Ternary complex formation between MADS-box transcription factors and the histone fold protein NF-YB.

Simona Masiero#, Carol Imbrianoǁ, Federica Ravasio*, Rebecca Favaro*, Nilla Pelucchi§, Mirella Sari Gorla*, Roberto Mantovaniǁ, Lucia Colombo§ and Martin. M. Kater*,#

*Dipartimento di Genetica e Biologia dei Microrganismi and §Dipartimento di Biologia, Universita’ di Milano, Via Celoria 26, 20133 Milano, Italy.
ǁDipartimento di Biologia Animale, Universita’ di Modena e Reggio, Via Campi 213/d, 41100 Modena, Italy.
#Present address: Max-Planck-Institut fuer Zuechtungsforschung, Carl-von-Linne-Weg 10, 50829 Koeln, Germany.

Corresponding author: tel. +39 02 50315013; fax. +39 02 50315044; e-mail: martin.kater@unimi.it

Running Title: MADS-box transcription factor interacts with NF-YB
SUMMARY

MADS-box proteins are transcription factors present in different eukaryotic kingdoms. In contrast to plants, for mammalian and yeast MADS-box proteins ternary complex formation with unrelated transcription factors was reported. We show here the first identification of such ternary interaction in plants. A rice seed specific NF-YB was identified as partner of OsMADS18 by two-hybrid screening. NF-YB contains a histone fold motif –HFM- and is part of the trimeric CCAAT-binding NF-Y complex. OsMADS18, alone or in combination with a natural partner, interacts with OsNF-YB1 through the MADS and I regions. The mouse NF-YB also associates with OsMADS18 in vivo, and in vitro as a NF-YB-NF-YC dimer. Other rice MADS-box proteins do not interact in these assays, indicating specificity for the interaction. OsNF-YB1 is capable to heterodimerize with NF-YC, but not to trimerize with NF-YA, thus precluding CCAAT-binding. Mutation of the variant D at position 99 of the HFM α2 helix into a conserved Serine recovers the capacity to interact with NF-YA, but not with DNA. This is the first indication that members of the NF-YB family work through mechanisms independent of the CCAAT box.
INTRODUCTION

MADS-box genes have been identified in a large number of different plant species (1). They have been shown to play key roles in plant development, regulating the transition from vegetative to reproductive growth (2-4), determining the identity of the floral meristem (5), floral organs (6, 7), ovules (8, 9), root development (10), shattering (11) and plant aging (12). The best-studied plant MADS-box genes are those determining floral organ identity in Arabidopsis and Antirrhinum, where many flower homeotic mutants –i.e. ag, ap3, pi and ap1 in Arabidopsis and squa, def, glo and ple in Antirrhinum- have been described and found to be caused by mutations in MADS-box genes (13, 14, 15).

Plant MADS-box proteins share a similar modular secondary structure. The MADS-box (M), at the N-terminus, is by far the most conserved part of these proteins and is the DNA binding domain (16). The MADS-box is followed by the I region (I), the K-box (K), and the C-terminus (C) which is the most divergent part (17, 18). The K and I are both involved in MADS-box protein dimer formation (19-23).

MADS-box proteins bind in vitro to a CArG box (23). This conservation in the binding sites does not reflect the specific functions that different MADS-box factors seem to have in vivo. The specific regulation of target genes will be dependent on the interactions MADS-box proteins make to form homo- or hetero-dimers and on the interactions these dimers make with ternary proteins. Using the two-hybrid system, it was shown that the Antirrhinum MADS-box proteins DEFICIENS (DEF) and GLOBOSA (GLO), exclusively interact with each other (19). On the contrary, the Antirrhinum PLENA (PLE) and Arabidopsis AGAMOUS (AG) proteins, both necessary for reproductive organ development, interact with several other MADS-box proteins (19, 22). The specificity of the interactions between the different MADS-box transcription factors is undoubtedly one of the mechanisms to enhance the selectivity for target gene activation. In addition, ternary complex formation between the Antirrhinum MADS-box proteins SQUAMOSA (SQUA), DEF and GLO has been described: the DEF-GLO heterodimer is able to form a ternary complex with the SQUA...
homodimer via the C-termini (24). A putative target promoter of DEF and GLO, containing two adjacent CArG boxes, showed that the ternary complex binds much stronger than the separate hetero- or homo-dimers.

