Running head: DI encodes GA3ox with dual localization

To whom correspondence should be addressed:
Bao-Cai Tan,
School of Life Sciences, Shandong University, Jinan, Shandong 250100, P. R. China
E-mail: bctan@sdu.edu.cn; Tel: (0531)-8836-3592;

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The Maize *DWARF1* Encodes a Gibberellin 3-Oxidase and Is Dual-Localized to the Nucleus and Cytosol

Yi Chen¹, Mingming Hou¹,², Lijuan Liu³, Shan Wu³, Yun Shen¹, Kanako Ishiyama⁴, Masatomo Kobayashi⁴, Donald R. McCarty³, Bao-Cai Tan¹,²*

¹Institute of Plant Molecular Biology and Agricultural Biotechnology, State Key Lab of Agrobiotechnology, The Chinese University of Hong Kong, Shatin, N.T., Hong Kong; ²Key laboratory of Plant Cell Engineering and Germplasm Innovation, Ministry of Education, School of Life Sciences, Shandong University, Jinan, Shandong 250100, P. R. China; ³Horticultural Sciences Department, University of Florida, Gainesville, FL 32611, USA; ⁴Experimental Plant Division, RIKEN BioResource Center, 3-1-1 Koyadai, Tsukuba, Ibaraki 305-0074, Japan;

*To whom correspondence should be addressed.

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**One-sentence Summary:** Bioactive gibberellins can be synthesized in the cytosol and the nucleus.
FOOTNOTES

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ABSTRACT

The maize GA deficient mutant *dwarf1 (d1)* displays dwarfism and andromonoecy (*i.e.* forming anthers in the female flower). Previous characterization indicated that the *d1* mutation blocked three steps in the GA biosynthesis; however, the locus has not been isolated and characterized. Here we report that *D1* encodes a GA 3-oxidase catalyzing the final step of bioactive GA synthesis. Recombinant DWARF1 (D1) is capable to convert GA$_{20}$ to GA$_{1}$, GA$_{20}$ to GA$_{3}$, GA$_{5}$ to GA$_{3}$ and GA$_{9}$ to GA$_{4}$ *in vitro*. These reactions are widely believed to take place in the cytosol. However, both *in vivo* GFP fusion analysis and western blotting of organelle fractions using a D1 specific antibody revealed that the D1 protein is dual-localized in the nucleus and the cytosol. Furthermore, the up-stream ZmGA20ox1 protein was found dual-localized in the nucleus and the cytosol as well. These results indicate that bioactive GA can be synthesized in the cytosol and the nucleus, two compartments where GA receptor GID1 exists. Furthermore, the D1 protein was found to be specifically expressed in the stamen primordia in the female floret, suggesting that the suppression of stamen development is mediated by locally synthesized GAs.
INTRODUCTION

The plant hormone gibberellins (GA) are diterpenoid compounds found in plants, fungi and bacteria. Among more than 130 species of GAs discovered in nature, only a few are bioactive; the rest are either biosynthetic intermediates or catabolites (Sponsel and Hedden, 2010; Yamaguchi, 2008). GA has multiple biological functions including promoting seed germination, stem elongation, flowering, pollen development and fruit growth (Sponsel and Hedden, 2010). The stem elongation function of GA contributed to the “Green Revolution” where mutations in GA signaling (Rht1) or biosynthesis (GA20ox) founded the semi-dwarf wheat and rice respectively (Peng et al., 1999; Sasaki et al., 2002). In maize, GA also plays an important role in sex determination. The immature flowers of maize are bisexual in the ear and the tassel. The unisexual flowers are achieved by selective arrest and abortion of the stamen in the ear and the pistil in the tassel (Dellaporta and Calderon-Urrea, 1994). GA-deficient mutants (dwarf1, dwarf3, dwarf5 and anther ear1) and GA-insensitive mutants (dwarf8 and dwarf9) carry bisexual flowers in the ear while maintain male flowers in the tassel, indicating GA mediates suppression of stamen primordium development in the female flower.

