Study of RAMOSA1 function during maize inflorescence development

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Study of RAMOSA1 function during maize inflorescence development

by

Xiang Yang

A dissertation submitted to the graduate faculty in partial fulfillment of the requirements for the degree of DOCTOR OF PHILOSOPHY

Major: Molecular, Cellular and Developmental Biology

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Ames, Iowa
2011

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CHAPTER 1. GENERAL INTRODUCTION

1.1 Inflorescence and branch architecture in plants

An inflorescence is a cluster or group of flowers arranged on a stem that is composed of a main branch or a complicated arrangement of branches. In botany, the term refers to the way individual flowers are arranged on the axis, the floral stem. It determines the external appearance of the flowering plants. Inflorescences can be described by many different characteristics such as the blooming order of the flower, how the flowers are arranged on the stem and how different clusters of flowers are grouped within it. The main groups of inflorescences are distinguished by branching architecture. In Figure 1.1, raceme, spike, corymb, umbel, spadix, head, catkin and botryoid are groups having simple branching while panicle, compound spike, anthela and compound umbel are the compound inflorescences which are composed of simple inflorescences (Weberling 1989). Within these groups, the most important characteristics are the intersection of the axes and different variations of the model, which represent different branching architecture.

A flower can have both staminate (male) and carpellate (female) reproductive structures, such plants are called hermaphrodite and monoecious. Plants having separate male and female flowers on the same individual are also called monoecious. Other plants, called dioecious, have unisexual reproductive structure such that all plants are either male or female. Maize is monoecious, having both male and female inflorescences on the same plant (Kiesselbach 1949). These inflorescences, as all of the other members of the grass family, have a peculiar architecture with many small spikes (spikelets) organized in panicles (Figure 1.2 A).
Figure 1.1 Different types of inflorescence
A spikelet consists of two bracts which are leaf-like organs at the base, also called *glumes*, followed by two florets (Figure 1.2 A). Each floret consists of three stamens and surrounded by a leaf-like lemma (the external one) and a leaf-like palea (the internal one).

In maize, the terminal male inflorescence is called the tassel, formed from the terminal shoot apical meristem while the female inflorescence called the ear is developed from axillary meristems several nodes below the tassel (Vollbrecht and Schmidt 2009) (Figure 1.2 A). The mature tassel consists of a symmetrical, many-rowed central axis and several asymmetrical long branches (Figure 1.2 B). Both the main spike and the lateral branches bear paired spikelets (McSteen, Laudencia-Chingcuanco et al. 2000). One spikelet is pedicellate and the other is sessile, each containing two functional staminate flowers. Each tassel spikelet consists of two glumes and two florets, an upper and lower floret. Each floret has one lemma, one palea, two lodicules and three stamens (Figure 1.2 B). The ear also has a symmetrical, many-rowed axis with paired spikelets, but no long, lateral branches. In the mature ear, it is difficult to distinguish the pedicellate from the sessile spikelet. Each ear spikelet produces a pair of glumes surrounding two florets. However in the mature ear, the lower floret degenerates so only the upper floret is functional. This phenomenon of the lower floret abortion in maize is unusual (Bonnett 1954). In the upper floret, two carpels fuse to form the long silk. The lemma, palea and lodicules are still present in the upper floret but obscured by the glumes. So in each mature ear spikelet, only silk, carpels and glumes appear (Figure 1.2 C). Although the mature tassel and ear appear to be different types of inflorescences, their
Figure 1.2  Structure of maize inflorescences

**Panel A.** Cartoons of maize male and female inflorescences.

**Panel B.** Mature maize inflorescences and the close-up of a tassel spikelet pair, spikelets and ear spikelets (Vollbrecht and Schmidt 2009).
development in the earliest stages is similar.

1.2 Meristems in maize inflorescence development

Meristems are groups of cells found at the growing tips of plants which can rapidly divide and further differentiate to form new organs (Doerner 2003). The apical meristem is a completely undifferentiated (indeterminate) tissue located in the buds and growing tips of roots, in which shoot apical meristems (SAM) produces all above-ground organs. In flowering plants, the primordia of leaves and all of the reproductive organs are initiated from SAMs.

During vegetative growth, the SAMs initiate leaves and each leaf surrounds a newly formed meristem at the axil. To form reproductive shoots, the flowers, a plant will go through a big transition from the vegetative meristem to inflorescence meristem. When the switch from vegetative to reproductive growth occurs, the SAM will stop initiating leaves but convert into an inflorescence meristem (IM). In maize, the IM then produces 2\textsuperscript{nd} order spikelet pair meristems (SPM) on the central spike and some 2\textsuperscript{nd} order branch meristems (BM) at the base of the tassel. Each SPM has a determinate fate to produce two 3\textsuperscript{rd} order spikelet meristems (SM) and each SM forms two 4\textsuperscript{th} order floral meristems (FM), so each SPM ends up with four FMs (Figure 1.3). But the BMs at the base of the tassel are indeterminate. They will keep growing to form lateral branches, and they have a similar function as IMs to produce SPMs which forms SMs and FMs on the lateral branches. In the ear (McSteen, Laudencia-Chingcuanco et al. 2000; Chuck, Muszynski et al. 2002; Laudencia-Chingcuanco and Hake 2002; Bommert, Satoh-Nagasawa et al. 2005), the central
Figure 1.3 Meristem transitions during maize inflorescence development

**Panel A.** Cartoons of meristem transitions during tassel development. Image modified from (McSteen, Laudencia-Chingcuano et al. 2000).

**Panel B.** SEM images (Laudencia-Chingcuano and Hake 2002) show meristem transitions in young tassel and ear.
spike has a similar development as the tassel central spike, but no branch
meristems are produced at the base. So no long branches are formed in the ear.

1.3 Genes functioning in maize inflorescence development

Many classical mutants that perturb aspects of maize inflorescence development
have been described. Interestingly, most of these mutant phenotypes were
consequences of altered meristem activities. That is, changes that affect meristem
initiation, maintenance and/or size, or meristem identity or determinacy lead to
mutant effects on inflorescence development. And different alterations of meristem
activity produce different mutant phenotypes. So far several important genes have
been identified and cloned and their functions on each stage of maize inflorescence
development have been investigated (Table 1.1).

Flowers are produced from IMs which derive from vegetative SAMs. If plants
have altered size or maintenance of the IM, they may produce an abnormal
inflorescence. For example, CLAVATA pathway genes in Arabidopsis function
together with WUSCHEL in regulating stem cell proliferation (Williams and Fletcher
2005). Several CLV related genes in maize have been cloned. They are the thick
tassel dwarf1 (td1) gene that is CLAVATA1-like and the fasciated ear2 (fea2) gene
that is CLAVATA2-like. These genes are also important in regulating inflorescence
meristem size and maintenance in maize (Lunde and Hake. 2005) (Figure 1.4 A). In
td1 mutants, the tassels have a thicker central spike and the ears are variably
fasciated, which are caused by enlarged IM (Bommert, Lunde et al. 2005). FEA2 has
a similar function as TD1 in controlling IM size. fea2 mutations result in enlarged IMs
Table 1.1 Selected mutants that affect maize inflorescence development

<table>
<thead>
<tr>
<th>Different staged meristems been affected</th>
<th>Mutated genes been cloned</th>
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<tr>
<td>Mutants affecting the size or maintenance of Inflorescence Meristems</td>
<td>thick tassel dwarf1 (td1), fascinated ear2 (fea2)</td>
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<tr>
<td>Mutants lacking an inflorescence</td>
<td>tasselless1 (tl1), barren stalk2 (ba2), barren stalk3 (ba3)</td>
</tr>
<tr>
<td>Mutants affecting the initiation or maintenance of axillary meristems</td>
<td>barren inflorescence1 (bif1), barren inflorescence2 (bif2), barren stalk1 (ba1), sparse inflorescence1 (spi1), unbranched1 (ub1), liguleless2 (lg2), Suppress of sessile spikelets1 (Sos1), tasselsheath4 (tsh4)</td>
</tr>
<tr>
<td>Mutants affecting the identity and determinacy of spikelet pair meristems</td>
<td>ramosa1 (ra1), ramosa2 (ra2), ramosa3 (ra3), ramosa1 enhancer locus2 (rel2)</td>
</tr>
<tr>
<td>Mutants affecting the identity and determinacy of spikelet meristems</td>
<td>branched silkless1 (bd1), indeterminate spikelet1 (ids1), tassel seed4 (ts4), tassel seed6 (ts6)</td>
</tr>
<tr>
<td>Mutants affecting floral meristem identity and floral organ specification</td>
<td>Vestigial glume1 (Vg1), silkless ears1 (sk1), silky1 (si1), zea agamous1 (zag1), indeterminate floral apex1 (ifa1)</td>
</tr>
</tbody>
</table>
and FMs in both tassel and ear while the SAMs appear normal (Taguchi-Shiobara, Yuan et al. 2001).

There are several curious mutants in maize that fail to form one or the other inflorescence. *tasselless1* (*tl1*) mutants fail to produce tassels but have normal ears. On the contrary, *barren stalk2* and *barren stalk3* lack ears and tillers but have normal tassels (Vollbrecht and Schmidt 2009).

Some mutants impact the initiation and/or maintenance of axillary meristems but have no significant effect on SAM and IM. These include *barren inflorescence1* (*bif1*), *barren inflorescence2* (*bif2*), *barren stalk1* (*ba1*), *sparse inflorescence1* (*spi1*), *unbranched1* (*ub1*), *liguleless2* (*lg2*), *Suppressor of sessile spikelets1* (*Sos1*) and *tasselsheath4* (*tsh4*) (Figure 1.4 B). *bif1* mutants, *ba1* mutants and *spi1* mutants fail to initiate all axillary meristems, so the mutant plants have no ears, no vegetative tillers, no tassel branches and spikelets (Gallavotti, Barazesh et al.; Ritter, Padilla et al. 2002; Barazesh and McSteen 2008). BIF2 also has an important function in axillary meristem and lateral organ primordium initiation in the inflorescence. *bif2* mutants typically produce some rudimentary tassels and ears which occasionally bear spikelets (McSteen, Malcomber et al. 2007). *ub1*, *lg2* and *sos1* are genes that affect initiation of specific meristems. *ub1* mutants cannot initiate branch meristems, resulting in no or only vestigial long branches on the tassels while the spikelet development along the central spike is normal (Maize database ID # 77211). *lg2* mutants have altered BM function. They either produce no long branches on the tassels or only produce one or two normal branches (Walsh and Freeling 1999). Both *ub1* and *lg2* mutants have normal ears. *tsh4* is required in BM initiation and
Pannel A. Mature inflorescences of wild type and mutants affecting the IMs.

Pannel B. Mature inflorescences of wild type and mutants either lacking one inflorescence (*tl1*) or affecting axillary meristems (all of the others). Images are adapted from their correspond publications.

Note: ta means mature tassel; ea means mature ear; tcp means the tips of the tassel central spike; sp means mature spikelet pairs; fl means two florets.

Figure 1.4 Mature maize inflorescences of wild type and mutants - I
maintenance while Sos1 only affects the sessile spikelet formation (Wu, Skirpan et al. 2009; Chuck, Whipple et al. 2010).

Aside from meristem initiation and maintenance, meristem identity is another aspect that controls how meristems behave in a certain context and affect inflorescence development. Three ramosa genes (ra1, ra2 and ra3) have specific functions in maintaining the determinate identity of the SPMs, thereby limiting branch outgrowth (Vollbrecht, Springer et al. 2005; Bortiri, Chuck et al. 2006; Satoh-Nagasawa, Nagasawa et al. 2006). When any of the three ramosa genes mutated, the SPMs on both the tassel and the ear become more indeterminate, showing a fate more like BMs and leading to a highly branched inflorescences (Figure 1.5 A). So the different phenotypes of long branches and spikelet pairs may be seen as the consequences of different meristem identities. If the 2nd order meristems produced by the IM are determinate SPMs, they will produce spikelet pairs. If the 2nd order meristems are indeterminate BMs, they will form lateral branches. The three ramosa genes control the 2nd order meristem determinacy and identity, regulating the switch between SPMs and BMs. This implies the normal function of the ramosa genes is to promotes the determinate fate of the 2nd order meristem. ramosa1 enhancer locus2 (rel2) is another gene, reported to interact with RA1, that regulates the determinacy of the 2nd order meristem (Gallavotti, Long et al. 2010).

Genes like branched silkless1 (bd1) and indeterminate spikelet1 (ids1) regulate the identity and determinacy of the higher order meristems, the SMs. bd1 encodes an ERF (ethylene-responsive element-binding factor) like transcription factor (Chuck, Muszynski et al. 2002). In bd1 mutants, the SMs on both inflorescences are
Panel A. Mature inflorescences of wt and mutants affecting SPMs and SMs (bd1).

Panel B. Mature inflorescences of wt and mutants affecting FMs. All images are adapted from their correspond publications.

Note: ta means mature tassel; ea means mature ear; sp means mutant spikelet pairs; fl means two florets. Arrows mark the mutant phenotypes.
indeterminate which led to extra spikelets and fertile flowers being produced on the
tassel and a complex branch phenotype with no flowers on the ear (Chuck,
Muszynski et al. 2002). IDS1 is one of an APETALA2 (AP2) class transcription factor.
In ids1 mutants, multiple florets instead of two are produced from one spikelet
(Chuck, Meeley et al. 1998).

Some ts4 and ts6 (tassel seed) mutants also affect SM initiation and normal
function. ts4 encodes a mir172 microRNA that target AP2 homeotic transcription
factors (Chuck, Meeley et al. 2007). In ts4 mutants SPMs fail to produce SMs while
in ts6 mutants the conversion from SMs to FMs is delayed, which leading to the
irregular branched inflorescences. And in both of these mutants, the pistil abortion is
failed so the tassels become feminized (Irish 1997).

There are several genes in maize that function specifically in the determinacy of
the floral meristem and the formation of floral organs. Vestigial glume1 (Vg1)
mutants have normal tassels and ears except the glumes on the spikelet are very
small which led to the early exposure of stamens on developing florets (Vollbrecht
and Schmidt 2009). silkless ears1 (sk1) mutants have ears without silks while silky1
(si1) mutants produce extra silks on both tassel and ear spikelets (Figure 1.5 B). si1
encodes a MADS-box gene which is related to the Arabidopsis MADS-box gene
APETALA3 (Ambrose, Lerner et al. 2000). Another MADS-box gene been cloned in
maize is zea agamous1 (zag1), which is a candidate ortholog of the Arabidopsis
AGAMOUS gene (Schmidt, Veit et al. 1993). zag1 mutants produce sterile silks
which greatly reduces the fertility of ears. indeterminate floral apex1 (ifa1) single
mutants have effects on floral organ development, but in ifa1; zag1 double mutants,
the FMs in the ears revert to a BM identity (Figure 1.5 B) suggesting a redundant role of *ifa1* and *zag1* in regulating floral meristem identity (Laudencia-Chingcuancio and Hake 2002).

### 1.4 The maize *ramosa1* gene and its specific function in inflorescence development

The name *ramosa* came from the Latin “*ramus*”, meaning “branch”, which greatly reflects the highly branched phenotype of the maize mutated inflorescence. So far, three *ramosa* genes (*ramosa1*, *ramosa2* and *ramosa3*) have been cloned to regulate the inflorescence branching systems in maize (Figure 1.5 A). The three genes are all expressed in the inflorescence and their mutants produce branches on the ear and an increased number of long branches on the tassel.

*ramosa1* (*ra1*) encodes a TF III A-type Cys$_2$-His$_2$ zinc finger protein with a QGLGGH motif around the C$_2$H$_2$ DNA binding domain which is similar to the QALGGH motif in petunia EPF family, so RA1 may interact with other DNA binding proteins to bind to specific target sequence (Kubo, Sakamoto et al. 1998; Takatsuji 1998; Vollbrecht, Springer et al. 2005). The recessive *ra1-R* allele contains a point mutation of C$_2$H$_2$ into C$_2$H$_1$ which causes a strong mutant phenotype with highly branched tassels and highly branched and functionally sterile ears (Gernert 1912). The C terminal DLQLRL motif of RA1 is a conserved EAR repression motif (Hiratsu, Mitsuda et al. 2004). A 9-amino acid-deletion at the *ra1* N terminal end (*ra1-RS* allele) causes a weak mutant phenotype. But when the N terminal deletion is combined with a point mutation at EAR motif (DLQLRL change to DFQLRL) in the
Figure 1.6  $ra1$ mutant phenotype and $ra1$ expression pattern

**Panel A.** Mature inflorescence of B73 and two $ra1-R$ mutants.

**Panel B.** SEMs showing young inflorescences of B73 and $ra1-R$ mutant.

**Panel C.** RNA in situ hybridization showed $ra1$ expression pattern.

All images are adapted from (Vollbrecht, Springer et al. 2005).
ra1-RSenh allele, the changes lead to a strong mutant phenotype that looks similar to ra1-R allele (Figure 1.6 A). All these data suggest that RA1 is a putative transcriptional repressor.

Scanning electron microscope (SEM) results show that in ra1 mutants, no extra 2nd order meristems are initiated from the central spike (Figure 1.6 B). The extra branches are actually produced by the 2nd order meristems that fail to maintain determinate SPM function but instead continue to grow into a protruding second order axes, exhibiting indeterminate BMs function (Vollbrecht, Springer et al. 2005). This suggests that normal RA1 functions in determining the fate of the 2nd order meristems, promoting determinate SPM function to impose spikelet-pair identity.

Previous research showed that ra1 RNA is highly expressed when second order SPMs are initiated and persists during the initiation of third order spikelet meristems. RNA in situ hybridization shows that ra1 is located at the junction between each determinate 2nd order meristem and the indeterminate main axis (Figure 1.6 C). It isn’t expressed exactly in the meristem cells, which suggests there might be a mobile signal involved in regulating meristem determinacy.

