UNIVERSITÀ DEGLI STUDI DI MILANO

Scuola di Dottorato in Scienze Biologiche e Molecolari

XXVI Ciclo

MOLECULAR CONTROL OF REPRODUCTIVE ORGAN DEVELOPMENT IN RICE (*ORYZA SATIVA* L.)

Alessandro Pilatone

Ph.D. Thesis

Scientific tutor: Prof. Martin Kater

Academic year: 2012-2013

SSD: BIO18

Thesis performed at Università degli Studi di Milano, Dipartimento di BioScienze

CONTENTS

PART I

SUMMARY

Understanding the function of the AGAMOUS subfamily genes in rice

In our Laboratory we are interested in the study of the genes that orchestrate the sexual organ formation and ovule development in rice (*Oryza Sativa* L), a model species for monocots. Several published works have shown that the MADS domain transcription factors belonging to the AGAMOUS subfamily of *Arabidopsis thaliana* play important roles during the development of stamens, carpel and ovules but also play roles in floral meristem determinacy. Despite the extensive knowledge that has become available about these MADS domain transcription factors in *Arabidopsis thaliana*, little was known about these genes in rice, a species that is quite distant from an evolutionary point of view.

Preliminary data for OsMADS3 and OsMADS58 gene functions

Analyzing the results published by Yamaguchi and colleagues in 2006 for the rice genes *OsMADS3* and *OsMADS58*, we had some doubts about the consistency of their data and the conclusion drawn for these. This first reason is that the *osmads3-2* mutant used by Yamaguchi to create the double mutant *osmads3-2 osmads58* RNAi, presents a milder phenotype than the *osmads3-3* knock-out mutant (Yamaguchi et al., 2006). In other words, they analyze the strong *osmads3-3* single mutant, but for making a double mutant with *osmads58,* they use a milder allele. Moreover, the phenotypic characteristics of the mutant *osmads3-2*, unlike the mutant *osmads3-3*, can be observed only in a low percentage of flowers.

The second reason is that the RNAi approach used by the Japanese group to obtain the *osmads58* mutant, can also lead to down-regulation of *OsMADS3*, which could make the obtained data unreliable. Alignments of the sequence used for silencing *OsMADS58* showed stretches of homology with *OsMADS3* (Figure 1). The RNase Dicer cuts the double-stranded RNA into fragments of about 21 nucleotides that are subsequently paired to the target mRNA, leading them to degradation (Schwab et al., 2006). In the *OsMADS3* and *OsMADS58* sequences, there are regions of 21 or more nucleotides that are the same and it is very likely that RNAi silence in the same way *OsMADS3* and *OsMADS58,* invalidating the obtained data. In our laboratory we used a mutant of the gene *osmads58* obtained by the insertion of a transposon of maize, coming from Sundaresan lab. (UC Davis), in this mutant it is not possible to observe a total shutdown of the gene *OsMADS58*, but a reduction in expression of about 35

times as compared to WT (qRT-PCR) (Figure 2), without interfering with the expression of the gene *OsMADS3*.
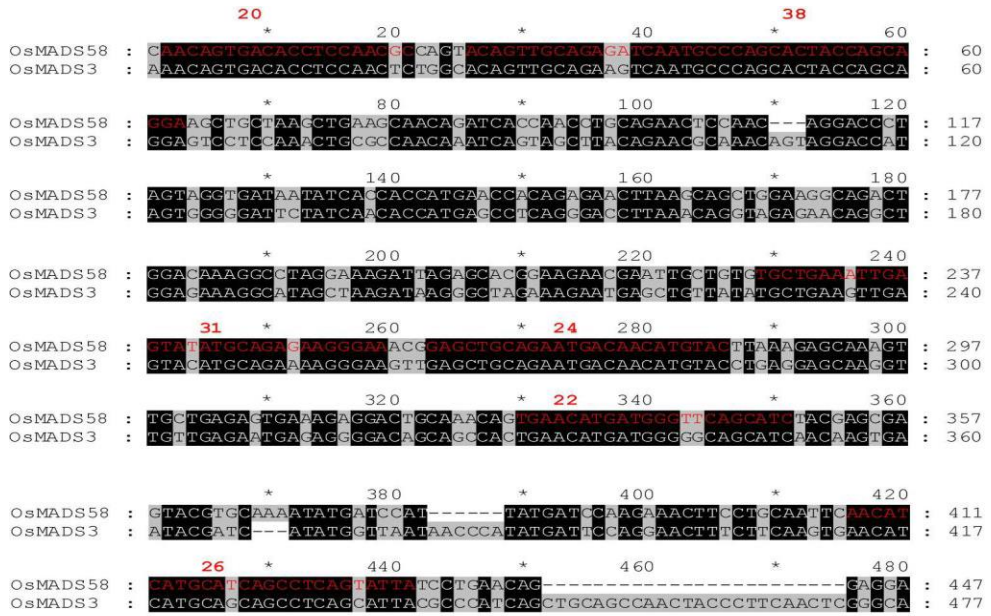
Figure 1 Alignment from the K-box region to the C-terminal of the genes *OsMADS3* and *OsMADS58*. The red indicates the number of consecutive identical nucleotides in areas of high similarity (Dreni et al., 2007).
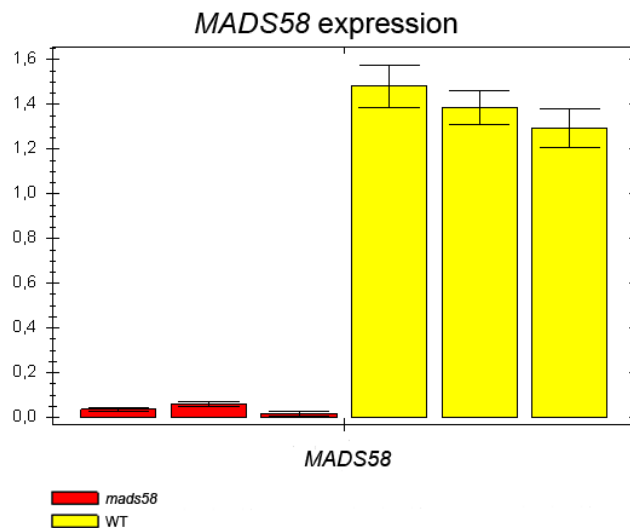
Figure 2 *OsMADS58* expression in *osmads58* mutant obtained by the insertion of a transposon of maize, coming from Sundaresan lab. (UC Davis).

The third reason is that expression analysis using an RNA in-situ hybridization approach of the genes *OsMADS3* and *OsMADS58* gave different results from those obtained by Yamaguchi and colleagues. It has been noted that the expression of both genes is the same during different stages of flower development, in contrast to what is currently published. The protein conservation between OsMADS3 OsMADS58 is 96% in the MADS domain and 67% the whole protein, this suggest a possible functional conservation of these proteins, which has been evaluated in several experiments carried out in our laboratory.

Based on these preliminary data, it was decided to verify the data published in literature for *OsMADS3* and *OsMADS58* starting from the analysis of the single mutants, and cross them to obtain the double mutant (*osmads3-3 osmads58*). Subsequently we crossed the single mutant *osmads3-3* with the mutant *osmads13*, to check the possible redundancy of genes in the determination of floral meristem activity and ovule development. The results of these studies are reported in Chapter II.

Study the molecular network involved in ovule development

In the second part of my project we moved our attention to rice ovule development trying to elucidate the molecular network involved in this complex process. The MADS domain transcription factors *OsMADS13* is the rice ovule identity gene, it is specifically expressed in the ovary during all developmental stages. The *osmads13* single mutant is female sterile because the ovules are converted into carpelloid structures. In Chapter III are reported the experiments that we have done to identify the genes that are modulated by

OsMADS13 in the ovary. Since MADS domain proteins recognize and bind CArG boxes [CC(A/T)6GG], tools to find the OsMADS13 putative direct target were shown.

STATE OF THE ART

Inflorescence development in rice

The transition from the vegetative to the reproductive phase takes place in response to environmental cues such as day length and temperature. In rice, after the last foliage leaf formed, called flag leaf, the shoot apical meristem (SAM) is converted to an inflorescence meristem (in rice this is called the rachis meristem) and later the first bract (bract 1) is formed. It is possible to distinguish the rachis meristem from the shoot apical meristem (SAM) because the rachis meristem is larger and taller than the SAM. Subsequently bract 2 and the primary branch primordia are formed in the same time, and the ten or more bracts and primary branches are produced in a spiral arrangement. When the bract 3 primordium is formed, the rachis meristem reaches its maximum size, and primary branch primordia start to be formed in a spiral arrangement. Therefore a gradual change from 1/2 alternate phyllotaxy (characteristic of vegetative phase) to spiral phyllotaxy can be observed in early reproductive stages. After producing ten or more primary branch primordia the rachis meristem is assumed to abort; in fact in the mature inflorescence we cannot find a terminal flower. *In situ* hybridization expression analysis of the gene *OSH1,* normally expressed in indeterminate cells in the meristem and not expressed in determinate or aborted cells (Sato et al., 1996), have shown that the rachis meristem is programmed to lose its activity after producing a cultivar specific number of primary branches (Ikeda et al., 2004).

The elongation of the primary branch inflorescence primordia start simultaneously when all primordia have been formed, subsequently

development is faster in distal branches than in proximal ones. The meristematic activity is maintained in the apex of each primary branch and produce later organs like bracts, secondary inflorescence branches and spikelets. The secondary branch meristem formed in the distal region of primary branch is immediately transformed without producing lateral organs. The fate of the rachis meristem differ from that of primary branch meristem and secondary branch meristem, in fact the rachis meristem is not converted to a spikelet meristem to form terminal spikelets.

Rachis and branches start rapid elongation when the inflorescence became 40mm long and all the floral organ primordia start to be formed.

Rice flower development

Rice flower development can be summarized in eight different stages (Ikeda et al., 2004). During the first phase, called Sp1, the spikelet meristem gives rise to the primordia of the rudimentary glumes. At stage Sp2, sterile glumes are formed, which are located in the same position of the rudimentary glumes. The origin of the primordium of the lemma occurs in the next step called Sp3, and the formation of the primordium of the palea in step Sp4. The lodicules are formed in phase Sp5 and the stamens in the next phase, called Sp6. During stage Sp7 the floral meristem gives rise to the carpel primordia, and stamens complete differentiation originating filaments and anthers. During the last phase called Sp8, three fused carpel primordia emerge laterally enclosing the floral meristem (FM). In many grasses including rice, only the two lateral carpel primordia produce a stigma, whereas in other grass species like some bamboos all carpel primordia develop stigmas. Inside the developing fourth whorl, the inner FM turns gradually downwards and differentiates into a hemianatropous ovule (Lopez-Dee et al., 1999; Itoh et al., 2005). Interestingly, in grasses, the ovule directly derives from FM cells (Lopez-Dee et al., 1999; Itoh et al., 2005; Dreni et al., 2007), which is clearly different from what happens in *Arabidopsis* and many other plant species, where ovules develop from newly emerging meristematic tissues (Colombo et al., 2008).

Pollen grain formation

The process of pollen grains formation starts from a cell called archespore, which differentiates into a pollen mother cell. Subsequently the differentiated cell undergoes meiosis, giving rise to 4 haploid cells very close to each other (pollen tetrad). The pollen tetrad cells distance themselves and initiate the differentiation of the pollen grain wall, and when the maturation is complete, a mitotic division originates a bi-nucleated cell. A new mitotic division of the nucleus leading to two sperm cells: the male gametes. In rice the process of pollen maturation is completed the day before flowering, when the pollen grain becomes perfectly round and the morphological differentiation has finished.

Ovule, embryo and kernel development

Rice ovule development starts when the length of the inflorescence is less than a centimeter and can be divided into nine stages (Lopez-Dee et al., 1999). The formation of the ovule primordium occurs from the inner layer of the floral apex thanks to the meristematic cells in active division (Ov1), most of these forms the nucellus, while a subepidermal cell develops into the archeospore (Ov2). The archeospore become polarized and differentiate generating the diploid megasporocite (Ov3), which can be easily recognized by its large nucleus. The diploid megasporocite, thanks to megasporogenesis (Ov4), leads to the formation of a linear tetrad of haploid megaspore, three of them degenerate, while the megaspore located at the chalazal pole of the nucellus becomes the functional cell (Ov5). The functional megaspore undergoes megagametogenesis, a process that consists in three mitotic divisions and leads to the generation of the so called embryo sac. The first mitotic division originates the two-nucleated female gametophyte (Ov6), the second forms the four-nucleated cell (Ov7) and the third the eight-nucleated megagametophyte, followed immediately by nuclear migration and cellularization (Ov8). During the maturation process of the embryo sac, there is the polarization of the egg cell and synergids, and the fusion of the two polar nuclei in the central cell. The antipodal cells divide forming a group of cells at the chalazal pole of the embryo sac (Ov9). After few years this model was proposed again with a small modification (Itoh et al., 2005), in fact in this papers the integuments primordium differentiation and division were grouped in two different stages. The formation of the integuments starts before megasporogenesis (between Ov2 and Ov4) and their development precedes the formation of the embryo sac. The inner integument, consisting of two cells layers, start to grow from

layer L1 of the primordium, while the outer integument, which differentiates early from epidermal and subepidermal cells, is composed by up to three cell layers. The development of the integuments is not synchronous, in fact, during megasporogenesis, the inner integument completely envelops the nucellus, but the outer integument does not keep up with the downward curvature of the ovule. Hence, the basal portion of the mature ovule is covered by both integuments, but the abaxial side of the ovule is enclosed only by the inner integument. The funiculus, containing a single vascular bundle that runs along it, develops on the adaxial side of the ovule. The rice ovule is ready to be fertilized one day before anthesis, causing almost complete self-fertilization. When the pollen falls on the stigma, it germinates forming a pollen tube able to grow into the style to reach the ovary and grows to the ovule which it enters through the micropyle to release the two sperm cells. At this stage the double fertilization process takes place: a sperm cell fuses with the egg cell giving rise to the diploid zygote, which through successive mitotic divisions will generate the embryo. The other sperm cell fuses with the bi-nucleate polar cell, giving rise to the triploid endosperm. The development of embryo and endosperm proceeds up to the formation of the kernel, consisting of mature ovary and the residues of lemma, palea, rachis and rudimentary glumes. Each kernel contains a single seed surrounded by some very compact layers of cells called pericarp, derived from the inner integument and from the nucellus. The endosperm is mainly composed of starch and has the function of feeding the embryo during development and germination stages, it occupies most of the caryopsis and is covered by a thin cell layer, called aleurone, in contact with the integuments. The embryo occupies only one fifth of the length of the caryopsis and it is located in its ventral part, in proximity of the base of the lemma. The embryo contains the shoot primordium enclosed in the coleoptiles

and the primordium of the root covered by the coleoriza. The coleoptile and coleoriza together form the embryonic axis, which is wrapped in a thick layer of cells called the cotyledon or scutellum.

Rice inflorescence and flower morphology

The rice inflorescence and flower morphology is quite different from those of model eudicots (*Arabidopsis thaliana*, *Antirrhinum majus*, and *Petunia*). In particular the rice inflorescence, called panicle, is formed by a grass specific structure named spikelet. The panicle is composed by a principal axis called the rachis divided by 6-20 knots from which originate the branches and subsequently the secondary branches. The rice flower is inserted on the secondary branches through a pedicel whose apex supports two rudimentary glumes and two sterile bracts, called sterile glumes. The flower is enclosed by two morphologically distinct bract-like organs that are the lemma and palea. These have an apiculo to their tip, but the lemma, unlike the palea, ends with a filiform protuberance called arista, probably providing a protective function for the floral structures. At the base of the flower, between the ovary and the glumes, there are two lodicule that become turgid during anthesis, allowing the opening of the flower. In the rice flower are six stamens present, each one consisting of a single anther, formed by four pollen chambers and supported by a filament. The gynoecium is formed by a pistil that is divided in an ovary containing a single ovule, a style and a bifurcated stigma (Yoshida and Nagato, 2011).

The ABC model

Flower development can be explained through the so-called 'ABC' model, which was proposed in the early '90 and based on observations of flower mutants in two eudicot model species: *A. thaliana* and *A. majus (Clark et al., 2002; Coen and Meyerowitz, 1991)*. In *Arabidopsis thaliana* the flowers are composed of four layers of ring-shaped regions called whorls, and the action and the interaction of A-, B-, and C-class genes is important for the determination of the floral organ identities. In *Arabidopsis thaliana* the sepals are specified in whorl 1 by A-class alone, the petals by A- and B-class together in whorl 2, the stamens by B- and C-class in whorl 3, and finally, the carpel by C-class alone in whorl 4. C-class genes also play important roles in floral meristem determinacy and repress A-class genes activity in the inner two whorls. More recently MADS-box genes have been studied in *Petunia hybrida*, which led to the discovery of a new class of genes responsible for ovule identity, called D class (Colombo et al., 1995); the ABC model was therefore extended to the ABCD model.

Some years later it was shown in *Arabidopsis* that the A, B, C and D class proteins are only functional when they establish interactions with a different group of MADS-domain proteins, which is the SEPALLATA subfamily (Davies et al., 1996; Honma and Goto, 2001; Pelaz et al., 2000; Favaro et al., 2003). SEP proteins act as molecular bridges allowing the combination of different MADS-domain proteins to form higher order complexes with different transcriptional regulation activities (de Folter et al., 2005; Immink et al., 2009). *SEP* genes are therefore indicated as class E and the original ABC model has finally become the ABCDE model (Theissen, 2001).

In *Arabidopsis thaliana* except for *APETALA 2* (*AP2*) as an A-class gene, all of the ABCDE functions are encoded by genes belonging to the MADS-box family. Notably, the A-class function in perianth organ specification is controversial because no recessive A-class mutant has been identified from other species than *Arabidopsis thaliana*, raising the recent proposal of an (A)BC model (Litt, 2007; Causier et al., 2010). In this model the (A) function acts to establish the floral meristem identity and to facilitate the production of the sepals, the ground state of floral organs. Later, (A) function is required for the activation and regulation of the B- and C- functions and for the establishment of the boundaries between the B- and C- functions. In conclusion, (A)-function is needed to enable the B- and C-functions to exert their control over floral organ identity (Causier et al., 2010).

Thanks to phylogenetic analysis it was possible to identify orthologous ABCDE genes in many other species. Studies carried in various species showed that the (A)BCDE model seems to be highly conserved even in distantly related monocot species, like for instance rice (Figure 3).
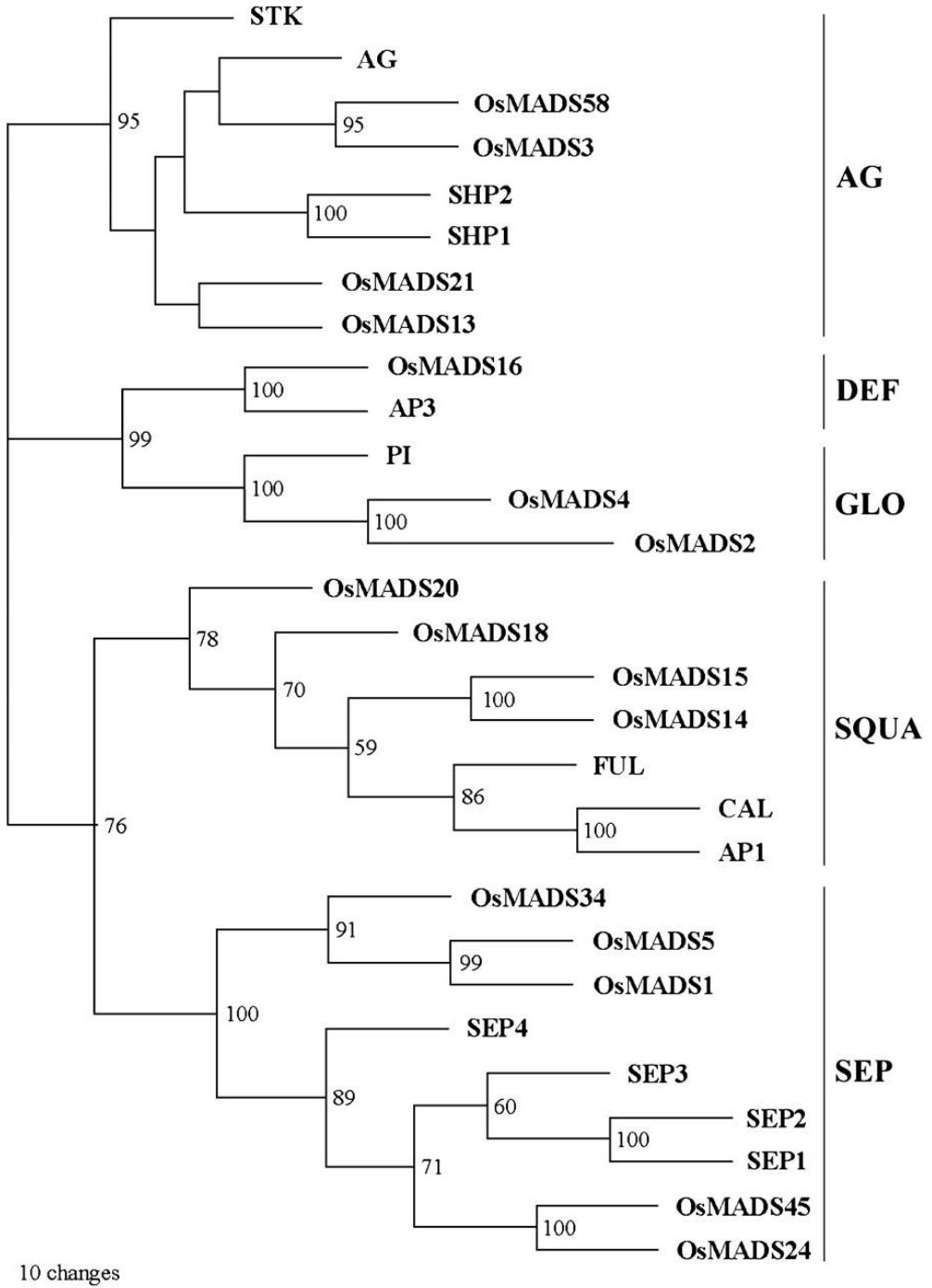
Figure 3 Phylogenetic tree of MADS-box proteins involved in rice and *Arabidopsis* flower development. (Kater et al., 2006).

A-class genes

The A-class genes of *Arabidopsis thaliana* are called *APETALA1 (AP1)* and *APETALA2 (AP2)*, the *ap1* mutant shows a homeotic conversion of sepals into bracts, and these flowers lack petals (Bowman et al., 1989). Whereas the *ap2* mutant shows the conversion of sepals into carpels and stamens into petals (Jofuku et al., 1994). The rice orthologs of *AP1* (class A) are *OsMADS14*, *OsMADS15* and *OsMADS18*, and according to the present knowledge, they are involved in the determination of the identity of the floral meristem rather than having an *Arabidopsis* type of A-class function (Kobayashi et al., 2012). *OsMADS14* and *OsMADS15* show an expression pattern similar to that of *AP1* of *Arabidopsis*: initially they are expressed throughout the floral meristem and subsequently their expression is confined to the primordia of the glumes, lemma, palea and lodicules (Kyozuka et al., 2000; Pelucchi et al., 2002). *OsMADS18* is widely expressed in all the plant tissues. Experimental data show that the overexpression of *OsMADS18* in *Arabidopsis* confers a phenotype similar to the *ap1* mutant. OsMADS18 is able to subtract AP1 protein partners from active transcription factor complexes, demonstrating that some proteins able to interact with AP1 are also able to interact with OsMADS18 (Pelucchi et al., 2002; Fornara et al., 2004). The overexpression of *OsMADS18* in rice, leads to the acceleration of the vegetative shoot meristems differentiation program, whereas silencing using RNA interference (RNAi) approach did not result in any visible phenotypic alteration.

B-class genes

The genes *APETALA3 (AP3)* and *PISTILLATA (PI)* are the B-class genes of *Arabidopsis thaliana.* In *ap3* and *pi* loss-of-function mutants, petals are replaced by sepals and stamens by carpels clearly showing that these genes control petal and stamen identity.

In rice are present three orthologs of the *Arabidopsis thaliana* B-class genes that are *OsMADS2*, *OsMADS4* e *OsMADS16. OsMADS4* is expressed in stamens and in lodicules primordia, and its suppression leads to the conversion of lodicules in paleas/lemmas and stamens into carpels. *OsMADS2* is also expressed in stamens and lodicules, but its expression is higher in lodicules. The *osmads2* mutant displays the conversion of lodicules into palea/lemma, but the development of stamens is not altered (Prasad and Vijayraghavan, 2003). Therefore it is supposed that *OsMADS2* is specialized in the determination of lodicule identity. The gene *OsMADS16*, also named *SUPERWOMAN1 (SPW1)*, is specifically expressed in lodicules and in stamens (Moon et al., 1999a, 1999b; Nagasawa et al., 2003). Recessive mutations in *SPW1*, lead to the conversion of the stamens into carpels and lodicules into palea/lemma (Nagasawa et al., 2003; Prasad and Vijayraghavan, 2003). A similar phenotype was obtained by RNAi based silencing of *SPW1* (Xiao et al., 2003). It was observed that the ectopic expression of *OsMADS16* leads to the homeotic transformation of carpels into stamens, but without any modification of the other whorls (Lee et al., 2003).

C-class genes

AGAMOUS (AG) is the only C-class gene of *Arabidopsis thaliana*, in the *ag* mutant we can observe the conversion of stamens into petals, and a new *ag* flower develops instead of the carpel. Therefore flowers are formed by a succession of sepals-petals-petals and are completely sterile (Bowman et al., 1991; Drews et al., 1991). *AG* also plays an important role in floral meristem determination, as can be noted by the repetition of the whorls present in the mutant.

The conservation of the C function was also confirmed in other plants. In *Antirrhinum majus,* the mutation of the C-class gene *PLENA* lead to the homeotic conversion of stamens into petals, while sepaloid or petaloid organs replace carpel. Moreover, like the *Arabidopsis ag* mutant, determinacy is lost in the most inner whorl (Bradley et al., 1993).

FBP6 and *pMADS3* are the C class genes of *Petunia hybrida*. The *fbp6-1* mutant flowers displays partial anther to petal conversions (Heijmans et al., 2012). Looking to the gynoecium, an incomplete fusion of the style and stigma inner tissues, and the transformation of the stigma into sepal- or leaf-like structures can be observed. The silencing of *pMADS3* by an RNAi approach, produced more severe homeotic conversions of stamens, with a reduction in the amount of pollen, but the gynoecium developed normally. Looking to plants homozygous for *fbp6* carrying the *pMADS3-RNAi* construct, it is possible to observe a full C function mutant phenotype, with the nearly complete or complete loss of reproductive organ development and the formation of a new flower in the fourth whorl.

The rice genome encodes two *AG* orthologs called *OsMADS3* and *OsMADS58* that will be presented later in detail in the paragraph related to the *AGAMOUS* subfamily.

D class genes

Two genes of *Petunia hybrida*, named *FLORAL BINDING PROTEIN7 (FBP7)* and *FLORAL BINDING PROTEIN11 (FBP11),* both expressed during ovule and seed development, were the first D-function genes discovered. Cosuppression lines in which both genes *FBP7* and *FBP11* were knocked down showed a transformation of ovules into carpel-like organs (Angenent et al., 1995). Later analyses suggested that the role of *FBP7* and *FBP11* may not be restricted to ovule identity specification; they also play a role in ovule differentiation and seed development (Colombo et al., 1997). In contrast with what was previously published by Angenent and colleagues in 1995, recent analysis on multiple transposon insertion alleles, have shown that ovule development was largely unaffected in *fbp7 fbp11* double mutants, concluding that ovule identity is not only specified by the D-class genes, but this function is redundantly shared among all *AG* members. In fact a strong loss of ovule identity was obtained combining the *fbp7 fbp11* double mutant with either the *PMADS3*-RNAi line or the *fbp6* mutant (Heijmans et al., 2012). Since cosuppression can lead to repression of homologous genes (Stam et al., 2000), C-class genes may also have been partly downregulated in the experiments performed by Angenent and colleagues in 1995. Some experiments demonstrated that the ectopic expression in the whole plant of only one of the *FBP7* or *FBP11* genes, lead to the formation of ectopic ovules on the sepals and rarely on the petals (Colombo et al., 1995). Analysis of the formation of the ovule on petals, showed that this likely occured independent of the formation of carpel tissue.

Studies on the similarity of the amino acid sequences showed that the gene of *Arabidopsis thaliana* gene orthologous to *FBP7* is *SEEDSTICK (STK)*

(Rounsley and Ditta, 1995). The *stk* mutant doesn't show loss of ovule identity, but the seeds are spaced in an irregular manner due to the excessive enlargement of the funiculus, and the detachment of the seed appears to be inhibited due to the absence of an adequate formation of the abscission zone (Pinyopich et al., 2003). Ectopic expression of *STK*, cause the conversion of sepals into carpelloid structures containing ectopic ovules (Favaro et al., 2003) (Figure 4).

Figure 4 Flower morphology of *CaMV 35S::STK* Transgenic Arabidopsis Plants (SEM images) (H) Curled carpeloid sepal with ectopic ovules. (I) and (J) Ectopic ovules, with outer (o) and inner (i) integuments and nucellus (n) (Favaro et al., 2003).

OsMADS13 is the rice D-class gene ortholog of *STK, FBP7,* and *FBP11,* this gene will be described in detail in the paragraph related to the *AGAMOUS* subfamily.

E class genes

Arabidopsis has four *SEP* subfamily genes, *SEP1*, *SEP2*, *SEP3* and *SEP4* that are largely redundant in their function. The *Arabidopsis abc* triple mutant and the quadruple mutant *sep1 spe2 sep3 sep4* display a very similar phenotype, showing the conversion of the floral organs into leaves (Ditta et al., 2004). *SEP* subfamily members are only found in angiosperms and can be divided into two subclades, namely the *AGL2/3/4* or *LOFSEP* group, and the *AGL9* or *SEP3* group; in *Arabidopsis*, *SEP3* belongs to the homonymous subclade, whereas *SEP1/2/4* are *LOFSEP* genes (Zahn et al., 2005; Malcomber and Kellogg, 2005).

Thanks to phylogenetic analysis (Figure 3) it was possible to identify *OsMADS1, OsMADS5, OsMADS7, OsMADS8* and *OsMADS34,* as the rice putative orthologs of the *Arabidopsis thaliana SEPALLATA* genes (Kater et al., 2006; Arora et al., 2007). *OsMADS1 (LEAFY HULL STERILE1 [LHS1]* has been well characterized, it is involved in determining the identity of lemma, palea and meristem of the inner floral organs (Jeon et al., 2000; Prasad et al., 2001, 2005; Agrawal et al., 2005; Chen et al., 2006). The *lhs1* mutant shows the conversion of lemma and palea in structures similar to leaves. Recent data have shown that the reduction in the expression of the *OsMADS7* and *OsMADS8* genes using a RNAi approach, lead to late flowering, and to the transformation of lodicules, stamens and carpel into palea/lemma like organs, and to floral indeterminacy (Cui et al., 2010). The quadruple mutant *osmads1 osmads5 osmads7 osmads8* shows the conversion of all floral organs, with the exception of the lemma, into structures similar to leaves (Cui et al., 2010), similarly to what happen in the *sep1 sep2 sep3 sep4* quadruple mutant. These data suggest a functional conservation of these genes in specifying

floral determinacy and organ identity between *Arabidopsis thaliana* and rice. The gene *OsMADS34* is expressed in all the vegetative and reproductive tissues. Analyzing the *osmads34* mutant, revealed the elongation of the sterile glumes and an increased number of primary branches and a decreased number of secondary branches (Gao et al., 2010). These results suggest that *OsMADS34* is involved in controlling rice inflorescence and spikelet morphology.

MADS Domain-Transcription Factors

MADS-domain transcription factors are involved in many biological processes in different organisms. They are able to modulate the transcription of their target genes by binding to the DNA as dimers. The acronym MADS (Schwarz-sommer et al., 1990) is derived from the initials of MINICHROMOSOME MAINTENANCE1 (MCM1, *Saccharomyces cerevisiae*; Passmore et al., 1988), AGAMOUS (*Arabidopsis thaliana*; Yanofsky et al., 1990), DEFICIENS (*Antirrhinum majus*; Sommer et al., 1990), and SERUM RESPONSE FACTOR (SRF, *Homo sapiens*; Norman et al., 1988). In eukaryotes MADS genes are divided into two subfamilies: the MADS-box type I or SRF-like and type II or MEF2-like (Alvarez-buylla et al., 2000), they differ for the position of the second α-helix responsible for the dimerization.

The MADS-box genes of plants have been isolated in different species and play important roles in the development of the reproductive organs. In particular, they are involved in the embryogenesis, in vegetative growth, in the determination of the floral meristem and in the development of the flower, ovule, fruit and seed. (Yanofsky et al., 1990; Colombo et al., 1995; Heck et al., 1995; Mandel and Yanofsky, 1995; Ferrándiz et al., 2000; Liljegren et al., 2000; Busi et al., 2003; Favaro et al., 2003; Pinyopich et al., 2003; Krizek and Fletcher, 2005; Brambilla et al., 2007; Dreni et al., 2007; Colombo et al., 2008; Gregis et al., 2008).

Studies using several model species, including *Arabidopsis*, *Antirrhinum majus*, *Petunia hybrida*, *Zea mays*, and *Oryza sativa*, have revealed that many of these functions are conserved among angiosperms (Schwarz-Sommer et al., 2003; Vandenbussche et al., 2003; Kater et al., 2006).

The largest part of the plant MADS-box type II family genes are formed by the MIKC-type genes. The MIKC proteins have a characteristic modular structure with, from the N to the C terminus of the protein, four characteristic domains: the MADS-box (M), Intervening (I), Keratin-like (K), and C-terminal (C) domains. In the AGAMOUS MIKC MADS-domain proteins genes involved in flower development, often an additional N-terminal region is present (Figure 5).

NH$_2$ — | MADS box | I | K box | C terminal | — COOH

Figure 5 Characteristic domains of a plant MIKC gene.