GA biosynthesis can be divided into three phases, believed to occur in three separate compartments (Yamaguchi, 2008). The first phase is the cyclisation of geranylgeranyl diphosphate (GGDP) in plastids (Supplemental Figure S1 online). The common diterpenoid precursor GGDP is converted to ent-kaurene through the functions of ent-copalyl diphosphate synthase (CPS) and ent-kaurene synthase (KS). CPS and KS are localized in plastids (Helliwell et al., 2001; Sun and Kamiya, 1994). The second phase is the formation of GA12 in ER. ent-kaurene is oxidized to form GA12 by cytochrome P450 monooxygenases (P450s) which is located on the plastid envelope and ER (Helliwell 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), GA 20-oxidase (GA20ox) and GA 3-oxidase (GA3ox). GA20ox catalyzes multiple oxygenation reactions at C-20 of GA12 to produce GA9, GA53 to GA20. GA9 is then
converted to bioactive GA₄, and GA₂₀ to GA₁ or GA₃ by GA3ox (reviewed by Yamaguchi, 2008). The third phase is widely believed to occur in the cytosol as GA3ox and GA20ox are soluble proteins and appear not containing any targeting sequences (Yamaguchi, 2008). However, direct experimental proof is lacking.

The GA signal transduction pathway involves a GA receptor (GID1) and several repressor proteins (DELLA). When GA level is low, DELL protein accumulate and interact with specific transcription factors that function to promote GA responsive gene expression (Arnaud et al., 2010; de Lucas et al., 2008; Feng et al., 2008; Hong et al., 2012). GA binding to GID1 facilitates the binding of GID1-GA with DELL, triggering degradation of the DELL proteins by 26S proteasome and release of the transcription factors (Sun, 2011). DELL proteins were localized in the nucleus (Ikeda et al., 2001; Peng et al., 1997; Silverstone et al., 1998). And the GID1 was found mostly in the nucleus, and with substantial abundance in the cytosol (Ueguchi-Tanaka et al., 2005; Willige et al., 2007). Thus, this brings into question how GA synthesis coordinates with the receptor in term of the compartmentation.

The d1 allele is a GA deficient mutant in maize known for many years (Fujioka et al., 1988; Phinney, 1956) and was studied biochemically and physiologically. The mutant was rescued by the application of GA₁, but not by GA₂₀ (Spray et al., 1996). It accumulated 10 times more GA₂₀ and GA₂₉ than the wild type (WT) (Fujioka et al., 1988). Metabolic analysis indicated that the mutation blocked three steps in GA biosynthesis, i.e. GA₂₀ to GA₅, GA₅ to GA₃, and GA₂₀ to GA₁ (Spray et al., 1996). It was speculated that D1 either encodes a GA 3-oxidase, or a regulator required for GA 3-oxidase expression. However, the identity of the mutation in d1 allele is not revealed.

In this study, we cloned the D1 gene and demonstrated that it encodes a GA 3-oxidase that catalyzes at least four reactions: GA₂₀ to GA₃, GA₂₀ to GA₁, GA₅ to GA₃ and GA₀ to GA₄. In contrast to the widely believed that bioactive GAs are synthesized in the cytosol, we prove that the D1 as well as the upstream GA20ox proteins are dual-localized to the nucleus and the cytosol, providing strong evidence that GA can be synthesized in the cytosol and the nucleus where the GA receptor GID1 is localized. By using a highly
specific D1 antibody, we revealed that specific GA production in the stamen primordia in the female floret mediates the suppression of stamen development, resulting in unisexual flower in maize.

RESULTS

Isolation and characterization of a dwarf mutant in maize

During a genetic analysis of a viviparous*3286 (vp*3286) mutant, we noticed that this line had an intriguingly high mutagenesis frequency. In one selfed progeny, it produced a dwarf mutant that exhibited recessive nuclear mutation pattern. The mutant is ~ one foot tall at maturity with shortened internodes, broad and dark-green leaves (Fig. 1A). Its tassel (male flowers) was erect and short with reduced branches. The anthers were fully developed, but shed poorly. The plant is andromonoecious, i.e. having bisexual flowers in the ear (female flowers in WT) and male flowers in the tassel (Fig. 1B). These phenotypes are reminiscent of typical GA deficient or insensitive mutants (Harberd and Freeling, 1989; Spray et al., 1996). To distinguish the two possibilities, we applied GA3 to the seedlings. The dwarfism was restored to WT by spraying 10 µM GA3 (Fig. 1C), indicating that the mutant is GA deficient. To determine which allele this mutant is, we crossed this mutant with other GA deficient mutants. The cross with the classic dwarf1 (d1) resulted dwarf plants, indicating that the new mutant was allelic to d1. We named this new allele d1-3286.