1.5 Thesis organization

Previous work showed that RA1 is a putative transcriptional repressor expressed at the base of the 2nd order meristems in maize young inflorescence to promote a determinate SPM fate (Vollbrecht, Springer et al. 2005). Here I sought to learn more about how RA1 works to regulate the fate of the 2nd order meristems.
I first examine relationships between \textit{ra1} and two other \textit{ramosa} genes, \textit{ra2} and \textit{ra3} (Chapter 2). Yeast two-hybrid analysis was used to test whether the three RAMOSA proteins can interact with each other to form a protein complex. The \textit{ra1}; \textit{ra2}; \textit{ra3} triple mutant plants were also produced and their young inflorescence was dissected for SEM (scanning electron microscope) to document the developmental basis of phenotypic differences. Then I screened the cDNA libraries made from immature 2 mm ears to identify RA1 interacting proteins (Chapter 3). Several transcription factors were isolated including Knotted-1 (KN1), which has an important function in regulating shoot apical meristem development (Vollbrecht, Reiser et al. 2000). The interaction between RA1 and KN1 was further confirmed by in vitro GST pull down and in vivo BiFC experiments. The \textit{ra1-RSenh}; \textit{kn1-e1} double mutants were made, the tassel branching phenotypes were characterized in detail and the genetic interaction between these two proteins was analyzed by statistical methods (Chapter 3). Since Knotted1 related homeobox proteins have been reported to control the plant hormone gibberellins (GA) level during plant development (Sakamoto, Kamiya et al. 2001; Hay, Kaur et al. 2002; Bolduc and Hake 2009), potential and known downstream genes of KN1 in maize were identified and their expression levels were analyzed in \textit{ra1-R} mutants and B73 (Chapter 3). A nuclear localization signal with RA1 was identified and analyzed (Chapter 3). In Chapter 4, I summarize our current findings and introduce analysis of other target genes for the RA1-KN1 protein complex and other proteins such as ARF8 that may interact with RA1 to regulate maize inflorescence branching architecture. These topics will likely be the basis of future work in investigating RA1 function and the ramosa pathway.
CHAPTER 2. RAMOSA GENES AND THE RAMOSA PATHWAY

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Keywords: maize inflorescence, *ramosa* gene, ramosa pathway
Contributions to this work

The experiments in this manuscript were performed by Ms. Xiang Yang and Dr. Erik Vollbrecht. Ms. Yang was responsible for carrying this project forward throughout the course of the project. Dr. Vollbrecht did the field work and consulted on the overall instructions and support for this work. In addition, Dr. Vollbrecht contributed to reviewing and editing of this work. This work was supported by the National Science Foundation (NSF 05-603) to Dr. Sarah Hake, Dr. David Jackson, Dr. Elizabeth Kellogg, Dr. Tobert Rocheford and Dr. Erik Vollbrecht.
Abstract

Three \textit{ramosa} genes, \textit{ramosa1} (ra1), \textit{ramosa2} (ra2) and \textit{ramosa3} (ra3), have been identified to regulate inflorescence branching architecture in maize. RA1 is a plant specific EPF-like protein with a Cys$_2$-His$_2$ zinc finger DNA binding domain and two EAR repression motifs. Its RNA is expresses at the junction between each 2$^{\text{nd}}$ order meristem and the main axis to regulate the branching architecture of maize inflorescence. In \textit{ra1-R} strong mutants, both the tassel and the ear become more branched. In \textit{ra1-RS} weak mutants, tassel branching is slightly increased and ears produce disordered rows. These phenotypes indicate that degree of branch outgrowth correlates with strength of RA1 activity. \textit{ra2} encodes a LOB-domain protein whose RNA is expressed in the edge of the bract and meristem early in inflorescence development. \textit{ra3} encodes a trehalose 6-phosphate phosphatase and is expressed in discrete domains subtending axillary inflorescence meristems. The expression patterns of the three \textit{ramosa} genes overlap in various tissues during early inflorescence development, and research suggests that the three \textit{ramosa} genes function in the same ramosa pathway to regulate 2$^{\text{nd}}$ order meristem determinacy and therefore inflorescence architecture. Genetic and molecular experiments place \textit{ra1} downstream of both \textit{ra2} and \textit{ra3}, and \textit{ra3} may act parallel with \textit{ra2}. To further elucidate the \textit{ramosa} pathway, yeast two-hybrid (Y2H) analysis was used to investigate the relationships between three RAMOSA proteins. The \textit{ra1}; \textit{ra2}; \textit{ra3} triple mutants were also made and the phenotypes of the young inflorescence were characterized by SEM.
2.1 Introduction

RA1 is a plant specific EPF-like protein which has one Cys$_{2}$-His$_{2}$ zinc finger DNA binding domain containing a variant QGLGHH region and two putative EAR repression motifs (Sigmon and Vollbrecht 2010). Thus, it is a putative transcriptional repressor. *ra1* is expressed at the junction between each 2$^{nd}$ order meristem and the main axis (Figure 2.2 A) and its RNA was only detected in maize developing inflorescences (Vollbrecht, Springer et al. 2005). It has a strong expression level in 2-4 mm young inflorescence then its expression level is highly reduced at the later stages. *ra1-R* is a strong mutant allele which contains two point mutations at the QGLGHH region in the C$_{2}$H$_{2}$ zinc finger domain (QGLGHH changes to QGLEGN) and causes highly branched tassels and ears after *ra1* was expressed (Figure 2.1). *ra1-RS* which lost N terminal 9 amino acids shows a weak mutant phenotype with more branches formed at the base of the tassel and disordered rows produced on the ear (Figure 1.6 A).

*ramosa 2* (*ra2*) encodes a putative transcription factor with a lateral organ boundary (LOB) domain (Bortiri, Chuck et al. 2006). In situ hybridization shows that *ra2* is expressed in the 2$^{nd}$ order meristem cells above the inflorescence bracts where it predicts the position of bract and spikelet pair meristems (Figure 2.2 A). In *ra2-R* null mutants, the tassels show a highly branched phenotype with acute branch angles and the ears form disorganized rows and several long branches (Figure 1.5 A).

*ra3* encodes a trehalose 6-phosphate phosphatase, or TPP enzyme (Satoh-Nagasawa, Nagasawa et al. 2006). RA3 is first expressed when the axillary
Figure 2.1 SEMs of B73 and \textit{ra1-R} tassel
meristem primordium is initiated in young inflorescences and its RNA localizes at the base of axillary meristems and SPMs. RNA expression continues until the later floral meristems are formed and it is expressed in a strip between upper and lower florets (Figure 2.2 A). In ra3-R mutants, both the tassel and ear show abnormal long branches and disordered rows (Figure 1.5 A).

The null mutation of ra2-R and ra3-R both have mature phenotypes similar to ra1-R mutants and they all make the 2nd order meristem exhibit BM activity. The expression patterns of the three ramosa genes in the SPMs of the ear are briefly overlapping (Figure 2.2 A). To further investigate the relationships between the three ramosa genes, their pairwise double mutants were made and analyzed. The double mutants of ra1-RS; ra2-R, ra1-RS; ra3-R and ra2-R; ra3-R all produced highly branched ears which resembled the ra1-R single mutant ear (Figure 2.2 B).

Molecular assays show that ra1 expression level is lowered in ra3-R mutants, is much less in ra2-R mutants and is considerably reduced in ra2-R; ra3-R double mutants (Figure 2.2 C). Furthermore, ra3 expression level and localization is not significantly changed in ra1-R or ra2-R single mutants (Satoh-Nagasawa, Nagasawa et al. 2006). ra2 also has the same expression pattern in tassels of ra1-R and ra3-R mutants (Vollbrecht, Springer et al. 2005; Bortiri, Chuck et al. 2006; Satoh-Nagasawa, Nagasawa et al. 2006). These results indicate that ra2 and ra3 regulate ra1 at the level of transcript accumulation. ra2 is upstream of ra1, perhaps acting in parallel with ra3 in the ramosa pathway to determine the fate of 2nd order meristems. But so far we still don’t know the action mechanisms of the 3 ramosa genes; how ra2 and ra3 regulate ra1 and how did they work to regulate the meristem determinacy.
**Figure 2.2 Relationships between 3 ramosa genes**

**Panel A.** RNA in situ hybridization results of the three ramosa genes in maize young inflorescences and a cartoon showing the overlapping expression domains of the three ramosa genes (Vollbrecht, Springer et al. 2005; Bortiri, Chuck et al. 2006; Satoh-Nagasawa, Nagasawa et al. 2006).

**Panel B.** Wild type and ramosa mutant mature ears. Compared with wild type B73, all of the ramosa mutants have extra long branches on the ear. The ra1-RS; ra2-R, ra1-RS; ra3-R, ra2-R; ra3-R and ra1-R; ra3-R double mutants have an extreme phenotype reminiscent of the ra1-R single mutants.

**Panel C.** QRT-PCR results of ra1 expression level in wild type and ramosa mutants. ra1 expression level is lowered in ra3-R and ra2-R single mutants (Satoh-Nagasawa, Nagasawa et al. 2006), and is considerably reduced in ra2-R; ra3-R double mutants.
Figure 2.2  Relationships between 3 *ramosa* genes
2.2 Materials and Methods

2.2.1 Materials

Unless otherwise noted, the chemicals used in these studies were obtained from either Sigma Chemical Co (www.sigmaaldrich.com) or Fisher Chemical Co (www.fishersci.com) and were of the highest quality available. The restriction enzymes and T4 DNA ligation enzyme used in this study were obtained from New England BioLabs (www.neb.com).

2.2.2 Plants

Plants were grown in the greenhouse or in the field, under standard conditions. B73 was used as wild-type line. ra1-R; ra2-R; ra3-R triple mutants were made from self-cross of ra3-R/ra3-R; ra2-R/+; ra1-R/+ plant. To genotype ra2-R homozygous mutants, one 262bp fragment was amplified from genomic DNA with the primers ra2-R-F and ra2-R-B (Appendix A). The PCR product was then digested by HaeIII (GGCC). The ra2-R homozygous plants produce three bands with 180bp, 56bp and 22bp while the wild type plants produce two bands with 206bp and 22bp. The digestions were running on 2% agarose gel to separate these different sized bands. To genotype ra1-R homozygous plants, one 765bp fragment was amplified from genomic DNA using primers RA8 and RA11 (Appendix A). The PCR product was then digested by AccI (GTMKAC). The ra1-R homozygous plants produce two bands with 429bp and 320bp while wild type plants only have one band with 765bp.

Young inflorescences were dissected from mutant plants grown in the greenhouse and fixed for SEM imaging.
2.2.3 Genes and accession numbers

For all clones used in this study, the complete sequencing was performed on both strands at the Iowa State University Nucleic Acid Facility. All of the primers used for cloning were synthesized at the Iowa State University Nucleic Acid Facility. The genes used in this study are *ra1* gene (DQ013174), *ra2* gene (DQ327701) and *ra3* gene (DQ436920).

2.2.4 Yeast two-hybrid analysis

Yeast host strains

The yeast host strain PJ69-4a was provided by Dr. Allen Meyer’s lab, Iowa State University. The vector systems used for this analysis are pBD-Gal4 and pAD-Gal4, obtained from Dr. Dan Voytas’s lab, University of Minnesota.

Constructs

The full length *ra1* cDNA sequence was PCR amplified from B73 genomic DNA using primers RA1-Eco-F and RA1-Sal-B (Appendix A). The PCR product was digested by EcoRI and SalI and ligated into similarly digested pBD-Gal4 and pAD-Gal4 vectors to generate pBD-RA1 and pAD-RA1 constructs. To produce pAD-RA1F and pBD-RA1F constructs, primers RA1-Eco-F and RA1F-Sal were used to PCR amplify *ra1F* fragment from B73 genomic DNA. The fragment was then digested and ligated into pAD-Gal4 and pBD-Gal4 empty vectors at EcoRI and SalI sites. To make pAD-RA2 construct, the full length *ra2* cDNA sequence was
PCR amplified from pET-RA2 construct (provided by Dr. Sarah Hake’s lab at UC Berkeley) using primers RA2-Eco-F and RA2-Xho-B (Appendix A). The PCR product was then digested by EcoRI and XhoI and ligated into similarly digested pAD-Gal4 vector to generate pAD-RA1 construct. pBD-RA2 construct was prepared with the similar method described above except using primer RA2-Sma instead of RA2-Xho-B for PCR amplification and using SmaI instead of XhoI to digest PCR product and pBD-Gal4 vector. The ra2-dm fragment was PCR amplified from pET-RA2 construct described above by using primers DM-Eco-F and RA2-Xho-B, DM-Eco-F and RA2-Sma separately (Appendix A). The PCR products were then digested by EcoRI and XhoI or EcoRI I and SmaI and ligated into similar digested pAD-Gal4 or pBD-Gal4 vector to produce pAD-RA2-DM and pBD-RA2-DM constructs. The full length ra3 was PCR amplified from RA3-pCRTOPO construct (provided by Dr. David Jackson’s lab at CSHL) using primers RA3-Eco-F and RA3-Sal-B (Appendix A) then cloned into pAD-Gal4 and pBD-Gal4 vector by using the similar method described above.

Yeast transformation and growth selection assay

Two constructs fused separately with the pAD-Gal4 and pBD-Gal4 vectors were co-transformed into yeast host strain PJ69-4a by using the yeast high efficiency transformation protocol provided by manufacturer (www.stratagene.com). The co-transformed yeast cells were first grown on the media with complete supplement mixture minus Leu and Trp (1xSC-LW) for 3-5 days at 30°C. When the colonies were
around 1-2 mm diameter, several colonies from each plate were picked up and re-streaked onto the –Adenine selective media with complete supplement mixture minus Leu, Trp and Ade (1xSC-ALW) for the protein-protein interaction assay. After incubating 3-5 days at 30°C, the re-streaked colonies grew big on the 1xSC-ALW selective media if the co-transformed fusion proteins interact with each other. The re-streaked colonies co-transformed with two empty vectors or two fusion proteins that don’t interact with each other didn’t grow on the –Adenine selective media.

Two or three co-transformed colonies from each 1xSC-LW plate were picked up again and grew into the 1xSC-LW liquid media at 30°C overnight until their OD600 reach 1.0. Then a serial dilution was made for each cell culture and let them grow on the –Adenine selective media for 3-5 days at 30°C.

Filter lift assay for lacZ expression analysis

Several co-transformed yeast colonies were picked up from each 1xSC-LW plate and re-streaked onto new 1xSC-LW plate and incubate for 2 days. After the re-streaked colonies were grown big, the filter lift assay was applied to each re-streaked colonies for the β-galactosidase (lacZ) gene expression analysis. The protocol for filter lift assay was provided by manufacturer (www.stratagene.com). For each sample, the colonies turned blue and the total tested colony number were counted and analyzed.

2.2.5 RA1 antisera preparation and purification

Constructs
The full length ra1 cDNA was PCR purified from B73 genomic DNA using primers RA8 and RA11. The PCR product was directly mixed with the pCR T7/NT-TOPO expression vector from Invitrogen (www.lifetechnologies.com) to make the 6×His-RA1 construct. The pAD-RA1 constructs described above were digested by BamHI and SalI and ligated into similarly digested pGEX-4T-3 expression vector (www.gelifesciences.com) separately to make GST-RA1 construct.

Fusion protein over-expression and purification

The 6×His-RA1 construct was transferred into E. coli BL21(DE3) chemically competent cells provided by Invitrogen and grown in 10ml LB liquid media with corresponding antibiotics at 37°C overnight. Next morning picked up 500 µl overnight culture into new 10 ml LB liquid media with antibiotics and incubated at 37°C until OD600 reached 0.25. The cell culture was then induced by 0.1% Isopropyl β-D-1 thiogalactopyranoside (IPTG) at 30°C for 4 hours to get over-expressed 6×His-RA1 fusion protein. The induced cell pellets were collected and total proteins in protein loading buffer were heated at 100°C for 5 minutes. The protein extracts were analyzed by running on the 12% SDS-PAGE gel. The GST-RA1 fusion protein was over-expressed by the same method.

The solubility of 6×His-RA1 fusion protein was analyzed by the repeated freeze-thaw method provided by manufacturer (www.lifetechnologies.com). As the 6×His-RA1 fusion protein turns out to be insoluble, the over-expressed 6×His-RA1 fusion protein was collected and purified by the ProBond Purification System under
denaturing conditions, following the provided protocol (www.lifetechnologies.com). The final purified proteins were eluted and the purified 6×His-RA1 band was detected by western blot using 6×His epitope tag (www.piercenet.com) as the primary antibody, goat anti-rabbit IgG-AP conjugate as the secondary antibody (www.bio-rad.com) and the Western Blue® Stabilized Substrate for Alkaline Phosphatase (www.promega.com) as the detection substrate.

RA1 polyclonal antisera production and purification

The purified 6×His-RA1 proteins were fractionated by running on 15% SDS-PAGE gel and stained with Coomassie blue. The correspond 6×His-RA1 band was cut from the gel and sent to Protein Facility Center at ISU for rabbit immunization. The antisera were collected from each rabbit after each immunization and the tilter was analyzed by using over-expressed GST-RA1 protein extracts for western blot. The total antisera were collected from the rabbits with higher tilter after three immunizations.

To purify RA1 polyclonal antibody from 6×His-RA1 antisera, the GST-RA1 fusion protein was over-expressed and purified by Glutathione Sepharose 4B beads (www.gelifesciences.com) following manufacturer’s protocol. After washing 4 times, the purified GST-RA1 fusion protein was eluted from the beads and mixed with CNBr activated Sepharose 4 Fast Flow beads following manufacture’s protocol (www.gelifesciences.com) to make a CNBr-GST-RA1 mixed column. Apply antisera to this column with totally 3 flow-throughs, and after washes the purified RA1 antibody was collected in fractions and analyzed by western blot.
2.2.6 GST pull-down analysis

Constructs

The pAD-RA1 construct described above was digested by EcoR I and Sal I and ligated into similarly digested pET28a expression vector (provided by Dr. Yanhai Yin’s lab, ISU) to make pET-RA1 construct which produces a lot of soluble 6×His-RA1 fusion proteins than the pCR-T7-RA1 construct which produced insoluble 6×His-RA1 fusion protein. The fragment ra1F was PCR amplified from ra1 cDNA using primers RA81Zm and RAF-B (Appendix A). The PCR product was directly mixed with the pCR T7/NT-TOPO expression vector described above to make the 6×His-RA1F construct. The pAD-RA2-DM constructs described above were digested by BamH I and Sal I and ligated into similarly digested pGEX-4T-3 expression vector described above to make the GST-RA2-DM construct.

Fusion protein transformation and over-expression in E.coli

The gst-ra1, gst-ra2-dm, 6×his-ra1 (on pET28a vector) and 6×his-ra1F fusion plasmids were transferred into E. coli BL21(DE3) chemical competent cells and induced by 0.1% IPTG with the same method described above. The cell pellet of each induced BL21(DE3) cell culture was collected and their total proteins were analyzed as by running on 12% or 15% SDS-PAGE gel. For each fusion protein, its solubility was analyzed by the repeated freeze-thaw experiment described by the manufacturer (www.lifetechnologies.com). All the fusion proteins used in this study were proved to be soluble.
**Fusion protein purification and pull-down analysis**

The over-expressed GST fusion proteins were purified by Glutathione Sepharose 4B beads following manufacturer’s protocol (www.gelifesciences.com). The 6×His fused proteins were over-expressed and purified under native condition by the ProBond Purification System described above. The concentration of each purified protein was determined by Bradford assay (Bradford 1976).

For pull-down analysis, the purified GST fusion proteins weren’t eluted from the Glutathione Sepharose 4B beads but left attached. 2-10 µg of purified GST fusion proteins with beads were used to mix with 2-10 µg purified 6×His fusion proteins and incubated at 4°C for 2 hours. After washing 4 times with the pull-down buffer, the final beads were re-suspended into the protein loading buffer, boiled at 100°C for 5 minutes and the isolated proteins were analyzed by western blot.

The fused RA1 and RA1F proteins were analyzed by using purified RA1 antibody as the primary antibody, using goat anti-rabbit IgG-AP conjugate as the secondary antibody and the Western Blue® Stabilized Substrate for Alkaline Phosphatase as the detection system. The 6×His fusion proteins were analyzed by using 6×His epitope tag as the primary antibody while the GST fusion proteins were analyzed by using anti-glutathione S-transferase, rabbit IgG fraction (www.probes.com) as the primary antibody. They both used the same secondary antibody and detection system as described above.
2.2.7 Scanning Electron Microscopy

2 mm, 3-4 mm young tassels and 2-3 mm, 4-5 mm and 6 mm young ears from $ra1-R; ra3-R, ra2-R; ra3-R$ double mutant plants and the $ra1-R; ra2-R; ra3-R$ triple mutant plants were collected for scanning electron microscopy.

All of these samples were fixed in a mixture of 2% paraformaldehyde and 2% glutaraldehyde in 0.1 M Cacodylate buffer, pH7.25 at 4°C overnight. The next day, the samples were washed with ddH2O for 5 minutes then dehydrated through an ethanol series from 25%, 50%, 70%, 95% to 3×100% ethanol. The samples were critical point dried, sputter coated with gold palladium for 45 seconds and viewed on the digital JEOL 5800LV scanning electron microscope with the voltage of 10 kV at the Microscopy and Nanoimaging Facility, ISU.

2.3 Results

The three RAMOSA proteins all function in promoting the determinate fate of 2nd order meristems during maize inflorescence development. Their expression patterns overlap and they are in the same ramosa pathway with $ra2$ and $ra3$ acting upstream of $ra1$. We therefore investigate whether or not the three RAMOSA proteins can interact with each other and possible function as a protein complex.