Despite this wealth of information, ternary complex formation between MADS-box proteins and unrelated polypeptides has not been described in plants. On the other hand the human MADS-box protein SRF, which activates numerous growth-factor-inducible genes such as c-fos (for review see 25), binds as a homodimer to the serum response element (SRE) and interacts with the ternary complex factors (TCFs) ELK-1 and SAP-1 (26, 27). The TCFs make additional contacts with DNA at an ETS motif, which is two base pairs 5’ of the SRE. Autonomous binding of ELK-1 and SAP-1 to the DNA is possible, however with restricted sequence specificity compared to the ternary complex.

Another transcription factor that is particularly abundant in plants is NF-Y, which interacts with high affinity and selectivity with the CCAAT box, one of the most widespread elements, present in 25% of eukaryotic promoters (28). NF-Y is a trimeric complex composed of NF-YA, NF-YB and NF-YC; NF-YB and NF-YC form a tight dimer through a histone fold motif -HFM- that offers a complex surface for NF-YA association (for review see 29, 30). In mammals and yeast only one gene for each subunit exists. A recent survey of transcription factors, made possible by the completion of the Arabidopsis genome sequencing project, revealed that 10 genes each for AtNF-YA, AtNF-YB and AtNF-YC exist (31), with hundreds of heterotrimeric combinations possible. We have cloned all of these genes and determined that some members of each subunit are expressed in a tissue specific way, while others are ubiquitous (32, 33).

In this paper, we report the identification of one of the rice NF-YB proteins as an interacting partner of rice OsMADS18. It is the first example of ternary complex formation between a plant MADS-box protein and an unrelated transcription factor. Surprisingly, this NF-YB is incapable to interact with NF-YA and therefore cannot bind to the CCAAT box.
EXPERIMENTAL PROCEDURES

Two-hybrid cDNA library construction and screening

A yeast two-hybrid cDNA library was made and screened following the instructions of the supplier (Stratagene) using mRNA extracted from leaves, inflorescences and developing kernels of rice Oryza sativa var Taipei 309. Yeast strain HF7c used for the library screening was supplied by Clontech (34). The interactions between OsMADS18, OsMADS14, OsMADS13 and OsMADS6 and OsNF-YB1, the bait deletions of OsMADS18 against OsNF-YB1 and the three-hybrid assays were assayed using yeast strain PJ69-4A (35). The yeast colonies were plated on media without triptophan, leucine and histidine. The positives were subsequently tested on media without - triptophan, leucine, and adenine or histidine with increasing concentrations of 3-AT.

Plasmid constructions

The OsMADS18 coding sequence, excluding the initial methionine was amplified with primers OL13: GAATTCGGGAGAGGGCCGGTGC; OL14: GTCGACCTCATGTGTGACTTGTCCGGAG to introduce the cloning sites EcoRI and SalI, the DNA fragment was cloned in pBDGAL4 (18-BD) and in pTFT (24). The bait deletions of OsMADS18 were also generated by PCR, subsequently digested and cloned in pBDGAL4. The M domain was amplified with primers OL13 and OL210 (GTCGACTCACAGTTTGAAACTCGTAGACTTGCCCTTGG), the M and I domains were amplified with OL13 and OL211 (GTCGACTCAATCCAGTTTGGACTTCAAAATTCC), the I, K and C domains with OL14 and OL207 (CGGAATTCTCCAGCCTCCCTCGAGTATGG), the K and C domains with OL14 and OL208 (CGGAATTCTGATGAATATGGAATTTTGAAGTCC) and the C domain with OL14 and OL209 (CGGAATTCAACCAGAATAATGTCTGCAAGC).
The complete *OsMADS18* coding region was also cloned in pET32a (Promega) to produce the recombinant protein (TRX-OsMADS18), using oligos OL13 and OL14. *OsMADS18* was fused in frame with thioredoxin at the N-terminal domain and with a tail of 6 histidine residues.

The coding region of *OsNF-YB1* was also cloned in pET32a to produce the recombinant protein TRX-OsNF-YB1. OsNF-YB1 was amplified using the oligos OL227 (CGGAATTCAATGGCAGGGAACAAAAAGCGTGC) and OL317 (CCGCTCGAGCAGACTAGACCTATTGACACC) and after cloning in pGEM-T Easy (Promega), digested with EcoRI and SmaI and cloned in pET32.

