Cloning and Characterization of *DWARF1* Gene and Study of Gibberellins Signaling in Maize

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Abstract of thesis entitled:

Cloning and characterization of *DWARF1* gene and study of gibberellins signaling in maize

Submitted by **Chen Yi**

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Gibberellins (GA) have multiple biological functions including promoting stem elongation, seed germination and flower development. The GA deficient *dwarf1* (*d1*) mutant in maize displays plant dwarfism and andromonoecy, *i.e.* forming anthers in the female flower. However, the molecular basis is not clear. Through molecular characterization of multiple *d1* alleles, I prove that the *d1* is caused by mutations in the GA 3-oxidase (ZmGA3ox2) that converts the inactive GA intermediates to bioactive GAs. The recombinant D1 protein catalyzes at least four reactions *in vitro*, converting GA_{20} to GA_3, GA_5 to GA_3, GA_{20} to GA_1 and GA_9 to GA_4. Subcellular localization analysis by two independent approaches which are *in vivo* D1-GFP analysis and western blot analysis of organelle fractions revealed that the D1 protein is dual-localized in the nucleus and the cytosol. ZmGA20ox was also localized in both the cytosol and the nucleus by the *in vivo* GFP fusion analysis. Interestingly, the dual-localization of D1 and ZmGA20ox coincides with the localization of the GA receptor GID1. In early phase of maize female flower development, the D1 protein was found specifically and highly expressed in the stamen primordia within the female florets. These results indicate that bioactive GAs can be synthesized in both the cytosol and the nucleus, and that the suppression of stamen in female florets is mediated by locally synthesized GAs. This finding provides new insights to the understanding of GA biosynthesis and signal transduction in plants.

DELLA proteins are repressors of GA signal transduction. DWARF8 (D8) is a DELLA protein in maize, Mutations in the N-terminal of D8 resulted in dominant GA insensitive *dwarf8* (*d8*)

phenotype. *d8* displayed similar phenotypes as the *d1*mutants; i.e. plant dwarfism, dark green leaves and andromonoecy, indicating that D8 is a master repressor mediating these GA functions. DELLA proteins suppressed the downstream signal transduction of GA by restricting their interacting protein functions through protein-protein interaction. Diverse GA responses require numerous DELLA interacting proteins. Based on the unique function of GA in regulating sex determination in maize, I hypothesize that D8 mediates the GA responses by interacting with yet unknown proteins in maize. Through yeast two hybrid screening of the maize ear cDNA library, I identified 14 proteins that showed genuine interaction in yeast system. Among these, a SPX domain containing protein named as ZmSPX1 was present. SPX domain containing proteins in yeast are implicated in cell cycle regulation; however, their functions in plants are unknown. GFP fusion analysis indicated that ZmSPX1 co-localizes with D8 in the nucleus and their interaction was confirmed by bimolecular fluorescence complementation (BiFC) and *in vitro* pull-down assay. To this point, I have identified several candidates for D8 interacting proteins and provided strong evidence that ZmSPX1 is a bona fide D8 interacting protein which set a foundation for further analysis of its function in mediating GA responses including sex determination, cell division and elongation.

克隆和鑒定玉米 DWARF1 基因和赤黴素生物信號通路的研究

摘要

赤黴素有多種生物學功能，包括促進莖的伸長、種子萌發以及花的發育。玉米赤黴素缺陷型突變體*dwarf1*（*d1*）表現出植株矮壯和雌雄兩性花，即原為雌花部位發育出雙性花。但是該突變的分子基礎尚不清楚。通過分析多個*d1*等位基因突變體的分子組成特征，我們證明d1 突變體是由能催化赤黴素中間代謝物轉變為活性赤黴素的赤黴素 3-氧化酶（ZmGA3ox2） 突變引起的。重組D1 蛋白能於體外催化至少 4 個反應，包括GA_{20} 轉變為GA_3，GA_5轉變為GA_3，GA_{20}轉變為GA_1 以及GA_9轉變為GA_4等。煙草細胞中D1-GFP 的瞬時表達和細胞組分蛋白質印染分析等兩個獨立的方法，揭示了D1 蛋白是雙定位於細胞核和細胞質中。此結果暗示活性赤黴素能夠在此兩種細胞器中合成。這個雙定位的結果與赤黴素受體GID1 蛋白的定位壹致。在早期的玉米雌花發育的過程中，D1 蛋白特異且大量地表達在雌花中的雄花原基細胞，揭示了赤黴素發揮其抑制雄花原基發育的功能需要在該組織大量合成。

DELLA 蛋白是赤黴素信號傳導的壹個阻遏物。玉米包含僅壹個 DELLA 蛋白命為 DWARF8（D8）。發生在 D8 蛋白 N 端的突變產生了赤黴素非敏感型突變體 *dwarf8* （*d8*）。*d8* 突變體與 *d1* 突變體有很多共同特征，包括侏儒植株，深綠色的葉片以及雌雄兩性花。這些特點都表明玉米對赤黴素的響應是被 DELLA 蛋白所抑制的。DELLA 蛋白是通過蛋白互作的方式來限制其互作蛋白功能，以達到抑制赤黴素下遊信號傳導的目的。多樣的赤黴素響應必然需要多樣的 DELLA 互作蛋白。基於赤黴素在調控玉米性別決定過程當中的獨特功能，我們提出假設：玉米當中存在未知的 D8 互作蛋白。通過酵母雙雜交方法篩選玉米雌花的 cDNA 文庫，找到 14 個在酵母系統與 D8 互作的蛋白。ZmSPX1 是這些蛋白中的壹個但不清楚功能。通過雙分子熒光互補實驗和蛋白質體外結合實驗，D8 和 ZmSPX1 之間的互作被進壹步確定。基于此，我们找到数个候选的 D8 互作蛋白以及证明了 ZmSPX1 是 D8 真正的互作蛋白；这些工作都为进一步研究 ZmSPX1 蛋白在调控性别分化、细胞分裂和分化等赤霉素响应中的作用提供了基础。

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Chen Yi

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List of Abbreviations

AGO	Argonaute 1 protein
ALC	Alcatraz
An1	anther ear 1
bHLH	basic helix-loop-helix
BiFC	bimolecular florescence complementation
BOI	Botrytis susceptible1 interactor
bp	Base pair
BR	Brassinosteroid
BSA	Bovine serum albumin
BZR1	Brassinozale-Resistant 1
ccdB	controller of cell division or death B
cDNA	complementary DNA
ChIP	Chromatin Immunoprecipitation
chr	chromosome
CPS	*ent*-copalyl diphosphate synthase
CYP	cytochrom P450
d	dwarf
D1	DWARF1
DET2	DE-ETIOLATED2
DMSO	dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
E1	primary ear floret
E2	secondary ear floret
eCFP	cyan fluorescent protein
ER	endoplasmic reticulum
EST	expressed sequence tag
eYFP	enhanced yellow florescence protein
FAA	Formalin–acetic acid–alcohol

GA	Gibberellic acid/gibberllin
GA3ox	GA3-oxidase
GA20ox	GA20-oxidase
GC-MS	Gas Chromatograph-Mass Spectrometer
GFP	green florescence protein
GGDP	geranylgeranyl diphosphate
GST	glutathione S-transferase
GUS	beta-glucuronidase
IPTG	Isopropyl β-D-Thiogalactoside
JA	jasmonic acid
JAZ1	JA ZIM-domain 1
KO	*ent*-kaurene oxidase
KS	*ent*-kaurene synthase
L	Long arm
LB	Luria-Bertani
LR	*att*L and *att*R sites
M	molar mass
MBP	maltose binding protein
mRNA	message RNA
2ODD	2-oxoglutarate dependent dioxygenases
PAC	Paclobutrazol
PBS	Phosphate Buffered Saline
PCR	Polymerase chain reaction
pGWB	gateway binary plasmid
PIF	phytochrome interacting protein
qRT-PCR	quantitative reverse transcription Polymerase chain reaction
Rh1	reduced hight 1
RNA	ribonucleic acid
S	short arm
SCL3	SCARECROW-LIKE 3
Sk1	Silkless1
T1	primary tassel floret

T2	secondary tassel floret
TF	transcription factor
WT	wild type
Y2H	yeast two hybrid

Chapter 1 Introduction

1.1 The functions and applications of Gibberellin

Gibberellin (GA) is one of the classic plant hormones. It was first recognized in 1926 but isolated later in 1936 (Reviewed by Stowe and Yamaki, 1957). GAs are diterpenoid compounds found in plants, fungi and bacteria. Among more than 130 species of GAs discovered in nature, the bioactive forms are GA_1, GA_3, GA_4 and GA_7 (Yamaguchi 2008; Sponsel and Hedden 2010). The rest are either intermediates of the biosynthesis or GA catabolites. GAs have various functions on the growth and development of a plant such as promoting seed germination, hypocotyl elongation, stem elongation, leaf expansion, floral induction, pollen development and fruit growth (Sponsel and Hedden 2010). The discovery of GA is contributed to its function in promoting stem elongation during the study of "the foolish seedling" disease in rice which is caused by over-production of GA by infected fungus *Gibberella fujikuroi*. Genetic alterations in the GA signal transduction (*Rht1*) and biosynthesis (*GA20ox*) founded the breeding of semi-dwarf wheat and rice respectively (Peng, Richards et al. 1999; Sasaki, Ashikari et al. 2002). These mutants largely contributed to the success of "Green Revolution". GA was once considered as a candidate of flowering stimulus as its ability in promoting bolting and flower formation in long-day (Lang, 1957). However, GA has less effect on short-day plants. At present, the promoting candidate of florigen was considered as Flowering Locus T (FT). In *Arabidopsis*, filament elongation was reduced in GA deficient mutant because of the reduced cell extension. Nonetheless, the pollen development and dehiscence were also required for GA function (Sanders et al., 1999; Cheng et al., 2004). GA has diverse functions in regulating flower development among species. For example, GA promotes filament and anther development in *Arabidopsis* and rice; in maize, however, GA suppresses stamen development (Dellaporta, 1994). In contrast to maize, GA promotes male organ development in cucumber (Pike and Peterson, 1969; Wittwer and Bukovac, 1962).

By utilizing the functions of GA, GA and its inhibitor were widely applied in agriculture. For example, in order to increase germination rate, GA is commonly applied in presoaking. The working concentrations are dependent on different species. The application of GA to table grape can

significantly increase berry size so that the yield is increased largely (Weaver, 1976). To obtain stable inheritant female line by self-cross, GA is applied to promote male flower development in cucumber. Paclobutrazol (PAC) is a GA biosynthetic inhibitor. Application of PAC in plants will result in shortened but thickered stem which will help strengthen the ability of lodging resistant and reduce the loss of yield.

1.2 GA biosynthesis and metabolism

The concentration, the location and the signal transduction of a plant hormone are usual ways for the hormone to regulate plant growth and development. The biosynthesis of GA can be divided into three phases (Yamaguchi 2008). They are believed to occur in separate compartments, which is a way to regulate the concentration of GA in the cellular level. The first phase is the cyclisation of geranylgeranyl diphosphate (GGDP) in plastids (Fig. 1). GGDP is converted to *ent*-kaurene through the functions of *ent*-copalyl diphosphate synthase (CPS) and *ent*-kaurene synthase (KS). CPS and KS were localized in plastids (Sun and Kamiya 1994; Helliwell, Chandler et al. 2001). The second phase is the formation of GA_{12} in endoplasmic reticulum (ER). GA_{12} is converted from *ent*-Kaurene by cytochrome P450 monoxygenases (P450s) which is located in the plastid envelope and ER (Helliwell, Chandler et al. 2001). The third phase is the formation of bioactive GAs. This involves the activities of two classes of 2-oxoglutarate dependent dioxygenases (2ODDs), GA20-oxidase (GA20ox) and GA3-oxidase (GA3ox). GA20ox catalyzes multiple oxygenation reactions at C-20 of GA_{12} to produce a C_{19}-GA (due to a loss of C-20 as CO_2), which is then converted to bioactive GAs by GA3ox (reviewed by Yamaguchi, 2008). These reactions were believed to occur in cytosol for the reasons that GA3ox and GA20ox were soluble proteins and they did not contain any apparent targeting sequences (Yamaguchi 2008). But, experimental proof is lacking. The conversion of bioactive GAs to inactive GA forms are catalyzing by GA 2-oxidase (GA2ox) (Olszewski et al., 2002). Inconsistent to the bioactive GA production, GA receptor GID1 protein is dually localized to the nucleus and the cytosol (Hartweck and Olszewski, 2006; Ueguchi-Tanaka et al., 2005). These inconsistent locations

between GA formation and perception raise questions: whether the last phase of GA biosynthesis occurs in both nucleus and cytosol or GA transporter exists between these two compartments?

In fact, in rice the predominant bioactive GA form is GA_1 instead of GA_4 which is the predominant form in *Arabidopsis*. GA_1 and GA_4 are synthesized from different pathways: the former from 13-OH and the latter from 13-H pathway. Two cytochrome P450 genes, *CYP714B1* and *CYP714B2*, were recently identified in rice encoding GA 13-oxidase that is the enzyme response for GA_1 biosynthesis (Magome et al., 2013). The two ways of GA biosynthesis are often coexist in flowering plant. This is another mechanism to regulate the concentration of GA in plant.

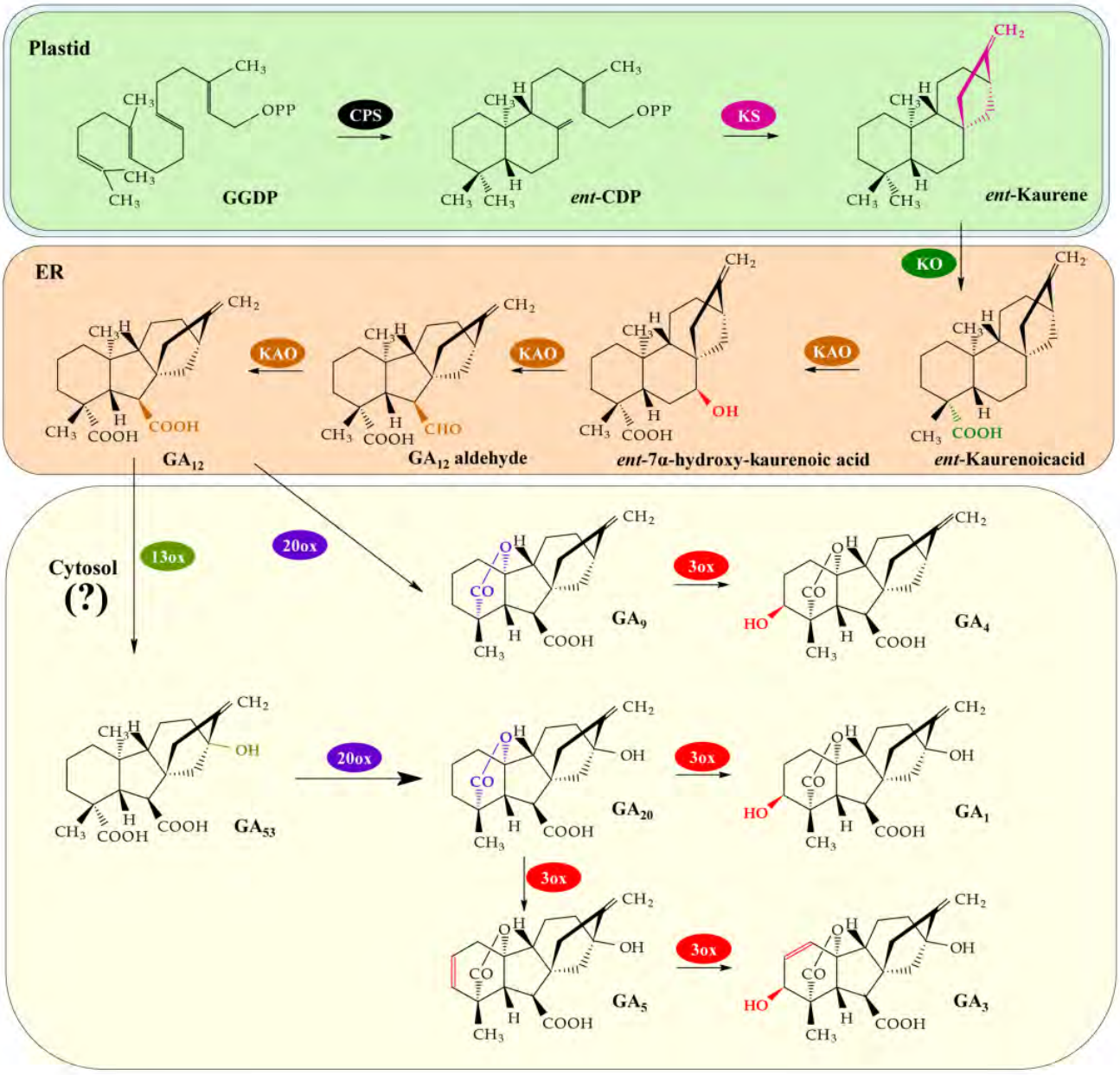

Figure 1. The GA biosynthetic pathway. The first phase of GA biosynthesis occurs in plastids. The second phase occurs in ER. The third phase was believed to occur in cytosol. But experimental data was lacking. GGDP, geranylgeranyl diphosphate; CPS, *ent*-copalyl diphosphate synthase; KS, *ent*-kaurene synthase; ER, endoplasmic reticulum; KO, *ent*-kaurene oxidase; KAO, *ent*-kaurene synthase; 13ox, GA 13-oxidase; 20ox, GA 20-oxidase; 3ox, GA 3-oxidase.

1.3 GA signal transduction pathway

The discovery of the GA receptor GID1 and the core repressor DELLA protein are the significant breakthrough for understanding the GA signal transduction mechanism. DELLAs were first discovered from *Arabidopsis* and were named after a highly conserved amino acid motif in their N-terminal domains belonging to GRAS family (Peng et al., 1997). Their C-terminal domains are also highly conserved included several conserved motifs. DELLAs are repressors in GA signaling and they locate in the center of a network between other related biological pathways and GA signaling pathway (Daviere et al., 2008). Without bioactive GA, DELLAs restrict downstream gene responses to GA. The complex formed by GA and its receptor GID1 will help DELLA proteins to be degraded by a series of ubiquitination. Structure analysis proposed that DELLA was a putative transcription factor and contained motifs for transcriptional coactivator (Peng et al., 1997). Biochemical data indicated that DELLA N-terminal domain is intrinsically unstructured without interactions with GA/GID1 complex (Sun et al., 2010). Intrinsically unstructured proteins (IUP) are able to be folded differently upon different partners. Because many aspects of plant growth and development such as dwarfism, dark green leaf, male sterile and late flowering are all related to DELLA protein functions, multiple binding activities are expected for DELLAs. Without obvious DNA binding domain and any DELLA binding gene found by performing CHIP-microarray (Zentella et al., 2007), DELLA should cooperate with other partners to suppress downstream gene responses to GA. This hypothesis was supported first by discoveries of two transcription factors PIF3 and PIF4 directly interacting with DELLA proteins (de Lucas et al., 2008; Feng et al., 2008). These discoveries demonstrated the coordination regulation of light and GA in plant.

1.4 DELLA interacting proteins

Two PHYTOCHROME INTERACTING PROTEIN (PIF), PIF3 and PIF4 were proved to be interacted with DELLA proteins (de Lucas et al., 2008; Feng et al., 2008). This is the first time to elucidate that DELLA proteins regulate the downstream of GA signal transduction through protein-

protein interaction. PIF3 and PIF4 were basic helix-loop-helix (bHLH) transcription factors. Recently, DELLA interacting proteins have been identified, which largely elevates our knowledge of the GA regulation mechanism and reveals the mechanisms of crosstalk between GA and other plant hormone or other environmental factors.