Full length $ra1$, $ra2$, $ra3$ and the truncations of $ra1$ ($ra1F$) and $ra2$ ($ra2-dm$) (Figure 2.3) were used to detect the protein-protein interactions by yeast two-hybrid analysis. The protein pairs tested in this study were listed in Table 2.1. The reporter genes used in this study were ADE2 and β-galactosidase ($lacZ$). Positive colonies which survived on the –Adenine selective media or turned blue after the filter lift
The *ra1* gene has one C2H2 zinc finger domain and two putative EAR repression motifs which suggests RA1 functions as transcriptional repressor. *ra1F* is a truncation of *ra1* without the C terminal EAR motif. It is used to detect the interactions with other RAMOSA proteins. The *ra2* gene encodes a putative transcription factor with a LOB domain. Its C terminal DM region alone causes a mutant phenotype with a normal *ra1* expression level (Bortiri, Chuck et al. 2006) so this region was used to test interactions with RA1 and RA3. *ra3* contains two conserved phosphatase boxes. It encodes a trehalose 6-phosphate phosphatase.
Figure 2.3 *ramosa* gene structures
assay (Figure 2.4) were counted and compared with the total colony number. The results of this comparison are listed in Table 2.1. A summary of the interactions between RAMOSA proteins tested in this study is listed in Table 2.2.

The RA1 antisera were prepared and purified and the purified antibody works well for the *E. coli* expression system (Figure 2.5). In vitro GST pull-down analysis was also performed to confirm the protein-protein interactions identified in yeast (Figure 2.6).

### 2.3.1 RA1 has a weak interaction with RA2

The full length RA1 showed a very weak interaction with the full length RA2 in yeast but its interaction with the C terminal RA2-DM truncation was stronger (Table 2.1). The RA1F truncation lacking the C terminal EAR motif showed a much stronger interaction with full length RA2 and RA2-DM but not with other RAMOSA proteins or truncations. The bait-pray swap test (Table 2.1) and the in vitro GST pull-down analysis (Figure 2.6 B) confirmed the interactions between RA1 and RA2, especially between the RA1F and RA2-DM domain. But compared with the positive controls (+ control), the interactions between RA1F and RA2-DM were still weak (Figure 2.4).

When the full length RA2 was fused with pBD-Gal4 vector and co-transformed with the pAD-Gal4 empty vector into yeast, all of the yeast colonies survived on the −Adenine selective media or turned blue after filter lift assay, which suggested that the full length RA2 may act as a transcription activator.
Table 2.1 Yeast two-hybrid results about RAMOSA proteins

<table>
<thead>
<tr>
<th></th>
<th>pAD-RA1</th>
<th>pAD-RA1F</th>
<th>pAD-RA2</th>
<th>pAD-RA2-DM</th>
<th>pAD-RA3</th>
<th>pAD - GAL4</th>
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</tr>
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<td>0/18</td>
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<td></td>
<td>1/36</td>
<td>0/12</td>
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</tbody>
</table>

Note:

L: The ratio of positive blue colonies to total colonies tested on lacZ selection.

A : The ratio of positive growing colonies to total colonies tested on –Adenine selection.

Blue text suggests a weak interaction between RA1-F and RA2-DM.

Blank column means did not test the paired proteins in this experiment.
Table 2.2  Summary of interactions between RAMOSA proteins in yeast

<table>
<thead>
<tr>
<th></th>
<th>pAD-RA1</th>
<th>pAD-RA1F</th>
<th>pAD-RA2</th>
<th>pAD-RA2-DM</th>
<th>pAD-RA3</th>
<th>pAD-Gal4</th>
</tr>
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<td>—</td>
<td>+, weak</td>
<td>+, weak</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>pBD-RA1F</td>
<td>—</td>
<td>—</td>
<td>+</td>
<td>+</td>
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</tr>
<tr>
<td>pBD-RA2</td>
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</tr>
<tr>
<td>pBD-Gal4</td>
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</table>

Note:

+  means the co-transformed proteins show interactions in yeast.
-  means the co-transformed proteins didn’t show any interaction with each other.
@  means colonies transformed with pBD-RA2 can turn on all of the reporter genes regardless of which pAD construct was co-transformed. It acts as a self-activator.
Figure 2.4  Yeast two-hybrid analysis of RAMOSA proteins

**Panel A.** Serial dilutions of yeast co-transformed colonies growing on –Adenine selective media. Results suggested that pBD-RA1 interacted with pAD-RA1 so RA1 may interact with itself. pBD-RA1F interacted with pAD-RA2-DM.

**Panel B.** Filter lift assay of yeast co-transformed colonies. Results suggested that pBD-RA1 interacted with pAD-RA1 and pBD-RA1F interacted with pAD-RA2-DM. But compared with + control, both of these interactions were weak.
Figure 2.4 Yeast two-hybrid analysis of RAMOSA proteins
Figure 2.5 Analysis of RA1 antisera and purified antibody

**Panel A.** Titer analysis of RA1 antisera after three immunizations. The over-expressed GST-RA1 protein extractions from *E.coli* were used as the inputs. Column 2 has 1:20 diluted input of column 1. Column M shows the marker protein bands with 78 KD, 48 KD, 38 KD and 25 KD.

**Panel B.** A comparison between RA1 antisera and purified RA1 antibody. The inputs used in this analysis were over-expressed GST-RA1 protein extractions and over-expressed 6×His-RA1 protein extractions. Arrows marked the GST-RA1 band with around 46 KD and 6×His-RA1 band with around 22 KD. Column M shows the marker protein bands with 78 KD, 48 KD, 38 KD and 25 KD.
Figure 2.5 Analysis of RA1 antisera and purified antibody
**Figure 2.6 GST pull-down analysis of RAMOSA proteins**

**Panel A.** In vitro GST pull-down analysis of RA1 interacting with itself. The input in the Sample column was purified GST-RA1 protein mixed with purified 6×His-RA1 protein. The input in the Neg column was purified GST-RA1 protein mixed with protein extracts from *E. coli* over-expressing 6×His empty vector as the negative control. The membranes were incubated separately with 6×His epitope tag antibody and purified RA1 antibody as the primary antibodies. The 6×His-RA1 band showed up in both the sample column while no 6×His-RA1 band showed up in the Neg column and the GST-RA1 band also showed up when incubated with RA1 antibody, which suggests RA1 interacts with itself.

**Panel B.** In vitro GST pull-down analysis of RA1F interacting with RA2-DM. The inputs in the Sample column were purified GST-RA2-DM protein mixed with purified 6×His-RA1F protein. The membranes with the same inputs were incubated separately with 6×His epitope tag, purified RA1 antibody and anti-glutathione S-transferase as the primary antibodies. The RA1 antibody didn't detect any band from the pull down results of purified GST protein with purified 6×His-RA1 protein, these results didn't show here.
Figure 2.6 GST pull-down analysis of RAMOSA proteins
2.3.2 RA1 may interact with itself

About half of the colonies co-transformed with two full-length RA1 grew on – Adenine selective media or turned blue after filter lift assay, but no colony became positive with full length RA1 and RA1F co-transformed (Table 2.1). This suggested that the full length RA1 can interact with itself in yeast, although this interaction seems weak when compared with the positive control (Figure 2.4). But it didn’t interact with the RA1F truncation in the yeast two-hybrid analysis. In vitro GST pull-down assay also confirmed the self-interaction of RA1 since purified RA1 antibody identified both the GST-RA1 band and the 6×His-RA1 band (Figure 2.6 A). This suggested that RA1 may function as a dimer or a more complicated protein polymer.

2.3.3 RA3 doesn’t interact with RA1 or RA2 in yeast

Very few yeast colonies co-transformed with RA3 and the other RAMOSA proteins and truncations grew on the –Adenine selective media or turned blue in the filter lift assay. Thus we don’t detect any protein-protein interactions between the full length RA3 with other RAMOSA proteins and truncations (Table 2.1) by the yeast two-hybrid technique.

2.3.4 ra1-R; ra2-R; ra3-R triple mutants look like double mutants

Here we dissected the young inflorescences of ra1-R; ra2-R; ra3-R triple mutants and ra1-R; ra3-R, ra2-R; ra3-R double mutants and studied the architecture of these early stage inflorescences by SEM. By comparing the tassel phenotypes on two different stages (2 mm and 3-4 mm) and the ear phenotypes on three different
Figure 2.7  SEMs of young inflorescences from *ramosa* mutants

**Pannel A.** 2 mm and 3-4 mm young tassels were collected from *ra1-R; ra3-R, ra2-R; ra3-R* double mutants and *ra1-R; ra2-R; ra3-R* triple mutants for SEM analysis. Bars equal 200 µm.

**Pannel B.** 3 mm, 4-5 mm and 6 mm young ears were collected from the double mutants of *ra1-R; ra3-R* and *ra2-R; ra3-R* and the *ra1-R; ra2-R; ra3-R* triple mutants for SEM analysis. Only the images for the tips of 6 mm ears were shown. Bars equal 200 µm.
Figure 2.7 SEMs of young inflorescences from *ramosa* mutants
stages (3 mm, 4-5 mm and 6 mm), we didn’t find any obvious developmental differences between each double mutant young inflorescences and the triple mutant young inflorescences.

From these SEM images, we can easily see that on *ramosa* double and triple mutants, the young inflorescence formed some spikelet pair meristems first, then one side of each SPM kept growing and formed a new branch while the other side seemed to stop growing and finally disappeared (Figure 2.7). In wild-type B73 (Figure 1.6 B), both two sides of each SPM kept growing although one side might grow a little bit faster than the other side, so finally each SPM formed two SMs instead of a branch.

2.4 Discussion

The three RAMOSA proteins have been shown to perform similar functions in regulating 2\textsuperscript{nd} order meristem development, which determines maize inflorescence branch architecture. RNA expression and genetic analysis put the three RAMOSA proteins in one *ramosa* pathway with RA2 acting parallel with RA3 and both acting upstream of RA1.

To investigate whether the three RAMOSA proteins interact with each other to form a protein complex, yeast two-hybrid analysis was used to test the direct interaction among the full length RAMOSA proteins and their truncations. Yeast strain PJ69-4a was used as the host and *ade2* and β-galactosidase (*lacZ*) were used as two reporter genes. *ade2* encodes AIR-carboxylase which catalyzes the sixth step of the Adenine biosynthetic pathway in *Saccharomyces cerevisiae*. The
yeast colonies co-transformed with the two detected plasmids were streaked on minus Adenine selective media. If the two co-transformed proteins interact with each other, the expression of ade2 was activated which allowed the synthesis of Adenine in yeast cells, so the colonies survived and grew on the –Adenine selective media. If the two co-transformed proteins didn’t interact with each other, no ADE2 was expressed and the yeast colonies couldn’t grow on the –Adenine selective media. In filter lift assay, X-gal was used as the substrate to reflect the expression of lacZ in co-transformed yeast colonies. If the expression of lacZ was activated in the co-transformed colonies, the active β-galactosidase hydrolyzed colorless X-gal to produce a blue product. So if the co-transformed colonies turned blue after filter lift assay, it suggested the co-transformed proteins interact with each other. Otherwise, the white colonies after filter lift assay suggested no interaction between the two co-transformed proteins.

In our study, we chose the three full length RAMOSA proteins and two RAMOSA truncations for yeast two-hybrid analysis. RA1F is a truncation of RA1 without the C terminal EAR motif. RA2-DM is a C terminal special region of RA2 (Figure 2.3). One allele of ra2 mutations contained only this region produced a highly branched phenotype but contained the normal ra1 expression level (Bortiri, Chuck et al. 2006). Since ra1 acts downstream of ra2 and its expression level was highly reduced in other ra2 mutants such as ra2-R, these results suggested that the ra2-dm region may have some functions in maintaining ra1 normal expression level. So here we chose this region to study its interaction with other RAMOSA proteins.
From yeast two-hybrid results, we found several but less than half of the RA1 and RA2 co-transformed colonies became positive on the –Adenine selective media and after filter lift assay, while a few more colonies became positive with RA1F and RA2 co-transformed. Bait-pray swap analysis supported the interaction between RA1 and RA2 especially between RA1F and RA2-DM truncations (Table 2.1). RA1 apparently can also interact with itself since almost half of the RA1 and RA1 co-transformed colonies became positive. From more than three repeated experiments, there were always only part of the RA1 and RA1 co-transformed colonies and RA1F and RA2-DM co-transformed colonies became positive, and these colonies grew well on the non-selective 1×SC-LW media, we think the interactions between RA1 with itself and RA1F with RA2-DM were weak. RA2 is a putative transcription factor. In this study, it acted like a transcriptional activator since co-transformed pBD-RA2 and pAD-Gal4 empty vector activated the expression of both reporter genes. RA3 didn’t show interaction with either RA1 or RA2 or their truncations.

To study more of RA1 function, we prepared polyclonal antisera against 6×His-RA1 and purified the antisera to get purified antibody against the full length RA1. We tested the specificity of the purified RA1 antibody with *E. coli* protein extracts and the quality and the specificity of RA1 antibody was much better than RA1 antisera. Only one band was recognized from the over-expressed 6×His-RA1 protein extracts, while one main band and two smaller bands were recognized from the over-expressed GST-RA1 protein extracts. We speculate that in the *E. coli* expression system, some truncations of the GST-RA1 fusion protein were produced that were recognized by the RA1 antibody. We also tried to extract total proteins from plant
tissue and detect RA1 with the purified RA1 antibody, but no signal was detected. There were several possible reasons why RA1 was not detected. Because RA1 is only expressed in a few cells in very young inflorescences, large amounts of plant tissues may be required to get enough RA1 in protein extracts for this experiment. If we didn’t analyze enough tissue for our experiment, then no band would be detected. Another possibility is that the titer of purified RA1 antibody is too low so that it cannot detect RA1 when its protein concentration is too low.

In vitro GST pull-down assay was performed to confirm the interactions between the two truncations of RA1F and RA2-DM, and RA1 with itself. The purified RA1 antibody, 6×His epitope tag, and anti-glutathione S-transferase were used to detect 6×His-RA1, GST-RA1, 6×His-RA1F and GST-RA2-DM bands. The confirmed interactions suggested RA1 may act as a dimer or multimer and that RA1 may interact with RA2 through the RA2-DM region when their expression patterns overlap during inflorescence development. However, no other experiments were done so far to further explain their working model. Perhaps other proteins are involved in their interactions to form a more complicated protein polymer.

Although some functions of the three RAMOSA proteins are similar and their expression patterns in young inflorescences partially overlap, they were reported to have their own special functions during maize inflorescence development (Vollbrecht, Springer et al. 2005; Bortiri, Chuck et al. 2006; Satoh-Nagasawa, Nagasawa et al. 2006). However by looking at the branch phenotypes of the mature ears, the initial data showed that each double mutant of ra1-RS; ra2-R, ra1-RS; ra3-R or ra2-R; ra3-R had the similar phenotype as the ra1-R single mutant. As ra1-R mutant ears were
almost sterile, no other \textit{ra1-R} related double mutants were made and analyzed before.

To further investigate the relationships of the three RAMOSA proteins, we made the \textit{ra1-R; ra2-R; ra3-R} triple mutant plants and \textit{ra1-R; ra3-R, ra2-R; ra3-R} double mutant plants. The young inflorescences from the triple and the two double mutants were collected for SEM. We compared the phenotypes of their 2\textsuperscript{nd} order meristems at two stages on young tassels and three stages on young ears. The triple mutants and the two double mutants looked highly similar to each other and no new phenotype was produced on the triple mutants during 2\textsuperscript{nd} order meristem development. This confirmed that RA2 and RA3 functioned through RA1 to determine the fate of 2\textsuperscript{nd} order meristems. From the SEM images, we also found that in these \textit{ramosa} mutants, the 2\textsuperscript{nd} order meristems weren’t completely switched from the spikelet pair meristems to the branch meristems as we describe above. However, the SPMs were initially formed on the central spike. But quickly one side of each SPM grew much faster and expanded to form a branch as would a meristem that bears BM functions, while the other side of this SPM stopped growing and finally disappeared. Since these SPMs in \textit{ramosa} mutants behaved totally different from those SPMs on the wild type plants, in order to better describe this mutant phenotype, we therefore describe the 2\textsuperscript{nd} order meristems in \textit{ramosa} mutants as BMs.
CHAPTER 3. RAMOSA1 INTERACTS WITH KNOTTED1 AND REGULATES MERISTEM DETERMINACY VIA GIBBERELLINS DURING MAIZE INFLORESCENCE DEVELOPMENT

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Contributions to this work

The experiments in this manuscript were performed by Ms. Xiang Yang and Dr. Erik Vollbrecht. Ms. Yang was responsible for carrying this project forward throughout the course of the project. Dr. Vollbrecht did all the field work for making the \textit{ra1-RSenh; kn1-e1} double mutants and consulted on the overall instructions and support for this work. In addition, Dr. Vollbrecht contributed to reviewing and editing of this work. This work was supported by the National Science Foundation (NSF 05-603) to Dr. Sarah Hake, Dr. David Jackson, Dr. Elizabeth Kellogg, Dr. Tobert Rocheford and Dr. Erik Vollbrecht.
Abstract

RAMOSA 1 (RA1) is a plant-specific EPF-like protein with a Cys$_2$-His$_2$ zinc finger DNA binding domain and two EAR repression motifs that regulates branch architecture of the maize inflorescence. In ra1-R and ra1-RSenh strong mutants, both the tassel and the ear become highly branched due to loss of meristem determinacy. To elucidate the mechanism of RA1 action, yeast two hybrid analysis was used to screen for RA1-interacting proteins encoded in young ear cDNA libraries. Several transcription factors including KNOTTED1 (KN1) were identified. The interaction between RA1 and KN1 was confirmed by GST pull down and bimolecular fluorescence complementation (BiFC) experiments, and mapped onto the domain structure of the two proteins. In tests for a genetic interaction, the tassel phenotypes of the ra1-RSenh; kn1-e1 double mutants were statistically analyzed, which also supported an interaction between RA1 and KN1 in regulating inflorescence branch architecture. KNOX proteins are known to regulate gibberellin levels in lateral organ initiation, and the ga2 oxidase1 gene is a direct target of KN1. We found altered transcript levels of gibberellin biosynthesis genes in developing ra1-R mutant inflorescences, and the exogenous gibberellic acid 3 (GA$_3$) partially corrected the ra1-R mutant phenotype. These results all suggest a role for gibberellins in regulating meristem determinacy during maize inflorescence development.
3.1 Introduction

The term inflorescence architecture refers to the way plant reproductive structures are arranged on the floral stem that is composed of a main branch or a complicated arrangement of branches. Inflorescence architecture determines the external appearance of a flowering plant. Maize is monoecious, having both a male inflorescence called the tassel and a female inflorescence called the ear on the same plant. In wild type maize, the mature tassel consists of a symmetrical, many-rowed central axis with several asymmetrical long branches at the base. Both the central axis and the lateral branches bear paired spikelets. The mature ear has only a symmetrical, many-rowed axis with paired spikelets, with no lateral branches.

In flowering plants, the inflorescences, leaves and all of the other above ground organs are produced by shoot apical meristems (SAMs). During vegetative growth, an SAM initiates leaves and each leaf subtends a meristem in its axil. When the plant transition from a vegetative stage to a reproductive stage, the SAM stops initiating leaves and converts into an inflorescence meristem (IM) (Wang and Li 2008). In maize, the IM produces the tassel by branching. It first forms determinate 2nd order spikelet pair meristems (SPM) on the central spike and indeterminate 2nd order branch meristems (BM) at the base of the tassel which led to SPM later. Each SPM produces two 3rd order spikelet meristems (SM) leading to four 4th order floral meristems (FM). An axillary meristem at the leaf axil produces the ear by progressing through the same meristem activities (Chuck, Muszynski et al. 2002; Laudencia-Chingcuanco and Hake 2002).
In maize, mutants with altered meristem initiation, identity, maintenance and/or size show effects on inflorescence development (Vollbrecht and Schmidt 2009). *ramosa1* (*ra1*) is reported to regulate maize inflorescence branching architecture by regulating the identity and determinacy of 2nd order meristems (Vollbrecht, Springer et al. 2005). *ra1* encodes a plant specific EPF-like protein which has one Cys$_2$-His$_2$ zinc finger DNA binding domain containing a variant QGLGGH region and two putative EAR repression motifs (Sigmon and Vollbrecht 2010). During maize inflorescence development, *ra1* is expressed at the junction between each 2nd order spikelet pair meristem (SPM) and the main axis to promote a determinate SPM fate. In *ra1-R* or *ra1-RSenh* strong mutants, the 2nd order meristems on the tassel and the ear assume indeterminate branch meristem (BM) identity instead of SPM identity which leads to a highly branched mutant phenotype (Gernert 1912; Vollbrecht, Springer et al. 2005; Gallavotti, Long et al. 2010). However, the mechanism by which RA1 determines the fate of the 2nd order meristems is still not understood.

