidopsis and other plant species, maize genes functioning in this step have not yet
been identified. Similarly, although amidase genes in Arabidopsis such as \textit{AMIDASE1 (AMII)} have been proposed to function in the final conversion of IAM to IAA, no AMII orthologs have been identified in maize.

The tryptamine (TAM) pathway in maize has been partially characterized. Similar to Arabidopsis, genes encoding tryptamine decarboxylases for the initial conversion of Trp to TAM have not been identified in maize, nor has the process by which N-hydroxyl tryptamine (HTAM) is converted to IAA in the final step of the pathway been determined. However, the cloning of an Arabidopsis \textit{YUCCA (YUC)} family ortholog has revealed the importance of this pathway in maize development. The \textit{sparse inflorescence1 (spi1)} mutant of maize was mapped and cloned by Gallavotti et al. (2008b) and shown to encode a flavin monooxygenase-like enzyme orthologous to Arabidopsis \textit{YUC1}. Since \textit{YUC} genes regulate the conversion of TAM to HTAM in the rate-limiting step of the pathway (Zhao et al., 2001), a crucial role for \textit{spi1} in maize auxin biosynthesis was revealed.

The importance of the \textit{spi1} locus was further evidenced by the phenotype of \textit{spi1} mutants (Gallavotti et al., 2008b). In vegetative growth, \textit{spi1} plants exhibited decreased height due to a reduction in the number of phytomers (leaves) produced. Reproductive growth was even more severely affected in \textit{spi1} mutants, as tassel inflorescences rarely produced functional spikelets and ear inflorescences had significant reductions in kernel number and defects in the apical inflorescence meristem. RT-PCR showed that \textit{spi1} is expressed in many different tissues of the plant, including developing tassels and ears, embryos, and leaves. Further expression analysis by RNA \textit{in situ} hybridization revealed that \textit{spi1} plays an important role in reproductive axillary meristem initiation. Highly localized expression was identified at the sites of newly forming meristems in both tassel and ear inflorescences, indicating that the normal initiation of axillary meristems in maize reproductive development requires very specific local auxin biosynthesis.
The phenotype of *spi1* single mutants resembles that of the Arabidopsis *yuc1;yuc2;yuc4;yuc6* quadruple mutant (Cheng et al., 2006). Thus, maize *YUC* genes exhibit less redundancy than those of the Arabidopsis *YUC* family, although their critical functions in auxin biosynthesis are conserved.

The indole-3-pyruvic acid (IPA) pathway has not yet been confirmed in maize. As in Arabidopsis, the genes regulating the conversion of IPA to indole-3-acetaldehyde (IAAld) and IAAld to IAA in the final two steps of the pathway have not been characterized. However, the recent identification and functional characterization of the *TAA1* and *TAR1/2/3/4* tryptophan aminotransferase genes has revealed the importance of this pathway in Arabidopsis development (Stepanova et al., 2008; Tao et al., 2008; Yamada et al., 2009). By regulating the conversion of Trp to IPA in the initial step of the IPA pathway, these genes have been shown to function in many aspects of vegetative and reproductive development. The cloning of the *vanishing tassel2* (*vt2*) locus has confirmed that this pathway is also functional in maize and is essential for normal vegetative and reproductive development in maize (Chapter 2). Severe morphological defects have been observed in both the tassel and ear inflorescences, and these defects are more dramatic than those identified in other auxin biosynthesis mutants thus far. In addition, *vt2* mutants show severe reductions in plant height and leaf number, indicating it plays a key role in normal vegetative development as well.

### 1.6 The role of auxin transport and signal transduction in maize vegetative and reproductive development

The role of auxin transport in maize development has been partially elucidated by identifying homologs of some of the known Arabidopsis transport genes. *Zea mays* *AUX1* (Zm*AUX1*) has been identified as an *AUX1* influx carrier homolog in maize, however its
functional characterization is not yet complete (Hochholdinger et al., 2000). Investigation of PIN1 efflux carrier homologs revealed three Zea mays PIN1 (ZmPIN1) proteins, ZmPIN1a/b/c, and analysis of ZmPIN1a by Gallavotti et al. (2008a) confirmed its auxin transport functionality. The role of ZmPIN1a in maize development was investigated by observing its expression pattern in various tissues, and results showed conservation with the patterns displayed by Arabidopsis PIN1. Distinct up-regulation of ZmPIN1a was observed at the location of all developing axillary meristems and branch primordia, indicating that polar auxin transport is essential for proper vegetative and reproductive development in maize (Gallavotti et al., 2008a).

An ortholog of Arabidopsis PINOID (PID), the regulator of PIN1 subcellular localization, has also been identified in maize. The barren inflorescence2 (bif2) mutant was characterized by its distinct barren phenotype in the tassel, including absence of lateral branches and reduced spikelet number, and also showed reductions in height and leaf number (McSteen and Hake, 2001; McSteen et al., 2007). RNA in situ hybridization showed that bif2 is expressed at the site of newly forming meristems in the inflorescence, indicating that bif2 plays an important role in the initiation of axillary meristems during reproductive development. Cloning and phylogenetic analysis of bif2 revealed that it encodes a serine/threonine protein kinase that is co-orthologous to PID (McSteen et al., 2007), and recent studies found that BIF2 can phosphorylate ZmPIN1a in vitro (Skirpan et al., 2009). Since similar phenotypes are observed in pid and bif2 mutants and the functionality of the genes has been shown to correlate, the importance of polar localization of auxin efflux carriers in plant development appears to be conserved across species.

Auxin signal transduction has been similarly characterized in maize through identification of some known Arabidopsis homologs. The ZmTIR1 gene has been identified as a homolog of the Arabidopsis F-box protein TIR1 and shows expression in developing leaf primordia, however its function in maize development has not been analyzed (Zhang et al., 2007). Aux/IAA homologs have also been preliminarily identified in maize, including the recessive
rootless with undetectable meristems1 (rum1) mutant and the semi-dominant Barren inflorescence1 (Bif1) mutant. rum1 mutants do not show vegetative morphological phenotypes except for a reduction in lateral and seminal root formation (Taramino et al., 2008). However, Bif1 mutants exhibit defects in both vegetative and inflorescence development such as reduced height, leaf number, branch number, and spikelet number (Barazesh and McSteen, 2008). The ARF family of transcription factors has not yet been characterized in maize but homologs of Arabidopsis ARF1, ARF2, and ARF5 have been identified (Brooks et al., 2009).

Thus, auxin transport and signal transduction mechanisms appear to be at least partially conserved across plant species. As in Arabidopsis, the importance of these processes in maize can be observed through the phenotypes of mutants lacking normal functionality. Identification of additional maize genes that function in either process will help further our understanding of their importance in different aspects of development.

1.7 Conclusions

In this thesis, the thorough characterization of two maize mutants will be presented. Both mutants exhibit severe defects in both vegetative and reproductive development, although the manner in which the two genes regulate these processes is divergent. vanishing tassel2 has been cloned and found to function in auxin biosynthesis (Chapter 2), while Developmental disaster1 has been fine-mapped and may play a role in auxin signaling (Chapter 3). The characterization of these two mutants helps illustrate the importance and divergence of these two genes in the genetic regulation of maize vegetative and reproductive development. Additional work to further elucidate the roles of vt2 and Dvd1 during maize development is discussed in Chapter 4.
Figure 1-1: Auxin biosynthesis in Arabidopsis.

One tryptophan-independent and four tryptophan-dependent pathways have been proposed. Thick lines indicate that genes encoding enzymes catalyzing these steps have been identified. Solid lines indicate that enzymatic activity has been detected. Dotted lines indicate the steps that are inferred. TRP, tryptophan; IAM, indole-3-acetamide; IAOX, indole-3-acetaldoximine; TAM, tryptamine; IPA, indole-3-pyruvic acid; IAN, indole-3-acetonitrile; HTAM, N-hydroxyl tryptamine; IAAlD, indole-3-acetaldehyde; IAA, indole-3-acetic acid.
Adapted from Sugawara et al., 2009.
One tryptophan-independent and four tryptophan-dependent pathways have been proposed. Thick lines indicate that genes encoding enzymes catalyzing these steps have been identified. Solid lines indicate that enzymatic activity has been detected. Dotted lines indicate the steps that are inferred. TRP, tryptophan; IAM, indole-3-acetamide; IAOx, indole-3-acetaldoximine; TAM, tryptamine; IPA, indole-3-pyruvic acid; IAN, indole-3-acetonitrile; HTAM, N-hydroxyl tryptamine; IAAl, indole-3-acetaldehyde; IAA, indole-3-acetic acid.
Adapted from Kriechbaumer et al., 2006; Sugawara et al., 2009.
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CHAPTER 2

*vanishing tassel2* encodes an auxin biosynthesis gene functioning in vegetative and inflorescence development in maize

This work is being prepared for submission:

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Notes:

Andrea Skirpan provided the cDNA used for RT-PCR expression analysis.

Tom Slewinski assisted with identification of candidate genes in the vt2 region.

Chris Hudson performed the provisional vt2 mapping.

Solmaz Barazesh constructed some of the original double mutant crosses.

The remaining work was completed by Kimberly Phillips.
2.1 Introduction

Auxin has been shown to play a critical role in all stages of plant development. Auxin, through its functions in cell division and cell expansion, is required for the initiation of lateral roots, vascular strands, leaves, flowers and floral organs (Benjamins and Scheres, 2008). Evidence from genetics, molecular biology and modeling has shown that auxin transport is crucial for providing the source of auxin required for organogenesis (Petrasek and Friml, 2009). More recently, the importance of auxin biosynthesis in providing a localized source of auxin for organogenesis has been appreciated (Chandler, 2009).

In plants, there are proposed to be four tryptophan (TRP)-dependent and one tryptophan-independent pathway for the biosynthesis of auxin, indole-3-acetic acid (IAA) (Figure 2-1) (Bartel, 1997; Woodward and Bartel, 2005; Pollmann et al., 2006; Kriechbaumer et al., 2008; Sugawara et al., 2009). Each pathway is named after an intermediate that it is proposed to utilize, but few genes encoding enzymes in each pathway have been identified. In addition, the extent to which the pathways share intermediates is not clear, indicating that much remains to be learned about how auxin biosynthesis occurs in plants. Furthermore, the relative importance of each pathway in different cell types, stages of development, and plant species is unknown.

What is known of the genetic control of the four TRP-dependent pathways in Arabidopsis and maize is indicated in Figure 2-1. (1) The IAM pathway. The conversion of TRP to IAA through an indole-3-acetamide (IAM) intermediate has been demonstrated in Arabidopsis (Pollmann et al., 2009). Genes encoding enzymes that catalyze the conversion of TRP to IAM are unknown but amidases (AMI1) that convert IAM to IAA have been identified in Arabidopsis (Pollmann et al., 2003). Amidase genes have not yet been identified from maize so the importance of this pathway remains unknown in the species. (2) The IAOx pathway. Genes encoding the cytochrome P450 enzymes, CYP79B2/CYP79B3, that convert TRP to indole-3-
acetaldoximine (IAOx) have been identified in Arabidopsis but are not present in any other non-cruciferous species (Zhao et al., 2002; Sugawara et al., 2009). IAOx is converted to indole-3-acetonitrile (IAN) by unknown means and IAN is converted to IAA by nitrilases. Genes encoding nitrilases have been identified from both maize and Arabidopsis (Park et al., 2003; Kriechbaumer et al., 2007). However, the existence of this pathway in maize has been questioned due to the absence of both orthologous CYP79B2/3 genes and detectable IAOx levels (Sugawara et al., 2009). (3) The TAM pathway. The enzymes converting TRP to Tryptamine (TAM) are not known but the conversion of TAM to N-hydroxyl tryptamine (HTAM) is catalyzed by the YUCCA (YUC) genes of Arabidopsis which play important roles in various aspects of development (Zhao et al., 2001). In maize, the sparse inflorescence1 (spi1) gene is a grass-specific member of the YUC gene family, indicating that this pathway is also important for maize development (Gallavotti et al., 2008b). (4) The IPA pathway. TRP is converted to indole-3-pyruvic acid (IPA) by the tryptophan aminotransferase gene of Arabidopsis, TAA1, and related genes, TAR1 and TAR2 (Stepanova et al., 2008; Tao et al., 2008; Yamada et al., 2009). It is not known how IPA is converted to indole-3-acetaldehyde (IAAld) but the conversion of IAAld to IAA is catalyzed by aldehyde oxidases which have been identified in both maize and Arabidopsis (Sekimoto et al., 1997; Sekimoto et al., 1998). Here, we identify vanishing tassel2 (vt2), a maize homolog of TAA1/TAR1/TAR2. The dramatic phenotype of vt2 loss of function mutants indicates that the IPA pathway plays a critical role in maize vegetative and reproductive development.

TAA1 was identified in three different genetic screens in Arabidopsis as indicated by the different phenotypes and nomenclature of respective mutants: insensitivity to ethylene-induced root shortening, weak ethylene insensitive8 (wei8) (Stepanova et al., 2008), insensitivity to shade-induced hypocotyl elongation, shade avoidance3 (sav3) (Tao et al., 2008) and insensitivity to NPA-induced root shortening, transport inhibitor response2 (tir2) (Yamada et al., 2009). Unlike the mild phenotype of taa1 single mutants, double mutants with the related gene tar2 show more
severe defects, producing dwarf, bushy plants with agravitropic roots, reduced vasculature and sterile flowers (Stepanova et al., 2008). *taa1;tar1;tar2* triple mutants are seedling lethal due to defects in embryogenesis (Stepanova et al., 2008). Similar phenotypes are seen in plants containing multiple knockouts of the *YUC* gene family (Cheng et al., 2006; Cheng et al., 2007a), raising the question of why the two pathways do not compensate for each other.

Defects in organogenesis are also seen in mutants with altered auxin transport. For example, mutations in the auxin efflux carrier *PINFORMED1 (PIN1)* and the *PINOID (PID)* kinase, which regulates PIN1 subcellular localization, produce an inflorescence with no flowers known as a pin inflorescence (Bennett et al., 1995; Galweiler et al., 1998; Christensen et al., 2000; Friml et al., 2004). Mutations in the *pid* co-ortholog in maize, *barren inflorescence2 (bif2)*, produce an equivalent phenotype called a barren inflorescence (bif) phenotype with no branches and few spikelets (small branches that bear the florets) in the male inflorescence known as the tassel, and few kernels in the female inflorescence known as the ear (McSteen and Hake, 2001). This pin/bif phenotype is also seen in *yuc1;yuc2* double mutants in Arabidopsis and *spi1* single mutants in maize (Cheng et al., 2006; Gallavotti et al., 2008b), indicating that both auxin transport and auxin biosynthesis are required for the initiation of flowers. The importance of these two processes in development is further illustrated by the synergistic interactions observed between auxin biosynthesis and transport mutants. For example, *yuc1;yuc4;pin1* triple mutants do not produce leaves and *spi1;bif2* double mutants have dramatically reduced leaf number, indicating that both auxin biosynthesis and transport are required for leaf initiation in addition to flower initiation (Cheng et al., 2007a; Gallavotti et al., 2008b).