Mature maize plants are diclinous, having a male inflorescence (tassel) on the top and a female inflorescence (ear) in the leaf axil. To understand the formation of andromonoecy in the d1 mutant, we examined the female flower development by SEM (Fig. 2). At early stage, the WT (A-D) and the d1 mutant (E-H) form two florets within one female spikelet, named E1 and E2 (Fig. 2). The early E1 and E2 florets are bisexual, each containing one central pistil primordium surrounded by three stamen primordia. At later stage, the E2 floret and the stamen of E1 abort in the WT while the pistil primordium of E1 extends into a long silk that serves for reception of pollen, resulting in a unisexual female flower (Fig. 2A-D). However in the d1 mutant, the development of
stamen primordia in E1 and E2 is not suppressed at the later stage, resulting in formation of anthers along with E1 elongated silks (Fig. 2E-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 ear 1 (Bensen et al., 1995) and GA insensitive mutant dwarf8 (Peng et al., 1997) indicates that suppression of anther development in the female flowers in maize is mediated by GA.

**Molecular characterization of the d1 alleles**

Previous study suggested that the d1 mutant was blocked in the GA20 to GA5, GA5 to GA3, and GA20 to GA1 conversion (Fujioka et al., 1988; Spray et al., 1996), leading to the notion that D1 encodes either a GA 3-oxidase or a factor required for GA 3-oxidase expression. Searching of the maize draft genome (AGPv2) by using rice GA 3-oxidases identified two homologs, named ZmGA3ox1 and ZmGA3ox2. In 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 are roughly the same size with conserved residues throughout the entire protein sequences (Fig. 3). At protein level, ZmGA3ox2 is more closely related to OsGA3ox2 (78% identity) than to ZmGA3ox1 (58%). At nucleotide level, ZmGA3ox2 and ZmGA3ox1 are quite divergent, sharing no significant similarity. This divergence suggests that the two genes were separated long time ago. In our experiments, DNA hybridization by using the ZmGA3ox2 as a probe did not hybridize to ZmGA3ox1 (Fig. 4A). 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, we 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 Genetic Stock Center.

DNA hybridization analysis by using the ZmGA3ox2 as a probe detected polymorphism
among four $d1$ alleles and inbreds (W22 and B73) (Fig. 4). With BamHI digestion, the inbred 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. 4A). Although the loading and fragment size detected in $d1$-6016 are 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 type element at position 69 (start from ATG) of the $ZmGA3ox2$ gene (Fig. 4B). 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, we 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 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. 4B). The $d1$-6016 allele contains a deletion of 2301 bps, covering 508 bps of upstream sequences and 1793 bps of $ZmGA3ox2$ gene (Fig. 4B). 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 presented in a ~2.2 kb BamHI fragment in $d1$-6016. This is the reason that a weak and similar WT size fragment was shown in the DNA hybridization result (Fig. 4B). Because all these four independent alleles contain mutations in the $ZmGA3ox2$ gene and crossed any two of these alleles gave rise to dwarf mutants, we 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 it is likely null mutation. This is consistent with the severe dwarf phenotype.

**Subcellular localization of the D1 protein**

Since GA3ox catalyzes the final step in producing bioactive GAs, the enzyme localization 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 epidermal cells. The tobacco epidermal cells contain a large vacuole in the middle and the cytosol is aligned along the cell wall. Strong GFP signals were found in both the cytosol and the nucleus by confocal laser microscopy (Fig. 5A). However, the control GFP protein was also showed dual-localization in the cytosol and the nucleus (Fig. 5A), raising a possibility that the nucleus localization of D1-GFP and GFP may be a result of passive diffusion if the size of fusion protein is smaller than the nuclear pore. To address this possibility, D1 was fused with GUS-GFP to yield D1-GUS-GFP (~132KDa), and tested with GUS-GFP (~91KDa) as a negative control. The result shows that D1-GUS-GFP is dual-localized to the cytosol and the nucleus, whereas GUS-GFP is localized to the cytosol only (Fig. 5B), ruling out the possibility of passive diffusion. The nucleus localization of GFP (26.9 KDa) may be subject to passive diffusion as it is smaller than the cut-off size (~40 KDa) of the nuclear pore (Marfori et al., 2010).