A PCR approach was used to mutate OsNF-YB1 into OsNF-YB1D99S. Two oligos introducing the mutation were used: OL400 (GGGTTCGTCGGCtccGAGGCCTCCGA) and OL401 (TCGGAGGCCTCggaGCCGACGAACCC). First PCR amplifications were performed with OL400 and OL227 and with OL 317 and OL401. Subsequently, the two PCR products were mixed and a second amplification was done with OL 317 and OL 227. The obtained template was cloned in pGEM-T Easy and sequenced, and subsequently cloned in pET32. *OsMADS14* was amplified with OL11 (GAATTCGGGCGGGCAAGCTGCAG) and OL12 (GTCGACTTTACCCTGTTGATGGC), digested with EcoRI and SalI and cloned in the pAD vector. The same strategy was employed for *OsMADS6* (6-BD) with primers OL87 (GAATTCGGGAGGGGAAGAGTTGAGC) and OL88 (GTCGACTCAAAGAACCACCATCCACGATGAAGC). pBDΔ6 was obtained by digesting 6-BD with SalI and XhoI to delete a fragment encoding 80 amino acids at the C-terminal end of the protein. This had to be done to prevent auto-activation of the reporter gene (36). Note that the M and I domains, necessary for interaction with OsNF-YB1, are present in this construct.

*OsMADS13* was amplified with OL174 (CGGAATTCCGGGAGGGCAGGATGAGATCAAG) and OL175 (ACGCGTCGACGTCATGAGGTTGTCAGAAGTGAG). pBDΔ6 was obtained by digesting 6-BD with SalI and XhoI to delete a fragment encoding 80 amino acids at the C-terminal end of the protein. This had to be done to prevent auto-activation of the reporter gene (36). Note that the M and I domains, necessary for interaction with OsNF-YB1, are present in this construct.
(CGAATTCATGATCATGAAGACACAAATGG) and OL226 (GTCGACTCATGAAAACTGAATTTGC), and cloned in pADGAL4. The mouse conserved HFM domain contains amino acids 51 to 140 and was cloned as EcoRI and SalI fragment in the pADGAL4 (37).

Expression analysis
Total RNA was extracted from rice variety Taipei 309 as described by Verwoerd et al. (36). Northern blotting was performed as described previously (39). RT-PCR reactions were performed as described previously (40).

Cloning of OsNF-YB1 full-length cDNA by RACE-PCR
To isolate the full-length OsNF-YB1 cDNA we performed according to manufacture’s instructions a RACE experiment employing the Marathon kit (Clontech).

Protein purification, co-immunoprecipitation and immunodetection
TRX-OsMADS18-PET32a and TRX-OsNF-YB1-PET32a plasmids were used to transform E. coli strain BL21 (DE3) plysS. To produce the recombinant proteins the overexpression system based on the T7 promoter was used (41). The His-tagged TRX recombinant proteins were purified using an affinity Ni-NTA Agarose column (Qiagen). The immunoprecipitations of the human NF-Y trimer and TRX-OsMADS18 or of the human NF-YA/NF-YC/OsNF-YB1 combinations were performed in NDB buffer plus 1 mM PMSF, 1 µg/µl BSA, 5 mM β-mercapto-ethanol and 0.1% NP-40. NDB consists of 20% glycerol, 100 mM KCl, 20 mM Tris-HCl (pH 7.8) and 0.5 mM EDTA. The proteins were incubated on ice to interact for two hours subsequently incubated at 4°C with anti-NF-YA or control anti-GATA1 antibodies (5 µg) linked to ProteinA-Sepharose. After two hours, the column was washed and eluted with SDS-buffer. The co-immunoprecipitation between OsMADS18 and the dimer BC were performed similarly with anti NF-YC antibody bound to ProteinG-Sepharose.
The anti-OsMADS18 antibodies were produced in rabbit immunising the animals with the C-terminal domain of the OsMADS18 protein. The polyclonal serum was purified on a Sepharose 4B-OsMADS18 affinity column (42). To detect the TRX-tagged OsNF-YB1 protein a purified rabbit antiserum against the TRX-tag was used.