The normal activities of class I KNOTTED-1 like homeobox (KNOX) transcription factors such as SHOOT MERISTEMLESS (STM) in *Arabidopsis thaliana* and KNOTTED1 (KN1) in maize (Vollbrecht, Veit et al. 1991) are required for vegetative shoot apical meristems (SAMs) initiation and maintenance. Null *stm* alleles produce terminal shoots comprised of cotyledons and only one or two leaves in *Arabidopsis thaliana* (Long, Moan et al. 1996; McConnell and Barton 1998). *kn1* loss-of-function mutants in maize lead to a shorter SAM that also produces a limited shoot, more commonly with one or two leaves (Vollbrecht, Reiser et al. 2000). Several reports indicate that one important function of KOX proteins in maintaining normal SAM
activity is to control a low active gibberellin (GA) level in SAMs (Hay, Kaur et al. 2002; Jasinski, Piazza et al. 2005).

Gibberellins are a class of plant hormones that function in many processes during plant growth and development, such as seed germination, stem elongation, cell division and flowering (Ruth and Jan 1994; Blazquez, Green et al. 1998; Thomas, Rieu et al. 2005). Normal functioning SAMs also require a low GA level. KNOX proteins in tobacco (Sakamoto, Kamiya et al. 2001) and potato (Chen, Banerjee et al. 2004) are reported to directly bind and inhibit the expression of \textit{ga20 oxidase} genes which encode a rate-limiting enzyme in GA biosynthesis. In maize, KN1 directly binds and promotes the expression of a \textit{ga2 oxidase} gene, which encodes an enzyme to degrade bioactive GAs (Bolduc and Hake 2009). Thus, either the reduced expression of \textit{ga20 oxidase} genes or the increased expression of \textit{ga2 oxidase} genes leads to a low GA level in SAMs.

Besides SAMs, KNOX proteins are also involved in plant reproductive development. STM in Arabidopsis was reported to play a role in regulating flower patterning, branching and internode growth (Endrizzi, Moussian et al. 1996; Scofield, Dewitte et al. 2007; Smith, Ung et al. 2011). Several recessive \textit{kn1} mutant alleles in maize also produce mutant phenotypes in the inflorescence, including reduced branching (Kerstetter, Vollbrecht et al. 1994; Vollbrecht, Reiser et al. 2000). Moreover, KN1 was reported to reduce bioactive GA during tassel development because exogenous GA$_3$ partially rescues the \textit{kn1-e1} mutant phenotypes of reduced spikelet density and altered pistil development (Bolduc and Hake 2009). However, there are no reports that explain how \textit{kn1} functions to regulate inflorescence
branching architecture apart from its effects on meristem maintenance.

In this study, we investigated nuclear localization of RA1 and identified that ra1 and kn1 interact during maize inflorescence development. Different expression levels of some of the maize ga20 oxidase genes and ga2 oxidases genes were detected between wild-type and ra1-R mutant young inflorescences. The results of GA3 rescue experiments further suggested that gibberellins were involved in controlling maize inflorescence branch architecture.

3.2 Materials and Methods

3.2.1 Materials

Unless otherwise noted, the chemicals used in these studies were obtained from either Sigma Chemical Co (www.sigmaaldrich.com) or Fisher Chemical Co (www.fishersci.com) or and were of the highest quality available. The restriction enzymes and T4 DNA ligation enzyme used in this study were obtained from New England BioLabs (www.neb.com).

3.2.2 Plants

ra1-R mutant allele has been previously described (Vollbrecht et al., 2005) and introgressed more than 6 times in the B73 background. Tassels and ears for RT-PCR, QPCR analysis were dissected from plants grown in the greenhouse in ISU. Plants for GA3 treatment were also grown in the greenhouse. ra1-RSenh; kn1-e1 double mutant plants were made from self-cross of kn1-e1/+; ra1-RSenh/+ plants in Mo17-5 background. To genotype kn1-e1 homozygous alleles, genomic DNA of
each plant were extracted from young leaves and a 704 bp fragment was PCR amplified from each plant’s genomic DNA by using primers kn1-e1-F3 and kn1-B (Appendix A). The PCR product was purified by ExoSAP-IT following manufacturer’s protocol (www.gelifesciences.com), and sent to the DNA Facility at ISU for sequencing. The *ra1-RSenh* homozygous alleles were genotyped using the similar method with primers RA8 and RA11 for PCR reaction.

YFP-RA1 transgenic plants were provided by Dr. David Jackson’s lab and the complimentary experiment were done to prove the normal function of YFP-RA1 fusion protein in maize inflorescence development. YFP-RA1 alleles were genotyped by using two primer-pairs EUO315 and EUO316, Bar-F and Bar-R for PCR reaction (Appendix A).

3.2.3 Subcellular localization analysis of GFP fusion proteins

Constructs

The full length cDNA of *ra1* and *ra1-R* was PCR amplified from B73, and *ra1-R* mutant genomic DNA respectively, using primers RA1-Sal-F and RA1-Xba-B (Appendix A). The full length cDNA of *ra1-RS* was PCR amplified from *ra1-RS* genomic DNA using primers RS-Sal-F and RA1-Xba-B. The PCR products were digested by Sal I and Xba I and ligated into similarly digested GFP empty vector, pZY212, to produce GFP-RA1, GFP-RA1-R and GFP-RA1-RS fusion constructs. To create GFP-RA1-P1920 construct, two primer pairs RA1-Sal-F and RA-P19, RA-P20 and RA1-Xba-B were used to amplify two parts of the *ra1* sequence from B73 genomic DNA. The two PCR products with the mutant sequence at one end were
then mixed together as the template to amplify \textit{ra1-P1920} whole fragment by using the primers RA1-Sal-F and RA1-Xba-B. The \textit{ra1-P1920} fragment was then cloned into pZY212 at the Sal I-Xba I site. The GFP-RA1-P2223, GFP-RA1-P2425, GFP-RA1-P1516, GFP-RA1-P1718, GFP-RA1-P2627, GFP-RA1-P2829, GFP-RA1-P3031, GFP-RA1-P3233, GFP-RA1-P3435, GFP-RA1-P1011, GFP-RA1-P1213 and GFP-RA1-P2829 constructs were produced by the same method as producing GFP-RA1-P1920 construct with their specific primers listed on (Appendix A). In all RA1 constructs, GFP was fused at the N-terminus. This arrangement is functional to complement \textit{ra1} mutants in transgenic maize (EV, unpublished). The GFP-KN1 construct was provided by David Jackson’s lab.

**Transient protein expression in \textit{N. benthamiana}**

The 3-week old \textit{N. benthamiana} leaves were isolated and put on the MS solid media (Murashige and Skoog 1962) for transient transformation and expression of all of the GFP fusion proteins. For the micro-projectile bombardment transformation system, 5 µg column purified plasmid DNA were precipitated onto 1.5 mg gold particles (1.0 micron, BIO-RAD) using 50 µl of 2.5 M CaCl$_2$ and 20 µl of 0.1 M spermidine (free base, Sigma). DNA coated particles were washed first with 70% ethanol then with 100% ethanol and finally re-suspended in 30 µl of cold 100% ethanol for two bombardments. The Model PDS-1000/He Biolistic Particle Delivery System (BIO-RAD) was used for the delivery with the helium pressure set at 1100 p.s.i.. After particle bombardment, Petri-dishes were sealed with parafilm and incubated in the dark at 25 to 28°C for 12 to 18 hours.
Fluorescence detection system

The *N. benthamiana* leaves after bombardment and incubation were imaged using Leica TCS SP5-X confocal microscope with the GFP filter (excitation at 489 nm). The TRITC filter (excitation at 610 nm) and the bright field were used for the background signals. For each bombardment, there were about 20-30 observations and each construct was bombarded at least three times to analyze the localizations. The cells shown for each construct are typical of all the bombardment experiments.

3.2.4 Screen for RA1 interacting proteins by yeast two-hybrid analysis

Yeast Host Strains

The yeast host strain PJ69-4a was provided by Dr. Allen Mayer's lab, ISU and was used to screen RA1 interacting proteins from cDNA libraries. Another yeast host strain YRG2 was obtained from Agilent Technologies and was used to quantify the protein-protein interactions.

Constructs and cDNA libraries

The full length *ra1* cDNA sequence was PCR amplified from B73 genomic DNA using primers RA1-Eco-F and RA1-Sal-B (Appendix A). The PCR product was digested by EcoR I and Sal I and ligated into pBD-Gal4 vector to produce pBD-RA1 construct.

The cDNA libraries from B73 2mm ears were constructed by HybriZAP-2.1 XR library construction kit and provided by Dr. Robert Schmidt’s lab, UCSD. The ExAssist Helper Phage was used to excise the pAD-GAL4 vectored phagemid
libraries from HybriZAP-2.1 vector and the plasmid cDNA libraries on the pAD-Gal4 vector were isolated and purified from the amplified phagemid libraries following manufacturer’s protocol (www.genomics.agilent.com).

RA1-interacting protein screening assay

For library screening, the full length pBD-RA1 construct was served as the bait and co-transferred with the pAD-Gal4 vectored plasmid cDNA libraries into yeast host strain PJ69-4a. The large scale yeast co-transferring experiments were performed by using the method listed on The Gietz Lab Yeast Transformation Home Page (home.cc.umanitoba.ca/~gietz). Totally 110 large (150×15 mm) plates were used with around $1 \times 10^8$ co-transformed yeast colonies to screen for the RA1 interacting proteins.

The co-transferred yeast cells were first cultured on the complete supplement mixture minus Leu and Trp solid media (1xSC-LW) for 3-5 days. When the colonies reached 1-2 mm big, they were filter transferred onto the complete supplement mixture minus Leu, Trp and Ade solid media (1xSC-ALW) for the protein-protein interaction assay. The colonies that obviously survived on the 1xSC-ALW media were picked up and streaked onto new 1xSC-ALW plates for re-selection. The whole plasmid DNAs were isolated from the yeast colonies that can grow on the 1xSC-ALW re-selection plates by using the glass beads (425-600 micron, Sigma) to break the yeast cells. Then the isolations were transferred into E. coli TOP10 chemical competent cell (www.lifetechnologies.com) and spread on the LB solid medium with Ampicillin (100 mg/L) to select for the plasmids on the pAD-Gal4 vector. The
plasmids were purified by using the QIAprep Spin Miniprep Kit and sequenced at the Iowa State University Nucleic Acid Facility. The interactions between RA1 and the isolated proteins were further confirmed by both straight and pair-switching yeast two-hybrid assay with *adenine* and *lacZ* as the reporter genes and using the similar methods described at Chapter 2.2.4. The pBD-KN1 fusion protein had a strong background in this yeast two-hybrid system, so I didn’t do the pair-switching assay to confirm its interaction with pAD-RA1.

The DNA sequences and the corresponding amino acid sequences of the putative RA1-interacting proteins were further analyzed by Blastx and Blastp against the Non-redundant protein sequences (nr) database in the National Center for Biotechnology Information (NCBI, blast.ncbi.nlm.nih.gov/Blast.cgi). The genes and proteins with the lowest E-values were selected. The cDNA sequences of the putative RA1 interacting proteins were also used as baits to Blastn against the Zm transcripts (V4a.53) database in PlantGDB (www.plantgdb.org/ZmGDB) to get the GRMZM number.

-Adenine selection and lacZ selection assay

Full length pBD-RA1 and pAD-KN1 isolated from protein screening assay were co-transformed into yeast host strain PJ69-4a by using the yeast high efficiency transformation protocol provided by manufacturer (www.stratagene.com). The co-transformed yeast cells were first grown on the media with complete supplement mixture minus Leu and Trp (1xSC-LW) for 3-5 days at 30°C. When the colonies were around 1-2 mm big, several colonies from each plate were picked up and re-
streaked onto the –Adenine selective media with complete supplement mixture minus Leu, Trp and Ade (1xSC-ALW) for the protein-protein interaction assay. After incubating 3-5 days at 30°C, the re-streaked colonies grew big on the 1xSC-ALW selective media if the co-transformed fusion proteins interact with each other. The re-streaked colonies co-transformed with two empty vectors or two fusion proteins that don’t interact with each other didn’t grow on the –Adenine selective media.

Two or three co-transformed colonies from each 1xSC-LW plate were picked up again and grew into the 1xSC-LW liquid media at 30°C overnight until their OD600 reach 1.0. Then a serial dilution was made for each cell culture and let them grow on the –Adenine selective media for 3-5 days at 30°C.

Filter lift assay was used to detect the expression of lacZ gene in yeast. Several co-transformed yeast colonies were picked up from each 1xSC-LW plate and re-streaked onto new 1xSC-LW plate and incubate for 2 days. After the re-streaked colonies were grown big, the filter lift assay was applied to each re-streaked colonies following manufacturer’s protocol (www.stratagene.com) for β-galactosidase (lacZ) gene expression analysis.

3.2.5 In vitro GST pull-down assay

Constructs

The full length ra1 cDNA was PCR amplified from B73 genomic DNA using primers RA1-Bam-F and RA1-Sal-B. The PCR product was digested by BamH I and Sal I and ligated into similar digested pGEX-4T-3 expression vector (www.gelifesciences.com) to make the GST-RA1 construct. The full length kn1
fragment was digested from pBD-KN1 construct by EcoR I and Sal I and ligated into similar digested pET28a vector (provided by Dr. Yanhai Yin’s lab, ISU) to produce the 6×His-KN1 construct.

Protein over-expression, purification and pull-down assay

The *gst-ra1* and 6×*his-kn1* fusion constructs were transferred into *E. coli BL21* (DE3) strain ([www.lifetechnologies.com](http://www.lifetechnologies.com)) and induced by 0.1% Isopropyl β-D-1 thiogalactopyranoside (IPTG) at 30°C for 4 hours. The solubility of the two fusion proteins were analyzed by the repeated freeze-thaw experiment following manufacturer’s protocol ([www.lifetechnologies.com](http://www.lifetechnologies.com)). The over-expressed GST-RA1 and 6×His-KN1 fusion proteins were purified using Glutathione Sepharose 4B beads and the ProBond Purification System, respectively, following manufacturers’ protocols ([www.gelifesciences.com](http://www.gelifesciences.com) and [www.lifetechnologies.com](http://www.lifetechnologies.com)).

For the pull-down assay, 2-10 µg purified GST-RA1 fusion protein bound on the glutathione sepharose 4B beads were mixed with 2-10 µg purified 6×His-KN1 fusion protein in 200 µl pull-down buffer (50mM Tris, pH7.4, 150mM NaCl, 1 mM EDTA, pH8.0, 1 mM DTT, 0.5% NP-40) and incubated at 4°C for 2 hours with gentle shaking. After washed 4 times with the pull-down buffer, the beads were re-suspended in the protein loading buffer, boiled and subjected to western blot assay. Purified RA1 antibodies described in Chapter 2.2.5 and 6×His epitope tag, affinity purified IgG from rabbit ([www.piercenet.com](http://www.piercenet.com)) were used as the primary antibodies, goat anti-rabbit (GAR)-HRP conjugate ([www.bio-rad.com](http://www.bio-rad.com)) was used as the secondary antibody and the ECL western blotting detection reagents was used as
the analysis system (www.gelifesciences.com). The over-expressed 6×His-RA1 fusion protein in BL21 (DE3) was insoluble, so I only did one way pull-down assay.

3.2.6 In vivo BiFC assay

Constructs

The full length cDNA of ra1 were PCR amplified from B73 genomic DNA using primers RA-Eco-F and RA-Bam-B. The PCR product was digested by EcoRI and BamHI and ligated into similar digested pBJ36+2×35S-SPYNE (N-terminal YFP fragment) and pBJ36+2×35S-SPYCE (C-terminal YFP fragment) vectors (provided by Dr. Robert Schmidt’s lab, UCSD) to produce pBJ-RA1-SPYNE and pBJ-RA1-SPYCE constructs. The full length kn1 sequence was PCR amplified from pBD-KN1 construct using primers KN-Eco-F and KN-Hind-B and cloned into the pBJ36+2×35S-SPYNE and pBJ36+2×35S-SPYCE vectors at the EcoRI and HindIII sites to produce pBJ-KN1-SPYNE and pBJ-KN1-SPYCE constructs. The fused fragments of RA1-SPYNE, RA1-SPYCE, KN1-SPYNE and KN1-SPYCE were then digested by NotI from the pBJ36 vectors and ligated into similar digested binary vector pML-BART to get the constructs for Agrobacterium tumefaciens transformation.

BiFC assay in N. benthamiana leaves

Each of the pML-RA1-SPYNE and pML-KN1-SPYCE constructs was transformed into A. tumefaciens strain C58C1, which together with the strain
containing suppressor p19 (Walter, Chaban et al. 2004) were incubated separately in 10ml LB liquid media with appropriate antibiotics at 28°C overnight. Next day, mixed the 3 Agrobacterium cells and infiltrated them into 4-6 weeks N. benthamiana leaves. Plants were grown in the greenhouse for 4-5 days. Then the A. tumefaciens infected leaves were isolated to detect and image the YFP signals using Leica TCS SP5-X confocal microscope with the YFP filter (excitation at 513 nm). The TRITC filter and the bright field were used to detect background signals.

3.2.7 Mapping RA1 and KN1 interacting regions

Constructs

The full length cDNA of ra1-R was PCR amplified from ra1-R mutant genomic DNA by using primers RA1-Eco-F and RA1-Sal-B and the pBD-RA1-R construct was produced by the similar method as making pBD-RA1 construct. To create pBD-RA1-P3 construct, the primers RA1-Eco-F and RA1-P3 was used to amplify the ra1-P3 fragment from B73 genomic DNA. The PCR product was then digested by EcoR I and Sal I and ligated into similar digested pBD-Gal4 vector. The ra1-P78 fragment was produced by using two primer-pairs RA1-Eco-F and RA1-P7, RA1-P8 and RA1-Sal-B to amplify two parts of ra1 sequence from B73 genomic DNA. The two PCR products were then mixed as the template and the primers RA1-Eco-F and RA1-Sal-B were used to PCR amplify the whole fragment. The ra1-P378 fragment was PCR amplified by using ra1-P78 fragment as template, RA1-Eco-F and RA1-P3 as primers. The ra1-F, ra1-P9, ra1-NZ, ra1-N1, ra1-Zinc and ra1-ZE fragment were PCR amplified from B73 genomic DNA using primer-pairs RA-Eco-F and RA-F-Sal,
RA1-Eco-F and RA1-P9, RA1-Eco-F and RA1-ZB, RA1-Eco-F and RA1-NB, RA1-ZF and RA1-ZB, RA1-ZF and RA1-P9, respectively. These fragments were cloned into the EcoRI-SalI site on the pBD-Gal4 and pAD-Gal4 vector with the similar method described above to create the fusion constructs. The pBD-KN1 construct was provided by Dr. Sara Hake’s lab and the kn1 fragment was digested by EcoRI and SalI and re-ligated into the similar digested pAD-Gal4 vector to create pAD-KN1. The kn1-Midd, kn1-CE, kn1-Homeo, kn1-MEX and kn1-N1 fragments were PCR amplified from pBD-KN1 construct using primer-pairs KN-MZX-F and KN-HB-Sal, KN-Ub-F and KN-Sal-B, KN-HF-Eco and KN-HB-Sal, KN-MZX-F and KN-MZX-B, KN-Eco-F and KN-NB-Sal, respectively. These fragments were cloned into the pBD-Gal4 and pAD-Gal4 vectors at EcoRI and SalI sites to get fusion constructs.

\[ \beta - \text{galactosidase quantitative assay} \]

The yeast host strain YRG2 was used to analyze the \( \beta \)-galactosidase activity by using chlorophenol red-\( \beta \)-D-galactopyranoside (CPRG, Sigma) as the substrate to perform the quantification (Serebriiskii and Golemis 2000).