The dual-localization of D1 in the nucleus and cytosol is surprising as GA3ox is widely believed to be in the cytosol (reviewed by Yamaguchi, 2008). To independently determine the subcellular localization of D1, we probed the D1 protein in subfractions of cytosol and nucleus by western blot analysis. A polyclonal D1 antibody was raised by using His-D1 recombinant protein. The specificity of the antibody was tested by western blot analysis on total proteins from the WT and the d1-6016 mutant seedlings which carries a deletion of the entire D1 gene. The antibody recognized a single band matching the expected size of D1 (41 KDa) in the WT, and no band in the d1-6016 mutant (Supplemental Figure S2 online), indicating that the D1 antibody is highly specific to the D1 protein. The WT seedling cells were fractionated into a nucleus and a cytosol fraction and the proteins were extracted. Western blot analysis detected presence of D1 protein both in the cytosol and the nucleus fraction (Fig. 5C). Antibodies against Heat Shock Protein 82 (α-HSP82, cytosol marker) and Histone 3 (α-H3, nucleus marker) were used to monitor cross contamination. No cross contamination between the two fractions was
detected. Thus, two independent approaches detected the consistent result that D1 is dual-localized to the cytosol and the nucleus.

The substrates of GA3ox are produced via the function of GA20ox, which convert GA_{12} to GA_9, and GA_{53} to GA_{20} (Hedden and Thomas, 2012). To test whether the upstream step is also localized in the nucleus and cytosol, we studied the subcellular localization of GA20ox. The maize ZmGA20ox1 cDNA was cloned and fused with GUS-GFP to create the ZmGA20ox1-GUS-GFP fusion. Using the same methodology, ZmGA20ox1-GUS-GFP was found to be dual-localized to the cytosol and the nucleus (Fig. 5D). In conjunction with the D1 localization, we conclude that bioactive GAs can be synthesized in both the cytosol and the nucleus.

**Determination of D1 enzymatic activity**

To determine the enzymatic function, the D1 protein was fused with glutathione S-transferase (GST) protein in pGEX-2T vector and expressed in *E. coli*. The fusion protein was purified and incubated with deuterium labeled GA substrates \( ^2H_3-GA_9, \) \( ^2H_2-GA_{20}, \) and not deuterium labeled \( ^2H_0-GA_5 \) in the presence of co-factors 2-ketoglutarate, ascorbate and FeSO₄ (Refer to Experimental procedures). The reaction products were analyzed by full-scan GC-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. 6). The MS results were provided online as Supplemental Figure S3 online. Different from *Arabidopsis* where the active GAs are GA_1 and GA_4 (Yamaguchi, 2006), maize, rice and wheat (monocots) synthesize GA_3 as an active form as well (Spray et al., 1996; Appleford et al., 2006). Although the biological function of GA_3 remains to be elucidated (Magome et al., 2013), 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 *dl* 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. 7A). In contrast, we could not detect the expression of ZmGA3ox1 in these tissues although the primers were approved working robustly on genomic DNA (data not shown). This result indicates that D1 is the predominant enzyme for GA production in most organs, whereas ZmGA3ox1 may be expressed in low level or in highly specialized cells. In addition, we analyzed whether a negative feedback regulation exists on the expression of ZmGA3ox1. Application of 100 µM GA3 in whole seedlings strongly inhibited the ZmGA3ox2 mRNA accumulation, suggesting that ZmGA3ox2 is subject to feedback regulation (Fig. 7B).

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 because 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. 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 predicts the potential sites of bioactive GA production, we determined the sites of D1 protein expression during maize female floret development by immunohistochemical analysis using the highly specific D1 antibody.

The WT and d1 ears (1 to 1.5 cm long) were fixed in wax and sectioned. The D1 protein was detected with Alexa-594 labeled secondary antibody which appeared red under confocal laser scanning microscope (Fig. 8). The florets in the ears were mostly at bisexual stage. Red fluorescent signals were detected exclusively in the stamen regions of E1 and E2 florets in the WT spikelets (Fig. 8A to C), but not in the d1 spikelets (Fig. 8G to I). The signal became much stronger at late stages than in early stage (Fig. 8A, C), probably due to the increased number of stamen cells. 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 bioactive GA production during maize sex determination. This result in conjunction with the SEM analysis suggests that GAs suppress stamen primordium development in female floret in a cell-autonomous manner.
Similar to florets in the ear, tassel florets are bisexual at early stages. During sex
determination, the stamen primordia develop into functional anthers while the pistil
primordium in the tassel floret is aborted through program cell death (Calderon-Urrea and
Dellaporta, 1999), resulting in only male florets in tassel. The d1 mutant contains normal
tassel florets. To test whether D1 plays a role in the tassel floret sex determination, we
performed immunohistochemical analysis on WT tassel (Fig. 9). We did not detected
strong signal of D1 in tassel florets from bisexual stage to unisexual stage (Fig. 9A to 9C).
Detection of Histone3 displayed strong signal indicating that the experiment system is
reliable (Fig. 9D to 9F). This result suggests that either D1 is not expressed in the tassel
floret or at a low level that beyond the limit of this immunohistochemical detection.