Here, we report on the identification of the *vt2* mutant of maize which has a similar phenotype to the *spi1;bif2* double mutant. *vt2* mutants exhibit a barren inflorescence, devoid of both branches and spikelets, as well as a dwarf vegetative phenotype due to the production of fewer leaves. Cloning of *vt2* indicates that it encodes a homolog of the *TAA1/TAR1/TAR2* genes
shown to catalyze the conversion of TRP to IPA in Arabidopsis auxin biosynthesis (Stepanova et al., 2008; Tao et al., 2008; Yamada et al., 2009). Our results indicate that the IPA pathway plays a critical role in maize vegetative and reproductive development. Furthermore, due to the reduced redundancy of the vt2 and spi1 genes in maize, we were able to test the relative contributions of the IPA and TAM pathways during development. The essentially additive nature of the spi1;vt2 double mutant implies that that the IPA and TAM pathways operate independently in maize.

2.2 Materials and Methods

2.2.1 Origin of vt2 alleles

The vt2-reference (vt2-ref) allele originated by Mutator transposon mutagenesis and was backcrossed to the B73 inbred line before phenotype analysis (Laurie Smith and Sarah Hake, Plant Gene Expression Center). vt2-TR799, vt2-GN21, vt2-GN210, and vt2-GN327 were obtained from the Maize Inflorescence Project (MIP) and arose via Ethylmethane Sulphonate (EMS) mutagenesis (http://www.maizegdb.org/ems-phenotype.php). vt2-123 was obtained from the RescueMu population (http://www.maizegdb.org/rescuemu-phenotype.php). vt2-1845 arose via spontaneous mutation in the B73 background (David Braun, Penn State University).

2.2.2 Mapping and cloning vt2

Two separate F2 mapping populations, vt2-ref-B73xMo17 and vt2-TR799-A619xB73, were constructed. Simple sequence repeat (SSR) and insertion-deletion polymorphism (IDP) markers from public databases (www.maizegdb.org and www.maizesequence.org) were used to
fine-map vt2 to two BAC contigs in bin 8.02. Single nucleotide polymorphism (SNP) markers (Table 2-1) from neighboring genes in the region were then designed to narrow the vt2 region further. Overlapping gene-specific PCR primers (Table 2-1) were designed to amplify and sequence the entire tryptophan aminotransferase candidate gene in all alleles.

2.2.3 RT-PCR expression analysis

cDNA was generated from total RNA for each tissue sample using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). 3 µl of cDNA was amplified by PCR using the vt2 gene-specific primers ex34-F and ex34-R (Table 2-1) for 40 cycles. GAPDH primers L4 and R4 (Table 2-1) were used as controls and amplified 1 µL of cDNA for 35 cycles.

2.2.4 Scanning Electron Microscopy

Ears were collected from field-grown plants after approximately 8 weeks, while tassels were collected from both field- and greenhouse-grown plants after approximately 5 weeks. Field-grown plants were exposed to an average daily minimum temperature of 12.9°C and an average daily maximum temperature of 23.7°C according to weather history data available for Pennsylvania Furnace, Pennsylvania from May 19th, 2009 through July 10th, 2009 (www.almanac.com). Greenhouse temperatures in typical maize growth rooms are held at a minimum temperature of 26.7°C for both day and night, with daytime temperatures typically increasing an additional 5-10°C. Cooler greenhouse rooms are held at a minimum temperature of 20°C and a maximum temperature of 26.7°C each day. Fixation and scanning electron microscopy of samples were performed as previously described (Wu and McSteen, 2007).
2.2.5 Taqman genotyping protocol

The Panzea database was used to identify single nucleotide polymorphisms (SNPs) in the vt2 region after preliminary fine-mapping (www.panzea.org). A SNP located on contig 327 was identified to be polymorphic between vt2 mutants (CGA) and the B73 background (CAA). Primers flanking the SNP were designed based on the supplied Panzea sequence and the SNP was confirmed to be linked to the vt2 mutant background via DNA sequencing. A Taqman SNP Genotyping Assay was then designed using the Custom Taqman SNP Genotyping Assays design program, File Builder v3.1 (Applied Biosystems, www.appliedbiosystems.com). Taqman assays were performed by the Penn State Huck Institutes Genomics Core Facility using an ABI 7300 Sequence Detection System.

2.2.6 Mature phenotype data

All mature phenotype data was obtained using the vt2-ref allele backcrossed into the B73 background six times. Data presented is representative of one field season.

Segregating families were planted in two separate field plantings, grown to maturity (10-12 weeks), and scored for phenotype. Mature tassel and ear phenotype data were obtained using 10 wild-type and 10 vt2/vt2 individuals from one segregating family. Tassel length was calculated by measuring from the tip of the tassel to the base of the flag leaf node and branch number was obtained by counting all visible lateral branches. Spikelet number was obtained prior to anthesis by removing and counting all spikelets from the branches and main spike. Kernel number was estimated by counting all spikelets on mature open-pollinated ears and ear length was obtained by measuring these ears from the base to the tip.
Plant height, leaf number, and ear number quantitative data were collected after full maturity (10-12 weeks) using all individuals from three segregating families (n = 150). Height was obtained by measuring from the ground to the tip of the tassel and ear number was scored by counting all visible ears on each plant. Leaf number was quantified by marking every 5\textsuperscript{th} leaf of developing field-grown plants beginning at four weeks until full maturity.

2.2.7 Leaf juvenile-to-adult transition analysis

Segregating families were greenhouse-grown for a total of nine weeks and genotyped for \textit{vt2} homozygous mutants using the Taqman SNP assay. Five \textit{vt2} mutants and five wild-type controls were used to count leaf number as described above, beginning with the first leaf and continuing through leaf ten. Upon total emergence from the whorl, leaves one through ten of each plant were studied for the presence or lack of epicuticular waxes on their blades. If a leaf was found to have epicuticular waxes present, it was deemed juvenile; lack of waxes, visible as a glossy appearance, on the leaf blade indicated that the leaf was adult. Leaves that contained some waxes (typically located on the outermost edges of the blades) were considered to be transitioning from juvenile to adult, and were classified as transitional.

2.2.8 Double mutant analysis

Segregating families were planted two times separated by a few weeks in two field seasons and grown to maturity (10-12 weeks). Data shown is representative of one planting.
2.2.8.1 vt2;spi1

Double mutant families were generated using the *spi1-ref* allele (Gallavotti et al., 2008b) in the B73 background. All individuals were genotyped for *vt2* using the SNP Taqman protocol and for *spi1* as previously described (Gallavotti et al., 2008b). Plant height, leaf number, and ear number were obtained for all individuals in three segregating F2 families (n=106) as described for *vt2* single mutants. Mature tassels from three segregating F2 families were collected before anthesis and tassel length, branch number, and spikelet number were obtained as described for *vt2* single mutants (n=10 individuals each for wild-type, *vt2*, and *bif2*; n=7 individuals for *vt2;spi1*). Open-pollinated ears from three segregating F2 families were collected and ear length and kernel number were obtained as described for *vt2* single mutants (n=2 wild-type, n=2 *spi1*, n=3 *vt2*, and n=4 *vt2;spi1* individuals). To confirm the ear phenotype results from the first field season, a second planting was utilized the following year. All individuals were genotyped for both *vt2* and *spi1* as above. Five ears representing each genetic class were obtained from five segregating F2 families to analyze ear length and kernel number, and results were consistent with those obtained during the first field season.

2.2.8.2 vt2;bif2

Double mutant families were generated using the *bif2-77* allele (McSteen et al., 2007) in the B73 background. All individuals were genotyped for *vt2* using the SNP Taqman protocol and for *bif2* as previously described (Skirpan et al., 2008). Plant height, leaf number, and ear number were obtained for all individuals in two segregating F2 families (n=97) as described for *vt2* single mutants. Mature tassels from four segregating F2 families were collected before anthesis and tassel length was obtained as described for *vt2* single mutants (n=10 individuals each for wild-
type, vt2, and bif2; n=7 individuals for vt2;bif2). Data for all vt2;bif2 double mutants was obtained about seven weeks after germination due to the drastically reduced lifespan of the plants. Photos display younger vt2;bif2 mutants next to siblings from a planting two weeks earlier in order to represent all individuals at full maturity.

2.2.8.3 vt2;tb1

Double mutant families were generated using the tb1-ref allele (Doebley et al., 1997) in the B73 background. All individuals from two segregating families (n=72) were scored and all vt2 mutants were genotyped as previously described (Hubbard et al., 2002) to identify double mutant combinations with either tb1/+ or tb1/tb1. Two individuals representing each remaining genetic class (wild-type, tb1/+ and tb1/tb1) were also genotyped as controls. Visible primary tillers were counted at maturity as those that were derived directly from one of the nodes on the main stalk. Visible secondary tillers were counted at maturity as those that were derived directly from a primary tiller.

2.2.8.4 vt2;ba1

Double mutant families were generated using the ba1-ref allele (Ritter et al., 2002) in the B73 background. All individuals from two segregating families (n=93) were scored for phenotype, with vt2;ba1 double mutants being identified by the lack of any ear shoots. Plant height, leaf number, and ear number were obtained for all individuals at maturity. Mature tassels from these families were collected before anthesis and tassel length was obtained as described for vt2 single mutants (n=10 individuals each for wild-type, vt2, and ba1; n=4 individuals for vt2;ba1).
2.2.9 Statistical analysis

Data was analyzed using Microsoft Excel 2003. Bar graphs were produced using the calculated mean of each data set and error bars are representative of the standard error of the mean. Data were considered statistically significant at p-value < 0.05.

2.3 Results

2.3.1 *vt2* functions in vegetative development

At maturity, *vt2* mutants were visibly shorter than wild-type siblings (Figure 2-2A). As expected, quantification revealed a statistically significant reduction in plant height in *vt2* mutants compared to wild-type (Figure 2-2B). To determine if this decrease in plant height was caused by a reduction in the number of phytomers produced, leaf number was counted. Wild-type maize plants produced about 20 leaves on average at maturity whereas *vt2* mutants produced an average of about 13 leaves (Figure 2-2C), confirming that the reduction in height of *vt2* mutants is caused by the production of fewer phytomers than normal.

The decrease in leaf number in *vt2* mutants could be due to the production of fewer juvenile leaves or fewer adult leaves. To determine which leaves were missing in *vt2* mutants, the juvenile-to-adult transition was analyzed through visual inspection of leaf waxes. Due to the production of certain epicuticular waxes, the surface of juvenile maize leaves appears dull while adult leaves appear glossy, and transitional leaves (with a glossy appearance at the tip and a matte appearance at the base and margins) are produced at the juvenile-to-adult transition (Kerstetter and Poethig, 1998). In wild-type siblings, the juvenile-to-adult transition began at leaf six when transitional leaves were produced, and the transition continued through leaf eight after which adult glossy leaves were produced (Figure 2-2D). *vt2* mutants showed no significant difference
in the transition point from juvenile to transitional leaves, or from transitional leaves to adult leaves (Figure 2-2E). As there is no difference in the timing of the juvenile-to-adult transition in vt2 mutants, this indicates that the later-formed adult leaves are those that are missing in vt2 mutants.

To test the role of vt2 in another aspect of vegetative development, we constructed double mutants with the vegetative axillary meristem mutant, teosinte branched1 (tb1). The tb1 gene functions to suppress the outgrowth of branches (tillers) from vegetative axillary meristems located in the axil of each leaf node (Doebley et al., 1997; Hubbard et al., 2002). Loss of function of tb1 allows the outgrowth of these vegetative axillary meristems, resulting in mutants that produce many tillers. vt2;tb1 double mutants produced more primary and secondary tillers than vt2 single mutants but fewer tillers than tb1 single mutants at maturity (Figure 2-3A,B). The reduction in the number of tillers produced in vt2;tb1 compared to tb1 alone is most likely due to the decreased number of phytomers available to produce additional tillers. This interpretation combined with the ability of vt2;tb1 double mutants to produce more tillers than vt2 single mutants suggests an additive genetic interaction, indicating that vt2 does not play a role in axillary meristem formation during vegetative development.

2.3.2 vt2 functions in inflorescence development

In maize development, tassel inflorescences normally produce a main spike with several long lateral branches extending near the base (Figure 2-4A) (McSteen et al., 2000). Short branches known as spikelet pairs house the florets and cover both the main spike and long branches (Figure 2-4A). vt2 mutant tassels were smaller at maturity and exhibited a severely barren phenotype compared to wild-type siblings, including a complete lack of lateral branches and functional spikelets (Figure 2-4A). Quantitative analysis of tassel length (Figure 2-4B),
branch number (Figure 2-4C), and spikelet number (Figure 2-4D) confirmed a significant reduction in vt2 mutants compared to wild-type.

In addition to male inflorescence defects, vt2 mutants also showed severe defects in the female inflorescence. vt2 mutant ears showed obvious defects in length and kernel number with a barren patch devoid of kernels often extending along the adaxial side of the ear (Figure 2-4E). Furthermore, kernels appeared to grow over the apex of the ear (Figure 2-4E) or both sides of the ear. Quantification of traits at maturity revealed a statistically significant reduction in both ear length (Figure 2-4F) and kernel number (Figure 2-4G) in vt2 mutants compared to wild-type. In addition, segregating families were scored to determine if the number of visible ear shoots produced by vt2 mutants was altered compared to wild-type siblings, and a statistically significant reduction in vt2 ear shoot number was detected (Figure 2-4H). These data show that vt2 ear inflorescences exhibit similar defects to those observed in tassel inflorescences, and together indicate that vt2 plays an important role in inflorescence development.

2.3.3 vt2 functions in axillary meristem formation during inflorescence development

To determine whether the reduction in branches and spikelets in vt2 inflorescences was caused by altered branch and spikelet pair meristem formation, we observed tassel inflorescences at early stages of development using scanning electron microscopy (SEM). Early in development, wild-type tassels produce branch meristems (BMs) at the base of the inflorescence and spikelet pair meristems (SPMs) in regular rows on the flanks of both the branches and main spike (Figure 2-5A, arrows) (Cheng et al., 1983). In contrast, vt2 mutant tassels showed a complete lack of formation of both BMs and SPMs early in development (Figure 2-5B). Later in development, a few spikelet pair meristems sometimes formed sporadically on the main axis, often located near the tip of the inflorescence (Figure 2-5C). SEM was also performed on
developing ear inflorescences, and similar defects in axillary meristem formation were observed (Figure 2-5D).