**Electrophoretic Mobility Shift Assay.**

For electrophoretic mobility shift assays $^{32}$P labelled fragments -10000 CPMs- are incubated in NF-Y Buffer (20 mM Hepes pH 7.9, 50 mM NaCl, 5% Glycerol, 5 mM MgCl$_2$, 5 mM β-ME) with the indicated amounts of recombinant proteins, in a total volume of 10 µl; the samples are loaded on a 4.5% Polyacrylamide in 0.5X TBE. The HSP40 CCAAT oligonucleotides used were the following:

5′AGGGCGGCGGCAGATTGGCCCGCGCGCGCGG3′

5′CCCAGCGGGCGCCGCAATCGCCCGCCGCCCT3′.
Results

OsMADS18 interacts with a member of the CCAAT binding factor family

In order to identify interactions between MADS-box and unrelated transcription factors in plants we performed a yeast two-hybrid screen using the rice MADS-box gene OsMADS18 (36). OsMADS18 is a widely expressed gene, its RNA can be detected in root, leaf, inflorescence and developing kernel tissues (Fig. 1A). Functional analysis indicated that OsMADS18 is involved in the control of the transition from vegetative growth to flowering (S. Masiero, F. Fornara, N. Pelucchi, M.M. Kater and L. Colombo, in preparation).

The complete OsMADS18 open reading frame was cloned in the DNA binding domain vector GAL4-BD and transformed to yeast strain HF7c (34). A rice cDNA expression library was constructed using RNA isolated from leaf, inflorescence and developing kernel tissues. The cDNAs were cloned as fusions to the activation domain of GAL4 (AD) and the library was used to transform the HF7c(18-BD) yeast strain. About $6 \times 10^6$ transformants were screened and colonies growing on medium lacking histidine supplemented with 5 mM 3-amino 1,2,4 triazole (3AT) were selected. Approximately 50 colonies were recovered and the pAD plasmids of those also able to activate the LacZ reporter gene were rescued. Sequence analysis of the cDNA inserts revealed that the majority of the clones encode, as expected, MADS-box protein. Among the non-MADS-box interactors, one had high homology with the CCAAT binding factor subunit NF-YB identified in mammals, yeast and Arabidopsis (43-45) and therefore named OsNF-YB1.

Characteristics of OsNF-YB1

The complete cDNA, encoding a putative protein of 186 amino acids, was cloned by race PCR. Comparison with NF-YB sequences from other species (32, 44) shows that OsNF-YB1 shares the highest sequence identity with Arabidopsis LEAFY COTILEDON1 (AtNF-YB9) and that the homology of proteins in different kingdoms is restricted to the evolutionarily conserved histone fold
domain (Fig. 2). This domain is composed of 3/4 α-helixes separated by short loops/strand regions L1 and L2. The comparison also shows that of all NF-YB proteins, the rice protein is the most divergent, especially in the α2 and α3-helix. Despite this, a prediction of the secondary structure indicates that also OsNF-YB1 can form an α-helix.

We investigated the tissue distribution of OsNF-YB1 by Northern blot analysis using RNA from roots, leaves, inflorescence containing developing flowers and developing seeds. This analysis showed that OsNF-YB1 transcripts can only be detected in developing seed tissue (Fig. 1A).

To show that the interaction between OsMADS18 and OsNF-YB1 can occur in vivo we investigated by RT-PCR if their mRNAs could be detected in the same tissues of developing kernels. RNA was extracted from glumes, endosperm and embryo tissues and used for RT-PCR analysis. This analysis (Fig.1B) revealed that the expression of OsMADS18 and OsNF-YB1 overlaps in the glumes and in endosperm tissue. RNA extracted from inflorescences for northern blot analysis also include relatively low amounts of glumes tissue, however no OsNF-YB1 transcript was detected by northern blot analysis in this tissue, probably due to a difference in sensitivity between these two methods. Another observation from the RT-PCR analysis is that OsNF-YB1 seems to be differentially spliced in endosperm tissue since also a smaller amplification product was detected. Future experiments are planned to study this splicing event in more detail.

OsMADS18 protein domains required for heterodimerisation with OsNF-YB1.

To investigate which of the domains of OsMADS18 are involved in the interaction with OsNF-YB1, combinations of the MIKC domains were fused to the binding domain of GAL4, resulting in 18M-BD, 18MI-BD, 18IKC-BD, 18KC-BD and 18C-BD (Fig. 3). These bait constructs were used to transform yeast strain PJ694A which allows selection for three different markers: adenine, histidine and LacZ (37). Co-transformation with ADNF-YB1 and assays for activation of the adenine, histidine or LacZ reporter genes demonstrated an interaction only with the 18MI-BD
vector (Fig. 3). The observation that OsNF-YB1 does not interact with the M or IKC domains suggests that the M and I domains are both necessary and together sufficient for interaction.