ALCATRAZ (ALC) which is a bHLH transcription factor contains the same H-E-R motif as PIF3 and PIF4 in their DNA interacting domains (Arnaud et al., 2013). ALC is required for the fruit patterning in *Arabidopsis*. The direct interaction between ALC and DELLA reveals that GA regulates valve margin development through release ALC from DELLA restriction. The interaction between DELLA and bHLH proteins, which connects GA and light responses and applies in tissue patterning, should be a versatile regulatory module also used in other GA responses in plant.

In fact, other regulatory modules were found downstream of DELLA. SCARECROW-LIKE 3 (SCL3) which is a GRAS protein like DELLA but without the GA-responsive DELLA domain, was found to be suppressed by DELLA and induced by GA suggesting that SCL3 should be a positive regulator of GA signaling. Interestingly, SCL3 interacted with DELLA proteins and regulated its own gene expression participating in the root and aboveground organ development (Zhang et al., 2011).

To inhibit GA signal transduction, DELLA may require partners: in *Arabidopsis* four RING domain proteins BOTRYTIS SUSCEPTIBLE1 INTERACTORs (BOIs) together with DELLAs through direct interaction repress the expression of a subset of GA responsive genes by binding the promoters of target genes (Park et al., 2013). This finding suggested that other co-regulators of DELLA in regulating GA signaling may exist and will be identified in the future.

Discoveries of some DELLA interacting proteins reveal that GA integrates with other plant hormone signaling transductions through direct interaction with core signal transduction components. The interaction between DELLA and the Brassinozale-Resistant 1 (BZR1) transcription factor elucidated that GA and Brassinosteroid (BR) act dependently through this direct interaction in the regulation of several aspects of plant growth and development (Hartwig et al., 2011). A key repressor of jasmonic acid (JA) signaling JA ZIM-domain 1 (JAZ1) was proved to be directly interacted with DELLA proteins *in vivo*. MYC2 that was a key transcriptional activator of JA responses interacted with JAZ1. DELLA competed with MYC2 to bind with JAZ1. This binding released MYC2 from

JAZ1 to regulate its target genes (Hou et al., 2010). Interestingly, another study demonstrated that DELLA interacted with MYC2 protein (Hong et al., 2012). Hong et al proposed that divergent physiological conditions may result in competitive binding between these proteins. Notably, most of the DELLA interacting proteins are transcription factors with the only exception JAZ1. As DELLA regulates many aspects of GA responses in plant, more and more new DELLA interacting proteins will be identified in the near future, which will help us clarify the mechanisms of GA regulation.

1.5 The advantages of maize as a model system to study GA biosynthesis and signaling

With plenty of genetic materials for biochemical and genetic research, maize has been a model system for several decades. Large population of transposons in maize genome causes a high frequency of mutation. In 2009, sequencing and assemble of the maize genome were finished (Schnable et al., 2009), which speeds up the study of maize in molecular biology and functional genomics. For example, the defining of GA biosynthesis pathway was credited for a set of GA sensitive mutants such as *dwarf1* (*d1*), *d2*, *d3*, *d4*, *d5* and *anther ear1* (*an1*) in maize.

d1 is an old mutant known for decades (Fujioka et al., 1988; Phinney, 1956). It can be rescued by the application of GA_1, but not by GA_{20} (Spray et al., 1996). It accumulated 10 times more GA_{20} and GA_{29} than the wild type (Fujioka et al., 1988). Metabolic analysis indicated that three steps in GA biosynthesis were blocked in *d1*, *i.e.* GA_{20} to GA_5, GA_5 to GA_3, and GA_{20} to GA_1 (Spray et al., 1996). The identity of D1 protein should be a GA3ox catalyzing the conversion from GA intermediates to bioactive forms or a positive regulator of GA3ox function. But the identity of the mutation in *d1* alleles is not resolved.

dwarf8 (*d8*) is a GA insensitive mutant in maize displaying similar phenotype with the *d1* mutant including dwarfism, dark-green leaf and andromonoecious ear (Fujioka et al., 1988). The D8 gene encodes a DELLA protein (Peng et al., 1999). Thus, GA responses in maize are mostly repressed by DELLA. In contrast to *Arabidopsis* who has four DELLA proteins with diverse or overlap

functions, maize contains one DELLA protein, which will simplify the work on studying the downstream pathway of DELLA.

1.6 Sex determination process in maize

The sexuality of plant is depended on their flowers. Most species of angiosperms carry perfect flowers that carry both female and male reproductive organs. However, some species carry unisexual flowers (only female or male reproductive organs) within the same plant, which are called monoecy; or unisexual flowers on separate plants, which are called dioecy. The most common mechanism found among monoecy and dioecy species to generate unisexual flowers is the selective developmental arrest of preformed organ primordia (Dellaporta and Calderon-Urrea, 1994).

Maize is monoecy grass with separate sexuality flowers: male flowers (tassel) on top and female flowers (ear) in the axial of the plant. Spikelet is the basic unit of maize as well as other grasses. Two florets are within one spikelet: in the tassel are called primary tassel floret (T1) and secondary tassel (T2) florets; while in the ear are called primary ear floret (E1) and secondary ear floret (E2), respectively. At the initial stage, every floret included pistil and stamen primordia which are able to develop as mature pistils or stamens. However, unisexual flowers are resulted from abolished pistil primordia in T1, T2 and E2 florets or stamen primordia in E1 and E2 florets, which are achieved by sex determination process. A model for maize sex determination proposed by Dellaporta and Calderon-Urrea, concluded that the sex determination process include genetic and hormonal determinants (Dellaporta and Calderon-Urrea, 1994).

During the last several decades, scientists have discovered or created certain important mutants involving to the sex determination process in maize, such as *tasselseed* (*ts1*, *ts2*, *ts4*, *ts5, ts6*), *dwarf* (*d1*, *d2*, *d3*, *d4*, *d5*, *d8*), *silkless* (*sk1*) and anther ear (*an1*). Based on the phenotypes of these mutants, it is clear that *tasselseed* genes promote gynoecial abortion; *dwarf*, *anther ear* and *silkless* genes promote stamen abortion and feminization. Gene *TASSELSEED2* (*TS2*) encodes a short-chain alcohol dehydrogenase (DeLong et al., 1993). Gene *TASSELSEED1* (*TS1*) encodes a lipoxygenase affecting JA signaling (Acosta et al., 2009). Application of JA can rescue the phenotype of *ts1* and *ts2*.

Thus, JA functions in pistil suppression in tassel florets. The product of *An1* gene functions as enzyme catalyzing the cyclization of GGPP to *ent*-kaurene which is the first tetracyclic intermediate in the GA biosynthetic pathway (Bensen et al., 1995). *d1*, *d2*, *d3*, *d4* and *d5* are GA sensitive mutants while *d8* carries gain-of-function DELLA protein. Stamens in the ear of these mutants can be developed. Thus, the biological function of GA in sex determination process is suppression of stamen development in the ear. Loss function of DET2 protein which is in the BR biosynthetic pathway results in the *tasselseed* phenotype of *na1* mutant. According to these discoveries, much more attentions should go to hormone interaction in the sex determination process.

In this study, cloning and characterization of the *D1* gene indicated that *D1* encodes a GA 3-oxidase catalyzing at least four reactions: GA_{20} to GA_5, GA_5 to GA_3, GA_{20} to GA_1 and GA_9 to GA_4. The D1 protein was found to be localized to both the nucleus and the cytosol, which suggests that bioactive GAs could be synthesized in both compartments within the cell. This finding coincides with the subcellular localization of GA receptor GID1 while in contrast to previous belief that bioactive GAs were synthesized in the cytosol. By detecting the sites of D1 protein expression in developing female florets, I provided evidence that GA was potentially synthesized in stamen primordia within female florets, resolving the sources of GA production at cellular levels. In order to identify new components downstream of DELLA in regulating GA responses in maize, I constructed a maize cDNA library in yeast and screened it by using D8 protein as bait protein. Several D8 interacting proteins were found through yeast two hybrid analyses. Their biological functions and roles in regulation of GA responses will be further studied in the future.

Chapter 2 Material and methods

2.1 DNA extraction and Southern blot analysis

Genomic DNA extraction and Southern blot analysis were carried out as previously reported (Tan et al., 2011). Details of procedures are as follows.

Fresh material was grinded to powder in liquid nitrogen. Then, DNA extraction buffer was added (5ml per gram) and mixed. All of them were transferred to centrifuge tube. After adding phenol:chloroform(1:1) (4ml per gram), shook it at least 30 min then centrifuge it at high speed. The upper layer supernatant was transferred to a fresh centrifuge tube. 0.1x volume 3 M NaOAc (pH5.2) was added to the supernatant. They were gently mixed; and then isopropanol (3.9 ml per gram) was added. After shaking gently for a few seconds, thread-like DNA was visible. The DNA was pelleted by centrifuge and washed with 1 ml 70% ethanol twice. After the ethanol was completely removed, the DNA was dried in a vacuum dryer and then dissolved in TE buffer.

To find the mutations of *D1* gene in four *d1* alleles, genomic DNAs from B73, W22, *d1-3286*, *d1-4*, *d1-6039* and *d1-6016* were digested with *Bam*HI enzyme at 37^{o}C for overnight. These samples were loaded equally. The probe used in Southern blot is probe I (Fig. 5A) and labeled with ^{32}P. Southern blot hybridization was performed by Prof. Tan.

2.2 Genomic library construction and screening

To clone the *d1-3286* allele, a size selected genomic library was constructed in λ-phage. The genomic DNA was digested with *Bam*HI enzyme, size-fractionated and ligated to a ZAP express vector as previously reported (Tan, Schwartz et al. 1997). Approximately 2.5x10^{5} plaques were screened with probe I of ZmGA3ox2 (Fig. 5A). Four clones were identified and excised into pBK-CMV plasmids according to the manufacturer's instruction (Stratagene, USA). This part is done by Prof. Tan.

2.3 D1 cDNA amplification

The full length of D1 cDNA was deduced from expressed sequence tag (EST) database in GenBank by using probe I sequence. To clone the full length cDNA from seedling, several pairs of primers were designed. Among them, D1-RTF1 (CTTCCTTCCCTCCTTCCTTG) and D1-RTR1 (CTAATTCCGTTCCGCAGCTA) worked well. The percentage of GC in *D1* cDNA is too high for regular PCR amplification. To solve this problem, dimethyl sulfoxide (DMSO) was added to the PCR reaction and PCR program was modified as follows. The polymerase was Phusion enzyme (Invitrogen).

Step 1, 98^{o}C, 30 sec; step 2, 98^{o}C, 10 sec; step 3, 64^{o}C, 30 sec; step 4, 72^{o}C, 45 sec; step 5, repeat step 2 to step 4 for 9 cycles; step 6, 98^{o}C, 10 sec; step 7, 58^{o}C, 30 sec; step 8, 72^{o}C, 45 sec; step 9, repeat step 6 to step 8 for 34 cycles; step 10, 72^{o}C, 7 min; step 11, 15^{o}C forever.

2.4 qRT-PCR analysis

Total RNAs were extracted by using RNeasy mini Kit (QIAGEN) from root, stem, leaf of 2-week-old seedlings, about 1.5 cm tassel and ear; whole seedlings treated with or without 100 μm GA_3. Prior to cDNA synthesis, total RNA was treated with DNase I (NEB). cDNA was synthesized by using reverse transcriptase (SuperScript III; Invitrogen). Primers specific for D1 were D1A-F6 (CGCCCATCTCCTCCTTCTTCT) and qPCRR1 (TCCATCACGTCACAGAAGCT). qRT-PCR was normalized with *Actin1* as a reference gene. The primers for maize *Actin1* gene are ZmACT-RTF1 (ATGGTCAAGGCCGGTTTCG) and ZmACT-RTR1 (TCAGGATGCCTCTCTTGGCC).

qRT-PCR was conducted with three technical replicates for each one of the three biological replicates of each tissue sample. The primers for *D1* and *Actin1* crossed intron in order to avoid amplification from genomic DNA.

2.5 Scan electronic microscopy analysis

Fresh *d1* and W22 ear about 1.5 cm to 3 cm were viewed on a Hitachi S-4700 scanning electron microscope at an accelerating voltage of 5 kV.

2.6 GA 3-oxidase Enzymic Assay

To demonstrate that *D1* encodes active GA 3-oxidases, the coding regions of *D1* was cloned in expression vector pGEX-2T and transformed it in the RIL strain of *E. coli* to produce in-frame GST-D1 translational fusion protein. Cells were induced by 0.1 mM IPTG at 28^{o}C for 4 hours. The same induction condition was used to produce GST protein. GST and GST-D1 recombinant protein were purified by using Glutathione Sepharose 4B (GE) following the manufacture's instruction. The recombinant protein was used for functional assays and GST protein as control. GC-MS was performed with an Auto Mass spectrometer (JEOL) connected to a Hewlett-Packard 5890 series II gas chromatograph. The analytical conditions used were described previously (Itoh et al., 2001). [17,17-$^{2}H_{2}$]GA_{20} was purchased from L. Mander, Australian National University (Canberra). [15,17,17-$^{2}H_{3}$]GA_{9} was synthesized from GA_{9}-norketone and (methyl d3) triphenylphosphonium-Br by Wittig reaction. All GAs used in this study were analyzed by full-scan GC-MS to show the absence of impurities. I collaborated with Prof. Masatomo Kobayashi (Riken) to perform the GC-MS analysis.

2.7 Chemically competent *E.coli* cells preparation

RIL, BL21, DB3.1 competent cells were prepared with $CaCl_2$ method as follows:
Active stock strains by streaking plates. Then, pick up an overnight culture colony from LB plates and cultured in LB medium at 37^{o}C for overnight. Inoculate cells to 100 ml LB medium by 1:100 dilution and culture at 37^{o}C until OD_{600} to 0.5. The cells were put on ice for 10 mins before collection. Collect the cells by centrifugation at 3000 g for 10 min at 4^{o}C and then gently resuspend the pellet with pre-

cooled 10 ml 0.05 mol/L $CaCl_2$. Keep on ice for 30 mins then centrifuge at 3000g for 10 mins at 4°C. Resuspend the pellet with 0.05 mol/L $CaCl_2$ containing 15% glycerol and aliquot to 200 µl per tube.

The strain of Agrobacteria used in my research is *EHA105*. The procedure of *EHA105* competent cells preparation is almost the same as above except for the culture temperature was 28°C.

To obtain large vectors, high transformation efficiency is required. To produce high efficient competent cell, I followed the method reported previously (Inoue et al., 1990).

2.8 Transient expression of D1-GFP, GUS-GFP, D1-GUS-GFP, ZmGA20ox1-GUS-GFP, D8-GFP and ZmSPX1-GFP fusion proteins in tobacco epidermal cells and laser confocal microscopy analyses

Full length of D1, GUS, D8 and ZmSPX1 cDNAs were cloned into pENTR/D vector (Invitrogen) respectively. The cDNAs of D1 and GUS, ZmGA20ox1 and GUS were ligated together through *Eco*RI. Then, D1-GUS and ZmGA20ox1-GUS were cloned into pENTR/D vector respectively. All above pENTR vectors were incubated with pGWB5 vector together with LR clonase (Invitrogen), resulting in D1-GFP, GUS-GFP, D1-GUS-GFP, ZmGA20ox1-GUS-GFP, D8-GFP and ZmSPX1-GFP fusion proteins. Leaves of 3- to 5-week-old *Nicotiana benthamiana* plants were infiltrated with *EHA105* strains containing above pGWB5 constructs respectively. Localization of fluorescent proteins was observed 36 hs after infiltration by using a confocal laser scanning microscope (Olympus FV1000-IX81). GFP signals were detected by using confocal fluorescence microscope. To obtain an empty pGWB5 vector which carries free GFP, pGWB5 vector was incubated with a self-ligated pENTR vector and LR clonase resulting in an empty pGWB5 vector without *ccdB* gene.
The pGWB5 and other pGWB vectors were kindly provided by Dr.Tsuyoshi Nakagawa. To preserve pGWB vectors, the pGWB vectors containing a *ccdB* gene must be transformed in DB3.1 strain, because the product of *ccdB* gene is toxic to DH5α strain.

2.9 Purification of His-D1 fusion protein and the D1 antibody

Due to the small size of His tag and simple purification method, protein fused with His tag is often used as antigen for antibody production. To purify His-D1 recombinant protein, the coding region of D1 was cloned in the expression vector pET-30a(+) (Novagen). The plasmid was transformed in the BL21 strain of *E. coli*. Cells were induced by 1 mM IPTG and cultured at 37^{o}C for overnight. Inclusion bodies were collected by centrifugation after sonication and dissolved in wash buffer (8 M Urea, 0.5 M NaCl, 10 mM Tris-HCl, pH8.0). BD TALON Metal Affinity Resins was used in the purification. Before binding with protein sample, resins were washed by H_2O, 10 mM Tris-HCl and wash buffer, respectively. This step was not stopped until the absorbance was stable and near zero. His-D1 fusion proteins were eluted from resins by incubation with elution buffer (500 mM Imidazole, 8 M Urea, 0.5 M NaCl, 10 mM Tris-HCl, pH8.0).

To be antigen, the concentration of His-D1 should be at least 2 μg/ml. Total amount of antigen should be at least 2 μg. The amount of antigen for immunity is one of important factors affecting the specificity and titer of the antibody. In general, the more antigens, the better antibody will be. The polyclonal antibody against D1 was raised in rabbit by immunizing His-D1 fusion protein. The specificity and purity of D1 antibody was tested by Western Blot. As the amount of endogenous D1 protein was largely divergent among different tissues, total proteins from highly expressed tissues such as 3-day-old germinated seedlings were isolated for antibody test. In order to confirm the specificity of the D1 antibody, a western blot of wild type versus *d1* mutant was carried out. To purify D1 antibody, D1 antibody was pre-incubated with the membrane containing *d1* total protein at 4^{o}C for overnight. Then the antibody was collected to perform Western Blot of WT vs *d1* protein. The result indicated that the purified D1 antibody could not recognize any protein in *d1* mutant and could recognize a single band in the WT. Moreover, the size is similar to expected size. Thus, this antibody was highly specific to endogenous D1 protein. It was used in the following western blot analysis and immunohistochemical analyses.

2.10 Fractionation of nuclei and cytosol

Cytosolic protein and nuclear protein were isolated from 3-day-old germinated seedlings where D1 was highly expressed. Seedlings were cut into pieces and incubated in cytosol isolation buffer (50 mM Tris-HCl pH7.6, 0.3 M sucrose, 0.8% Triton X-100, 15 mM KCl, 5 mM MgCl2, 0.1 mM EDTA and 1 mM DTT) on ice for 10 mins. The supernatant was transferred to a new tube and then centrifuged at 12000 g for 10 mins at 4 ℃. The supernatant

Isolation of intact nuclei from maize was followed previously reported (Pandey et al. 2006). To prevent contamination from the supernatant, the nuclei pellet was washed three times with wash buffer (0.1 M Tris, 0.8 M KCl, 0.1 M EDTA, 10 mM spermidine, 10 mM spermine, pH 9.4) and pelleted by centrifugation at 1800 g at 4°C for 15 mins. The purity of the nuclear protein and cytosolic protein were monitored by western blot with Histone3 antibody (α-H3) and heat shot protein (α-HSP) antibody. Total protein extraction was performed as previous description (Xu et al., 2006).