The MADS-domain consists of approximately 58 amino acids and is able to recognize and bind consensus sequences known as CArG-boxes [CC(A/T)$_6$GG], some studies demonstrated that MADS domain protein complexes often interact with DNA by contacting multiple nearby CArG box sequences, separated by less than 300 base pair (Egea-cortines et al., 1999; Zhang et al., 2008; Mendes et al., 2013). The MADS-box domain is also involved in the dimerization of the MADS-box transcription factors and in the nuclear localization of the protein complexes, and it is the most conserved region of the protein (Pellegrini et al., 1995; Santelli and Richmond, 2000; Hassler and Richmond, 2001). The 'Intervening' domain consists of about 30 amino acids, but its length is variable and it has not a clear biochemical function, but in *Arabidopsis* it is critical for the specificity in the formation of the dimers able to bind the DNA (Krizek and Meyerowitz, 1996; Riechmann et al., 1996b; Riechmann and Meyerowitz, 1997). The amino acid sequence of the K domain is not particularly conserved in contrast to the secondary structure of this domain. It contains several "abcdefg" repetitions, where "aed" are hydrophobic amino acids involved in the formation of amphipathic α-

helices (Riechmann and Meyerowitz, 1997). Some publications show that the K domain is essential for protein-protein interactions between different MADS-domain transcription factors (Davies et al., 1996; Fan et al., 1997; Egea-cortines et al., 1999; Riechmann et al., 1996a; Zachgo et al., 1995). The C-terminal domain is highly variable in sequence and in length and its function is fundamental for the proteins. In fact, the overexpression of MADS-domain genes without the C-terminal region cause dominant negative phenotypes (Mizukami et al., 1996). The C-terminal domain may contain a transcriptional activation domain (Cho et al., 1999), or can play important roles for the formation of functional multimeric complexes, able to control the transcription of the genes modulated by the MADS-domain proteins (Egea-cortines et al., 1999; Masiero et al., 2002).

MADS-box protein interactions

The MADS-domain transcription factors interact forming homo-or hetero-dimers and these protein-protein interactions are essential for the binding to the CArG box consensus sequence (Shore and Sharrocks, 1995). It has been shown that B class proteins of *Antirrhinum majus*, denominated DEFICIENS (DEF) and GLOBOSA (GLO), form a ternary complex with the protein SQUAMOSA to control the flower architecture (Egea-cortines et al., 1999). Other recent work demonstrated that SEP proteins act as molecular bridges allowing the combination of different MADS-domain proteins to form higher-order complexes with different transcriptional regulation activities (Immink et al., 2009). Thanks to two-hybrid interaction experiments between proteins in yeast, it was possible to achieve a complete map of protein-protein interactions between the MADS-domain transcription factors in *Arabidopsis thaliana* (de Folter et al., 2005), but unfortunately this large scale analysis was not carried out for rice's MADS-domain transcription factors (Figure 6).
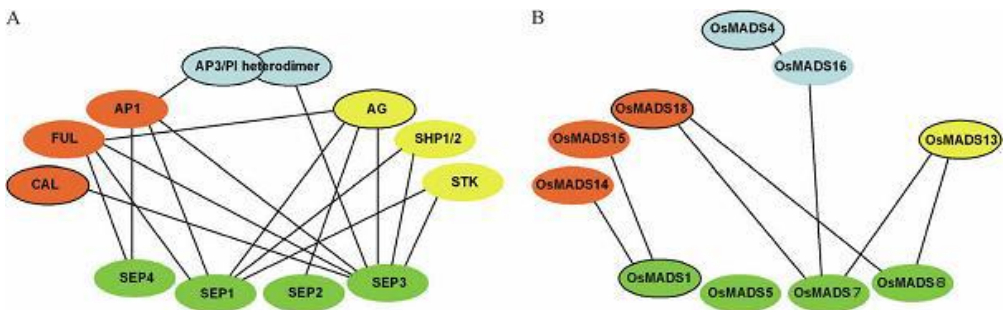

Figure 6 MADS-box protein interactions in yeast two-hybrid screens in *Arabidopsis* and rice (Kater et al., 2006)

In *Arabidopsis thaliana*, the AP3-PI dimer interacts with AP1 and SEP3, AG interacts with SEP3, which acts as a mediator in the interaction with the dimer PI-AP3 (Honma and Goto, 2001). These interactions are the basis of the so-

called "quartet model", which says that the complexes formed by AP1-AP3-SEP3-PI and PI-AP3-SEP3-AG, respectively, specify the identity of petals and stamens(Honma and Goto, 2001; Theissen and Saedler, 2001).

The model of interactions between MADS-domain transcription factors is also similar for rice, in fact the SEP-like proteins OsMADS8 (also named OsMADS45) and OsMADS7 (also named OsMADS24), show an interaction profile similar to the SEP proteins in *Arabidopsis thaliana*. They interact with OsMADS18 (similar to AP1), with OsMADS16 (similar to AP3) and with OsMADS13 (similar to STK). Recent data have shown that OsMADS8 and OsMADS7 are able to interact with STK of *Arabidopsis thaliana* and FBP7 of *Petunia*, demonstrating that the interactions between the SEP and D-class proteins are conserved from an evolutionary point of view (Favaro et al., 2002, 2003). A recent yeast-3-hybrid assay shows that in *Arabidopsis*, the homeodomain transcription factor involved in the formation of integuments BEL1 (Reiser et al., 1995), interacts with the ovule identity MADS-domain factors when they dimerize with SEPALLATA proteins. Therefore in the quadruple mutant *bel1 stk shp1 shp2* the integument is transformed into a carpelloid leaf-like structure and development of the female gametophyte arrests before meiosis (Brambilla et al., 2007). Concluding, SEP proteins are important for the formation of ternary complexes and it has been shown that the SEP3 protein is able to activate transcription in yeast and in plant cells (Honma and Goto, 2001) and therefore SEP proteins are probably not only important for higher order MADS-domain protein complex formation but also to provide transcriptional activation activity to these complexes.

The *AGAMOUS* subfamily and the development of reproductive organs

The C- and D-class genes, wich are involved in reproductive organ identity determination, fruit, and seed development, belong to the same MADS-box phylogenetic group, which has been named the *AGAMOUS* subfamily (Becker and Theissen, 2003; Kramer and Jaramillo, 2004; Zahn et al. 2006).

An ancient gene duplication event, that predated the diversification of extant flowering plants, generated two lineages within the *AGAMOUS* subfamily, which are conserved in all angiosperms, the *AGAMOUS* lineage and the *AGL11* lineage. Subsequently, in core eudicots, the AGAMOUS lineage was further divided into *PLE* lineage and *euAG* lineage (Kramer and Jaramillo, 2004; Zahn et al.).

In general, it was show that the C function is conserved among *AGAMOUS* lineage genes. A single *AGAMOUS* lineage gene can play all the aspects of the C function, like the *Arabidopsis AG* gene; (Yanofsky et al., 1990) or multiple *AGAMOUS* lineage paralogs can show different degrees of redundancy/subfunctionalization, like in petunia (Heijmans et al., 2012). In the *AGAMOUS* lineage of *Arabidopsis thaliana,* besides *AGAMOUS*, there are two other members of this lineage named *SHATTERPROOF1* (*SHP1*) and *SHP2* (Liljegren et al., 2000). Despite *AG* that belong to the *euAG* lineage, the genes *SHP1* and *SHP2*, belong to the *PLE* lineage and not directly contribute to the C function. Therefore *SHP1* and *SHP2* are important for dehiscence zone formation in the siliques and thereby for pod shattering.

In *Arabidopsis* the fourth member of the *AGAMOUS* subfamily, and the only member of the *AGL11* lineage, is *STK.* As said before, it is involved in regulating seed detachment, seed coat development and embryosac cell

identity (Pinyopich et al., 2003; Mizzotti et al., 2012; Matias-Hernandez et al., 2010). *STK* is specifically expressed in the ovule (Rounsley and Ditta, 1995; Pinyopich et al., 2003) like others *AGL11* lineage genes found in other core eudicots (Kramer and Jaramillo, 2004).

It was published that the genes belonging to the *AGL11* lineage genes share redundancy with the ones belonging to the *AGAMOUS* lineage in specifying ovule identity (Pinyopich et al., 2003; Heijmans et al., 2012). The analysis of the triple mutant *stk shp1 shp2,* showed that ovule integuments were converted into carpelloid leaves (Pinyopich et al., 2003; Brambilla et al., 2007). The *ag* mutants completely lacks stamens and carpels, but the *ap2 ag* double mutant develops carpeloid sepals with ectopic ovules (Bowman et al., 1991). This indicates that there are alternative pathways or other genes that can promote the development of carpels and ovules without *AG*. The quadruple mutant *ap2 ag shp1 shp2* however, lacks ovule and carpels (Pinyopich et al., 2003); which shows that *SHP1* and *SHP2* are responsible for the formation of carpels and ovules in the a*p2 ag* double mutant. It's possible to complement the *ag* mutant phenotype, transforming *Arabidopsis* with a *35S::SHP2* (Pinyopich et al., 2003). These experiments show that *SHP1* and *SHP2* are partially redundant with *AG* and the explanation for their different activities is that their function in plant development is highly dependent on the difference in expression pattern. In wild-type plants *SHP1/SHP2* are already expressed in the ovule before fertilization and then in the dehiscence zone of the fruit, whereas *STK* is specifically expressed in the ovule and in the funiculus. Airoldi and Davies in 2012 concluded that AG is the only C function factor of *Arabidopsis*, whereas SHP proteins fully maintain a similar potential activity but they are not physically present in the meristem and primordia cells at the appropriate time. Their functions are rather restricted to regulate specific tissue types after

carpel identity has been established (Airoldi and Davies, 2012). It is possible to conclude that *AGAMOUS* lineage genes can regulate C, D, or both these functions, whereas *AGL11* lineage genes are normally specialized in the D function. In addition to *A. thaliana*, *AGAMOUS* subfamily members have been functionally characterized in several other species.

In monocots it is possible to find data for C and D function genes only for rice and maize, where it was possible to generate loss of function mutants. In the Poaceae (grass family) both *AGAMOUS* and *AGL11* lineages are further duplicated within this family (Kramer and Jaramillo, 2004; Zahn et al.; Dreni et al., 2007) (Figure 8).

The *AGAMOUS* subfamily of *Oryza sativa* L. is composed of four genes, namely *OsMADS3*, *OsMADS58*, *OsMADS13* and *OsMADS21*. *OsMADS3* and *OsMADS58* belong to the *AGAMOUS* lineage and play roles similar to *AGAMOUS*, but their functions in meristem determinacy and floral organs identity are differently distributed between them and their predominant action is also carried out in different whorls (Kang et al., 1998; Yamaguchi et al., 2006). The knock-out mutant *osmads3-2* shows the partial conversion of stamens into lodicules, but the defects in whorl 4 were very weak, and an increase in carpel number was observed in only a few flowers (Yamaguchi et al., 2006). Whereas in the severe loss-of-function allele *osmads3-3*, almost all stamens in whorl 3 were homeotically transformed into lodicule-like organs. Some stamens were completely transformed into lodicules that were indistinguishable from those in the second whorl.

From studies conducted on the *osmads3-4* intermediate mutant, it was shown that *OsMADS3* also plays a pivotal role in late anther development at least in part by regulating ROS (Reactive Oxygen Species) homeostasis. It was observed that OsMADS3 is able to directly bind the promoter of *MT-1-4b*, a

gene that encodes a metallothionein involved in ROS-scavenging (Hu et al., 2011).

The *osmads58* RNAi mutant has indeterminate flowers and a reiteration of the floral organs including stamens and carpelloid structures were observed. So it seems that *OsMADS3* plays a predominant role in determining stamen identity, while *OsMADS58* in the determination of carpel identity and floral meristem determinacy (Yamaguchi et al., 2006). The suppression of *OsMADS58* using an RNAi approach in the *osmads3-2* (a weak mutant allele of *OsMADS3)* mutant background, doesn't lead to the homeotic conversion of carpels into palea/lemma as predicted by the ABCDE model. On the other hand, the homeotic mutation of carpels into stamens and failure of midrib formation in leaves was observed when the gene *DROOPING LEAF (DL)* was mutated (Nagasawa et al., 2003; Yamaguchi et al., 2004). From the analysis of the flower of the *dl* mutant and the data obtained from the silencing of *OsMADS58* by RNAi in a *osmads3-2* weak allele mutant background, Yamaguchi et al., 2006 proposed that, in rice, the *AGAMOUS* orthologs *OsMADS3* and *OsMADS58* do not regulate carpel identity. They proposed that *DL*, the ortholog of the *Arabidopsis CRABS CLAW (CRC)*, is involved in regulating carpel identity in rice (Yamaguchi et al., 2004). Moreover, DL/CRC interacts antagonistically with class B genes repressing them in the fourth whorl (Alvarez and Smyth, 1999; Yamaguchi et al., 2004), suggesting that DL and CRC play a conserved and diversified role in controlling carpel identity in rice and *Arabidopsis*. Recently, Li and coworkers (2011a) observed genetic interactions between *DROOPING LEAF* and the *AGAMOUS* subfamily genes *OsMADS3* and *OsMADS13*. Their results show that the loss of FM determinacy is increased in the *osmads3 dl* double mutant and that *DL* and *OsMADS13* may function in the same pathway specifying the identity of

carpel/ovule and floral meristem. *DL* may act upstream of *OsMADS13*, positively regulating *OsMADS13* expression, while *OsMADS13* may repress the ectopic expression of *DL* in the ovule(Li et al., 2011).

The genes *OsMADS13* and *OsMADS21,* belonging to the *AGL11* lineage, are the other two members of the rice *AGAMOUS* subfamily. Their expression is quite different; in fact *OsMADS13* is ovule-specific, whereas *OsMADS21* is also expressed in anthers, carpel and stigmas (Lopez-Dee et al., 1999; Dreni et al., 2007). The *osmads13* knock-out mutant (Figure 7 F-G-H-I-J) is female sterile and ovules are converted into carpelloid structures. We can also observe the formation of carpels inside carpels phenotype, suggesting that *OsMADS13* plays an important role in floral meristem determinacy (Dreni et al., 2007; Arora et al., 2007). The knock-out mutant *osmads21* is fully fertile and indistinguishable from wild-type plants, in the double mutant *osmads13 osmads21*, the loss of functionality of the gene *OsMADS21* does not cause an increase of the *osmads13* phenotype. The amino acid sequence of the protein encoded by the gene *OsMADS21* is very similar to that of *OsMADS13*, but despite this, the gene *OsMADS21* seem not to be involved in ovule development (Dreni et al., 2007) but it plays a role during seed development (Dreni personal communication). So it seems that *OsMADS13* and *OsMADS21* are not redundant in their function, and that the only gene responsible for the ovule formation in rice is *OsMADS13*.

The rice *AGAMOUS* subfamily was recently expanded with a new member named *OsMADS66* (Arora et al., 2007), which is closely linked to the *OsMADS58* locus and seems to represents a tandem gene duplication event. Therefore, *OsMADS66* is most likely a non-functional pseudogene in the *Oryza sativa* subspecies, whereas its status in most of the other *Oryza* species has yet to be determined.
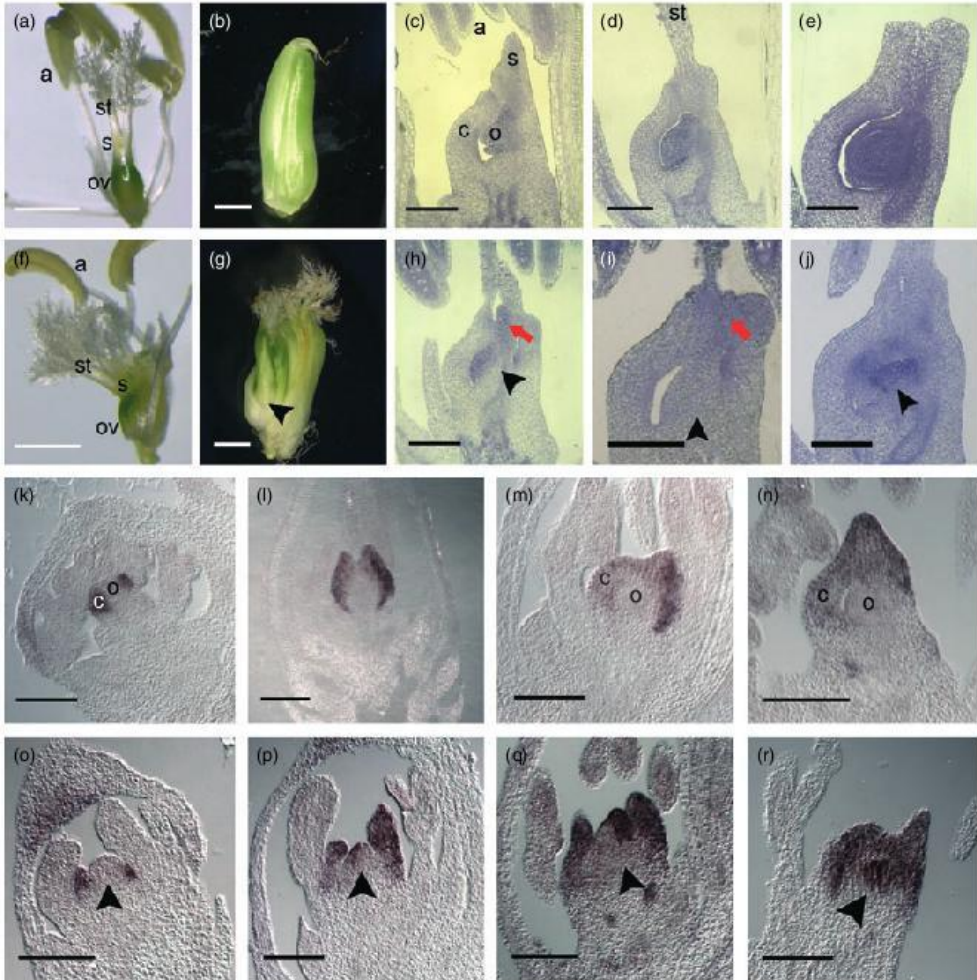
Figure 7 Analysis of the *osmads13* mutant phenotype. (a) Wild-type carpel at about 1 day before flowering. (b) Wild-type kernel at 6 days after pollination. (c–e) Histologic analysis of wild-type developing carpels. (f) *osmads13* carpel with extra stigmas protruding from the carpel. (g) *osmads13* carpel at later stages (after flowering) in which the indeterminate formation of carpels can be observed (indicated with an arrowhead). (h, i) histologic analysis of *osmads13* pistils in which the ovule is homeotically converted into a carpel (indicated with arrowheads). (j) Histologic analysis of *osmads13* (k–r) *DL* expression in wild-type and *osmads13* mutant flowers at different stages of development: (k–n) wild-type flowers, (o–r) mutant flowers. White and black scale bars represent 1 mm and 100 μm, respectively (Dreni et al., 2007).
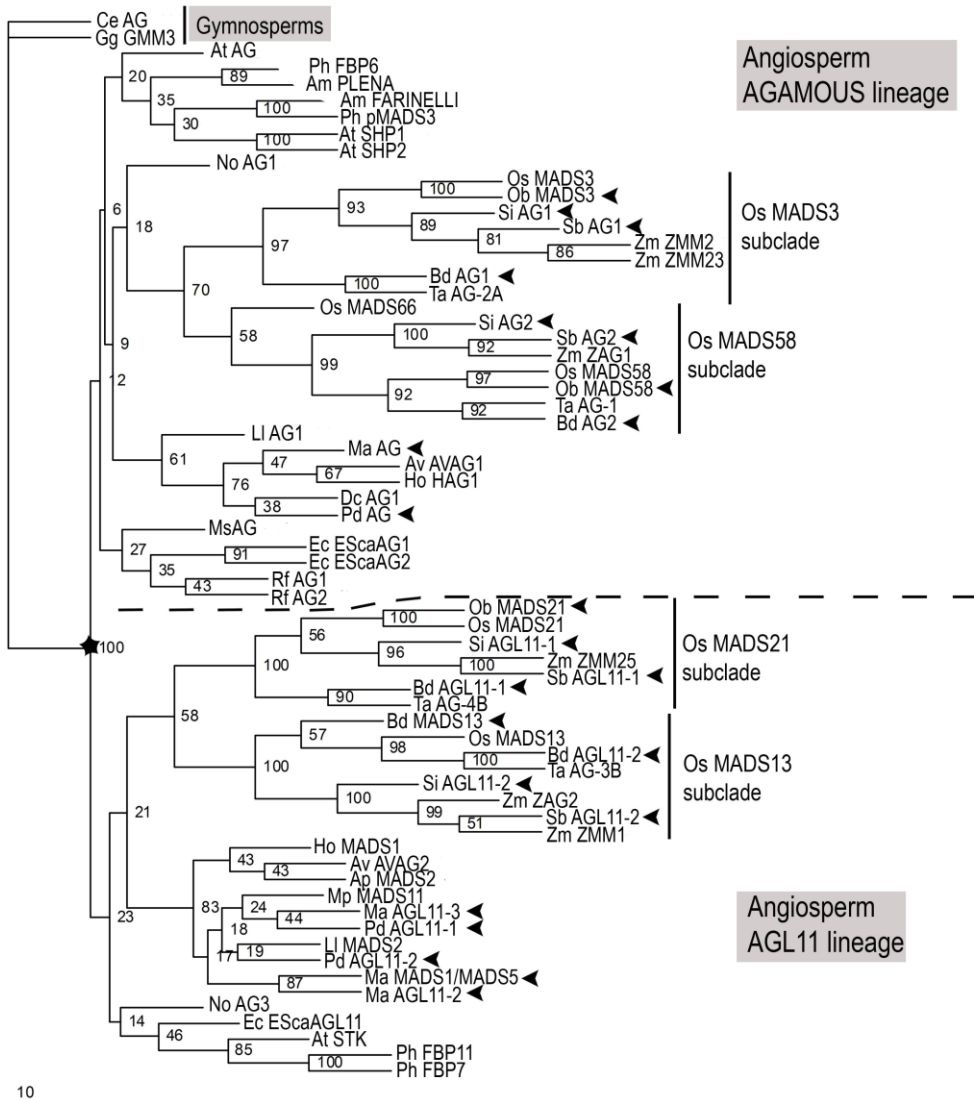
Figure 8 Phylogenetic tree of AGAMOUS subfamily proteins. The dichotomy between *AGAMOUS* and *AGL11* lineages is marked with a black stars(Dreni et al., 2007).

REFERENCES

Agrawal, G.K., Abe, K., Yamazaki, M., Miyao, A., and Hirochika, H. (2005). Conservation of the E-function for floral organ identity in rice revealed by the analysis of tissue culture-induced loss-of-function mutants of the OsMADS1 gene. Plant Mol. Biol. **59**: 125–35.

Airoldi, C.A. and Davies, B. (2012). Gene duplication and the evolution of plant MADS-box transcription factors. J. Genet. Genomics **39**: 157–65.

Alvarez, J. and Smyth, D.R. (1999). CRABS CLAW and SPATULA, two Arabidopsis genes that control carpel development in parallel with AGAMOUS. Development **126**: 2377–86.

Alvarez-buylla, E.R., Pelaz, S., Liljegren, S.J., Gold, S.E., Burgeff, C., Ditta, G.S., Yanofsky, M.F., and Pouplana, L.R. De (2000). An ancestral MADS-box gene duplication occurred before the divergence of plants and animals. **97**.

Angenent, G.C., Franken, J., Busscher, M., Dijken, A. Van, Dons, H.J.M., and Tunena, A.J. Van (1995). A Novel Class of MADS Box Genes 1 s Involved in Ovule Development in Petunia. **7**: 1569–1582.

Arora, R., Agarwal, P., Ray, S., Singh, A.K., Singh, V.P., Tyagi, A.K., and Kapoor, S. (2007). MADS-box gene family in rice: genome-wide identification, organization and expression profiling during reproductive development and stress. BMC Genomics **8**: 242.

Becker, A. and Theissen, G. (2003). The major clades of MADS-box genes and their role in the development and evolution of flowering plants. Mol. Phylogenet. Evol. **29**: 464–89.

Bowman, J.L., Drews, G.N., and Meyerowitz, E.M. (1991). Expression of the Arabidopsis floral homeotic gene AGAMOUS is restricted to specific cell types late in flower development. Plant Cell **3**: 749–58.

Bowman, J.L., Smyth, D.R., and Meyerowitz, E.M. (1989). Genes directing flower development in Arabidopsis. Plant Cell **1**: 37–52.

Bradley, D., Carpenter, R., Sommer, H., Hartley, N., and Coen, E. (1993). Complementary floral homeotic phenotypes result from opposite orientations of a transposon at the plena locus of Antirrhinum. Cell **72**: 85–95.

Brambilla, V., Battaglia, R., Colombo, M., Masiero, S., Bencivenga, S., Kater, M.M., and Colombo, L. (2007). Genetic and molecular interactions between BELL1 and MADS box factors support ovule development in Arabidopsis. Plant Cell **19**: 2544–56.

Busi, M.V., Bustamante, C., D'Angelo, C., Hidalgo-Cuevas, M., Boggio, S.B., Valle, E.M., and Zabaleta, E. (2003). MADS-box genes expressed during tomato seed and fruit development. Plant Mol. Biol. **52**: 801–15.

Causier, B., Schwarz-Sommer, Z., and Davies, B. (2010). Floral organ identity: 20 years of ABCs. Semin. Cell Dev. Biol. **21**: 73–9.

Chen, Z.-X., Wu, J.-G., Ding, W.-N., Chen, H.-M., Wu, P., and Shi, C.-H. (2006). Morphogenesis and molecular basis on naked seed rice, a novel homeotic mutation of OsMADS1 regulating transcript level of AP3 homologue in rice. Planta **223**: 882–90.

Cho, S., Jang, S., Chae, S., Chung, K.M., and Moon, Y. (1999). Analysis of the C-terminal region of Arabidopsis thaliana APETALA1 as a transcription activation domain. 419–429.

Clark, J.I., Coen, E.S., and Centre, J.I. (2002). The cycloidea gene can respond to a common dorsoventral prepattern in Antirrhinum. **30**.

Coen, E.S. and Meyerowitz, E.M. (1991). The war of the whorls: genetic interactions controlling flower development. Nature **353**: 31–7.

Colombo, L., Battaglia, R., and Kater, M.M. (2008). Arabidopsis ovule development and its evolutionary conservation. Trends Plant Sci. **13**: 444–50.

Colombo, L., Franken, J., Koetje, E., van Went, J., Dons, H.J., Angenent, G.C., and van Tunen, A.J. (1995). The petunia MADS box gene FBP11 determines ovule identity. Plant Cell **7**: 1859–68.

Colombo, L., Franken, J., Van der Krol, A.R., Wittich, P.E., Dons, H.J., and Angenent, G.C. (1997). Downregulation of ovule-specific MADS box genes from petunia results in maternally controlled defects in seed development. Plant Cell **9**: 703–15.

Cui, R., Han, J., Zhao, S., Su, K., Wu, F., Du, X., Xu, Q., Chong, K., Theissen, G., and Meng, Z. (2010). Functional conservation and diversification of class E floral homeotic genes in rice (Oryza sativa). Plant J. **61**: 767–81.

Davies, B., Egea-cortines, M., Silva, E.D.A., Saedler, H., and Sommer, H. (1996). Multiple interactions amongst floral homeotic MADS box proteins. **15**: 4330–4343.

Ditta, G., Pinyopich, A., Robles, P., Pelaz, S., and Yanofsky, M.F. (2004). The SEP4 gene of Arabidopsis thaliana functions in floral organ and meristem identity. Curr. Biol. **14**: 1935–40.

Dreni, L., Jacchia, S., Fornara, F., Fornari, M., Ouwerkerk, P.B.F., An, G., Colombo, L., Kater, M.M., and Celoria, V. (2007). The D-lineage MADS-box gene OsMADS13 controls ovule identity in rice. 690–699.

Dreni, L. and Kater, M.M. (2013). MADS reloaded: evolution of the AGAMOUS subfamily genes. New Phytol.

Drews, G.N., Weigel, D., and Meyerowitz, E.M. (1991). Floral patterning. Curr. Opin. Genet. Dev. **1**: 174–8.

Egea-cortines, M., Saedler, H., and Sommer, H. (1999). Ternary complex formation between the MADS-box proteins SQUAMOSA , DEFICIENS and GLOBOSA is involved in the control of floral architecture in Antirrhinum majus. **18**: 5370–5379.

Fan, H., Hu, Y., Tudor, M., and Ma, H. (1997). Specific interactions between the K domains of AG and AGLs , members of the MADS domain family of DNA binding proteins. **12**: 999–1010.

Favaro, R., Immink, R.G.H., Ferioli, V., Bernasconi, B., Byzova, M., Angenent, G.C., Kater, M., and Colombo, L. (2002). Ovule-specific MADS-box proteins have conserved protein-protein interactions in monocot and dicot plants. Mol. Genet. Genomics **268**: 152–9.

Favaro, R., Pinyopich, A., Battaglia, R., Kooiker, M., Borghi, L., Ditta, G., Yanofsky, M.F., Kater, M.M., and Colombo, L. (2003). MADS-box protein complexes control carpel and ovule development in Arabidopsis. Plant Cell **15**: 2603–11.

Ferrándiz, C., Gu, Q., Martienssen, R., and Yanofsky, M.F. (2000). Redundant regulation of meristem identity and plant architecture by FRUITFULL, APETALA1 and CAULIFLOWER. Development **127**: 725–34.

De Folter, S., Immink, R.G.H., Kieffer, M., Parenicová, L., Henz, S.R., Weigel, D., Busscher, M., Kooiker, M., Colombo, L., Kater, M.M., Davies, B., and Angenent, G.C. (2005). Comprehensive interaction map of the Arabidopsis MADS Box transcription factors. Plant Cell **17**: 1424–33.

Fornara, F., Parenicová, L., Falasca, G., Pelucchi, N., Masiero, S., Ciannamea, S., Lopez-Dee, Z., Altamura, M.M., Colombo, L., and Kater, M.M. (2004). Functional characterization of OsMADS18, a member of the AP1/SQUA subfamily of MADS box genes. Plant Physiol. **135**: 2207–19.

Gao, X., Liang, W., Yin, C., Ji, S., Wang, H., Su, X., Guo, C., Kong, H., Xue, H., and Zhang, D. (2010). The SEPALLATA-like gene OsMADS34 is required for rice inflorescence and spikelet development. Plant Physiol. **153**: 728–40.

Gregis, V., Sessa, A., Colombo, L., and Kater, M.M. (2008). AGAMOUS-LIKE24 and SHORT VEGETATIVE PHASE determine floral meristem identity in Arabidopsis. Plant J. **56**: 891–902.

Hassler, M. and Richmond, T.J. (2001). The B-box dominates SAP-1-SRF interactions in the structure of the ternary complex. EMBO J. **20**: 3018–28.

Heck, G.R., Perry, S.E., and Nichols, K.W. (1995). AGL15 , a MADS Domain Protein Expressed in Developing Embryos. **7**: 1271–1282.

Heijmans, K., Ament, K., Rijpkema, A.S., Zethof, J., Wolters-arts, M., Gerats, T., and Vandenbussche, M. (2012). Rede fi ning C and D in the Petunia ABC. **24**: 2305–2317.

Honma, T. and Goto, K. (2001). Complexes of MADS-box proteins are sufficient to convert leaves into floral organs. Nature **409**: 525–9.

Hu, L., Liang, W., Yin, C., Cui, X., Zong, J., Wang, X., Hu, J., and Zhang, D. (2011). Rice MADS3 regulates ROS homeostasis during late anther development. Plant Cell **23**: 515–33.

Ikeda, K., Sunohara, H., and Nagato, Y. (2004). Developmental Course of Inflorescence and Spikelet in Rice. **156**: 147–156.

Immink, R.G.H., Tonaco, I.A.N., de Folter, S., Shchennikova, A., van Dijk, A.D.J., Busscher-Lange, J., Borst, J.W., and Angenent, G.C. (2009). SEPALLATA3: the "glue" for MADS box transcription factor complex formation. Genome Biol. **10**: R24.

Itoh, J.-I., Nonomura, K.-I., Ikeda, K., Yamaki, S., Inukai, Y., Yamagishi, H., Kitano, H., and Nagato, Y. (2005). Rice plant development: from zygote to spikelet. Plant Cell Physiol. **46**: 23–47.

Jeon, J.S., Jang, S., Lee, S., Nam, J., Kim, C., Lee, S.H., Chung, Y.Y., Kim, S.R., Lee, Y.H., Cho, Y.G., and An, G. (2000). leafy hull sterile1 is a homeotic mutation in a rice MADS box gene affecting rice flower development. Plant Cell **12**: 871–84.

Jofuku, K.D., Boerib, B.G.W. Den, Montaguib, M. Van, and Okamuroa, J.K. (1994). Control of Arabidopsis Flower and Seed Development by the Homeotic Gene APETALA2. **6**: 1211–1225.

Kang, H.G., Jeon, J.S., Lee, S., and An, G. (1998). Identification of class B and class C floral organ identity genes from rice plants. Plant Mol. Biol. **38**: 1021–9.

Kater, M.M., Dreni, L., and Colombo, L. (2006). Functional conservation of MADS-box factors controlling floral organ identity in rice and Arabidopsis. J. Exp. Bot. **57**: 3433–44.

Kobayashi, K., Yasuno, N., Sato, Y., Yoda, M., Yamazaki, R., Kimizu, M., Yoshida, H., Nagamura, Y., and Kyozuka, J. (2012). In fl orescence Meristem Identity in Rice Is Speci fi ed by Overlapping Functions of Three AP1 / FUL -Like MADS Box Genes and PAP2 , a SEPALLATA MADS Box Gene. **24**: 1848–1859.

Kramer, E.M. and Jaramillo, M.A. (2004). Patterns of Gene Duplication and Functional Evolution During the Diversification of the AGAMOUS Subfamily of MADS Box Genes in Angiosperms.

Krizek, B.A. and Fletcher, J.C. (2005). Molecular mechanisms of flower development: an armchair guide. Nat. Rev. Genet. **6**: 688–98.