DISCUSSION

D1 encodes a GA 3-oxidase catalyzing the final step of GA biosynthesis in maize.
We characterized a dwarf mutant in maize isolated during the study of vp*3286. Genetic
analysis confirms that this mutant is an allele of d1. Biochemical characterization on d1
indicates that the mutation blocks three steps of GA biosynthesis (Fujioka et al., 1988;
Spray et al., 1996). This raises two possibilities on the molecular identity of D1 gene, 1)
it encodes a GA 3-oxidase (ZmGA3ox); 2) it encodes a proteins required for ZmGA3ox
activity or expression.

We tested the first possibility by identifying mutations in the d1 alleles in
candidate genes. The maize genome draft (AGPv2) contains two putative GA 3-oxidases,
ZmGA3ox1 on chr 6L and ZmGA3ox2 on chr 3S. Because the d1 allele was mapped to
chr 3S (Neuffer and England, 1995), ZmGA3ox2 stands as the most likely candidate.
Analyzing four alleles of d1 including the d1-4 allele that was previously considered as
d4 identified independent mutations in the ZmGA3ox2 gene in each allele (Fig. 4). In
vitro enzymatic activity analysis confirmed that the recombinant ZmGA3ox2 protein
catalyzes four reactions converting GA9 to GA4, GA20 to GA1, GA20 to GA3, GA5 to GA3
(Fig. 6). This extends the previous analysis that the d1 mutation blocked three steps in
GA biosynthesis (Spray et al., 1996). Together with the report that ZmGA3ox2 is a
candidate gene for a major QTL for plant height, qPH3.1 (Teng et al., 2013), these results indicate that \(D1\) encodes ZmGA3ox2, a GA 3-oxidase that catalyzes the final step of GA biosynthesis.

Although two GA 3-oxidases exist in maize, we did not detect the expression of ZmGA3ox1 in the major tissues tested (Fig. 7), suggesting that ZmGA3ox1 plays a minor role in GA biosynthesis in major tissues. It was reported that ZmGA3ox1 was expressed in the immature tassel (Teng et al., 2013). Thus it is possible that ZmGA3ox1 is expressed at a very low level or in limited cell types, for example in a few cells in immature tassels. In contrast, ZmGA3ox2 is highly expressed in all tissues tested, suggesting that ZmGA3ox2 plays a predominant role in GA biosynthesis through plant growth and development. The sever dwarf phenotype of \(d1\) mutants provides strong genetic evidence that a functional redundancy by ZmGA3ox1 is not evident. Interestingly the rice genome contains two GA3ox genes. OsGA3ox2 is predominantly expressed and OsGA3ox1 is only found in unopened flowers in rice (Itoh et al., 2001). However, this pattern is different from Arabidopsis where four GA3ox genes have overlapping expressions (Hu et al., 2008; Mitchum et al., 2006). To address the specific role of GA3ox1 in maize tassels or rice immature flowers requires the analysis of corresponding loss of function mutants in either species.

**Bioactive GA can be potentially synthesized in the nucleus and the cytosol.**

The compartment of bioactive GA synthesis inside the cell has not been resolved so far. It is widely believed to be in the cytosol based on the evidence that GA3ox is a water soluble enzyme and does not appear to contain any obvious targeting signals (Yamaguchi, 2008). Because GA3ox catalyzes the last step resulting in bioactive GA production, the site of GA3ox predicts at least the potential site of GA synthesis. By this logic, we examined the localization of GA3ox2 by two independent approaches. First, the D1-GFP fusion protein was found in both compartments although a NLS signal was not found in the protein sequence (Fig. 3). To rule out the possibility that nucleus localization may be resulted from non-specific diffusion due to a smaller size of D1-GFP (~68kD), we tested
D1-GUS-GFP (~132kD). This experiment showed the dual-targeting to the nucleus and the cytosol as well. Second, we raised a highly specific D1 antibody which reacted to a single band in the WT that matches the D1 protein size and did not react to any band in the d1-6016 deletion mutant (Supplemental Figure S2 online). The antibody detected presence of D1 in both the cytosol and the nucleus fraction (Fig. 5C). These results lead us to conclude the GA3ox is dual-localized in the cytosol and the nucleus, thus these two compartments as the potential sites for GA biosynthesis.