2.11 Immunohistochemical analyses of D1 protein in developoing female and male florets

The maize ears and tassels about 1 to 1.5 cm long were fixed in FAA solution (45% ethanol, 5% acetic acid and 1.9% formaldehyde). Dehydration and paraffin embedment steps were as follows: 30% ethanol for 30 mins, 50% ethanol for 30 mins, 70% ethanol for 30 mins, 80% ethanol for 30 mins, 90% ethanol for 30 mins, 95% ethanol for 30 mins, 100% ethanol for 30 mins, 100% ethanol for 1h, 100% xylene for 1h, 100% xylene for 1h, 100% xylene for 1h, 50% xylene/50% paraffin wax for 2h, 100% paraffin wax for 3h, 100% paraffin wax for 3h. After sectioning, the samples were put in xylene for 5mins, xylene for 5mins again, 100% ethanol for 1 min, 95% ethanol for 1 min, 75% ethanol for 1 min, 50% ethanol for 1 min, 30% ethanol for 1 min and running tap water for 1 min. Then the samples were blocked in 3% BSA in 1xPBS buffer for at least 2hrs and incubated with anti-D1 antibody or Histone3 (1:100 dilution) at 4°C for overnight. After washing with wash buffer (1% BSA in 1xPBS buffer) for 3 times (each for 10 mins), the samples were incubated with fluorescent secondary antibody (Alexa®594 goat anti-rabbit, Invitrogen) at room temperature for 1h. The

secondary antibody was removed and washed extensively with wash buffer for 3 times (each for 20 mins). The fluorescent signals were observed and imaged under a confocal laser scanning microscope (Olympus FV1000-IX81).

2.12 Yeast two hybridization

2.12.1 cDNA library construction

B73 is one of maize inbreed line. Its genome was sequenced and widely used as maize reference genome sequence. The maize cDNA library in yeast was constructed from B73 cDNA. 1.0 cm to 1.5 cm ears were harvested for total RNA isolation. TRIzol reagent (Invitrogen) was used during RNA isolation. mRNA was isolated from total RNA following the manufacturer's protocol (Promega). To construct cDNA library, Clontech SMART technology was utilized. Because full-length D8 protein was toxic to yeast host cell based on the result of toxicity test, C-terminal of D8 protein was used as bait protein during screening. A standard protocol of yeast two hybrid screening (Clontech) was followed to screen the ear cDNA library. The cDNA library contains more than 2 million independent clones. In this study, more than 1 million independent clones were screened.

2.12.2 Yeast transformation

In order to obtain high transformation efficiency, I used the medium provided by the kit of Yeastmaker Yeast Transformation System 2 (Clontech) and followed the manufacture's instruction. Fresh yeast competent cell has the highest transformation efficiency. Unlike *E.coli* competent cell, yeast competent cell will lose all the ability to obtain DNA if they are stored at -80 ^{o}C.

2.12.3 Yeast plasmid isolation

As yeast contains cell wall, the bacteria plasmid isolation method is not suitable for yeast plasmid. The method I used was modified based on the QIAGEN "QiaPrep" Kit. Details are listed below.

4 ml overnight cultured cells in selective media were collected. The pellet was resuspended in 500 µl sterile distilled H_2O. Cells were pelleted again and then resuspended in 250 µl Qiagen buffer P1 (containing RNAse A). Importantly, they were added 250 µl Qiagen buffer P2 together with 250 µl acid washed glass beads and swirled for 2 mins. Let them stay 5 mins in P2 at 4^{o}C. 350 µl chilled Qiagen buffer N3 was added and mixed by inverting. Then they were incubated on ice for 5 mins. After spinning down for 10 mins, the supernatant was added to the Qiaprep-spin column. The residual steps were followed the manufacture's instruction.

2.12.4 Sequence analyses of putative interacting proteins

The conversion of first strand cDNA using SMART technology is not ideal as the manufacturer's claim. In fact, most clones of the cDNA library contain partial cDNA. To gain the intact sequence information of the clones after screening, it is necessary to pay effort on sequence analysis. Generally, search the inserted sequences of the clones in EST database; then assemble the EST sequences as long as possible. Further, search the translated amino acid sequences in protein non-redundant database in order to find specific domains, which will be helpful for speculating their functions.

2.13 Pull down assay

Full-length cDNA of ZmSPX1 was cloned in pMal-C2X vector, creating a fusion with the maltose-binding protein (MBP). RIL Cells contained empty pMal-C2X and pMal-C2X-ZmSPX1 were induced by 0.2 mM IPTG and cultured at 28^{o}C for 6 hours. MBP-ZmSPX1 and MBP proteins are purified by amylose resin beads. Full-length cDNA of D8 was cloned in pGEX-4T-1, creating a GST-D8 fusion protein. The GST-D8 recombinant protein was purified from RIL by sepharose beads (GE). Then, equal amount of MBP-ZmSPX1 and MBP proteins were added to the GST-D8 bound Glutathione-Sepharose 4B beads and incubated for 4 hours at 4^{o}C. After sufficient washing (up to 8 times) with

1XPBS buffer, the bound proteins were eluted with Glutathione and analyzed by Western Blot with MBP antibody.

2.14 Bimolecular fluorescence complementation assays

By applying Gateway technology, full length cDNA of ZmSPX1 and D8 were cloned in binary pBiFC vectors containing the amino-terminal fragment of the eYFP fluorescent protein or carboxy-terminal fragment of the eCFP fluorescent protein ($eYFP^N$ and $eCFP^C$) (Gampala, Kim et al. 2007). CFP^C is modified from YFP^C in order to enhance the fluorescent signal in BiFC assay. All these constructs were transformed in *EHA105*. All eight pairwise combinations of these constructs were mixed at a 1:1 OD_{600} ratio and injected into 3-4-week-old tobacco epidermal cells. After 36 hs, fluorescent signals were observed under confocal fluorescence microscope.

Chapter 3 Results

3.1 Isolation and characterization of a dwarf mutant in maize

The *viviparous*3286* (*vp*3286*) mutant showed an intriguingly high mutagenesis frequency in selfed progenies during the genetic analysis. The *vp*3286* line was from the Donald Robertson collection. A dwarf mutant was generated in one of the selfed progenies. The mutant plant exhibited severe dwarfism (~ one foot tall at maturity) with shortened internodes (Fig. 2A). The leaves are broad, erect and dark green. With fully developed anthers, its tassels are erect and short with reduced branches. The plant is andromonoecious, ***i.e.*** having bisexual flowers in the ear and male flowers in the tassel. In the female flower (ear), male organs (stamens) are developed (Fig. 2B). These phenotypes resemble typical GA deficient or insensitive mutants (Harberd and Freeling, 1989; Spray et al., 1996). To distinguish the two, the mutant seedlings were sprayed with 10 μM GA_3. Exogenous GA restored the dwarf seedling to WT height, while the untreated mutant seedling remained dwarf (Fig. 2C). This result indicates that this mutant is GA deficient. Then this mutant was crossed with the classic *dwarf1* (*d1*) allele obtained from the Maize Genetics Cooperation Stock Center (Phinney, 1956). Their offsprings showed the dwarf plant phenotypes as the two mutants, indicating that the new mutant was allelic to *d1*. This new allele was named as *d1-3286*.

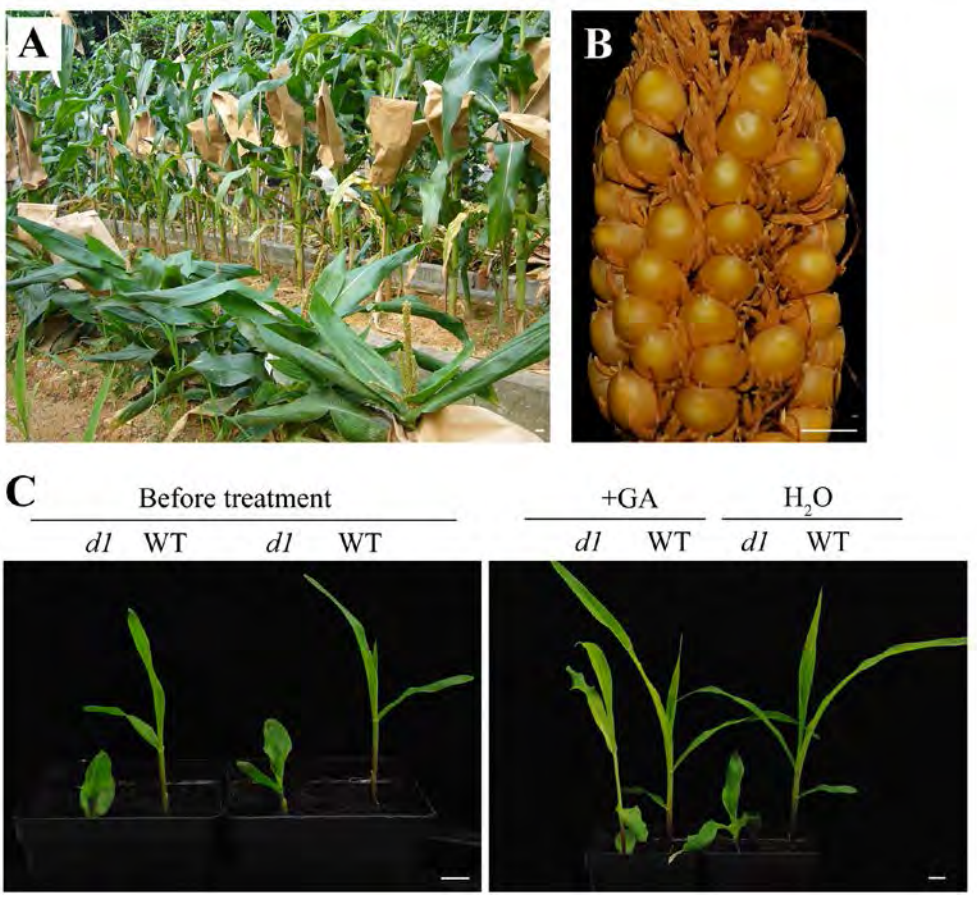

Figure 2. Phenotypes of the *d1* allele and its response to GA treatment.

A. Homozygous adult *d1-3286* plants showed dwarfism with wide and compacted dark green leaves (front row) in contrast to the WT (back row).

B. A homozygous ear of *d1-3286* displayed andromonoecy, formation of anthers in the ear.

C. The dwarf phenotype of *d1-3286* seedling before and after spray with 10μM GA_3 for 7 days. Bars, 1 cm.

3.2 Comparison of ear floret development between *d1* and WT

In maize, male flowers (tassel) are on the top while female flowers (ear) are in a leaf axil of the plant. To understand the andromonoecy in *d1* mutant, I compared the floral development between WT and the *d1* mutant by Scanning Electron Microscope (SEM). Spikelet is the basic unit of maize inflorescence. In early stage of WT floral development, two florets are formed within one female spikelet, named primary ear floret (E1) and secondary ear floret (E2). E1 and E2 florets are bisexual at this stage, each containing one central pistil primordium surrounded by three stamen primordia. As flower develops, the pistil primordium further extends into a long silk that serves for pollen landing and conducts for pollen tube growth. But the pistil primordium in E2 and stamen primordia in E1 and E2 are aborted, resulting from the effect of sex determination (Fig. 3A-D). In the *d1* mutant, the female spikelet at early developmental stage is similar to the WT. But at the later stage, the development of stamen primordia in E1 and E2 is not suppressed, resulting in formation of anthers along with elongated silks (Fig. 3E-H). The relief of suppression on anther growth and development results in andromonoecy. This result as well as the observed andromonoecy in GA deficient mutant *anther ear1* (Bensen *et al*., 1995) and GA insensitive mutant *dwarf8* (Peng *et al*., 1997) indicates that the suppression of anther development in the female flowers in maize is mediated by GA.

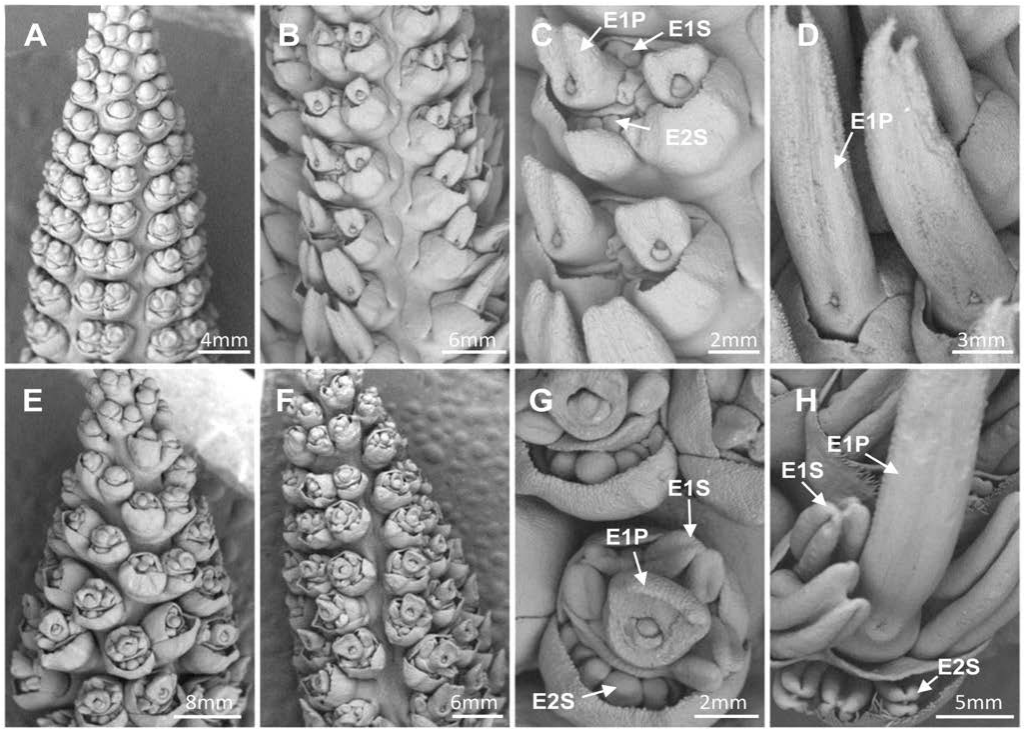

Figure 3. Female and male organ development in ear florets in WT and *d1-3286* allele.

The ears (female flowers) of WT (**A-D**) and the *d1-3286* allele (**E-H**) were analyzed at different stages of flower development by scanning electron microscope. E1, primary ear floret; E2, secondary ear floret; E1P, pistil of E1; E1S, stamen of E1; E2S, stamen of E2.

3.3 Molecular characterization of the *d1* alleles

The *d1* locus is a recessive nuclear mutation. Although it was isolated many years ago (Phinney, 1956), the molecular basis for this mutation has not been identified. Biochemical evidence suggested that it was blocked in converting GA_{20} to GA_5, GA_5 to GA_3, and GA_{20} to GA_1 (Fujioka et al., 1988; Spray et al., 1996). This suggests that D1 encodes either a GA 3-oxidase or a factor that is required for GA 3-oxidase expression. The possibility that D1 encoded a GA 3-oxidase in maize was first explored.

Two homologs were identified in the maize draft genome (AGPv2) by searching with rice GA 3-oxidases, named ZmGA3ox1 and ZmGA3ox2. In an alignment with related GA3ox proteins from rice OsGA3ox1, OsGA3ox2 (Itoh *et al*., 2001), pea PsGA3ox1 (Lester *et al*., 1997; Martin *et al*., 1997), *Arabidopsis* AtGA3ox1 (Chiang *et al*., 1995), barley HvGA3ox2 (Spielmeyer *et al*., 2004), wheat TaGA3ox2 (Appleford *et al,*. 2006) and brachipodium BdGA3ox2, the nine proteins have roughly the same size with conserved residues throughout the entire protein sequences (Fig. 4). At the protein level, ZmGA3ox2 is more closely related to OsGA3ox2 (78% identity) than to ZmGA3ox1 (58%). At nucleotide level, *ZmGA3ox2* and *ZmGA3ox1* are very divergent, sharing low similarity. This divergence suggests that the two genes were separated long time ago. Indeed, DNA hybridization by using the *ZmGA3ox2* as a probe did not recognize *ZmGA3ox1* (Fig. 5A). *ZmGA3ox2* is located on Chr 3S and *ZmGA3ox1* on Chr 6L. Because the classic *d1* mutation was mapped on Chr 3S (Neuffer and England, 1995), the *ZmGA3ox2* gene remains a good candidate for the *D1* gene. To test this notion, I analyzed four independent alleles of *d1* (*d1-3286*, *d1-6039, d1-4* and *d1-6016*). The *d1-6039* allele was isolated from a targeted direct tagging experiment with *d1-3286*. The *d1-4* allele was previously known as *d4*, but later was proven to be allelic to *d1* (Stinard, 2009). The *d1-6016* allele was also known as *302A* in the Maize Genetics Cooperation Stock Center. DNA hybridization analysis by using the *ZmGA3ox2* as a probe detected polymorphism among four *d1* alleles and inbreds (W22 and B73) (Fig. 5). Digested by *Bam*HI, B73 and W22 were predicted to yield a 2.2kb fragment. A similar size fragment was detected in *d1-6039*, B73 and W22; a 6.7kb fragment in *d1-3286*; and a

1.6kb fragment in *d1-4* (Fig. 5A). Although the loading and fragment size detected in *d1-6016* were similar to *d1-6039*, the signal in *d1-6016* was much weaker than *d1-6039*. Molecular cloning and sequencing indicate that the *d1-3286* allele contains an insertion of a 4.5kb *Copia*-like retrotransposon at position 69 (start from ATG) of the *ZmGA3ox2* gene (Fig. 5B). The *d1-6039* allele contains a deletion of "C" residue at position 399 in the first exon, causing a frame-shift which ends the translation at 163 amino acids. In addition, I also detected two short deletions of 15 bps and 7bps in the 5'-UTR region, six indels in the first intron, and several SNPs in the coding region. However, all these changes did not change the protein sequence, suggesting that the *d1-6039* allele is likely derived from a different maize background rather than B73. The *d1-4* allele contains a deletion of 487 bps, covering 389 bps of the first exon and 98 bps of the first intron (Fig. 5B). The *d1-6016* allele contains a deletion of 2301 bps, covering 508 bps of upstream sequences and 1793 bps of ZmGA3ox2 gene (Fig. 5B). The residual sequences of ZmGA3ox2 in *d1-6016* contain a 291 bp fragment overlapping with the probe used in the DNA hybridization analysis. These 291 bps were also present in a ~2.2 kb *Bam*HI fragment in *d1-6016.* This is the reason that a weak and similar wild type size fragment was shown in the DNA hybridization result (Fig. 5B). Because all these four independent alleles contain mutations in the *ZmGA3ox2* gene, I conclude that the maize *D1* locus encodes ZmGA3ox2, a putative GA 3-oxidase in the biosynthesis of GAs. The nature of mutation in each allele appears to abolish the ZmGA3ox2 function; hence they are likely null mutations. This is consistent with the severe dwarf phenotype.