Although field-grown vt2 mutant tassels never produced functional spikelets, we observed that temperature conditions during development can greatly impact the severity of the observed phenotype. In vt2 mutant tassels grown under typical maize greenhouse growing conditions, we observed the production of several branch meristems and irregularly placed spikelet pair meristems (data not shown). To further explore the role of temperature variations in vt2 inflorescence development, we grew plants under much cooler greenhouse growing conditions. In wild-type greenhouse plants, SPMs on the branches and main spike gave rise to two spikelet meristems (SMs) (Figure 2-5E). In contrast, vt2 mutants grown in cooler greenhouse conditions typically did not produce branch meristems, although numerous spikelet pair meristems developed along the main axis (Figure 2-5F). Often, these spikelet pair meristems would give rise to single spikelet meristems (Figure 2-5F). Hence, in cooler greenhouse conditions, the vt2 mutant phenotype was more severe than that observed under warmer greenhouse conditions, although it was still much weaker than the phenotypes of field-grown plants which are exposed to very cold minimum temperatures.

Therefore, vt2 mutants produce few branches and spikelets due to defects in BM, SPM, and SM formation. Furthermore, the phenotype is temperature dependent. In addition to vt2-ref, this temperature dependence was also observed in the vt2-123 and vt2-1845 alleles; hence, this phenomenon is not due to vt2-ref being a temperature-sensitive allele.

2.3.4 Positional cloning of vt2

vt2 was proposed to be either allelic or closely linked to the semi-dominant Bif1 mutant (Smith and Hake, 1993; Barazesh and McSteen, 2008). To test if vt2 was allelic to Bif1, plants
heterozygous for vt2 were crossed by plants heterozygous for Bif1 and mutant plants in the F1 were self pollinated to generate 12 F2 families. If vt2 was allelic to Bif1 then no wild-type plants would be recovered in the F2, while if vt2 was unlinked to Bif1 then 3/16 wild-type plants would be recovered. As a small percentage of wild-type plants (3%, 7/233) were recovered in the F2, it was concluded that vt2 was closely linked to Bif1 on chromosome 8.

To further define the map position of vt2, two F2 mapping populations, vt2-ref-B73 x Mo17 and vt2-TR799-A619 x B73, were constructed. Using simple sequence repeat (SSR) and insertion-deletion polymorphism (IDP) markers from public databases, vt2 was fine-mapped to within two BAC contigs in bin 8.02. Marker idp98 was identified as the closest public flanking marker available on the north side of vt2 with seven recombinant chromosomes out of 513 chromosomes (1.36cM, contig 326) and umc1974 was identified as the closest public marker on the south side of vt2 with one recombinant chromosome out of 431 chromosomes (0.12cM, contig 327) (Figure 2-6A). Between these two flanking markers, a third public marker, umc1872, was found to be polymorphic but identified zero recombinant chromosomes out of 488 chromosomes (0cM, contig 327). Using single nucleotide polymorphism (SNP) markers identified in neighboring genes in the region, the number of recombinants on the north (idp98) side was narrowed from seven to two (WD1 SNP, two recombinant chromosomes out of 462 chromosomes, 0.22cM), placing vt2 on contig 327 within a region containing three overlapping BAC clones (Figure 2-6A). A candidate gene search in the region revealed a tryptophan aminotransferase-like gene (Stepanova et al., 2008; Tao et al., 2008; Yamada et al., 2009) located on the overlapping portion of two of the three BAC clones, one of which contained marker umc1872 (0cM) (Figure 2-6A).

To test if the tryptophan aminotransferase-like candidate gene was vt2, overlapping gene-specific PCR primers (Table 2-1) were designed to amplify and sequence the gene from all vt2 alleles. Point mutations that were not present in the progenitor backgrounds were identified in the
coding regions of each of four EMS-induced alleles, three of which caused a single amino acid substitution and one of which caused a premature stop codon in the predicted protein (Figure 2-6B,C). PCR amplification of the vt2-1845 allele using primers near the 3’ end of the gene revealed a size polymorphism of about 300bp in the 5th exon between the mutant and progenitor backgrounds (Figure 2-6B). The Mutator (Mu) transposon-induced alleles, vt2-ref and vt2-123, were screened with a conserved terminal inverted repeat primer (Mu3456) and gene-specific primers to identify potential Mu insertions. Sequencing of the PCR products revealed a Mu1 insertion in the first exon of vt2-123 and a Mu1 insertion in the first intron of vt2-ref (Figure 2-6B). These data indicate significant sequence changes in seven independent alleles, and confirm that the vt2 gene encodes a tryptophan aminotransferase.

### 2.3.5 vt2 encodes an enzyme functioning in the IPA pathway of Trp-dependent auxin biosynthesis

Sequence analysis of vt2 revealed highest similarity to the tryptophan aminotransferase gene of Arabidopsis, TAA1 and two tryptophan aminotransferase-related genes, TAR1 and TAR2 (Figure 2-6C) (Stepanova et al., 2008; Tao et al., 2008; Yamada et al., 2009). These genes have been categorized in the superfamily of pyridoxal-5’-phosphate (PLP)-dependent enzymes, which differ from other typical allinases like TAR3 and TAR4 by their lack of a C-terminal EGF domain (Stepanova et al., 2008; Tao et al., 2008). TAA1 has been demonstrated in vitro to catalyze the conversion of TRP to IPA (Tao et al., 2008). Conservation was highest in the C-terminal allinase domain (Figure 2-6C), and sequence alignment indicated that VT2 shared 51.9 % amino acid identity with TAA1, 50.8 % identity with TAR1, and 56% identity with TAR2 in this region. The predicted amino acid sequence of VT2 was longer than TAA1/TAR1/TAR2 due to an extended N terminus. Sequence alignment also indicated that the vt2 EMS-induced alleles had mutations in
conserved regions. Notably, the vt2-GN21 allele had an Arginine to Trpophan amino acid substitution at one of the known enzyme active sites (Tao et al., 2008). As all alleles have a similar phenotype to that of vt2-GN21, all alleles are assumed to be null.

2.3.6 vt2 is broadly expressed

Sequence alignment of vt2 with its closest homologs in maize allowed the design of gene-specific primers. RT-PCR revealed that vt2 is broadly expressed, with expression found in all tissues tested (Figure 2-7A). Expression was thought to be at low levels as a high concentration of cDNA and 40 PCR cycles were required. We could not detect expression of vt2 by RNA in situ hybridization presumably due to the low level of expression (data not shown).

2.3.7 vt2 and spi1 exhibit an additive interaction

In Arabidopsis, the knockout of multiple YUC or TAA genes is required to produce a significant phenotype (Cheng et al., 2006; Cheng et al., 2007a; Stepanova et al., 2008). Therefore, the effect of eliminating two pathways of TRP-dependent auxin biosynthesis has not yet been examined. Thus, we constructed double mutants between vt2 and spi1, a YUC-like gene functioning in the TAM pathway of auxin biosynthesis (Gallavotti et al., 2008b) to determine if these two genes have overlapping functions.

spi1 mutants are slightly shorter and produce a few less leaves than wild-type, hence the overall vegetative spi1 phenotype is not nearly as severe as that of vt2 mutants (Figure 2-8A) (Gallavotti et al., 2008b). vt2;spi1 double mutants revealed a vegetative phenotype very similar to that of vt2 alone (Figure 2-8A). Quantitative analysis of plant height revealed a statistically significant reduction in double mutants compared to vt2 or spi1 alone (Figure 2-8B). To
determine if the reduction in height was due to decreased phytomer number, we counted leaf number and found that double mutants had a small but statistically significant reduction compared to either single mutant alone (Figure 2-8C). The reduction in plant height of double mutants is thus due in part to a reduction in phytomer number, however reduced tassel length is likely attributing to the defect in height as well (see below).

The tassel inflorescence phenotype of spi1 mutants is less severe than vt2 mutants, with nonetheless a strong reduction in branch and functional spikelet number (Figure 2-8D; (Gallavotti et al., 2008b). vt2;spi1 double mutant tassels resembled vt2 single mutants, with the complete absence of branches (Figure 2-8G) or spikelets (Figure 2-8H). However, tassel length was found to be significantly reduced compared to either single mutant alone (Figure 2-8F). In the female inflorescence, spi1 mutant ears resemble vt2 ears but are typically much less severe (Figure 2-8E). In contrast, vt2;spi1 double mutant ears revealed a phenotype that was more severe than either single mutant alone (Figure 2-8E). Quantification revealed a statistically significant reduction of both ear length (Figure 2-8I) and kernel number in double mutants compared to either single mutant (Figure 2-8J). Since double mutants could produce ears, we also tested whether they produced an altered number of visible ear shoots. Quantification revealed that vt2;spi1 plants produced an equivalent number of ear shoots as vt2 plants, whereas spi1 plants produced a similar number as wild-type plants (Figure 2-8K).

Although at first glance the vegetative and reproductive phenotype data appeared to indicate that vt2 might be epistatic to spi1, quantification revealed that vt2 and spi1 actually exhibited an additive genetic interaction. Since each mutation caused a reduction in plant height, leaf number, tassel length, ear length, and kernel number on its own and we found that eliminating the function of both genes resulted in a phenotype that ‘combines’ each separate reduction to produce a slightly more severe phenotype, we interpret the interaction as additive. This interaction indicates that spi1 and vt2 function independently.
2.3.8 vt2 and bif2 exhibit a synergistic interaction

The effects of eliminating both a gene in auxin biosynthesis and a gene in auxin transport have already been examined through double mutant combinations with spi1 and bif2 (Gallavotti et al., 2008b). The results of these studies revealed a synergistic interaction that produced plants with a very similar vegetative phenotype to that of vt2 mutants. To test the impact of eliminating a major auxin transport gene and an auxin biosynthesis gene playing an even greater role in vegetative development than spi1, we constructed double mutants between vt2 and bif2. The bif2 vegetative phenotype is not dramatic, with mutants showing only a slight reduction in height and leaf number compared to wild-type plants (Figure 2-9A) (McSteen et al., 2007). In contrast, the vt2;bif2 double mutant vegetative phenotype was extremely severe (Figure 2-9A arrow). Double mutants showed a significant reduction in plant height compared to all other genetic classes (Figure 2-9B). Leaf number was counted to determine whether the reduction in plant height was caused by the production of fewer phytomers, and quantification revealed that vt2;bif2 double mutants produced significantly fewer leaves than all other genetic classes, with only about seven total leaves produced (Figure 2-9C). In fact, the phenotype was so severe that the double mutants died weeks before siblings flowered.

bif2 single mutants typically produce tassels with a reduced number of branches, spikelets, florets and floral organs (Figure 2-9D) (McSteen and Hake, 2001). vt2;bif2 double mutants produced completely barren tassels similar to vt2 single mutants, although they also had an obvious reduction in size (Figure 2-9D). Quantification of tassel length confirmed a significant reduction in vt2;bif2 mutants compared to either single mutant alone (Figure 2-9E). In addition, vt2;bif2 mutants never produced visible ear shoots (Figure 2-9F).

Both vegetative and reproductive data for vt2;bif2 double mutants show a phenotype that is significantly more severe than either single mutant alone. This can be interpreted as a
synergistic interaction, indicating that vt2 and bif2 have overlapping functions to regulate vegetative and reproductive development in maize.

2.3.9 vt2 is epistatic to ba1

To investigate the interaction of vt2 with a gene functioning in auxin response, we constructed double mutants with barren stalk1 (ba1) (Ritter et al., 2002; Gallavotti et al., 2004). Although ba1 mutants still transport auxin normally, they fail to develop normal tassel and ear inflorescences since ba1 regulates the initiation of all lateral meristems (Ritter et al., 2002; Gallavotti et al., 2008a). ba1 mutants do not show an obvious vegetative phenotype except for a reduction in height (Figures 2-10A,B), which is due to a decrease in tassel length (Figure 2-10E) rather than leaf number (Figure 2-10C). The vt2;ba1 double mutant vegetative phenotype clearly resembled that of vt2 single mutants (Figure 2-10A). Quantification revealed that there was no significant reduction in plant height (Figure 2-10B) or leaf number (Figure 2-10C) in vt2;ba1 double mutants compared to vt2 alone.

ba1 mutants produce tassel inflorescences similar to those of vt2 mutants, exhibiting a complete lack of branches and spikelets (Figure 2-10D). However, unlike vt2 mutants, ba1 tassels produce suppressed bract primordia (visible as bumps) in regular rows along the rachis of the tassel (Figure 2-10D arrow) (Ritter et al., 2002). These bumps indicate pools of auxin that are produced and transported normally to the inflorescence, but can not be utilized to produce spikelets due to the absence of ba1 gene function (Gallavotti et al., 2008a). vt2;ba1 double mutants produced tassels that resembled vt2 single mutants, with no evidence of bract primordia that are normally observed in ba1 single mutants (Figure 2-10D). Tassel length of vt2;ba1 double mutants was not significantly different than either single mutant alone (Figure 2-10E). Finally,
bal mutants never produce ears since they lack the ability to initiate ear axillary meristems. vt2;bal double mutants similarly never produced an ear shoot (Figure 2-10F).

These results illustrate that vt2 is completely epistatic to bal during both vegetative and tassel inflorescence development. As bal mutants do not produce ear shoots, bal is epistatic to vt2 during ear inflorescence development. These results support the idea that vt2 is functioning upstream in auxin production during development, while bal is functioning downstream in auxin response.

2.4 Discussion

Here, we show that the vt2 plays a significant role in axillary meristem formation during inflorescence development in maize. During vegetative development, vt2 does not play a role in axillary meristem formation but functions in leaf/phytomer initiation. vt2 encodes an enzyme with significant homology to tryptophan aminotransferases that have been demonstrated to convert TRP to IPA in Arabidopsis (Stepanova et al., 2008; Tao et al., 2008; Yamada et al., 2009). Our results suggest that the IPA pathway for TRP-dependent auxin biosynthesis contributes significantly to vegetative and reproductive development in maize.