**Specificity of the OsNF-YB1 - OsMADS18 interaction**

To test for specificity of the OsNF-YB1 - OsMADS18 interaction a two-hybrid assay was performed using various MADS-box proteins as bait. In particular, three MADS-box genes all expressed in developing kernels were of interest: (i) *OsMADS14* has, of all published rice MADS-box sequences, the greatest similarity with *OsMADS18*; (ii) *OsMADS6* is one of the MADS-box proteins that forms heterodimers with *OsMADS18* (36); (iii) *OsMADS13* is exclusively expressed in ovules and developing kernels (46). All three selected genes are divergent from each other and belong to different phylogenetic clusters. Comparing the M and I domains of these MADS-box proteins shows that they are most divergent in the I domain (Fig. 4).

Except for the control pBD18-pADNF-YB1, none of the double transformants were able to activate the adenine or LacZ reporter genes. Within the limits of the members of the family tested here, these results indicate that the interaction between OsNF-YB1 and OsMADS18 is highly specific, particularly since OsMADS14, which is closely related to OsMADS18, does not interact.

**Ternary complex formation between OsMADS18, OsMADS6 and OsNF-YB1**

The two-hybrid assays described above show that OsNF-YB1 interacts with OsMADS18 but not with OsMADS6, which is one of the MADS-box proteins that physiologically interacts with OsMADS18 (36). It was therefore important to verify whether the MADS-box dimer composed of OsMADS18 and OsMADS6 is also able to interact with OsNF-YB1. In order to test this, the three proteins were co-expressed in yeast. OsMADS18 was cloned in the pTFT vector under the control of the ADH1 promoter (24). The yeast strain PJ694A was co-transformed with pBDΔ6, pTFT-18 and pADNFY-B1 and yeast cells containing these three plasmids grow well in medium lacking histidine, whereas yeast cells containing different combinations of control plasmids only showed
residual growth on the selective medium (Fig. 5). This indicates that OsMADS18, which is able to interact with both OsNF-YB1 and OsMADS6, brings the GAL4-BD and -AD domains in close vicinity, resulting in activation. These ‘three-hybrid’ analyses prove that the MADS-box dimer is able to form a ternary complex with OsNF-YB1.

**OsMADS18 interacts with the NF-YB subunit of mouse**

In *Arabidopsis* multiple genes encoding NF-Y subunits are identified with several of them showing tissue specific expression (32, 33, 43, 45). Because of the plethora of NF-YB genes in plants, this raised the question whether OsMADS18 is also able to interact with the distantly related NF-YB of mouse (44). Using the two-hybrid assay, we showed indeed that OsMADS18 interacts with wild-type mouse NF-YB and with a mutant containing only the mouse HFM, as growth was observed on histidine dropout medium containing 3mM 3AT. The result was also positive using the LacZ reporter gene. On the other hand, OsMADS14 scored negative in this assay (not shown).

The interactions observed *in vivo* raised the question whether OsMADS18 also interacts with the NF-Y trimer. Investigating this with the plant trimer was not feasible, since plants encode several NF-Y A and C subunits, and therefore it was impracticable to determine the correct constitution of the trimer that binds to OsMADS18, if any. We then used recombinant NF-Y from mammals to test for the interaction with OsMADS18 *in vitro*. The NF-Y recombinant trimer was incubated with OsMADS18, immunoprecipitated with a monoclonal anti-NF-YA antibody and analysed by Western blotting using anti-OsMADS18 antibodies (Fig. 6). The results show that OsMADS18 was to some extent bound to the NF-Y trimer, whereas a control with an irrelevant antibody shows the OsMADS18 protein can only be detected in the unbound fraction (Fig. 6). Within the NF-Y trimer, subunit B and C form a tight heterodimeric complex (47). We next investigated whether such dimer can interact with OsMADS18, by performing an immunoprecipitation with a polyclonal anti-NF-YC antibody, using equal quantities of OsMADS18 and mouse NF-YB-NF-YC. This experiment showed that OsMADS18 is capable to bind the mouse NF-YB/NF-YC dimer (Fig. 6). The
interaction with the NF-YB-NF-YC dimer appears to be stronger than with the trimer, since all the OsMADS18 is found in the bound fraction.