Formation of bioactive GA in the nucleus required its precursor. ZmGA20ox1 catalyzes the penultimate reaction of bioactive GA synthesis leading to the formation of the substrate of D1. We found that ZmGA20ox1 was dual-localized to the nucleus and the cytosol as well (Fig.5D), which suggests that the precursor of bioactive GA may be synthesized in the nucleus and the cytosol. These results provide strong evidence that bioactive GA can be synthesized in the cytosol and the nucleus.

Interestingly, this conclusion coincides with the subcellular localization of GA receptor GID1. The rice GID1, also a soluble protein, is primarily targeted to the nucleus, the proposed site of action. 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). The similar dual-localization of D1 and GID1 raises interesting questions as to whether GA is synthesized and perceived in both compartments, and how GA synthesis is coordinated with the GA signal transduction components between the two compartments. Especially, the DELLA proteins, the negative GA signal transduction components, are only found in the nucleus (Peng et al., 1997; Silverstone et al., 1998; Willige et al., 2007).

**The suppression of stamen primordia in maize female florets is probably mediated by locally synthesized GAs.**

GA promotes floral organ development in bisexual flower system such as Arabidopsis and rice (Koornneef and van der Veen, 1980; Aya et al., 2009). Although GA3ox genes are widely expressed in actively growing tissues (Kaneko et al., 2003), the sites of GA
biosynthesis are not necessarily the locations of GA action. For instance, the development of Arabidopsis petal was defective in GA deficient mutant, but no GA3ox gene expression was found in the petal of WT flowers (Hu et al., 2008). In contrast to the promotion function in bisexual flower system, GA has a suppression role in maize unisexual flower development. The presence of D1 protein in stamen initials in maize E1 and E2 florets (Fig. 8) is consistent with the andromonoecy phenomenon where stamen growth and development are not suppressed in the E1 and E2 florets in d1 mutant (Fig. 2E to H). This result suggests that locally synthesis of bioactive GAs is required by the suppression of stamen cell development in E1 and E2 florets.

The influence of GA deficiency in maize male florets is not as severe as female florets, because the tassel of d1 mutant carries fertile male florets. At least two possibilities offer explanation to this phenomenon, 1) GA does not affect the stamen primordium development in male floret; or 2) the level of GAs in tassel is too low to induce the suppression of stamen primordia. Previous study reported that the amount of GA-like substances in tassel was much lower than in ear (Rood and Pharis, 1980). Consistently, no strong signal was detected by immunohistochemical analysis with D1 antibody in tassel florets (Fig.9), indicating that the abundance of D1 in tassel florets is much lower than in the ear. Furthermore, exogenous GA converts male florets in tassel to female florets (Nickerson, 1959). Considering the above evidence, we speculate that the stamen development in tassel florets may be the result of deficiency in de novo GA biosynthesis as indicated by the low expression of the D1 protein (ZmGA3ox2). In that case, the sex determination in maize is achieved by the regulation of bioactive GA synthesis.

Maize dwarf8 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, downstream component of DELLA proteins which are able to induce
WEE1 gene expression are needed to be identified in order to understand the underlying mechanism of GA function in sex determination in maize.

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

Retrotransposable elements are abundant in many genomes. In maize, it accounts for more than 80% of the genome (Baucom et al., 2009; Schnable et al., 2009). The vast majority of retrotransposable elements are kept quiescent by epigenetic regulation (Slotkin and Martienssen, 2007), such that active retrotransposon elements are rarely found in nature (Picault et al., 2009). However, they can be re-activated by means of stresses and ‘genomic shock’ (McClintock, 1984). In rice, tissue culture can activate Tos17 transposition, a type I or II transposable element (Hirochika, 2001). The isolation of d1-3286 suggested that the copia-like element inserted in the d1-3286 might be active. 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.

**MATERIALS AND METHODS**

**DNA extraction and Southern analysis**

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

**Protein sequence alignment and Phylogenetic Analysis**

Sequence data was retrieved from the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov). Homology searches in GenBank were done using the Basic Local Alignment Search Tool server (http://www.ncbi.nlm.nih.gov/BLAST/).
Multiple alignments of protein sequences were performed by CLUSTALW program and The Phylogenetic tree performed by MEGA 4.1 (Beta 3) software (Kumar et al., 2004) (http://www.megasoftware.net) using the Clustal X and N-J plot programs (Saitou and Nei, 1987).