The phenotype of vt2 mutants shares many similarities with the phenotype of spi1 mutants which are defective in the TAM pathway for TRP-dependent auxin biosynthesis (Gallavotti et al., 2008b). Both mutants have defects in axillary meristem formation during reproductive development, shorter inflorescences due to defects in cell elongation (data not shown) and shorter plant height due to defects in phytomer/leaf initiation during vegetative development (Gallavotti et al., 2008b; Barazesh et al., 2009). The vt2 mutant, however, has more severe defects than spi1 although both are presumed to be null alleles. Another difference between vt2 and spi1 is the interaction of each gene with tbl. spi1;tbl double mutants have fewer tillers than
would be expected considering the defect of $spi1$ in leaf number, indicating that $spi1$ plays a role in vegetative axillary meristem formation (Gallavotti et al., 2008b). On the other hand, the effect of $vt2$ on tiller number in the $vt2;tb1$ double mutant can be explained by the effect of $vt2$ on phytomer/leaf number, leading to the conclusion that $vt2$ does not play a role in vegetative axillary meristem formation. The striking similarities in phenotype between $spi1$ and $vt2$ suggest that both the TAM and IPA pathways contribute auxin for organogenesis.

In Arabidopsis, multiple knockouts of the $TAA$ genes or the $YUC$ genes are required to exhibit a severe phenotype, while in maize, single knockouts of either $vt2$ or $spi1$ have a dramatic effect on development (Cheng et al., 2006; Cheng et al., 2007a; Stepanova et al., 2008). Phylogenetic analysis indicates that there is a gene family for both $vt2$ and $spi1$ in monocots and eudicots, indicating that the differences in redundancy between maize and Arabidopsis are not obviously due to differences in gene copy number; however, it is clear that the $spi1$ and $vt2$ genes alone play a more important role in maize than the $yuc1/taa1$ genes in Arabidopsis (Gallavotti et al., 2008b; Yamada et al., 2009). The availability of single gene knockouts with dramatic phenotypes in maize enabled us to test the relative contribution of the TAM and IPA pathways to development through the construction of double mutants. The results show that $vt2;spi1$ double mutants have a slightly more severe phenotype than the $vt2$ single mutant. We interpret this interaction as additive. Synergism, epistasis and additivity can be difficult to distinguish when mutants have similar phenotypes. The argument for additivity can be made by analyzing, for example, the effect on leaf number. The reduction in leaf number caused by the $spi1$ mutation is about one fewer leaf than normal, while the reduction caused by the $vt2$ mutation is about five leaves. The $spi1;vt2$ double mutants have about seven fewer leaves than normal, which can thus be accounted for by an additive contribution of loss of $spi1$ and $vt2$ gene function. Therefore, we conclude that $spi1$ and $vt2$ act independently. Similarly, Arabidopsis $taa1;tir7$ double mutants eliminate both the IPA pathway and the ability to produce TRP from indole, and produce a more
severe phenotype which indicates that additional pathways also act in parallel to the IPA pathway in Arabidopsis (Yamada et al., 2009).

The presence of multiple parallel pathways raises the question of why the different pathways do not compensate for each other in either maize or Arabidopsis. In fact, in Arabidopsis, upregulation of the IAOx pathway has been shown to compensate for defects in the IPA pathway (Stepanova et al., 2008). Furthermore, expression of the bacterial iaaM gene, which catalyzes the conversion of TRP to IAM, can rescue the yuc1;yuc4 double mutant in Arabidopsis (Cheng et al., 2006). Therefore, other auxin biosynthetic pathways, when misregulated, can compensate for deficiencies in the TAM or IPA pathways in Arabidopsis. As knockout of the TAM and IPA pathways have a phenotype, these alternate pathways do not appear to compensate normally in either maize or Arabidopsis. This indicates that lack of compensation may be due to differences in expression pattern or availability of intermediates in different cell types.

In contrast to the similarity in phenotype seen between spi1 and vt2 mutants, vt2 mutants have few similarities with the phenotypes of taa1, tar1 and tar2 single, double and triple mutants in Arabidopsis (Stepanova et al., 2008). Some of these differences may be superficial due to the fact that, for example, leaf and flower number have not been quantified in taa1;tar double mutants, and vt2 mutants have similarly not been tested for insensitivity to ethylene, shade or NPA. One clear difference between maize and Arabidopsis is that the taa1;tar2 double mutants have very significant defects in apical dominance, exhibiting a bushy phenotype presumably due to outgrowth of secondary branches. In contrast, bushiness is not a characteristic of vt2 mutants. In fact, testing the interaction of vt2 with tb1 showed that vt2 did not appear to play a role in suppression or promotion of axillary branch outgrowth. These apparent differences between vt2 and taa1 mutants could be due to sub-functionalization of different gene family members which has not yet been fully addressed in maize or Arabidopsis.
Another difference between the vt2 and taa1 mutants is that vt2 mutants have a weaker phenotype at higher temperature, while some defects in taa1 mutants can only be detected at higher temperature (Stepanova et al., 2008; Yamada et al., 2009). Stepanova et al. (2008) found that increasing temperatures resulted in increased hypocotyl length in both wild-type and single taa1/wei8, tar1, and tar2 mutant individuals. However, the wei8 mutant response was significantly less than that of wild-type individuals, which appeared to indicate that taa1 shows a more severe phenotype under higher temperature. It has previously been determined that ethylene may mediate increased hypocotyl elongation in response to auxin (Smalle et al., 1997). We thus speculate that the ability of tar1 and tar2 mutants to produce equivalent hypocotyls to wild-type under increased temperatures could be due to their normal responsiveness to ethylene (Stepanova et al., 2008), whereas the reduced elongation observed in taa1/wei8 could be explained by the wei8 mutant's characteristic insensitivity to ethylene.

Our results indicate that vt2 is more similar to TAR2 than TAA1. As the TAA1 gene is temperature-induced (Yamada et al., 2009), one possible explanation for the weaker phenotype of vt2 mutants at higher temperature is that closely related paralogs more similar to TAA1 may be capable of providing increased activity. Future work is required to address the role of these gene family members and their relative contributions to auxin biosynthesis, including at varying temperatures. It has also been shown in Arabidopsis that free auxin levels increase at higher temperature (Gray et al., 1998), although the pathway(s) which may be contributing to these increased levels have not been determined. In the IAOx pathway, cyp79b2;cyp79b3 double mutants were shown to have reduced auxin levels at higher temperature compared to wild-type (Zhao et al., 2002). Therefore, it is possible that both the IPA and IAOx pathways may be responsible for increasing free auxin levels at higher temperatures.

In contrast to the additive interaction observed in vt2;spi1 double mutants, synergistic interactions are seen when both auxin biosynthesis and auxin transport are reduced. This was
first demonstrated in maize by \textit{spi1; bif2} double mutants, as disruption of each single gene has mild effects on vegetative development but dramatic effects are observed in the double mutant combination (Gallavotti et al., 2008b). \textit{vt2; bif2} double mutants show even more dramatic effects, with plant height reduced to 10\% that of normal, leaf number reduced by 38\%, and the start of senescence weeks earlier than normal. These synergistic interactions indicate that both auxin transport and biosynthesis are required for normal development.

Synergistic interactions between auxin biosynthesis and auxin transport have also been seen in Arabidopsis. For example, \textit{yuc1; yuc4; pin1} triple mutants do not produce any leaves (Cheng et al., 2007a) and \textit{yuc1; yuc4; pid} triple mutants do not produce cotyledons (Cheng et al., 2007b; Cheng et al., 2008). Synergistic interactions are similarly seen when auxin biosynthesis and auxin influx components are knocked out; for example, \textit{yuc1; yuc2; yuc4; yuc6; aux1} quintuple mutants similarly do not produce leaves (Cheng et al., 2007a). Thus, the intertwined roles of auxin biosynthesis and transport in organogenesis may be common to many plant species.
One tryptophan-independent and four tryptophan-dependent pathways have been proposed. Thick lines indicate that genes encoding enzymes catalyzing these steps have been identified. Solid lines indicate that enzymatic activity has been detected. Dotted lines indicate the steps that are inferred. TRP, tryptophan; IAM, indole-3-acetamide; IAOx, indole-3-acetaldoximine; TAM, tryptamine; IPA, indole-3-pyruvic acid; IAN, indole-3-acetonitrile; HTAM, N-hydroxy tryptamine; IAAld, indole-3-acetaldehyde; IAA, indole-3-acetic acid.

Adapted from Bartel, 1997; Woodward and Bartel, 2005; Kriechbaumer et al., 2006; Sugawara et al., 2009.
Figure 2-2: Mature vegetative phenotype analysis of vt2 mutants.

(A) vt2 mutants are visibly shorter than their wild-type siblings. (B) Quantification of plant height. (C) Quantification of leaf number. (D) Percentage of wild-type individuals for each leaf trait by leaf number. (E) Percentage of vt2 individuals for each leaf trait by leaf number. Asterisk (*) indicates significant reduction at p<0.05; error bars represent the standard error of the mean.
Figure 2-3: vt2;tb1 double mutant analysis.

(A) Mature vegetative phenotype of all genetic classes in a segregating family. (B) Quantification of primary (grey bars) and secondary (white bars) tillers. Error bars represent the standard error of the mean.
Figure 2-4: Mature inflorescence phenotype analysis of vt2 mutants.

(A) Wild-type tassels normally produce multiple lateral branches at the base, and both the branches and the main spike are covered in pairs of spikelets, while vt2 mutant tassels produce no lateral branches or functional spikelets. (B) Quantification of tassel length. (C) Quantification of tassel branch number. (D) Quantification of spikelet number from entire tassel. (E) Wild-type ears normally produce hundreds of kernels in regular rows from the base to the tip, while vt2 mutant ears are smaller in size, produce very few kernels, and typically have barren patches on one or both sides. (F) Quantification of ear length. (G) Quantification of kernel number. (H) Quantification of visible ear shoot number. Asterisk (*) indicates significant reduction at p<0.05; error bars represent the standard error of the mean.
Figure 2-5: Scanning electron micrographs of developing inflorescences.

(A) Wild-type field-grown tassel at 3mm stage exhibiting BMs at the base and SPMs covering the branches and main spike.  
(B) vt2 field-grown tassel at 3mm stage exhibiting complete lack of BM and SPM initiation.  
(C) vt2 field-grown tassel at 5mm stage exhibiting a few SPMs produced near the tip.  
(D) vt2 field-grown ear at 7.5mm stage exhibiting sporadic SPM initiation, single SM formation, barren patches on the main axis, and a fasciated IM.  
(E) Wild-type greenhouse-grown tassel at 7mm stage exhibiting the production of paired SMs in regular rows on all branches and the main spike.  
(F) vt2 greenhouse-grown tassel at 7mm stage exhibiting a weaker mutant phenotype at increased growing temperatures compared to field samples. No lateral branches are produced while some SPMs are produced in irregular fashion along the main spike. Some SPMs initiate paired SMs while others abnormally form single SMs. 
BM, branch meristem; SPM, spikelet pair meristem; SM, spikelet meristem; IM, inflorescence apical meristem; PS, paired spikelets; SS, single spikelet.  Scale bars = 250 µm.
Figure 2-6: Cloning and sequence analysis of vt2.

(A) Diagram representing the vt2 region in maize after mapping with public and single nucleotide polymorphism (SNP) markers (not to scale). The number of recombinant chromosomes (R) out of the total number of chromosomes is displayed below each marker. Maize BAC clones within this region are represented by rectangles, with the shaded rectangles indicating the overlapping clones on which vt2 was identified. 

(B) Schematic of the vt2 gene structure including the position and mutations in seven alleles. Exons are represented by boxes and insertions are represented by triangles. Gly, Glycine; Glu, Glutamic acid; Arg, Arginine; Trp, Tryptophan. 

(C) Sequence alignment of the predicted vt2 protein and three Arabidopsis tryptophan aminotransferases. vt2 shows the highest similarity to TAR2. Asterisks indicate the position of mutations in each EMS allele.
Figure 2-7: vt2 is broadly expressed.

Qualitative RT-PCR expression shows that vt2 is expressed in all tissues tested. GAPDH, Glyceraldehyde 3-phosphate dehydrogenase control.
Figure 2-8: vt2;spi1 double mutant analysis.

(A) vt2;spi1 plants resemble vt2 single mutants with a reduction in height. (B) Quantification of plant height. (C) Quantification of leaf number. (D) vt2;spi1 tassels resemble vt2 single mutants except with a reduction in length. (E) vt2;spi1 ears show reduced length and kernel number. (F) Quantification of tassel length. (G) Quantification of branch number. (H) Quantification of spikelet number. (I) Quantification of ear length. (J) Quantification of kernel number. (K) Quantification of ear number. Asterisk (*) indicates significant reduction at p<0.05 compared to either single mutant alone; error bars represent the standard error of the mean.
Figure 2-9: vt2; bif2 double mutant analysis.

(A) vt2; bif2 plants (arrow) exhibit a drastic reduction in vegetative growth compared to vt2 or bif2 single mutants. (B) Quantification of plant height. (C) Quantification of leaf number. (D) vt2; bif2 tassels are severely underdeveloped compared to vt2 or bif2 single mutants. (E) Quantification of tassel length. (F) Quantification of ear number. Asterisk (*) indicates significant reduction at p<0.05 compared to either single mutant alone; error bars represent the standard error of the mean.
Figure 2-10: vt2;ba1 double mutant analysis.

(A) vt2;ba1 plants resemble vt2 single mutants. (B) Quantification of plant height. (C) Quantification of leaf number. (D) vt2;ba1 tassels resemble vt2 single mutants. (E) Quantification of tassel length. (F) Quantification of ear number. Error bars represent the standard error of the mean.
Table 2-1: Primers used for vt2 map-based cloning, sequencing alleles, and expression analysis.

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2.5 Literature Cited


CHAPTER 3

*Developmental disaster*1: A novel mutation causing defects during vegetative and inflorescence development in maize (*Zea mays*, Poaceae)

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Notes:

Andrea Skirpan assisted with mapping *Dvd1* and preliminary phenotype analyses.

Nick Kaplinsky originally identified the *Dvd1* mutant and did preliminary characterization and rough-mapping.

The remaining work was completed by Kimberly Phillips.
3.1 Introduction

In all plants, shoot growth is modular and is based on repeating units called phytomers (Steeves and Sussex, 1989; McSteen and Leyser, 2005). Phytomers consist of a leaf, a node which is the attachment site for the leaf, an axillary meristem which is produced in the axil of the leaf, and an internode which forms the stem between the nodes. During vegetative development, the internodes are short, the leaves are large and the axillary meristems are often suppressed. In maize, the outgrowth of vegetative axillary meristems is suppressed by the *teosinte branched1* (*tb1*) gene (Doebley et al., 1997; Hubbard et al., 2002). In *tb1* mutants, all basal branches grow out to produce vegetative branches called tillers. In many plants, including maize, the transition to flowering triggers a rapid elongation of internodes, suppression of leaves to form bract leaves, and the outgrowth of axillary meristems to produce flowers or flowering branches called inflorescences. Therefore, the regulation of the relative growth and activity of the components of the phytomer controls plant morphology.