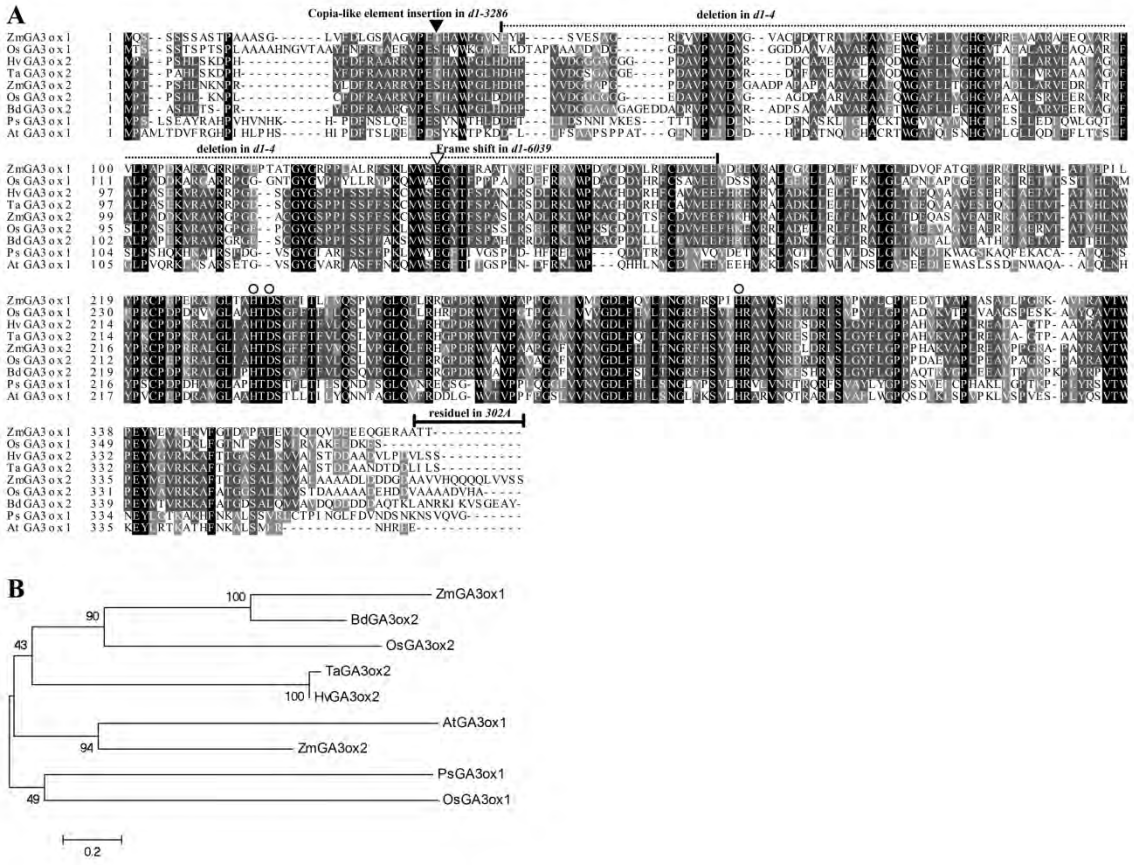

Figure 4. CLUSTALW sequence alignment and phylogenetic analysis of ZmGA3ox1 and ZmGA3ox2 with related GA 3-oxidases.

Amino acid alignment (A) and a phylogenetic tree (B) showing the relationships among maize ZmGA3ox2 (AFS50158), ZmGA3ox1 (AFS50160), *Arabidopsis* AtGA3ox1(NP_173008), rice OsGA3ox1(BAB62073) and OsGA3ox2(BAB17075), pea PsGA3ox1(AAB65829), barley HvGA3ox2(AAT49061), brachipodium BdGA3ox2 (XP_003569638) and wheat TaGA3ox2(Q3I409). Mutations (deletion or insertion) in later characterized *d1* alleles were marked (referring to Fig. 5). The highly conserved ferrous iron ligand binding residues for 2ODDs were marked as circles.

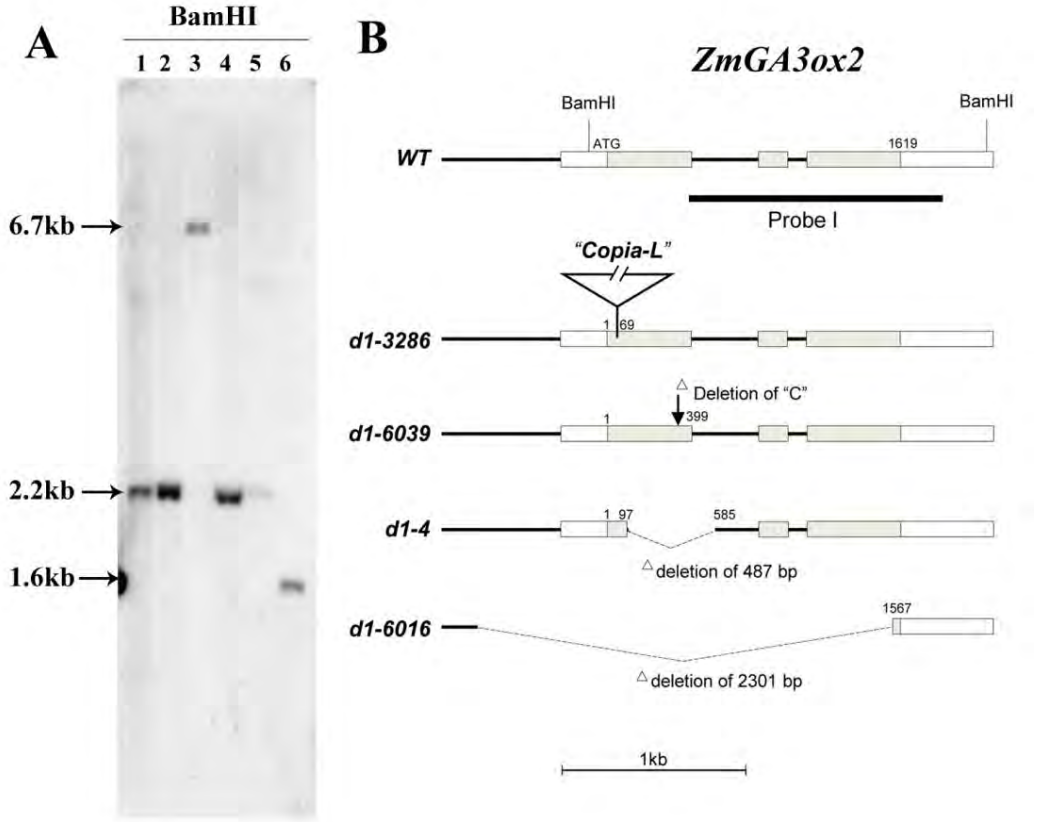

Figure 5. Molecular characterization of *d1* alleles.

A. DNA hybridization analysis on the *d1* alleles with *ZmGA3ox2* probe I. 1, B73; 2, W22; 3, *d1-3286*; 4, *d1-6039*; 5, *d1-6016*; 6, *d1-4*.

B. *ZmGA3ox2* gene structure and different *d1* alleles. Position of probe I was indicated. Exons are boxes. Shaded boxes are translated regions, empty boxes 5' or 3' UTR. Introns and non-coding regions are solid lines. A *Copia*-like element (acc number: JX307642) was inserted at position 69 of the *d1-3286* allele. One base "C" was deleted at position 399 of the *d1-6039* allele. A 487 bp fragment was deleted in the *d1-4* allele. A 2301 bp fragment was deleted in *d1-6016* allele.

3.4 Subcellular localization of the D1 and ZmGA20ox1 protein

The GA biosynthetic pathway has been placed in three compartments, with early steps in the plastid, mid-steps in the ER and late steps in the cytosol (Yamaguchi, 2008). Evidences supporting the belief that bioactive GAs were produced in the cytosol included: 1) GA3ox was a water soluble protein; 2) its sequence lacked any obvious organelle targeting signals (Yamaguchi, 2008). However, the GA signal transduction components including the receptor GID1 and the repressor DELLA proteins are primarily localized in the nucleus (Hartweck and Olszewski, 2006; Ueguchi-Tanaka et al., 2005; Willige et al., 2007). Since GA3ox catalyzes the final step of GA biosynthesis leading to production of bioactive GAs, conceivably its location determines the potential sites of bioactive GA production, given that the substrates are present. To determine the subcellular localization, D1 protein was fused with the green fluorescence protein (GFP) and transiently expressed in tobacco leaves. GFP signals were detected in both the cytosol and the nucleus by confocal laser microscope (Fig. 6A). The tobacco epidermal cells contained a large vacuole in the middle and the cytosol aligned along the cell wall. However, free GFP protein was also dual-localized to the cytosol and the nucleus (Fig.6A). Thus, it is not clear what contribute to the dual localization of D1-GFP fusion protein. In order to exclude the passive diffusion of GFP protein in the nucleus, a large fusion protein GUS-GFP about 91KDa was used as negative control (Grebenok et al., 1997) and D1 protein was fused with GUS-GFP protein. The results showed that D1-GUS-GFP was dual localized to the cytosol and the nucleus while the negative control GUS-GFP was localized to the cytosol (Fig.6B). Thus, it is concluded that D1 is dual localized to the cytosol and the nucleus when transiently expressed in tobacco leaves.

To further confirm the subcellular localization of D1 protein, western blot analysis was performed with D1 antibody to detect the presence of D1 protein in the cytosol and the nucleus. The polyclonal D1 antibody was raised against His-D1 recombinant protein. To confirm the specificity of D1 antibody, western blot analysis was performed on total proteins from *d1-6016* and wild type seedlings. The size of the endogenous D1 protein was predicted to be 41 KDa. The D1 antibody recognized a single band matching the expected size in the wild type, and no band in the *d1-6016* mutant (Fig.7), indicating that the D1 antibody is highly specific to the D1 protein. The wild type

seedling cells were fractionated into a nucleus and a cytosol fraction and the proteins were extracted. Western blot analysis detected the presence of D1 protein both in the cytosol and the nucleus fraction (Fig. 6C). Antibodies against Heat Shock Protein 82 (α-HSP82) and Histone3 (α-H3) were used to monitor cross contamination of cytosol and nucleus fraction. Western blot analysis did not detect the presence of the HSP82 in the nucleus fraction and the H3 protein in the cytosol fraction, indicating that there was no cross contamination between the two fractions. Together these results indicate that D1 is dual-localized to the cytosol and the nucleus.

The enzyme GA20ox converts the precursors of bioactive GAs, which provides the substrates for GA3ox. It was interesting that ZmGA20ox1-GUS-GFP was also dual localized to the cytosol and the nucleus (Fig. 6D). This finding further supports the hypothesis that bioactive GAs are synthesized both in the cytosol and the nucleus.

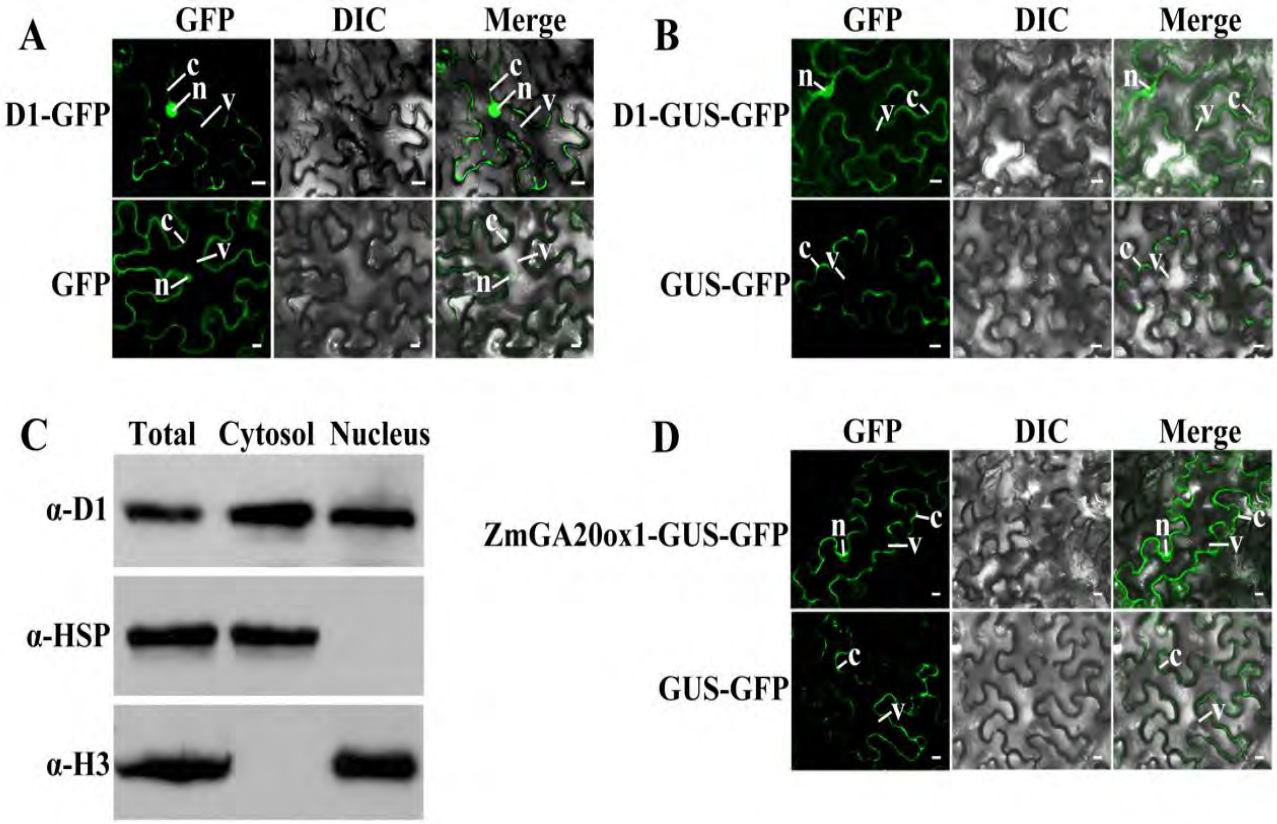

Figure 6. Subcellular localization of D1 and ZmGA20ox1 protein.

(A) D1-GFP fusion and GFP proteins,

(B) D1-GUS-GFP and GUS-GFP fusion proteins,

(D) ZmGA20ox1-GUS-GFP (NP_001241783) and GUS-GFP fusion proteins were transiently expressed in tobacco leaf epidermal cells and were analyzed by fluorescent laser confocal microscope. c, cytosol; n, nucleus; v, vacuole. Bars, 10 μm.

(C) Western blot detection of D1 protein in nucleus and cytosol fractions of maize seedlings, total, total proteins from shoot and root; α-D1, anti-D1 (ZmGA3ox2) antibody; α-H3, anti-histone 3 antibody (nuclear marker); α-HSP82, anti-heat shock protein 82 antibody (cytosolic marker). The protein amount was loaded identical for each antibody.

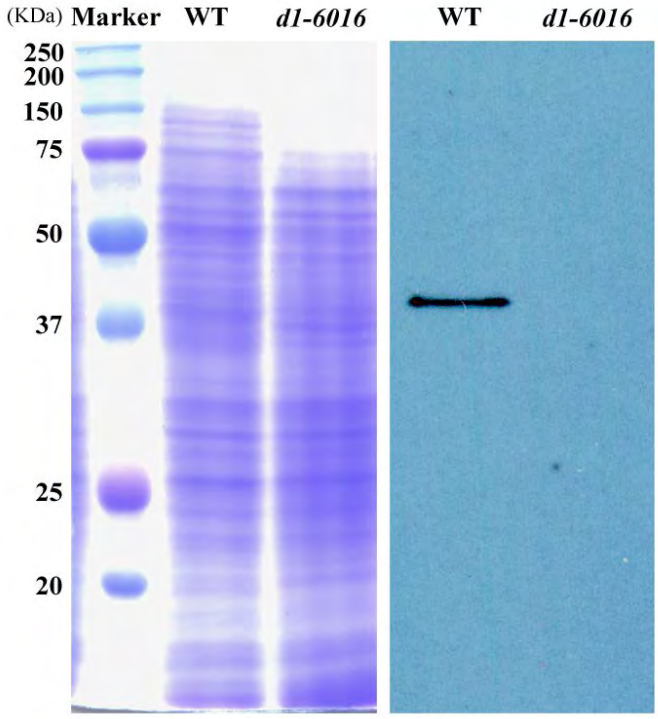

Figure 7. Specificity of the D1 antibody.

Western blot analysis with D1 antibody on total proteins from the *d1-6016* and WT seedlings (right panel) and Coomassie Blue staining of the loading amount of the two samples (Left panel).

3.5 Determination of D1 enzymatic activity

To determine the enzymatic function, D1 was fused with glutathione S-transferase (GST) protein in pGEX-2T vector and expressed in *E. coli*. The fusion protein was purified by glutathione sepharose 4B column. The protein was incubated with deuterium labeled GA substrates; $^{2}H_3$-GA_9, $^{2}H_2$-GA_{20} and $^{2}H_0$-GA_5 in the presence of co-factors 2-ketoglutarate, ascorbate and $FeSO_4$ (Refer to Chapter 2). The reaction products were analyzed by full-scan GS-MS and identified with Kovats retention indices (KRI). Recombinant GST-D1 protein is capable to catalyze four reactions, converting GA_9 to GA_4, GA_{20} to GA_1, GA_{20} to GA_3, and GA_5 to GA_3 (Table 1, Fig. 9). The MS results were provided as Figure 8. The conversion data was presented in Table 1. This result confirmed the previous biochemical analysis of *d1* mutants (Spray et al., 1996). It also showed similarity to the rice OsGA3ox activity (Itoh et al., 2001). Different from *Arabidopsis* where the active GAs are GA_1 and GA_4 (Yamaguchi, 2006), maize and rice (monocots) synthesize GA_3 as an active form as well (Spray et al., 1996). Although the biological function of GA_3 remains to be elucidated, it is concluded that D1 (ZmGA3ox2) protein catalyzes the final step of biologically active GAs in maize. This conclusion is well consistent with the previous studies on physiological and biochemical features of the *d1* mutant (Phinney, 1956; Fujioka *et al*., 1988; Spray *et al*., 1996).

The expression pattern of *ZmGA3ox2* gene in different organs was determined by qRT-PCR analysis. *D1* (*ZmGA3ox2*) was found to be expressed in most tissues, ***e.g.*** roots, stems, leaves, tassels and ears (Fig. 10A). In contrast, I could not detect the expression of *ZmGA3ox1* in these tissues although the primers used were approved working robustly on genomic DNA. This result indicates that D1 is mostly responsible for the production of GA in organs, whereas *ZmGA3ox1* may be expressed at low level or in highly specialized cells. In addition, I analyzed whether a negative feedback regulation exists on the expression of *ZmGA3ox2*. Application of 100 µM GA_3 in whole seedlings strongly inhibited the *ZmGA3ox2* mRNA accumulation, suggesting that *ZmGA3ox2* is subject to feed-back regulation (Fig. 10B).

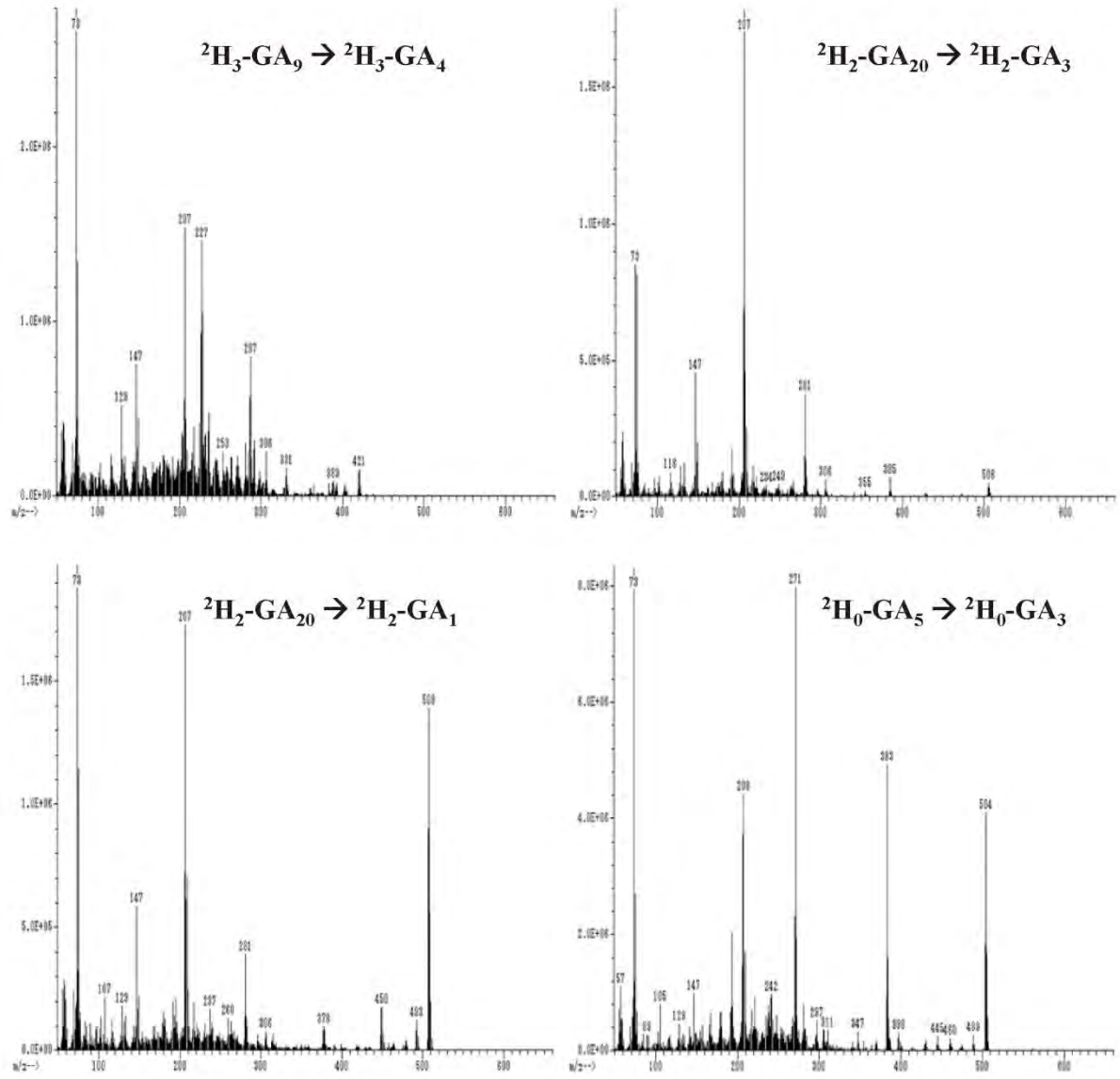

Figure 8. Mass spectrometry analysis of the *in vitro* activity of recombinant GST-ZmGA3ox2 fusion protein.