Krizek, B.A. and Meyerowitz, E.M. (1996). Mapping the protein regions responsible for the functional specificities of the Arabidopsis MADS domain organ-identity proteins. Proc. Natl. Acad. Sci. U. S. A. **93**: 4063–70.

Kyozuka, J., Kobayashi, T., Morita, M., and Shimamoto, K. (2000). Spatially and Temporally Regulated Expression of Rice MADS Box Genes with Similarity to Arabidopsis Class A , B and C Genes. **41**: 710–718.

Lee, S., Kyungsook, Æ.J.J.Æ., Sanghee, Y.M.Æ., and Chung, L.Æ.Y. (2003). Alteration of floral organ identity in rice through ectopic expression of OsMADS16. 904–911.

Li, H., Liang, W., Yin, C., Zhu, L., and Zhang, D. (2011). Genetic interaction of OsMADS3, DROOPING LEAF, and OsMADS13 in specifying rice floral organ identities and meristem determinacy. Plant Physiol. **156**: 263–74.

Liljegren, S.J., Ditta, G.S., Eshed, Y., Savidge, B., Bowman, J.L., and Yanofsky, M.F. (2000). SHATTERPROOF MADS-box genes control seed dispersal in Arabidopsis. Nature **404**: 766–70.

Litt, A. (2007). An Evaluation of A-Function: Evidence from the APETALA1 and APETALA2 Gene Lineages. Int. J. Plant Sci. **168**: 73–91.

Lopez-Dee, Z.P., Wittich, P., Enrico Pè, M., Rigola, D., Del Buono, I., Gorla, M.S., Kater, M.M., and Colombo, L. (1999). OsMADS13, a novel rice MADS-box gene expressed during ovule development. Dev. Genet. **25**: 237–44.

Malcomber, S.T. and Kellogg, E.A. (2005). SEPALLATA gene diversification: brave new whorls. Trends Plant Sci. **10**: 427–35.

Mandel, M.A. and Yanofsky, M.F. (1995). A gene triggering flower formation in Arabidopsis. Nature **377**: 522–4.

Masiero, S., Imbriano, C., Ravasio, F., Favaro, R., Pelucchi, N., Gorla, M.S., Mantovani, R., Colombo, L., and Kater, M.M. (2002). Ternary complex formation between MADS-box transcription factors and the histone fold protein NF-YB. J. Biol. Chem. **277**: 26429–35.

Matias-Hernandez, L., Battaglia, R., Galbiati, F., Rubes, M., Eichenberger, C., Grossniklaus, U., Kater, M.M., and Colombo, L. (2010). VERDANDI is a direct

target of the MADS domain ovule identity complex and affects embryo sac differentiation in Arabidopsis. Plant Cell **22**: 1702–15.

Mendes, M.A., Guerra, R.F., Berns, M.C., Manzo, C., Masiero, S., Finzi, L., Kater, M.M., and Colombo, L. (2013). MADS domain transcription factors mediate short-range DNA looping that is essential for target gene expression in Arabidopsis. Plant Cell **25**: 2560–72.

Mizukami, Y., Huang, H., and Tudor, M. (1996). Functional Domains of the Floral Regulator AGAMOUS: Characterization of the DNA Binding Domain and Analysis of Dominant Negative Mutations. **8**: 831–845.

Mizzotti, C., Mendes, M.A., Caporali, E., Schnittger, A., Kater, M.M., Battaglia, R., and Colombo, L. (2012). The MADS box genes SEEDSTICK and ARABIDOPSIS Bsister play a maternal role in fertilization and seed development. Plant J. **70**: 409–20.

Moon, Y., Jung, J., Kang, H., and An, G. (1999a). Identification of a rice APETALA3 homologue by yeast two-hybrid screening. **1**: 167–177.

Moon, Y., Kang, H., Jung, J., Jeon, J., Sung, S., and An, G. (1999b). Determination of the Motif Responsible for Interaction between the Rice APETALA1 / AGAMOUS-LIKE9 Family Proteins Using a Yeast Two-Hybrid System 1. **120**: 1193–1203.

Nagasawa, N., Miyoshi, M., Sano, Y., Satoh, H., Hirano, H., Sakai, H., and Nagato, Y. (2003). SUPERWOMAN1 and DROOPING LEAF genes control floral organ identity in rice. Development **130**: 705–18.

Norman, C., Runswick, M., Pollock, R., and Treisman, R. (1988). Isolation and properties of cDNA clones encoding SRF, a transcription factor that binds to the c-fos serum response element. Cell **55**: 989–1003.

Passmore, S., Maine, G.T., Elble, R., Christ, C., and Tye, B.K. (1988). Saccharomyces cerevisiae protein involved in plasmid maintenance is necessary for mating of MAT alpha cells. J. Mol. Biol. **204**: 593–606.

Pelaz, S., Ditta, G.S., and Yanofsky, M.F. (2000). B and C ‾ oral organ identity functions require SEPALLATA MADS-box genes. **405**: 9–12.

Pellegrini, L., Tan, S., and Richmond, T.J. (1995). Structure of serum response factor core bound to DNA. Nature **376**: 490–8.

Pelucchi, N., Fornara, F., Favalli, C., Masiero, S., Lago, C., P�, E.M., Colombo, L., and Kater, M.M. (2002). Comparative analysis of rice MADS-box genes expressed during flower development. Sex. Plant Reprod. **15**: 113–122.

Pinyopich, A., Ditta, G.S., Savidge, B., Liljegren, S.J., Baumann, E., Wisman, E., and Yanofsky, M.F. (2003). Assessing the redundancy of MADS-box genes during carpel and ovule development. 85–88.

Prasad, K., P., S., Kumar, S., Kushalappa, K., and Vijayraghavan, U. (2001). Ectopic expression of rice OsMADS1 reveals a role in specifying the lemma and palea, grass floral organs analogous to sepals. Dev. Genes Evol. **211**: 281–290.

Prasad, K., Parameswaran, S., and Vijayraghavan, U. (2005). OsMADS1, a rice MADS-box factor, controls differentiation of specific cell types in the lemma and palea and is an early-acting regulator of inner floral organs. Plant J. **43**: 915–28.

Prasad, K. and Vijayraghavan, U. (2003). Note Uncovers Its Second-Whorl-Specific Function in Floral Organ Patterning. **2305**: 2301–2305.

Reiser, L., Modrusan, Z., Margossian, L., Samach, A., Ohad, N., Haughn, G.W., and Fischer, R.L. (1995). The BELL1 gene encodes a homeodomain protein involved in pattern formation in the Arabidopsis ovule primordium. Cell **83**: 735–42.

Riechmann, J.L. and Meyerowitz, E.M. (1997). MADS domain proteins in plant development. Biol. Chem. **378**: 1079–101.

Riechmann, J.L., Wang, M., and Meyerowitz, E.M. (1996a). DNA-binding properties of Arabidopsis MADS domain homeotic proteins APETALA1 , APETALA3 , PISTILLATA and AGAMOUS. **24**: 3134–3141.

Riechmann, J.L., Wang, M., and Meyerowitz, E.M. (1996b). DNA-binding properties of Arabidopsis MADS domain homeotic proteins APETALA1, APETALA3, PISTILLATA and AGAMOUS. Nucleic Acids Res. **24**: 3134–41.

Rounsley, S.D. and Ditta, G.S. (1995). Diverse Roles for MADS Box Genes in Arabidopsis Development. **7**: 1259–1269.

Santelli, E. and Richmond, T.J. (2000). Crystal structure of MEF2A core bound to DNA at 1.5 A resolution. J. Mol. Biol. **297**: 437–49.

Sato, Y., Hong, S., Tagiri, A., Kitano, H., Yamamoto, N., Nagato, Y., and Matsuoka, M. (1996). A rice homeobox gene,. **93**: 8117–8122.

Schwab, R., Ossowski, S., Riester, M., Warthmann, N., and Weigel, D. (2006). Highly Specific Gene Silencing by Artificial MicroRNAs in Arabidopsis. **18**: 1121–1133.

Schwarz-Sommer, Z., Davies, B., and Hudson, A. (2003). An everlasting pioneer: the story of Antirrhinum research. Nat. Rev. Genet. **4**: 657–66.

Schwarz-sommer, Z., Huijser, P., and Nacgen, W. (1990). Genetic Control of Flower Development by Homeotic Genes in Antirrhinum majus.

Shore, P. and Sharrocks, A.D. (1995). The MADS-box family of transcription factors. **13**: 1–13.

Sommer, H., Beltrán, J.P., Huijser, P., Pape, H., Lönnig, W.E., Saedler, H., and Schwarz-Sommer, Z. (1990). Deficiens, a homeotic gene involved in the control of flower morphogenesis in Antirrhinum majus: the protein shows homology to transcription factors. EMBO J. **9**: 605–13.

Stam, M., de Bruin, R., van Blokland, R., van der Hoorn, R.A., Mol, J.N., and Kooter, J.M. (2000). Distinct features of post-transcriptional gene silencing by antisense transgenes in single copy and inverted T-DNA repeat loci. Plant J. **21**: 27–42.

Theissen, G. (2001). Development of floral organ identity: stories from the MADS house. Curr. Opin. Plant Biol. **4**: 75–85.

Theissen, G. and Saedler, H. (2001). Plant biology. Floral quartets. Nature **409**: 469–71.

Vandenbussche, M., Zethof, J., Souer, E., Koes, R., Tornielli, G.B., Pezzotti, M., Ferrario, S., Angenent, G.C., and Gerats, T. (2003). Toward the analysis of the petunia MADS box gene family by reverse and forward transposon insertion mutagenesis approaches: B, C, and D floral organ identity functions require SEPALLATA-like MADS box genes in petunia. Plant Cell **15**: 2680–93.

Xiao, H., Wang, Y., Liu, D., Wang, W., Li, X., Zhao, X., and Xu, J. (2003). Functional analysis of the rice AP3 homologue OsMADS16 by RNA interference. 957–966.

Yamaguchi, T., Lee, D.Y., Miyao, A., Hirochika, H., An, G., and Hirano, H. (2006). Functional Diversification of the Two C-Class MADS Box Genes OSMADS3 and OSMADS58 in Oryza sativa. **18**: 15–28.

Yamaguchi, T., Nagasawa, N., Kawasaki, S., Matsuoka, M., Nagato, Y., and Hirano, H.-Y. (2004). The YABBY gene DROOPING LEAF regulates carpel specification and midrib development in Oryza sativa. Plant Cell **16**: 500–9.

Yanofsky, M.F., Ma, H., Bowman, J.L., Drews, G.N., Feldmann, K.A., and Meyerowitz, E.M. (1990). The protein encoded by the Arabidopsis homeotic gene agamous resembles transcription factors. Nature **346**: 35–9.

Yoshida, H. and Nagato, Y. (2011). Flower development in rice. J. Exp. Bot. **62**: 4719–30.

Zachgo, S., Silva, E.D.A., Motte, P., Tröbner, W., and Saedler, H. (1995). Functional analysis of the Antirrhinum floral homeotic DEFICIENS gene in vivo and in vitro by using a temperature-sensitive mutant. **2875**: 2861–2875.

Zahn, L.M., Kong, H., Leebens-Mack, J.H., Kim, S., Soltis, P.S., Landherr, L.L., Soltis, D.E., Depamphilis, C.W., and Ma, H. (2005). The evolution of the SEPALLATA subfamily of MADS-box genes: a preangiosperm origin with multiple duplications throughout angiosperm history. Genetics **169**: 2209–23.

Zahn, L.M., Leebens-Mack, J.H., Arrington, J.M., Hu, Y., Landherr, L.L., dePamphilis, C.W., Becker, A., Theissen, G., and Ma, H. (2006) Conservation and divergence in the AGAMOUS subfamily of MADS-box genes: evidence of independent sub- and neofunctionalization events. Evol. Dev. **8**: 30–45.

Zhang, A., David, J.J., Subramanian, S. V, Liu, X., Fuerst, M.D., Zhao, X., Leier, C. V, Orosz, C.G., Kelm, R.J., and Strauch, A.R. (2008). Serum response factor neutralizes Pur alpha- and Pur beta-mediated repression of the fetal vascular smooth muscle alpha-actin gene in stressed adult cardiomyocytes. Am. J. Physiol. Cell Physiol. **294**: C702–14.

PART II

PUBLISHED PAPER: Functional analysis of all AGAMOUS subfamily members in rice reveals their roles in reproductive organ identity determination and meristem determinacy

Dreni L, **Pilatone A**, Yun D, Erreni S, Pajoro A, Caporali E, Zhang D, Kater MM. **Functional analysis of all AGAMOUS subfamily members in rice reveals their roles in reproductive organ identity determination and meristem determinacy**. Plant Cell. **Published in** 2011 Aug;23(8):2850-63. doi: 10.1105/tpc.111.087007. Epub 2011 Aug 2.PubMed PMID: 21810995; PubMed Central PMCID: PMC3180796.

Functional Analysis of All AGAMOUS Subfamily Members in Rice Reveals Their Roles in Reproductive Organ Identity Determination and Meristem Determinacy [W]

Ludovico Dreni,[a] Alessandro Pilatone,[a] Dapeng Yun,[b] Stefano Erreni,[a] Alice Pajoro,[a,1] Elisabetta Caporali,[c] Dabing Zhang,[b] and Martin M. Kater[a,2]

[a] Dipartimento di Scienze Biomolecolari e Biotecnologie, Università degli Studi di Milano, 20133 Milan, Italy
[b] School of Life Sciences and Biotechnology, Shanghai Jiao Tong University, Shanghai 200240, China
[c] Dipartimento di Biologia, Università degli Studi di Milano, 20133 Milan, Italy

Reproductive organ development is one of the most important steps in the life cycle of plants. Studies using core eudicot species like thale cress (*Arabidopsis thaliana*) and snapdragon (*Antirrhinum majus*) have shown that MADS domain transcription factors belonging to the AGAMOUS (AG) subfamily regulate the identity of stamens, carpels, and ovules and that they are important for floral meristem determinacy. Here, we investigate the genetic interactions between the four rice (*Oryza sativa*) AG subfamily members, *MADS3*, *MADS13*, *MADS21*, and *MADS58*. Our data show that, in contrast with previous reports, *MADS3* and *MADS58* determine stamen and carpel identity and, together with *MADS13*, are important for floral meristem determinacy. In the *mads3 mads58* double mutant, we observed a complete loss of reproductive organ identity and massive accumulation of lodicules in the third and fourth floral whorls. *MADS21* is an *AGL11* lineage gene whose expression is not restricted to ovules. Instead, its expression profile is similar to those of class C genes. However, our genetic analysis shows that *MADS21* has no function in stamen, carpel, or ovule identity determination.

INTRODUCTION

Twenty years ago, Coen and Meyerowitz (1991) proposed, based on the analysis of thale cress (*Arabidopsis thaliana*) and snapdragon (*Antirrhinum majus*) floral homeotic mutants, the genetic ABC model. This model explains how three classes of floral homeotic genes, termed class A, B, and C genes, determine the identity of the four floral organ types: sepals, petals, stamens, and carpels. C-function genes regulate stamen and carpel identity in whorl 3 and 4. Furthermore, they are important to prevent meristem indeterminacy in whorl 4. The *Arabidopsis agamous* (*ag*) mutant has normal sepals and petals, whereas the stamens in whorl 3 are homeotically transformed into petals and in whorl 4 instead of a pistil, which in *Arabidopsis* is composed of two fused carpels, a new *ag* flower develops (Bowman et al., 1989). *AG* was the first class C gene cloned and encodes a MIKC-type MADS domain transcription factor (Yanofsky et al., 1990; Parenicová et al., 2003). *AG* expression starts at stage 3 of flower development in the domains of the floral meristem (FM) where stamen and carpel primordia develop, and its expression remains during all stages of stamen and carpel development. *AG*

is also expressed during ovule development and plays a role in ovule identity determination (Pinyopich et al., 2003; Brambilla et al., 2007).

In *Arabidopsis*, four genes, *AG*, *SHATTERPROOF1* (*SHP1*), *SHP2*, and *SEEDSTICK* (*STK*; previously known as *AGL11*), form the *AG* monophyletic subfamily within the MADS box gene phylogeny (Parenicová et al., 2003; Kramer et al., 2004; Zahn et al., 2006). Analysis of the evolutionary history of gene duplications showed that the *SHP1* and *SHP2* genes are the products of a recent duplication event, which probably occurred after the divergence of rosid and asterid eudicots (Vision et al., 2000). These two *Arabidopsis* genes are expressed in developing pistils, fruit, and ovules. They are redundantly involved in stigma, style, and medial tissue development and specify the dehiscence zone, which is important for the dispersal of seeds (Liljegren et al., 2000; Colombo et al., 2010). *SHP1* and *SHP2* are also redundant with *STK* in determining ovule identity, since in the *stk shp1 shp2* triple mutant, ovules are converted into carpel-like structures (Pinyopich et al., 2003; Brambilla et al., 2007). *STK* expression is restricted to the placenta and developing ovules, and the *stk* single mutant develops ovules with a longer funiculus, which upon seed maturation does not abscise from the seeds (Rounsley et al., 1995; Favaro et al., 2003; Pinyopich et al., 2003).

The duplication event that gave rise to the *AG* (eu*AG* clade) and the *SHP* genes (*PLENA* clade) took place early in the history of the core eudicots (Kramer et al., 2004; Zahn et al., 2006), whereas a more ancient duplication event occurred early in angiosperm evolution, marking the origin of the *AG* and *AGL11* lineages (Kramer et al., 2004; Zahn et al., 2006).

In rice (*Oryza sativa*), 75 MADS box genes have been identified (Arora et al., 2007) of which four genes, *MADS3*, *MADS58*, *MADS13*, and *MADS21*, belong to the *AG* subfamily. *MADS3* and *MADS58* fall into the *AG* lineage, whereas *MADS13* and *MADS21* are *AGL11* lineage genes (Kramer et al., 2004; Yamaguchi et al., 2006; Dreni et al., 2007). Although Zahn et al. (2006) placed *MADS13* in the *AG* lineage, other phylogenetic analyses, gene structure, and specific amino acid residues strongly suggest that *MADS13* belongs to the *AGL11* lineage (Kramer et al., 2004; Dreni et al., 2007).

In rice, the basal unit of the inflorescence is the spikelet, which bears one single floret. The two outer basal bracts, named glumes, are extremely reduced in rice and are called rudimentary glumes. Subsequently, there are two empty glumes, or sterile lemmas, that are homologous to lemmas but fail to initiate florets. The floret is composed of a lemma and palea, which are considered the first-whorl organs and enclose the other floral organs: two lodicules (second whorl), six stamens (third whorl), and a carpel containing a single ovule (fourth whorl).

Recently, rice *MADS3* and *MADS58*, which are considered to be paralogous genes, were characterized (Yamaguchi et al., 2006). Disruption of *MADS3* by a T-DNA insertion caused homeotic transformations of stamens into lodicules and ectopic development of lodicules in the second whorl near the palea, whereas carpels developed almost normally. The silencing of *MADS58* by an RNA interference (RNAi) approach resulted in carpel developmental defects and FM indeterminacy. From these observations, the authors concluded that during rice evolution subfunctionalization occurred between these two genes. The subfunctionalization of *AG* clade genes might be a more common feature of grasses. In maize (*Zea mays*), there are three *AG* lineage genes, namely, *ZAG1* (ortholog of rice *MADS58*) and *ZMM2* and *ZMM23* (both orthologs of rice *MADS3*). *ZAG1* and *ZMM2* transcripts are detectable in the ear and tassel reproductive organs; however, *ZAG1* transcript levels are higher in carpels, whereas those of *ZMM2* are more abundant in stamens (Schmidt et al., 1993; Mena et al., 1996). A transposon-induced mutation in *ZAG1* resulted in the loss of female FM determinacy, but carpel identity was not affected and male flowers developed normally (Mena et al., 1996). Functional analysis of *ZMM2* has not been reported yet, but its expression profile suggests a role in reproductive organ development. Functional and expression analyses of *ZMM23* (Münster et al., 2002) have not been reported yet.

Interestingly, in none of the rice *AG* gene mutants, including the *mads3 mads58*-RNAi double mutant, was carpel identity lost, suggesting that other genes might regulate carpel identity. This would be in contrast with core eudicot plants where carpel identity has shown to be determined by the *AG* lineage genes. *DROOPING LEAF* (*DL*), a gene belonging to the YABBY transcription factor family, has been suggested as a candidate to determine carpel identity in rice (Nagasawa et al., 2003; Yamaguchi et al., 2006). *DL* is essential for the development of carpels since in the *dl* mutant, carpels are homeotically transformed into multiple stamens (Nagasawa et al., 2003). *DL* is expressed in the carpel anlagen and subsequently during carpel development (Yamaguchi et al., 2004). In the *mads58*-RNAi and the *mads3 mads58*-RNAi double mutant lines, *DL* was still expressed in the

carpels, indicating that its expression is independent of *MADS3* and *MADS58* activity.

Of the rice *AGL11* lineage genes, *MADS13* has been characterized in most detail. Its expression is, like *STK* of *Arabidopsis*, restricted to the ovule primordium and carpel inner epidermis, and its expression remains during all phases of ovule development (Lopez-Dee et al., 1999). In the *mads13* mutant, ovules are homeotically transformed into carpelloid structures (Dreni et al., 2007). Based on this phenotype, *MADS13* was classified as a class D homeotic gene (Colombo et al., 1995). In the *mads13* mutant, a "carpels inside carpels" phenotype was often observed, indicating a loss of FM determinacy. This phenotype was not observed in the *Arabidopsis stk shp1 shp2* triple mutant, which also shows homeotic conversions of ovules into carpels (Pinyopich et al., 2003). This might be due to the fact that the ovule in the rice floret develops directly from the FM, whereas in *Arabidopsis* the developing carpels completely use up the FM, and ovules develop later from meristematic protrusions arising from the placenta (Dreni et al., 2007; Colombo et al., 2008).

MADS13 and *MADS21* are paralogous genes; however, *MADS21* expression is not restricted to ovules. Its expression is also observed in stamens and carpel tissues, although at low levels (Arora et al., 2007; Dreni et al., 2007). The *mads21* single mutant did not show any aberrant phenotype, and when combined with *mads13*, it did not enhance the *mads13* phenotype, indicating that this gene does not have a role in ovule identity determination.

Here, we report a detailed characterization of the rice *AG* subfamily. For our analysis, we used insertion mutants for all four genes and our results provide important new insights into the function of these genes in reproductive organ development in rice. We show, in contrast with Yamaguchi et al. (2006), that *mads58* mutant flowers do not have significant developmental defects. However, when the *mads58* mutant was combined with the *mads3* mutant, reproductive organ identity was completely lost, very similar to what has been described for the *ag* mutant in *Arabidopsis*. This confirms that the C-function is also conserved in monocotyledonous plants. Furthermore, generating the *mads3 mads13* and *mads13 mads58* double mutants confirmed an important role for *MADS13* in FM determinacy since an astonishing proliferation of carpels was observed in the center of these double mutant flowers. Interestingly, when the *mads21* mutant was combined with these double mutants, none of the higher order mutant combinations showed an enhanced phenotype, which suggests that *MADS21* does not contribute to the determination of reproductive organ identity.

RESULTS

Expression Analysis of Rice *AG* Subfamily Genes

By in situ hybridization, we analyzed in detail the expression of the four rice *AG* subfamily genes during flower development. *MADS3* expression was first detected in the third-whorl founder cells positioned laterally to the FM and remained expressed in the emerging stamen primordia (Figures 1A to 1C). By contrast, *MADS58* expression was observed uniformly throughout the FM; in the stamen
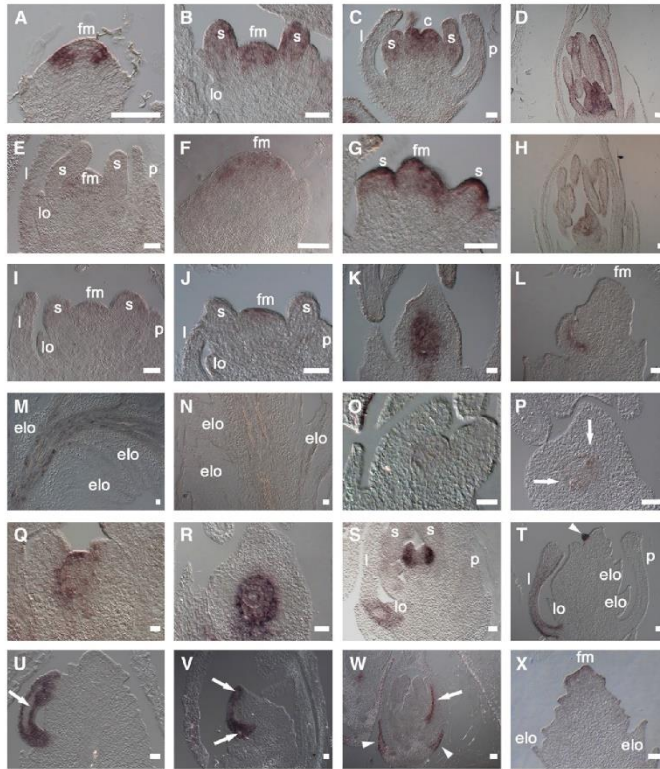
Figure 1. Expression Analysis of AG Family MADS Box Genes.

(A) to **(E)** Expression profile of *MADS3* in wild-type floral buds (**[A]** to **[D]**) and in the *mads3-3* mutant as negative control **(E)**.

(F) to **(I)** Expression profile of *MADS58* in wild-type floral buds (**[F]** to **[H]**) and in the *mads58* mutant as negative control **(I)**.

(J) to **(N)** *MADS13* activation in the wild-type FM **(J)** and its expression in wild-type ovules **(K)**, at the adaxial side of a *mads3-3 mads58* double mutant palea-like primordium **(L)** and at a later developmental stage of the same mutant in the central vasculature parenchyma **(M)**. As expected, no signal is visible in the *mads3-3 mads13 mads58* triple mutant vasculature **(N)**.

(O) to **(R)** Comparative expression of *MADS21* at similar stages of wild-type (**[O]** and **[P]**) and *pMADS13:MADS21* (**[Q]** and **[R]**) ovule primordia. Arrows in **(P)** indicate the integument primordia.

(S) to **(U)** *DL* expression in the wild-type carpel **(S)** and in the *mads3-3 mads58* double mutant palea-like primordia (**[T]** and **[U]**). In **(S)** and **(T)**, the expression is clearly visible also in the lemma midrib. The early palea-like primordium **(T)** and the palea-like vasculature **(U)** are indicated with an arrowhead and an arrow, respectively. There is no obvious expression in the vasculature.

(V) *DL* expression in the *mads3-3 mads13 mads58* triple mutant palea-like primordium; no expression is observed in the differentiating vascular region (arrows).

(W) and **(X)** *G1* expression profile.

(W) Expression in the wild-type sterile glumes (arrowheads) and palea (arrow).

(X) Expression in the *mads3-3 mads58* double mutant floral apex during the formation of the fourth whorl.

c, carpel; elo, ectopic lodicule; fm, floral meristem; l, lemma; lo, lodicule; p, palea; s, stamen. Bars = 50 μm.

50

primordia, it seemed to be preferentially expressed in the epidermal cells (Figures 1F and 1G). During early stages of stamen development and differentiation, *MADS3* and *MADS58* were expressed in the filament and in the anther wall (Figures 1D and 1H). In the microspore mother cells, we observed only a weak *MADS3* expression, whereas *MADS58* transcripts were not detected.

As soon as the stamen primordia emerged, both *MADS3* and *MADS58* were expressed in the FM and remained stably expressed in the carpel and ovule primordia with a very similar expression pattern (Figures 1B to 1D and 1G to 1H). In general, *MADS3* and *MADS58* have a very similar expression profile, which is reminiscent of *AG* expression in *Arabidopsis* (Drews et al., 1991). Yamaguchi et al. (2006) reported a significantly different expression profile for *MADS3*, since they found that its expression was only transient during initial stages of stamen, carpel, and ovule primordia formation. For our experiments we used specific probes spanning the K-box and C-terminal region of *MADS3* and *MADS58* (see Supplemental Figure 1 online). To completely exclude cross-hybridization of our probes, we also performed in situ hybridizations using the *mads3-3* and *mads58* knockout mutants (see below), which confirmed specificity of the probes for each of the two genes (Figures 1E and 1I). The *MADS3* expression profile was also confirmed using another antisense RNA probe targeting a region between the C terminus and 3′ untranslated region, which has no significant sequence similarity with *MADS58* (see Supplemental Figures 1 and 2M online). These expression profiles are in agreement with data from Arora et al. (2007), who observed by microarray analysis that both *MADS3* and *MADS58* were continuously and increasingly expressed during panicle development. Furthermore, the presence of *MADS3/RAG* transcripts in stamen primordia was also reported by Hu et al. (2011), Ikeda et al. (2005), Kyozuka et al. (2000), and Kang et al. (1995).

The expression profiles of the two *STK* orthologs, *MADS13* and *MADS21*, have been reported previously (Lopez-Dee et al., 1999; Dreni et al., 2007). The ovule identity gene *MADS13* starts to be expressed in the apical part of the FM just before the differentiation of the carpel and ovule primordia. Subsequently, its expression continues in the ovule primordium and in the inner layer of the developing carpel wall (Figures 1J and 1K). During panicle and flower development, *MADS21* is by far the lowest expressed *AG* subfamily gene of rice (Arora et al., 2007; Dreni et al., 2007). Although *MADS21* belongs to the *AGL11* lineage, its expression profile is not ovule specific and is reminiscent of a typical C-function gene (Dreni et al., 2007). *MADS21* expression is observed in developing stamens, carpels, and ovules. Expression in stamens appeared very weak, and the strongest signal was detected in ovule integument primordia, but not in the developing nucellus (Figure 1P).

Analysis of the expression of the four rice *AG* subfamily genes shows that during the differentiation of the FM into the ovule primordium, the expression of all four genes overlap in this domain.

Analysis of *mads3* and *mads58* Single Mutants

To characterize the function of *MADS3*, we analyzed plants homozygous for the *mads3-3* or *mads3-4* mutant alleles (Yamaguchi et al., 2006; Hu et al., 2011).

The *mads3-3* allele carries a *T-DNA* insertion in the second intron of *MADS3*. This intron typically contains conserved regulatory elements important for the proper expression of *AG*-like genes (Causier et al., 2009). *mads3-3* is the only *MADS3* full knockout mutant allele that has been reported thus far. However, the original line that we received from Postech (line 1A-19842, Dongjin cultivar background) was not suitable for our genetic studies, since all the plants showed a dwarf phenotype and tiny flowers producing almost no pollen and were almost completely sterile (95%). This phenotype was also observed in wild-type segregants. To eliminate this background effect, we performed backcrosses, using plants heterozygous for the *mads3-3* allele as male parent, to Dongjin wild-type plants. By selfing plants across a few generations, we obtained a *mads3-3* mutant line in a restored genetic background that did not show this dwarf phenotype. The *mads3-4* mutant contains a frame-shift allele caused by a 2-bp deletion in the fifth exon encoding the second K-box α-helix, resulting in a C-terminal truncation of 100 amino acids.

Under our growing conditions and in the restored genetic background, the *mads3-3* knockout mutant had a milder phenotype than previously reported by Yamaguchi et al. (2006). Half of the flowers had six floral organs in the third whorl and a single carpel in the fourth whorl (Figure 2C), whereas the other flowers showed a weak loss of FM determinacy, leading to one to three additional floral organs in both the third and fourth whorls (Figure 2D). All of the third-whorl stamens of *mads3-3* mutant flowers were partially or totally converted into lodicule-like structures. When the homeotic conversion was incomplete, we observed the formation of chimeric lodicule filaments bearing anther-like structures (Figures 2C and 2D). Furthermore, these mutant anthers did not develop pollen grains. In comparison with wild-type flowers, the gynoecium was often altered in shape, showing enlarged or elongated ovaries and multiple stigmas. Consistently with the *MADS3* expression profile and with *MADS3* being an *AG* subfamily member, we never observed defects in second-whorl lodicules, which developed normally at the lemma side of the *mads3-3* mutant flowers. This is in contrast with the previous observations made by Yamaguchi et al. (2006), which reported the formation of ectopic lodicules at the palea side of the second whorl. We also observed a fully developed third lodicule at the palea side of most *mads3-3* flowers; however, this ectopic organ clearly developed instead of a third whorl stamen and not from the second whorl (Figure 2C). Interestingly, identity determination of this palea side stamen seems to be particularly sensitive to a reduction in *MADS3* activity. This became especially clear from the analysis of the milder *mads3-4* mutant allele, which shows a less severe conversion of stamens into lodicules, a narrow ovary, and usually no loss of FM determinacy. In each flower, a variable number of anthers were able to form a normal amount of pollen grains (Hu et al., 2011), although these pollen grains failed to reach maturity. Though the *mads3-4* mutant shows a milder phenotype, the identity of the palea-side stamen was much more affected relative to the other stamens, but it was never completely converted into a lodicule (Figure 2B). This partial conversion of the palea-side stamen nicely confirms that the extra lodicule, which is usually observed in the more severe *mads3-3* mutant, derives from the homeotic conversion of the third-whorl
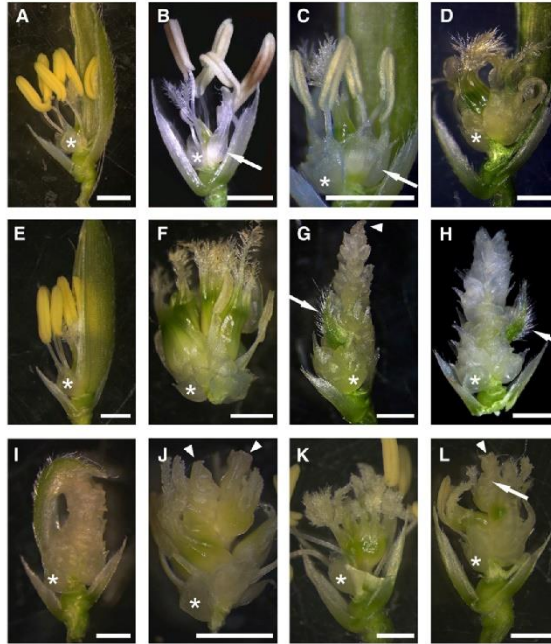
Figure 2. Rice *AG* Family Gene Mutant Phenotypes.