3.2.8 Analysis of ra1-RSenh; kn1-e1 double mutants

All plants derived from kn1-e1+/; ra1-RSenh/+; Mo17-5 F2 populations that segregated the ra1-RSenh and kn1-e1 alleles in the Mo17 inbred background. Mature tassels were collected from wild type Mo17, ra1-RSenh single mutant, kn1-e1 single mutant and ra1-RSenh; kn1-e1 double mutant plants. The number of
compound branches, main branches, mixed branches, spikelet multimers, spikelet pairs and single spikelets on each tassel were counted. MANOVA analysis was first used to analyze the possible interaction between RA1 and KN1 from the data of all of the 2nd order meristems. ANOVA with the linear model \( Y = K + a^{*}ra1-RSenh + b^{*}kn1-e1 + c^{*}ra1-RSenh\cdot kn1-e1 \) was then used to analyze each type of the 2nd order meristems for the possible interaction between RA1 and KN1. R software (version 2.12.0) was used to do these analyses.

3.2.9 Identification and phylogenetic analysis of maize GA20oxs and GA2oxs

To identify GA20 oxidase and GA2 oxidase family genes in maize, each of the published GA20 oxidase and GA2 oxidase family proteins in rice (Sakamoto, Miura et al. 2004; Lo, Yang et al. 2008) and their conserved domains were used as baits to do tBLASTn against maize genomic sequences in the NCBI HTGS database (blast.ncbi.nlm.nih.gov) and MAGI database (magi.plantgenomics.iastate.edu). From these genomic sequences, the start codon, stop codon and the exons of each gene were carefully identified and analyzed by comparing with the bait protein sequences to predict the amino acid sequences of all the maize putative GA20 oxidase and GA2 oxidase proteins. The amino acid sequences of all the GA20 oxidase and GA2 oxidase family genes in maize and rice were aligned separately by using MUSCLE program (www.ebi.ac.uk/Tools/muscle). Evolutionary relationships of each family genes were analyzed by using the PAUP4.0 program to produce the “best” midpoint rooted phylogenetic tree with the maximum parsimony. The bootstrap support value of each node was produced by PAUP4.0 program with 1000 replicates.
3.2.10 RT-PCR and quantitative RT-PCR analysis

RT-PCR

Tissues were carefully dissected and quickly frozen in liquid nitrogen or -80°C. Total RNA was extracted from each sample using TRIzol reagent, the concentration of extracted RNA was detected by using the NanoDrop ND-1000 Spectrophotometer, and treated with DNase I under manufacturer’s protocol (www.lifetechnologies.com). The concentration of each DNase-treated RNA sample was detected again using the NanoDrop ND-1000 Spectrophotometer and the same amount of RNA was used for the semi-quantitative RT-PCR analysis. The first strand cDNAs were produced by using SuperScript™ III First-Strand Synthesis System under manufacturer’s protocol (www.lifetechnologies.com). All primers used in this analysis were listed in Appendix A. The RT-PCR reactions performed in Appendix B and C all had 35 PCR cycles. RT-PCR products were detected in a 1% agarose gel stained with ethidium bromide.

Quantitative RT-PCR

Tissues used for this analysis were collected and treated the same way as described above. The preparation of the total RNA and the synthesis of the first strand cDNA were using the same method as mentioned above. For those genes that showed different expression levels between B73 and ra1-R mutants in semi-quantitative RT-PCR analysis were further analyzed by QRT-PCR. Primers used in this study were listed in Appendix A. The QPCR reactions were performed by using Brilliant II SYBR Green QPCR Master Mix with ROX on the MX 4000 Multiplex
Quantitative PCR system (Stratagene). Each sample was performed 6 independent replications with 3 independent RNA isolations and 2 replicas for each of the RNA isolations. Ubiqitin (Ubi) was used as an internal reference to monitor and normalize the relative level of each transcript. The block effects in every QPCR experiment were removed before analyzing the average value and the standard deviation for each gene.

3.2.11 Gibberellic acid 3 (GA₃) treatments

Plant

ra₁-R/B73 heterozygous and ra₁-R homozygous mutant plants in the B73 inbred background were used for Gibberellic Acid, GA₃ (www.sigmaaldrich.com) treatment. All the plants were planted at the same time and located at the same area in the greenhouse to make sure they grew under the same conditions.

GA₃ treatment

After 14 DAG, about 2 plants from each group were dissected everyday to detect the tassel development stages. At 21 DAG, the tassels from each plant were reached around 2 mm. At this stage, the tassel has produced several lateral branches and some spikelet pair meristems at the base but has no spikelet meristems. Then some ra₁-R homozygous plants and ra₁-B73/ra₁-R heterozygous plants were randomly chosen to be treated with GA₃, the others were treated only with water as the controls. For each treatment, 1 ug GA₃ was used to spray into the leaf whorl of the plant since it was turned out to be the best amount for tassel
treatment within the 0.1 ug, 1 ug, 10 ug and 100 ug range. The next treatment was performed 3 days later and 1 or 2 plants from each group were dissected before the treatment to detect the tassel development. The plants were treated around 10 days with totally four treatments performed until the tassels reached 6-7 mm. Then all the plants were grown under normal conditions until maturity. The mature tassels were dissected carefully to count the number of each 2nd order meristem. In total, 23 tassels from ra1-R homozygous plants treated with GA3, 15 tassels from ra1-R homozygous plants planted treated with water, 15 tassels from ra1-B73/ra1-R heterozygous plants treated with GA3 and 20 tassels from ra1-B73/ra1-R heterozygous plants treated with water were counted for statistical analysis.

3.3 Results

3.3.1 Differential nuclear localization of RA1 and RA1 mutant forms in planta

To visualize the subcellular localization pattern of full-length ra1 and ra1-R mutant protein in planta, green fluorescent protein tagged fusions of RA1 and RA1-R (GFP-RA1 and GFP-RA1-R) (Figure 3.1A) were transiently expressed in 3-week-old N. benthamiana leaves by microprojectile bombardment. Protein localization was then detected using confocal microscopy. Most of the GFP-RA1 fusion protein localized in the cell nucleus with some diffuse in the cytoplasm (Figure 3.1B). This pattern was distinct from that of the GFP-RA1-R, in which most of the fusion protein was diffused throughout the cytoplasm while very little was localized in the cell nucleus. ra1-R mutant protein contains two amino acid differences (G62E and H64N) within the Cys2-His2 zinc finger domain of RA1 such that the QGLGGH motif in RA1
is QGGLEN in RA1-R, and the C$_2$H$_2$ motif becomes C$_2$H$_1$ (Vollbrecht, Springer et al. 2005). To analyze the amino acid sequence requirements for nuclear localization, two GFP fused RA1 mutant proteins were made: GFP-RA1-P1920 (G62E) and GFP-RA1-P2223 (H64N) were mutated within the QGLGGH motif to separately investigate the polymorphisms in the $ra1$-$R$ mutant. Subcellular localization of the fusion proteins was detected by the same method. GFP-RA1-P2223 failed to localize GFP signal inside of the nucleus while GFP-RA1-P1920 showed normal nuclear localization (Figure 3.1B).

Since His64 is also the first His in the C$_2$H$_2$ zinc finger domain, to investigate whether the C$_2$H$_2$ zinc finger structure is required for RA1 nuclear localization, two GFP fused proteins, GFP-RA1-P1516 (C51S) and GFP-RA1-P1718 (H68N) were made which have mutations at the 2$^{nd}$ Cys and the 2$^{nd}$ His of the C$_2$H$_2$ zinc finger domain, respectively. However, these two fusion proteins showed normal nuclear localization which suggests that an intact zinc finger per se is not required in localizing RA1 into the cell nucleus. We further made four other GFP fused proteins with the single amino acid mutations at the QGLGGH motif: GFP-RA1-P2627 (Q59G), GFP-RA1-P3031 (G60D), GFP-RA1-P3233 (G60A) and GFP-RA1-P3435 (L61E). The nuclear results showed that the L61E mutation greatly affected the protein nuclear localization while the Q59G and G60D mutations partially affected nuclear localization. On the other hand, G60A mutation showed normal protein nuclear localization. These suggest that in addition to its putative role in DNA binding (Isernia, Bucci et al. 2003), the QGLGGH motif also serves as a nuclear localization signal in RA1.
Figure 3.1  Subcellular localization of RA1 and RA1 mutants in planta

Panel A. Constructs used in these studies. Mutated amino acids are labeled in red and bold font above construct schematics.

Panel B. Confocal images of the GFP fusion constructs transiently expressed in *N. benthamiana* leaves. a, c, e, g, i, k, m, o, q, s, o, images taken with the GFP filter only. b, d, f, h, l, n, p, r, t, v, images taken with GFP filter, the TRITC filter and the bright field to show the GFP signal and additional cellular features. a-b, images of pZY212 empty vector containing the *mgfp6* sequence as the control. c-d, images of GFP-RA1. e-f, images of GFP-RA1-R. g-h, images of GFP-RA1-P1920. i-j, images of GFP-RA1-P2223. k-l, images of GFP-RA1-P1516. m-n, images of GFP-RA1-P1718. o-p, images of GFP-RA1-P2627. q-r, images of GFP-RA1-P3031. s-t, images of GFP-RA1-P3233. u-v, images of GFP-RA1-P3435. Bars = 50 µm.
Figure 3.1 Subcellular localization of RA1 and RA1 mutants in planta
3.3.2 RA1 physically interacts with KN1

We applied a yeast two-hybrid approach to identify RA1 interacting proteins functioning in early maize inflorescence development. Specifically, we screened a cDNA library prepared from 2mm B73 ears, utilizing a RA1 full-length construct as the bait. As a result, from $10^8$ primary yeast transformants, we identified 8 transcription factor genes including *knotted-1* (*kn1*), and some others that encode putative RA1 interacting proteins. Most of their interactions with RA1 were reconfirmed by directly testing for a yeast two-hybrid interaction assay and/or by doing bait-prey swap analysis if the pBD-Gal4 fused protein had no background on yeast two-hybrid assay (Table 3.1).

RA1 and KN1 interacted in yeast as the co-transformed colonies showed positive results on –Adenine selection and lacZ selection assay (Figure 3.2A). To confirm their interactions, glutathione S-transferase was fused to full length *ra1* and a 6xHis tag was fused to *kn1* for in vitro protein pull-down assays. Because 6xHis-RA1 fusion protein produced in *Escherichia coli* was insoluble, we only did this test in one direction. GST-RA1 was purified from *E. coli* extracts onto Glutathione Sepharose 4B beads and the beads were mixed with protein extracts obtained from *E. coli* cells over-expressing 6xHis-KN1. The proteins pulled down were analyzed by protein gel blotting using anti-6xHis antibody (Figure 3.2B). GST-RA1, but not GST alone, precipitated from the extracts a 46-kD protein species that was recognized by anti-6xHis antibody and was of a similar size as 6xHis-KN1. These results indicate RA1 binds to KN1 in vitro.
Table 3.1 RA1-interacting proteins identified by yeast two-hybrid analysis

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"Times Isolated" indicates the total number of times a related cDNA was isolated followed by the number of screening experiments in parentheses. The interaction with RA1 was re-confirmed by direct testing in yeast PJ69-4a strain. The interaction with RA1 was re-confirmed by direct testing and bait-prey swap assay. The bait-prey swap assay showed background in yeast only the direct testing was performed. *More than one GRMZM number qualified as the best hit but only one of them is listed in the Table.
Figure 3.2 RA1 and KN1 interact in vitro and in vivo

**Panel A.** Yeast two-hybrid analysis showed the interaction between pBD-RA1 and pAd-KN1 with –Adenine selection and lacZ selection.

**Panel B.** GST pull down assay with full length RA1 and KN1. The input are purified proteins were run on an SDS-PAGE gel. The pull downs are western blots probed with the purified anti-RA1 antibody and the anti-6xHis antibody. Protein molecular weights are expected as follows: GST-RA1 = 44.7 KD, 6xHis-KN1 = 43.3 KD. +, protein expressed; - empty vector control.

**Panel C.** BiFC analysis of RA1-KN1 interactions in *N. benthamiana* leaves. a, c, e, confocal images taken with the YFP filter only. b, d, f, confocal images taken with the YFP filter, the TRITC filter and the bright field to show the YFP signal and additional cellular features. a-b, co-transform RA1-SPYNE and KN1-SPYCE. c-d, co-transform RA1-R-SPYNE and KN1-SPYCE. e-f, co-transform pML-SPYNE and KN1-SPYCE as a negative control. Bars = 50 µm.
Figure 3.2 RA1 and KN1 interact in vitro and in vivo
We next used the biomolecular fluorescence complementation (BiFC) assay to monitor the interaction between RA1 and KN1 in \textit{planta}. For the BiFC assay, the N-terminal domain and the C-terminal domain of YFP (SPYNE and SPYCE) were fused to the C-terminal ends of RA1 and KN1, respectively. Interactions between the two respective fusion proteins were tested in \textit{N. benthamiana} leaves using the transient expression assay mediated by \textit{Agrobacterium} \cite{Voinnet, Rivas et al. 2003}. Confocal microscopy images demonstrated that coexpression of RA1-SPYNE and KN1-SPYCE resulted in bright fluorescence in the cell nucleus (Figure 3.2C). And co-injection of KN1-SPYNE and RA1-SPYCE gave the same results. Thus, this assay indicated that RA1 and KN1 interact in the plant cell nucleus. However, as RA1-R mutant protein doesn’t enter into the plant cell nucleus (Figure 3.1B), when RA1-R-SPYNE and KN1-SPYCE were co-transformed into \textit{N. benthamiana} leaves, only a weak YFP signals were detected outside of the cell nucleus, suggesting that the mutated RA1-R protein interacts with KN1 in the cytoplasm instead of the cell nucleus.

\subsection*{3.3.3 RA1 and KN1 interact through zinc finger region and homeodomain}

To investigate the interacting domains of RA1 and KN1, we made several mutations and truncations of RA1 and KN1 (Figure 3.3A), and directly tested each interaction in yeast by quantitative $\beta$–galactosidase assay (Figure 3.3B). The KN1-N1 construct, which contains the N terminal 97 amino acids, showed no interaction with RA1. The KN1-Midd construct, which contains the middle region of KN1 from MEINOX domain to homeodomain, and the KN1-CE construct, which contains the
region from homeodomain to the C terminal end, only showed a partial interaction with RA1. The homeodomain alone showed a partial but much stronger interaction with RA1, but none of the KN1 truncations interacted as strongly with RA1 as the full-length KN1. We also tested several truncations of RA1, a putative transcription factor that contains two EAR repression motifs (Vollbrecht, Springer et al. 2005). RA1-P3 has a site mutation at the 1st EAR motif (L168F). RA1-P378 has one site mutation at the 1st EAR motif (L168F) and another four site-mutations at the 2nd EAR motif (L102F, L104F, S105F, L106F). RA1-F is a truncation of RA1 in which the C-terminal EAR motif (the 1st EAR motif) been removed. RA1-P9 is another truncation of RA1 with both EAR motifs removed. All four of these mutant proteins showed strong interactions with the full-length KN1, which means the EAR motifs in RA1 are not required for the interaction. RA1-NZ contains N terminal 71 amino acids of RA1 including the zinc finger, and RA1-Zinc includes only 26 amino acids from Y46 to D71 of RA1, which only contains the C_2H_2 zinc finger domain. The β–galactosidase activity assay showed that both of them interacted with KN1, although not as strongly as the full length RA1 with KN1. But RA1-ZE which contains 53 amino acids from C_2H_2 zinc finger domain to the beginning the secondary EAR motif showed strong interactions with either the full length KN1 or the KN1 homeodomain. These data imply that the 53 amino acids of RA1 including the C_2H_2 zinc finger region and the homeodomain of KN1 are critical for the protein-protein interaction between RA1 and KN1.
Figure 3.3 Identify interacting domains in RA1 and KN1

**Panel A.** Gene structures used in this experiment. The length of each fragment in base pairs is labeled under each structure. Mutated amino acids are labeled in red and bold font. These fragments were fused in frame into pBD-Gal4 and pAD-Gal4 vector respectively as showed in B.

**Panel B.** Quantitative assay for β-galactosidase activity in yeast. Constructs are identified to the left of the bars. β-galactosidase activity was quantified (means ±SD, n = 3 with three replicates for each sample) for each pair of the constructs. The activity of the positive control was assigned at 100%, and the activities of other samples were calculated relative to this value. The black bars show the relative activity of each pair of construct and the gray bars show the relative activity of the pBD-Gal4 fused protein with the pAD-Gal4 empty vector which was used as the negative control for each pair of constructs.
Figure 3.3 Identify interacting domains in RA1 and KN1
3.3.4 The tassel branch phenotype of *ra1-RSenh; kn1-e1* double mutants support the interaction between RA1 and KN1

To test the genetic relationship between *ra1* and *kn1*, we made *ra1-RSenh; kn1-e1* double mutant plants and analyzed the branch phenotypes of their mature tassels in comparison to wild type and each single mutant (Figure 3.4A, B). In the mature tassel of wild type Mo17 or *ra1-RSenh* single mutants, several types of branches developed from 2\(^{nd}\) order meristems: From base to apex, the main axis bore a few compound branches (bearing some 3\(^{rd}\) order branches, plus or minus some spikelets), followed by main branches (bearing only spikelet pairs). Proceeding acropetally, the axis then bore some mixed branches (bearing both single spikelets and spikelet pairs), then spikelet multimers (bearing only single spikelets and having three or more of them), then spikelet pairs. Single spikelets and spikelet pairs were interspersed near the tip of the main axis. In the tassels of plants with different genotypes, these 2\(^{nd}\) order meristem types developed but in much different relative numbers. The mean of the total 2\(^{nd}\) order meristems in *kn1-e1* single mutant tassel was much smaller than wild type Mo17 (49.7 vs 125.6) and *kn1-e1* single mutant tassel produces no single spikelets (Appendix B). *ra1-RSenh* single mutant tassel produces much more compound branches, mixed branches and spikelet multimers but less spikelet pairs compared with wild type Mo17 (Appendix B). The tassel branch phenotype of *ra1-RSenh; kn1-e1* double mutant plants was complicated. It has fewer 2\(^{nd}\) order meristems and no single spikelets, but more compound branches, mixed branches and spikelet multimers. Although this looks like an additive phenotype, the actual mean number of some meristem types such as
Figure 3.4 Analyze *ra1-RSenh; kn1-e1* double mutant plants

**Pannel A.** Mature tassels from each group. a-d, mature tassels in the field. e-h, dried tassels on the map, bars = 5 cm. a, e, tassels of wild-type Mo17. b, f, tassels of *kn1-e1* single mutant. c, g, tassels of *ra1-RSenh* single mutant. d, h, tassels of *ra1-RSenh; kn1-e1* double mutant.

**Pannel B.** MANOVA analysis results for total 2\textsuperscript{nd} order meristems and the tassel length.

**Pannel C.** ANOVA analysis results for the *ra1-RSenh·kn1- e1* term (the genetic interaction) on each type of the 2\textsuperscript{nd} order meristems.
Figure 3.4 Analyze *ra1-RSenh; kn1-e1* double mutant plants
spikelet multimers couldn’t be explained simply by addition. Therefore, statistical methods were used to analyze the possible interactions between \( ra1 \) and \( kn1 \) based on the tassel branch phenotypes of the wild type, two single and the double mutant plants.