Maize produces highly modified phytomers in the inflorescence (Irish, 1997; McSteen and Leyser, 2005). There are two types of inflorescence in maize: the male inflorescence, called the tassel, produced after the conversion of the shoot apical meristem to an inflorescence meristem; and the female inflorescence, called the ear, produced from an axillary meristem in the axil of a leaf on the main stalk (Kiesselbach, 1949). During inflorescence development, four types of axillary meristem are produced (Cheng et al., 1983; Irish, 1997; McSteen et al., 2000). Branch meristems (BMs) give rise to the long branches at the base of the tassel. Spikelet pair meristems (SPMs) produce short branches bearing two spikelets. Spikelet meristems (SMs) produce the spikelets which consist of two leaf-like glumes enclosing two florets. Lastly, floral meristems (FMs) produce the floral organs. In the inflorescence, the phytomers have very short
internodes and the subtending leaves are suppressed. For example, BMs and SPMs form in the axils of bract leaf primordia which do not grow out (McSteen and Leyser, 2005).

Genes required for the initiation of axillary meristems in the inflorescence have been identified by the characterization of the *barren* class of mutants in maize. *Barren inflorescence 1 (Bif1), barren inflorescence 2 (bif2), barren stalk 1 (ba1) and sparse inflorescence 1 (spi1)* mutants produce fewer branches, spikelets, florets and floral organs in the tassel, fewer kernels in the ear and fewer ears overall (McSteen and Hake, 2001; Ritter et al., 2002; Barazesh and McSteen, 2008; Gallavotti et al., 2008c). The analogous mutants in Arabidopsis have *pinformed*-like inflorescences (Okada et al., 1991; Bennett et al., 1995; Przemeck et al., 1996; Cheng et al., 2006). Both the *barren* and *pinformed*-like mutants are caused by defects in auxin biosynthesis, transport or response (Galweiler et al., 1998; Hardtke and Berleth, 1998; Christensen et al., 2000; Benjamins et al., 2001; Zhao et al., 2001; Gallavotti et al., 2004; McSteen et al., 2007; Barazesh and McSteen, 2008; Gallavotti et al., 2008c). *bif2* encodes a serine/threonine protein kinase co-orthologous to *PINOID* which regulates auxin transport in Arabidopsis (Christensen et al., 2000; Benjamins et al., 2001; Friml et al., 2004; Lee and Cho, 2006; McSteen et al., 2007; Michniewicz et al., 2007), while *Bif1* has a very similar phenotype to *bif2* and is proposed to regulate auxin transport (Barazesh and McSteen, 2008; Gallavotti et al., 2008a). *spi1* encodes a *YUCCA*-like flavin mono-oxygenase involved in auxin biosynthesis (Gallavotti et al., 2008c). *ba1* encodes a bHLH transcription factor that functions in axillary meristem initiation (Gallavotti et al., 2004). Although the relationship of *ba1* with auxin transport is debated (Wu and McSteen, 2007; Gallavotti et al., 2008a), biochemical, cellular and genetic evidence suggest that BA1 is a target of BIF2 (Skirpan et al., 2008).

In addition, the *barren* mutants have defects in vegetative development. For example, *bif2, ba1* and *spi1* produce fewer tillers in double mutant combination with *tb1* indicating that they also function in vegetative axillary meristems (Ritter et al., 2002; McSteen et al., 2007;
Gallavotti et al., 2008c). In addition, *Bif1, bif2* and *spi1* mutants are slightly shorter than normal due to the production of fewer leaves (McSteen et al., 2007; Barazesh and McSteen, 2008; Gallavotti et al., 2008c). Double mutant combinations of either *Bif1* or *spi1* with *bif2* have synergistic effects resulting in dwarfed plants, illustrating the redundant roles of *Bif1, bif2* and *spi1* in vegetative development (Barazesh and McSteen, 2008; Gallavotti et al., 2008c).

Our understanding of how axillary meristems develop has been greatly enhanced by characterizing the *barren* class of mutants. Here, we introduce a new member of this class of mutants, *Developmental disaster 1 (Dvd1)*, so named because of the pleiotropic defects in plant development caused by the mutation. *Dvd1* mutants have defects in axillary meristem formation during vegetative and reproductive development similar to the *barren* mutants, with the exception that bract leaves grow out in the inflorescence. In addition, unlike the *barren* mutants, the semi-dwarf stature of *Dvd1* mutants is due to the production of shorter internodes rather than fewer leaves. The suite of defects in *Dvd1* mutants together with the interaction of *Dvd1* with *bif2* suggest that we have identified a novel regulator of axillary meristem, internode and bract leaf development. Moreover, the *Dvd1* phenotype suggests that these three aspects of phytomer development are coordinately regulated in the control of plant morphology.

3.2 Materials and Methods

3.2.1 *Dvd1* origin and mapping

*Dvd1* was identified as a semi-dominant *reversed germ orientation (rgo)* mutant in a screen of the *Mutator* Maize Targeted Mutagenesis (MTM) population (Kaplinsky, 2002; May et al., 2003). The mutation was provisionally mapped to the short arm of chromosome 5 by the Maize Mapping Project (www.maizemap.org) with simple sequence repeat (SSR) primers
(Kaplinsky, 2002). To more accurately map \textit{Dvd1}, we constructed new mapping populations and identified other SSR markers from MaizeGDB (http://www.maizegdb.org/) (Lawrence et al., 2005) and from BAC contigs in the region (http://www.maizesequence.org/ and http://www.genome.arizona.edu/fpc/) (Coe et al., 2002). Additional insertion deletion polymorphism (IDP) markers in the region were identified from the MAGI database (http://magi.plantgenomics.iastate.edu/) (Emrich et al., 2004). For the first mapping population, \textit{Dvd1} was backcrossed to Mo17 six times, crossed to B73 and then backcrossed to B73. Mapping with this population revealed that \textit{Dvd1} resides between markers \textit{umc1870} (3/791 recombinants) and \textit{umc1591} (41/1335 recombinants). However, the region around \textit{Dvd1} was not polymorphic indicating that \textit{Dvd1} possibly arose in the B73 background. Hence, \textit{Dvd1} introgressed into Mo17 (eight times) was used as a second mapping population which allowed us to map \textit{Dvd1} to between \textit{idp3995} (9/1039 recombinants) and \textit{bnlg1902} (22/963 recombinants) on chromosome 5 bin 3.

\textbf{3.2.2 Mature phenotype data}

\textit{Dvd1} was backcrossed seven times into both the B73 and Mo17 inbred lines before phenotypic analysis. Data was collected from field-grown plants at maturity (10-12 weeks). Tassel branch number was quantified by scoring all individuals in two families from both B73 and Mo17 (n = 76 and 110, respectively). Five individuals representing each genetic class (normal, heterozygous mutant and homozygous mutant) were used for quantification of inflorescence phenotypes before anthesis. For each individual, all spikelets from the branches and main spike were counted and scored as single, paired, or triple. The spikelets were then dissected to quantify floret and stamen number. Ear number was obtained by counting visible ears on all individuals from two B73 families (n = 97) and two Mo17 families (n = 58). Kernel
number was counted on mature open-pollinated ears from two families in the Mo17 background (n = 16).

Data for plant height was collected at maturity in two B73 families (n = 97) and was obtained by measuring from the ground to the tip of the tassel. Leaf number was counted beginning soon after germination so that senesced leaves would be included in the total leaf count (n = 85). For internode length quantification, 10 individuals from each genetic class were collected, leaves were removed, and measurements were taken from the base of one node to the base of the next node.

3.2.3 SEM and histology

Developing tassels from 5-6 week old greenhouse-grown plants were dissected from segregating families and immediately fixed in FAA (3.7% formalin, 50% ethanol, 5% glacial acetic acid). Samples were kept in fix at 4°C overnight then dehydrated through an ethanol series. Basal internodes below the tassel were also obtained from these plants and cut into approximately 0.5-1.0 cm pieces before being fixed and dehydrated in the same manner.

For SEM, samples stored in 100% ethanol were critical point dried, sputter coated, and mounted as described previously (Wu and McSteen, 2007). Samples were viewed and photographed using a JSM 5400 scanning electron microscope (JEOL, Peabody, Massachusetts, USA) at an accelerating voltage of 10-20 keV.

For histology, samples in 100% ethanol were embedded into wax, sectioned, mounted on slides, de-waxed, and Toluidine Blue O (TBO)-stained as previously described (Barazesh and McSteen, 2008). Images were obtained on a Nikon Eclipse 80i upright microscope under bright field conditions with a DXM1200F digital camera (Nikon, Melville, New York, USA). Internode
cell size was determined by measuring the length and width of 10 cells per section of five internode sections of each genetic class.

3.2.4 Double mutant analyses

Double mutant families were grown to maturity (10-12 weeks) in two summer field seasons. Data presented here is representative of one field season.

3.2.4.1 Dvd1;tb1

Double mutant families were generated using the tb1-ref allele (Doebley et al., 1997) in the B73 genetic background. Segregating F₂ families were planted in two separate field locations and grown to maturity (n = 209 and 172). Genotyping for tb1 was performed as previously described (Hubbard et al., 2002). The number of visible primary and secondary tillers were counted at maturity (n = 49).

3.2.4.2 Dvd1;bif2

Double mutant families were generated using the bif2-77 allele in the B73 genetic background (McSteen et al., 2007). Four segregating F₂ families were planted (n = 205). Individuals were genotyped for bif2 as previously described (Skirpan et al., 2008). Plant height (n = 200) and leaf number (n = 199) were measured as described for Dvd1 single mutants. Tassels were collected for quantification of spikelet and bract leaf number (n = 54).
3.2.5 Statistical analysis

Students $t$-tests were performed using Minitab v.15 (State College, PA) at 95% confidence intervals. In all graphs, bars represent the mean of each data set and error bars represent the standard error of the mean.

3.3 Results

3.3.1 Dvd1 maps to chromosome 5

$Dvd1$ was previously mapped to the short arm of chromosome 5 with simple sequence repeat (SSR) markers (Kaplinsky, 2002). We fine mapped $Dvd1$ using additional public molecular genetic markers and narrowed the region down to two BAC contigs between $umc1870$ (0.38 cM) and $bnlg1902$ (2.28 cM) in bin 5.03. No mutants with similar phenotypes have been mapped in this region indicating that $Dvd1$ is a novel mutant. To analyze the effects of the mutation, $Dvd1$ was backcrossed into two different inbred lines: B73 and Mo17. Analysis of segregation ratio showed that $Dvd1$ is a semi-dominant mutation with homozygous $Dvd1/Dvd1$ individuals having a more severe phenotype than heterozygous $Dvd1/+\) mutants (Figure 3-1).

3.3.2 Dvd1 mutants have defects in inflorescence development

Normal tassels produce a main spike with several long branches at the base (Figure 3-1A, D). Both the branches and main spike are covered by short branches called spikelet pairs which contain the florets. The mature tassel phenotype of $Dvd1$ mutants was sparse compared to normal siblings (Figure 3-1A, D). In B73, $Dvd1/+\) tassels produced no branches and a reduced number of spikelets while $Dvd1/Dvd1$ mutants were even more severe, producing very few functional
spikelets (Figure 3-1A). In Mo17, Dvd1 mutants also produced fewer branches and spikelets in the tassel but the phenotype was less severe than in B73 (Figure 3-1D).

Quantification of the tassel phenotypes confirmed a statistically significant reduction in tassel branch number in Dvd1 heterozygous and homozygous mutants in both B73 (Figure 3-2A, \( t = 17.40, \text{d.f.} = 21, P < 0.001 \) and \( t = 17.40, \text{d.f.} = 21, P < 0.001 \)) and Mo17 backgrounds (Figure 3-2B, \( t = 5.49, \text{d.f.} = 84, P < 0.001 \) and \( t = 10.14, \text{d.f.} = 42, P < 0.001 \)). As the tassel phenotype was more severe in B73, subsequent analysis was carried out in this genetic background. Total spikelet number was significantly reduced in both Dvd1/+ and Dvd1/Dvd1 mutant tassels (Figure 3-2E, \( t = 14.84, \text{d.f.} = 4, P < 0.001 \) and \( t = 18.16, \text{d.f.} = 4, P < 0.001 \)). In normal plants, spikelets form in pairs. However, in Dvd1/+ mutants some spikelets formed singly and in triplets while all of the spikelets produced in Dvd1/Dvd1 mutants formed singly (Figure 3-2F). Defects were also seen within spikelets: Dvd1/Dvd1 mutants had a reduction in the number of florets per spikelet although the reduction was not statistically significantly different from normal (Figure 3-2G, \( t = 2.47, \text{d.f.} = 4, P = 0.069 \)). Furthermore, Dvd1/Dvd1 mutants had a significant reduction in the number of stamens per floret compared to normal (Figure 3-2H, \( t = 5.14, \text{d.f.} = 4, P = 0.007 \)). In addition, bract leaves that would otherwise be suppressed from growing out in normal individuals developed in Dvd1 mutants (Figure 3-1C). Quantification showed an increase in bract leaf outgrowth in Dvd1/+ and Dvd1/Dvd1 mutants compared to no outgrowth in normal plants (Figure 3-2I, \( t = -2.36, \text{d.f.} = 4, P = 0.078 \) and \( t = -10.49, \text{d.f.} = 4, P < 0.001 \)). The failure of Dvd1 mutants to produce the normal number of tassel branches, spikelets, florets and floral organs suggests that the initiation or maintenance of all types of axillary meristems are defective in Dvd1 inflorescences.