(A) Wild-type mature rice flower.
(B) *Mads3-4* mutant flower. The arrow indicates the more severely affected palea-side stamen.
(C) Mild *mads3-3* mutant phenotype. The arrow indicates the third-whorl ectopic lodicule replacing the palea-side stamen.
(D) Severe *mads3-3* mutant phenotype.
(E) *Mads3-3/+ mads21 mads58* mutant flower showing no phenotype.
(F) *Mads3-3 mads58/+* flower.
(G) *Mads3-3 mads58* double mutant. A palea-like organ developed in place of the carpel toward the lemma side (arrow).
(H) *Mads3-4 mads58* double mutant. Rarely, like in this picture, the palea-like organ does not develop at the lemma side (arrow).
(I) *Mads3-3 mads13 mads58* triple mutant showing an increased development of the palea-like organ.
(J) and **(K)** *Mads3-3 mads13* **(J)** and *mads13 mads58* **(K)** double mutants.
(L) *Mads3-3 mads13/+ mads58/+* flower. After producing a few ectopic carpelloid structures, the indeterminate FM switched to the differentiation of ectopic lodicule primordia (arrow).
To show the inner whorls, lemma and palea were partially or completely removed. In all of the pictures, the asterisk marks the second whorl lodicule, whereas the arrowheads in **(G)**, **(J)**, and **(L)** indicate the visible indeterminate FM. Bars = 100 μm.

stamen and not from the second whorl. Despite the fact that the second-whorl lodicules do not seem to be affected in these mutants, anthesis never occurred in *mads3-3* and *mads3-4* mutant flowers.

To characterize *MADS58* functionally, we obtained a *mads58* insertion mutant carrying a *dSpm* element in the second intron. Quantitative RT-PCR analysis, using RNA extracted from 2-cm-long panicles of the *mads58* homozygous mutant, showed that *MADS58* mRNA levels were strongly reduced with respect to the

wild type during floral organogenesis (nearly 35-fold reduction) (see Supplemental Figure 3A online). Yamaguchi et al. (2006) reported dramatic phenotypes in both strong and weak *MADS58* RNAi silencing lines, resembling those of intermediate *Arabidopsis ag* mutants (Mizukami and Ma, 1995; Sieburth et al., 1995). Surprisingly, despite the drastic reduction in mRNA levels observed in our *mads58* knockout mutant, we did not detect any of the previous reported phenotypes. Plants were highly fertile and flowers developed normally, apart from 5% of the flowers in

which one or both stigmas were bifurcated (see Supplemental Figure 2B online). This ratio increased to 20% when plants were simultaneously heterozygous for the *mads3-3* allele.

MADS3 and *MADS58* Redundantly Regulate Reproductive Organ Identity

The *mads3* single mutant phenotype shows the importance of *MADS3* for correct stamen development, although stamen identity was only partially lost. Yamaguchi et al. (2006) assessed the phenotypic effects when combining the *mads3* mutant with a *MADS58* knockdown construct. However, for this analysis, they used the mild *mads3-2* mutant, and based on our *mads58* knockout mutant data, it is difficult to judge the specificity of their *mads58*-RNAi construct. Therefore, we decided to analyze functional redundancy between *MADS3* and *MADS58* using the stable *mads3-3* and *mads58* knockout alleles. Plants homozygous for *mads3-3* and heterozygous for *mads58* already showed a strong increase in the *mads3-3* mutant phenotype (i.e., loss of stamen identity, FM indeterminacy, and alterations in carpel morphology) (Figure 2F). The phenotype of *mads3-3 mads58* homozygous double mutant flowers was very dramatic. They showed a complete loss of sexual organ identity and FM determinacy (Figure 2G). In addition, the size of the FM was strongly increased (Figure 3A), and the ortholog of the *Arabidopsis CLV3* gene *FON2/FON4* (Chu et al., 2006; Suzaki et al., 2006) remained stably expressed in the indeterminate FM (see Supplemental Figure 2D online). The combination of these features resulted in a striking enlargement of the third whorl, which frequently consisted of even more than 20 ectopic lodicules arranged in a spiral phyllotaxis (Figure 3A). During early stages of development, the second-whorl lodicules were forced against the lemma wall due to the development of this ectopic mass, and as a consequence they obtained an enlarged and flattened appearance (Figure 2G). In 52% of the flowers (*n* = 71 spikelets), mostly at the lemma side of the floral axis, a small green lemma/palea-like structure replaced the carpel in the fourth whorl (Figures 2G and 3A). Furthermore, the FM continued to produce lodicule-like organs, and frequently we observed branching of the FM (see Supplemental Figure 2E online). Twenty percent of the flowers had two to three lemma/palea-like organs in and after the fourth whorl, whereas the remaining 28% produced only lodicules. The phenotype of the *mads3-4 mads58* double mutant (*n* = 61 spikelets) was nearly identical (Figure 2H), with indeterminate flowers showing one (64%) or two to three (8%) lemma/palea-like organs in place of the carpel, or only lodicules (28%).

In the most indeterminate flowers of the *mads3-3 mads58* double mutant plants, the FM remained strongly active for even more than 1 month after heading. In these flowers, the ectopic mass of hundreds of lodicules forced the lemma and palea away and they protruded outside the flower (see Supplemental Figure 2E online). At these late stages, the FM sometimes produced a number of small, well-developed sterile lemma- and lemma/palea-like structures, from which axillary indeterminate FMs developed that produced again only lodicules (see Supplemental Figure 2F online). These *mads3 mads58* double mutant phenotypes revealed that a double knockout of *MADS3* and *MADS58* produces a rice mutant flower in which reproductive organ

identity is completely lost and in which the FM becomes indeterminate, which is very similar to the *ag* mutant phenotype in *Arabidopsis* (Bowman et al., 1989).

As described above, a lemma/palea-like organ often replaced the gynoecium in the *mads3-3 mads58* double mutant. We carefully analyzed the cellular and molecular identity of this ectopic organ by means of microscopy and by in situ RNA hybridization experiments.

Lemma and palea have a similar epidermal cell morphology, with rounded projections and trichomes on the abaxial surface and smooth, wide, rectangular cells on the adaxial surface. However, the palea has a distinctive marginal tissue that is smooth and trichome-less, which is similar to the outer glume epidermis (Prasad et al., 2001; Prasad and Vijayraghavan, 2003; Figures 3B to 3D). The glume-like organs that developed in place of carpels in *mads3-3 mads58* double mutant flowers showed rounded projections and trichomes on the abaxial surface and smooth trichome-less marginal tissue, which strongly supports palea identity for this organ (Figure 3E). However, in situ hybridization analysis showed that *DL* was expressed in these palea-like structures. *DL* expression is typical for the fourth-whorl carpel tissue, and it seems that its expression remained unaffected in this ectopic organ, except for the marginal tissues and the midvein where no *DL* expression was observed (Figures 1S to 1U; see Supplemental Figure 2J online). In the first-whorl organs, *DL* expression occurs only in the lemma rudimentary midrib but never in the palea (Yamaguchi et al., 2004; Figures 1S and 1T). As expected, the B-class gene *MADS2* was expressed in the ectopic lodicules but not in the palea-like primordium where *DL* remained expressed (see Supplemental Figure 2L online). Using in situ hybridization experiments, we also investigated the expression of *G1/ELE*, which is a specific marker for the sterile lemmas and palea that is not expressed in the lemma (Yoshida et al., 2009; Hong et al., 2010). *G1* is initially highly expressed in the sterile lemmas and palea primordia, and subsequently its expression gradually fades during the development of these floral organs (Figure 1W). Even if we observed *G1* activation in the *mads3-3 mads58* indeterminate floral apices, its expression did not clearly mark the palea-like primordia (Figure 1X).

Thus, in the rice *mads3 mads58* double mutant, the carpel is converted into an organ with a palea-like appearance, which, however, markedly differs from the first-whorl palea at the molecular level and retains expression of genes normally expressed in the fourth whorl.

Genetic Interactions between *MADS3*, *MADS13*, and *MADS58*

Our previous analysis of the *mads13* mutant revealed a carpel-in-a-carpel phenotype, which points to indeterminacy of the FM (Dreni et al., 2007). However, this phenotype could also be a consequence of the homeotic conversion of the ovule into a carpel that itself tries to make an ovule, which in turn is transformed in a carpel again. Since our data and those of Yamaguchi et al. (2006) clearly showed that *MADS3* and *MADS58* redundantly regulate FM determinacy, we investigated whether *MADS13* shares this role with these genes in the center of the fourth whorl where they all are coexpressed during the transition
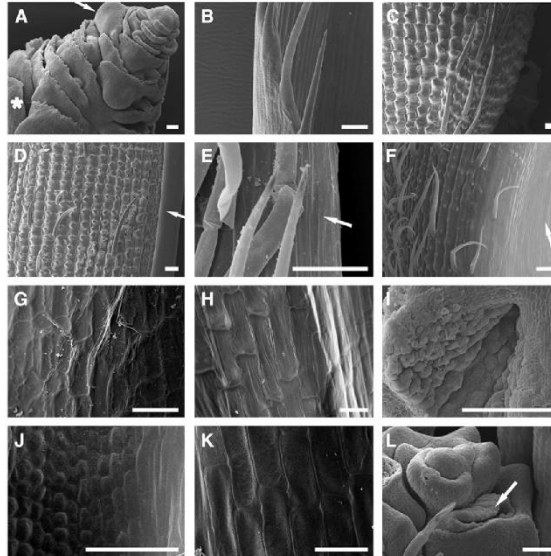
Figure 3. Scanning Electron Microscopy Analysis of Mutant Flowers.

(A) Inner organs of a *mads3-3 mads58* double mutant floral bud showing a second-whorl lodicule (asterisk), a fourth-whorl lemma-side palea-like primordium (arrow), several ectopic lodicules, and the enlarged FM.
(B) Wild-type sterile lemma with the abaxial surface oriented on the left.
(C) to **(F)** Abaxial surface of wild-type lemma **(C)**, wild-type palea **(D)**, *mads3-3 mads58* double mutant palea-like organ **(E)**, and *mads3-3 mads13 mads58* triple mutant palea-like organ **(F)**. The palea and palea-like marginal regions are shown in **(D)** to **(F)** (arrows).
(G) to **(K)** Adaxial surface of wild-type lemma **(G)**, wild-type palea **(H)**, *mads3-3 mads58* double mutant palea-like organ (**[I]** and **[J]**), and *mads3-3 mads13 mads58* triple mutant palea-like organ **(K)**.
(L) *Mads3-3 mads13* double mutant indeterminate FM surrounded by several developing carpel primordia and also an ectopic lodicule primordium (arrow).
Bars = 100 μm in **(D)** and 50 μm in the other pictures.

of the FM into the ovule primordium. Therefore, we analyzed double and triple mutant combinations involving these three *AG* subfamily genes.

The *mads3-3 mads13* double mutant showed a complete loss of FM determinacy inside the fourth whorl (Figure 2J). As expected, the stamen phenotype remained the same as in the *mads3-3* single mutant, since *MADS13* is not expressed in the third whorl. The carpel was mostly unclosed and contained a reiteration of similar ectopic carpels; often this ectopic mass was able to force the lemma and palea away from each other within a few weeks after heading (see Supplemental Figure 2G online). The FM often branched (Figure 2J), but its size was smaller than that observed in the *mads3-3 mads58* double mutant (Figure 3L), and it produced a minor number of ectopic organs.

The *mads13 mads58* double mutant flowers showed a similar but milder phenotype, and carpel morphology was less affected

(Figure 2K). Curiously, in both the *mads3-3 mads13* and the *mads13 mads58* double mutants, sometimes the first ectopic organs arising from the carpel were two small stamens, with a subsequent reiteration of ectopic carpels (see Supplemental Figures 2H and 2I online). Scanning electron microscopy analysis revealed that, in rare events, the *mads3-3 mads13* double mutant formed ectopic lodicules between the ectopic carpels (Figure 3L).

As described above, in plants homozygous for *mads3-3* and heterozygous for *mads58*, FM determinacy was partially lost in the inner two whorls (Figure 2F). However, ovule-like structures usually developed inside of the ovary. This effect was greatly enhanced in plants homozygous for *mads3-3* and heterozygous for both *mads58* and *mads13*. In this genetic background, the FM remained active inside the gynoecium, producing a reiteration of ectopic carpels and often also lodicules, indicating a partial ectopic expression of B-function genes (Figure 2L).

54

Molecular control of reproductive organ development in rice (Oryza Sativa L.)

Analysis of the AGAMOUS Subfamily Genes of Rice   2857

Since reproductive floral organs completely disappeared in the mads3-3 mads58 double mutant, we expected that MADS13 expression would be completely absent from these double mutant flowers. To test this hypothesis, we performed quantitative RT-PCR (qRT-PCR) experiments using RNA extracted from pools of mads3-3 mads58 double mutant and from wild-type developing panicles of 1 to 14 cm long. Surprisingly, MADS13 expression in the double mutant showed only a reduction of nearly fourfold, whereas MADS21, which is already weakly expressed in wild-type panicles, was reduced by 23-fold (see Supplemental Figure 3B online). To localize the residual MADS13 expression in the mads3-3 mads58 double mutant, we performed in situ RNA hybridization experiments. Surprisingly, MADS13 mRNAs were not detected in the indeterminate FM during fourth whorl formation and also not during later developmental stages, but transcripts were detected only in the adaxial epidermis of the ectopic palea-like lateral organ primordium, exactly as in the corresponding epidermis of the fourth-whorl carpel in wild-type plants (Figure 1L). At later stages, we observed an irregular spotted expression in the parenchyma cells of the central vascular bundle developing at the center of the elongating floral axis, indicating that this ectopic vascular system might correspond to the ovule dorsal vascular bundle, where MADS13 is normally highly expressed in the wild type (Figure 1M). In agreement with this expression pattern, the mads3-3 mads13 mads58 triple mutant differed from the mads3-3 mads58 double mutant only in the palea-like organ, which was more enlarged (Figure 2I). Scanning electron microscopy analysis revealed that at the basal part of the adaxial surface of the mads3-3 mads58 double mutant palea-like organ, often the epidermal layer did not develop, thus exposing the subepidermal cells (Figure 3I). In the rest of the adaxial surface, some of the cells were converted into palea epidermis cells, but often patches of smaller polyhedral cells still resembling those of carpels were visible (Figure 3J). By contrast, in the mads3-3 mads13 mads58 triple mutant, the ectopic palea adaxial side was more regular and completely converted into a first-whorl palea epidermis (Figure 3K). This means that MADS3, MADS13, and MADS58 redundantly specify the identity of the carpel inner epidermis, since in the mads13 single mutant, the identity of these cells was not lost.

The DL expression pattern in the palea-like organs did not markedly differ between the mads3-3 mads58 double and the mads3-3 mads13 mads58 triple mutants (Figures 1T to 1V). Also, the G1 activation in the indeterminate floral apex was similar between these two mutants (see Supplemental Figure 2O online).

Analysis of the Role of MADS21 in Reproductive Organ Development

Since the AGL11 lineage gene MADS21 has a similar expression profile as MADS3 and MADS58, we tested the hypothesis that this gene also contributes to the C-function in rice. Since the mads3-3 mads21 mads58 triple mutant is not informative (because MADS21 expression is almost completely suppressed in mads3-3 mads58 flowers [see Supplemental Figure 3B online] and because reproductive organ identity is already completely lost in this double mutant), we analyzed the consequences of the loss of MADS21 activity in a reduced C-function background.

Flowers of the mads21 mutant were completely normal, in agreement with our previous observations (Dreni et al., 2007), and the mads3-3 and mads58 phenotypes were not enhanced in mads3-3 mads21 and mads21 mads58 double mutants, respectively. Furthermore, mads3-3 mads58/+ and mads3-3 mads21 mads58/+ plants had the same phenotype. Most mads3-3/+ mads21 mads58/+ flowers were normal (Figure 2E), and the frequency of bifurcated stigmas did not increase when compared with mads3-3/+ mads58 mutant plants.

Similarly, the mads13 mads21 mads58 triple mutant flowers phenocopied those of the mads13 mads58 double mutant. Taken together, these data strongly suggest that MADS21 does not play a crucial role in the formation of reproductive organs and FM determinacy.

MADS21 Retains Ovule Identity Determination Activity

The higher order mutants described above show that MADS21 does not contribute to the C-function. We previously reported that the mads13 phenotype was not enhanced in the mads13 mads21 double mutant (Dreni et al., 2007). This suggests that the AGL11 lineage gene MADS21 has no class C activity and also does not determine ovule identity (class D activity). This is curious considering its phylogenetic classification. A possible explanation for the absence of class D gene activity might be its low expression level. To test this, we made a gene construct in which the MADS21 gene was expressed under the control of the MADS13 upstream region. Five independent transgenic rice lines were obtained, and in none of these lines did MADS21 upregulation cause a phenotypic effect. One of these lines showed an ~10-fold increase in MADS21 expression in developing panicles (see Supplemental Figure 3C online), and this line was used for crosses with the mads13 mutant. In situ RNA hybridization confirmed that the MADS13 upstream region is able to drive strong expression in developing ovules (Figures 1Q and 1R), although the expression seemed variable between different flowers. In contrast with wild-type samples, a strong MADS21 signal was visible even in the developing nucellus (Figure 1R). In the F2 generation, we analyzed ovule development in mads13 mutant plants carrying the pMADS13:MADS21 construct. In the mads13 mutant, amorphic or normal appearing carpels replace ovules (Dreni et al., 2007; Figures 4C and 4F). In the mads13/pMADS13:MADS21 plants, 37% of the flowers (n = 35 spikelets) showed partial complementation of the mads13 phenotype. Nucellus and integument-like structures or naked nucella without embryo sacs were observed. Some ovaries developed complete ovule-like structures enveloped by integuments, in which surprisingly the megaspore mother cell was replaced by a short tracheary element (Figures 4B and 4E). This preliminary result shows that higher expression levels of MADS21 can partially compensate for the loss of MADS13 function, although ovule development was not completely restored, suggesting that the protein might have lost some specific characteristics for correct ovule development. This hypothesis will be tested in the future by the analysis of more independent pMADS13:MADS21 transformants and using transgenic pMADS13:MADS13 control lines in the mads13 mutant background.
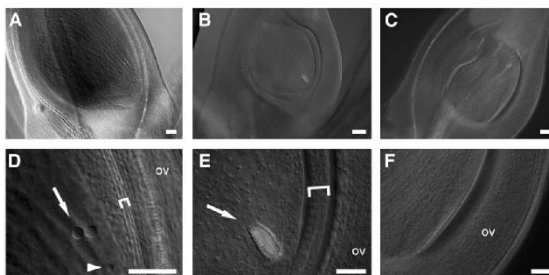
Figure 4. Whole-Mount Tissue Clearing Analysis.

(A) and **(D)** Wild-type mature ovule showing details **(D)** of the central cell nuclei (arrow), the egg cell nucleus (arrowhead), and the inner integument, which is laterally ~4 μm thick (bracket).

(B) and **(E)** *mads13/pMADS13:MADS21* mutant ovule showing details **(E)** of the tracheary element replacing the embryo sac (arrow) and the inner integument, which is laterally ~16 μm thick (bracket).

(C) and **(F)** A *mads13* mutant ovary that is filled with carpelloid cells.

ov, ovary wall. Bars = 50 μm for **(A)** to **(C)** and 20 μm for **(D)** to **(F)**.

DISCUSSION

The C-Function

Our analysis using the model plant rice clearly shows that the C-function is highly conserved between core eudicot and monocot plants. Similarly, rice *DEF/GLO* orthologs have been shown to determine the identity of second and third whorl floral organs (B-function), whereas *SQUA*-like MADS box genes are more likely required to influence FM identity rather than having a true A function, although further analysis will be required (reviewed in Kater et al., 2006). Recessive A-function mutants, clearly showing identity defects in first- and second-floral whorl organs, have thus far been reported only in *Arabidopsis*, and probably only a BC model can be applied to other model core eudicots (Schwarz-Sommer et al., 2003; Causier et al., 2010).

Recently, the functional conservation of *AG*-like genes in mediating the C-function, which means the determination of stamen and carpel identity, and the regulation of FM determinacy has also been demonstrated by virus-induced gene silencing experiments in the basal eudicots California poppy (*Eschscholzia californica*) and opium poppy (*Papaver somniferum*), both belonging to the Papaveraceae family (Hands et al., 2011; Yellina et al., 2010). Interestingly, in both these basal eudicots and in the asterid *A. majus*, the inhibition of *AG*-like gene function led to the homeotic conversion of the gynoecium into petal-like organs, rather than into sepal-like organs as would be predicted by the classic ABC model, suggesting an involvement of *AG*-like genes in the repression of B-function genes in the fourth whorl of these species (Davies et al., 1999; Yellina et al., 2010; Hands et al., 2011).

Based on our mutant analysis, the two rice *AG* lineage genes *MADS3* and *MADS58* redundantly mediate the C-function (Figure 5). Although the *mads58* mutant might not be a complete knockout since some transcript was detected by real-time PCR

analysis, the contribution of *MADS3* seems to be more important since strong *mads3* single mutants already showed some defects, whereas most of the *mads58* flowers were indistinguishable from wild-type flowers. In particular, the *mads3* knockout causes a partial loss of stamen identity, suggesting a major role in third-whorl organ identity specification when compared with *MADS58*, as already reported by Yamaguchi et al. (2006). This functional difference might be explained by protein subfunctionalization. Analysis of the amino acid sequences highlighted interesting differences between MADS3 and MADS58. For example, the AG motif II (Kramer et al., 2004) is poorly conserved in the grass MADS58 subclade. However, the level of gene expression and the differences in timing of the onset of gene expression might also contribute to this partial subfunctionalization. *MADS3* is strongly and specifically activated in the stamen anlagen of the floral apex, whereas at the same stage, *MADS58* is activated at a lower level uniformly throughout the FM. After the third whorl is differentiated, *MADS3* and *MADS58* show a very similar expression profile at the center of the FM and in the deriving gynoecium. Consistent with this, FM determinacy and carpel morphogenesis are only weakly affected in the *mads3* knockout mutant, and there is no loss in carpel identity. Interestingly, although *MADS21* is weakly expressed in the developing reproductive organs, it does not seem to contribute to the C-function, which is consistent with its classification as an *AGL11* lineage gene.

Although we show that the rice *mads3 mads58* double mutant largely corresponds to the *ag* mutant of *Arabidopsis*, there are also important differences between their phenotypes. *mads3 mads58* double mutant flowers displayed an enlargement of the third whorl, bearing even more than 20 ectopic lodicules arranged in a spiral phyllotaxis. Fifty percent of the mutant flowers developed a single palea-like structure in place of the carpel, mostly from the lemma side of the floral axis (Figure 2G), meaning that the position of the fourth-whorl primordium was maintained.
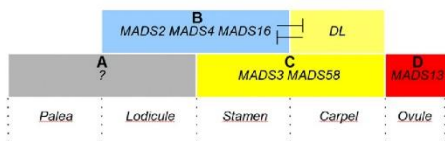
56

Figure 5. Genetic Model for Floral Organ Identity Determination in Rice.

The existence of a true rice A function determining the identity of the first- and second-whorl organs has, like in most flowering plants, not been identified yet. The homeotic conversion of the carpel into a palea-like organ in the *mads3 mads58* double mutant suggests that only the palea, and not the lemma, can be considered as a first-whorl organ. The class B genes regulate lodicule identity in the second whorl, and together with class C genes, they determine stamen identity in the third whorl. In the fourth whorl, *DL* represses the expression of class B genes. The main class C genes of rice are *MADS3* and *MADS58*. Since the ovule develops directly from the FM, it can be considered a fifth-whorl organ. Based on current data, *MADS13* seems to be the only *AG* subfamily gene regulating ovule identity in rice. Among the four *AG* subfamily genes of rice, *MADS3*, *MADS13*, and *MADS58* regulate also FM determinacy, whereas *MADS21* seems not to play important functions during flower development. The MADS domain proteins shown in this model might interact with SEP-like and/or AGL6-like MADS domain proteins providing E-function (Favaro et al., 2002; Kater et al., 2006; Ohmori et al., 2009; Cui et al., 2010; Li et al., 2010; Li et al., 2011a), which for simplicity are not shown in this scheme.

Subsequently, the rest of the FM was consumed by the production of a large number of ectopic lodicules. The other 50% of the *mads3 mads58* double mutant flowers developed zero or two to three palea-like structures instead of the carpel, and again a large number of ectopic lodicules developed in the center of the flower. Thus, there is no regular alternation of first- and second-whorl floral organs like in the *ag* mutant. Furthermore, in the *ag* mutant, the conversion of the carpel into a new *ag* flower is complete, whereas in the rice *mads3 mads13 mads58* triple mutant, the ectopic palea-like structure remains much smaller than the first-whorl palea, and even though its morphology and cell shapes resemble those of a first-whorl palea, it is still not a true palea from a molecular point of view. In particular, in both the *mads3 mads58* double and the *mads3 mads13 mads58* triple mutants, *DL* expression remains unaffected in this palea-like organ. Furthermore, *G1* appears irregularly activated in the region of the mutant floral apex, but its expression does not clearly mark the arising palea-like primordium as normally occurs in the wild-type palea.

Interestingly, *DL* expression in the palea-like organ provides new insights about its true functions during carpel development. It seems that *DL* cannot specify carpel identity without *MADS3* and *MADS58*, and it is possible that it has no C-function at all. *DL* is required as negative regulator of class B gene expression in the fourth whorl (Nagasawa et al., 2003). This function is enough to explain the *dl* mutant phenotype, since the ABC model predicts that the simultaneous expression of B and C genes leads to stamen identity. From this point of view, the *dl* mutant is not a true class C mutant; otherwise, a conversion of the carpel into sterile perianth organs like a palea (or a lodicule due to class B ectopic

expression) should be expected. In this context, it might be better to consider *DL* as a fourth-whorl marker rather than a carpel identity marker, of which its transcriptional regulation is at least in part independent of the true C-function genes *MADS3* and *MADS58*. Based on the observation that in *dl* mutant flowers the carpel is replaced by two to seven stamens, a direct function of *DL* in FM determinacy has been proposed (Nagasawa et al., 2003; Yamaguchi et al., 2004). However, this partial loss of FM determinacy might be an indirect effect. In agreement with this hypothesis, it has recently been shown that *MADS13*, which regulates FM determinacy, is not expressed in *dl* mutant flowers (Li et al., 2011b). Furthermore, the *dl* phenotype did not change in the *dl mads13* double mutant, whereas the loss of FM determinacy seen in *dl* flowers was strongly enhanced in the *dl mads3* double mutant (Li et al., 2011b), most likely due to the simultaneous repression of *MADS3* and *MADS13*. These data therefore suggest that the main function of *DL* in carpel development is to repress the B-function genes (Figure 5), a function that its *Arabidopsis* ortholog *CRABS CLAW* (*CRC*), and its ortholog in the basal eudicot *E. californica*, *EcCRC*, do not have (Alvarez and Smyth, 1999; Bowman and Smyth, 1999; Orashakova et al., 2009). However, these studies clearly indicate that in basal and core eudicots, the *CRC*-like genes regulate important aspects of carpel morphogenesis. Based on current data, it is not possible to exclude a similar function also for *DL* in rice.

FM Determinacy

Among the four *Arabidopsis AG* subfamily genes, only *AG* seems to regulate FM determinacy. We previously suggested that in rice the ovule identity gene *MADS13* also participates in mediating FM determinacy (Dreni et al., 2007). On the contrary, Yamaki et al. (2011) proposed that *MADS13*, like its orthologs in *Arabidopsis* and petunia (*Petunia hybrida*), might be simply a master regulator of ovule identity and not involved in determining FM activity. Here, we show that in rice, three of the four *AG* subfamily genes (*MADS3*, *MADS13*, and *MADS58*) redundantly regulate FM determinacy. Like in *Arabidopsis* (Mizukami and Ma, 1995), FM determinacy in rice seems to be sensitive to the available amount of *AG*-like protein(s), since all the three possible double mutant combinations (*mads3 mads58*, *mads3 mads13*, and *mads13 mads58*) resulted in an enhanced FM indeterminacy. Based on our double and triple mutant combinations, it seems that the contribution of *MADS21* to FM determinacy is not significant. *MADS3* and *MADS13* appear to be the most important genes because FM determinacy is already partially lost in the single knockout mutants.

The Role of *AG* Subfamily Genes in Ovule Development

Rice possesses two *AGL11* lineage genes, namely, *MADS13* and *MADS21*. Interestingly, the determination of ovule identity seems to be an exclusive function of *MADS13*. This is in contrast with *Arabidopsis*, where all four *AG* subfamily genes are largely redundant in carrying out this function (Pinyopich et al., 2003; Brambilla et al., 2007). When expressing *MADS21* at high levels inside the carpel under the control of the putative *MADS13* promoter, partial complementation of the *mads13* mutant

phenotype was observed. This shows that *MADS21* is potentially able to induce the pathway that leads to the formation of ovules but that this activity normally remains unobserved due to its low expression in the ovule. Since the complementation of the *mads13* mutant phenotype was only partial, it is possible that *MADS21* has limited D-function activity. However, a higher number of independent transformants and the necessary controls will be needed to confirm this hypothesis. The expression of *MADS21* strongly increases in developing kernels (Arora et al., 2007), suggesting that this gene might have an important function only after fertilization.

By contrast, *MADS3* and *MADS58* are both strongly expressed at all stages of ovule development; thus, they are likely required for ovule development, possibly in *MADS13*-independent pathways. It could be that they are needed to establish a genetic background necessary for *MADS13* functions. Interestingly, *MADS13* expression in the *mads3 mads58* double mutant flowers was restricted to the palea-like fourth-whorl organ and the central vascular bundle of the floral axis, which suggests that *MADS13* expression is only dependent on class C gene activity for its early activation in the FM.

In the wild type, *MADS13* is expressed in developing ovules and in the ovary inner epidermis; however, no developmental defects are evident in the epidermal layer in the *mads13* mutant. Indeed, when comparing the *mads3 mads58* double mutant with the *mads3 mads13 mads58* triple mutant, we demonstrated that these three genes redundantly determine the identity of the ovary adaxial epidermis. Therefore, the *mads3 mads58* mutant will provide a good genetic background in which to study *MADS13* functions in the development of the ovary adaxial epidermis, since in this way these functions will not be masked by redundancy.

Conservation of Expression of *AG* and *AGL11* Lineage Genes

Since both *AG* and *AGL11* lineage genes have been isolated in the main Angiosperm taxa, it is thought that these two lineages of the *AG* subfamily arose from a gene duplication event that occurred in the most recent common ancestor of flowering plants (Kramer et al., 2004). Many dicot and monocot *AG* lineage genes are expressed in stamen, carpel, and ovule, although a restriction of the expression domain is not uncommon, especially in the core eudicot *PLENA* subclade. It is thus likely that the *AG* lineage ancestor gene had an expression profile similar to *AG* of *Arabidopsis* (see Supplemental Figure 4 online) (Zahn et al., 2006).

In core eudicots, *AGL11* lineage genes are specifically expressed in developing ovules and annexed ovary tissues with only a few exceptions, so very likely this expression profile has been inherited and conserved from the early eudicot plants (Zahn et al., 2006). In monocots, a similar expression profile has been reported for the rice gene *MADS13*, its maize ortholog *ZAG2*, the lily (*Lilium longiflorum*) gene *LMADS2*, and *MADS1* from hyacinth (*Hyacinthus orientalis*). However, expression in stamens has also been shown for monocot *AGL11* lineage genes, for example, *MADS21* in rice (Dreni et al., 2007) and *AVAG2* in asparagus (*Asparagus officinalis*; Yun et al., 2004). Thus, it seems plausible that the angiosperm *AGL11* lineage ancestral gene had an

expression pattern already restricted to the ovules and annexed ovary tissues and that the conservation of this expression domain, and the related D-function, is less strict in monocots when compared with core eudicots. However, this assumption can be confirmed only by expression analysis of *AGL11* lineage genes in basal angiosperms like *Amborella* and *Nymphaea*, in basal eudicots, and basal monocots, whose data are still completely missing.

METHODS

Plant Material and Growth Conditions

We used plants of rice (*Oryza sativa* ssp *japonica*) of the following lines: cultivars Nipponbare and Dongjin, *mads13* and *mads21* mutants (Dreni et al., 2007); *mads3-4* mutant (Hu et al., 2011); *mads3-3* mutant (line 1A-19842, with a *T-DNA* insertion 4656 bp after the 5′-splice site of the second intron, from the mutant population of Pohang University of Science and Technology, Republic of Korea; Jeon et al., 2000; Jeong et al., 2006); *mads58* mutant (line RdSpm2035B_3.1, with a *dSpm* insertion 117 bp after the 5′-splice site of the second intron, from the Nipponbare mutant population of the Sundaresan Lab, Department of Plant Biology, University of California, Davis, CA).

Plants were grown in a phytotron under 28°C during the light cycle and under 24°C during the dark cycle.

Primers Used for PCR Genotyping of Mutant Plants

The following primers were used: *mads3-3* mutant, OsP297F and OsP298R for the wild-type allele and OsP95/T-DNALB and OsP298R for the mutant allele; *mads3-4* mutant, OsP270F and OsP272R for the wild-type allele and OsP271F and Osp272R for the mutant allele (alternatively, plant genotyping was performed by PCR amplification and sequencing of the fifth exon); *mads58* mutant, OsP201F and OsP202R for the wild-type allele and Osp201F and OsP196/Spm3′-3 for the mutant allele. The PCR genotyping of *mads13* and *mads21* mutants has been described previously (Dreni et al., 2007).