Purified GST-ZmGA3ox2 was incubated with [$^2H_0$], [$^2H_2$] and [$^2H_3$] GA substrates. The reaction product was analyzed by mass spectrometry.

Table 1. Identification of the metabolites from GAs incubated with recombinant maize GA 3-oxidase 2 by full-scan GC-MS and Kovats retention indices (KRI)

GA Substrate	GA Product	KRI	Characteristic ions at m/z (% relative intensity of base peak)
$^{2}H_3$-GA_9	$^{2}H_3$-GA_4	2515	421(100), 406(16), 403(42), 393(51), 389(57)
$^{2}H_2$-GA_{20}	$^{2}H_2$-GA_1	2674	508(100), 493(9), 449(12), 418(2), 392(1)
$^{2}H_2$-GA_{20}	$^{2}H_2$-GA_3	2697	506(100), 491(5), 475(3), 447(6), 433(4), 416(1)
$^{2}H_0$-GA_5	$^{2}H_0$-GA_3	2699	504(100), 489(7), 473(2), 445(7), 431(3), 414(2)

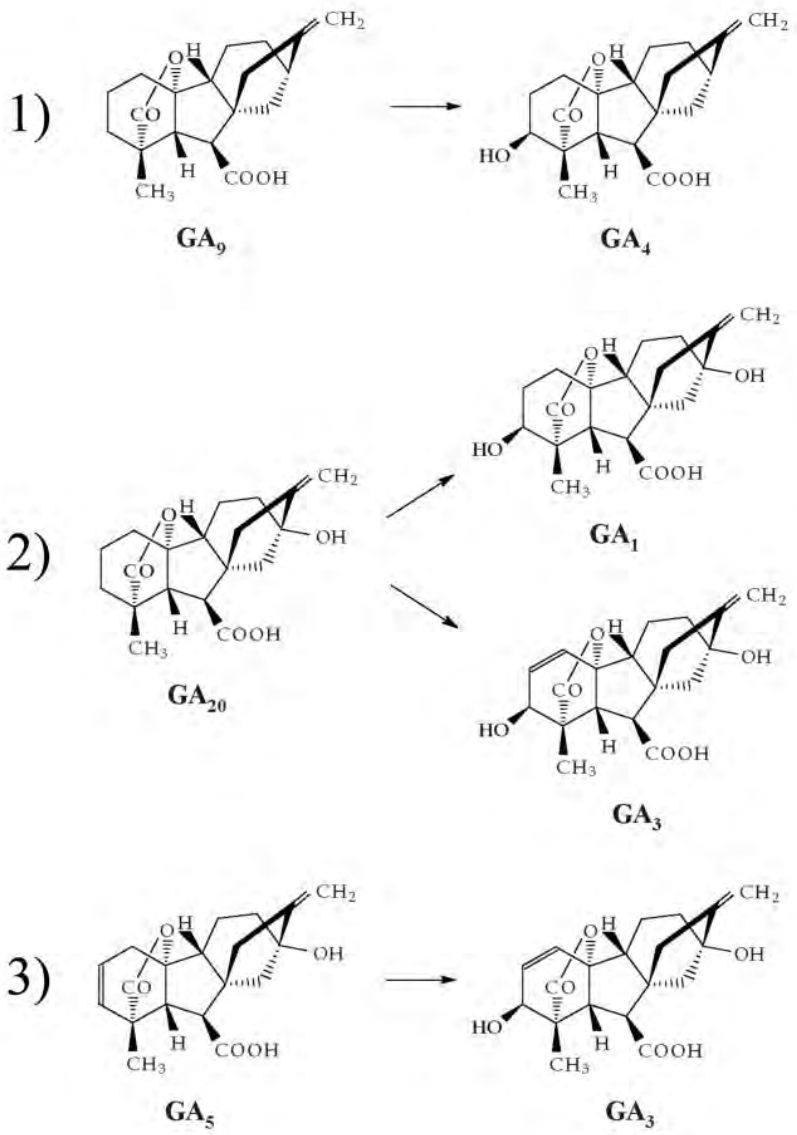

Figure 9. Recombinant D1 protein possesses four GA 3-oxidase activities *in vitro*.

Recombinant GST-D1 protein catalyzes the reaction from GA_9 to GA_4, GA_{20} to GA_1, GA_{20} to GA_3, and GA_5 to GA_3.

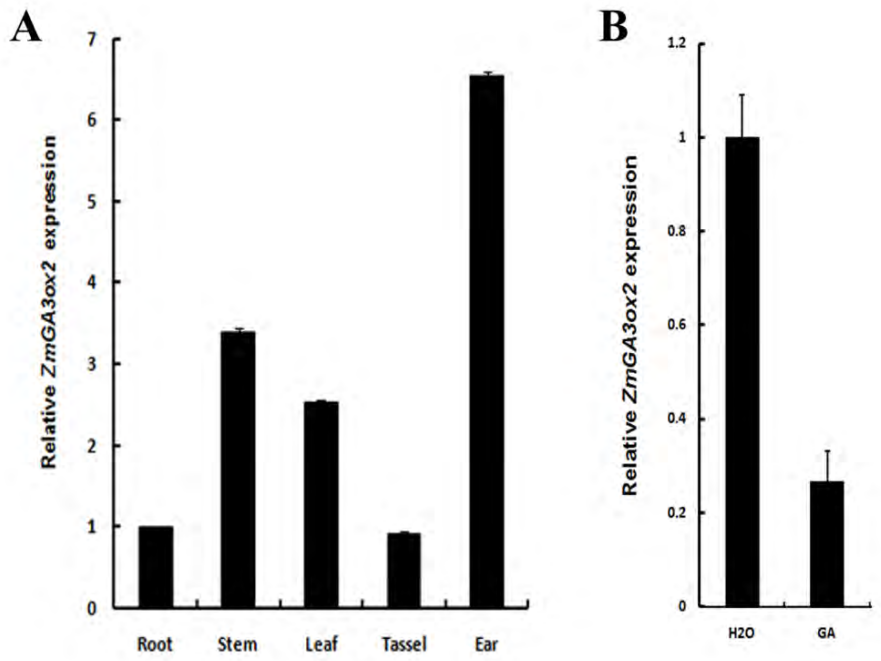

Figure 10. Expression of *ZmGA3ox2* in maize.

A. qRT-PCR analysis of *ZmGA3ox2* in different tissues. *Actin1* gene was reference gene.

B. qRT-PCR analysis of *ZmGA3ox2* expression in WT whole seedlings after treatment with GA_3. *Actin1* gene was reference gene. Error bar stands for SE.

3.6 Sites of D1 protein expression during maize female and male floret development

GA is believed to suppress the development of stamen primordia in the ear in light of the evidence that GA deficient or insensitive mutants show andromonoecy in maize (Dellaporta and Calderon-Urrea, 1994). But, it is unclear whether GA plays this function in a cell autonomous or non-autonomous fashion. To answer this question, determination of the sites of bioactive GA production in the female floret is necessary. However, because bioactive GAs exist at extremely low concentration in plant tissues and can be readily transported, detection of GAs in specific cells is difficult. As GA3ox catalyzes the final step in GA biosynthesis converting inactive GAs to active GAs, the location of GA3ox enzyme pinpoints the potential sites of bioactive GA production. Hence, I used the highly specific D1 antibody to detect the D1 protein expression sites in maize developing female florets through immunohistochemical analysis.

Maize WT and *d1* ears (about 1 to 1.5 cm long) were fixed in wax and sectioned. The D1 protein was recognized by D1 antibody labeled with Alexa-594 labeled secondary antibody. Positive signals were red under confocal laser scanning microscope (Fig. 11). The ear florets examined were mostly at bisexual stage. Red fluorescent signals were detected exclusively in the stamen regions of E1 and E2 florets in the WT spikelet (Fig. 11A to C), but not in the *d1* spikelet (Fig. 11G to I). The signals became much stronger at late developmental stage than at early stage (Fig. 11A, C). As the stamen primordia in ear florets have the abilities to differentiate and expand at early stage, it is possible that the increased stamen cells cause the signal become stronger at late stage. This result indicates that the D1 protein is specifically expressed in the stamen initials during female floret development. Thus, stamen cells in both E1 and E2 are the potential sites for the formation of bioactive GAs during sex determination. Taken together, the SEM and immunohistochemical analysis data strongly suggest that GAs suppress stamen primordium development in female floret in a cell-autonomous manner.

At the initial stage, maize tassel florets are bisexual similar to the ear florets. But during sex determination process, the stamen primordia survive and develop into functional organs while pistil

primordium in tassel floret is abolished through program cell death (Calderon-Urrea and Dellaporta, 1999), resulting in male florets in tassel. Mature tassel florets in *d1* mutant are male florets and the pollen are fertile. Hence, GA deficiency may have little effect on male floret development. I performed immunohistochemical analysis on WT tassel with D1 antibody to visualize the D1 location in tassel florets (Fig.12). No strong signal of D1 in tassel florets was detected from bisexual stage to unisexual stage (Fig.12A to 12C). Detection of Histone3 displayed strong signals indicating that the experiment system was reliable (Fig.12D to 12F). This result suggested that the accumulation of D1 in tassel florets is too low to reach the limit of detection.

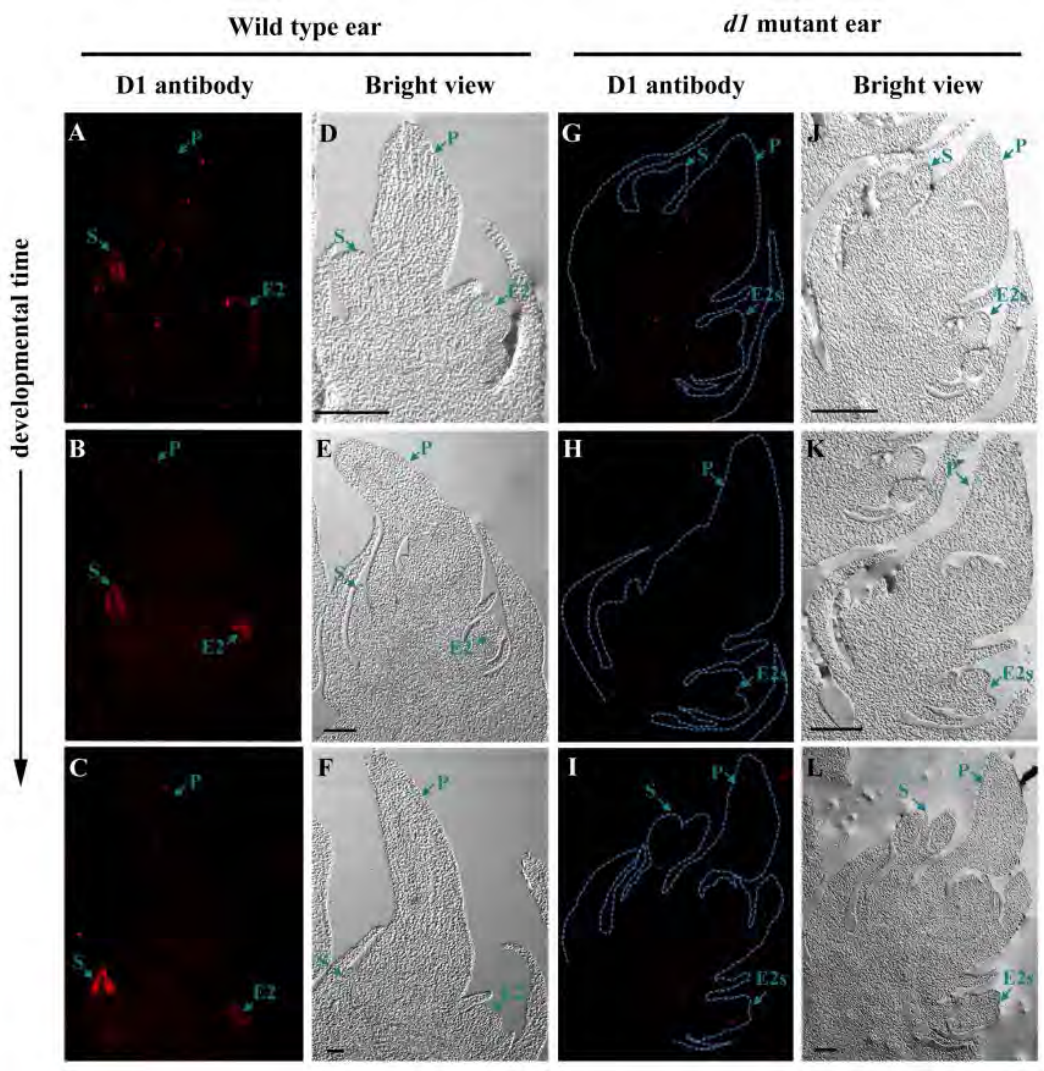

Figure 11. Immunohistochemical detection of D1 protein in developing maize female florets.

Sections of WT and *d1* ears from 1 cm to 1.5 cm were incubated with anti-D1 antibody and followed with secondary antibody labeled with Alexa-594 which produces red fluorescent signals under fluorescent microscope. **A-C**, WT female florets incubated with D1 antibody; **D-F**, bright views of A-C; **G-I**, *d1* female florets incubated with D1 antibody; **J-L**, bright views of G-I. S, stamen of E1 florets; P, pistil of E1 florets; E2, secondary ear florets; E2s, stamen of E2 florets. Bars, 200 μm.

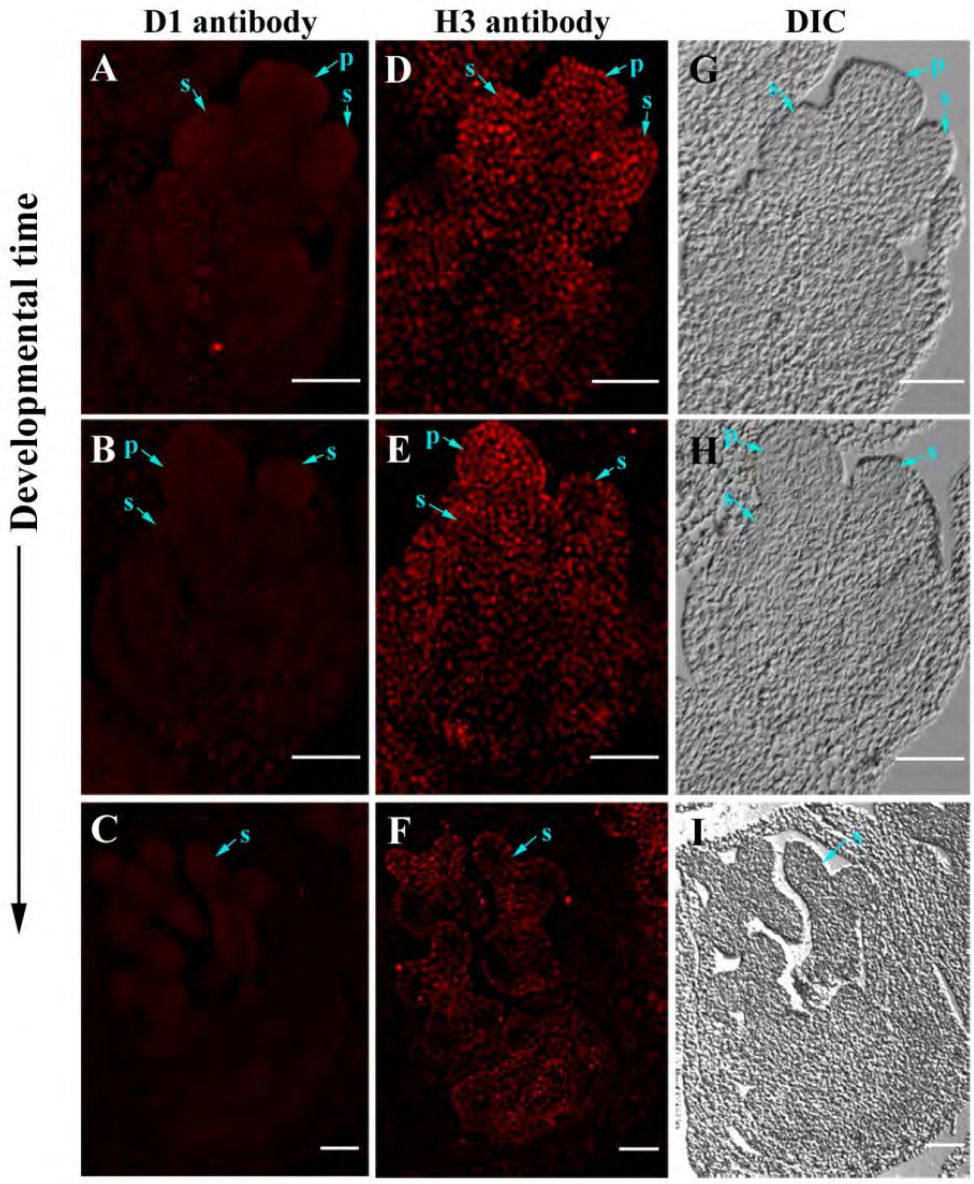

Figure 12. Immunohistochemical detection of D1 protein in developing maize male florets.

Sections of WT tassels from 1 cm to 1.5 cm were incubated with anti-D1 antibody and anti-H3 antibody followed with secondary antibody labeled with Alexa-594 which produces red fluorescent signals under fluorescent microscope. **A-C**, WT male florets incubated with D1 antibody; **D-F**, WT male florets incubated with H3 antibody; **G-I**, bright views of A-C and D-F. S, stamen; P, pistil. Developmental time arrow points to late stage. Bars, 100 μm.

3.7 Candidates of DELLA interacting proteins in maize

In maize, GAs regulate several aspects of growth and development through the downstream of DELLA protein. To screen DELLA interacting proteins, applications of yeast two hybrid (Y2H) were successful for screening *Arabidopsis* cDNA libraries (Feng et al., 2008; Hartwig et al., 2011; Park et al., 2013). Maize contains one DELLA protein rather than five DELLA proteins in *Arabidopsis*, which reduces the workload of identification of DELLA interacting protein candidates. As GAs regulate sex determination process in maize, I expected new DELLA-related regulators existed in the ear of maize. The genome of maize B73 inbreed line was sequenced and assembled. Thus, I isolated B73 mRNAs from the developing ears and constructed a cDNA library in yeast following the manufacturer's instruction (Clontech). Using D8 protein as the bait protein, more than 1 million independent clones were screened and 89 positive clones were isolated in the Y2H screening (Table 2, Fig.13). Sequence analysis showed that these clones covered 29 proteins. So far, 14 kinds of proteins have been confirmed as genuine interaction in yeast.