**OsNF-YB1 is impaired in NF-YA association and CCAAT-box binding.**

The relative divergence of OsNF-YB1 prompted us to determined whether the latter indeed binds to the CCAAT sequence. To this aim, we used EMSA with an oligonucleotide containing a high affinity NF-Y site (48). Recombinant His-tagged OsNF-YB1 was purified and a dose-response experiment performed with recombinant mammalian NF-YA and NF-YC; as a control, EMSA with recombinant mammalian NF-YB was concomitantly run. Fig. 7A shows that even at very high doses –20 ng- of OsNF-YB1 only an extremely weak band was obtained (lanes 4-6), whereas, as expected, a 20/30-fold lower amount of mammalian NF-YB is sufficient to generate the NF-Y complex (lanes 1-3). We conclude that OsNF-YB1 is severely crippled in CCAAT-binding capacity. There could be three reasons for such behaviour: alteration in the binding to NF-YC, in the trimerization with NF-YA, or in the association with DNA. To distinguish between these possibilities, we incubated the OsNF-YB1 His-tagged protein with recombinant YC5, an NF-YC mutant devoid of sequences outside the HFM and of His-tags (49). The proteins were loaded on an NTA-Agarose Nickel column, eluted and assayed in Western blots. The results in Fig. 7B show that both the OsNF-YB1 and the His-less YC5 coeluted from the column after Imidazole addition. Since YC5 has no intrinsic affinity for NTA-Agarose (49), this result indicates that OsNF-YB1 heterodimerizes with NF-YC, presumably through the HFM, since the YC5 mutant only contains this domain. Next, we assayed a NF-YC-OsNF-YB1 heterodimer for NF-YA binding. Because NF-YA has intrinsic affinity for NTA-Agarose, we switched to immunoprecipitations with the anti-NF-YA Mab7 antibody, that recognizes the Q-rich region outside of the subunits interaction moiety (42). The results of this experiment are shown in Fig. 7C: as expected, NF-YA was
immunoprecipitated by the Mab7, but not by the control anti-Gata1 antibody, whereas neither NF-YC, nor OsNF-YB1 were found in the bound fraction, indicating a lack of association between the HFM dimer and NF-YA. Taking these data together, we conclude that the incapacity of OsNF-YB1 to bind to DNA is probably due to alterations in trimer formation. Structure-function analysis of the mammalian NF-YB protein identified only two residues that are apparently involved in NF-YA association: E90 and S97 in the α-2 helix of the HFM (50). Comparison with the OsNF-YB1 sequence indicates that while E90 is conserved, S97 corresponds to a Aspartic Acid at position 99 of the rice sequence; we therefore mutated OsNF-YB1 at this position into a Serine to potentially recover the NF-YA association capacity. The corresponding recombinant protein was purified and used in EMSA and immunoprecipitation assays as described above for the wild-type protein. As shown in Fig. 7C, the NF-YC-OsNF-YB1D99S dimer does recover NF-YA binding, as tested in immunoprecipitation assays. We conclude that deviation at this particular α2 NF-YB residue is the cause for the lack of NF-YA binding in Os-NF-YB1. We then wished to verify whether the gain of function mutant also recovered CCAAT-binding: dose-response analysis of the Os-NF-YB1D99S with NF-YA and NF-YC in EMSA failed to show any improvement in the CCAAT-binding capacity with respect to the wild-type OsNF-YB1 (Fig. 7A). Therefore, deviant residues in the DNA-binding subdomains of OsNF-YB1, such as α1, L1 or L2, are responsible for the lack of CCAAT-box binding.
DISCUSSION

The two most notable conclusions of our experiments are: (i) the identification of the first non-MADS interacting partner of a MADS-box protein and (ii) the documentation of the first non-CCAAT binding member of the NF-YB family.

Specificity of the interaction between OsMADS18 and OsNF-YB1

Our *in vivo* data showed that none of the other MADS proteins, including OsMADS14 which is closely related to OsMADS18, interacts with OsNF-YB1. This is a clear indication for specificity and one might speculate that the interaction is limited to obligate heterodimerisation.