Plasmid Construction and Plant Transformation

A 3435-bp fragment of the *MADS13* upstream region (p*MADS13*), comprising the 5′ untranslated region and leader intron sequences, but not the start codon, was amplified with primers OsP131F-SacI and OsP132R-SpeI, using Phusion High-Fidelity DNA polymerase (Finnzymes). The PCR product was digested with SacI (partial digestion) and SpeI restriction enzymes. The binary vector pK2GW7 (Karimi et al., 2002), carrying the *NptII* selectable marker gene for in planta selection, was digested with the same enzymes to excise the *35S* promoter. The p*MADS13* fragment was then ligated into the digested plasmid to generate the NOB1186 vector. The *MADS21* coding sequence was amplified from Nipponbare panicle cDNA using the primers OsP48-attB1 and OsP49-attB2 and recombined into NOB1186 using Gateway Technology (Invitrogen). Finally, this p*MADS13:MADS21* vector was introduced in the *Agrobacterium tumefaciens* strain LBA 4404. Rice transformation was performed using geneticin (G418) selection and the protocol of Hiei et al. (1994).

cDNA Preparation and qRT-PCR Analysis

Total RNA from three biological replicates was extracted with the LiCl method, and its integrity was checked on agarose gels. Total RNA was then treated with recombinant DNase I, RNase-free (Roche Diagnostics) in 1× TAB II buffer, after which ~0.5 μg of RNA was reverse transcribed

with the iScript cDNA synthesis kit (Bio-Rad). Negative controls were performed without the addition of reverse transcriptase into the mix. Tenfold dilutions of cDNA were tested in RT-PCR and qRT-PCR experiments using reference genes. No amplification was observed in negative controls. For qRT-PCR analyses, cDNA templates (10-fold dilutions) and primers (OsP274F and OL121R for *MADS13*; OsP20F and OsP57R for *MADS21*; OsP226F and OsP222R for *MADS58*) were added to iQ SYBR Green Supermix (Bio-Rad). qRT-PCR reactions were performed with three technical replicates/samples using a Bio-Rad CFX96 real-time PCR detection system, and data were managed using the CFX Manager software based on the delta-delta Ct method. Data were normalized using the two reference genes LOC_Os06g11170.1 (coding for a putative nucleic acid binding protein) and LOC_Os06g48970.1 (coding for a putative protein kinase) with the same primers published by Narsai et al. (2010) (see Supplemental Table 1 online). Before performing expression analysis, the primers' efficiency was estimated through a five-point standard curve (10-, 40-, 160-, 640-, and 2560- fold dilutions of cDNA). We obtained the following amplification efficiencies: 81% for *MADS13*, 95% for *MADS21*, 96% for *MADS58*, 92% for LOC_Os06g11170.1, and 95% for LOC_Os06g48970.1.

Scanning Electron Microscopy Analysis

Samples were prepared and analyzed as described previously (Favaro et al., 2003).

In Situ RNA Hybridization Experiments and Whole-Mount Tissue Clearing

In situ hybridization experiments were performed essentially as described previously (Dreni et al., 2007) with the only difference that sections were pretreated following the method described by Jackson (1991). The preparation of *MADS13*, *MADS21*, *DL*, and *FON4* digoxygenin-labeled antisense RNA probes was described previously (Chu et al., 2006; Dreni et al., 2007). The cDNA fragments for the in vitro transcription of the other RNA probes were amplified with the following primers: OsP16F and OsP17R-T7 for *MADS3* (first probe), OsP72F and OsP73R-T7 for *MADS3* (second probe), OsP63F OsP64R-T7 for *MADS58*, OsP307F and OsP308R-T7 for *G1*, and MADS2F and MADS2R for *MADS2*.

For clearing of ovaries, a 2-d treatment with Herr's 4½ clearing solution (Herr 1971), followed by 2 d in lactic acid saturated with chloral hydrate, was used. Samples where then transferred and mounted in chloral hydrate/glycerol/water (8:1:2, w/v/v) to improve the contrast.

Samples were subsequently observed using a Zeiss Axiophot D1 microscope equipped with differential interface contrast optics (Carl Zeiss MicroImaging). Images were captured on a Zeiss Axiocam MRc5 camera using AxioVision software.

Primers Used in This Work

The sequences are listed in Supplemental Table 1 online.

Images

The multifocus pictures were made using CombineZP software (http://combinezp.software.informer.com/). Figures and panels were assembled and processed using Adobe Photoshop CS3.

Accession Numbers

Sequence data from this article can be found in the GenBank/EMBL database under the following accession numbers: *MADS3* (L37528), *MADS13* (AF151693), *MADS21* (AY551913), and *MADS58* (AB232157).

Supplemental Data

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

Supplemental Figure 1. Alignment of *MADS3* and *MADS58* Coding Sequences.

Supplemental Figure 2. Morphological and Molecular Analyses.

Supplemental Figure 3. Quantitative RT-PCR Expression Analysis.

Supplemental Figure 4. Scheme of the Ancient Gene Duplication That Led to the Formation of the *AG* and *AGL11* Lineages.

Supplemental Table 1. Sequence of Primers Used in This Work.

AUTHOR CONTRIBUTIONS

L.D. performed genetic, histological, and molecular experiments and wrote the manuscript. A. Pilatone and S.E. performed genotyping and phenotyping experiments. D.Y. and D.Z. provided the *mads3-4* single and *mads3-4 mads58* double mutants and rice *MADS2* in situ data. A. Pajoro did rice transformation experiments. E.C. did the scanning electron microscopy analysis. M.M.K. coordinated the research and wrote the manuscript.

REFERENCES

Alvarez, J., and Smyth, D.R. (1999). *CRABS CLAW* and *SPATULA*, two *Arabidopsis* genes that control carpel development in parallel with *AGAMOUS*. Development **126:** 2377–2386.

Arora, R., Agarwal, P., Ray, S., Singh, A.K., Singh, V.P., Tyagi, A.K., and Kapoor, S. (2007). MADS-box gene family in rice: Genome-wide identification, organization and expression profiling during reproductive development and stress. BMC Genomics **8:** 242.

Bowman, J.L., and Smyth, D.R. (1999). *CRABS CLAW*, a gene that regulates carpel and nectary development in *Arabidopsis*, encodes a novel protein with zinc finger and helix-loop-helix domains. Development **126:** 2387–2396.

Bowman, J.L., Smyth, D.R., and Meyerowitz, E.M. (1989). Genes directing flower development in *Arabidopsis*. Plant Cell **1:** 37–52.

Brambilla, V., Battaglia, R., Colombo, M., Maslero, S., Bencivenga, S., Kater, M.M., and Colombo, L. (2007). Genetic and molecular interactions between BELL1 and MADS box factors support ovule development in *Arabidopsis*. Plant Cell **19:** 2544–2556.

Causier, B., Bradley, D., Cook, H., and Davies, B. (2009). Conserved intragenic elements were critical for the evolution of the floral C-function. Plant J. **58:** 41–52.

Causier, B., Schwarz-Sommer, Z., and Davies, B. (2010). Floral organ identity: 20 years of ABCs. Semin. Cell Dev. Biol. **21:** 73–79.

2862   The Plant Cell

Chu, H., Qian, Q., Liang, W., Yin, C., Tan, H., Yao, X., Yuan, Z., Yang, J., Huang, H., Luo, D., Ma, H., and Zhang, D. (2006). The *floral organ number4* gene encoding a putative ortholog of Arabidopsis CLAV-ATA3 regulates apical meristem size in rice. Plant Physiol. **142:** 1039–1052.

Coen, E.S., and Meyerowitz, E.M. (1991). The war of the whorls: Genetic interactions controlling flower development. Nature **353:** 31–37.

Colombo, L., Battaglia, R., and Kater, M.M. (2008). *Arabidopsis* ovule development and its evolutionary conservation. Trends Plant Sci. **13:** 444–450.

Colombo, L., Franken, J., Koetje, E., van Went, J., Dons, H.J., Angenent, G.C., and van Tunen, A.J. (1995). The petunia MADS box gene *FBP11* determines ovule identity. Plant Cell **7:** 1859–1868.

Colombo, M., Brambilla, V., Marcheselli, R., Caporali, E., Kater, M.M., and Colombo, L. (2010). A new role for the *SHATTERPROOF* genes during *Arabidopsis* gynoecium development. Dev. Biol. **337:** 294–302.

Cui, R., Han, J., Zhao, S., Su, K., Wu, F., Du, X., Xu, Q., Chong, K., Theissen, G., Meng, Z. (2010). Functional conservation and diversification of class E floral homeotic genes in rice (*Oryza sativa*). Plant J. **61:** 767–781.

Davies, B., Motte, P., Keck, E., Saedler, H., Sommer, H., and Schwarz-Sommer, Z. (1999). *PLENA* and *FARINELLI*: Redundancy and regulatory interactions between two *Antirrhinum* MADS-box factors controlling flower development. EMBO J. **18:** 4023–4034.

Dreni, L., Jacchia, S., Fornara, F., Fornari, M., Ouwerkerk, P.B., An, G., Colombo, L., and Kater, M.M. (2007). The D-lineage MADS-box gene *OsMADS13* controls ovule identity in rice. Plant J. **52:** 690–699.

Drews, G.N., Bowman, J.L., and Meyerowitz, E.M. (1991). Negative regulation of the Arabidopsis homeotic gene *AGAMOUS* by the *APETALA2* product. Cell **65:** 991–1002.

Favaro, R., Immink, R.G., Ferioli, V., Bernasconi, B., Byzova, M., Angenent, G.C., Kater, M., Colombo, L. (2002). Ovule-specific MADS-box proteins have conserved protein-protein interactions in monocot and dicot plants. Mol. Genet. Genomics **268:** 152–159.

Favaro, R., Pinyopich, A., Battaglia, R., Kooiker, M., Borghi, L., Ditta, G., Yanofsky, M.F., Kater, M.M., and Colombo, L. (2003). MADS-box protein complexes control carpel and ovule development in *Arabidopsis*. Plant Cell **15:** 2603–2611.

Hands, P., Vosnakis, N., Betts, D., Irish, V.F., Drea, S. (2011). Alternate transcripts of a floral developmental regulator have both distinct and redundant functions in opium poppy. Ann. Bot. **107:** 1557–1566.

Herr, J.M., Jr. (1971). A new clearing-squash technique for the study of ovule development in angiosperms. Am. J. Bot. **58:** 785–790.

Hiei, Y., Ohta, S., Komari, T., and Kumashiro, T. (1994). Efficient transformation of rice (*Oryza sativa* L.) mediated by Agrobacterium and sequence analysis of the boundaries of the T-DNA. Plant J. **6:** 271–282.

Hong, L., Qian, Q., Zhu, K., Tang, D., Huang, Z., Gao, L., Li, M., Gu, M., and Cheng, Z. (2010). ELE restrains empty glumes from developing into lemmas. J. Genet. Genomics **37:** 101–115.

Hu, L., Liang, W., Yin, C., Cui, X., Zong, J., Wang, X., Hu, J., and Zhang, D. (2011). Rice MADS3 regulates ROS homeostasis during late anther development. Plant Cell **23:** 515–533.

Ikeda, K., Nagasawa, N., and Nagato, Y. (2005). *ABERRANT PANICLE ORGANIZATION 1* temporally regulates meristem identity in rice. Dev. Biol. **282:** 349–360.

Jackson, D. (1991). *In-situ* hybridisation in plants. In Molecular Plant Pathology: A Practical Approach. D.J. Bowles, S.J. Gurr, and M.J. McPherson, eds (Oxford, UK: Oxford University Press), pp. 163–174.

Jeon, J.S., et al. (2000). T-DNA insertional mutagenesis for functional genomics in rice. Plant J. **22:** 561–570.

Jeong, D.H., et al. (2006). Generation of a flanking sequence-tag database for activation-tagging lines in japonica rice. Plant J. **45:** 123–132.

Kang, H.G., Noh, Y.S., Chung, Y.Y., Costa, M.A., An, K., and An, G. (1995). Phenotypic alterations of petal and sepal by ectopic expression of a rice MADS box gene in tobacco. Plant Mol. Biol. **29:** 1–10.

Karimi, M., Inzé, D., and Depicker, A. (2002). GATEWAY vectors for *Agrobacterium*-mediated plant transformation. Trends Plant Sci. **7:** 193–195.

Kater, M.M., Dreni, L., and Colombo, L. (2006). Functional conservation of MADS-box factors controlling floral organ identity in rice and *Arabidopsis*. J. Exp. Bot. **57:** 3433–3444.

Kramer, E.M., Jaramillo, M.A., and Di Stilio, V.S. (2004). Patterns of gene duplication and functional evolution during the diversification of the *AGAMOUS* subfamily of MADS box genes in angiosperms. Genetics **166:** 1011–1023.

Kyozuka, J., Kobayashi, T., Morita, M., and Shimamoto, K. (2000). Spatially and temporally regulated expression of rice MADS box genes with similarity to *Arabidopsis* class A, B and C genes. Plant Cell Physiol. **41:** 710–718.

Li, H., Liang, W., Hu, Y., Zhu, L., Yin, C., Xu, J., Dreni, L., Kater, M.M., Zhang, D. (2011a). Rice *MADS6* interacts with the floral homeotic genes *SUPERWOMAN1*, *MADS3*, *MADS58*, *MADS13*, and *DROOPING LEAF* in specifying floral organ identities and meristem fate. Plant Cell **23:** 2536–2552.

Li, H., Liang, W., Jia, R., Yin, C., Zong, J., Kong, H., Zhang, D. (2010). The *AGL6*-like gene *OsMADS6* regulates floral organ and meristem identities in rice. Cell Res. **20:** 299–313.

Li, H., Liang, W., Yin, C., Zhu, L., and Zhang, D. (2011b). Genetic interaction of *OsMADS3*, *DROOPING LEAF* and *OsMADS13* in specifying rice floral organs identities and meristem determinacy. Plant Physiol. **156:** 263–274.

Liljegren, S.J., Ditta, G.S., Eshed, Y., Savidge, B., Bowman, J.L., and Yanofsky, M.F. (2000). *SHATTERPROOF* MADS-box genes control seed dispersal in *Arabidopsis*. Nature **404:** 766–770.

Lopez-Dee, Z.P., Wittich, P., Enrico Pè, M., Rigola, D., Del Buono, I., Gorla, M.S., Kater, M.M., and Colombo, L. (1999). *OsMADS13*, a novel rice MADS-box gene expressed during ovule development. Dev. Genet. **25:** 237–244.

Mena, M., Ambrose, B.A., Meeley, R.B., Briggs, S.P., Yanofsky, M.F., and Schmidt, R.J. (1996). Diversification of C-function activity in maize flower development. Science **274:** 1537–1540.

Mizukami, Y., and Ma, H. (1995). Separation of *AG* function in floral meristem determinacy from that in reproductive organ identity by expressing antisense AG RNA. Plant Mol. Biol. **28:** 767–784.

Münster, T., Deleu, W., Wingen, L.U., Ouzunova, M., Cacharrón, J., Faigl, W., Werth, S., Kim, J.T.T., Saedler, H., and Theißen, G. (2002). Maize MADS-box genes galore. Maydica **47:** 287–301.

Nagasawa, N., Miyoshi, M., Sano, Y., Satoh, H., Hirano, H., Sakai, H., and Nagato, Y. (2003). *SUPERWOMAN1* and *DROOPING LEAF* genes control floral organ identity in rice. Development **130:** 705–718.

Narsai, R., Ivanova, A., Ng, S., and Whelan, J. (2010). Defining reference genes in *Oryza sativa* using organ, development, biotic and abiotic transcriptome datasets. BMC Plant Biol. **10:** 56.

Ohmori, S., Kimizu, M., Sugita, M., Miyao, A., Hirochika, H., Uchida, E., Nagato, Y., Yoshida, H. (2009). *MOSAIC FLORAL ORGANS1*, an *AGL6*-like MADS box gene, regulates floral organ identity and meristem fate in rice. Plant Cell **21:** 3008–3025.

Orashakova, S., Lange, M., Lange, S., Wege, S., and Becker, A. (2009). The *CRABS CLAW* ortholog from California poppy (*Eschscholzia californica*, Papaveraceae), *EcCRC*, is involved in floral meristem termination, gynoecium differentiation and ovule initiation. Plant J. **58:** 682–693.

Parenicová, L., de Folter, S., Kieffer, M., Horner, D.S., Favalli, C., Busscher, J., Cook, H.E., Ingram, R.M., Kater, M.M., Davies, B., Angenent, G.C., and Colombo, L. (2003). Molecular and phylogenetic analyses of the complete MADS-box transcription factor family in Arabidopsis: New openings to the MADS world. Plant Cell **15:** 1538–1551.

Pinyopich, A., Ditta, G.S., Savidge, B., Liljegren, S.J., Baumann, E., Wisman, E., and Yanofsky, M.F. (2003). Assessing the redundancy of MADS-box genes during carpel and ovule development. Nature **424:** 85–88.

Prasad, K., Sriram, P., Kumar, C.S., Kushalappa, K., and Vijayraghavan, U. (2001). Ectopic expression of rice OsMADS1 reveals a role in specifying the lemma and palea, grass floral organs analogous to sepals. Dev. Genes Evol. **211:** 281–290.

Prasad, K., and Vijayraghavan, U. (2003). Double-stranded RNA interference of a rice *PI/GLO* paralog, *OsMADS2*, uncovers its second-whorl-specific function in floral organ patterning. Genetics **165:** 2301–2305.

Rounsley, S.D., Ditta, G.S., and Yanofsky, M.F. (1995). Diverse roles for MADS box genes in Arabidopsis development. Plant Cell **7:** 1259–1269.

Schmidt, R.J., Veit, B., Mandel, M.A., Mena, M., Hake, S., Yanofsky, M.F. (1993). Identification and molecular characterization of ZAG1, the maize homolog of the *Arabidopsis* floral homeotic gene *AGAMOUS*. Plant Cell **5:** 729–737.

Schwarz-Sommer, Z., Davies, B., and Hudson, A. (2003). An everlasting pioneer: The story of *Antirrhinum* research. Nat. Rev. Genet. **4:** 657–666.

Sieburth, L.E., Running, M.P., and Meyerowitz, E.M. (1995). Genetic separation of third and fourth whorl functions of AGAMOUS. Plant Cell **7:** 1249–1258.

Suzaki, T., Toriba, T., Fujimoto, M., Tsutsumi, N., Kitano, H., and Hirano, H.Y. (2006). Conservation and diversification of meristem maintenance mechanism in *Oryza sativa*: Function of the *FLORAL ORGAN NUMBER2* gene. Plant Cell Physiol. **47:** 1591–1602.

Vision, T.J., Brown, D.G., and Tanksley, S.D. (2000). The origins of genomic duplications in *Arabidopsis*. Science **290:** 2114–2117.

Yamaguchi, T., Lee, D.Y., Miyao, A., Hirochika, H., An, G., and Hirano, H.Y. (2006). Functional diversification of the two C-class MADS box genes OSMADS3 and OSMADS58 in *Oryza sativa*. Plant Cell **18:** 15–28.

Yamaguchi, T., Nagasawa, N., Kawasaki, S., Matsuoka, M., Nagato, Y., and Hirano, H.Y. (2004). The YABBY gene DROOPING LEAF regulates carpel specification and midrib development in *Oryza sativa*. Plant Cell **16:** 500–509.

Yamaki, S., Nagato, Y., Kurata, N., and Nonomura, K. (2011). Ovule is a lateral organ finally differentiated from the terminating floral meristem in rice. Dev. Biol. **351:** 208–216.

Yanofsky, M.F., Ma, H., Bowman, J.L., Drews, G.N., Feldmann, K.A., and Meyerowitz, E.M. (1990). The protein encoded by the *Arabidopsis* homeotic gene *agamous* resembles transcription factors. Nature **346:** 35–39.

Yellina, A.L., Orashakova, S., Lange, S., Erdmann, R., Leebens-Mack, J., and Becker, A. (2010). Floral homeotic C function genes repress specific B function genes in the carpel whorl of the basal eudicot California poppy (*Eschscholzia californica*). Evodevo. **1:** 13.

Yoshida, A., Suzaki, T., Tanaka, W., and Hirano, H.Y. (2009). The homeotic gene *long sterile lemma* (*G1*) specifies sterile lemma identity in the rice spikelet. Proc. Natl. Acad. Sci. USA **106:** 20103–20108.

Yun, P.Y., Kim, S.Y., Ochiai, T., Fukuda, T., Ito, T., Kanno, A., and Kameya, T. (2004). AVAG2 is a putative D-class gene from an ornamental asparagus. Sex. Plant Reprod. **17:** 107–116.

Zahn, L.M., Leebens-Mack, J.H., Arrington, J.M., Hu, Y., Landherr, L.L., dePamphilis, C.W., Becker, A., Theissen, G., and Ma, H. (2006). Conservation and divergence in the AGAMOUS subfamily of MADS-box genes: Evidence of independent sub- and neofunctionalization events. Evol. Dev. **8:** 30–45.

PART III

Study the molecular network involved in ovule development

Introduction

During the floral transition of *Arabidopsis thaliana*, the shoot apical meristem (SAM) is converted into an Inflorescence Meristem (IM) that subsequently generates floral meristems (FMs). The FM is composed of a population of indeterminate cells that will give origin to all floral organs. As proposed by the ABCDE model (Bowman et al., 2012), floral organ identity in *Arabidopsis thaliana* is specified by the combined action of different classes of homeotic genes mainly encoding MADS-domain transcription factors. These transcription factors are able to bind as homo- or hetero-dimers (Pellegrini et al., 1995; Shore and Sharrocks, 1995; Riechmann et al., 1996; de Folter et al., 2005) to DNA consensus sequences known as CArG-boxes [CC(A/T)$_6$GG] (Egea-cortines et al., 1999; Nurrish and Treisman, 1995; Mendes et al., 2013) modulating the transcription of their target genes. In *Arabidopsis*, the formation of sexual reproductive organs is orchestrated by the interaction of B- and C class genes for the specification of stamen, C-class genes alone for carpel identity determination, and class D- gene for ovule identity specification. *AGAMOUS* (*AG*) is the C-class gene of *Arabidopsis,* therefore in the *ag* mutant it is possible to observe the conversion of stamens into petals, and a new *ag* flower develops instead of the carpel. Moreover flowers are formed by a succession of sepals-petals-petals and are completely sterile (Bowman et al., 1991; Drews et al., 1991). *AG* also plays an important role in floral meristem determination, as can be noted by the repetition of the floral whorls present in the mutant.

The MADS domain transcription factor SEEDSTICK (STK) of *Arabidopsis,* is considered to be a D-class ovule identity gene, and is specifically expressed in ovules. However, its loss of function affects only the development of

funiculus, an umbilical-cord-like structure that connects the ovule to the placenta. Ten years ago, Pinyopich and colleagues discovered that *STK* is involved in determining ovule identity redundantly with *SHP1 SHP2* and *AG*. Furthermore, genetic and protein interaction studies have shown that these ovule identity factors interact with SEPALLATA (SEP) MADS domain transcription factors and that these interactions are essential for their function in ovule development (Favaro et al., 2003). Recent data have shown that *VERDANDI* (*VDD*), a gene belonging to the *REPRODUCTIVE MERISTEM* family (Romanel et al., 2009), is a direct target of *STK* (Matias-Hernandez et al., 2010). *VDD* is involved in the regulation of the formation of antipodal and synergid cells, and its loss of function results in semi-sterility.

In situ hybridization studies have shown that the expression of *VDD* is redundantly modulated by the ovule identity factors STK, SHP1, and SHP2, since the expression of *VDD* is strictly dependent on the activity of the three MADS domain ovule identity factors (Matias-Hernandez et al., 2010). Only in the triple mutant *stk shp1 shp2* it is not possible to detect *VDD* expression during ovule development (Matias-Hernandez et al., 2010).

OsMADS3 and *OsMADS58,* are the rice orthologs of *AGAMOUS.* In Chapter II we have shown that they are the C-class genes of rice, involved in stamen and carpel identity and floral meristem determination. (Dreni et al., 2011). *OsMADS13* is the rice D-class gene orthologous to *STK* (Dreni et al., 2007). In the *osmads13* mutant ovules are homeotically converted into carpelloid structures, similarly to what happens in the *Arabidopsis stk shp1 shp2* triple mutant.

In the last few years, the transcriptional networks controlling the development of the female gametophyte have been studied in *Arabidopsis thaliana*. In particular, the transcriptomes of individual cell types in the mature embryo

sac was recently published, and large-scale transcriptome analysis was done to find the genes involved in the development of the female gametophyte (Wuest et al., 2010; Ohnishi et al., 2011; Schmid et al., 2012; Sánchez-León et al., 2012). On the other hand, in rice the genome-wide expression profiling of mature ovules from the megaspore mother cell at pre-meiotic stage to the mature embryo sac has been recently investigated by using Laser Microdissection (LMD) followed by microarrays analysis (Kubo et al., 2013). Despite the data obtained by Kubo and colleagues, little is known in rice regarding the regulatory pathways controlling very early stages of ovule development, specifically in cells of the FMs that give origin to ovules. In this Chapter we show the strategies that we used to try to get insight into the molecular network underlying FM determinacy and Ovule Primordia (OP) formation in rice. We also discuss the putative roles of genes already described in literature and novel genes that are putatively involved in the correct formation of the ovule.

Identification of genes that are differentially expressed in *osmads13* mutant as compared to wild-type using an RNA sequencing approach.

If we look at the rice inflorescences, it is possible to observe a basipetal gradient for panicle development, where we can find late stage of floral organ formation (Ovule Primordia, OP) at the top of the inflorescence, and early stage (Floral Meristems, FM) at the bottom of the inflorescence (Itoh et al., 2005). Due to the inaccessibility, low abundance and small size of cells of FM and OP, we decided to start the work doing an exome-sequencing of whole inflorescences at very early stages of development in order to identify genes that are mis-regulated in the *osmads13* female sterile mutant. Young inflorescences 16 days after floral induction (LD-SD shift), with a length between 0,5cm and 0,8cm from *osmads13* mutant and wild-type plants were collected (Figure 9). We selected this stage because there are no visible phenotypic alterations between wild-type and *osmads13* inflorescences. Panicles longer than 0,8 cm were discarded because in later stages the integument primordium differentiates into inner and outer integuments in wild-type flowers, whereas, ectopic carpels form instead of the mature ovule in *osmads13* mutant flowers, giving rise to a carpel-in-a-carpel structure.
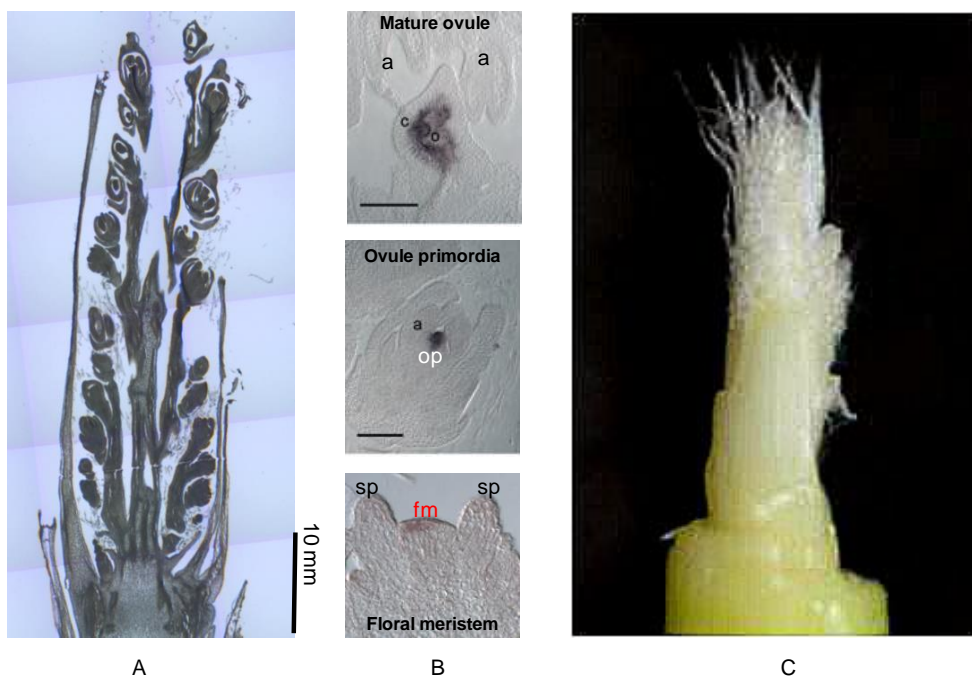
Figure 9 (A) In rice inflorescence is possible to observe a basipetal gradient for panicle development where we can find late stage of floral organ formation (Ovule Primordia, OP) at the top of the inflorescence, and early stage (Floral Meristems, FM) at the bottom of the inflorescence. (B) *OsMADS13* expression was evaluated by *in-situ* hybridization in different stages of flower development (C). An example of inflorescences with a length between 0,5cm and 0,8cm that we collected from *osmads13* mutant and wild-type for RNA-seq experiment. (a) anther, (c) carpel, (fm) floral meristem, (o) ovule, (op) ovule primordia, (sp) stamen primordia.

Material collected from wild-type and *osmads13* mutant inflorescence's was pooled and three biological replicates were used by IGA sequencing in Udine. Poly(A) libraries were made from total RNA, and were sequenced using the 'Illumina' technology.

The obtained datasets were analyzed using the DE-seq e BTC Suite software, aligning the reads to the reference genome (*Oryza sativa,* sub. *Japonica,* cv Nipponbare [MSU 7.0 release]). Gene expression was quantified by normalizing for total read length and the number of sequencing reads, evaluating the RPKM (Reads Per Kilobase per Million mapped reads) for each gene.

From this analysis, 811 genes were found to be differentially expressed in the *osmads13* mutant background as compared to wild type, from which 475 genes displayed a "False Discovery Rate" (FDR) cut-off value lower than 0.05.

Analyzing the dataset of 475 genes using a simplified tool for Gene Ontology analysis called GO-slims, we found 379 genes with an annotated function, 85 genes that encode for proteins with an unknown function and 11 genes related to transposable element (Figure 10). It is also possible to divide these genes in functional categories as follows: Nucleic Acid Binding, Protein Binding, Hormonal Pathways, Signal Transduction Pathways, Metabolism, and Structural Components.
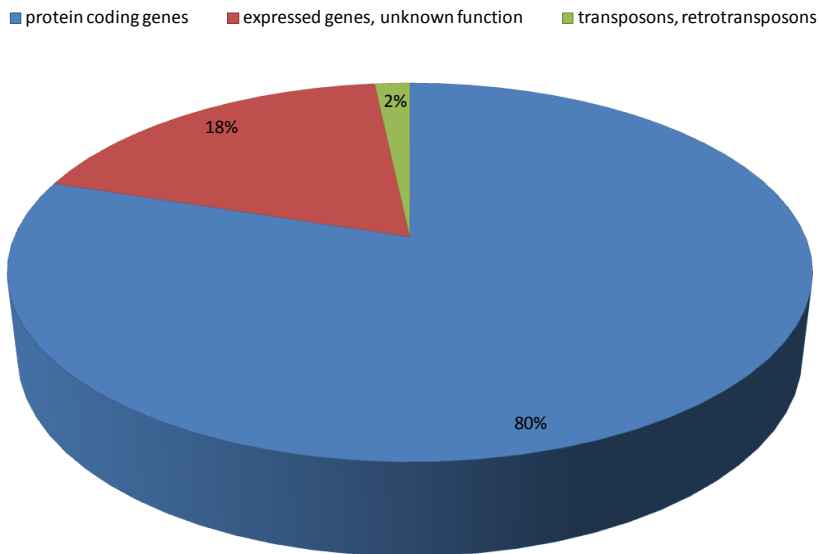
Figure 10 Output of Gene Ontology results obtained using the GO-slims software.

A Gene Ontology (GO) analysis for molecular function was also performed on the 379 mis-regulated genes with an annotated function, by using the

AgriGO tool. Interestingly, we found that the dataset was enriched for the Nucleic Acid Binding category and in particular for transcription regulatory activity genes (TFs) (Figure 11).

	Non TE genes	TFs genes	%
RNA-seq	380	81	21%
Rice Genome	39045*	2384**	6,10%
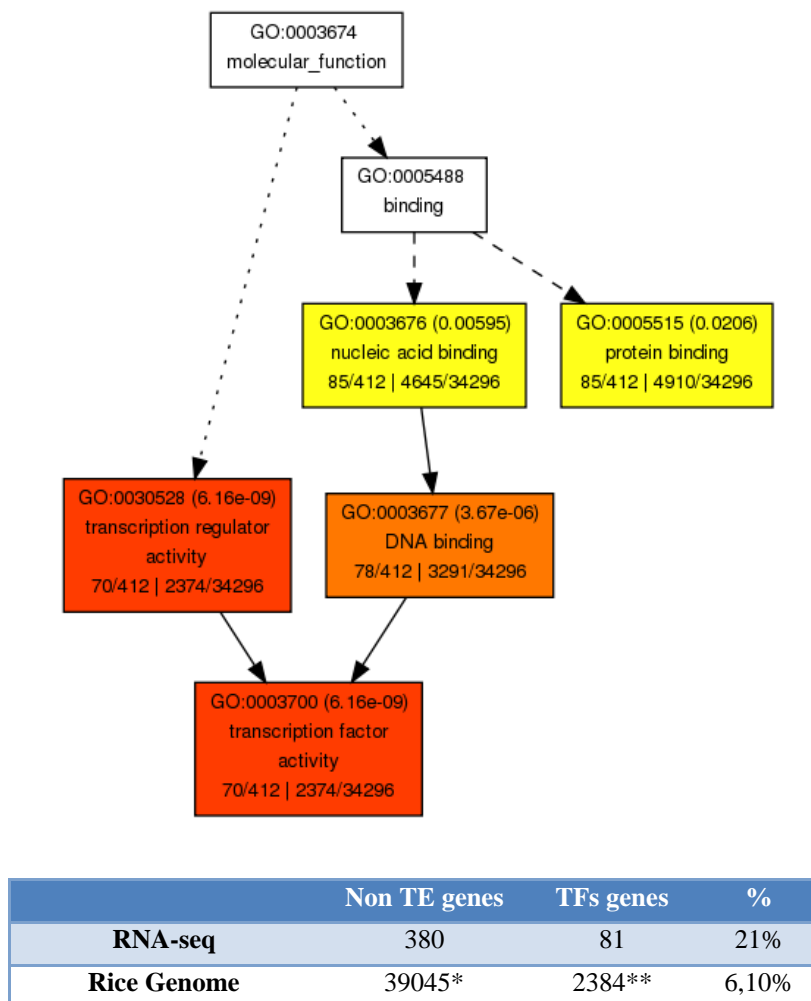
Figure 11 Gene Ontology (GO) enrichment analysis for molecular function using the software AgriGO. An enrichment for transcription regulatory activity genes and in particular for transcription factors (TFs) was found (* Taken from Rice Genome Annotation Project - RGAP; taken from Database of Rice Transcription Factors - DRTF).