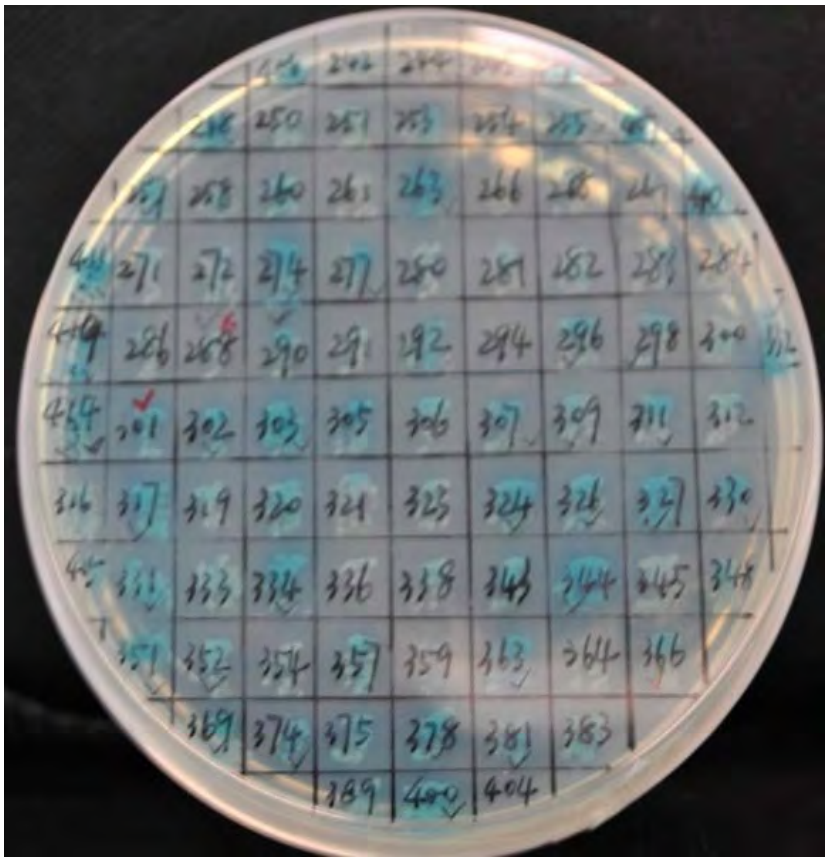

Figure 13. Results of Yeast two Hybrid screening. Blue colonies were positive interaction in yeast.

Table 2. Records of Yeast two hybrid screening

Colony No.	Positive (P) or Negative (N)	Colony No.	Positive (P) or Negative (N)	Colony No.	Positive (P) or Negative (N)	Colony No.	Positive (P) or Negative (N)	Colony No.	Positive (P) or Negative (N)
242	N	**244**	N	**245**	N	**248**	P	**250**	N
251	N	**253**	N	**254**	N	**255**	P	**257**	P
258	N	**260**	N	**261**	N	**263**	P	**266**	N
268	N	**269**	N	**271**	N	**272**	N	**274**	P
277	P	**280**	N	**281**	N	**282**	N	**283**	N
284	N	**286**	N	**288**	N	**290**	P	**291**	N
292	N	**294**	N	**296**	P	**298**	P	**300**	N
301	P	**302**	P	**303**	P	**305**	N	**306**	N
307	P	**309**	P	**311**	P	**312**	N	**316**	N
317	P	**319**	N	**320**	N	**321**	N	**323**	N
324	P	**326**	P	**327**	P	**330**	P	**331**	P
332	P	**333**	N	**334**	P	**336**	N	**338**	N
343	N	**344**	P	**345**	N	**348**	N	**351**	P
352	P	**354**	N	**357**	N	**359**	N	**363**	P
364	N	**366**	N	**369**	P	**374**	P	**375**	N
378	P	**381**	P	**383**	N	**389**	N	**400**	P
406	P	**408**	N	**409**	P	**410**	P	**411**	P
414	P	**419**	P						

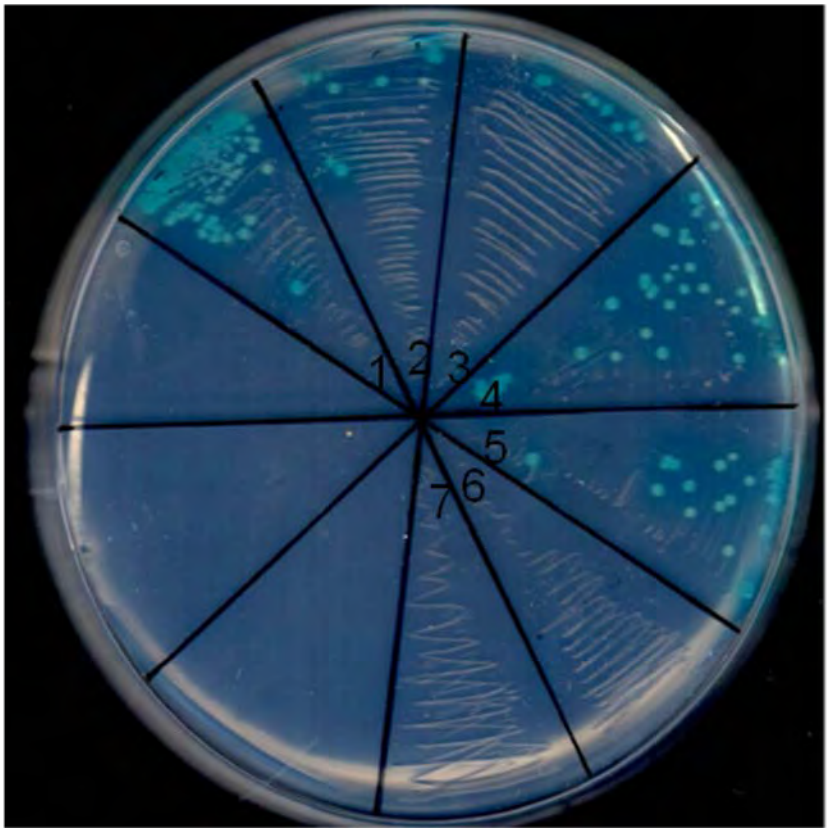

Figure 14. D8 interacts with ZmSPX1 in yeast. Blue colonies were yeast cells containing both D8 and ZmSPX1on the QDO/X/A plate (without Ade/His/Leu/Trp and supplemented with Aureobasidin A and X-α-Gal). 1,2,3,4 and 5 were yeast cells containing both pGBKT7-$D8^{C}$ and pGADT7-ZmSPX1 vectors; 6 and 7 were yeast cells containing both empty pGBKT7 and pGADT7-ZmSPX1 vectors.

3.8 Confirmation of candidate interaction proteins with D8

3.8.1 Subcellular localization of D8 and its interacting protein

To determine the D8 localization, D8-GFP fusion was constructed in pGWB5 and transiently expressed in tobacco epidermal cells. By confocal fluorescence microscopic analysis, GFP signals were detected in the nucleus (Fig. 15). Thus, D8 is localized in the nucleus, which is consistent to other DELLA proteins in *Arabidopsis* and rice (Silverstone et al. 2001; Itoh et al. 2002). Due to the location of D8, its interacting proteins should be also localized in the nucleus. Among those fourteen candidates found in Y2H, some of them were chosen to test first. ZmSPX1 interacting with D8 in yeast (Fig. 14) was found to be localized in the nucleus (Fig. 16).

The expression of ZmSPX1 was diverse in different tissues and the highest in the ear (Fig. 17). The expression of ZmSPX1 in the ear was much higher than in other tissue. This result leads me to speculate that ZmSPX1 may play important function in the developing ear.

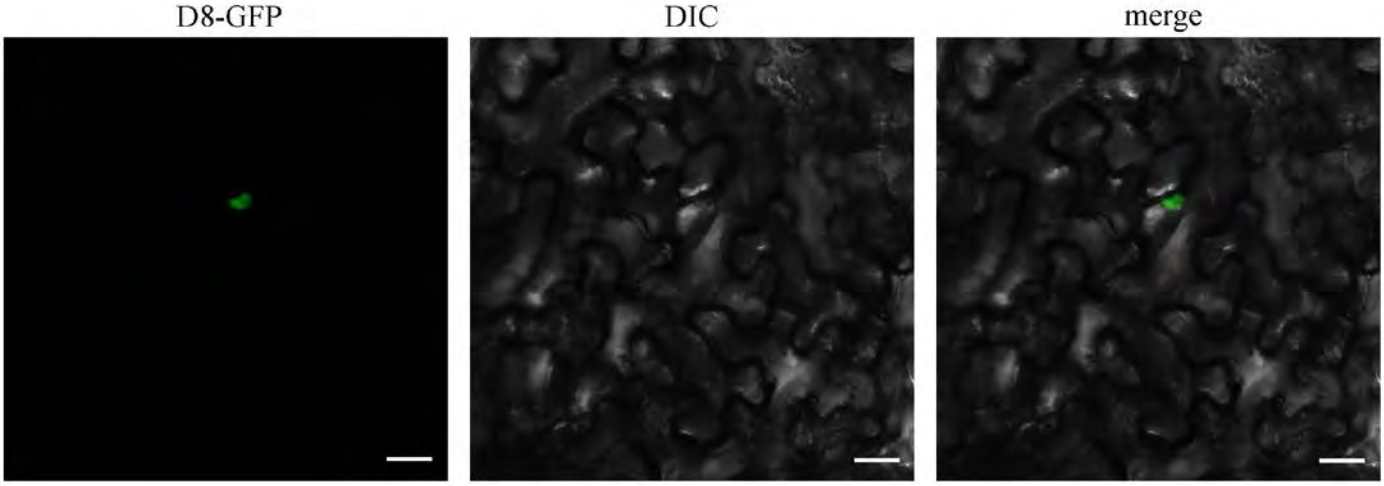

Figure 15. Subcellular Localization of D8 protein. D8-GFP signals were detected in the nucleus in tobacco epidermal cells. DIC, Differential Interference Contrast. Bars, 25 μm

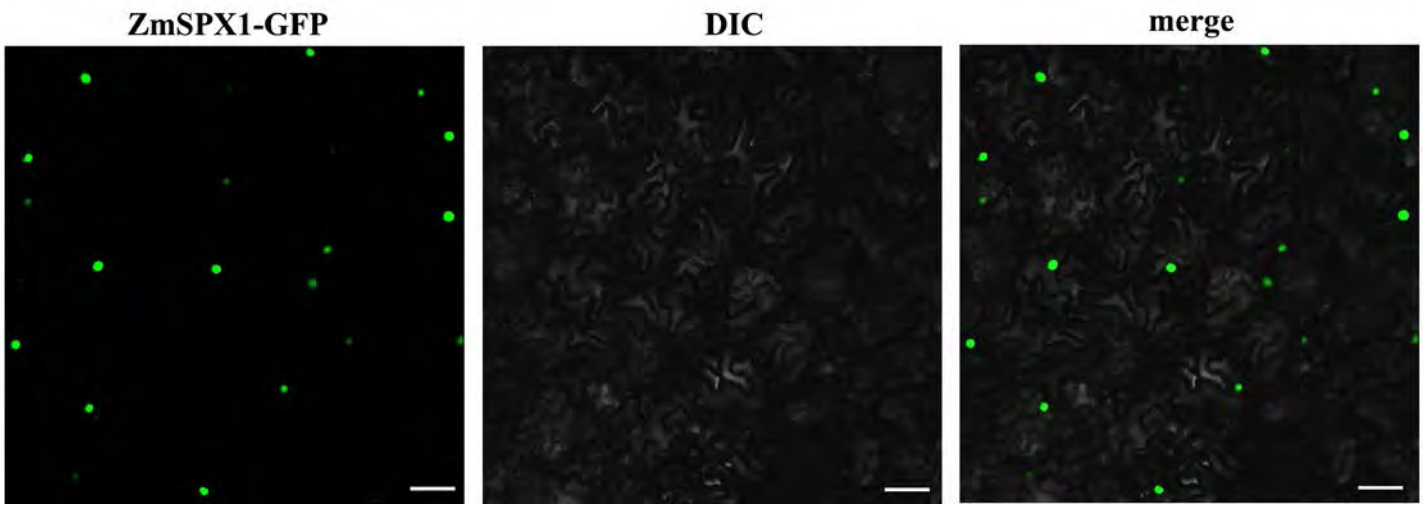

Figure 16. Subcellular Localization of ZmSPX1 protein. ZmSPX1-GFP signals are detected in the nucleus in tobacco epidermal cells. Bars, 50 μm.

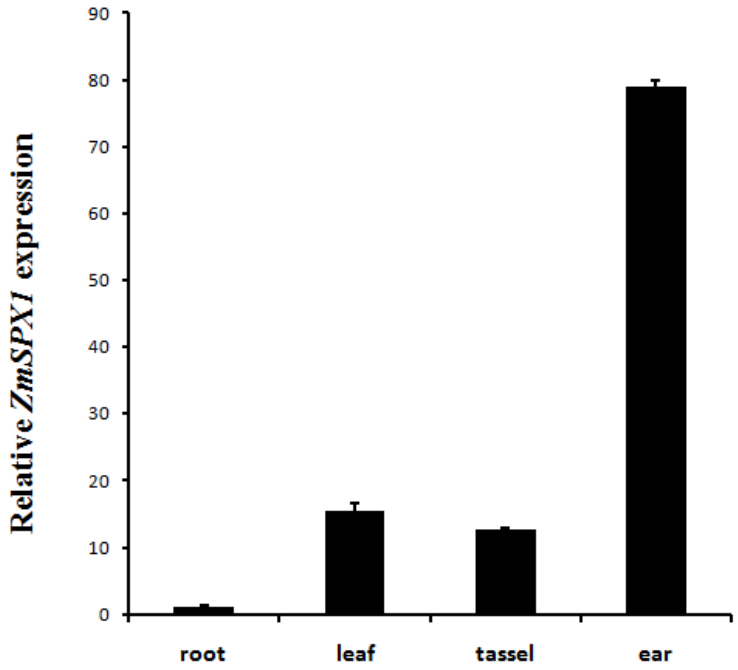

Figure 17. Expression of *ZmSPX1* in different tissuses. qRT-PCR analysis of ZmSPX1 in different tissue. *Actin1* gene was reference gene. Error bar stands for SE.

3.8.2 Bimolecular fluorescence complementation assays.

To confirm the interaction of ZmSPX1 and D8, full length cDNA of ZmSPX1 and D8 were cloned in binary pBiFC vectors containing the amino-terminal fragment of the eYFP fluorescent protein or carboxy-terminal fragments of the eCFP fluorescent protein ($eYFP^N$ and $eCFP^C$) (Gampala et al. 2007). All these constructs were transformed in *Agrobacterium tumefaciens*. All eight pairwise combinations of these constructs were mixed at an OD_{600} 1:1 ration and injected into 2-3-week-old tobacco epidermal cells. Green fluorescence signals can be observed in the nucleus under confocal microscope, if the two proteins interact with each other. Positive nuclear BiFC interaction signals in four combinations (YFP^N–D8 with CFP^C–ZmSPX1, YFP^N–D8 with ZmSPX1–CFP^C, CFP^C -D8 with YFP^N–ZmSPX1 and CFP^C -D8with YFP^N -ZmSPX1) were detected under confocal fluorescence microscope (Fig. 18). Meanwhile, full length cDNA of AGO was cloned in binary pBiFC vectors. The combinations between AGO and D8 were used as negative control for the BiFC assay. None of their combinations showed green fluorescence signal in tobacco epidermal cells. These results suggested that D8 and ZmSPX1 directly interacted *in vivo*.

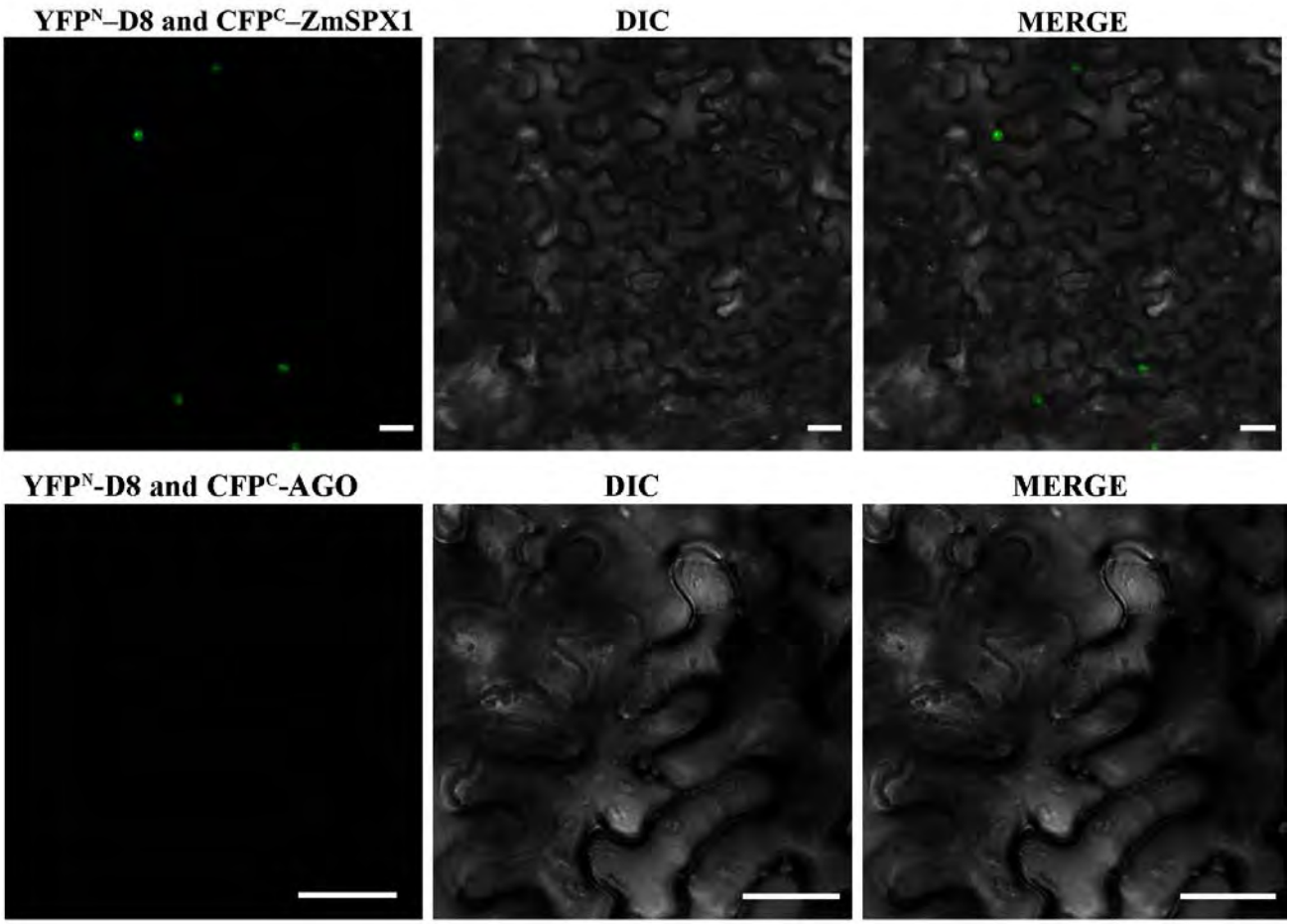

Figure 18. Bimolecular fluorescence complementation (BiFC) analysis of D8 and ZmSPX1.

Green fluorescence signals were displayed in the nucleus. YFP^N-D8, D8 fused with N-terminal of YFP; CFP^C-ZmSPX1, ZmSPX1 fused with C terminal of CFP; CFP^C-AGO, AGO protein fused with C terminal of CFP. CFP^C is modified from C-terminal of YFP in order to enhance fluorescence signal. Bars, 30 μm.