Where is the specificity stemming from? At first sight, it would seem logical to assume that the MADS-box protein is responsible for it, especially since the distantly related mouse NF-YB interacts with OsMADS18 and not with OsMADS14. The dissection of the OsMADS18 domains required for interaction with OsNF-YB1 showed that the binding of these proteins depends upon both the MADS and I regions. It is formally possible that the highly conserved MADS-box imparts specificity, since two residues in the MADS-box are divergent between OsMADS18 and the related family members, P5 and H33. However, we consider it far more likely that the divergent I region is responsible for it, since in this domain the four rice MADS-box proteins have only two residues in common (Fig. 4). Therefore, only the surface of OsMADS18 might have the correct structure to stabilize binding. On the other hand, the apparent divergence of plant NF-Ys suggests that specificity is also built in NF-YB, a notion further strengthened by the peculiar lack of NF-YA binding by OsNF-YB1. The part that contacts the MADS-box protein is clearly the conserved HFM domain and inspection of alignments identifies amino acid stretches that are significantly at variance with other members of the family in plants, especially in the α2 helix (see below). In this respect, the mammalian homologue might be regarded as an “average” NF-YB which has been subjected to selection against specificity. The fact that unlike in mammals, plants have several tissue specifically expressed MADS-box and NF-Y genes, also suggests that they might be more
limited in the interactions they can make. Thus, we favour a scenario whereby even within generally conserved domains subtle differences in MADS and NF-YB genes impart selectivity for special partnerships. To test this hypothesis, additional OsNF-YBs must be tested with the panel of MADS-box proteins described here.

**Ternary complex formation between MADS-box and NF-Y subunits.**

Ternary interactions of MADS-box proteins are well studied in mammals and yeast. For the mammalian MADS-box proteins belonging to the SRF and myocyte enhancer factor 2 (MEF2) subfamilies, it has been shown that the regions C-terminal of the MADS-box, called respectively SAM domain—a 22 residues domain found in SRF, AGAMOUS and MCM1- and MEFS2 domain—a 15 residues domain found in the MEF2 family—are required for interaction with other proteins. In the case of SRF, the binding surface for the transcription factor Elk-1 has been mapped to a hydrophobic patch formed by the second β-sheet located at the end of the MADS-box and the N-terminal coil of the SAM domain (27). Interestingly, the region of OsMADS18, which is required for interaction with OsNF-YB1, is equivalent to the domains utilised by SRF, MEF2A and MCM1 for interaction with other polypeptides. This implies that the domains used by MADS-box proteins to make interactions with unrelated proteins are positional conserved between mammals, yeast and plants.

The sequence similarity between OsNF-YB1 and other NF-YB proteins is restricted to the histone fold motif. Our co-immunoprecipitation experiments showed that OsMADS18 binds to the NF-YB-NF-YC dimer and, less efficiently, to the trimer. Our analysis of the subunits interaction and DNA-binding features of OsNF-YB1 conclusively determines that the latter efficiently heterodimerizes with mouse NF-YC, but that the resulting dimer is very inefficient to associate NF-YA and, by consequence, in CCAAT binding. Might this be due to the use of mammalian NF-Y subunits? Certainly not, since NF-YC heterodimerizes normally with OsNF-YB1 and we could derive a gain of function mutant of OsNF-YB1 by simply restoring the conserved Serine at position 97 of NF-
YB: this protein recovers NF-YB-like capacity to bind to NF-YA, but still lacked DNA-binding. Mammalian NF-YB/CBF-A and yeast HAP3 have been extensively mutagenized (50, 51). Only two mutations in the HFM -E90 and S97- selectively abrogated the binding of NF-YA, while the vast majority affected either the dimerization or the DNA-binding capacity. E90 is conserved in OsNF-YB1 and in all NF-YB genes identified so far, whereas S97 is a Glycine in many NF-YBs – including yeast HAP3- but it is an Aspartic acid in OsNF-YB1 (see Fig. 2). It should be emphasized that other rice OsNF-YBs do have a Serine at this position and are thus possibly fit in terms of NF-YA association (32). The lack of CCAAT-binding of the trimer containing the OsNF-YBD99S mutant is evidently due to other residues in NF-YB that are essential for this function: it is difficult a priori to pinpoint which, as several residues in the α1 and L2 are different with respect to the mammalian protein. During evolution, it is likely that once D99 has changed, one or more of the CCAAT-contacting amino acids have stopped to be constrained by the necessity to interact with a specific sequence, accumulating changes in residues of the histone fold that would not alter the overall structure and heterodimerization capacity of the protein, but only its DNA-binding.