Dvd1 mutants also had defects in the development of the ear, which arises from an axillary meristem a few nodes below the tassel. In B73, Dvd1/+ ears were morphologically unaffected but ear number was significantly reduced, while Dvd1/Dvd1 mutants failed to produce
any ears (Figure 3-1B, 3-2C, $t = 4.03$, d.f. = 80, $P < 0.001$ and $t = 19.98$, d.f. = 29, $P < 0.001$). As $Dvd1/Dvd1$ mutants occasionally produced an ear in the Mo17 genetic background (Figure 3-2D), analysis of the ear phenotype was carried out in this background. Normal maize ears typically produce regular rows of kernels as a result of the initiation of paired spikelets which each produce a single floret (the lower floret aborts) (Kiesselbach, 1949; Cheng et al., 1983). In Mo17, however, $Dvd1$ heterozygous ears exhibited irregular kernel rows (Figure 3-1E), reduced size (70% of normal length) and reversed germ orientation (rgo) (Figure 3-1F, asterisk). Quantification revealed a statistically significant reduction in the number of kernels in $Dvd1/+\text{ mutants compared to normal (Figure 3-2J, } t = 4.50\text{, d.f. = 12, } P = 0.001\text{). In homozygous } Dvd1\text{ mutants, ears that were occasionally produced were small and had very few kernels (Figures 3-1E, 3-2J). Bract leaf outgrowth was also visible in the ear (arrow in Figure 3-1F). Hence, } Dvd1\text{ tassels and ears have similar defects in bract leaf and axillary meristem development although the severity of the defect differs between B73 and Mo17.}

### 3.3.3 $Dvd1$ mutants have defects in axillary meristem formation during inflorescence development

To investigate if the reduced number of branches and spikelets in $Dvd1$ mutants was due to defective BM and SPM formation, we performed scanning electron microscopy (SEM) on tassel inflorescences at various stages of development in the B73 background. Early in development, normal inflorescences developed lateral branches at the base of the main spike and the flanks of both the branches and main spike were covered by SPMs (visible as bumps) in regular rows (Figure 3-3A). Both $Dvd1/+\text{ and } Dvd1/Dvd1\text{ inflorescences at similar developmental stages had no evidence of BM initiation (Figure 3-3B, C). SPM formation was observed in } Dvd1/+\text{ mutants, although there were patches without SPMs (Figure 3-3B). In
Dvd1/Dvd1 mutants, SPMs often failed to initiate (Figure 3-3C). Bract primordia were visible in regular phyllotaxy in Dvd1/Dvd1 mutants (Figure 3-3C) but were hidden by SPMs in normal inflorescences. Furthermore, unlike normal, bract leaves continued to grow out in Dvd1/Dvd1 mutants (Figure 3-3C).

In normal inflorescences, most SPMs gave rise to two SMs (Figure 3-3D). Dvd1/+ inflorescences at the same stage failed to initiate some SMs, leading to the production of single spikelets (Figure 3-3E). Dvd1/Dvd1 inflorescences mainly produced single SMs (Figure 3-3F). In addition, in Dvd1/+ and Dvd1/Dvd1, some SPMs aborted after initiation (Figure 3-3E, G, H). The outgrowth of bract leaves was also observed, especially in Dvd1/Dvd1 mutants (Figure 3-3F, G, H). Bract leaves occasionally subtended developing spikelets (Figure 3-3G) while other bract leaves were solitary (Figure 3-3C, H). Later in development, bract leaves continued to grow in Dvd1 mutants (Figure 3-3H). Normal tassels typically develop an upper and a lower FM in each spikelet later in development (not shown). Although some FMs appeared to develop normally in Dvd1/+ mutants, abnormalities in the production of floral organs were seen in other FMs (Figure 3-3I).

In summary, Dvd1 mutants have defects in the initiation and outgrowth of all axillary meristems produced during inflorescence development similar to the barren mutants. However, distinct from the barren mutants, Dvd1 mutants exhibit the abnormal outgrowth of bract leaves.

3.3.4 Dvd1 mutants have defects in vegetative development

In addition to defects in inflorescence development, Dvd1 mutants also exhibited defects during vegetative development as they were semi-dwarf. In both B73 and Mo17 inbred lines, Dvd1 mutant plants were markedly shorter than their normal siblings, with homozygous mutants having an even more severe height reduction than heterozygotes (B73 shown in Figure 3-4A).
Quantification of mature plant height in B73 showed that Dvd1/Dvd1 mutants were less than half the height of normal siblings and confirmed that there was a statistically significant reduction in plant height in both Dvd1/+ and Dvd1/Dvd1 compared to normal (Figure 3-4B, $t = 5.24$, d.f. = 35, $P < 0.001$ and $t = 9.46$, d.f. = 25, $P < 0.001$).

To determine if the significant reduction in plant height of Dvd1 mutants was due to the production of fewer phytomers, we quantified leaf number. Interestingly, there was no significant difference in leaf number in either heterozygous or homozygous Dvd1 mutants compared to their normal siblings (Figure 3-4C, $t = -1.07$, d.f. = 39, $P = 0.291$ and $t = 1.21$, d.f. = 7, $P = 0.266$). Thus, reduced plant height in Dvd1 mutants is not caused by a reduction in the number of phytomers produced.

Since leaf number was not affected in Dvd1 mutants, the defect in plant height was further investigated by analyzing the internodes. Leaves were removed from individuals at maturity which revealed that the internodes from heterozygous and homozygous mutants were shorter and much more irregular in length compared to those of normal individuals (Figure 3-5A). Quantification of mature internode length showed a significant difference at all (except one) internodes measured in Dvd1/Dvd1 individuals compared to normal (Figure 3-5B, Table 3-1) and a significant reduction in half of the internodes in Dvd1/+ individuals compared to normal (Figure 3-5B, Table 3-1). Thus, the reduction of plant height in Dvd1 mutants is due to defects in internode length.

To establish if the observed reduction in internode length of Dvd1 mutants was due to differences in cell elongation, we sectioned developing internodes and stained with TBO. Normal internodes develop regular cell files in longitudinal section (Figure 3-6A). Surprisingly, Dvd1/+ mutants had larger cells and Dvd1/Dvd1 mutants had very irregular cells with both larger and smaller cells than normal (Figure 3-6B, C). Quantification of these defects showed that the cells in Dvd1 mutants were indeed significantly longer than normal (Figure 3-6D, $t = -4.93$, d.f. =
72, \( P < 0.001 \) and \( t = -4.87, \text{ d.f.} = 80, P < 0.001 \) and significantly wider than normal (Figure 3-6E, \( t = -14.0, \text{ d.f.} = 94, P < 0.001 \) and \( t = -9.6, \text{ d.f.} = 74, P < 0.001 \)). As cells in Dvd1 internodes are larger than normal, we infer that the reduction in internode length is due to reduced cell proliferation.

**3.3.5 Dvd1 mutants have defects in axillary meristem formation during vegetative development**

Dvd1 mutants have defects in all types of axillary meristems during inflorescence development. To determine if Dvd1 functioned in axillary meristem formation during vegetative development, we constructed double mutants with teosinte branched1 (tb1). Outgrowth of tillers from vegetative axillary meristems in maize is normally suppressed by tb1, so tb1 mutants have a highly branched (tillered) phenotype (Doebley et al., 1997; Hubbard et al., 2002). In Dvd1; tb1 double mutants, we observed a severe reduction in overall tiller number (Figure 3-7A). Genotyping individuals for tb1 revealed that Dvd1 often completely suppressed the tb1 phenotype. Quantification of tiller number confirmed a statistically significant reduction of tiller number in both Dvd1/+; tb1/tb1 and Dvd1/Dvd1; tb1/tb1 double mutants compared to tb1/tb1 single mutants (Figure 3-7B, Primary tiller number \( t = 7.39, \text{ d.f.} = 9, P < 0.001 \) and \( t = 7.0, \text{ d.f.} = 2, P = 0.02 \)). Thus, Dvd1 also plays a role in axillary meristem formation during vegetative development.

**3.3.6 Dvd1 and bif2 exhibit an additive genetic interaction**

Double mutant combinations of bif2 with some of the other barren mutants have synergistic effects which further illustrate the roles of these genes in vegetative development (Barazesh and McSteen, 2008; Gallavotti et al., 2008c). For example, Bif1 and spi1, which
regulate auxin transport and biosynthesis respectively, produce extremely dwarf plants in combination with \textit{bif2} (Barazesh and McSteen, 2008; Gallavotti et al., 2008c). To test whether \textit{Dvd1} had a similar interaction with \textit{bif2}, we constructed double mutants. \textit{bif2} mutant tassels have some similarities to those of \textit{Dvd1} including fewer branches, spikelets, florets and floral organs (Figure 3-8A). \textit{Dvd1}/\textit{Dvd1}; \textit{bif2}/\textit{bif2} double mutants produced barren tassels with no lateral branches, no spikelets and a few bract leaves (Figure 3-8A). Quantification revealed that the number of spikelets was not significantly different from \textit{Dvd1}/\textit{Dvd1} (Figure 3-8B, \( t = -0.18, \) d.f. = 4, \( P = 0.868 \)), but the number of bract leaves was significantly reduced compared to \textit{Dvd1}/\textit{Dvd1} (Figure 3-8C, \( t = 11.98, \) d.f. = 11, \( P < 0.001 \)). Furthermore, there was no enhancement of vegetative phenotypes as \textit{Dvd1}/\textit{Dvd1}; \textit{bif2}/\textit{bif2} double mutants were the same height as \textit{Dvd1}/\textit{Dvd1} (Figure 3-8D, \( t = -0.76, \) d.f. = 3, \( P = 0.5 \)) and had the same number of leaves as \textit{bif2} (Figure 3-8E, \( t = -0.26, \) d.f. = 3, \( P = 0.813 \)). We interpret these data as an additive interaction between \textit{Dvd1} and \textit{bif2}, suggesting that \textit{dvd1} functions independently of \textit{bif2}.

### 3.4 Discussion

We have identified and characterized a novel maize mutant with defects in both vegetative and reproductive development. We show using SEM analysis that \textit{Dvd1} mutants produce fewer branches, spikelets, florets and floral organs due to defects in the production of axillary meristems in the inflorescence. SEM analysis also shows that the leaves visible in the inflorescence of \textit{Dvd1} mutants are due to the outgrowth of bract leaf primordia which are normally suppressed. In addition, \textit{Dvd1} mutants have defects during vegetative development due to shortened internodes. Genetic interaction studies with \textit{tb1} further illustrate the function of \textit{Dvd1} in axillary meristems during vegetative development. Therefore, we have identified a novel
player in the regulation of axillary meristem, bract leaf and internode development illustrating that all three aspects of phytomer development are under common genetic control.

3.4.1 Role of dvd1 in axillary meristem development

*Dvd1* mutants are most similar to the *barren* class of mutants in maize which have defects in axillary meristem initiation during vegetative and inflorescence development. The inflorescence defects of *Dvd1* mutants such as fewer branches, spikelets, florets and floral organs are also seen in *Bif1, bif2, bal* and *spi1* mutants (McSteen and Hake, 2001; Ritter et al., 2002; Barazesh and McSteen, 2008; Gallavotti et al., 2008c). Single spikelets and a reduction in the number of organs at the center of the floret, which are observed in *Dvd1* mutants, are also characteristic of the *barren* class of mutants. During ear development, fewer ear shoots and fewer kernels in the ear are seen in the *barren* mutants; in particular, *bal* mutants never produce an ear shoot (Ritter et al., 2002), which is similar to the effects of the *Dvd1* mutation in B73. *Dvd1* mutants produce aborted SPMs which are also seen when normal plants are treated with auxin transport inhibitors (Wu and McSteen, 2007). Furthermore, double mutant combinations between *Dvd1* and *tb1* show that *Dvd1* mutants have defects in vegetative axillary meristems. The magnitude of the effect of *Dvd1* on tiller outgrowth is very similar to the effect of *bif2* and *bal* double mutant combinations with *tb1* (Ritter et al., 2002; McSteen et al., 2007). Thus, *Dvd1* is a new member of the *barren* class of mutants.

The *barren* mutants all have defects in auxin biosynthesis, transport or response (McSteen et al., 2007; Wu and McSteen, 2007; Barazesh and McSteen, 2008; Gallavotti et al., 2008c; Gallavotti et al., 2008a; Skirpan et al., 2008). A key difference between the mutants that are defective in auxin transport or biosynthesis (*Bif1, bif2* and *spi1*) and the *bal* mutant, is the phenotype of the inflorescence rachis. In *bal* mutants, bract primordia are produced with normal
phyllotaxy resulting in the production of regular bumps along the surface of the inflorescence rachis (Ritter et al., 2002). On the other hand, in \textit{Bif1, bif2} and \textit{spi1} mutants, bract primordia are not visible in a regular pattern on the surface of the inflorescence rachis resulting in a smooth or ridged inflorescence rachis (McSteen and Hake, 2001; Barazesh and McSteen, 2008; Gallavotti et al., 2008c; Skirpan et al., 2008). The interpretation of the \textit{Bif1, bif2} and \textit{spi1} inflorescence rachis phenotype is that phyllotaxy is abolished due to the defects in auxin biosynthesis and transport, resulting in auxin being unavailable to specify the position of the bract primordia (Reinhardt et al., 2003; Skirpan et al., 2008). The interpretation of the \textit{ba1} bract phenotype is that auxin transport is normal during the initiation of bract primordia and subsequently there are defects in the initiation of SPM in the axils of bract primordia (Gallavotti et al., 2008a; Skirpan et al., 2008). In this paper, SEM analysis shows that homozygous \textit{Dvd1} mutants differ from \textit{Bif1, bif2} and \textit{spi1} mutants and instead, are more similar to \textit{ba1} mutants as bract primordia are produced with regular phyllotaxy along the surface of the inflorescence.

Another mechanism to distinguish the \textit{barren} mutants from each other is through their genetic interaction with \textit{bif2}. \textit{Bif1} and \textit{spi1} mutants have a synergistic interaction with \textit{bif2}, while \textit{ba1; bif2} double mutants resemble \textit{bif2} (Barazesh and McSteen, 2008; Gallavotti et al., 2008c; Skirpan et al., 2008). Double mutants between \textit{Dvd1} and \textit{bif2} do not have synergistic defects and instead appear to be somewhat additive. The genetic interaction of \textit{Dvd1} with \textit{bif2} suggests that the \textit{Dvd1} mutant does not have general defects in auxin transport or biosynthesis similar to \textit{Bif1} and \textit{spi1}.

3.4.2 Role of \textit{dvd1} in bract leaf outgrowth

The compensatory relationship between the axillary meristem and the subtending leaf has long been recognized (Steeves and Sussex, 1989). For example, during vegetative development
the leaf is large and the axillary meristem is suppressed, while during floral development the
axillary meristem is large and the subtending bract leaf is suppressed (Steeves and Sussex, 1989;
Long and Barton, 2000). The compensatory relationship between the axillary meristem and the
subtending bract leaf was experimentally demonstrated in Arabidopsis by expressing diphtheria
toxin under the control of the leafy (lfy) promoter which is expressed in floral meristems (Nilsson
et al., 1998). Ablation of floral meristems in these plants resulted in the outgrowth of bract
leaves.