Further analyses have shown that in the *osmads13* mutant, 68% of genes encoding for TFs were up-regulated (Figure 12), which indicates that OsMADS13 can be a master regulator for the repression of several TFs.
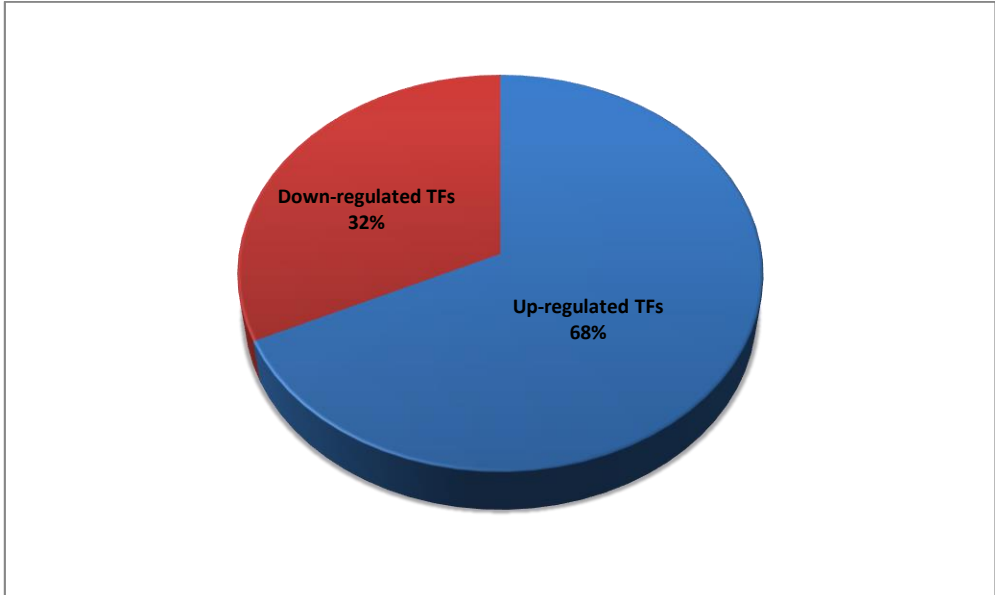
Figure 12 We found from RNA-seq results that the 68% of genes encoding for transcription factors are up-regulated in the *osmads13* mutant.

Selection of a subset of Deregulated Expressed genes (DEGs)

Since we are interested in transcription factors involved in flower development and since transcription factor encoding genes are most enriched in our dataset, we made the first selection based on TFs molecular function; in particular we selected genes classified in the "Nucleic Acid Binding" category. Subsequently, to focus attention on putative direct targets of OsMADS13, a second selection based on the CArG-box consensus sequences found in the regulatory regions of selected genes was done. For this purpose, we used a free online tool called "Match", based on a free library of the TRANS-FAC software, able to find the CArG-box consensus motif in the regulatory regions of selected genes. For the analysis we improved the software search according to the CArG-boxes of the *VERDANDI* promoter recognized and bound by SEEDSTICK (Hernandez et al., 2010; Mendes et al., 2013).

Finally, to select genes that are mainly expressed during reproductive stages, their *in-silico* expression pattern was predicted using the online software RiceXPro (http://*ricexpro.dna.affrc.go.jp*) and we also looked for the role of putative *Arabidopsis* orthologs.

Validation of RNA-Seq results and expression analysis in the ovary by large-scale RT-qPCR

From RNA-seq experiment we obtained a list of mis-expressed genes in the *osmads13* mutant. Based on the criteria explained in the previous chapter, we selected 45 interesting genes (Table 1) to be validated using the Microfluidic Dynamic Array (Fluidigm Corporation), a new technology for high-throughput qPCR (Spurgeon et al., 2008). For this purpose, we designed and tested primers that were specific for genes of interest (Table in Material and Methods). Furthermore, we tried to go deeper in the analysis confirming alterations in expression levels of selected genes in the *OsMADS13* expression domain. For this reason, we fixed and embedded inflorescences at the same developmental stage as used for RNA-seq analysis, because in these stages it's possible to collect material from FM and OP.

Subsequently, using Laser Microdissection (LMD), cells of the floral meristem and ovule primordia were collected from wild-type and mutant flowers (Figure 13).

Locus ID	MSU Rice Genome Annotation Project
Os06g11170	formin-binding protein-related, putative, expressed
Os01g10504	OsMADS3 - MADS-box family gene with MIKCc type-box, expressed
Os09g32948	OsMADS8 - MADS-box family gene with MIKCc type-box, expressed
Os12g10540	OsMADS13 - MADS-box family gene with MIKCc type-box, expressed
Os05g11414	OsMADS58 - MADS-box family gene with MIKCc type-box, expressed
Os03g11600	YABBY domain containing protein, putative, expressed
Os03g15270	gibberellin receptor GID1L2, putative, expressed
Os02g42990	OsSAUR11 - Auxin-responsive SAUR gene family member, expressed

Os04g51890	OsSAUR20 - Auxin-responsive SAUR gene family member, expressed
Os07g29310	OsSAUR30 - Auxin-responsive SAUR gene family member, expressed
Os03g58600	PAZ domain containing protein, putative, expressed
Os09g36700	ribonuclease T2 family domain containing protein, expressed
Os05g13630	RNA recognition motif containing protein, putative, expressed
Os07g01530	expressed protein
Os04g58810	CAF1 family ribonuclease containing protein, putative, expressed
Os03g57240	ZOS3-19 - C2H2 zinc finger protein, expressed
Os04g35500	ZF-HD protein dimerisation region containing protein, expressed
Os08g34010	ZF-HD protein dimerisation region containing protein, expressed
Os02g13310	homeobox domain containing protein, expressed
Os06g45140	bZIP transcription factor domain containing protein, expressed
Os09g36910	bZIP transcription factor domain containing protein, expressed
Os01g49830	B3 DNA binding domain containing protein, expressed
Os03g09170	ethylene-responsive transcription factor, putative, expressed
Os09g35010	dehydration-responsive element-binding protein, putative, expressed
Os04g52090	AP2 domain containing protein, expressed
Os05g41760	AP2 domain containing protein, expressed
Os11g07460	TCP family transcription factor, putative, expressed
Os01g11550	TCP family transcription factor, putative, expressed
Os01g50940	helix-loop-helix DNA-binding domain containing protein, expressed
Os08g37290	basic helix-loop-helix, putative, expressed
Os07g39220	BES1/BZR1 homolog protein, putative, expressed
Os04g43680	MYB family transcription factor, putative, expressed
Os01g32770	DUF260 domain containing protein, putative, expressed
Os07g12340	NAC domain-containing protein 67, putative, expressed
Os04g51000	transcription factor FL, putative, expressed

Os08g39890	OsSPL14 - SBP-box gene family member, expressed
Os03g53340	HSF-type DNA-binding domain containing protein, expressed
Os09g35790	HSF-type DNA-binding domain containing protein, expressed
Os03g04240	glutathione S-transferase, putative, expressed
Os03g04220	glutathione S-transferase, putative, expressed
Os11g02240	CAMK_KIN1/SNF1/Nim1_like.4 - CAMK includes calcium/calmodulin depedent protein kinases, expressed
Os03g12730	receptor protein kinase CLAVATA1 precursor, putative, expressed
Os01g08860	hsp20/alpha crystallin family protein, putative, expressed
Os02g02410	DnaK family protein, putative, expressed

Table 1 Interesting genes selected from RNA-seq analysis output.
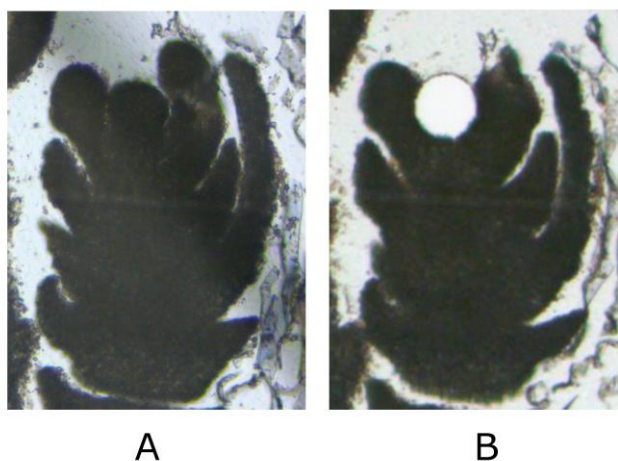

Figure 13 An example of cut of the rice Ovule Primordia (OP) done by Laser Microdissection. Images of flower (A) before cut and (B) after cut.

RNA was extracted using the kit Pico Pure (NuGen) that is used for extracting low amounts of RNA, and the quality (RIN value) was assessed using the Agilent Bioanalyzer (see material and methods). The quality of the extracted

RNA was very low (RIN about 1.6), and for reliable RT-qPCR analyses, a RIN value between 6.0 and 7.0 is needed (Schroeder et al., 2006; Takahashi et al., 2010).

FM wild-type   FM *osmads13* mutant

RIN: 7.70   RIN: 7.40

OP wild-type   OP *osmads13* mutant

RIN: 7.10   RIN: 7.20
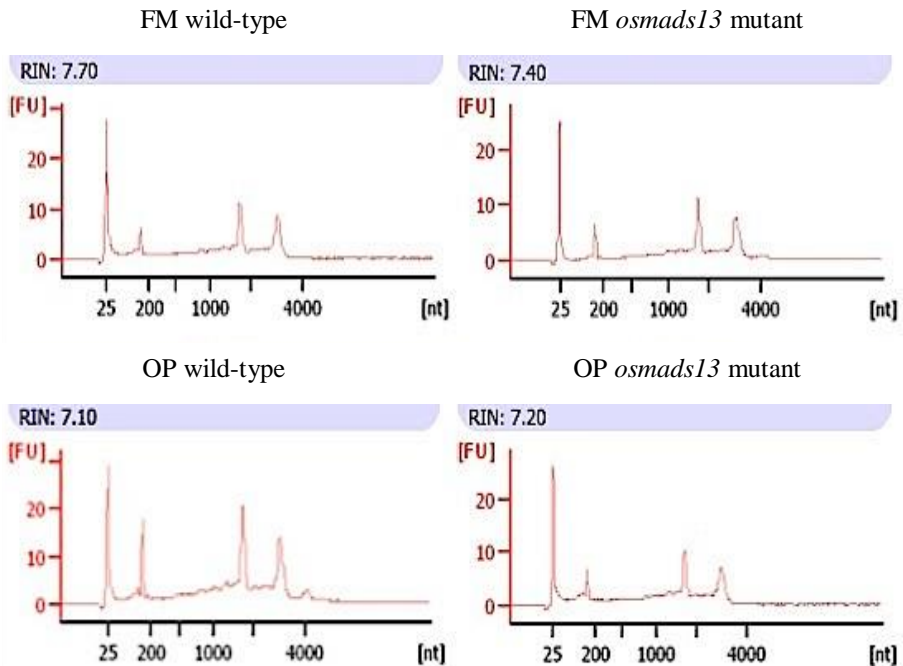
Figure 14 RIN of the RNA purified from tissue obtained by Laser Microdissection.

Therefore, the Laser Microdissection protocol was improved in order to obtain a good RNA quality for downstream applications. In particular, the fixation was done using 100% acetone (Takahashi et al., 2010), instead of 75% (v/v) ethanol and 25% (v/v) acetic acid (Yu et al., 2007). The dehydration and eosine-staining steps were skipped and the embedding time was considerably reduced from 48h to 24h.

With the modified protocol, we improved the RIN (RNA integrity Number) of Laser Microdissected samples from approximately 1,6 to 7,0 (Figure 14). To get a sufficient amount of RNA for down-stream application, 80-100 cuts by Laser Microdissection were performed and pooled together. RNA was

extracted and quality (RIN) (Table 2) and quantity were evaluated. As expected, the amount of the RNA extracted from LMD material was to low as input for high-throughput RT-qPCR, so we amplified it using the kit OvationPicoSL WTAv2, based on retrotranscription and linear-amplification (for more details see materials and methods), that allow us to obtain several micrograms of cDNA from picograms of total RNA extracted from samples.

Sample	RIN
Floral Meristem wild-type	7,7
Floral Meristem wild-type	7,7
Floral Meristem wild-type	7,7
Floral Meristem *osmads13* mutant	7,4
Floral Meristem *osmads13* mutant	7,4
Floral Meristem *osmads13* mutant	7,4
Ovule Primordia wild-type	7,2
Ovule Primordia wild-type	7,2
Ovule Primordia wild-type	7,2
Ovule Primordia *osmads13* mutant	7,1
Ovule Primordia *osmads13* mutant	7,1
Ovule Primordia *osmads13* mutant	7,1

Table 2 Rna quality of Laser Microdissected material.

The data-output coming from this experiment were normalized using *OsEF1* gene as reference, and summarized in a "heat map" (Figure 15) that show the different expression level of selected genes in *osmads13* mutant and wild type ovule. In this experiment we also confirmed the data obtained from RNA sequencing experiment.
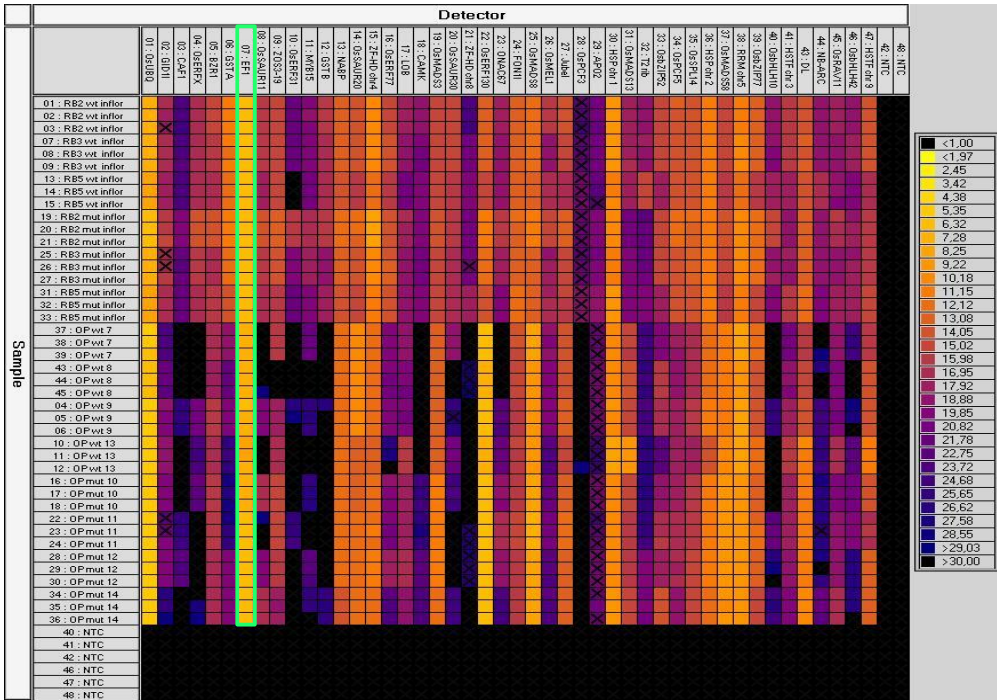
Figure 15 "Heat map" obtained from the analysis of the data obtained from the Microfluidic Dynamic Array (Fluidigm Corporation) experiment. The expression was normalized using *OsEF1* gene as reference (indicated in the green square).

PCF5 is a putative target of OsMADS13

Members of the TCP family of plant-specific transcription factors regulate plant architecture through control of cell proliferation and differentiation, originated in the Streptophyta about 650–800 million years ago (MYA) (Navaud et al., 2007). The TCP acronym derives from the first members discovered in this family: *teosinte-branched 1* (*tb1*) from *Zea mays* (Doebley et al., 1997), *CYCLOIDEA (CYC)* from *Antirrhinum majus* (Luo et al., 1996) and *PROLIFERATION CELL FACTOR 1* and *2* (*PCF1* and *PCF2)* from *Oryza sativa* (Kosugi and Ohashi, 2002). The genes that belong to this family, encode proteins sharing the so called TCP domain, a 59-amino acid basic helix–loop–helix (bHLH) motif that allows DNA binding and protein–protein interaction (Kosugi and Ohashil, 1997; Cubas et al., 1999).

If we look at the function of these genes, we found that *tb1* of maize controls developmental switches that contributed to the evolution from its wild ancestor teosinte (Doebley et al., 1995, 1997). Maize, differently from teosinte that is highly branched, has one dominant axis of growth (Hubbard et al., 2002). The maize *tb1* mutants resemble teosinte in their overall architecture, due to the presence of secondary and tertiary axillary branching, as well as to an increase in the length of each node, rather than to an increase in the number of nodes (Hubbard et al., 2002).

On the other hand, the gene *CYCLOIDEA (CYC)* (Cubas et al., 1999) of *Antirrhinum majus,* redundantly with *DICHOTOMA (DICH)*, is involved in the establishment of the dorsoventral asymmetry of the flower, reducing cell proliferation at the dorsal region of young floral meristems (Clark et al., 2002). Recently, *PCF1* and *PCF2* have been isolated in *Oryza Sativa* as DNA-binding proteins that specifically interact with site IIa and site IIb, which are

conserved sequences located in the promoter of the Proliferating Cell Nuclear Antigen (PCNA). These specific sequences are necessary for the meristematic tissue-specific expression of the rice PCNA, which encodes a protein involved in DNA replication and repair, maintenance of chromatin structure, chromosome segregation and cell-cycle progression (Kosugi and Ohashil, 1997; Kosugi et al., 1995). Phylogenetic analyses based on amino acid sequence similarity of the TCP domain showed that the TCP family can be divided in two major clades with slightly different TCP domains: Class I (Kosugi and Ohashi, 2002) also known as PCF class (Cubas, 2002), or TCP-P class, and Class II also known as TCP-C class (Navaud et al., 2007).

Most class I genes are involved in promoting plant growth and proliferation, despite the single mutants that have been analysed have mild or no phenotypic defects, probably as a result of genetic redundancy (Li et al., 2005; Hervé et al., 2009; Tatematsu et al., 2008; Sasazawa et al., 2006). Whereas in *Arabidopsis*, ectopic expression of *AtTCP20* fused to the EAR repressor domain (which probably inactivates several class I genes) leads to severe phenotypes, suggesting complex but as yet unclear roles in the regulation of cell division, expansion and differentiation (Hervé et al., 2009). By contrast, the proposed role of class II genes in preventing growth and proliferation is based directly on phenotypes observed in single and multiple mutants.

Interestingly, analyzing the results obtained from RNA sequencing on whole inflorescences we found the deregulation of the *PCF5* gene in the sample.
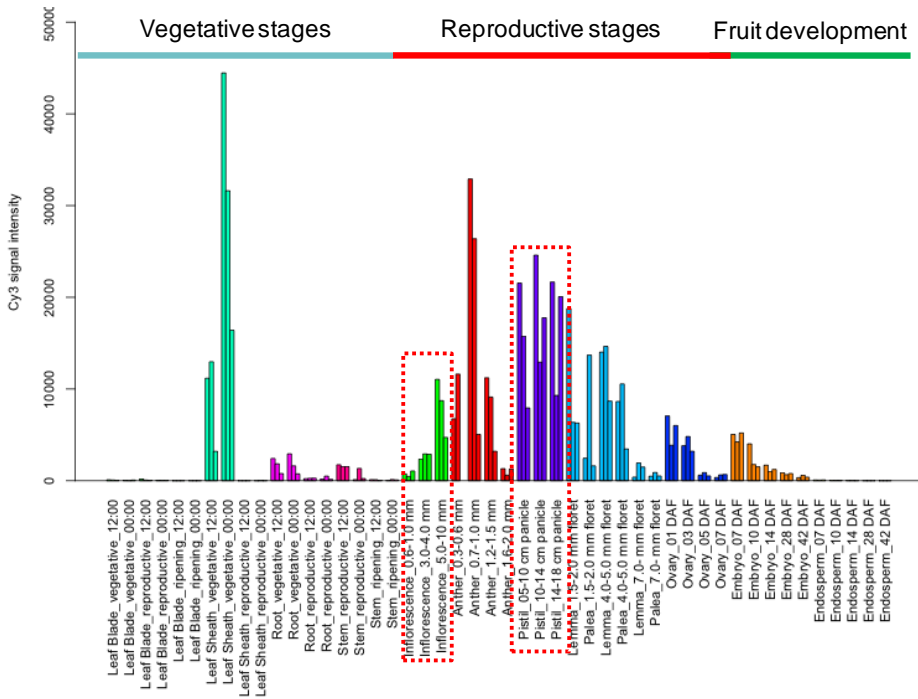
Figure 16 Expression profile of the TCP trascription factor PCF5 in tissues and organs. *In silico* data predicted using the online software RiceXPro (http://ricexpro.dna.affrc.go.jp).

This gene belongs to the Class II of TCP transcription factor family of rice and is differentially expressed in the *osmads13* mutant (fold change *osmads13* mutant / wild type = 1,380) (Table 3). We also confirmed its deregulation by RT-qPCR analysis on Ovule Primordia (OP) Laser Microdissected material, with a fold change *osmads13* mutant / wild type of 1,60, and Floral Meristem (FM) with a fold change *osmads13* mutant / wild type of 1,93 (Figure 17).

PCF5 RPKM wild-type	RPKM *osmads13* mutant	mutant/wild-type RPKM
20,857	28,792	1,38

Table 3 RPKM of *PCF5* from RNA-seq data analysis.

Figure 17 qRT-PCR expression of *PCF5* in wild-type and *osmads13* mutant ovule primordia and floral meristem.

OsMADS8 is a putative target of OsMADS13

The *SEPALLATA-like* MADS-box genes grouped in the E-class (Theissen and Saedler, 2001) are involved in the determination of sepals, petals, stamens and carpels, and play also roles in floral meristem determinacy. The *Arabidopsis thaliana*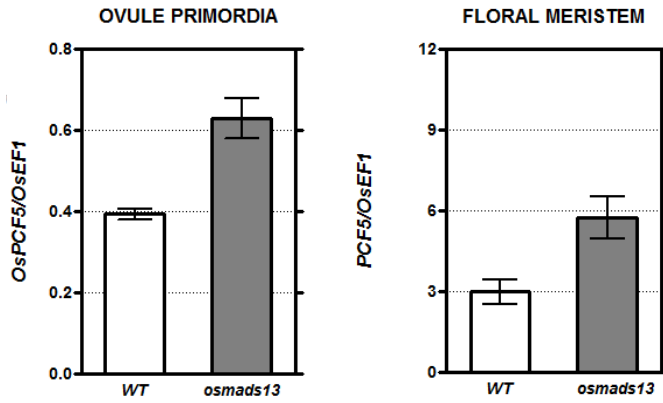 genome encodes four *SEPALLATA* genes, called *SEP1*, *SEP2*, *SEP3* and *SEP4*. On the other hand, in rice five *SEP*-like genes are present, named *OsMADS1, OsMADS5, OsMADS7, OsMADS8* and *OsMADS34* (Arora et al., 2007). Interestingly, the multiple knockdown mutants of the rice genes *OsMADS1*, *OsMADS5*, *OsMADS7* and *OsMADS8*, shows the homeotic transformation of all floral organs except the lemma into leaf-like organs (Cui et al., 2010) and this phenotype resemble that of the *sep1 sep2 sep3 sep4* quadruple mutant of *Arabidopsis thaliana* (Ditta et al., 2004; Honma and Goto, 2001; Pelaz et al., 2000). It was recently shown that *SEP3* is more important for the class E gene function than any of the other three *SEP* genes in *Arabidopsis* (Melzer et al., 2009). *OsMADS7* and *OsMADS8* are classified in the *SEP3*-clade (Kang et al., 1997; Zahn et al., 2005) and are very similar in terms of phylogenetic relationship and expression patterns (Münster et al., 2002; Becker and Theissen, 2003; Malcomber and Kellogg, 2004, 2005; Nam et al., 2004; Prasad et al., 2005). *In situ* hybridization showed that during flower development *OsMADS8* and *OsMADS7* were expressed in the part of the floral meristem where the lodicule and stamen primordia originate. At later stages, the transcripts of *OsMADS8* and *OsMADS7* were localized in the developing lodicules, stamens and pistils. In the mature florets, the transcripts of both *OsMADS8* and *OsMADS7* were confined to the reproductive organs (i.e. the stamens and ovary). In conclusion, looking at the expression of these genes, we can observe that they are overlapping during spikelet development

but their spatial distributions appeared to be slightly different during the early development of floral meristems.
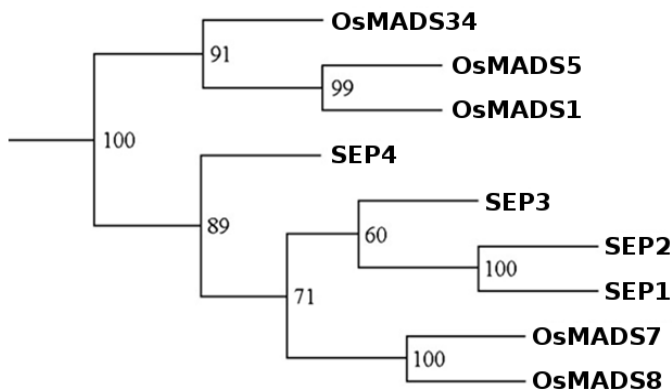
Figure 18 Phylogenetic tree of SEPALLATA proteins of rice and *Arabidopsis thaliana* (Kater et al., 2006).

The expression patterns of *OsMADS8* and *OsMADS7* are quite similar to those of the *Arabidopsis SEP1*, *SEP2*, and *SEP3* genes, suggesting that *OsMADS8* and *OsMADS7* might play a corresponding role in rice. The silencing of both genes using an RNA interference approach, leads to a variable range of aberrant carpels phenotypes. In plants with the strongest phenotypes, the carpels were completely unfused and the floral meristem determinacy was lost, leading to the formation of additional reproductive organ-like structures inside the carpels. Protein-protein interactions studies using two-hybrid approach, shows that OsMADS7 and OsMADS8 proteins are able to physically interact with class A, class B and class C floral organ identity proteins (Moon et al., 1999a, 1999b; Lim et al., 2000), but more interestingly, they are able to interact with OsMADS13 (class D) (Dreni et al., 2007), the ovule identity gene of rice.

From RNA sequencing analysis on whole inflorescences, we found that *OsMADS8* is down-regulated in *osmads13* mutant inflorescences (fold change *osmads13* mutant / wild-type =0,744) (Table 4), but this was not statistically significant (FDR: 0.15). On the contrary, an up-regulation of *OsMADS8* was found in the *osmads13* mutant Ovule Primordia (OP) (fold change *osmads13* mutant / wild-type = 1,61), and Floral Meristem (FM) (fold change *osmads13* mutant / wild-type = 1,38, not statistically significant) (Figure 19), using the material obtained with the Laser Microdissection approach.

OsMADS8 RPKM wild-type	RPKM *osmads13* mutant	mutant/wild-type RPKM
57,119	42,474	0,744

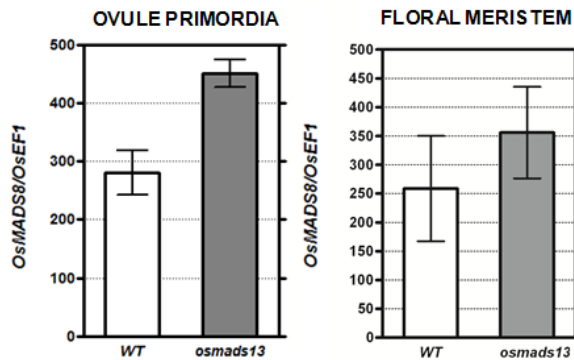Table 4 RPKM of *OsMADS8* from RNA-seq data analysis.

Figure 19 qRT-PCR expression of *OsMADS8* in wild-type and *osmads13* mutant ovule primordia and floral meristem.

Chip-seq technology

A widely used technology to detect the direct binding of a transcription factor (TF) to DNA and modifications of histone proteins, is the Chromatin Immunoprecipitation (ChIP) experiment. Combining ChIP with massively parallel DNA sequencing allows the identification of the genome-wide binding sites of a transcription factor (ChIP-seq). Interestingly, ChIP-seq is one of the first techniques that used the Next Generation Sequencing (NGS) technologies; in fact, the first related work was published in 2007 (Robertson et al., 2007). Differently from ChIP-chip, in ChIP-seq experiments the DNA fragments were directly sequenced instead of hybridized to a microarray. To obtain reliable data from this kind of experiment it is necessary to use a highly specific antibody, that needs to be validated for interacting with the bound protein or specific modifications of proteins. In the last few years, this technology was applied in several works to generate genome-wide DNA-binding maps of transcription factors in different species. Recently in our group, a Chip-seq experiment was successfully adopted to identify the direct target genes of the MADS-domain transcription factor SVP (Short Vegetative Phase) in *Arabidopsis thaliana* (Gregis et al., 2013). For rice not many ChIP-seq experiments have been published. Recently, it was used to detect histone modifications in rice: methylation and acetylation and to study the epigenetic status of telomeres (Vaquero-Sedas et al., 2012; Zong et al., 2013; Du et al., 2013; Nallamilli et al., 2013).

Since MADS-box transcription factors are able to bind CArG-box consensus sequences located in the regulatory regions of their target genes (Egea-cortines et al., 1999; Nurrish and Treisman, 1995), we would like to use this technology

to find direct targets, that are actually unknown, of the ovule identity factor OsMADS13 at genome-wide resolution.

Testing αOsMADS13 antibodies

In a previous study, polyclonal antibodies specific for the peptide NH_2-KATIDRYKKAHACGS-COOH, were tested by dot blotting with native and denatured form of the OsMADS13 protein. From this analysis, a good signal was found in the sample of total denaturated proteins extracted from rice wild type inflorescences, whereas a signal was not found in the same sample using the native form of OsMADS13 protein. Probably, the epitope of the OsMADS13 native form is not accessible and cannot be recognized by α-OsMADS13.

As expected, a signal was not found in the negative controls of protein-extracted from *osmads13* mutant inflorescences and using proteins extracted from *Arabidopsis thaliana* siliques.

GFP tagged OsMADS13 for ChIP-seq experiments

Since the α-OsMADS13 antibodies didn't work *in vivo*, we transformed rice calli (*osmads13* mutant background) (Figure 20 A) with the *pOsMADS13::OsMADS13 (CDS)::GFP* construct, in order to use commercial α-GFP antibodies for ChIP assays. We also performed another transformation of the same calli background, using the construct *pOsMADS13::OsMADS13 (CDS)* to verify the rescue of the *osmads13* mutant phenotype with the non-GFP tagged protein (Figure 20 B). Thirteen *pOsMADS13::OsMADS13 (CDS)::GFP* independently transformed regenerated plants were generated, and frequently more than a single plant for each lines was found. Subsequently, plants were grown in a greenhouse for about two months under Long Day (LD) conditions (16 h light/8 h dark), and moved fifteen days under Short Day (SD) conditions (12 h light/12 h dark) to induce flowering. Afterwards, we analyzed GFP expression for each independent transformed line, and we found GFP expression in the ovary of a few lines (Figure 24 A-B), but we didn't obtain seeds from these plants.

Clearing analysis to verify correct ovule formation in flowers collected during the heading stage from *pOsMADS13::OsMADS13 (CDS)* transformed plant was done. In particular, eleven independently transformed lines were analyzed in details and we concluded that the transgenic cassette wasn't able to fully complement the *osmads13* phenotype. In these lines, ovule development was completely restored and the embryo-sac seemed to develop normally, however, a tracheid like structure developed in the region where synergids and egg-cell are normally located in wild type ovaries (Figure 21). From these data we concluded that the construct *pOsMADS13::OsMADS13 (CDS)* is not able to fully complement the *osmads13* mutant phenotype.

A   •   ***pOsMADS13::OsMADS13(<u>CDS</u>)::GFP***   ➡️   **In *osmads13* mutant background**

B   •   ***pOsMADS13::OsMADS13(<u>CDS</u>)***   ➡️   **In *osmads13* mutant background**
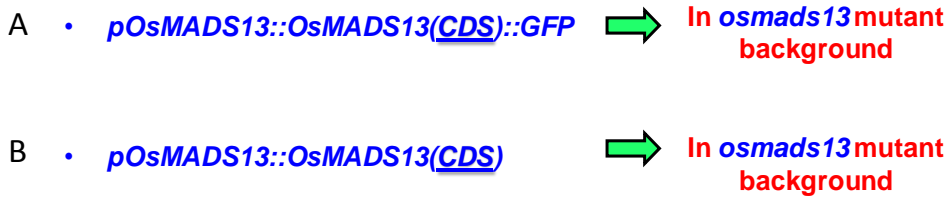
Figure 20 Scheme of the transformations with the constructs *pOsMADS13::OsMADS(CDS)* and *pOsMADS13::OsMADS13 (CDS)::GFP.*

Figure 21 Clearing analysis to verify the correct ovule formation in *pOsMADS13::OsMADS13 (CDS)* positive lines.

Cis-Regulatory elements controlling gene expression are preferentially found in the promoters of target genes. However, there are also several examples of important regulatory sequences in introns, particularly near the 5' end of genes (Rose, 2008). A typical example is the second intron in the MADS-box gene *AG*, which is bound by multiple factors (Sieburth and Meyerowitz, 1997; Deyholos and Sieburth, 2000; Hong et al., 2003; Bao et al., 2004; Gómez-Mena et al., 2005). One of these is the floral meristem identity gene of *Arabidopsis LEAFY (LFY)*, that positively regulates *AG* by binding to sites located within this second intron (Busch et al., 1999; Lohmann et al., 2001).