**qRT-PCR analysis**
Total RNA was extracted from root, stem, leave of 2-week-old seedlings, about 1.5 cm tassel and ear. Total RNA was isolated from whole seedlings treated with or without 100 μM GA3. Total RNA was isolated using RNeasy mini Kit (QIAGEN) and treated with DNase I (NEB) prior to cDNA synthesis. cDNA was synthesized by using reverse transcriptase (SuperScript III; Invitrogen). qRT-PCR were performed by using primers specific for D1 (D1A-F6, CGCCCATCTCCTCCTTCTTCT; qPCR R1, TCCATCACGTCACAGAAGCT) and normalized with *Actin1* as a reference gene. The primers for maize *Actin1* gene are ZmACT-RTF1 (ATGGTCAAGGCCGGTTTCG) and ZmACT-RTR1 (TCAGGATGCCTCTCTTGGCC). qRT-PCR were conducted with three technical replicates for each one of the three biological replicates of each tissue sample.

**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.

**GA 3-oxidase Enzyme 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 0.1mM IPTG at 28°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) as the manuscript described. The recombinant protein was used for functional assays. GC-MS was performed with an Auto Mass spectrometer (JEOL) connected to a Hewlett-Packard 5890 series II gas chromatograph. The analytical
conditions used are described previously (Itoh et al., 2001). [17, 17-²H₂]GA₂₀ was purchased from L. Mander, Australian National University (Canberra). [15, 17, 17-²H₃]GA₉ was synthesized from GA₉-norketone and (methyl d₃) triphenylphosphonium-Br by Wittig reaction (Takahashi et al., 1986). All GAs used in this study were analyzed by full-scan GC-MS to show the absence of impurities.

**Transient expression of D1-GFP, D1-GUS-GFP, ZmGA20ox1-GUS-GFP and GUS-GFP fusion in tobacco epidermal cells and laser confocal microscopy analysis**

*DI* and *GUS* cDNA were respectively cloned into pGWB5 vector and fused with a GFP protein in the C-terminal by using Gateway technology (Invitrogen). *DI* and *GUS*, ZmGA20ox1 and *GUS* were fused through EcoRI and cloned into pGWB5 vector respectively. Leaves of 3- to 5-week-old Nicotiana benthamiana plants were infiltrated with *A. tumefaciens* EHA105 strains containing the pGWB5, pGWB5-D1, pGWB5-D1-GUS, pGWB5-ZmGA20ox1-GUS and pGWB5-GUS construct, respectively. Localization of fluorescent proteins was observed 36 h after infiltration by using a confocal laser scanning microscope (Olympus FV1000-IX81). Vector pGWB5 without insertion was created by LR reaction with a self-ligated pENTR vector. Empty pGWB5 and pGWB5-GUS vectors were used as negative control.

**Genomic library construction and screening**

To clone the *ZmGA3ox2* gene in the *d1-3286* allele, a size selected genomic library was constructed in λ-phage. The genomic DNA was digested with *BamH*I restriction enzyme, size-fractionated and ligated to a ZAP Express vector as previously reported (Tan et al., 1997). Approximately 2.5x10⁵ plaques were screened with probe I of *ZmGA3ox2* (Fig. 5A). Four clones were identified and excised into pCMV plasmids according to the manufacture instruction (Stratagene, USA).

**Purification of His-D1 fusion protein**

To purified His-D1 recombinant protein, the coding region of *DI* was cloned in the expression vector pET-30a(+) (Novagen). The plasmid was transformed in the BL21
strain of *E. coli*. Cells were induced by 1mM IPTG and cultured at 37°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 (Clontech) was used in the purification. Purification procedure was followed the manufacture instruction with some modification. Briefly, before binding with protein sample, resins were washed by H2O, 10 mM Tris-HCl and wash buffer, respectively. Washing step was not stopped until the absorbance is 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).

**Fractionation of nuclei and cytosol and the western blot analysis**

Polyclonal antibody against D1 was raised in rabbit by using His-D1 fusion protein. In order to confirm the specificity of the D1 antibody, a western blot of WT versus *d1* mutant was carried out. Western blot result indicated that the D1 antibody could not recognize any protein in *d1* mutant. This antibody was used in the following western blot analysis and immunohistochemical analysis.

To isolate cytosolic protein, 3-day-old germinated 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 min. The supernatant was transferred to a new tube and then centrifuged by 12000 g for 10 min at 4°C. The supernatant was collected for western blot analysis.