3.8.3 *In vitro* pull-down assays.

To verify the interaction between two proteins, independent evidences are convinced. For the case of ZmSPX1 and D8, a pull-down assay was employed. Full-length ZmSPX1 was tagged with MBP protein and D8 was tagged with GST protein. The GST-D8 bound Glutathione-Sepharose 4B beads were incubated with equal amount of MBP–ZmSPX1 and MBP proteins. After sufficient washing, the bound proteins were eluted by Glutathione and analyzed by western blot with MBP antibody. MBP-ZmSPX1 was recognized by anti-MBP antibody while MBP protein was not detected (Fig.19). This result strongly suggested that D8 protein interacted with ZmSPX1 *in vitro*.

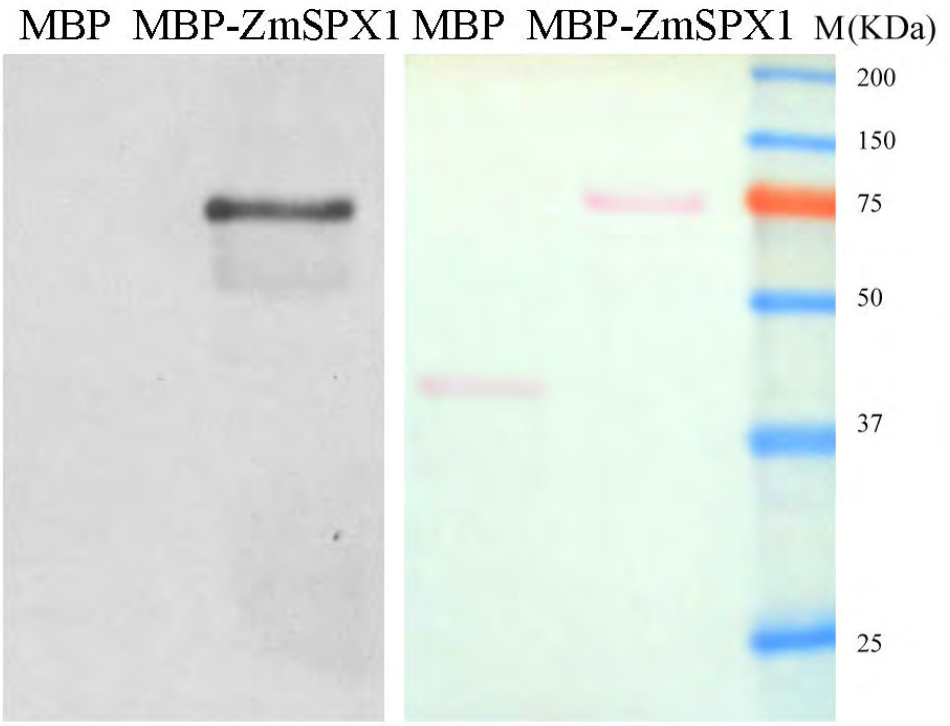

Figure 19. *In vitro* pull down assay of GST-D8 and MBP-ZmSPX1. Supernatant from the Sepharose beads incubated with GST-D8 and MBP or MBP-ZmSPX1 (left panel) was detected by anti-MBP antibody. The loading of MBP and MBP-ZmSPX1 was stained with Ponceau S (right panel). M, marker.

Chapter 4 Discussion

4.1 D1 encodes a GA 3-oxidase catalyzing the final step of GA biosynthesis in maize

A maize dwarf mutant was isolated by Prof. Tan during the study of *vp*3286*. Genetic analysis confirmed that this mutant is an allele of classic *d1* mutant. *d1* mutant has been known for decades (Fujioka et al., 1988; Phinney, 1956), but the causative gene had never been cloned. Based on previous biochemical results (Fujioka et al., 1988; Spray et al., 1996), two possibilities were put forward to account for the block of bioactive GA synthesis in the *d1* mutant. *D1* encodes either a GA 3-oxidase (ZmGA3ox) or a factor required for GA 3-oxidase function. Two putative GA 3-oxidases identified through analysis of the maize genome draft (version AGPv2) were *ZmGA3ox1* on chr 6L and *ZmGA3ox2* on chr 3S. The classic *d1* allele was mapped on chr 3S (Neuffer and England, 1995), thus making *ZmGA3ox2* as the most likely candidate gene for the *d1* mutation. By analyzing multiple alleles of *d1*, mutations in the *ZmGA3ox2* gene were identified in each allele. The newly isolated allele *d1-3286* carried insertion of a Copia-like retrotransposon; the *d1-4* allele contained a 487bp deletion that removed part of the first exon and part of the first intron. The *d1-6039* allele had a 1bp deletion that caused a frame-shift in the coding protein. The *d1-6016* allele contained a 2301bp deletion that removed most part of the gene with only 64bp left before stop codon (Fig. 5). The mutation of *ZmGA3ox2* in *d1-6016* was found by another group independently (Teng et al., 2013). *In vitro* enzymatic activity analysis confirmed that the recombinant ZmGA3ox2 protein catalyzed at least four reactions converting GA_9 to GA_4, GA_{20} to GA_1, GA_{20} to GA_3 and GA_5 to GA_3 (Fig. 9). This result indicated that ZmGA3ox2 catalyzed final step of bioactive GA formation, consistent to the previous biochemical analysis that the *d1* mutation blocked three steps in GA biosynthesis (Spray et al., 1996).

Although two GA 3-oxidases exist in maize, the severe GA-deficient phenotype of *d1* and the expression analysis indicates that the *D1* (*ZmGA3ox2*) gene plays a predominant role in GA biosynthesis throughout maize plant growth and development. I did not detect the expression of *ZmGA3ox1* in the major tissues and during plant development, implying that it may express at a low

level or localized to a few specific cells. In fact, Teng et al found *ZmGA3ox1* was expressed in young tassel (Teng et al., 2013). In contrast to four GA3ox genes in the Arabidopsis genome with overlapping functions (Hu et al., 2008b; Mitchum et al., 2006), the maize and rice genome contains two GA3ox genes with one predominantly expressed (Itoh et al., 2001). Thus, maize and rice may provide a simpler model than *Arabidopsis* in studying the bioactive GA biosynthesis and regulation.

4.2 Bioactive GA can be potentially synthesized in the nucleus and the cytosol

The compartment of bioactive GA synthesis inside the cell is believed to be in the cytosol based on two pieces of information, 1) GA3ox is a water soluble enzyme; 2) the predicted protein sequence does not contain any obvious targeting signals (Yamaguchi, 2008). Because D1 catalyzes the conversion of inactive intermediates to bioactive GAs, the localization of D1 protein defines the site of GA production. Analyses by two independent approaches, i.e. transient expression of D1-GFP and D1-GUS-GFP fusion *in vivo* and immunohistodetection of the endogenous D1 in subcellular fractions, revealed that D1 is localized in the nucleus and the cytosol (Fig. 6). The antibody was proven to be highly specific to D1 (Fig. 7), ruling out the possibility of false recognition. Formation of bioactive GA in the nucleus required its precursor. As GAs are hydrophobic molecules, it is possible that they can cross the plasma and the nuclear membrane by passive diffusion. Another possibility is that the substrate of D1 is synthesis in the nucleus. ZmGA20ox1 catalyzes the penultimate reaction of bioactive GA synthesis leading to the formation of the substrate of D1. I found that ZmGA20ox1 was dual localized to the nucleus and the cytosol as well (Fig.6D), which suggested that the precursor of bioactive GA may be synthesized in the nucleus and supported the hypothesis that bioactive GA was synthesized not only in the cytosol but also in the nucleus. Interestingly, the dual-localization of D1 coincides with the subcellular localization of GA receptor GID1. The rice GID1 which is also a soluble protein is primarily targeted to the nucleus where the proposed site of action is. However, GID1 was also detected in the cytosol (Ueguchi-Tanaka et al., 2005). Consistently, the *Arabidopsis* GA receptor AtGID1 was also dual-localized in the nucleus and the cytosol (Willige et al., 2007).

Hence, the potential GA production compartmentation as indicated by the D1 localization perfectly matches the compartmentation of GA perceptions. DELLA proteins that are the negative GA signal transduction components, however, are only found in the nucleus (Peng et al., 1997; Silverstone et al., 1998; Willige et al., 2007). The inconsistent localizations of the core components of GA signal transduction raise a few vital questions about the regulation of GA biosynthesis and signaling:

1. Why do the enzymes co-exist in two compartments?

2. How do cells regulate the expression of the enzymes between two compartments?

3. Will GA be transported between cytosol and nucleus?

4. Are there any new components of GA perception?

4.3 The suppression of stamen primordia in female florets is probably mediated by locally synthesized GAs

Due to the numerous GA intermediates and the trace of bioactive GAs, it is difficult to define the accurate cell types of GA biosynthesis by detecting bioactive GAs. Because GA3ox catalyzes the last step of bioactive GA formation, the location of GA3ox is an indicator to the sites of bioactive GA synthesis. In *Arabidopsis* developing flowers, GA3ox genes are expressed in stamen filament, anther and flower receptacles (Hu et al., 2008). In rice, the *OsGA3ox2* is highly expressed in stamen primordia and other floral organs such as glume, lemma, palea and pistil during floral development (Kaneko et al., 2003). These findings suggest that GA is widely synthesized in actively growing tissue during floral development in *Arabidopsis* and rice. In contrast to the promotion function in bisexual flower system, GA plays suppression function in unisexual flower system. For example, in maize, GA suppresses the development of stamen organs in female florets. Furthermore, the sites of GA biosynthesis do not pinpoint to the location of GA action. For instance, the development of petal in GA deficient mutant was defected but no GA3ox gene expression was detected in WT flowers in *Arabidopsis* (Hu et al., 2008). In order to find out whether the production of bioactive GA link to the

site of its action when it acts as a suppression signal, I defined the location of D1 in developing maize florets. Because the accumulation level of mRNA does not directly mean the presence of its product, a highly specific D1 antibody provided direct information about the location of D1. I detected the presence of D1 protein in stamen initials in E1 and E2 florets (Fig. 11). This result is consistent with the andromonoecy phenomenon where stamen growth and development are not suppressed in the E1 and E2 florets in *d1* mutant (Fig. 3E to H), suggesting that the suppression of stamen cell development in E1 and E2 florets requires synthesis of bioactive GA locally.

Maize *d8* mutant with impaired DELLA protein displays similar andromonoecious phenotype as *d1* (Peng et al., 1999; Dellaporta and Calderon-Urrea, 1994), indicating that GA regulates the stamen primordium development through DELLA proteins. The sites of D1 expression in female floret are precisely the sites where the *WEE1* gene is expressed (Kim et al., 2007). WEE1 is a negative regulator of cell cycle in maize. Thus, the identification of downstream components of DELLA proteins which are able to induce *WEE1* gene expression will be great help for understanding the underlying mechanism of GA function in sex determination in maize.

4.4 GA antagonistic to JA in regulating stamen development in the tassel

D1 was highly expressed in the stamen cells of ear florets indicating that locally synthesized GA is necessary for the suppression of stamen cell development in the ear florets. In other hand, the expression of D1 in tassel florets is too low to be detected (Fig.12). Considering the effect of GA on the suppression of stamen cells in the ear, the low concentration of GA in tassel (Nickerson, 1959), I speculated that high level of bioactive GAs was necessary for the suppression of the stamen development in tassel florets. Nonetheless, the effects of exogenous GA on the tassel included two aspects: the development of pistil and the suppression of stamen (Nickerson, 1959). Interestingly, the florets in the tassel of *tasselseed* (*ts*) mutant were converted to female florets similar to the application of GA (Dellaporta and Calderon-Urrea, 1994). The *ts1*and *ts2* mutant can be rescued by JA treatment (Acosta et al., 2009). Thus, GA and JA act antagonistically in regulating stamen cell development

(Dellaporta and Calderon-Urrea, 1994). This antagonism may be indirect or direct though affecting the synthesis and/or the signaling. GA regulates the stamen development in the ear through suppressing the function of DELLA protein (Dellaporta and Calderon-Urrea, 1994; Peng, 1999).

In *Arabidopsis*, GA and JA crosstalk with each other through direct interaction of their core signaling components DELLA (GA) and JAZs (JA) (Hou et al., 2010).Inspired by this model, I proposed a model here to explain how GA and JA crosstalk to regulate stamen and pistil development in the tassel floret. GA suppresses stamen development via an unknown factor (protein A) restricted by DELLA, while JA suppresses pistil development via an unknown factor (protein B) restricted by JAZs (Fig.20). When GA level is low, abundant DELLAs will compete with protein B to bind with JAZs resulting in some free protein Bs to suppress pistil development. When JA level is low, JAZs are accumulated and bind with some DELLAs, which releasing some free protein As from DELLAs to suppress stamen development. The identification of protein A and protein B will be a breakthrough for unraveling the regulation mechanism of maize sex determination.

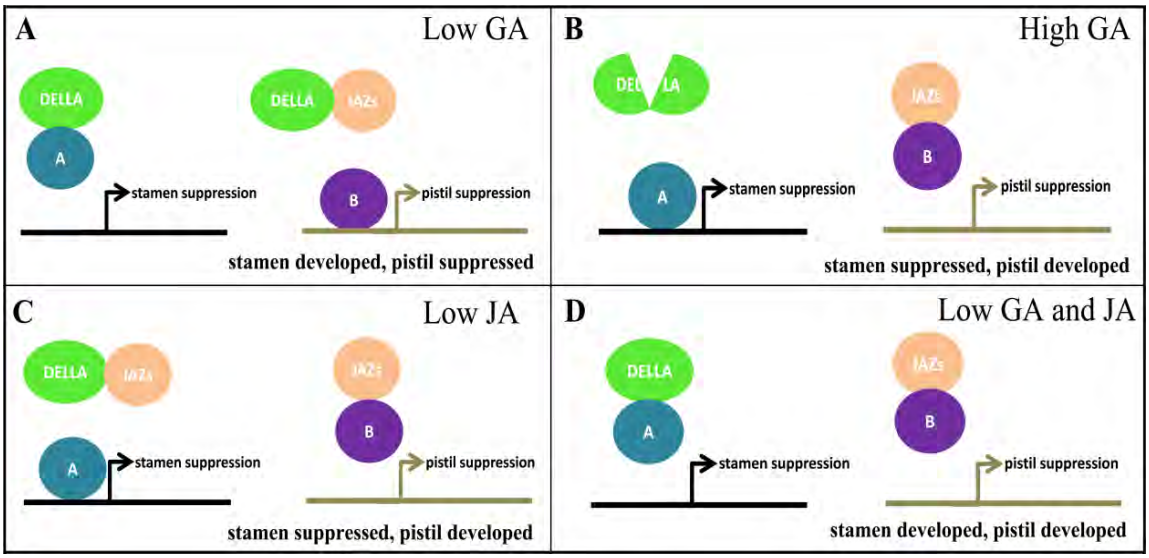

Figure 20. The model of GA and JA crosstalk by direct interaction in regulating tassel floret development.

A. When GA level is low (e.g. WT and *d1* mutant), abundant DELLA proteins interact with protein A resulting in stamen development; and compete with protein B to interact with JAZs so that residual protein Bs induce pistil suppression.

B. When GA level is high (e.g. exogenous GA in WT tassel), DELLAs are degraded and protein As are released from DELLAs to induce the stamen suppression; in other hand, protein Bs binds to JAZs so that pistils develop in the tassel florets.

C. When JA level is low (e.g. *ts1*or *ts2* mutant), abundant JAZs restrict protein B's function to induce pistil suppression; and competitively bind with DELLAs so that some free protein As are released from DELLAs to suppress pistil development.

D. When both GA and JA levels are low (e.g. *ts2* and *d1* double mutant), accumulated DELLAs and JAZs restrict protein A and protein B respectively, so that the development of stamen and pistil is not suppressed.

4.5 An active Copia-like element may exist in the progenitors of *d1-3286* allele

Transposable elements contain two types: DNA-type transposon and retrotransposon. Transposable elements are abundant in maize genome and seem to be genetic regulated (Tan et al., 2011). In maize, retrotransposon accounts for more than 80% of the genome (Baucom et al., 2009; Schnable et al., 2009). As the vast majority of retrotransposable elements are kept quiescent by epigenetic regulation (Slotkin and Martienssen, 2007), active retrotransposons are rarely found in nature (Picault et al., 2009). However, they can be re-activated by means of stresses and 'genomic shock' (McClintock, 1984). *Tos17* is an well-known active retrotransposon which can be reactivated in rice during tissue culture (Hirochika, 2001). It was observed that the line *vp*3286* that produced the *d1-3286* allele had a higher mutagenic activity than similar lines derived from the active Mutator line. In addition to the *d1-3286* mutation, a big embryo allele was isolated from the selfed *vp*3286* lines (Suzuki M., et al., unpublished). Hence, an active retrotransposon such as the one identified in the *d1-3286* allele would be a plausible explanation for the high mutagenic activity in the *vp*3286* line. Active transposons were widely utilized to construct mutant library, such as the utilization of *Tos17*, *Ac*/*Ds* and *Mu*/*MuDR*. The new Copia-like element found in *d1-3286* may be developed to be a new tool for creating mutants.

4.6 The quality of maize cDNA library is satisfied for screening

To find a real interacting protein of a target protein, a high quality cDNA library is the most important element. The factors for evaluating the quality of a cDNA library include the size distribution of double strand cDNA, the composition of clones and the titer of the library. Considering the results of Y2H, BiFC and pull down assay, ZmSPX1 is an genuine interacting protein of D8. This successful case indicated the quality of this cDNA library constructed by myself is good enough for application. ZmSPX1 is a newly identified DELLA interacting protein which has not been reported in public. The biological function of ZmSPX1 remains unknown and is waiting to be analyzed. In addition, some

proteins identified in this screen are worth to further studying as they belong to important protein families.

4.7 The potential function of ZmSPX1

SPX domain was named after three proteins Suppressor of Yeast gpa1 (Syg1), the yeast Phosphatase 81 (Pho81) and the human Xenotropic and Polytropic Retrovirus receptor 1 (Xpr1) (Reviewed by Secoo et al., 2011). In yeast, SPX domain related to cell cycle regulation (Schneider et al., 1994). In rice and *Arabidopsis*, SPX proteins related to the regulation of Pi homeostasis (Duan et al., 2008). However, the regulation mechanism is not clear. In maize, the biological function of SPX proteins has not been reported. Microarray data found that the expression of ZmSPX1 was significantly reduced under nitrogen (N) and phosphate deprivation in maize (Schluter et al., 2012). However, I found that the expression of ZmSPX1 was increased under nitrate deprivation. In other hand, ZmSPX1 should be responsive to GA as it interacts with D8. Thus, ZmSPX1 may involve to a regulation mechanism integrated by GA and N deficiency. To dig out the function of ZmSPX1, it is necessary to obtain the mutant of ZmSPX1. However, it is not easy to observe its phenotype if the mutant is a weak allele of *spx*. As the expression of ZmSPX1 is the highest in the developing ear, it may functions in the ear development. In maize, N deficiency affect the grain yield by reducing the ear size and the biomass accumulation. Meanwhile, ears and seeds of *d1* mutant are much smaller than WT. Considering these information, I speculated that ZmSPX1 may relate to cell expansion during ear development, which was regulated by GA and N.

Reference

Acosta, I.F., Laparra, H., Romero, S.P., Schmelz, E., Hamberg, M., Mottinger, J.P., Moreno, M.A., and Dellaporta, S.L. (2009). tasselseed1 is a lipoxygenase affecting jasmonic acid signaling in sex determination of maize. *Science* 323, 262-265.

Appleford, N. E., Evans, D. J., Lenton, J. R., Gaskin, P., Croker, S. J., Devos,K.M. Phillips, A. L., Hedden, P. (2006). Function and transcript analysis of gibberellin-biosynthetic enzymes in wheat. *Planta* 223, 568-582.