Thus we cloned the complete genomic region of *OsMADS13* to generate the construct *pOsMADS13::OsMADS13 (genomic)*, and we removed the stop codon of *OsMADS13* to create the GFP fusion construct *pOsMADS13::OsMADS13 (genomic)::GFP*. Subsequently we repeated the transformation inserting each construct in *osmads13* mutant and heterozygous background (Figure 23). In this case, we also transformed *osmads13/OsMADS13* heterozygous calli to be able to obtain T0 seeds even if the transgenic cassette will not be able to fully complement the *osmads13* phenotype leading to sterility. ChIP experiments will be eventually performed on T1 panicles of GFP-lines in heterozygous or wild type background.

We repeated the same transformation twice. From the first transformation, we analyzed six lines of heterozygous calli transformed with the construct *pOsMADS13::OsMADS13 (genomic)::GFP,* but we observed GFP expression in the ovary only in one of these positive lines. Differently from the transformation done with the construct *pOsMADS13::OsMADS13 (CDS)::GFP* (Figure 24 B)*, we observed a different GFP expression pattern in the positive line for the construct *pOsMADS13::OsMADS13(genomic)::GFP* (Figure 24 C). In particular the GFP signal in *pOsMADS13::OsMADS13 (genomic)::GFP* positive line seems to be stronger in the integuments and weaker in the nucellus tissues as compared to *pOsMADS13::OsMADS13 (CDS)::GFP* positive lines (Figure 24 C-D). The seeds obtained from this line were sowed and PCR analysis on T1 plants was done to find the plants positive for the construct *pOsMADS13::OsMADS13 (genomic)::GFP* in homozygous state in *osmads13* mutant background. To discriminate wild-type *OsMADS13* allele from the construct *pOsMADS13::OsMADS13 (genomic)::GFP*, we set-up a digestion of the PCR amplified region between primers Osp66 and Osp311

with the restriction enzyme SpeI, able to cut only the fragment amplificated from T-DNA insertion (Figure 22).
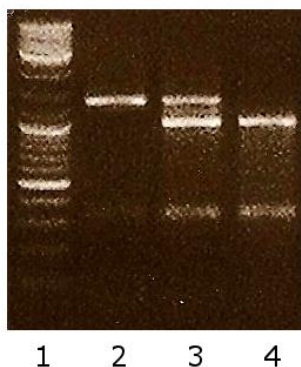
Figure 22 Digestion to discriminate wild type *OsMADS13* allele from the construct *pOsMADS13::OsMADS13 (genomic)::GFP.* 1: Marker, 2 wild-type control, 3 *pOsMADS13::OsMADS13 (genomic)::GFP* in *osmads13/+* calli background, 4: *pOsMADS13::OsMADS13 (genomic)::GFP* in *osmads13* calli background.

From this analysis we obtained nine plants (*osmads13* mutant background) carrying the construct *pOsMADS13::OsMADS13 (genomic)::GFP* in homozygous state and we are waiting for seeds, that will be collected in 6 weeks, to confirm the full complementation of the *osmads13* mutant phenotype.

From the second transformation we obtained fifteen independent lines for the construct *pOsMADS13::OsMADS13 (genomic)::GFP* for both the *osmads13* mutant background and the heterozygous background. Thirty-four lines for the control *pOsMADS13::OsMADS13 (genomic)* in *osmads13* mutant background and eight lines for the same construct in heterozygous background were obtained. Now we are growing these positive regenerant lines in phytotron and soon we will induce flowering and GFP expression will be analyzed in the ovary. In this second experiment we obtained plants with the construct in the *osmads13* mutant background and therefore we will be able

to verify the full-complementation of the *osmads13* mutant phenotype directly in T0 lines.

In conclusion, for the ChIP-seq experiments we are going to select a positive line carrying the construct *pOsMADS13::OsMADS13 (genomic)::GFP* introduced into the *osmads13* mutant background, expressing GFP in the ovary, and able to make seeds (phenotype complementation). Subsequently, this line will be propagated to obtain a large amount of plants to extract enough chromatin from young panicle for ChIP-seq experiment.

- *pOsMADS13::OsMADS13(genomic)::GFP*

  In *osmads13* mutant background

  In *osmads13/+* background

- *pOsMADS13::OsMADS13(genomic)*

  In *osmads13* mutant background

  In *osmads13/+* background
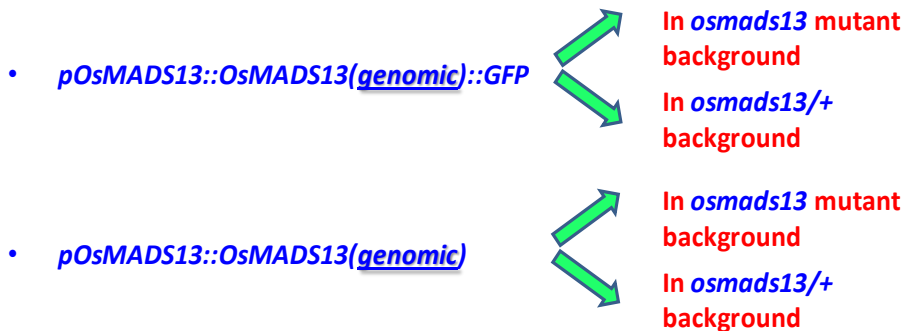
Figure 23 Scheme of the transformations with the constructs *pOsMADS13::OsMADS13(genomic)* and *pOsMADS13::OsMADS13 (genomic)::GFP.*
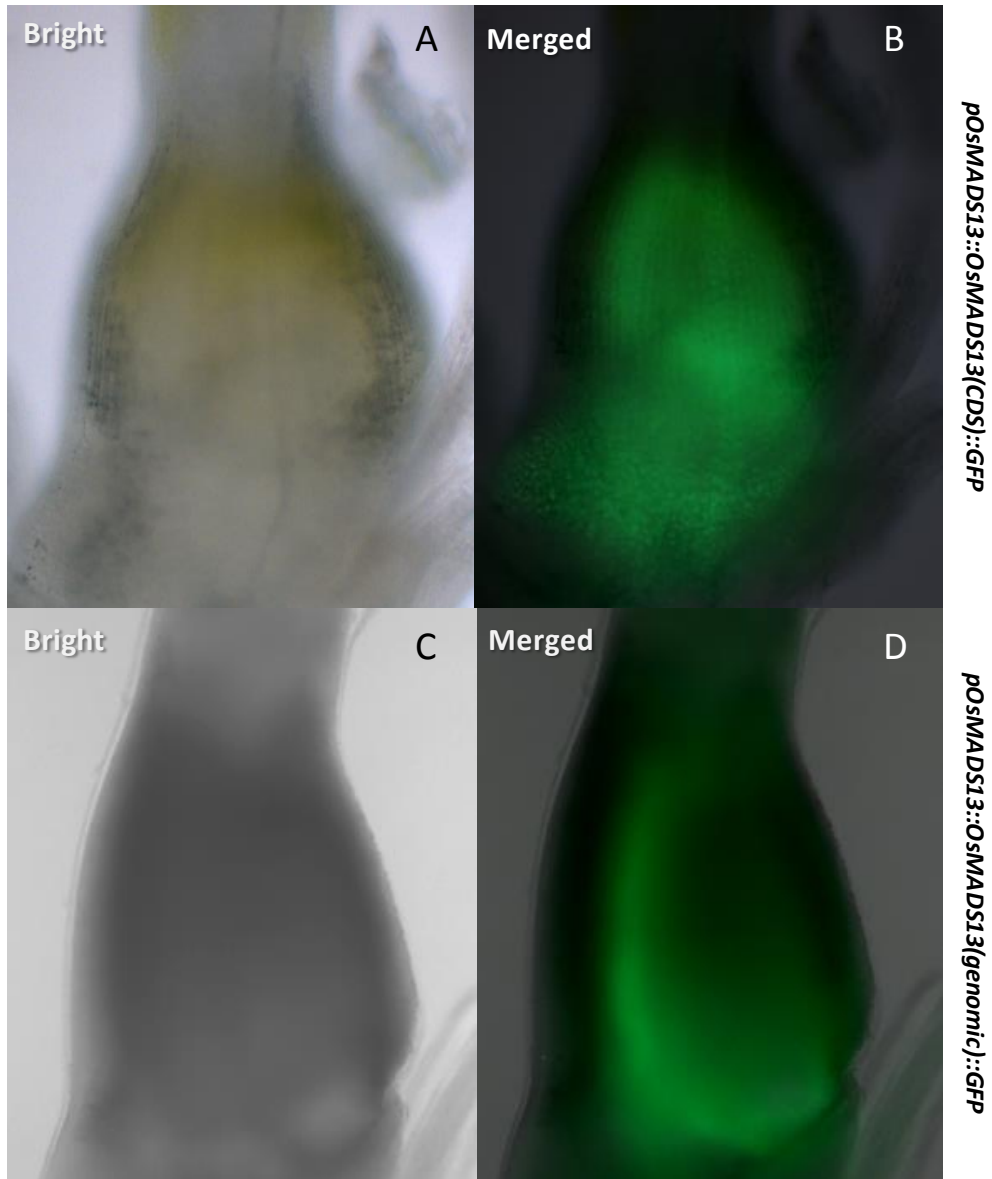
Figure 24 Comparison between GFP expression in *pOsMADS13::OsMADS13 (CDS)::GFP* (B) and *pOsMADS13::OsMADS13 (genomic)::GFP* (D) positive lines. The GFP expression pattern is quite different in *pOsMADS13::OsMADS13 (CDS)::GFP* as compared to *pOsMADS13::OsMADS13 (genomic)::GFP* positive lines. In particular the GFP signal seems to be stronger in the integuments and weaker in the nucellus tissues in the *pOsMADS13::OsMADS13 (genomic)::GFP* positive line.

DISCUSSION

The role of *AGAMOUS* subfamily genes in rice

Several studies conducted in *Arabidopsis thaliana* and *Antirrhinum majus* have shown that genes belonging to the *AGAMOUS* subfamily are involved in stamen, carpel and ovule development, and they also play an important role in floral meristem determinacy. The *AGAMOUS* subfamily of *Arabidopsis thaliana* is composed of: *SEEDSTICK (STK)*, *SHATTERPROOF1 (SHP1)*, *SHP2* and *AGAMOUS (AG)*.

Phylogenetic analysis has shown that *AG*, *SHP1* and *SHP2* belong to the same evolutionary clade, named *AG-lineage*, whereas *STK*, is the only member of the *AGL11-lineage*. Pinyopich and colleagues in 2003 studied in detail the roles of the *AGAMOUS* subfamily genes in *Arabidopsis,* and they found that *STK, SHP1, SHP2* and *AG,* redundantly regulate ovule development. According to the ABCDE model, the C Class gene *AGAMOUS* of *Arabidopsis* is involved in the determination of stamens and carpel identity, and it also plays an important role in floral meristem determinacy (Bowman et al., 1991; Drews et al., 1991). Phylogenetic studies have shown that two *AGAMOUS* orthologs, named *OsMADS3* and *OsMADS58,* are present in the rice genome. Yamaguchi and colleagues in 2006, starting from the analysis of the single mutants *osmads3* and *osmads58-RNAi* silencing line and of the double mutant os*mads3 osmads58-RNAi,* tried to demonstrate the functional conservation of the C- function in rice. From these analyses, they concluded that the stamen identity was partially lost in all the *AG-like* mutants, whereas carpel identity was maintained. These data are in disagreement with what is predicted by the ABCDE model, in which due to the lack of the C- function, we expect to

obtain flowers that are indeterminate and having in the third whorl stamens homeotically transformed into lodicules and carpel transformed into a palea/lemma. The data obtained by Yamaguchi et al., (2006) would implicate that the C function is not conserved in the monocot species rice which would be a great surprise also because of the strong structural conservation of carpels between monocot and dicot species. Considering that Yamaguchi et al., (2006) used for their double mutant a mild *osmads3* mutant allele and a RNAi approach to silence *OsMADS58*, makes it likely that the observed phenotype is a rather mild double mutant phenotype and might have lead to incorrect conclusions. Therefore, we repeated the functional characterization of *OsMADS3* and *OsMADS58*. We also studied in detail the genetic interactions with the other two members of the rice *AGAMOUS* subfamily, named *OsMADS13* and *OsMADS21*, which belong to the *AGL11-lineage.*

In **Chapter II** we demonstrated that *OsMADS3* and *OsMADS58* are the C-class genes of rice and the lack of both genes resulted in a phenotype that is in agreement with what is predicted by the ABCDE model. Moreover, our analysis also confirmed the role of *OsMADS3* and *OsMADS58* in floral meristem determinacy, similar to the *AG* function in *Arabidopsis thaliana.*

Looking at the *stk* mutant of *Arabidopsis* it is possible to observe an enlargement of the funiculus and the inhibition of seed detachment after maturation, whereas ovule identity was not affected. Differently from *Arabidopsis*, *OsMADS13* that is the *STK* ortholog in rice, was not redundant with other genes in determining ovule identity. From previous analysis conducted in our laboratory on the *osmads13* single mutant, it was shown that it is completely sterile because ovules are converted into carpelloid structures. Analyzing the mutant of the other member of the *AGL11-lineage* named *OsMADS21,* we didn't find a clear role for this gene during ovule

development. In fact, the *osmads21* single mutant didn't show visible alterations in respect to wild-type, and the *osmads13 osmads21* double mutant didn't display an enhancement of the *osmads13* single mutant phenotype.

From the analysis of the phenotype of transformed *osmads13* lines carrying the construct *pOsMADS13::OsMADS21*, we found a restoration of ovule identity although these ovules had a gametophyte defect strongly suggesting that *OsMADS21* has no role in wild-type ovule identity determination due to its low expression level.

In **Chapter II,** I also show that differently from the exclusive function of *AG* in the determinacy of floral meristem activity in *Arabidopsis thaliana,* in rice *OsMADS3* and *OsMADS58*, but also *OsMADS13* act redundantly in this process. It is important to notice that ovule developmental stages show some differences as we compare rice with *Arabidopsis thaliana.* In particular, in rice from the floral meristem also the ovule develops, whereas in *Arabidopsis thaliana* the FM was completely consumed by the development of the two carpels. Therefore, in *Arabidopsis thaliana* ovules are most likely originating from 'new' meristematic cells located in the placenta. The evolutionary distance between rice and *Arabidopsis* might explain these differences during ovule development, but it is still possible to find some similarities comparing these two species. *AG* of *Arabidopsis* is involved in floral meristem determinacy, and *STK* plays roles in differentiation of carpel margin to ovule founder cells. In fact, ovules were homeotically converted into secondary ectopic carpel-like structures when the *stk* mutant was combined with mutations of *SHP1* and *SHP2* (Pinyopich et al., 2003). On the other hand, in *Petunia* and rice, placenta and ovules arise directly from the inner part of the floral meristem, and differently from *Arabidopsis*, the floral meristem is maintained after carpel primordia formation. In *Petunia* two genes

orthologous to *SEEDSTICK* are present, named *FBP7* and *FBP11*. It was shown that the co-suppression of *FBP7* and *FBP11*, leads to the transformation of ovules into carpel-like structures developed directly from the placenta (Angenent et al., 1995; Colombo et al., 1995). On the contrary, more recent analysis based on multiple transposon insertion alleles, have shown that the ovule development was not largely affected in *fbp7 fbp11* double mutants, and that a strong loss of ovule identity was obtained combining the *fbp7 fbp11* double mutant with either the *PMADS3*-RNAi line or the *fbp6* mutant (Heijmans et al., 2012). From these analysis Heijmans and colleagues in 2012 concluded that the ovule identity in *Petunia hybrida* was redundantly specified by *AG-lineage* and *AGL11-lineage, and this characteristic* seems to be conserved within core eudicots.

OsMADS13 is a master regulator of several downstream processes

Laser Microdissection could be considered a very powerful approach to isolate specific tissues or cell-type, in order to extract RNA, DNA or protein from microdissected tissues. A Laser Microdissection approach followed by a microarray analysis, was used to report the expression profiles of the megaspore mother cell (MMC) (Schmidt et al., 2011), and of the three cell types of mature female gametophytes of *Arabidopsis thaliana* (Wuest et al., 2010). On the other hand, a similar experiment was done in rice to study the genome-wide expression profile of mature ovules from the megaspore mother cell at pre-meiotic stage to the mature embryo sac (Kubo et al., 2013). Recently, Schmid and colleagues published an interesting work, in which Laser Microdissection followed by RNA-sequencing were successfully applied to study the transcriptome of the central cells of the mature embryo sac of *Arabidopsis thaliana* (Schmid et al., 2012). Later, another experiment was done to evaluate the transcriptional changes during pollination in papilla cells of three different species of Brassicaceae, in order to study the process of pollination at the molecular level (Osaka et al., 2013).

Our idea was to perform a similar experiment in rice using Laser Microdissection to collect material from floral meristem (FM) and ovule primordia (OP), of both *osmads13* mutant and wild type inflorescences. Subsequently, we wanted to study the deregulation of interesting genes in the *OsMADS13* expression domain doing an RNA-seq analysis.

Since a similar experiment was never done before in rice using the RNA extracted from FM and OP material by Laser Microdissection, we needed to improve the fixation and embedding protocol in order to obtain a good RNA

quality for downstream applications. The set-up of the experiment required few mouths of work, and in the meantime we sequenced the RNA extracted from *osmads13* mutant and wild-type whole inflorescences at early stages of ovule development, to speed up the identification of genes that are differentially expressed (**Chapter III**). Later good quality of the RNA extracted from Laser Microdissected material was obtained, which allowed starting collecting samples from floral meristem (FM) and ovule primordia (OP), of both *osmads13* mutant and wild type inflorescences, in order to study the deregulation of interesting genes in the *OsMADS13* expression domain. Subsequently, a selection of the most interesting genes based on the criteria shown in **Chapter III** was done. Enrichment for transcription factors (TFs) was found in the subset of selected genes and 68% of these TFs are up-regulated in the *osmads13* mutant. These data suggest that during early stages of rice ovule development OsMADS13 is a key regulator of downstream regulatory pathways and mostly represses these pathways. In particular, based on Gene-Ontology (GO) analysis, and looking for the role of putative *Arabidopsis* orthologs, we found that some of these TFs could be involved in floral meristem activity. One interesting gene that could plays such a role is *PCF5,* which belongs to the class II of TCP transcription factors, and is up-regulated in the ovule primordia (OP) and floral meristem (FM) in the *osmads13* mutant. Since now, *PCF5* has not been described in literature, whereas from phylogenetic studies conducted in 2002, Kosuki and Ohashi have shown that this gene belongs to the Class II of TCP transcription factors, which are supposed to encode for protein involved in repressing several growth-associated genes. Two homologous Class II TCP transcription factors, named CINCINNATA (CIN) of *Antirrhinum majus* and TCP4 of *Arabidopsis thaliana* are involved in repressing cell division, controlling in this way leaf

morphogenesis (Nath et al., 2003; Palatnik et al., 2003). Recent work have also demonstrated that the expression of *TCP4* in budding yeast, lead to a block in cell division at the transition from G1 to S stages (Aggarwal et al., 2011). Taken together these data, we hypothesized that *PCF5* could be involved in a similar role repressing cell division in proliferating cells of the rice floral meristem. Due to the little knowledge available about this gene, further analysis will be required to study in details the role of PCF5 during ovule development. MADS-domain transcription factors play important roles during flower development, therefore the up-regulation of *OsMADS8* in the ovule primordia (OP) of the *osmads13* mutant, as described in **Chapter III,** is of interest. It was previously shown that *OsMADS8* and *OsMADS7* belong to the *SEP3*-clade (Kang et al., 1997; Zahn et al., 2005). From the analysis of the plants in which both genes were silenced using an RNAi approach, some interesting phenotypic defects were observed: aberrant carpels, late flowering, transformation of lodicules, stamens and carpel into palea/lemma like organs and the floral meristem was indeterminate (Cui et al., 2010). Since in **Chapter II** it was shown that OsMADS3, OsMADS58 and OsMADS13 are redundantly involved in determining floral meristem determinacy, it might be that OsMADS7 and OsMADS8 could play roles in making functional multimeric complexes interacting with C and D class proteins. Yeast three-hybrid experiment have previously shown that SEP3 is necessary for the formation of functional ovule identity complexes in *Arabidopsis thaliana,* which is composed by the proteins SHP1, SHP2 and STK. A similar role for OSMADS7 and OsMADS8 in rice is further supported by the observation that OsMADS13 physically interacts with these MADS-domain factors (Favaro et al., 2002, 2003; Cui et al., 2010).

Given that we found four CArG-like consensus sequences in the *OsMADS8* promoter, it might be that OsMADS13 directly regulates its expression. In the future interesting experiment could be done to verify if OsMADS8 is also able to interact with the rice C-function proteins OsMADS3 and OsMADS58.

In **Chapter II** it was also shown that *OsMADS3* and *OsMADS58* are redundantly involved in rice carpel development. Since in *osmads13* ovules are converted into carpelloid structures, it could be hypothesized that OsMADS13 is a repressor of C-class genes. From RT-qPCR on Laser Micro-Dissected material, an up-regulation of these genes was found in ovule primordia (OP) and floral meristem (FM) of the *osmads13* mutant. Also the YABBY transcription factor *DROOPING LEAF (DL)*, which is supposed to be a carpel identity gene of rice (Nagasawa et al., 2003; Yamaguchi et al., 2004), was up-regulated in the *osmads13* mutant. In conclusion, the MADS-domain transcription factor OsMADS13 in a master regulator of several downstream genes leading to correct ovule development. Most of the deregulated genes are transcription factors (TFs), and most of them are up-regulated in the *osmads13* mutant. Further analysis will be necessary to elucidate the functions of these genes during different stages of ovule development, starting from analysis of the phenotype of some available mutants, or using an RNAi based approach for silencing. Taken the advantages of the histological analysis, we will also be able to detect alteration during ovule development of the mutant lines.

Thanks to our RNA-seq analysis now we have the transcriptome of the early stages of rice ovule development, and these data could be used as a starting point for future experiments that will focus on the elucidating the roles of downstream genes in this complicated but fascinating process.

Complementation of the *osmads13* mutant phenotype

From a Chromatin Immuno-Precipitation (ChIP) experiment done using an antibody against STK, it was shown that *VERDANDI (VDD)* is a direct target of SEEDSTICK (STK) (Matias-Hernandez et al., 2010). Our idea was to follow a similar strategy to find direct targets of the rice ovule identity factor OsMADS13. In previous studies, the polyclonal antibody against OsMADS13 was tested by dot blotting with native and denatured form of the OsMADS13 protein. From this analysis, it was concluded that the antibodies were not suitable for ChIP experiments. Our alternative approach was to express under its native promoter a OsMADS13-GFP fusion protein in order to use commercial αGFP antibodies for ChIP experiment. To verify if the fusion protein works in the same way as the endogen OsMADS13 protein, we transformed using this contruct *osmads13* mutant rice calli. From these positive plants, we expected to obtain seeds due to the complementation of the *osmads13* mutant phenotype. Moreover, transforming the *osmads13* mutant calli, it was possible to avoid the competition between the OsMADS13 fusion and native proteins for binding to the target DNA sequences. Analyzing the positive lines we found GFP expression in the ovary, although, the plants were completely sterile. From the analysis of the ovule morphology of the transformed plants carrying the construct *pOsMADS13::OsMADS13 (CDS),* we found that the embryo sac seemed to develop normally, however a tracheid-like structure developed at the basal portion of the ovule. From clearing analysis we concluded that the *pOsMADS13::OsMADS13 (CDS)* was not able to fully complement the *osmads13* mutant phenotype, but we didn't observe the formation of carpeloid structures instead of ovules, as normally happens in *osmads13* mutant (Figure 25). These data indicate that

OsMADS13 can also be involved in the development of the female gametophyte, and this was never observed previously due to the severe *osmads13* mutant phenotype. Interestingly, it was previously shown that also STK of *Arabidopsis thaliana*, with SHP1 and SHP2, play crucial roles in the development of the embryo sac. In particular, from the analysis of the *stk shp1 shp2* triple mutant, it was shown that the development of the female gametophyte is arrested after megasporogenesis (Brambilla et al., 2007; Colombo et al., 2008). Moreover, the only direct target of STK with a known function is *VERDANDI (VDD),* which belongs to the REM family of transcription factors, and is involved in the determination of cell identity in the female gametophyte (Matias-Hernandez et al., 2010). Unfortunately, from nucleotide alignment analysis, no putative orthologs of *VDD* gene were present in the rice genome, furthermore we didn't found members of the REM family in the dataset of the RNA-seq analysis. However, we cannot exclude that other OsMADS13 uncharacterized target genes could be involved in the female gametophyte development of rice. Another hypothesis attributes the lack of the introns in the construct *pOsMADS13::OsMADS13 (CDS)* as the cause of defects in the female gametophyte of the positive transformed lines.

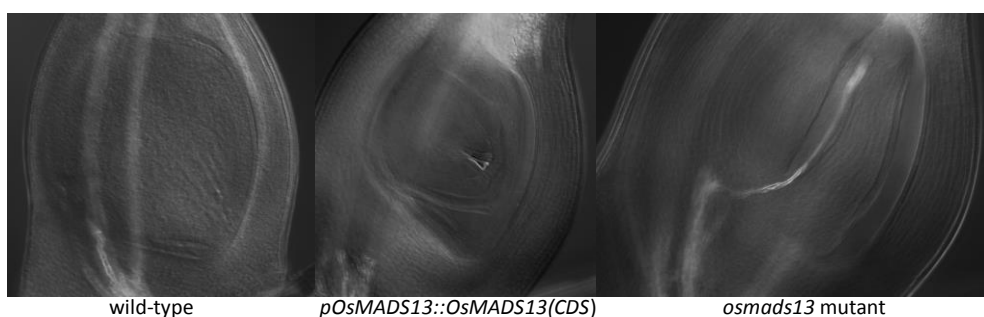wild-type   *pOsMADS13::OsMADS13(CDS)*   *osmads13* mutant

Figure 25 Clearing analysis of wild-type, *pOsMADS13::OsMADS13 (CDS)* positive line, and osmads13 mutant control ovules.

Searching in literature, we found that some regulatory sequences are frequently located in introns (Rose, 2008). In particular, the second intron in

the MADS-box gene *AG*, is bound by multiple factors (Sieburth and Meyerowitz, 1997; Deyholos and Sieburth, 2000; Hong et al., 2003; Bao et al., 2004; Gómez-Mena et al., 2005). Kooiker and colleagues in 2005, have shown that the leader-intron of *SEEDSTICK*, is important for the ovule-specific expression of *STK*. In particular, the β-glucoronidase (GUS) reporter gene was expressed in all floral organs of *Arabidopsis* transformed with a construct in which the first intron of *STK* was deleted (Kooiker et al., 2005). In the same work it was also shown that BASIC PENTACYSTEINE (BPC) factors, binds the target *GAGA* boxes across the *SEEDSTICK* promoter and the leader intron restricting *STK* expression specifically in the ovule (Simonini et al., 2012; Kooiker et al., 2005). From previous analysis, it was shown that *GAGA* boxes are also present in the *OsMADS13* leader intron, therefore we suppose that a regulatory mechanism similar to that of *Arabidopsis* could be present in rice.

Taken together these published data, we decided to repeat the transformation using the whole locus of *OsMADS13* fused to *GFP* under the *OsMADS13* promoter, and subsequently we introduced this construct into the *osmads13* mutant and heterozygous background.

Also in these positive lines we found GFP expression in the ovary (see **Chapter III**), whereas the expression pattern was quite different as compared to the positive plants transformed with *pOsMADS13::OsMADS13 (CDS)::GFP*. From the analysis of positive lines transformed with the whole *OsMADS13* locus, it was possible to observe a stronger GFP expression in the integuments, and a weaker GFP expression in the nucellus, as compared to the lines carrying the construct *pOsMADS13::OsMADS13 (CDS)::GFP*. Furthermore, we observed that the GFP expression pattern of positive lines for *pOsMADS13::OsMADS13 (genomic)::GFP*, is more similar to the

OsMADS13 expression evaluated by *in situ* hybridization in comparable developmental stages (Lopez-Dee et al., 1999; Dreni et al., 2007).

Taken together these data, we hypothesized that *OsMADS13* expression pattern could be important for the correct development of female gametophyte, and the introns might contain some regulatory regions that play crucial role in determining the spatial GFP expression in the ovary.

To increase optical resolution, we are going to do a Confocal Microscopy analysis to study in detail the GFP expression in the lines positive for the construct *pOsMADS13::OsMADS13 (genomic)::GFP*. At the same time, clearing analysis will be performed to verify if ovule development is correct in plants carrying the construct *pOsMADS13::OsMADS13 (genomic)* introduced into the *osmads13* mutant background. To prove the full complementation of the *osmads13* mutant phenotype, we are going to select positive lines that will be able to make seeds. In particular we are interested in fertile *osmads13* plants carrying the *pOsMADS13::OsMADS13 (genomic)::GFP* construct, that we will use to perform ChIP experiments to verify if putative downstream target genes are directly regulated by OsMADS13. In case no complete restore of ovule development is observed then these lines will still be useful since ovule identity determination and meristem determinacy are restored. This therefore allows us to use these lines to study the regulatory pathways regulating these processes and that are under direct control of OsMADS13.

In conclusion, the work presented in my thesis elucidates the genetic interactions between rice *AG* subfamily members and shows that *OsMADS13* is important in the control of ovule identity determination, floral meristem determinacy and probably also embryo sac development. The identification

and functional analysis of regulatory pathways downstream of OsMADS13 has been initiated during my thesis project and this work promises to generate interesting insights in this complicated process in the near future.

MATERIAL & METHODS

Plant materials

Oryza sativa L. ssp. *Japonica* of cultivars Nipponbare and Dongjin, and mutant lines 2D-003-37 (Tos17 insertion in *OsMADS13*, cv. Dongjin), were grown in a greenhouse for about two months under Long Day (LD) conditions (16 h light at 28°C/8 h dark at 22°C), and moved for fifteen days to Short Day (SD) conditions (12 h light at 28°C/12 h dark at 22°C) to induce flowering.

Genotyping of mutant plant

For genotyping of the knock-out line 2D-003-37 we used the following primers: Osp66 (forward) with Osp67 (reverse) to detect the *OsMADS13* wild-type allele, and Osp66 with Osp14 (Tos17R) for the *osmads13* mutant allele (Table 5).

Primer used for genotyping

CODE	SEQUENCE (5' → 3')	DETAILS
Osp 14	CCAGTCCATTGGATCTTGTATCTTGTATATAC	Reverse Tos17
Osp 66	CTTTGATCTTGTGAGGAGACAG	Forward 5' UTR
Osp 67	GCCTAGATGCAAGTATAGAGTG	Reverse leader intron

Table 5 Primer used for genotyping.

Plasmid Construction and Plant Transformation

A 3435-bp fragment of the Os*MADS13* upstream region (*pOsMADS13*), comprising the 5' untranslated region and leader intron sequences, but not the start codon, was amplified with primers Osp131F-SacI and Osp132R-SpeI, using Phusion High-Fidelity DNA polymerase (Finnzymes). The PCR product was digested with SacI (partial digestion) and SpeI restriction enzymes. The binary vector pK2GW7 (Karimi et al., 2002), carrying the *NptII* selectable marker gene for in planta selection, was digested with the same enzymes to excise the *35S* promoter. The *pOsMADS13* fragment was then ligated into the digested plasmid to generate the NOB1186 vector. The Os*MADS13* genomic sequence was amplified from Nipponbare leaf genomic DNA using the primers Osp46-attB1 and Osp47-attB2 and recombined into NOB1186 using Gateway Technology (Invitrogen). To obtain the construct *pOsMADS13:OsMADS13:GFP*, the Os*MADS13* genomic sequence was amplified in two fragments, the first part using the primers Osp46F-AttB1 and Osp397R and the second using the primers Osp396F and Osp312R+SpeI (able to remove the stop codon). The *GFP* fragment was amplified using the *pGREEN II* plasmid carrying the *GFP* gene as template, using primers Atp2718F-SpeI-AatII and Vep124R-attB2. The fragments amplified using the primers Osp396F and Osp312R+SpeI and the *GFP* fragment were digested with SpeI restriction enzyme and ligated. A second ligation was done to complete the construct linking the fragment amplified using the primers Osp46F-AttB1 and Osp397R. The ligation product was recombined into NOB1186 using Gateway Technology (Invitrogen). Finally, *pOsMADS13:OsMADS13* and *pOsMADS13:OsMADS13:GFP* vectors were introduced in the *Agrobacterium tumefaciens* strain EHA105. Rice

transformation was performed using geneticin (G418) selection and the protocol of Hiei et al. (1994).

Primer used for cloning

CODE	SEQUENCE (5' → 3')	DETAILS
Osp 46	GGGGACAAGTTTGTACAAAAAAGCAGGCTACATGGGGAGGGGCAGGATTGAGATC	OsMADS13 Gateway (attB1) forward
Osp 47	GGGGACCACTTTGTACAAGAAAGCTGGGTGAAGTTCATGAGGTTCAGAAGTG	OsMADS13 Gateway (attB2) reverse
Osp 131	TAGAGCTCATCCATCTCCATCCGATCGAC	OsMADS13 promoter forward + SacI
Osp 132	CCACTAGTCTTCTTGCTCTCAACTGATC	OsMADS13 5'UTR reverse + SpeI
Osp 312	CCACTAGTGAAGTGAGGAGGCGGCGCGT	OsMADS13 CDS reverse + SpeI
Osp 396	CAATGGCTACCTTTCTTCTGC	OsMADS13 forward for locus cloning
Osp 397	GAAGTTGAGCCACAAGCATG	OsMADS13 reverse for locus cloning

Atp 2718	CACCACTAGTGACGTCATGGTGAGCAAGGG CGAGGAG	GFP forward + SpeI-AatII
Vep 124	GGGGACCACTTTGTACAAGAAAGCTGGGTT TACTTGTACAGCTCGTCCA	GFP Gateway (attB2) reverse

Table 6 Primer used for cloning.