The compensatory relationship between the axillary meristem and the subtending bract
leaf is also seen in the maize inflorescence as demonstrated by the bal mutant. bal mutants have
defects in axillary meristem initiation and have larger than normal bract primordia (Ritter et al.,
2002). However, Dvd1 mutants are distinct from bal mutants as bract primordia do not grow out
to produce bract leaves in bal. The suppression of bract leaves in maize is controlled by the
tasselsheath1 (tsh1) gene (McSteen and Hake, 2001). tsh1 mutants have elongated bract leaves
subtending the branches and the spikelet pairs at the base of the tassel (McSteen and Hake, 2001).
ba1; tsh1 double mutants produce a ba1 tassel with bract leaves (P.M. unpublished results)
indicating that tsh1 suppresses bract leaf outgrowth in ba1 mutants. We propose that the
differences between the Dvd1 and ba1 mutant phenotypes could be explained by the expression
of tsh1 in ba1 but not in Dvd1, which could be tested once tsh1 is cloned. A further indication
that the Dvd1 mutant is distinct from ba1 is that ba1 has an epistatic interaction with bif2 while
Dvd1 has an additive interaction with bif2. Therefore, Dvd1 represents a distinct type of barren
mutant.
3.4.3 Role of *dvd1* in germ orientation

The *Dvd1* mutant was originally isolated based on the rgo phenotype in the ear. An rgo phenotype can develop in one of three ways which can be explained by defects in the development of florets. Normally, the spikelet produces two florets, an upper and a lower floret. Due to the alternate phyllotaxis of floret initiation these florets are mirror images of each other. In the ear, the lower floret aborts leaving only the upper floret (Cheng et al., 1983). Analysis of *rgo1* mutants in maize showed that an rgo phenotype can occur due to the production of three florets (Kaplinsky and Freeling, 2003). In this case, the lower two florets abort, leaving the third floret in an inverse orientation compared to normal so that when the ovary is pollinated, the embryo (germ) forms on the opposite face of the endosperm (Kaplinsky and Freeling, 2003). Another way of obtaining an rgo phenotype is through the production of single florets. If only the lower floret forms and it does not abort, then the germ would be in an inverse orientation compared to normal. This phenotype is seen in *Bif1* and *bif2* mutants (PM, unpublished results). A third mechanism of obtaining an rgo phenotype is through changes in floral symmetry. If the floret is twisted compared to normal or if the axis of adaxial - abaxial symmetry is not set up correctly, as in the *wandering carpel* mutant of maize, then an rgo phenotype could form (Irish et al., 2003). As *Dvd1* mutants have fewer florets than normal, it is likely that the rgo phenotype in *Dvd1* is caused by the production of single florets.

3.4.4 Role of *dvd1* in internode development

*Dvd1* plants are semi-dwarf due to the production of shorter internodes. As the cells in mutant internodes are significantly larger than normal, we infer that the defect in *Dvd1* is caused by reduced cell proliferation and that the cells expand to compensate for the reduction. There are
many examples in which reduced cell proliferation has been shown to result in compensatory increases in cell expansion (Haber and Foard, 1964; Hemerly et al., 1995; Doonan, 2000; Shpak et al., 2004).

Mutants with defects in various hormone pathways cause plants to be shorter than normal due to a reduction in the size of the internodes. Dwarf mutants in rice and wheat are caused by defects in gibberellic acid (GA) or brassinosteroid pathways (Hedden, 2003; Morinaka et al., 2006). However, short internodes in these mutants are caused by reduced cell elongation in contrast to $Dvd1$ mutants which have larger cells. Furthermore, $Dvd1$ does not exhibit other characteristics of GA- or brassinosteroid- insensitive mutants, indicating that it is unlikely that $dvd1$ is involved in GA or brassinosteroid hormone pathways.

Multiple mutants have been identified that are dwarf due to reduced auxin transport. $brachytic2$ ($br2$) mutants in maize and $dwarf3$ mutants in sorghum are semi-dwarf due to the reduced length of internodes (Multani et al., 2003). $br2$ encodes an ABC transporter protein which functions in regulating auxin transport. $roughsheath2$ ($rs2$) and $semaphore$ ($sem$) mutants in maize also have short internodes and reduced polar auxin transport (Schneeberger et al., 1998; Tsiantis et al., 1999; Scanlon et al., 2002). Furthermore, treatment of maize plants with auxin transport inhibitors causes dwarfism (Tsiantis et al., 1999). Mutants with short internodes and defects in auxin transport have also been seen in Arabidopsis (Gil et al., 2001; Geisler et al., 2003). As auxin is known to control cell expansion (Jones et al., 1998; Christian et al., 2006), some of these cases have been shown to be caused by reduced cell elongation (Multani et al., 2003). However, auxin also plays a role in regulating cell division (del Pozo et al., 2005; Li et al., 2005; Vanneste et al., 2005; Hartig and Beck, 2006; David et al., 2007). We speculate that many of the defects in $Dvd1$ mutants could be explained by the $dvd1$ gene functioning in auxin-mediated cell proliferation.
3.4.5 Conclusions

*Dvd1* mutants exhibit pleiotropic defects in phytomers produced during both vegetative and reproductive development. There are differences in the severity of the defects in two different genetic backgrounds, B73 and Mo17, implying that there are other genetic factors influencing the phenotype which would be interesting to pursue in the future. The defects in axillary meristem initiation and outgrowth indicate that *Dvd1* plays an important role in axillary meristems during both vegetative and reproductive development. The defect in bract leaf outgrowth is likely an indirect effect of the lack of axillary meristem initiation. Furthermore, *Dvd1* mutants have defects in internode development. The *Dvd1* mutant illustrates that axillary meristem and internode development are under common genetic control.

Interestingly, selection on both axillary meristem activity and internode length have been instrumental in the domestication of crop plants. For example, axillary meristems were suppressed during the domestication of maize leading to a single axis of growth compared to its wild relative teosinte which is bushy (Doebley et al., 1997). Furthermore, selection of semi-dwarf varieties of wheat, sorghum and rice has been critical to reduce lodging (plants falling over) and increase yield which led to the “green revolution” in agriculture (Hedden, 2003; Multani et al., 2003; Morinaka et al., 2006). These examples illustrate the importance of understanding the regulation of axillary meristem and internode development for agriculture and for plant morphology in general.

As *Dvd1* is a dominant mutant, it could be either a loss or a gain of function mutation, so the *dvd1* gene may be either a positive or a negative regulator of axillary meristem and internode development. We have mapped *Dvd1* to two BAC contigs on chromosome 5. Positional cloning of the locus will clarify the mechanism by which the *dvd1* gene plays such an important role in vegetative and reproductive development.
Figure 3-1: Dvl mature inflorescence phenotypes.

(A) Tassels in the B73 genetic background. Dvl+/ and Dvl+/Dvl mutants have no lateral branches, reduced spikelet number, and elongated bract leaves compared to normal. (B) Open-pollinated ears in the B73 background. Dvl+/ ears resemble normal, while Dvl+/Dvl mutants fail to produce ears. (C) Close-up of bract leaf outgrowth on a Dvl/Dvl mutant tassel in B73. (D) Tassels in the Mo17 background. Dvl+/ and Dvl+/Dvl mutants produce fewer branches and spikelets than normal. (E) Open-pollinated ears in the Mo17 background. Dvl+/ ears are shorter and have irregular rows of kernels. When ears are produced in Dvl/Dvl mutants, size, and kernel number are reduced. (F) Close-up of Dvl+/ ear in Mo17. Arrowhead indicates normal germ orientation, asterisk indicates reversed germ orientation, and arrow indicates that bract leaves are also visible in the ear.
Figure 3-2: Quantification of Dvd1 mature inflorescence phenotypes.

(A) Tassel branch number in B73. (B) Tassel branch number in Mo17. (C) Number of ears in B73. (D) Number of ears in Mo17. (E) Spikelet number in B73. (F) Percentage of spikelets that are single (white), paired (gray) or triplet (hatched) in B73. (G) Number of florets per spikelet in B73. (H) Number of stamens per floret in B73. (I) Bract leaf outgrowth in B73. (J) Kernel number in Mo17.
Figure 3-3: Scanning electron micrographs of developing Dvd1 inflorescences in the B73 background.

(A) Normal tassel with long lateral branches visible at the base. SPM are produced near the tip of the inflorescence and produce two SMs soon afterwards. (B) Dvd1/+ mutant tassel with no lateral branches. SPMs are produced at the tip and some produce paired SMs (PS) while others produce only a single SM (SS). (C) Dvd1/Dvd1 mutant tassel. Bract primordia (BR) are visible in regular phyllotaxy and bract leaves elongate at the base of the tassel. Some bract primordia produce SPMs in their axils while many bract primordia do not produce SPMs. (D) Normal tassel showing the development of paired SMs (PS). The outer and inner glumes (GL) are the leaf-like organs produced by the SMs. Image provided by X. Wu. (E) Dvd1/+ mutant tassel showing that single SMs (SS) and aborted SPMs (AS) can form instead of paired SMs. (F) Dvd1/Dvd1 mutant tassel showing several single SMs (SS) being produced. (G) Dvd1/Dvd1 mutant tassel showing that some SPMs abort later in development (AS). Single spikelets are sometimes produced in the axils of elongated bract leaves (B). (H) Dvd1/Dvd1 later in development showing elongated bract leaves and aborted SPMs (AS). (I) Dvd1/+ later in development showing the irregular arrangement of floral organs. FMs with normal arrangement of floral organs (NF) are produced along with abnormal FMs (AF). Scale bars = 250µm. AF, abnormal FM; AS, aborted spikelet pair meristem; BR, bract; BM, branch meristem; GL, glume; NF, normal floral meristem; PS, paired spikelet meristem; SM, spikelet meristem; SPM, spikelet pair meristem; SS, single spikelet meristem.
Figure 3-4: *Dvd1* vegetative phenotype in the B73 background.

(A) *Dvd1/+* and *Dvd1/Dvd1* mutants are markedly shorter than normal siblings. Note that some leaves have already senesced at maturity. (B) Quantification of plant height. (C) Quantification of leaf number.
Figure 3-5: *Dvl1* internode analyses.

(A) Internodes at the base of mature plants in the B73 background after the removal of all leaves. Brackets indicate the length of each internode. *Dvl1* mutant internodes are reduced compared to normal resulting in semi-dwarf stature. (B) Quantification of internode length between consecutive leaves in B73. Leaf number is from base of the plant (leaf 9-10) to the tip of the plant (Leaf 20-21). Internodes below leaf 9 are not shown. * indicates statistically significant difference from normal at *P*-value < 0.05.
Figure 3-6: Histology of developing Dvd1 internodes.

(A-C) Longitudinal sections from developing internodes stained with TBO in the B73 background. (A) Normal internode showing regular cell size and cell shape resulting in elongated files of cells. (B) Dvd1/+ internode showing enlarged cells. (C) Dvd1/Dvd1 internode showing highly irregular cell size and shape. (D) Quantification of cell length. (E) Quantification of cell width. Scale bars = 100µm.
Figure 3-7: Dvd1; tb1 double mutant analysis.

(A) Mature whole plant phenotype of individuals in a segregating family in the B73 background. Dvd1 suppresses the highly branched tb1 phenotype in double mutants. (B) Quantification of primary (grey bar) and secondary (white bar) tiller number of individuals from each genetic class in a segregating family.
Figure 3-8: *Dvl1; bif2* double mutant analysis.

(A) Mature tassel phenotype of all genetic classes from a segregating family in the B73 background. (B) Quantification of tassel spikelet number in individuals from each genetic class. (C) Quantification of bract number. (D) Quantification of plant height. (E) Quantification of leaf number.
Table 3-1: Statistical values for internode lengths shown in Figure 3-5B.

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CHAPTER 4

Summary and discussion of the roles of *vanishing tassel2* and *developmental disaster1* in maize vegetative and reproductive development
The work presented in this thesis offers new insights into the genetic regulation of vegetative and reproductive development in maize. Positional cloning of the *vanishing tassel2* locus represents a significant contribution to the maize community as it provides the first evidence of a functional IPA auxin biosynthesis pathway in maize or any other monocot (Chapter 2). Furthermore, characterization of the *vt2* mutant phenotype has helped elucidate its critical role in many aspects of development (Chapter 2). In addition, characterization of the novel *Developmental disaster1* maize mutant has revealed pleiotropic defects with disruption of a single locus; specifically, the *Dvd1* phenotype revealed that both axillary meristem and internode development are under common genetic control in maize (Chapter 3).

The *vt2* and *Dvd1* characterization projects have also revealed how two mutants with similar phenotypes can be functioning in different aspects of development. For instance, both mutants exhibit dramatic reductions in height compared to wild-type; in fact, both *vt2* and homozygous *Dvd1* mutants typically show about a 50% height reduction compared to normal siblings. However, by investigating the development of the mutants’ phytomers, it was determined that *vt2* mutants are shorter because they produce fewer leaves while *Dvd1* mutants are shorter due to reductions in internode elongation. Similarly, phytomers produced in the inflorescence exhibit differences during *vt2* and *Dvd1* mutant development. Utilizing scanning electron microscopy, it was determined that young *vt2* inflorescences are defective in the formation of axillary meristem and bract primordia, as evidenced by a ‘smooth’ inflorescence rachis. Conversely, *Dvd1* mutants show regular formation of bract primordia on the surface of the inflorescence early in development. Such a phenotype is more comparable to the *barren stalk1* maize mutant which was concluded to transport auxin normally and be deficient only in the actual initiation of axillary meristems during development. This suggests that the lack of
meristem development in \textit{Dvd1} mutants may also be due to a defect in auxin response rather than biosynthesis, as in the case of \textit{vt2}.

Additional support for diverse functions of \textit{vt2} and \textit{Dvd1} during maize development comes from their genetic interactions with other auxin-related maize mutants. For instance, a dramatic synergistic phenotype was observed in \textit{vt2};\textit{bif2} double mutants, suggesting that \textit{vt2} and \textit{bif2} function together to regulate vegetative and reproductive development. However, \textit{Dvd1};\textit{bif2} double mutants showed a clear additive interaction in both vegetative and reproductive phenotypes, suggesting the two genes function independently of one another in these aspects of development.

4.2 Future Work

4.2.1 \textit{vanishing tassel2}

Although the \textit{vt2} phenotype has been thoroughly characterized and genetic interactions with other auxin-related mutants have been examined, additional work to further elucidate the function of \textit{vt2} in maize development could be performed.

Identifying and characterizing the closely related maize duplicates of \textit{vt2} could reveal more about the genetic redundancy of auxin biosynthesis genes in maize development. Through BLAST analysis of the available maize genome data, we have identified at least five closely-related paralogs of \textit{vt2}. The most closely related gene (‘\textit{vt2-like}’) is located on chromosome 3, and the remaining paralogs are located on chromosomes 2, 3, and 6. \textit{vt2} and \textit{vt2-like} showed about 83\% similarity at the amino acid level for their predicted proteins.