From a segregating population, in total 11 \( ra1\text{-}RSenh; kn1\text{-}e1 \) double mutant plants, 9 \( ra1\text{-}RSenh; + \) single mutants, 13 +; \( kn1\text{-}e1 \) single mutants and 14 +; + wild-type plants were analyzed. Every 2\(^{\text{nd}}\) order branch was classified into one of the six 2\(^{\text{nd}}\) order meristem types. MANOVA analysis was applied on all 2\(^{\text{nd}}\) order meristem types and the results indicated that \( ra1 \), \( kn1 \) and their interaction were all involved in regulating the development of 2\(^{\text{nd}}\) order meristems and the tassel length (Figure 3.4B). The ANOVA analysis applied on each type of the 2\(^{\text{nd}}\) order meristems (Appendix C) also suggested a genetic interaction between RA1 and KN1, especially in regulating the development of compound branches (p value = 1.9e-05), spikelet multimers (p value = 1.6e-04) and spikelet pairs (p value = 1.3e-04) (Figure 3.4C). Large scale plants including all of the heterozygous plants in each group, which produced a similar phenotype as the homozygous single mutants or the wild-type plants, by the same statistical methods gave very similar results, which supported the genetic interaction between RA1 and KN1.

3.3.5 KNOX targets \textit{GA20 Oxidase} and \textit{GA2 Oxidase} family genes in maize

\( kn1 \) related homeobox genes (\textit{knox} genes) are known to regulate gibberellin levels by controlling the expression of \textit{GA20 oxidase} genes and \textit{GA2 oxidase} genes in the shoot apical meristem and in leaf and seed development in many dicot plants.
such as Arabidopsis, tobacco, tomato and potato (Sakamoto, Kamiya et al. 2001; Hay, Kaur et al. 2002; Chen, Banerjee et al. 2004; Jasinski, Piazza et al. 2005; Singh, Filardo et al. 2010). Furthermore kn1 in maize was reported to directly regulate the gibberellin catabolism gene GA2ox-1 (Bolduc and Hake 2009) and this is the only direct target yet reported. We used the available maize genome sequence databases to identify all of the putative GA20 oxidase family gibberellin biosynthesis genes (GA20ox) and GA2 oxidase family gibberellin catabolism genes (GA2ox) in maize. Totally four putative ZmGA20ox family genes and twelve putative ZmGA2ox family genes were identified based on the reported four OsGA20ox genes (Oikawa, Koshioka et al. 2004) and ten OsGA2ox genes from rice (Lo, Yang et al. 2008). Phylogenetic analysis indicated we identified the maize orthologs for each rice gene (Figure 3.5). The ZmGA family genes were named according to their relationships with OsGA genes and previously published results (Bolduc and Hake 2009), and their transcript accumulation levels in young inflorescences were analyzed.

3.3.6 ZmGA20 Oxidase and ZmGA2 Oxidase genes expression levels differ between ramosa1 mutants and B73 wild type

Based on the interaction between RA1 and KN1, we first tested all the putative ZmGA20ox and ZmGA2ox family genes for expression in developing inflorescences (4 mm tassels and ears) of wild type B73 and ra1-R mutants by semi-quantitative RT-PCR (Appendix D, E). While expression of several genes was not detected in young inflorescences, the ZmGA20ox-2, ZmGA2ox-6, ZmGA2ox-8 and ZmGA2ox-
Figure 3.5  Phylogenetic analysis of *GA20 oxidase* genes and *GA2 oxidase* genes from *Zea mays* (Zm) and *Oryza sativa* (Os)

**Panel A.** Mid-point rooted phylogenetic tree of the putative *GA20 oxidase* genes.

**Panel B.** Mid-point rooted phylogenetic tree of the putative *GA2 oxidase* genes. Bootstrap support values are shown on each node.
Figure 3.5 Phylogenetic analysis of GA20 oxidase genes and GA2 oxidase genes from Zea mays (Zm) and Oryza sativa (Os)
11 genes had about the same expression levels in wild type B73 and ra1-R mutants. On the other hand, ZmGA20ox-1, ZmGA2ox-1, ZmGA2ox-7, ZmGA2ox-9 and ZmGA2ox-12 showed different expression levels in wild type B73 and ra1-R mutant tassels (Appendix F). These genes also show similar expression pattern in ra1-RSenh mutated young tassels (Appendix G).

To better understand the different expression levels of ZmGA20ox-1, ZmGA2ox-1, ZmGA2ox-7 and ZmGA2ox-12 in the young tassels of B73 and ra1-R mutants, quantitative RT-PCR was performed using ubiquitin as the control. Based on the similarity of ZmGA2ox-9 to OsGA2ox-9 which is reported to encode a C_{20}GA2ox (Lo, Yang et al. 2008), and the fact that 19-carbon gibberellins are the biologically active forms of gibberellins, we didn’t do quantitative RT-PCR on this gene. 2-3 mm and 4-5 mm tassels of B73 and ra1-R mutants were carefully staged and collected for extracting mRNA. Expression levels of ra1 and kn1 were also tested. Levels of ZmGA20ox-1 showed no difference between B73 and ra1-R mutants in 2-3 mm tassels but were significantly reduced in 4-5 mm tassels of ra1-R mutants (P value = 0.002). Conversely, levels of ZmGA2ox-1, ZmGA2ox-7 and ZmGA2ox-12 were significantly increased in the 2-3 mm tassels of ra1-R mutants (p value equals 0.0002, 0.0003 and 0.0008 respectively) and were also increased in the 4-5 mm tassels (p value equals 0.01, 0.02 and 0.03 respectively) (Figure 3.6).

Because GA20 oxidase proteins synthesize bioactive GAs while GA2 oxidase proteins modify active GAs into inactive forms (Hedden and Phillips 2000), these results suggested that the bioactive gibberellin levels are reduced in ra1-R mutants. Transcript levels of ra1 were increased in both stages of ra1-R mutant tassels,
Figure 3.6  QRT-PCR results of select ZmGA20 oxidase and ZmGA2 oxidase genes in B73 and ra1-R mutants

The transcript level of each gene in wild-type B73 at each stage was assigned the value of 1 and the transcript level of that gene in corresponding ra1-R mutants was calculated relative to this value. Error bars indicate the relative SD (n=3 with two replicates for each sample). *p value is between 0.01 and 0.05. **p value is less than 0.01.
Figure 3.6  QRT-PCR results of select ZmGA20 oxidase and ZmGA2 oxidase genes in B73 and ra1-R mutants
consistent with a previous report (Vollbrecht, Springer et al. 2005). The *kn1* transcript level was also significantly increased in both the 2-3 mm and 4-5 mm tassels of *ra1-R* mutants (p value equal 0.0002 and 0.002 respectively).

### 3.3.7 Gibberellic acid treatment partially corrects the highly branched phenotype of *ra1-R* mutants

Compared with B73, in *ra1-R* mutants both the tassel and the ear exhibit a highly branched phenotype. qRT-PCR results suggested the hypothesis that reduced levels of bioactive gibberellins correlate with increased branching of *ra1-R* mutants. To further test this hypothesis, gibberellic acid (GA$_3$) was used to treat *ra1-R* mutants and sibling, normal *ra1-B73/*ra1-R* heterozygous plants, and the branch phenotype of their mature tassels was carefully analyzed after the treatment. GA$_3$ treatment was started when the plants were around 2 or 3 weeks old and the tassels were around 2 mm (Figure 3.7A). In this stage, other than a few lateral branch meristems formed at the base of the central spike, all of the other 2$^{nd}$ order meristems on the central spike are just beginning to initiate and they will be subsequently determined to form spikelet pair meristems in *ra1-B73/*ra1-R* heterozygotes and branch meristems in *ra1-R* mutants. This period of time is also when *ra1* is expressed. The plants were treated every 3 days with 1 ug GA$_3$ per plant per treatment and the treatments were stopped around 10 days later when tassels reached 6.5 mm length, by which point in time 2$^{nd}$ order branching on the main axis had ceased. Some plants were treated only with water and used as controls.
In the *ra1-R* mutant, mature tassel, there are several types of branches developed from 2\textsuperscript{nd} order meristems which match those types produced on *ra1-RSenh* single mutants (Vollbrecht, Springer et al. 2005). All of these 2\textsuperscript{nd} order meristem types also developed in the tassel of nonmutant, *ra1-B73/ra1-R* heterozygous plants, but in much different relative numbers. The occurrences of compound branches, mixed branches and spikelet multimers were much lower and the proportion of main branches and spikelet pairs were much higher than in *ra1-R* mutant tassel (Figure 3.7B, C).

After treatment with GA\textsubscript{3}, the total number of 2\textsuperscript{nd} order branches was unchanged in both genotypes (for *ra1-B73/ra1-R* heterozygous plants, mean is 96.7 for non-treated plants and 87.6 for GA\textsubscript{3} treated plants, p value = 0.1; for *ra1-R* homozygous mutants, mean is 63.9 for non-treated plants and 57.6 for GA\textsubscript{3} treated plants, p value = 0.15.) In nonmutant, *ra1-B73/ra1-R* heterozygous tassels there were more mixed branches after GA\textsubscript{3} treatment (p value = 0.0006) but the effects on the number of all other 2\textsuperscript{nd} order meristem types were not significant. In treated tassels of *ra1-R* mutants on the other hand, the occurrence of main branches (p value = 0.04), mixed branches (p value = 0.005) and spikelet multimers (p value = 0.03) were significantly reduced. In addition, the number of single spikelets on the main axis of *ra1-R* treated tassel was significantly increased (p value = 0.03), while the number of the other 2\textsuperscript{nd} order meristems was relatively unchanged (Figure 3.7B). As exogenous GA\textsubscript{3} application reduced the number of some relatively indeterminate branch types on the main axis of the *ra1-R* tassel and increased the number of determinate single spikelets, the branched phenotype in *ra1-R* was partially rescued.
Figure 3.7  GA$_3$ treatment alters the 2$^{nd}$ order meristem development

**Pannel A.** SEM images of representative young tassels used in this experiment. a-b, tassels from $ra1$-$B73/ra1$-$R$ heterozygous plants. c-d, tassels from $ra1$-$R$ mutant plants. a, c, 2 mm tassels. GA$_3$ treatment was started at this stage. b, d, 4 mm tassels. At this stage, $ra1$-$R$ mutant tassel showed a different phenotype from the $ra1$-$B73/ra1$-$R$ heterozygous tassel. Bars = 200 µm.

**Pannel B.** Table showed the results of the average branch and spikelet number after GA$_3$ treatment. Each type of 2$^{nd}$ order meristems is labeled with a specific color. Significant p values are labeled with red type.

**Pannel C.** Apical-basal distribution of all 2$^{nd}$ order meristems with or without GA$_3$ treatment. Each color represents one type of the 2$^{nd}$ order meristems which has the same representation as in B.

**Pannel D.** GA$_3$ treatment produces more single spikelets on $ra1$-$R$ mutant tassels. e-f, tassel images of $ra1$-$R$ mutant without GA$_3$ treatment. g-i, tassel images of $ra1$-$R$ mutant offer treatment by GA$_3$. f, g, images of mature tassels. e, h, i, images showed the tip areas of each tassel. h, i, tassel tips showing the single spikelets with all of the spikelet pairs been removed. e, Red arrow shows the single spikelet produced on an untreated, $ra1$-$R$ mutant.
Figure 3.7 GA3 treatment alters the 2nd order meristem development
3.4 Discussion

RA1 is a putative transcription factor with one C$_2$H$_2$ zinc finger domain and two EAR repression motifs. In this study, we detected the nuclear localization ability of RA1 protein by fusing it with a GFP tag. Most of the GFP-RA1 fusion protein entered into the cell nucleus which is an important character for transcription factors. However, in the RA1 amino acid sequence, there is no traditional nuclear localization signal (NLS). The N terminal region has no NLS in RA1 since the nine amino acid deletion at RA1 N terminal end didn’t affect protein nuclear localization (Appendix H). And there is an F exists within the Arg/Lys hexapeptide which might break the nuclear localization function of the Arg/Lys hexapeptide (Boulikas 1994; Cokol, Nair et al. 2000). Single amino acid mutations and deletions at RA1 Arg/Lys hexapeptide also didn’t affect the nuclear localization of the mutated GFP-RA1 protein (Appendix H). By detecting the nuclear localization of GFP-RA1-R mutant protein, we found that the His to Asn mutation at the QGLGGH motif destroyed the protein nuclear localization ability. As the zinc finger domains in some proteins are responsible for protein nuclear localization (Matheny, Day et al. 1994; Hatayama, Tomizawa et al. 2008; Ito, Azumano et al. 2009) and this H64 is also the first His in the C$_2$H$_2$ zinc finger, we mutated the second His (H68N) and the second Cys (C51S) in the C$_2$H$_2$ zinc finger and used these GFP-fused RA1 mutant proteins to test whether the C$_2$H$_2$ zinc finger structure is responsible for RA1 nuclear localization. Single amino acid mutations at the zinc-chelating residues had no effect on nuclear localization, so for RA1 the C$_2$H$_2$ zinc finger structure doesn’t serve as the nuclear localization signal. Since the single QALGGH zinc finger domain is capable of DNA
binding in EPF-like proteins (Takatsuji and Matsumoto 1996; Carla Isernia 2003) and for those proteins containing both NLS and DNA-binding region, 90% of them have an overlapping DNA binding region and NLS (Cokol, Nair et al. 2000), we made several other RA1 single amino acid mutations at the QGLGGH motif and tested their nuclear localization abilities. The mutation of G60A has no effect on the nuclear localization. The mutations of Q59G and G60D partially affect the protein nuclear localization while the L61E mutation greatly destroyed the protein nuclear localization. So for RA1, the nuclear localization signal overlaps with the QGLGGH motif and within it the Leu and His each play an important function in importing RA1 into cell nucleus.

RA1 functions by imposing a determinate fate on 2nd order meristems. However, according to RNA in situ hybridization results, *ra1* is expressed at a junction between each determinate 2nd order meristem and the indeterminate main axis (Vollbrecht, Springer et al. 2005), not in 2nd order meristems proper. Therefore, other proteins or hormone signals may propagate a RA1 derived signal. We tried to detect RA1 protein localization pattern in young inflorescences by making antisera against RA1 and purifying RA1 antibodies. However, this purified antibody detects recombinant protein expressed in *E. coli* but does not recognize an antigen from young inflorescence protein extracts. Similarly, no specific pattern was detected by protein immunolocalization.

KN1 was identified as one of the RA1 interacting proteins from the yeast two-hybrid screening assay. Their interaction has been confirmed by several methods and mapped to the zinc finger region of RA1 and the homeodomain of KN1.
According to published reports of kn1 RNA and KN1 protein accumulating in the inflorescences (Jackson, Veit et al. 1994; Barazesh and McSteen 2008), the expression domains overlap, suggesting KN1 and RA1 are in the same cells and potentially able to reside in the same complex. The tassel branch phenotype of ra1-RSenh; kn1-e1 double mutant plants also supported a genetic interaction between ra1 and kn1. KNOX proteins are known to interact with BEL1-like homeodomain family proteins at the MEINOX domain and form a protein complex functioning in determining KNOX subcellular localization and their target genes (Bhatt, Etchells et al. 2004; Hay and Tsiantis 2010). Here we reported that RA1 interacts with KN1 and may work as a KN1 binding partners in regulating the 2nd order meristems during maize inflorescence development.

As the GA2ox-1 gene is reported to be a direct target of KN1 in maize (Bolduc and Hake 2009), we further investigated whether or not the maize GA20 oxidase genes and GA2 oxidase genes, and therefore gibberellins, are involved in regulating 2nd order meristems. Four ZmGA20 oxidase genes and twelve ZmGA2 oxidase genes were identified in the maize genome and most of the ZmGA2 oxidase genes contain the TGAC motif which underlies the functional KNOX binding site (Bolduc and Hake 2009). Expression of only some of these genes was detected in young inflorescences. Some genes showed similar expression level in B73 and ra1-R mutants but some showed differential expression (Supplemental E). Interestingly, ZmGA20ox-1 showed a reduced expression level while three ZmGA2 oxidase genes showed increased expression levels in the young tassels of ra1-R mutant compared with wild-type B73. Since at the 2-3 mm stage, the tassels in ra1-R mutants look
similar as those in B73, the detected expression differences were not likely caused by any gross, morphological, differences in phenotype. Although it is not known whether or not these ZmGA genes are direct targets of RA1, since RA1 is a putative transcriptional repressor, we hypothesize that RA1 is involved in regulating the expression of at least some of these genes as a binding partner of KN1. For example, KN1 is purported to activate GA2ox1 expression in the vegetative SAM (Bolduc and Hake 2009), where RA1 is not expressed. In one possible working model (Figure 3.8) in SPMs of wild type B73, RA1 interacts with KN1 and the complex works as a transcriptional repressor to inhibit the expression of KN1 target genes such as ZmGA2ox-1. As a result, active gibberellins levels are increased in a small group of cells around the ra1 expression domain. In the ra1-R mutant, the mutated RA1-R protein doesn’t enter into the cell nucleus because of the H64N mutation at the QGLGGH motif. RA1-R still has the ability to interact with KN1, but not in the nucleus. Thus KN1 exerts its function without RA1, and activates the expression of its target genes such as ZmGA2ox-1 or binds with other proteins and regulates the transcription of the target genes. These gene products enter into the cytosol and convert some active gibberellins into inactive forms. In other RA1 strong mutants such as ra1-RSenh, the mutated RA1 protein may enter into the cell nucleus and bind with KN1, but the repression function is destroyed so KN1 still exerts its RA1-independent function.

The expression differences of the ZmGA20ox-1 gene and the three ZmGA2 oxidase genes are all consistent with a reduced level of bioactive gibberellins in ra1-R mutants. To test whether gibberellins are involved in regulating 2nd order
Figure 3.8  Possible working model of RA1-KN1 protein complex
meristems in inflorescence, we did exogenous gibberellic acid 3 (GA$_3$) treatment experiments. *ra1-B73/ra1-R* heterozygous plants and *ra1-R* homozygous mutants with the same genetic background and at the same growing stage were used for the treatment. Since GA is involved in regulating several developmental processes such as seed germination, shoot apical meristem activity, flowering time, reproductive organ formation (Evans and Poethig 1995; Thornsberry, Goodman et al. 2001; Bolduc and Hake 2009), in these experiment, GA$_3$ solution was sprayed into the leaf whorl of the treated plants at discrete stages to avoid influencing the development of other tissues or organs. To determine appropriate amounts of GA$_3$ per plant, we did several experiments and tested different amounts of GA$_3$ from 0.01 µg to 500 µg for each plant per treatment. 1 µg GA$_3$ for each plant per treatment turned out to be the best amount for this study since this amount had a minimal influences on the normal development of the whole plant. Exogenous gibberellins had no effect on the total number of 2$^{nd}$ order meristems but in *ra1-R* mutant tassels, GA$_3$ application partially reduced the number of mixed branches and produced a lot of single spikelets instead of the spikelet multimers or mixed branches, while the spikelet pairs were not affected (Figure 3.7D). By this shift to more determinate 2$^{nd}$ order meristem fates, the *ra1-R* mutant phenotype was partially corrected. Gibberellins have been reported to regulate several developmental processes of plant reproductive organs, such as to induce flower initiation and promote flowering in Arabidopsis and tobacco (Gallego-Giraldo, García-Martínez et al. 2007; Mutasa-Göttgens and Hedden 2009), reduce spikelet density of maize tassels (Bolduc and Hake 2009), promote male flower development in cucumber but promote female flower development in maize
and castor bean (Aya, Ueguchi-Tanaka et al. 2009), regulate anther development in Arabidopsis and pollen tube growth in rice (Chhun, Aya et al. 2007; Aya, Ueguchi-Tanaka et al. 2009). Here we report that gibberellins also function in regulating meristem determinacy in maize tassels. However, GAs are not the only mechanism to regulate this aspect of inflorescence branch architecture. For example, other proteins such as REL2 (Gallavotti, Long et al. 2010) have been reported to interact with RA1 to regulate the fate of the 2nd order meristems and auxin may also be involved in this working model (Gallavotti, Long et al. 2010). Similarly, we are now working on other candidate RA1 interacting proteins listed on Table 3.1. New protein complexes and working models will likely be identified from these experiments.
CHAPTER 4. SUMMARY AND FUTURE WORK

4.1 Summary of previous work

The previous two chapters (Chapter 2 and 3) showed our progress toward investigating the mechanism of RAMOSA1 action during maize inflorescence development. First, we tested whether the three RAMOSA proteins (RA1, RA2, RA3) interact with each other to form a protein complex. We used yeast two hybrid analysis to directly test the interactions between the three full length proteins and their truncations (Table 2.2). We detected no interaction between RA3 and RA1 or RA2 but there was a weak interaction between RA1 and RA2, especially between RA1-F and RA2-DM domain (Figure 2.4). In vitro GST pull down experiment also confirmed this interaction (Figure 2.6). But both experiments also showed that the interaction between RA1 and RA2 was weak, so we hypothesize there are other proteins involved in the RA1-RA2 protein complex to form a more complicated protein complex. We also made the triple mutant of ra1-R; ra2-R; ra3-R and compared the SEM images of the young inflorescences from the triple mutant with the double mutants (Figure 2.7). However, no new phenotypes were identified from these triple mutants, which supported the current ramosa pathway where RA2 and RA3 have parallel functions but both act upstream of RA1 (McSteen 2006).