Total RNA Extraction and RNA-sequencing

Three biological replicates from wild-type and *osmads13* mutant whole inflorescences at early stages of development (length between 0,5cm and 0,8cm) were collected. Total RNA was extracted using the PureLink RNA Mini Kit - Ambion, and subsequently treated with TURBO DNase Ambion according to manufacturer's instructions. The sequencing libraries were prepared at IGA Technology Services (Udine, Italy) using 4ug of Total RNA from each sample, and PolyA-selected RNA was then sequenced on a HiSeq 2000 Illumina sequencing machine in 6plex. Resulting in 20 to 40 million 50 bp long single end reads for each sample.

RNA-sequencing analysis:

DE genes

Transcript abundance quantification and differential expression (DE) analyses were performed using the Bowtie/Tophat/Cufflinks pipeline (BTC) (Trapnell et al., 2012) with the MSU Osa1 Release 7 Annotation and standard

parameters. Given the nature of the data (50 bp single end reads) we quantified transcripts considering the reference annotation, disabling the option for reference genome-based assembly of the transcriptome.

GO Analysis

A custom script was used to determine the GO enrichment according to the GOslim categorization. A hypergeometric distribution statistical testing method was applied to determinate the enriched genes and the Benjamini and Hochberg false discovery rate (FDR) correction was performed in order to limit the number of false positives. The FDR was set to 0.05.

CArG-box search

The Match™ public version 1.0 tool available at the "Gene Regulation" website (http://www.gene-regulation.com/pub/programs.html), was used to find DNA sequences for predicted transcription factor binding sites. Sequences of interesting genes from 3 kb upstream of the Transcription Start Site (TSS) to 3' UTRs were analysed for AGAMOUS-like binding sites. The default parameters were modified based on the CArG-boxes sequences bound by *SEEDSTICK* (Hernandez et al., 2010; Mendes et al., 2013) of the *VERDANDI* promoter.

Tissue embedding for Laser Microdissection

Whole inflorescences with length between 0,5cm and 0,8cm were harvested from wild-type and *osmads13* mutant in ice cold Acetone, infiltrated with vacuum for 15', and stored overnight in fresh fixative at 4°C. The next day ethanol was slowly substituted by xylene (xylene : ethanol (v:v) 1:3, 1:1, 1:3,

pure xylene, pure xylene) at room temperature (RT), changing solution every 60 minutes. Flakes of Paraplast Xtra® (Sigma – Aldrich) were then added and left to dissolve in xylene o/n at RT. The next day inflorescence were moved to 54°C for 10 minutes and then xylene was substituted with fused Paraplast Xtra® with 2 hours changes for 24 hours at 54°C. The next day samples in Paraplast Xtra® were lodged in Petri dishes and stored at 4°C

Laser Microdissection

Tissue was sectioned into slices with a Leica RM 2155 microtome, distended on pure methanol (Schmid et al., 2012) on Leica PET membrane frameslides, dried at 37°C for 30 minutes, washed twice (5 minutes each) in pure xylene to remove Paraplast Xtra®, dried at room temperature for 10 minutes and immediately dissected with a Leica LMD6000 laser microdissector.

RNA extraction, retrotranscription and amplification

To get a sufficient amount of RNA for down-stream application, 80-100 sections were performed for each sample by using the LMD 6000 system (Leica). After microdissection 10 µl of XB buffer from the Arcturus Picopure RNA extraction Kit (Life Technologies) was added. Samples were then heated at 42°C for 30 minutes, centrifuged at 800 r.c.f for 2 minutes and stored at -80. Before proceeding with the extraction, material from 3 different tubes were pooled in one; and RNA extraction was then performed using the Arcturus Picopure RNA extraction kit as specified by manufacturer's instructions. RNA integrity and concentration was tested using the RNA 6000 Pico assay kit on

an Agilent 2100 Bioanalyzer (in collaboration with "Fondazione Filarete"). Retro-transcription and amplification were performed using the Ovation PicoSL WTA System V2 (NuGen technologies) as specified by the manufacturer's instructions. Three biological replicates were obtained for each sample: wild-type Floral Meristem (FM), wild-type Ovule Primordia (OP), *osmads13* mutant FM and *osmads13* mutant OP.

Expression analysis by standard and large-scale RT-qPCR

Specific primers for selected genes were designed and tested by quantitative Reverse Transcription PCR (RT–qPCR). RNA was extracted using Pure Link RNA Mini Kit –Ambion, from wild type and *osmads13* mutant pools of early developing inflorescence with a length between 0,5cm and 0,8cm. Subsequently the RNA was treated with TURBO DNase – Ambion, according to manufacturer's instructions. The retro-transcription was done starting from ~0.5 μg of RNA with the iScript cDNA synthesis kit (Bio-Rad). Negative controls were performed without the addition of reverse transcriptase into the mix. Tenfold dilutions of cDNA were tested in RT-PCR and qRT-PCR experiments and no amplification was observed in negative controls. For qRT-PCR analyses, cDNA templates (10-fold dilutions) and primers (Table 7) were added to iQ SYBR Green Supermix (Bio-Rad). qRT-PCR reactions were performed with three technical replicates/samples using a Bio-Rad CFX96 real-time PCR detection system, and primers set efficiency was calculated using the CFX Manager 2.1 software based on the delta-delta Ct method.

Primer used for RT-qPCR

LOCUS ID	PRIMER CODE	PRIMER SEQUENCE (5' → 3')
Os02g06640	Osp 355	GAGCCTCTGTTCGTCAAGTA
	Osp 356	ACTCGATGGTCCATTAAACC
Os03g08010	RT 1212	TGGTATGGTGGTGACCTTTG
	RT 1213	GTACCCACGCTTCAGATCCT
Os06g11170	RT 418	GGAATGTGGACGGTGACACT
	RT 419	TCAAAATAGAGTCCAGTAGATTTGTCA
Os01g10504	RT 973	GGGATTCTATCAACACCATGAG
	RT 974	CTCAACTTCAGCATATAACAGC
Os09g32948	Osp 655	CACCTTGCAGATCGGGTTTA
	Osp 656	ATCTGTGTCGTCACATCCGT
Os12g10540	Osp 671	GCTTGTGGCTCAACTTCTGG
	Osp 672	CAACCAGGTGCTTGTTGGTG
Os05g11414	RT 975	CTGCTAAGCTGAAGCAACAG
	RT 976	CTTCCAGCTGCTTAAGTTCTC

Os03g11600	Osp 426	AGTCCCATGCAAGAGGCTGATG
	Osp 427	TTCCTCCTGCAGTCAGTGCAAG
Os03g15270	Osp 459	ACTTCCTCGTGTGCATCTCC
	Osp 460	GCTCTGACGAAAGCCTTGAT
Os02g42990	Osp 623	ACGTGTCATGTGTGCTCGAT
	Osp 624	GCATCCACACAGAAAGGGGA
Os04g51890	Osp 667	GGATGGTGAGGGATGATTTG
	Osp 668	ATCCATGAGGGCTGCATTAC
Os07g29310	Osp 621	GTTCGGATTGGTGATCCATGC
	Osp 622	GCACGCGTTAAACTCACCAG
Os03g58600	Osp 458	CCTGAAATCACCAAATACCG
	Osp 522	TTCTGATGGCATCCATTTC
Os09g36700	Osp 489	AGCTCTGCTCTGCCTTCTTG
	Osp 490	ACAGGGAAGAGTTGGTGGTG
Os05g13630	Osp 663	GACGAGCAAACCCTCATGGA

	Osp 664	TCCCCATCTCACTGTCCCAA
Os07g01530	Osp 557	AGCTATATATGGCGAGCGAGC
	Osp 658	GTCGTCCTTCTTGTCCCCTG
Os04g58810	Osp 659	GTGGCAGCGAGATGATACGA
	RT 1371	CCACCACACGAGAAAATCAACC
Os03g57240	Osp 617	ATGTTTGCCTCTGCTGCTGTA
	Osp 618	TGGCGATTCGAGCATGAGAT
Os04g35500	Osp 531	CACACTGTCACTGGCACACA
	Osp 532	ATTAACGCGGAACTGAGCGA
Os08g34010	Osp 533	GCGGTGAGCTGAGTTGATCT
	Osp 534	TTCAAGCACTCCCTGTACACC
Os02g13310	Osp 481	TTTGATGGCGATGTTAGCTG
	Osp 482	ATCGATCTCCCAATGAGGTG
Os06g45140	Osp 535	ACTCGAATCACAGGTCGAGCAACT
	Osp 536	TGTCCGTGACCGCTGTATTGAACT

Os09g36910	Osp 537	CAAGTACGAGCAGCTGAGGA
	Osp 538	GAAGAATCAGAATGGCGCGG
Os01g49830	Osp 625	TTCGCCCATGTTCTTGCTTG
	Osp 626	GGGAAATATCTCCATTGGTTGTGG
Os03g09170	Osp 545	GGACGCGATCCTTTCCTGAT
	Osp 546	AAGCAGACCAGGGGTTGATG
Os09g35010	Osp 547	CTCGCACTGAAAAGTGTGGAC
	Osp 548	AGCTCTTTGGAGAGGAGAAGT
Os04g52090	Osp 553	CAAACCAACCACCCACCATC
	Osp 554	TTCTTGTTCGGGTCGAGCAC
Os05g41760	Osp 551	AATGTGGGAGTGGAGAGCAC
	Osp 552	GGGGCAATGTGAGAACGCTA
Os11g07460	Osp 479	ATCAGCACCACCCGTTCTAC
	Osp 480	CCACCACCGCTTTCTTCTC
Os01g11550	Osp 477	CATCACGGAATTGTCGATTG

	Osp 478	CAACAGACACAGAGGCTTGC
Os01g50940	Osp 520	TCGTGCGTACGGTATAGCTG
	Osp 521	CCCCTATACGATGCTTCAGG
Os08g37290	Osp 539	TTTTAAACCCCACGACAACC
	Osp 540	GACCCACCCTTCCTCACC
Os07g39220	Osp 501	CCAGCTGATCATGGTTGTTG
	Osp 502	GATGCACATGACAGGTACGG
Os04g43680	Osp 631	TCCAACCTTACTCTGCAGCC
	Osp 632	GACCCTTCTTGAGCCCCATC
Os01g32770	Osp 603	ACTTGTGTGATGTCTGCCTGT
	Osp 604	GGTGCGAAACAAGGAAATAACC
Os07g12340	Osp 607	TTCATCAGAGCTGTGTCAAGTG
	Osp 608	AGCAATGCCAATTCAAGGATGGA
Os04g51000	Osp 524	AGAAGGCGAGGAGGAAGAAG
	Osp 525	GCACTGCTCGTACAGATGGA

Os08g39890	Osp 609	GGATATGGTGCCAACACATACAG
	Osp 610	GACATGGCTGCAGCCTGGTTGTG
Os03g53340	RT 1196	TTCTGGATGCAGCTGCTCAG
	RT 1197	TCATCCTCCTCGTCGTTGTC
Os09g35790	RT 1214	AGCAACTTCGTGATGCACTG
	RT 1215	CACCATTTGCTGTTGTCTGC
Os03g04240	RT 1374	CACGGCTTTAATTCGTCGGG
	RT 1375	GAGAGGAGACCTCAAGAAGGC
Os03g04220	RT 1376	AGTTCCAAGGGGTTGTCACG
	Osp 640	ACTCATGGAAAGGACACAATCAA
Os11g02240	Osp 643	CTGTCCTCGCATCCACTCC
	Osp 644	CCTTGAGGCTTTCACTGCTTG
Os03g12730	Osp 463	CACCTTCCTCGACCTCTCTG
	Osp 464	GCTGTTAGGCTCTGCATTCC
Os01g08860	Osp 611	ATTCCGCATCTCTCTGCATC

	Osp 612	CAGACCACCATGTCCTCACA
Os02g02410	Osp 613	CACCAAGAAGTCCCAGGTGT
	Osp 614	GTCGAACTTGCCGAGAAGAC

Table 7 Primer used for RT-qPCR.

Microfluidic Dynamic Array

A Microfluidic Dynamic Array (Spungeon et al., 2008) experiment to evaluate the differential expression levels of selected genes between wild-type and *osmads13* mutant was done in collaboration with the Genomics Platform of CRAG (Barcelona, SPAIN), according to manufacturer's instructions. RNAs extracted from whole inflorescences used for the RNA-seq experiment, and from Laser Microdissection samples: wild-type Ovule Primordia (OP) and *osmads13* mutant OP, were analysed on a single chip. *OsEF1* reference gene was used for data normalization and relative gene expression values were determined using the delta-delta Ct method.

ACKNOWLEDGEMENTS

REFERENCES

Aggarwal, P., Padmanabhan, B., Bhat, A., Sarvepalli, K., Sadhale, P.P., and Nath, U. (2011). The TCP4 transcription factor of Arabidopsis blocks cell division in yeast at G1→S transition. Biochem. Biophys. Res. Commun. **410**: 276–81.

Angenent, G.C., Franken, J., Busscher, M., Dijken, A. Van, Dons, H.J.M., and Tunena, A.J. Van (1995). A Novel Class of MADS Box Genes 1 s lnvolved in Ovule Development in Petunia. **7**: 1569–1582.

Arora, R., Agarwal, P., Ray, S., Singh, A.K., Singh, V.P., Tyagi, A.K., and Kapoor, S. (2007). MADS-box gene family in rice: genome-wide identification, organization and expression profiling during reproductive development and stress. BMC Genomics **8**: 242.

Bao, X., Franks, R.G., Levin, J.Z., and Liu, Z. (2004). Repression of AGAMOUS by BELLRINGER in floral and inflorescence meristems. Plant Cell **16**: 1478–89.

Becker, A. and Theissen, G. (2003). The major clades of MADS-box genes and their role in the development and evolution of flowering plants. Mol. Phylogenet. Evol. **29**: 464–89.

Bowman, J.L., Drews, G.N., and Meyerowitz, E.M. (1991). Expression of the Arabidopsis floral homeotic gene AGAMOUS is restricted to specific cell types late in flower development. Plant Cell **3**: 749–58.

Bowman, J.L., Smyth, D.R., and Meyerowitz, E.M. (2012). The ABC model of flower development: then and now. Development **139**: 4095–8.

Brambilla, V., Battaglia, R., Colombo, M., Masiero, S., Bencivenga, S., Kater, M.M., and Colombo, L. (2007). Genetic and molecular interactions between BELL1 and MADS box factors support ovule development in Arabidopsis. Plant Cell **19**: 2544–56.

Busch, M.A., Bomblies, K., and Weigel, D. (1999). Activation of a floral homeotic gene in Arabidopsis. Science **285**: 585–7.

Clark, J.I., Coen, E.S., and Centre, J.I. (2002). The cycloidea gene can respond to a common dorsoventral prepattern in Antirrhinum. **30**.

Colombo, L., Battaglia, R., and Kater, M.M. (2008). Arabidopsis ovule development and its evolutionary conservation. Trends Plant Sci. **13**: 444–50.

Colombo, L., Franken, J., Koetje, E., van Went, J., Dons, H.J., Angenent, G.C., and van Tunen, A.J. (1995). The petunia MADS box gene FBP11 determines ovule identity. Plant Cell **7**: 1859–68.

Cubas, P., Lauter, N., Doebley, J., and Coen, E. (1999). The TCP domain: a motif found in proteins regulating plant growth and development. Plant J. **18**: 215–22.

Cui, R., Han, J., Zhao, S., Su, K., Wu, F., Du, X., Xu, Q., Chong, K., Theissen, G., and Meng, Z. (2010). Functional conservation and diversification of class E floral homeotic genes in rice (Oryza sativa). Plant J. **61**: 767–81.

Deyholos, M.K. and Sieburth, L.E. (2000). Separable whorl-specific expression and negative regulation by enhancer elements within the AGAMOUS second intron. Plant Cell **12**: 1799–810.

Ditta, G., Pinyopich, A., Robles, P., Pelaz, S., and Yanofsky, M.F. (2004). The SEP4 gene of Arabidopsis thaliana functions in floral organ and meristem identity. Curr. Biol. **14**: 1935–40.

Doebley, J., Stec, A., and Gustus, C. (1995). teosinte branched1 and the origin of maize: evidence for epistasis and the evolution of dominance. Genetics **141**: 333–46.

Doebley, J., Stec, A., and Hubbard, L. (1997). The evolution of apical dominance in maize. Nature **386**: 485–8.

Dreni, L., Jacchia, S., Fornara, F., Fornari, M., Ouwerkerk, P.B.F., An, G., Colombo, L., Kater, M.M., and Celoria, V. (2007). The D-lineage MADS-box gene OsMADS13 controls ovule identity in rice. 690–699.

Dreni, L., Pilatone, A., Yun, D., Erreni, S., Pajoro, A., Caporali, E., Zhang, D., and Kater, M.M. (2011). Functional analysis of all AGAMOUS subfamily members in rice reveals their roles in reproductive organ identity determination and meristem determinacy. Plant Cell **23**: 2850–63.

Drews, G.N., Weigel, D., and Meyerowitz, E.M. (1991). Floral patterning. Curr. Opin. Genet. Dev. **1**: 174–8.

Du, Z., Li, H., Wei, Q., Zhao, X., Wang, C., Zhu, Q., Yi, X., Xu, W., Liu, X.S., Jin, W., and Su, Z. (2013). Genome-wide analysis of histone modifications: H3K4me2, H3K4me3, H3K9ac, and H3K27ac in Oryza sativa L. Japonica. Mol. Plant **6**: 1463–72.

Egea-cortines, M., Saedler, H., and Sommer, H. (1999). Ternary complex formation between the MADS-box proteins SQUAMOSA , DEFICIENS and GLOBOSA is involved in the control of floral architecture in Antirrhinum majus. **18**: 5370–5379.

Favaro, R., Immink, R.G.H., Ferioli, V., Bernasconi, B., Byzova, M., Angenent, G.C., Kater, M., and Colombo, L. (2002). Ovule-specific MADS-box proteins have conserved protein-protein interactions in monocot and dicot plants. Mol. Genet. Genomics **268**: 152–9.

Favaro, R., Pinyopich, A., Battaglia, R., Kooiker, M., Borghi, L., Ditta, G., Yanofsky, M.F., Kater, M.M., and Colombo, L. (2003). MADS-box protein complexes control carpel and ovule development in Arabidopsis. Plant Cell **15**: 2603–11.

De Folter, S., Immink, R.G.H., Kieffer, M., Parenicová, L., Henz, S.R., Weigel, D., Busscher, M., Kooiker, M., Colombo, L., Kater, M.M., Davies, B., and Angenent, G.C. (2005). Comprehensive interaction map of the Arabidopsis MADS Box transcription factors. Plant Cell **17**: 1424–33.

Gómez-Mena, C., de Folter, S., Costa, M.M.R., Angenent, G.C., and Sablowski, R. (2005). Transcriptional program controlled by the floral homeotic gene AGAMOUS during early organogenesis. Development **132**: 429–38.

Gregis, V. et al. (2013). Identification of pathways directly regulated by SHORT VEGETATIVE PHASE during vegetative and reproductive development in Arabidopsis. Genome Biol. **14**: R56.

Heijmans, K., Ament, K., Rijpkema, A.S., Zethof, J., Wolters-arts, M., Gerats, T., and Vandenbussche, M. (2012). Rede fi ning C and D in the Petunia ABC. **24**: 2305–2317.

Hervé, C., Dabos, P., Bardet, C., Jauneau, A., Auriac, M.C., Ramboer, A., Lacout, F., and Tremousaygue, D. (2009). In vivo interference with AtTCP20 function induces severe plant growth alterations and deregulates the expression of many genes important for development. Plant Physiol. **149**: 1462–77.

Hong, R.L., Hamaguchi, L., Busch, M.A., and Weigel, D. (2003). Regulatory elements of the floral homeotic gene AGAMOUS identified by phylogenetic footprinting and shadowing. Plant Cell **15**: 1296–309.

Honma, T. and Goto, K. (2001). Complexes of MADS-box proteins are sufficient to convert leaves into floral organs. Nature **409**: 525–9.

Hubbard, L., Mcsteen, P., Doebley, J., and Hake, S. (2002). Expression Patterns and Mutant Phenotype of teosinte branched1 Correlate With Growth Suppression in Maize and Teosinte. **1**: 1927–1935.

Itoh, J.-I., Nonomura, K.-I., Ikeda, K., Yamaki, S., Inukai, Y., Yamagishi, H., Kitano, H., and Nagato, Y. (2005). Rice plant development: from zygote to spikelet. Plant Cell Physiol. **46**: 23–47.

Kang, H.G., Jang, S., Chung, J.E., Cho, Y.G., and An, G. (1997). Characterization of two rice MADS box genes that control flowering time. Mol. Cells **7**: 559–66.

Karimi, M., Inzé, D., and Depicker, A. (2002). GATEWAY vectors for Agrobacterium-mediated plant transformation. Trends Plant Sci. **7**: 193–5.

Kater, M.M., Dreni, L., and Colombo, L. (2006). Functional conservation of MADS-box factors controlling floral organ identity in rice and Arabidopsis. J. Exp. Bot. **57**: 3433–44.

Kooiker, M., Airoldi, C.A., Losa, A., Manzotti, P.S., Finzi, L., Kater, M.M., and Colombo, L. (2005). BASIC PENTACYSTEINE1, a GA binding protein that induces conformational changes in the regulatory region of the homeotic Arabidopsis gene SEEDSTICK. Plant Cell **17**: 722–9.

Kosugi, S. and Ohashi, Y. (2002). DNA binding and dimerization speci ® city and potential targets for the TCP protein family. **30**.

Kosugi, S. and Ohashil, Y. (1997). PCFI and PCF2 Specifically Bind to cis Elements in the Rice Proliferating Cell Nuclear Antigen Gene. **9**.

Kosugi, S., Suzuka, I., and Ohashi, Y. (1995). Two of three promoter elements identified in a rice gene for proliferating cell nuclear antigen are essential for meristematic tissue-specific expression. Plant J. **7**: 877–86.

Kubo, T., Fujita, M., Takahashi, H., Nakazono, M., Tsutsumi, N., and Kurata, N. (2013). Transcriptome analysis of developing ovules in rice isolated by laser microdissection. Plant Cell Physiol. **54**: 750–65.

Li, C., Potuschak, T., Colón-Carmona, A., Gutiérrez, R.A., and Doerner, P. (2005). Arabidopsis TCP20 links regulation of growth and cell division control pathways. Proc. Natl. Acad. Sci. U. S. A. **102**: 12978–83.

Lim, J., Moon, Y.H., An, G., and Jang, S.K. (2000). Two rice MADS domain proteins interact with OsMADS1. Plant Mol. Biol. **44**: 513–27.

Lohmann, J.U., Hong, R.L., Hobe, M., Busch, M.A., Parcy, F., Simon, R., and Weigel, D. (2001). A molecular link between stem cell regulation and floral patterning in Arabidopsis. Cell **105**: 793–803.

Lopez-Dee, Z.P., Wittich, P., Enrico Pè, M., Rigola, D., Del Buono, I., Gorla, M.S., Kater, M.M., and Colombo, L. (1999). OsMADS13, a novel rice MADS-box gene expressed during ovule development. Dev. Genet. **25**: 237–44.

Luo, D., Carpenter, R., Vincent, C., Copsey, L., and Coen, E. (1996). Origin of floral asymmetry in Antirrhinum. Nature **383**: 794–9.

Malcomber, S.T. and Kellogg, E.A. (2004). Heterogeneous expression patterns and separate roles of the SEPALLATA gene LEAFY HULL STERILE1 in grasses. Plant Cell **16**: 1692–706.

Malcomber, S.T. and Kellogg, E.A. (2005). SEPALLATA gene diversification: brave new whorls. Trends Plant Sci. **10**: 427–35.

Matias-Hernandez, L., Battaglia, R., Galbiati, F., Rubes, M., Eichenberger, C., Grossniklaus, U., Kater, M.M., and Colombo, L. (2010). VERDANDI is a direct target of the MADS domain ovule identity complex and affects embryo sac differentiation in Arabidopsis. Plant Cell **22**: 1702–15.

Melzer, R., Verelst, W., and Theissen, G. (2009). The class E floral homeotic protein SEPALLATA3 is sufficient to loop DNA in "floral quartet"-like complexes in vitro. Nucleic Acids Res. **37**: 144–57.

Mendes, M.A., Guerra, R.F., Berns, M.C., Manzo, C., Masiero, S., Finzi, L., Kater, M.M., and Colombo, L. (2013). MADS domain transcription factors mediate short-range DNA looping that is essential for target gene expression in Arabidopsis. Plant Cell **25**: 2560–72.

Moon, Y., Jung, J., Kang, H., and An, G. (1999a). Identification of a rice APETALA3 homologue by yeast two-hybrid screening. **1**: 167–177.

Moon, Y., Kang, H., Jung, J., Jeon, J., Sung, S., and An, G. (1999b). Determination of the Motif Responsible for Interaction between the Rice APETALA1 / AGAMOUS-LIKE9 Family Proteins Using a Yeast Two-Hybrid System 1. **120**: 1193–1203.

Münster, T., Faigl, W., Saedler, H., and Theißen, G. (2002). Evolutionary Aspects of MADS-box Genes in the Eusporangiate Fern Ophioglossum. Plant Biol. **4**: 474–483.

Nagasawa, N., Miyoshi, M., Sano, Y., Satoh, H., Hirano, H., Sakai, H., and Nagato, Y. (2003). SUPERWOMAN1 and DROOPING LEAF genes control floral organ identity in rice. Development **130**: 705–18.

Nallamilli, B.R.R., Zhang, J., Mujahid, H., Malone, B.M., Bridges, S.M., and Peng, Z. (2013). Polycomb group gene OsFIE2 regulates rice (Oryza sativa) seed development and grain filling via a mechanism distinct from Arabidopsis. PLoS Genet. **9**: e1003322.

Nam, J., Kim, J., Lee, S., An, G., Ma, H., and Nei, M. (2004). Type I MADS-box genes have experienced faster birth-and-death evolution than type II MADS-box genes in angiosperms. Proc. Natl. Acad. Sci. U. S. A. **101**: 1910–5.

Nath, U., Crawford, B.C.W., Carpenter, R., and Coen, E. (2003). Genetic control of surface curvature. Science **299**: 1404–7.

Navaud, O., Dabos, P., Carnus, E., Tremousaygue, D., and Hervé, C. (2007). TCP transcription factors predate the emergence of land plants. J. Mol. Evol. **65**: 23–33.

Nurrish, S.J. and Treisman, R. (1995). DNA binding specificity determinants in MADS-box transcription factors. Mol. Cell. Biol. **15**: 4076–85.

Ohnishi, T., Takanashi, H., Mogi, M., Takahashi, H., Kikuchi, S., Yano, K., Okamoto, T., Fujita, M., Kurata, N., and Tsutsumi, N. (2011). Distinct gene expression profiles in egg and synergid cells of rice as revealed by cell type-specific microarrays. Plant Physiol. **155**: 881–91.

Osaka, M. et al. (2013). Cell type-specific transcriptome of Brassicaceae stigmatic papilla cells from a combination of laser microdissection and RNA sequencing. Plant Cell Physiol. **54**: 1894–906.

Palatnik, J.F., Allen, E., Wu, X., Schommer, C., Schwab, R., Carrington, J.C., and Weigel, D. (2003). Control of leaf morphogenesis by microRNAs. Nature **425**: 257–63.

Pelaz, S., Ditta, G.S., and Yanofsky, M.F. (2000). B and C ⁻ oral organ identity functions require SEPALLATA MADS-box genes. **405**: 9–12.

Pellegrini, L., Tan, S., and Richmond, T.J. (1995). Structure of serum response factor core bound to DNA. Nature **376**: 490–8.

Pinyopich, A., Ditta, G.S., Savidge, B., Liljegren, S.J., Baumann, E., Wisman, E., and Yanofsky, M.F. (2003). Assessing the redundancy of MADS-box genes during carpel and ovule development. 85–88.

Prasad, K., Parameswaran, S., and Vijayraghavan, U. (2005). OsMADS1, a rice MADS-box factor, controls differentiation of specific cell types in the lemma and palea and is an early-acting regulator of inner floral organs. Plant J. **43**: 915–28.

Riechmann, J.L., Wang, M., and Meyerowitz, E.M. (1996). DNA-binding properties of Arabidopsis MADS domain homeotic proteins APETALA1, APETALA3, PISTILLATA and AGAMOUS. Nucleic Acids Res. **24**: 3134–41.

Robertson, G. et al. (2007). Genome-wide profiles of STAT1 DNA association using chromatin immunoprecipitation and massively parallel sequencing. Nat. Methods **4**: 651–7.

Romanel, E.A.C., Schrago, C.G., Couñago, R.M., Russo, C.A.M., and Alves-Ferreira, M. (2009). Evolution of the B3 DNA binding superfamily: new insights into REM family gene diversification. PLoS One **4**: e5791.

Rose, A.B. (2008). Intron-mediated regulation of gene expression. Curr. Top. Microbiol. Immunol. **326**: 277–90.

Sánchez-León, N. et al. (2012). Transcriptional analysis of the Arabidopsis ovule by massively parallel signature sequencing. J. Exp. Bot. **63**: 3829–42.

Sasazawa, T., Yoshie, N., Watanabe, F., and Hata, Y. (2006). [Effect of beta-TCP containing the bone-growth promoting compound, TAK-778, on the vertex bone of rat--change of volmetric bone formation corresponding to the containing volume of TAK-778]. Nihon Hotetsu Shika Gakkai Zasshi **50**: 422–31.

Schmid, M.W., Schmidt, A., Klostermeier, U.C., Barann, M., Rosenstiel, P., and Grossniklaus, U. (2012). A powerful method for transcriptional profiling of specific cell types in eukaryotes: laser-assisted microdissection and RNA sequencing. PLoS One **7**: e29685.

Schmidt, A., Wuest, S.E., Vijverberg, K., Baroux, C., Kleen, D., and Grossniklaus, U. (2011). Transcriptome analysis of the Arabidopsis megaspore mother cell uncovers the importance of RNA helicases for plant germline development. PLoS Biol. **9**: e1001155.

Schroeder, A., Mueller, O., Stocker, S., Salowsky, R., Leiber, M., Gassmann, M., Lightfoot, S., Menzel, W., Granzow, M., and Ragg, T. (2006). The RIN: an RNA integrity number for assigning integrity values to RNA measurements. BMC Mol. Biol. **7**: 3.

Shore, P. and Sharrocks, A.D. (1995). The MADS-box family of transcription factors. **13**: 1–13.

Sieburth, L.E. and Meyerowitz, E.M. (1997). Molecular dissection of the AGAMOUS control region shows that cis elements for spatial regulation are located intragenically. Plant Cell **9**: 355–65.

Simonini, S., Roig-Villanova, I., Gregis, V., Colombo, B., Colombo, L., and Kater, M.M. (2012). Basic pentacysteine proteins mediate MADS domain complex binding to the DNA for tissue-specific expression of target genes in Arabidopsis. Plant Cell **24**: 4163–72.

Spurgeon, S.L., Jones, R.C., and Ramakrishnan, R. (2008). High throughput gene expression measurement with real time PCR in a microfluidic dynamic array. PLoS One **3**: e1662.

Takahashi, H., Kamakura, H., Sato, Y., Shiono, K., Abiko, T., Tsutsumi, N., Nagamura, Y., Nishizawa, N.K., and Nakazono, M. (2010). A method for obtaining high quality RNA from paraffin sections of plant tissues by laser microdissection. J. Plant Res. **123**: 807–13.

Tatematsu, K., Nakabayashi, K., Kamiya, Y., and Nambara, E. (2008). Transcription factor AtTCP14 regulates embryonic growth potential during seed germination in Arabidopsis thaliana. Plant J. **53**: 42–52.

Theissen, G. and Saedler, H. (2001). Plant biology. Floral quartets. Nature **409**: 469–71.

Trapnell, C., Roberts, A., Goff, L., Pertea, G., Kim, D., Kelley, D.R., Pimentel, H., Salzberg, S.L., Rinn, J.L., and Pachter, L. (2012). Differential gene and transcript expression analysis of RNA-seq experiments with TopHat and Cufflinks. Nat. Protoc. **7**: 562–78.

Vaquero-Sedas, M.I., Luo, C., and Vega-Palas, M.A. (2012). Analysis of the epigenetic status of telomeres by using ChIP-seq data. Nucleic Acids Res. **40**: e163.

Wuest, S.E., Vijverberg, K., Schmidt, A., Weiss, M., Gheyselinck, J., Lohr, M., Wellmer, F., Rahnenführer, J., von Mering, C., and Grossniklaus, U. (2010). Arabidopsis female gametophyte gene expression map reveals similarities between plant and animal gametes. Curr. Biol. **20**: 506–12.

Yamaguchi, T., Lee, D.Y., Miyao, A., Hirochika, H., An, G., and Hirano, H. (2006). Functional Diversification of the Two C-Class MADS Box Genes OSMADS3 and OSMADS58 in Oryza sativa. **18**: 15–28.

Yamaguchi, T., Nagasawa, N., Kawasaki, S., Matsuoka, M., Nagato, Y., and Hirano, H.-Y. (2004). The YABBY gene DROOPING LEAF regulates carpel specification and midrib development in Oryza sativa. Plant Cell **16**: 500–9.

Yu, Y., Lashbrook, C.C., and Hannapel, D.J. (2007). Tissue integrity and RNA quality of laser microdissected phloem of potato. Planta **226**: 797–803.

Zahn, L.M., Kong, H., Leebens-Mack, J.H., Kim, S., Soltis, P.S., Landherr, L.L., Soltis, D.E., Depamphilis, C.W., and Ma, H. (2005). The evolution of the SEPALLATA subfamily of MADS-box genes: a preangiosperm origin with multiple duplications throughout angiosperm history. Genetics **169**: 2209–23.

Zong, W., Zhong, X., You, J., and Xiong, L. (2013). Genome-wide profiling of histone H3K4-tri-methylation and gene expression in rice under drought stress. Plant Mol. Biol. **81**: 175–88.