Isolation of intact nuclei from maize was as previously reported (Pandey et al., 2006). 3-day-old germinated maize seedlings were used. To prevent contamination from the supernatant, the nuclei pellet was washed three times by resuspension in 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 min. The integrity and purity of the nuclei were monitored by microscopic observation. Total protein extraction and western blot detection were performed as described previously (Xu et al., 2006).
Immunohistochemical analysis of D1 protein in developing female and male florets

The maize ear and tassel about 1 to 1.5 cm long were fixed in a FAA solution (45% ethanol, 5% acetic acid and 1.9% formaldehyde), then were embedded in paraffin. After section and de-paraffin, the samples were blocked in 3% BSA in 1xPBS buffer for at least 2 h and then incubated with anti-D1 antibody or anti-Histone3 (1:100 dilution) at 4°C for overnight. After washing with 1% BSA in 1xPBS buffer for 3 times (each for 10 min), the samples were incubated with fluorescent secondary antibody (Alexa®594 goat anti-rabbit, Invitrogen) at room temperature for 1 h. The secondary antibody was removed and washed extensively with 1% BSA for 3 times (each for 20 min). The fluorescent signals were observed and imaged under a confocal laser scanning microscope (Olympus FV1000-IX81).

Accession Number

Sequence data for ZmGA3ox2 genomic DNA and cDNA, ZmGA3ox1 genomic DNA, alleles d1-4, d1-6039, d1-3286 and d1-6016 can be found in the GenBank/EMBL database under accession numbers JX307637, JX307638, JX307639, JX307640, JX307641, JX307642 and KC867701, respectively.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. The GA biosynthetic pathway in plants.
Supplemental Figure S2. Specificity of the D1 antibody.
Supplemental Figure S3. Mass spectrometry analysis of the in vitro activity of recombinant GST-ZmGA3ox2 fusion protein.

ACKNOWLEDGEMENTS

The authors wish to thank the maize genetic stock center for providing the d1 alleles. Special thanks to Phillip Stinard and Marty Sachs for the d1-4 allele. The authors wish to thank Dr. Tsuyoshi Nakagawa (Shimane University, Japan) for the pGWB vectors.
LITERATURE CITED


are differently expressed during the growth of rice. Proc. Natl. Acad. Sci. USA 98, 8909-8914.


FIGURE LEGENDS

Figure 1. Phenotypes of the dI allele and its response to GA treatment.
A. Homozygous adult dI-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 dI-3286 displayed andromonoecy, formation of anthers in the ear.
C. The dwarf phenotype of dI-3286 seedling before and after spray with 10 µM GA3 for 7 days. Bars, 1 cm.

Figure 2. Female and male organ development in ear florets in WT and dI-3286 allele.
The ears (female flowers) of WT (A-D) and the dI-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.

Figure 3. 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 dI alleles were marked (referring to Fig. 4). The highly conserved ferrous iron ligand binding residues for 2ODDs were marked as circles.

Figure 4. Molecular characterization of dI alleles.
A. DNA hybridization analysis on the dI alleles with ZmGA3ox2 probe I. 1, B73; 2, W22; 3, dI-3286; 4, dI-6039; 5, dI-6016; 6, dI-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.

**Figure 5. Subcellular localization of D1 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, 10μm. (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 6. Recombinant D1 protein possesses four GA 3-oxidase activities in vitro.**

**Figure 7. Expression of ZmGA3ox2 in maize.**

qRT-PCR analysis of *ZmGA3ox2* in different tissues (A) and in WT whole seedlings after treatment with GA3 (B). *Actin1* gene was reference gene. Error bars represent SE.

**Figure 8. 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
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Figure 9. Immunohistochemical detection of D1 protein in developing maize male florets.
Sections of WT tassels from 1cm to 1.5cm 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.
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)

<table>
<thead>
<tr>
<th>GA Substrate</th>
<th>GA Product</th>
<th>KRI</th>
<th>Characteristic ions at m/z (% relative intensity of base peak)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$^2$H$_3$-GA$_9$</td>
<td>$^2$H$_3$-GA$_4$</td>
<td>2515</td>
<td>421(100), 406(16), 403(42), 393(51), 389(57)</td>
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<tr>
<td>$^2$H$<em>2$-GA$</em>{20}$</td>
<td>$^2$H$_2$-GA$_1$</td>
<td>2674</td>
<td>508(100), 493(9), 449(12), 418(2), 392(1)</td>
</tr>
<tr>
<td>$^2$H$<em>2$-GA$</em>{20}$</td>
<td>$^2$H$_2$-GA$_3$</td>
<td>2697</td>
<td>506(100), 491(5), 475(3), 447(6), 433(4), 416(1)</td>
</tr>
<tr>
<td>$^2$H$_0$-GA$_5$</td>
<td>$^2$H$_0$-GA$_3$</td>
<td>2699</td>
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