Phenotypic characterization of the close \textit{vt2} paralogs could be very beneficial if loss of function mutants can be identified. In addition to comparing the single mutant phenotypes of \textit{vt2}
and the duplicate genes, double mutants between vt2 and its paralogs could also be produced. Based on the double and triple mutants constructed in Arabidopsis (Stepanova et al., 2008), it would be expected that eliminating the functions of multiple maize tryptophan aminotransferases would result in a significantly more severe phenotype than either single mutant alone. If this is the case, the genetic redundancy of vt2 and its paralogs would be further supported.

As we hypothesize that the weaker phenotypes of vt2 mutants grown under increased temperature could be explained by increased expression of other tryptophan aminotransferases, it is important to test whether or not this is the case. Real-time RT-PCR could be conducted to quantify the transcript levels of vt2 paralogs in wild-type and vt2 tissue samples grown under different temperature conditions. It would be expected that transcript levels of vt2 paralogs grown under higher temperatures would be significantly increased compared to the their transcript levels in vt2 samples grown under low temperatures.

A question that remains following the characterization of vt2 is why one pathway of auxin biosynthesis cannot compensate for loss of another during development, and one of the simplest explanations is that the pathways are simply functioning in different areas of the plant. For instance, spi1 does not show dramatic defects during vegetative development however vt2 mutants show severe vegetative phenotypes, indicating that vt2 plays a significant role in vegetative development while spi1 does not. RNA in situ hybridization has also revealed a very localized expression pattern of spi1 at certain stages of development in inflorescences (Gallavotti et al., 2008b). Hence, if vt2 is functioning in a similar manner but exhibits different spatial or temporal localization, the lack of compensation of the two pathways could be explained. Preliminary work to perform RNA in situ hybridization using vt2 as a probe has been conducted but thus far has not been successful. It is suspected that the very low levels of expression of vt2 that were observed by RT-PCR analysis could be contributing to the difficulty in obtaining an in situ signal.
Availability or lack of substrates in each pathway could also play a role in the ability of different IAA biosynthesis routes to compensate for one another. Since these factors could be cell-specific, as well as regulated by temperature and/or light, thorough biochemical analysis of available intermediates similar to the analysis conducted by Sugawara et al. (2009) could be performed to determine the similarities and differences among the pathways.

Additional phenotype work to determine if vt2 plays a role in the shade avoidance response, root ethylene sensitivity, or NPA resistance could also be conducted. These studies could allow easier comparison of vt2 function in maize to the function of Arabidopsis tryptophan aminotransferases, since these were the key phenotypes observed in the sav3, wei8, and tir2 Arabidopsis mutants (Stepanova et al., 2008; Tao et al., 2008; Yamada et al., 2009).

Finally, numerous auxin-related experiments could be performed with vt2 to further elucidate its roles in auxin function. For instance, ZmPIN1 localization studies could be used to determine if location or expression levels are affected as an indicator of auxin transport in vt2 mutants (Gallavotti et al., 2008a). Free IAA levels could also be measured to test if endogenous auxin levels are reduced in vt2 mutants, as would be expected for a mutant deficient in auxin biosynthesis (Skirpan et al., 2009). In addition, DR5 auxin-reporter fusion studies could be used to infer auxin concentrations in different tissues (Gallavotti et al., 2008a).

4.2.2 Developmental disaster

In addition to cloning Dvd1 to determine the nature of the DVD1 protein, additional analyses could be performed to further elucidate Dvd1’s role in development. A summary of the current mapping status of Dvd1 is provided in Appendix A.

Further phenotypic characterization could shed additional light on the function of Dvd1. For instance, preliminary data has been found which indicates that Dvd1 mutants may have
defects in root development. Adventitious roots appear to be reduced in number in *Dvd1* mutants compared to wild-type, and mutants may have defects in elongation as well. It is possible that the agravitropic development observed in *Dvd1* shoots could be occurring during root growth as well. Phyllotaxy patterning has also been found to be severely disrupted in *Dvd1* mutants depending on genetic background. In fact, the overall severity of *Dvd1* vegetative growth was significantly greater in the Mo17 genetic background compared to B73, so it is possible that this background contains a genetic modifier that can account for these differences. It would be interesting to introgress *Dvd1* into additional maize backgrounds and further investigate the extent of the background-dependence of the *Dvd1* phenotype.

Our hypothesis that *Dvd1* functions in a similar manner to *bal* could be investigated by determining whether auxin transport and synthesis are similarly unaffected in *Dvd1* mutants. Studies to investigate the localization and expression levels of *ZmPIN1* in *Dvd1* mutants could be performed as was accomplished for *bal* mutants by Gallavotti et al. (2008). If the results do not show significant differences in mutants compared to normal, it could be concluded that polar auxin transport is functioning normally in *Dvd1* mutants to produce the observed axillary meristem and bract primordia, thus supporting the hypothesis that *Dvd1* may play a role in auxin signaling.

It is also entirely possible that *Dvd1* is not an auxin-related gene at all. The pleiotropic defects observed in *Dvd1* development indicate that hormones are likely involved, but growth hormones other than auxin have been found to disrupt multiple aspects of development as well (Taiz and Zeiger, 2006). For instance, the dwarf (*d1*) mutant of maize is defective in gibberellin biosynthesis and shows a severe reduction in internode length, tassel length, and floral development (Spray et al., 1996). Cytokinins also play important roles in development; for instance, the maize mutant *abphyll* is defective in the negative regulation of cytokinin signaling and has altered phyllotaxy due to defects in its shoot apical meristem (Giulini et al., 2004).
Hence, numerous other phytohormones in plants have overlapping functions with those of auxin during development, and defects in their synthesis or signaling could likewise cause the phenotypes observed in Dvd1 mutants.

Thus, in order to further understand the function of Dvd1, it is critical to clone the corresponding gene. Although all available public markers in the Dvd1 region have been exhausted, additional markers can be designed from neighboring genes to identify linked single nucleotide polymorphisms (SNPs), which was a beneficial technique in the cloning of vt2. Since mapping populations have been constructed in two different genetic backgrounds, it is possible that enough polymorphic SNP markers could be developed to narrow the Dvd1 region to within only a few genes. Cloning Dvd1 could reveal an important gene functioning in hormonal control of plant development.

4.3 Future Perspectives

The work presented in this thesis has focused on the genetic regulation of vegetative and reproductive development in maize. The characterization of two maize mutants, vt2 and Dvd1, has revealed that genes causing similar loss of function phenotypes can act in divergent processes of development. Such severe phenotypes indicate that hormones could be disrupted in these mutants, such as the critical growth hormone auxin. vt2 has been found to play a key role in auxin biosynthesis, and while many aspects of Dvd1’s phenotype suggest it may function in auxin signaling, it is also possible that Dvd1 plays a role in the regulation of a different hormone. Characterization of additional vt2 and Dvd1 phenotypes, as well as the cloning of the Dvd1 locus, will provide even further insight into the regulation of vegetative and reproductive growth in plants.
4.4 Literature Cited


APPENDIX

Additional data on the characterization and positional cloning of

*Developmental disaster1*
A.1 Introduction

*Dvd1*’s interaction with other maize inflorescence mutants was analyzed but not included in the published work presented in Chapter 3. Double mutant combinations of *Dvd1* with the meristem initiation mutant *barren stalk1 (ba1)* and with the meristem determinacy mutant *ramosa1 (ra1)* were constructed and analyzed for tassel and ear phenotypes. *Dvd1* mapping work subsequent to that presented in Chapter 3 (Section 3.3.1) has also been performed to further narrow the *Dvd1* region and enable a feasible candidate gene search in the future.

A.2 Results

A.2.1 *Dvd1;ba1* double mutants

Since *Dvd1* appeared to play a role in axillary meristem initiation, we constructed double mutants with another maize mutant defective in this process, *ba1*, to determine if there was any phenotype enhancement. Double mutant plants were constructed using the *ba1-ref* allele (Ritter et al., 2002) in the B73 background and were found to resemble *Dvd1* single mutants during vegetative growth (data not shown), indicating that *Dvd1* is epistatic to *ba1* during vegetative growth. Conversely, *Dvd1/+;ba1/ba1* and *Dvd1/Dvd1;ba1/ba1* double mutants did not produce ears, indicating that *ba1* is epistatic to *Dvd1* during ear inflorescence development. In some cases, the *ba1-ref* allele has been found to initiate some meristems in the inflorescence and can thus produce functional spikelets at maturity. The cause of these differences in phenotypic severity is likely due to background-dependent modifiers. Using a *ba1-ref* line in which mutants displayed this weaker *ba1* phenotype, it was found that *Dvd1;ba1* double mutants did not produce
any functional spikelets in mature tassels (Figure A-1). In addition, bract primordia in the form of bumps were clearly visible on the rachis of both Dvd1/+;bal/bal and Dvd1/Dvd1;bal/bal mutants. This complete abolishment of functional spikelets can be interpreted as a synergistic interaction since the double mutants produced a significantly more severe phenotype than either single mutant. This is especially evident in the Dvd1/+;bal/bal double mutant since both the weak bal and Dvd1/+ single mutants were able to produce many functional spikelets. Considering the severity of the Dvd1;bal double mutant phenotypes, it is possible that the two genes function in overlapping pathways.

A.2.2 Dvd1;ra1 double mutants

Since Dvd1 mutants show strong inhibition of meristem initiation and ra1 mutants show indeterminate growth of reproductive meristems, we constructed Dvd1;ra1 double mutants to test the interaction of these two genes using the ra1-ref allele (Vollbrecht et al., 2005). ra1 single mutants exhibit a highly branched tassel and ear phenotype due to indeterminate growth, however Dvd1;ra1 double mutants did not show any branching in the tassel inflorescence (Figure A-2A). This data suggests that Dvd1 is completely epistatic to ra1 in the tassel. In the ear, Dvd1/+;ra1/ra1 double mutants resembled Dvd1/+ ears but also exhibited branching similar to ra1 mutants (Figure A-2B). As Dvd1/Dvd1 mutants never produce ears in the B73 background, the production of one Dvd1/Dvd1;ra1/ra1 double mutant ear was surprising (Figure A-2B). Nonetheless, this double mutant ear supported the findings of the Dvd1/+;ra1/ra1 ears since it resembled a Dvd1/Dvd1 ear (as observed in Mo17 background, Section 3.3.2 and Figure 3-1) and also displayed branching like ra1 single mutants. This additive interaction in the ear indicates that Dvd1 and ra1 function in separate pathways for ear inflorescence development.


A.2.3 Current Dvd1 mapping summary

Following exhaustion of publicly available markers in the Dvd1 region, single nucleotide polymorphism (SNP) markers were utilized to narrow the region further. Using a public database (www.Panzea.org), known SNPs were found in the Dvd1 region and utilized in both mapping populations. Two SNPs, SNP297 and SNP294, proved very useful in narrowing the region on the south side of Dvd1 (Figure A-3). Although the newly-narrowed region was still large, two candidate genes were identified and sequenced for potentially significant changes in Dvd1 mutants: an AUXIN RESPONSE FACTOR (ARF) gene and a SQUAMOSA PROMOTOR BINDING-LIKE (SQUA) gene (Figure A-3). Although neither was found to be the Dvd1 locus, a useful SNP was identified in the SQUA sequence and used to eliminate recombinants, and this marker also helped confirm the location of the Panzea SNP markers.

A more recent search for newly-added public markers has also been performed. Although many markers had in fact been added to the databases, idp8125 was the only one that proved useful in either mapping population. However, this marker was very beneficial since it helped narrow the Dvd1 region on the north side by more than 50% (Figure A-4A). The recent work completed using both SNP markers and idp8125 has narrowed the Dvd1 region to within the end portions of two BAC contigs encompassing less than 25 overlapping BAC clones (Figure A-4B).

A.3 Discussion

The synergistic interaction of Dvd1 with ba1 indicates that the two genes function together to regulate the initiation of axillary meristems. However, Dvd1 functions upstream of ba1 to regulate vegetative growth, while ba1 functions upstream of Dvd1 during ear inflorescence
development. It was also found that *Dvd1* is completely epistatic to *ral* in the tassel, indicating that *Dvd1* functions upstream of *ral* to regulate axillary meristem initiation. *Dvd1;ra1* double mutants showed an additive interaction in the ear, which suggests that the two genes function independently in ear inflorescence development.

The mapping of *Dvd1* has been complicated by the fairly rapid exhaustion of public markers in the region. After utilizing all of the original public markers that were available, the *Dvd1* region still spanned a total of three BAC contigs and hundreds of BAC clones. The region also appeared to be poorly annotated in the public genome browser and did not show good synteny with rice or sorghum. However, since two mapping populations have been constructed, plenty of potential still exists to proceed with mapping via SNP markers from neighboring genes. For instance, utilization of the SNP identified in the *SQUA* gene has already reduced the number of recombinants on the south side of *Dvd1* by more than 50%, and this was reduced again by nearly 50% with a Panzea SNP. Although all known Panzea SNPs have been exhausted, new features were recently added to another public database (www.MaizeGDB.org) which provide the location and sequence of known SNPs in the genome between the B73 and Mo17 genetic backgrounds. The next step for mapping *Dvd1* should be to utilize these databases, since this will save a great deal of time compared to designing primers and sequencing inbreds to identify potentially useful SNPs.

Once the *Dvd1* region has been narrowed to fewer BACs, a search can be performed for suitable candidate genes that could explain *Dvd1*'s suspected roles in auxin signaling or another aspect of hormonal control. Cloning of the locus will reveal the manner by which *Dvd1* regulates so many important aspects of maize vegetative and reproductive development.
Figure A-1: *Dvd1;ba1* double mutant analysis.

Mature tassel inflorescence phenotype of a segregating double mutant family. Similar to *ba1*, double mutants did not produce ears.
Figure A-2: $Dvd1;ra1$ double mutant analysis.

(A) Mature tassel inflorescence phenotype.  (B) Mature ear inflorescence phenotype. $Dvd1/Dvd1$ mutants did not produce any visible ears.
Figure A-3: The use of single nucleotide polymorphism (SNP) markers in *Dvd1* mapping.

**A** The original recombinant data for the *Dvd1* region between *umc1870* and *bnilg1902*, with the addition of SNP markers that have been utilized to narrow the region further. **B** The known location of some markers on the maize genome browser (www.maizesequence.org).
Figure A-4: Current Dvd1 mapping summary.

(A) The number of recombinants for each of the closest markers utilized thus far in the Dvd1 region. (B) Screenshot of the maize genome browser showing the current narrowed Dvd1 region on chromosome 5 (www.maizezsequence.org). The BAC clones which contain the two closest flanking markers, idp8125 and SNP294, are indicated.
A.4 Literature Cited