Second, as ra1 encodes an EPF-like transcription factor, we tested its nuclear localization ability by fusing it with a GFP tag and bombarded the fusion protein into N. benthamiana leaves. Most of the GFP-RA1 proteins entered into cell nucleus which supported its character as a transcription factor (Figure 3.1). However, the mutant fusion protein GFP-RA1-R didn't enter into the cell nucleus. As RA1-R
mutants have a $C_2H_2$ zinc finger domain changed into $C_2H_1$, we first tested whether or not the $C_2H_2$ zinc finger domain was involved in localizing RA1 protein into the cell nucleus. However, single amino acid mutants of the Cys and another His in the $C_2H_2$ zinc finger domain had no effect on their nuclear localization ability. By analyzing the amino acid sequence of RA1, no traditional nuclear localization signals such as the arginine-lysine hexapeptide were identified. The nuclear localization signal (NLS) also didn’t locate at the N terminal end since GFP-RA1-RS protein still entered into the cell nucleus (Figure 3.1). Altogether, our results showed that there is an NSL in RA1 that overlaps with its DNA binding motif, the QGLGHH motif.

Third, we used yeast two hybrid analysis to screen for RA1 interacting proteins from the cDNA libraries made from B73 2 mm ears. Several proteins were identified from this experiment. All of the transcription factors and other proteins isolated several times were listed in Table 3.1. The interactions between RA1 and these candidates were confirmed by direct test in yeast two-hybrid analysis and by bait-pray swap analysis. Because RA1 is a putative transcription factor and KNOTTED1 is a well known transcription factor among these candidates, we decided to focus on the interaction between RA1 and KN1.

Fourth, we used several techniques (yeast two hybrid, GST pull-down and BiFC) to confirm the interaction between RA1 and KN1 in vitro and in vivo (Figure 3.2). In the yeast system, the interaction was mapped to the RA1 zinc finger region and the KN1 homeodomain (Figure 3.3). We also made $ra1-RSenh; kn1-e1$ double mutant plants and analyzed the tassel branch phenotype of these double mutants. Statistical analysis results supported the existence of a genetic interaction between
RA1 and KN1 during tassel development, and this interaction had a significant effect on producing more compound branches and spikelet pairs and reducing spikelet multimers (Figure 3.4).

Fifth, based on the interaction between RA1 and KN1 and the relationships among KN1, the expression of the \textit{GA2ox-1} gene and gibberellin levels during plant development (Bolduc and Hake 2009), we tested whether or not RA1 functions through this KN1-GA pathway during maize inflorescence development. We first identified \textit{GA20 oxidase} and \textit{GA2 oxidase} family genes in maize with rice genes as queries (Figure 3.5). Then we did semi-quantitative RT-PCR to analyze the expression of each gene in young inflorescences of B73 and \textit{ra1-R} mutant (Appendix D and E). Three \textit{ZmGA2ox} genes and one \textit{ZmGA20ox} gene showed different expression in young tassels of B73 compared to the \textit{ra1-R} mutant. These differences were confirmed by doing quantitative RT-PCR (Figure 3.6). As these different expression levels of the 4 GA genes suggested reduced gibberellins levels in \textit{ra1-R} mutant tassels, we finally used exogenous GA$_3$ to treat the \textit{ra1-R} mutant tassels during tassel development and analyzed the tassel branch phenotypes of the treated and non-treated plants. Statistical analysis results showed that GA$_3$ had significant effects on increasing single spikelets and reducing the spikelet multimers and mixed branches (Figure 3.7). So the exogenous GA$_3$ partially rescued the \textit{ra1-R} mutant phenotype.

We also made RA1 antiserum and produced affinity purified RA1 antibodies. However, the purified RA1 antibodies seemed to work only in the \textit{E. coli} expression system. No antigen was recognized in the protein extractions isolated from young
tassels.

4.2 Future work

Our current work focused on the interactions between RA1 and KN1 and their relationships with the GA biosynthetic genes in regulating inflorescence branching phenotype. However, KNOX proteins have other target genes such as IPT genes which may also involved in the RA1-KN1 working model. And other plant hormones such as cytokinin and auxin may also play functions during inflorescence development. We also identified other candidate proteins listed in Table 3.1 that showed interactions with RA1, so RA1 may play its function through other proteins in common or distinct protein complexes.

4.2.1 Other target genes for a protein complex that contains RA1 and KN1

Plant adenosine phosphate IPT (isopentenyltransferase) genes were reported to be a direct target of KNOX proteins in Arabidopsis, rice and other species. KNOX proteins activate the transcription of IPT genes to increase de novo cytokinin level since IPTs catalyze the rate-limiting step during cytokinin biosynthesis (Yanai, Shani et al. 2005; Sakamoto, Sakakibara et al. 2006).

To identify IPT family genes in maize, we used the reported IPT family genes in rice (Sakamoto, Sakakibara et al. 2006) as queries to identify corresponding maize family genes by using the same method described above (Chapter 3). Totally 12 ZmiIPT genes were identified and their phylogenetic relationships with the rice family
Figure 4.1 Phylogenetic analysis of IPT genes in rice and maize
Table 4.1 Expression differences of ZmIPT genes in B73 and ra1-R

<table>
<thead>
<tr>
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<tr>
<td>UDZmIPT1</td>
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</tr>
<tr>
<td>UDZmIPT2</td>
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<tr>
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</tr>
<tr>
<td>UDZmIPT12</td>
<td>Expression was not detected</td>
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</table>
genes were analyzed by the same methods described above (Chapter 3) (Figure 4.1).

Then we detected the transcription level of each \textit{ZmIPT} gene in 4-6 mm tassels of B73 and \textit{ra1-R} mutant. The primers used in this test were listed in Appendix A. Semi-quantitative RT-PCR results showed that the \textit{ZmIPT9} gene had different expression level in B73 and \textit{ra1-R} mutant (Table 4.1) so the \textit{IPT} genes and cytokinin might also be involved in the RA1-KN1 working model.

\subsection*{4.2.2 Other proteins interact with RA1}

\textit{rel2} (\textit{ramosa1 enhancer locus2}) gene was identified from a genetic screen for the enhancement of maize inflorescence branching architecture and \textit{rel2} mutants significantly increase the branching phenotype of \textit{ra1-RS} weak mutant (Gallavotti, Long et al. 2010). REL2 acts as a transcriptional co-repressor and it interacts with RA1 to form a protein complex. We made several RA1 amino acid mutations at the two EAR repression motifs and detected their interaction with the full length REL2 and the REL2 N terminal region (REL<WD40). The results showed that the two EAR motifs physically interact with REL2 especially its N terminal region (Figure 4.2).

Maize \textit{rel2} is a homolog of \textit{TPL} (TOPLESS) in Arabidopsis which interacts with the AUX/IAA-ARF protein complex to regulate the transcription of \textit{ARF} target genes (Szemenyei, Hannon et al. 2008). AUX/IAA proteins have the EAR repression motif which is similar with RA1 and we also identified two paralogous \textit{ARF8} proteins from the yeast two-hybrid screening experiment (Table 3.1). These results suggested a possible function model of REL2-RA1-ARF8 protein complex. We have ordered the
**Figure 4.2 Identifying the region of RA1 that interacts with REL2**

**Panel A.** Gene structures of *ra1* and *rel2* related constructs tested in yeast two-hybrid analysis. The mutated amino acids were labeled in red, bold.

**Panel B.** Yeast two-hybrid analysis of interactions between RA1 and REL2. The −Adenine selective media were used to test the interactions between each set of paired proteins.
Figure 4.2 Identifying the region of RA1 that interacts with REL2
mutant seeds of the two paralogous ARF8 proteins and other members of the Vollbrecht lab will continue investigating the RA1-ARF8 protein complex.
### Appendix A. Oligonucleotides used in these studies.

<table>
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<td>To genotype ra2-R homozygous alleles</td>
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<tr>
<td>ra2-R-B</td>
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</tr>
<tr>
<td>RA8</td>
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<td>To genotype ra1-R and ra1-RSenh homozygous alleles</td>
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<td>kn1-e1-F3</td>
<td>5’ AGAGCTCGATCGCTCTTC 3’</td>
<td>To genotype kn1-e1 homozygous alleles.</td>
</tr>
<tr>
<td>kn1-B</td>
<td>5’ CTCTTGCGCTATCTCCGTAG 3’</td>
<td></td>
</tr>
<tr>
<td>EUO315</td>
<td>5’ GTGGAGCTGTGAGCAAGGGGCAGG 3’</td>
<td>To genotype YFP-RA1 allele.</td>
</tr>
<tr>
<td>EUO316</td>
<td>5’ GTCGCGAGCTGCACGTCGC 3’</td>
<td></td>
</tr>
<tr>
<td>Bar-F</td>
<td>5’ CAACTTCCGTACCGAGCCGC 3’</td>
<td>To genotype YFP-RA1 allele.</td>
</tr>
<tr>
<td>Bar-R</td>
<td>5’ GCTGCCAGAAACCACGTCA 3’</td>
<td></td>
</tr>
<tr>
<td><strong>Yeast two-hybrid analysis</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RA1-Eco-F</td>
<td>5’ GATTGGAATTCATGGAGGGAGAAGAT GACGG 3’</td>
<td>Cloning of ra1 full length cDNA into pAD-Gal4 and pBD-Gal4 vectors.</td>
</tr>
<tr>
<td>RA1-Sal-B</td>
<td>5’ ATCGGTCGACTTACGTGCACGTACCC ATTTGTAG 3’</td>
<td></td>
</tr>
<tr>
<td>RA1F-Sal</td>
<td>5’ TATAGTCGACACGCTTTCCCGGCA TC 3’</td>
<td>To create ra1-F fragment.</td>
</tr>
<tr>
<td>RA1-P3</td>
<td>5’ ATAGTCGACTCAGTAGCCAGCTCT AAGCTGAAAATCCAGACG 3’</td>
<td>To create ra1-P3 fragment.</td>
</tr>
<tr>
<td>RA1-P7</td>
<td>5’ CGAAATCTGAAACTCAAAGTCAAGAC 3’</td>
<td>To create ra1-P78 fragment.</td>
</tr>
<tr>
<td>RA1-P8</td>
<td>5’ GACTTTTAGTTTCAAGATTTTCTGTCGCTG 3’</td>
<td></td>
</tr>
<tr>
<td>RA1-P9</td>
<td>5’ CGTGTCGACCTATGAGGTGCAACTAGG ATTAGG 3’</td>
<td>To create ra1-P9 fragment.</td>
</tr>
<tr>
<td>RA1-ZB</td>
<td>5’ ATAGTCGACCTAGTCCAGCCTTGGA TGTT 3’</td>
<td>To create ra1-NZ fragment.</td>
</tr>
<tr>
<td><strong>RA1-NB</strong></td>
<td>5' ATTGTCGACTCATGTTACAGCA GACGACG AC G 3'</td>
<td>To create <em>ra1-N1</em> fragment.</td>
</tr>
<tr>
<td><strong>RA1-ZF</strong></td>
<td>5' CGTGAATTCTACACCTGCGGGTATTGC 3'</td>
<td>To create <em>ra1-Zinc</em> fragment.</td>
</tr>
<tr>
<td><strong>RA1-ZB</strong></td>
<td>5' ATAGTCGACCTAGTCCAGCCTGGA TAG TTT 3'</td>
<td></td>
</tr>
<tr>
<td><strong>KN-MZX-F</strong></td>
<td>5' ATAGAATCCCCTACGCGACGACGT 3'</td>
<td>To create <em>kn1-Midd</em> fragment.</td>
</tr>
<tr>
<td><strong>KN-HB-Sal</strong></td>
<td>5' ATTGTCGACCTGGATGCGTCCGATG 3'</td>
<td></td>
</tr>
<tr>
<td><strong>KN-Ub-F</strong></td>
<td>5' AATGAATTCAGGTCGTGCCGAACATC 3'</td>
<td>To create <em>kn1-CE</em> fragment.</td>
</tr>
<tr>
<td><strong>KN-Sal-B</strong></td>
<td>5' ATTGTCGACCTAGCCGAGCCGGTGACGAG 3'</td>
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</tr>
<tr>
<td><strong>KN-HF-Eco</strong></td>
<td>5' GCCGAAATTCCTCAAGCAAGAAGACTGCTCA AAG 3'</td>
<td>Paired with <strong>KN-HB-Sal</strong> to create <em>kn1-Homeo</em> fragment.</td>
</tr>
<tr>
<td><strong>KN-MZX-B</strong></td>
<td>5' ATTGTCGACCGAAAAGGGAAGGTTGACGT GCCG 3'</td>
<td>Paired with <strong>KN-MZX-F</strong> to create <em>kn1-MEX</em> fragment.</td>
</tr>
<tr>
<td><strong>KN-Eco-F</strong></td>
<td>5' GCCGAAATTCATGAGAGAGATACCGACA AC 3'</td>
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</tr>
<tr>
<td><strong>KN-NB-Sal</strong></td>
<td>5' ATTGTCGACCTTAGTTAGGAGACGACG AC G 3'</td>
<td></td>
</tr>
<tr>
<td><strong>RA2-Eco-F</strong></td>
<td>5' CTATGGAATTCATGCTGCCCGTCG AGC 3'</td>
<td>Cloning of <em>ra2</em> full length cDNA into pAD-Gal4 vector.</td>
</tr>
<tr>
<td><strong>RA2-Xho-B</strong></td>
<td>5' TATCCTCGAGTTACATGCTGCTGTC TCTCCCTT 3'</td>
<td></td>
</tr>
<tr>
<td><strong>RA2-Sma</strong></td>
<td>5' TATACCCGGGTATACGTGGGTCGTTGCTTCCCTT 3'</td>
<td>Paired with <strong>RA2-Eco-F</strong> or DM- Eco-F to clone <em>ra2</em> full length or <em>ra2-dm</em> into pBD-Gal4 vector.</td>
</tr>
<tr>
<td><strong>DM-Eco-F</strong></td>
<td>5' CTAAAGATTCATGATAGCGGCATCT CATGGCTTCCCTT 3'</td>
<td>Paired with <strong>RA2-Xho-B</strong> or <strong>RA2-Sma</strong> for making pAD-RA2-DM or pBD-RA2-DM constructs.</td>
</tr>
<tr>
<td><strong>RA3-Eco-F</strong></td>
<td>5' CCTGAATTCAGGCTCGCAGAGATCA CCAACC 3'</td>
<td>Cloning of <em>ra3</em> full length cDNA into pAD-Gal4 and pBD-Gal4 vectors.</td>
</tr>
<tr>
<td><strong>RA3-Sal-B</strong></td>
<td>5' ATCGAGGACTCATGCAGAGGCTGTC TATCA 3'</td>
<td></td>
</tr>
</tbody>
</table>

**Fusion proteins for GST pull-down analysis**

<p>| <strong>RA81Zm</strong> | 5' ATGGAGGAGAAGATGACGGC 3' | Cloning of <em>ra1</em> full length cDNA into pCR-T7/NT-TOPO vector. |
| <strong>RA11</strong> | 5' TGCAGCTCAGCTACCGATTAG 3' | |
| <strong>RA1F-B</strong> | 5' CTAAGCGCTTCCCGCCGATC 3' | Paired with <strong>RA81Zm</strong> for making 6×His-RA1F construct. |</p>
<table>
<thead>
<tr>
<th>DNA Sequence</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>RA1-Bam-F 5' ATAGGATCCGAGGAGGAGATGACG GC 3'</td>
<td>Paired with RA1-Sal-B for making GST-RA1 construct</td>
</tr>
<tr>
<td>RA1-Bam-B 5' ATAGGATCCCAAGTAGTAGCCAGTCTA AGC 3'</td>
<td>Paired with RA1-Eco for making pBJ-RA1 related constructs</td>
</tr>
<tr>
<td>KN-Hind-B 5' TAAAAGGTTATGCCGAGCCGTACAG CCC 3'</td>
<td>Paired with KN1-Eco for making pBJ-KN1 related constructs</td>
</tr>
<tr>
<td>RA1-Sal-F 5' CACGCGTCTGACATGGAGGAGAGATGACG GAC 3'</td>
<td>Cloning of full length ra1 and ra1-R cDNA into pZY212 vector.</td>
</tr>
<tr>
<td>RA1-Xba-B 5' GCTCTAGAGCATACAGAATAAATGACATCC 3'</td>
<td>Paired with RA1-Xba-B to produce GFP-RA1-RS construct.</td>
</tr>
<tr>
<td>RS-Sal-F 5' ATCGTGACATGAAACTGCAGCAAC AAC 3'</td>
<td>Paired with RA1-Xba-B to produce GFP-RA1-RS construct.</td>
</tr>
<tr>
<td>RA-P19 5' ATGTGCCCTTCCAGCCCTTGCTGAT 3'</td>
<td>To create ra1-P1920 fragment.</td>
</tr>
<tr>
<td>RA-P20 5' GGGCTGGAAGGCCACATGAACATCC 3'</td>
<td>To create ra1-P2223 fragment.</td>
</tr>
<tr>
<td>RA-P22 5' GTTCATGTTGCCTCCAGCCCTT 3'</td>
<td>To create ra1-P2223 fragment.</td>
</tr>
<tr>
<td>RA-P23 5' GGGAGGGAACATGAACATCCACA 3'</td>
<td>To create ra1-P2425 fragment.</td>
</tr>
<tr>
<td>RA-P24 5' TTGCAGCTTCCAGCCCTTGCTGAT 3'</td>
<td>To create ra1-P2425 fragment.</td>
</tr>
<tr>
<td>RA-P25 5' GGGAGGCACCATGAACATCCACAG 3'</td>
<td>To create ra1-P2425 fragment.</td>
</tr>
<tr>
<td>RA-P26 5' CAGCCTGTTGATGTTCAATGACATCCACAG 3'</td>
<td>To create ra1-P1516 fragment.</td>
</tr>
<tr>
<td>RA-P27 5' ATGAAACATCAACAGGCTGGACAG 3'</td>
<td>To create ra1-P1718 fragment.</td>
</tr>
<tr>
<td>RA-P28 5' GGCTGGAAGGCCACATGAACATCCACA 3'</td>
<td>To create ra1-P1718 fragment.</td>
</tr>
<tr>
<td>RA-P29 5' TGGCCATGTTGATGTTCAATGACATCCACAG 3'</td>
<td>To create ra1-P1718 fragment.</td>
</tr>
<tr>
<td>RA-P30 5' CAGCCTGTTGATGTTCAATGACATCCACAG 3'</td>
<td>To create ra1-P1718 fragment.</td>
</tr>
<tr>
<td>RA-P31 5' GCACAAGACCTGGGAGGCCACAT 3'</td>
<td>To create ra1-P3031 fragment.</td>
</tr>
<tr>
<td>Sequence Name</td>
<td>Reverse Complement Sequence</td>
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<tr>
<td>---------------</td>
<td>-----------------------------</td>
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<tr>
<td>RA-P32</td>
<td>5' CCTCCCAGCGCTTGTGCTGATCTGA 3'</td>
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<tr>
<td>RA-P33</td>
<td>5' GCACAAGCGCTGGGAGGCACAT 3'</td>
</tr>
<tr>
<td>RA-P34</td>
<td>5' GCCCTCCCTCCCTTTGTGCTGATCTG 3'</td>
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<tr>
<td>RA-P35</td>
<td>5' ACAAGGGAGGGAGGCCACATGAAC 3'</td>
</tr>
<tr>
<td>RA-P28</td>
<td>5' CTGAACCTCCTGGAATACCCGCAG 3'</td>
</tr>
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<td>RA-P29</td>
<td>5' TATTGCAAGAGGAGGATGCAGAC 3'</td>
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<tr>
<td>RA-P10</td>
<td>5' TCTGCGCTCGTCCAGCCCT 3'</td>
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<tr>
<td>RA1-P11</td>
<td>5' GGCTGGACGAGGCCAGAC 3'</td>
</tr>
<tr>
<td>RA1-P12</td>
<td>5' GAACCTCCTCCTGCAATACCC 3'</td>
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<tr>
<td>RA1-P13</td>
<td>5' GTATTGCAAGGAGGATGCAGAC 3'</td>
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**RT-PCR analysis**