Arnaud, N., Girin, T., Sorefan, K., Fuentes, S., Wood, T.A., Lawrenson, T., Sablowski, R., and Ostergaard, L. (2010). Gibberellins control fruit patterning in Arabidopsis thaliana. *Genes Dev* 24, 2127-2132.

Baucom, R.S., Estill, J.C., Chaparro, C., Upshaw, N., Jogi, A., Deragon, J.M., Westerman, R.P., Sanmiguel, P.J., and Bennetzen, J.L. (2009). Exceptional diversity, non-random distribution, and rapid evolution of retroelements in the B73 maize genome. *PLoS genetics* 5, e1000732.

Bensen, R. J., Johal, G. S., Crane, V. C., Tossberg, J. T., Schnable, P. S., Meeley, R. B., Briggs, S. P. (1995). Cloning and characterization of the maize An1 gene. *Plant Cell* 7, 75-84.

Calderon-Urrea, A., and Dellaporta, S.L. (1999). Cell death and cell protection genes determine the fate of pistils in maize. *Development 126*, 435-441.

Cheng H, Qin LJ, Lee SC, Fu XD, Richards DE, Cao DN, Luo D, Harberd NP, Peng JR. (2004). Gibberellin regulates Arabidopsis floral development via suppression of DELLA protein function. *Development* 131, 1055–1064.

Chiang, H.H., Hwang, I., and Goodman, H.M. (1995). Isolation of the Arabidopsis GA4 locus. *Plant Cell* 7, 195-201.

Daviere, J.M., de Lucas, M., Prat, S. (2008).Transcriptional factor interaction: a central step in DELLA function. *Curr Opin Genet Dev* 18, 296–303.

Dellaporta, S.L. and Calderon-Urrea, A. (1994). The sex determination process in maize. *Science* 266, 1501-1505.

DeLong, A., Calderon-Urrea, A., Dellaporta, S.L. (1993). Sex determination gene TASSELSEED2 of maize encodes a short-chain alcohol dehydrogenase required for stage-specific floral organ abortion. *Cell* 74, 757-768.

de Lucas, M., Daviere, J.M., Rodriguez-Falcon, M., Pontin, M., Iglesias-Pedraz, J.M., Lorrain, S., Fankhauser, C., Blazquez, M.A., Titarenko, E., and Prat, S. (2008). A molecular framework for light and gibberellin control of cell elongation. *Nature* 451, 480-484.

Duan K., Yi K., Dang L., Huang H., Wu W., Wu P. (2008). Characterization of a sub-family of Arabidopsis genes with the SPX domain reveals their diverse functions in plant tolerance to phosphorus starvation. *Plant J* 54: 965–975.

Feng, S., Martinez, C., Gusmaroli, G., Wang, Y., Zhou, J., Wang, F., Chen, L., Yu, L., Iglesias-Pedraz, J.M., Kircher, S., *et al.* (2008). Coordinated regulation of Arabidopsis thaliana development by light and gibberellins. *Nature* 451, 475-479.

Fujioka,S., Yamane, H., Spray, C.R., Katsumi, M., Phinney, B.O., Gaskin, P., Macmillan, J., Takahashi, N. (1988). The dominant non-gibberellin-responding dwarf mutant (D8) of maize accumulates native gibberellins. *Proc Natl Acad Sci U.S.A.* 85,9031-9035.

Fujioka, S., Yamane, H., Spray, C.R., Gaskin, P., MacMillan, J., Phinney, B.O., and Takahashi, N. (1988). Qualitative and Quantitative Analyses of Gibberellins in Vegetative Shoots of Normal, dwarf-1, dwarf-2, dwarf-3, and dwarf-5 Seedlings of Zea mays L. *Plant Physiol* 88, 1367-1372.

Gampala, S.S., Kim, T.W., He, J.X., Tang, W., Deng, Z., Bai, M.Y., Guan, S., Lalonde, S., Sun, Y., Gendron, J.M., Chen, H., Shibagaki, N., Ferl, R.J., Ehrhardt, D., Chong, K., Burlingame, A.L., Wang, Z.Y. (2007). An essential role for 14-3-3 proteins in brassinosteroid signal transduction in Arabidopsis. *Dev Cell* 13, 177-189.

Grebenok, R.J., Pierson, E., Lambert, G.M., Gong, F.C., Afonso, C.L., Haldeman-Cahill, R., Carrington, J.C., and Galbraith, D.W. (1997). Green-fluorescent protein fusions for efficient characterization of nuclear targeting. *Plant J* 11, 573-586.

Harberd, N.P., and Freeling, M. (1989). Genetics of dominant gibberellin-insensitive dwarfism in maize. *Genetics* 121, 827-838.

Hartweck, L.M., and Olszewski, N.E. (2006). Rice GIBBERELLIN INSENSITIVE DWARF1 is a gibberellin receptor that illuminates and raises questions about GA signaling. *Plant Cell* 18, 278-282.

Hartwig, T., Chuck, G. S., Fujioka, S., Klempien, A., Weizbauer, R., Potluri, D. P., Choe, S., Johal, G. S., Schulz, B. (2011). Brassinosteroid control of sex determination in maize. *Proc.Natl.Acad.Sci.USA* 108, 19814-19819.

Hedden, P. and Phillips, A. L. (2000). Gibberellin metabolism: new insights revealed by the genes. *Trends Plant Sci* 5,523-30.

Helliwell, C.A., Chandler, P.M., Poole, A., Dennis, E.S., and Peacock, W.J. (2001). The CYP88A cytochrome P450, ent-kaurenoic acid oxidase, catalyzes three steps of the gibberellin biosynthesis pathway. *Proc. Natl. Acad.Sci.USA* 98, 2065-2070.

Hirochika, H. (2001). Contribution of the Tos17 retrotransposon to rice functional genomics. *Curr Opin Plant Biol* 4, 118-122.

Hong, G.J., Xue, X.Y., Mao, Y.B., Wang, L.J., and Chen, X.Y. (2012). Arabidopsis MYC2 Interacts with DELLA Proteins in Regulating Sesquiterpene Synthase Gene Expression. *Plant Cell* 24, 2635-2648.

Hou, X., Lee, L.Y., Xia, K., Yan, Y., and Yu, H. (2010). DELLAs modulate jasmonate signaling via competitive binding to JAZs. *Dev Cell* 19, 884-894.

Hu, J., Mitchum, M.G., Barnaby, N., Ayele, B.T., Ogawa, M., Nam, E., Lai, W.C., Hanada, A., Alonso, J.M., Ecker, J.R., *et al.* (2008). Potential sites of bioactive gibberellin production during reproductive growth in Arabidopsis. *Plant Cell* 20, 320-336.

Ikeda, A., Ueguchi-Tanaka, M., Sonoda, Y., Kitano, H., Koshioka, M., Futsuhara, Y., Matsuoka, M., and Yamaguchi, J. (2001). slender rice, a constitutive gibberellin response mutant, is caused by a null mutation of the SLR1 gene, an ortholog of the height-regulating gene GAI/RGA/RHT/D8. *Plant Cell* 13, 999-1010.

Inoue, H., H. Nojima, and H. Okayama. (1990). High efficiency transformation of Escherichia coli with plasmids. *Gene* 96:23-28.

Itoh, H., Ueguchi-Tanaka, M., Sentoku, N., Kitano, H., Matsuoka, M., and Kobayashi, M. (2001). Cloning and functional analysis of two gibberellin 3 beta -hydroxylase genes that are differently expressed during the growth of rice. *Proc.Natl.Acad.Sci.USA* 98, 8909-8914.

Itoh, H., Ueguchi-Tanaka, M., Sato, Y., Ashikari, M., Matsuoka, M. (2002). The Gibberellin Signaling Pathway Is Regulated by the Appearance and Disappearance of SLENDER RICE1 in Nuclei. *Plant Cell* 14, 57-70.

Kaneko, M., Itoh, H., Inukai, Y., Sakamoto, T., Ueguchi-Tanaka, M., Ashikari, M., and Matsuoka, M. (2003). Where do gibberellin biosynthesis and gibberellin signaling occur in rice plants? *Plant J* 35, 104-115.

Kim, J.C., Laparra, H., Calderon-Urrea, A., Mottinger, J.P., Moreno, M.A., and Dellaporta, S.L. (2007). Cell cycle arrest of stamen initials in maize sex determination. *Genetics* 177, 2547-2551.

Kumar, S., Tamura, K., and Nei, M. (2004). MEGA3: Integrated software for Molecular Evolutionary Genetics Analysis and sequence alignment. *Brief Bioinform* 5, 150-163.

Lang A. 1957. The effect of gibberellin upon flower formation. *Proc Natl Acad Sci USA* 43, 709–717.

Lester, D.R., Ross, J.J., Davies, P.J., and Reid, J.B. (1997). Mendel's stem length gene (Le) encodes a gibberellin 3 beta-hydroxylase. *Plant Cell* 9, 1435-1443.

Magome, H., Nomura, T., Hanada, A., Takeda-Kamiya, N., Ohnishi, T., Shinma, Y., Katsumata, T., Kawaide, H., Kamiya, Y., Yamaguchi, S. (2013). CYP714B1 and CYP714B2 encode gibberellin 13-oxidases that reduce gibberellin activity in rice. *Proc. Natl. Acad. Sci. USA* 110, 1947-1952.

Martin, D.N., Proebsting, W.M., and Hedden, P. (1997). Mendel's dwarfing gene: cDNAs from the Le alleles and function of the expressed proteins. *Proc.Natl.Acad.Sci.USA* 94, 8907-8911.

McClintock, B. (1984). The significance of responses of the genome to challenge. *Science* 226, 792-801.

Mitchum, M.G., Yamaguchi, S., Hanada, A., Kuwahara, A., Yoshioka, Y., Kato, T., Tabata, S., Kamiya, Y., and Sun, T.P. (2006). Distinct and overlapping roles of two gibberellin 3-oxidases in Arabidopsis development. *Plant J* 45, 804-818.

Neuffer, M.G., and England, D. (1995). Induced mutations with confirmed locations. *Maize Genetics Cooperation Newsletter* 69, 43-46.

Nickerson, N.H. (1959).Sustained treatment with gibberellic acid of five different kinds of maize. *Annals of the missouri botanical garden* 46, 19-37.

Olszewski N, Sun TP, Gubler F. (2002). Gibberellin signaling: biosynthesis, catabolism, and response pathways. *Plant Cell* 14, S61-80.

Pandey A., Choudhary M.K., Bhushan D., Chattopadhyay A., Chakraborty S., Datta A., Chakraborty N. (2006). The nuclear proteome of chickpea (Cicer arietinum L.) reveals predicted and unexpected proteins. *J Proteome Res* 5, 3301-3311.

Park, J., Nguyen, K. T., Park, E., Jeon, J. S., Choi, G. (2013). DELLA proteins and their interacting RING Finger proteins repress gibberellin responses by binding to the promoters of a subset of gibberellin-responsive genes in Arabidopsis. *Plant Cell* 25, 927-943.

Peng, J., Carol, P., Richards, D.E., King, K.E., Cowling, R.J., Murphy, G.P., and Harberd, N.P. (1997). The Arabidopsis GAI gene defines a signaling pathway that negatively regulates gibberellin responses. *Genes Dev* 11, 3194-3205.

Peng, J., Richards, D.E., Hartley, N.M., Murphy, G.P., Devos, K.M., Flintham, J.E., Beales, J., Fish, L.J., Worland, A.J., Pelica, F., *et al.* (1999). 'Green revolution' genes encode mutant gibberellin response modulators. *Nature* 400, 256-261.

Phinney, B.O. (1956). GROWTH RESPONSE OF SINGLE-GENE DWARF MUTANTS IN MAIZE TO GIBBERELLIC ACID. *Proc.Natl.Acad.Sci.USA* 42, 185-189.

Picault, N., Chaparro, C., Piegu, B., Stenger, W., Formey, D., Llauro, C., Descombin, J., Sabot, F., Lasserre, E., Meynard, D., *et al.* (2009). Identification of an active LTR retrotransposon in rice. *Plant J* 58, 754-765.

Pike, L.M. and Peterson, C.E. (1969). Gibberellin A4/A7, for induction of staminate flowers on the gynoecious cucumber (*Cucumis sativus L.*). *Euphytica* 18, 106–109.

Rood, S.B. and Pharis, R.P. (1980). Changes of endogenous gibberellin-like substances with sex reversal of the apical inflorescence of corn. *Plant Physiol* 66, 793-796.

Saitou, N. and Nei, M. (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol* 4, 406-25.

Sanders PM, Bui AQ, Weterings K, McIntire KN, Hsu YC, Lee PY, Truong MT, Beals TP, Goldberg RB. (1999). Anther developmental defects in Arabidopsis thaliana male-sterile mutants. *Sex Plant Reprod* 11, 297–322.

Sasaki, A., Ashikari, M., Ueguchi-Tanaka, M., Itoh, H., Nishimura, A., Swapan, D., Ishiyama, K., Saito, T., Kobayashi, M., Khush, G.S., *et al.* (2002). Green revolution: a mutant gibberellin-synthesis gene in rice. *Nature* 416, 701-702.

Schnable, P.S., Ware, D., Fulton, R.S., Stein, J.C., and al., e. (2009). The B73 maize genome: complexity, diversity, and dynamics. *Science* 326, 1112-1115.

Schneider, K.R., Smith, R.L., O'Shea, E.K. (1994). Phosphate-regulated inactivation of the kinase PHO80-PHO85 by the CDK inhibitor PHO81. *Science* 266,122-126.

Secco, D., Wang, C., Arpat, B. A., Wang, Z., Poirier, Y., Tyerman, S. D., Wu, P., Shou, H., Whelan, J. (2012). The emerging importance of the SPX domain-containing proteins in phosphate homeostasis. *New Phytol* 193, 842-51.

Silverstone, A.L., Ciampaglio, C.N., and Sun, T. (1998). The Arabidopsis RGA gene encodes a transcriptional regulator repressing the gibberellin signal transduction pathway. *Plant Cell* 10, 155-169.

Silverstone, A.L., Jung, H.S., Dill, A., Kawaide, H., Kamiya, Y., Sun, T.P. (2001). Repressing a repressor: gibberellin-induced rapid reduction of the RGA protein in Arabidopsis. *Plant Cell* 13, 1555-1566.

Slotkin, R.K., and Martienssen, R. (2007). Transposable elements and the epigenetic regulation of the genome. *Nature reviews Genetics* 8, 272-285.

Sponsel, V.M., and Hedden, P. (2010). Gibberellin Biosynthesis and Inactivation. *In* "Plant Hormones: Biosynthesis, Signal Transduction, Action!" Peter J. Davies (*ed*), Springer, New York. 63-94.

Spray, C.R., Kobayashi, M., Suzuki, Y., Phinney, B.O., Gaskin, P., and MacMillan, J. (1996). The dwarf-1 (dt) Mutant of Zea mays blocks three steps in the gibberellin-biosynthetic pathway. *Proc.Natl.Acad.Sci.USA* 93, 10515-10518.

Spielmeyer, W., Ellis, M., Robertson, M., Ali, S., Lenton, J. R., Chandler, P. M. (2004). Isolation of gibberellin metabolic pathway genes from barley and comparative mapping in barley, wheat and rice. *Theor Appl Genet* 109,847-855.

Stinard, P.S. (2009). d4 is allelic to d1. *Maize Genetics Cooperation Newsletter 83*, 51-51.

Stowe, B.B., Yamaki, T. (1957). The history and physiological action of the gibberellins. *Annual Review of Plant Physiology* 8,181-216.

Sun, X., Jones, W.T., Harvey, D., Edwards, P.J., Pascal, S.M., Kirk, C., Considine, T., Sheerin, D.J., Rakonjac, J., Oldfield, C.J., et al. (2010). N-terminal domains of DELLA proteins are intrinsically unstructured in the absence of interaction with GID1/gibberellic acid receptors. *J Biol Chem* 285, 11557-11571.

Sun, T.P. (2011). The molecular mechanism and evolution of the GA-GID1-DELLA signaling module in plants. *Curr Biol* 21, R338-R345.

Sun, T.P., and Kamiya, Y. (1994). The Arabidopsis GA1 locus encodes the cyclase ent-kaurene synthetase A of gibberellin biosynthesis. *Plant Cell* 6, 1509-1518.

Tan, B.C., Chen, Z., Shen, Y., Zhang, Y., Lai, J., and Sun, S.S. (2011). Identification of an active new mutator transposable element in maize. *G3 (Bethesda)* 1, 293-302.

Tan, B.C., Schwartz, S.H., Zeevaart, J.A., and McCarty, D.R. (1997). Genetic control of abscisic acid biosynthesis in maize. *Proc.Natl. Acad.Sci. U.S.A.* 94, 12235-12240.

Teng F., Zhai L., Liu R., Bai W., Wang L., Huo D., Tao Y., Zheng Y., Zhang Z. (2013). ZmGA3ox2, a candidate gene for a major QTL, qPH3.1, for plant height in maize. *Plant J* 73, 405-416.

Ueguchi-Tanaka, M., Ashikari, M., Nakajima, M., Itoh, H., Katoh, E., Kobayashi, M., Chow, T.Y., Hsing, Y.I., Kitano, H., Yamaguchi, I., *et al.* (2005). GIBBERELLIN INSENSITIVE DWARF1 encodes a soluble receptor for gibberellin. *Nature* 437, 693-698.

WEAVER R.J. (1976). Grape growing. John Wiley & Sons Inc, San Francisco, CA, USA. 384 pp.

Willige, B.C., Ghosh, S., Nill, C., Zourelidou, M., Dohmann, E.M., Maier, A., and Schwechheimer, C. (2007). The DELLA domain of GA INSENSITIVE mediates the interaction with the GA INSENSITIVE DWARF1A gibberellin receptor of Arabidopsis. *Plant Cell* 19, 1209-1220.

Wittwer, S.H. and Bukovac, M.I. (1962). Staminate flower formation on gynoecious cucumber as influenced by the various gibberellins. Naturwissenshaften 49, 305–306.

Xu, W.H., Wang, Y.S., Liu, G.Z., Chen, X., Tinjuangjun, P., Pi, L.Y., and Song, W.Y. (2006). The autophosphorylated Ser686, Thr688, and Ser689 residues in the intracellular juxtamembrane domain of XA21 are implicated in stability control of rice receptor-like kinase. *Plant J* 45, 740-751.

Yamaguchi, S. (2006). Gibberellin biosynthesis in Arabidopsis. *Phytochemistry Reviews* 5, 39–47.

Yamaguchi, S. (2008). Gibberellin metabolism and its regulation. *Annu.Rev.Plant. Biol.* 59, 225-251.

Zentella, R., Zhang, Z.L., Park, M., Thomas, S.G., Endo, A., Murase, K., Fleet, C.M., Jikumaru, Y., Nambara, E., Kamiya, Y., and Sun, T.P. (2007). Global analysis of della direct targets in early gibberellin signaling in Arabidopsis. *Plant Cell* 19, 3037-3057.

Zhang, Z. L., Ogawa, M., Fleet, C. M., Zentella, R., Hu, J., Heo, J. O., Lim, J., Kamiya, Y., Yamaguchi, S., Sun, T. P. (2011). Scarecrow-like 3 promotes gibberellin signaling by antagonizing master growth repressor DELLA in Arabidopsis. *Proc.Natl. Acad.Sci.U.S.A.*108, 2160-2165.

Appendix

1. Publication

Yi Chen, Mingming Hou, Lijuan Liu, Shan Wu, Yun Shen, Kanako Ishiyama, Masatomo Kobayashi, Donald R. McCarty, Bao-Cai Tan (2014). The Maize *DWARF1* Encodes a Gibberellin 3-Oxidase and Is Dual-Localized to the Nucleus and Cytosol. *Plant Physiol* 166, 2028-2039.

Yi Chen and Bao-Cai Tan (2014). New insight in the Gibberellin biosynthesis and signal transduction. Plant signaling and behavior (accepted).