<table>
<thead>
<tr>
<th>ZmGA2ox1-F</th>
<th>5' CAGTTTCAGGATCGTGTC 3'</th>
<th>For testing the transcription of ZmGA2ox-1 gene</th>
</tr>
</thead>
<tbody>
<tr>
<td>ZmGA2ox1-R</td>
<td>5' ACTCTCTGCTGCAGTTC 3'</td>
<td></td>
</tr>
<tr>
<td>ZmGA2ox2-F</td>
<td>5' GCGGTACAGGACCTTCA 3'</td>
<td>For testing the transcription of ZmGA2ox-2 gene</td>
</tr>
<tr>
<td>ZmGA2ox2-R</td>
<td>5' TCGCTACCTAGACCTCG 3'</td>
<td></td>
</tr>
<tr>
<td>ZmGA2ox3-F</td>
<td>5' TCGGGAGTGCGGGTGTC 3'</td>
<td>For testing the transcription of ZmGA2ox-3 gene</td>
</tr>
<tr>
<td>ZmGA2ox3-R</td>
<td>5' GTGGTGCTGACGCGCTG 3'</td>
<td></td>
</tr>
<tr>
<td>ZmGA2ox4-F</td>
<td>5' AGCGCCTGCCCAGACTGTG 3'</td>
<td>For testing the transcription of ZmGA2ox-4 gene</td>
</tr>
<tr>
<td>ZmGA2ox4-B</td>
<td>5' CTCTGCTTCGGCGTCCC 3'</td>
<td></td>
</tr>
<tr>
<td>ZmGA2ox5-F</td>
<td>5' GGACCTCCTCCTCGCTAT 3'</td>
<td>For testing the transcription of ZmGA2ox-5 gene</td>
</tr>
<tr>
<td>ZmGA2ox5-R</td>
<td>5' CCATTTAGACTCTGGGCTC 3'</td>
<td></td>
</tr>
<tr>
<td>ZmGA2ox6-F</td>
<td>5' GCCTACTTCCTGAGGCCG 3'</td>
<td>For testing the transcription of ZmGA2ox-6 gene</td>
</tr>
<tr>
<td>ZmGA2ox6-B</td>
<td>5' CAGTGAGGTCGTGCTCGC 3'</td>
<td></td>
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<tr>
<td>------------</td>
<td>----------------------------</td>
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</tr>
<tr>
<td>ZmGA2ox7-F</td>
<td>5' GCTAGAGGCTACGTCTTTGC 3'</td>
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</tr>
<tr>
<td>ZmGA2ox7-R</td>
<td>5' CATTGGTGGCTGTGCGTAATAC 3'</td>
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</tr>
<tr>
<td>ZmGA2ox8-F</td>
<td>5' GGTAGCAGTGCCAAGGC 3'</td>
<td></td>
</tr>
<tr>
<td>ZmGA2ox8-R</td>
<td>5' GCAGCCAGAACAGCAGG 3'</td>
<td></td>
</tr>
<tr>
<td>ZmGA2ox9-F</td>
<td>5' TCGCCTACTTTTCTCTGC 3'</td>
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<tr>
<td>ZmGA2ox9-R</td>
<td>5' ACACGACTCTCTCTCTC 3'</td>
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<tr>
<td>ZmGA2ox10-F</td>
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<td>ZmGA2ox10-R</td>
<td>5' GATGTAGCTACTGCAGGATG 3'</td>
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<tr>
<td>ZmGA2ox11-F</td>
<td>5' CAAGTCCAGGGTTTTCCTC 3'</td>
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<tr>
<td>ZmGA2ox11-R</td>
<td>5' CGTCCCTGAAGCGTCACG 3'</td>
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<tr>
<td>ZmGA2ox12-F</td>
<td>5' CAGCTCAGGGAGACGAGC 3'</td>
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</tr>
<tr>
<td>ZmGA2ox12-R</td>
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</tr>
<tr>
<td>ZmGA20ox1-F</td>
<td>5' TGGTGGACGACGCCACC 3'</td>
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</tr>
<tr>
<td>ZmGA20ox1-R</td>
<td>5' TGGAGGAGGGAGTCAGGG 3'</td>
<td></td>
</tr>
<tr>
<td>ZmGA20ox2-F</td>
<td>5' CGGCGCTACCGGGACTTC 3'</td>
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</tr>
<tr>
<td>ZmGA20ox2-R</td>
<td>5' GTAGCACAGCAGTAGGGC 3'</td>
<td></td>
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<tr>
<td>ZmGA20ox3-F</td>
<td>5' CCAGGAGGACAAGCGAGC 3'</td>
<td></td>
</tr>
<tr>
<td>ZmGA20ox3-R</td>
<td>5' TTGTGGACGACGCCACC 3'</td>
<td></td>
</tr>
<tr>
<td>ZmGA20ox4-F</td>
<td>5' CTGTCCAACGGCCGGGTAC 3'</td>
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</tr>
<tr>
<td>ZmGA20ox4-R</td>
<td>5' AGACCCGCGCTCGCTTAGG 3'</td>
<td></td>
</tr>
<tr>
<td>ZmlPT1-F</td>
<td>5' GCCTCAGCACGCTCGACG 3'</td>
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</table>

For testing the transcription of ZmGA2ox-7 gene
For testing the transcription of ZmGA2ox-8 gene
For testing the transcription of ZmGA2ox-9 gene
For testing the transcription of ZmGA2ox-10 gene
For testing the transcription of ZmGA2ox-11 gene
For testing the transcription of ZmGA2ox-12 gene
For testing the transcription of ZmGA20ox-1 gene
For testing the transcription of ZmGA20ox-2 gene
For testing the transcription of ZmGA20ox-3 gene
For testing the transcription of ZmGA20ox-4 gene
For testing the transcription of ZmlPT-1 gene
<table>
<thead>
<tr>
<th>ZmIPT1-B</th>
<th>5' CTCTCCCATGGACGCAGCTAC 3'</th>
<th>For testing the transcription of ZmIPT-2 gene</th>
</tr>
</thead>
<tbody>
<tr>
<td>ZmIPT2-F</td>
<td>5' CAGGCATGCGGAGCGCAAG 3'</td>
<td>For testing the transcription of ZmIPT-2 gene</td>
</tr>
<tr>
<td>ZmIPT2-B</td>
<td>5' CGTCGTCCGTATCCTGCAGC 3'</td>
<td>For testing the transcription of ZmIPT-2 gene</td>
</tr>
<tr>
<td>ZmIPT3-F</td>
<td>5' GATGATCAGCTGCAGTGTG 3'</td>
<td>For testing the transcription of ZmIPT-3 gene</td>
</tr>
<tr>
<td>ZmIPT3-B</td>
<td>5' ATCAACAACCTTATTCCCTAC 3'</td>
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</tr>
<tr>
<td>ZmIPT4-F</td>
<td>5' GAGGCCGTACAGAGTGCTGC 3'</td>
<td>For testing the transcription of ZmIPT-4 gene</td>
</tr>
<tr>
<td>ZmIPT4-B</td>
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<td>For testing the transcription of ZmIPT-4 gene</td>
</tr>
<tr>
<td>ZmIPT5-F</td>
<td>5' ATCAAGGTCAACACGTCGCG 3'</td>
<td>For testing the transcription of ZmIPT-5 gene</td>
</tr>
<tr>
<td>ZmIPT5-B</td>
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<td>For testing the transcription of ZmIPT-5 gene</td>
</tr>
<tr>
<td>ZmIPT6-F</td>
<td>5' AGGCGGACGAGGCTTGCTTG 3'</td>
<td>For testing the transcription of ZmIPT-6 gene</td>
</tr>
<tr>
<td>ZmIPT6-B</td>
<td>5' CAGCGGCCACCTTCTCTTG 3'</td>
<td>For testing the transcription of ZmIPT-6 gene</td>
</tr>
<tr>
<td>ZmIPT7-F</td>
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<td>For testing the transcription of ZmIPT-7 gene</td>
</tr>
<tr>
<td>ZmIPT7-B</td>
<td>5' CTTATCCTCGCCCTCTACAT 3'</td>
<td>For testing the transcription of ZmIPT-7 gene</td>
</tr>
<tr>
<td>ZmIPT8-F</td>
<td>5' AGCGTCCCGGTGCTCTCT 3'</td>
<td>For testing the transcription of ZmIPT-8 gene</td>
</tr>
<tr>
<td>ZmIPT8-B</td>
<td>5' CAGGCTAGGAGGCTGCTGCT 3'</td>
<td>For testing the transcription of ZmIPT-8 gene</td>
</tr>
<tr>
<td>ZmIPT9-F</td>
<td>5' TCGATTGCATGAGGATGG 3'</td>
<td>For testing the transcription of ZmIPT-9 gene</td>
</tr>
<tr>
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<td>5' AGTGCTTTGACGACCTG 3'</td>
<td>For testing the transcription of ZmIPT-9 gene</td>
</tr>
<tr>
<td>ZmIPT10-F</td>
<td>5' TGCCTGCTCAAGAAGCCGT 3'</td>
<td>For testing the transcription of ZmIPT-10 gene</td>
</tr>
<tr>
<td>ZmIPT10-B</td>
<td>5' GTCTAATTGGCTCAACACC 3'</td>
<td>For testing the transcription of ZmIPT-10 gene</td>
</tr>
<tr>
<td>ZmIPT11-F</td>
<td>5' GCTTGGTGACTGCTGATG 3'</td>
<td>For testing the transcription of ZmIPT-11 gene</td>
</tr>
<tr>
<td>ZmIPT11-B</td>
<td>5' TCCCTGCTCCCTAATC 3'</td>
<td>For testing the transcription of ZmIPT-11 gene</td>
</tr>
<tr>
<td>ZmIPT12-F</td>
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</tr>
<tr>
<td>Primer</td>
<td>Sequence</td>
<td>Function</td>
</tr>
<tr>
<td>----------------</td>
<td>-------------------------------</td>
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</tr>
<tr>
<td>ZmIPT12-B</td>
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<tr>
<td>Actin-F</td>
<td>5' GMARCAACTGGGAYGACATGGA GAA 3'</td>
<td>For testing the transcription of auxin gene</td>
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<tr>
<td>Actin-R</td>
<td>5' ACRTRCACTTTCAATGAGTTGT ABGT 3'</td>
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<tr>
<td>QRT-PCR analysis</td>
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</tr>
<tr>
<td>GA2ox1-FQ</td>
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<td>For testing the transcription of ZmGA2ox-1 gene</td>
</tr>
<tr>
<td>GA2ox1-RQ</td>
<td>5' CAGGCAATAGTACATCAGTCCA 3'</td>
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<tr>
<td>GA2ox7-FQ</td>
<td>5' GCTAGAGGCTACGTACATTGCC 3'</td>
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</tr>
<tr>
<td>GA2ox7-RQ</td>
<td>5' CATTGGTGCGTGCTGTTCAATAC 3'</td>
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<tr>
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<td>For testing the transcription of ZmGA2ox-12 gene</td>
</tr>
<tr>
<td>GA2ox12-RQ</td>
<td>5' AGGATATATGCAGGACACAGCAG 3'</td>
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<td>GA20ox1-FQ</td>
<td>5' TGGTGGACGACGCAACC 3'</td>
<td>For testing the transcription of ZmGA20ox-1 gene</td>
</tr>
<tr>
<td>GA20ox1-RQ</td>
<td>5' TGGAGGAGGGAGTCAGGG 3'</td>
<td></td>
</tr>
<tr>
<td>Kn1-FQ</td>
<td>5' ACCGAGCTCCTGAAGTTGATG 3'</td>
<td>For testing the transcription of kn1 gene</td>
</tr>
<tr>
<td>Kn1-RQ</td>
<td>5' TAGGCCGTGGGATGAAATG 3'</td>
<td></td>
</tr>
<tr>
<td>RA1-FQ</td>
<td>5' GGTAATTGCTAGGTTAGGCACACG 3'</td>
<td>For testing the transcription of ra1 gene</td>
</tr>
<tr>
<td>RA1-RQ</td>
<td>5' GCCCTTGTGCTGATCTGAATCCTT 3'</td>
<td></td>
</tr>
<tr>
<td>Ubi-FQ</td>
<td>5' TAAGCTGCCGATGGCTGCCTGC 3'</td>
<td>For testing the transcription of ubiquitin gene</td>
</tr>
<tr>
<td>Ubi-RQ</td>
<td>5' CTGAAGACAGAACACATAATGACACA 3'</td>
<td></td>
</tr>
</tbody>
</table>
Appendix B. The average number of each 2<sup>nd</sup> order meristems in single and double mutant tassels

<table>
<thead>
<tr>
<th>2&lt;sup&gt;nd&lt;/sup&gt; order meristem</th>
<th>Mo17</th>
<th>kn1-e1 single</th>
<th>ra1-RSenh single</th>
<th>ra1-RSenh; kn1-e1 double</th>
</tr>
</thead>
<tbody>
<tr>
<td>Compound branches</td>
<td>1.7</td>
<td>1</td>
<td>5.2</td>
<td>7.9</td>
</tr>
<tr>
<td>Main branches</td>
<td>3.2</td>
<td>4.8</td>
<td>3.4</td>
<td>3.5</td>
</tr>
<tr>
<td>Mixed branches</td>
<td>1.9</td>
<td>1.5</td>
<td>12.7</td>
<td>9.1</td>
</tr>
<tr>
<td>Spikelet multimers</td>
<td>2.1</td>
<td>1.1</td>
<td>21.3</td>
<td>7.5</td>
</tr>
<tr>
<td>Spikelet pairs</td>
<td>115.4</td>
<td>41.4</td>
<td>59.6</td>
<td>18</td>
</tr>
<tr>
<td>Single spikelets</td>
<td>1.2</td>
<td>0</td>
<td>1.2</td>
<td>0</td>
</tr>
<tr>
<td>Total 2&lt;sup&gt;nd&lt;/sup&gt; order meristems</td>
<td>125.6</td>
<td>49.7</td>
<td>103.4</td>
<td>46</td>
</tr>
<tr>
<td>Tassel length</td>
<td>39.2</td>
<td>27.3</td>
<td>33.4</td>
<td>28.6</td>
</tr>
</tbody>
</table>
Appendix C. The function of \textit{ra1-RSenh}, \textit{kn1-e1} and \textit{ra1-RSenh\cdot kn1-e1} in different types of 2\textsuperscript{nd} order meristems

<table>
<thead>
<tr>
<th></th>
<th>\textit{ra1-RSenh}</th>
<th>\textit{kn1-e1}</th>
<th>\textit{ra1-RSenh\cdot kn1-e1}</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Coefficient (a)</td>
<td>P value</td>
<td>Coefficient (b)</td>
</tr>
<tr>
<td>Compound branches</td>
<td>3.5</td>
<td>3.3e-08***</td>
<td>-0.8</td>
</tr>
<tr>
<td>Main branches</td>
<td>0.2</td>
<td>0.7</td>
<td>1.6</td>
</tr>
<tr>
<td>Mixed branches</td>
<td>10.7</td>
<td>3.3e-09***</td>
<td>-0.5</td>
</tr>
<tr>
<td>Spikelet multimers</td>
<td>19.2</td>
<td>6.7e-11***</td>
<td>-1</td>
</tr>
<tr>
<td>Spikelet pairs</td>
<td>-55.9</td>
<td>7.7e-13***</td>
<td>-74</td>
</tr>
<tr>
<td>Single spikelets</td>
<td>0.008</td>
<td>1</td>
<td>-1.2</td>
</tr>
</tbody>
</table>
Appendix D. RT-PCR results of *ZmGA20oxs* and *ZmGA2oxs* in 4-6 mm tassel
Appendix E. RT-PCR results of \textit{ZmGA20oxs} and \textit{ZmGA2oxs} in 4-6 mm ear
Appendix F. Summary of expression differences among B73 and ra1-R mutants

<table>
<thead>
<tr>
<th>Gene</th>
<th>4-6 mm tassel</th>
<th>4-6 mm ear</th>
<th>Gene</th>
<th>4-6 mm tassel</th>
<th>4-6 mm ear</th>
</tr>
</thead>
<tbody>
<tr>
<td>ZmGA20ox-1</td>
<td>different</td>
<td>same</td>
<td>ZmGA20ox-5</td>
<td>none</td>
<td>none</td>
</tr>
<tr>
<td>ZmGA20ox-2</td>
<td>same</td>
<td>same</td>
<td>ZmGA20ox-6</td>
<td>same</td>
<td>different</td>
</tr>
<tr>
<td>ZmGA20ox-3</td>
<td>none</td>
<td>none</td>
<td>ZmGA20ox-7</td>
<td>different</td>
<td>same</td>
</tr>
<tr>
<td>ZmGA20ox-4</td>
<td>none</td>
<td>same</td>
<td>ZmGA20ox-8</td>
<td>same</td>
<td>different</td>
</tr>
<tr>
<td>ZmGA20ox-1</td>
<td>different</td>
<td>different</td>
<td>ZmGA20ox-9</td>
<td>different</td>
<td>different</td>
</tr>
<tr>
<td>ZmGA20ox-2</td>
<td>none</td>
<td>none</td>
<td>ZmGA20ox-10</td>
<td>none</td>
<td>none</td>
</tr>
<tr>
<td>ZmGA20ox-3</td>
<td>none</td>
<td>none</td>
<td>ZmGA20ox-11</td>
<td>same</td>
<td>same</td>
</tr>
<tr>
<td>ZmGA20ox-4</td>
<td>none</td>
<td>none</td>
<td>ZmGA20ox-12</td>
<td>different</td>
<td>different</td>
</tr>
</tbody>
</table>
Appendix G. RT-PCR results of \textit{ZmGA20ox} and \textit{ZmGA2oxs} in 4-6 mm tassel
Appendix H Subcellular localization of RA1 mutated proteins in planta

(A) Constructs used in these studies. Mutated amino acids are labeled in red and bold font above construct schematics. (B) Confocal images of the GFP fusion constructs transiently expressed in *N. benthamiana* leaves. Images were taken the same way as Figure 3.1.
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Jackson, D., B. Veit, et al. (1994). Expression of maize KNOTTED1 related homeobox genes in the shoot apical meristem predicts patterns of morphogenesis in the vegetative shoot. 120: 405-413.


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