Several plant NF-YAs exist that show little variation in the amino acids required for HFM binding (50, 51, for a complete discussion see 32) and are indeed capable to bind to CCAAT-boxes (B. Testoni, G. Gusmaroli, C. Tonelli R.M., in preparation). Therefore, our data suggest that there is a set of selected NF-YBs in plants that exert their functions away from the classical CCAAT target sites. This finding has potentially far reaching consequences in other systems as well, most notably in mammalian cells, where NF-YA was shown to be absent, such as in peripheral monocytes and the differentiated myotubes (30). There is increasing evidence for a role of the NF-YB and NF-YC subunits in the absence of NF-YA. Results obtained by us evidenced that the dimer also interact with other proteins in the absence of NF-YA: for example, by immunoprecipitations and glycerol gradients experiments NF-YB, probably together with NF-YC, interacts in the absence of NF-YA, with TFIID, the TATA box binding complex and in particular with some of the HFM TAF$_{16}$s (37, 52).
We propose that (i) the MADS-box and the NF-Y HFM dimers form a complex on promoters that have a CArG box, and/or (ii) that an NF-Y trimer, containing selective NF-YA-binding OsNF-YBs, can associate a MADS-box dimer on a CCAAT-box containing promoter. While the data presented here lend support to the first hypothesis, to prove the second model other rice OsNF-YBs should be identified that have the capacity to interact with specific MADS-box proteins. In both the proposed situations, interaction with the NF-Y dimer or trimer, binding to the CArG box can only be established when the MADS-box proteins are able to dimerise when interacting with NF-YB. Our ‘yeast three-hybrid’ assays, in which we examined the interaction between OsMADS6, OsMADS18 and OsNF-YB1, indeed confirm that the MADS-box dimer is able to interact with OsNF-YB1, making the proposed hypothesis realistic. Further work is required to clarify this point and to identify target genes that are specifically activated by the MADS-box-NF-YB connection.
REFERENCES


Footnotes

We would like to thank Hans Sommer for providing us the pTFT ‘three-hybrid vector’. Martin Kater was supported by E.U. grant BIO4-CT.98.5015. This project was further supported by EU-BIOTECH n° BIO4-CT97-2217 to Lucia Colombo and by grants PRIN99 and PRIN2000 to Roberto Mantovani.
**Figure legends:**

**Fig. 1 Expression analysis of OsMADS18 and OsNF-YB1.** A, Northern blot analysis of OsMADS18 and OsNF-YB1. Total RNA was extracted from roots (r), leaves (l), inflorescences (i) (which include developing flowers of different stages) and developing kernels (dk). The probes are indicated to the right of each panel. B, RT-PCR expression analysis of OsMADS18 and OsNF-YB1 in different parts of the seed. RNA was extracted from embryos (e), glumes (g), endosperm (en) and from complete seeds. PCR+: positive control using a cloned cDNA as template, PCR-: negative control.

**Fig. 2 Amino acid sequence alignment of NF-YB proteins.** Dashes represent gaps introduced to maximize the alignment, amino acids conserved in all the five proteins are indicated in red, amino acids conserved in OsNF-YB1 and others are indicated in yellow, amino acids that are conserved in others but not in OsNF-YB1 are indicated in green and blue. The three α-helices of the histone fold are indicated with + symbols. L1 and L2 are the loop regions. The included NF-YB amino acid sequences are mNF-YB from mouse; HAP3 from yeast; AtNF-YB9 (LEC1), AtNF-YB1 and AtNF-YB2 from Arabidopsis, and OsNF-YB1 from rice. The Aspartic acid residue (D) in OsNF-YB1 preventing NF-YA association is indicated with an arrow.

**Fig. 3 Overview of two-hybrid assays to study the interaction between OsNF-YB1 and protein domains of OsMADS18.** The various OsMADS18 domain combinations that are fused to the GAL4 binding domain, are indicated as bait on the left. The indicated MADS-box, I region, K-box and C region are indicated and are as defined in Ma et al, 1991 (17). The ‘target’ sequence is OsNF-YB1 fused to the GAL4 activation domain (AD). The rightmost column indicates whether an interaction was observed indicated by growth of the yeast on selective media.
Fig. 4 Sequence comparison between OsMADS18 and other rice MADS-box proteins tested for interaction with OsNF-YB1. Panel A: Alignment of the MADS-box sequences of OsMADS18, OsMADS14, OsMADS6 and OsMADS13. Panel B: Alignment of the I region sequences. Identical amino acids are not coloured, amino acids that differ from OsMADS18 but belonging to the same chemical grouping are indicated in light grey and amino acids not falling in the same group are indicated in dark grey. Amino acids were grouped as follows: GASTP, ILVM, FYW, QN, ED, HKR, and C. The two amino acids in the MADS-box that are divergent between OsMADS18 and the other three MADS-box proteins are indicated with an arrowhead.

Fig. 5 Interactions between OsMADS18, OsMADS6 and OsNF-YB1 detected by "three-hybrid analysis". Panel A and B show the ‘yeast three-hybrid analysis’ by which we tested the interaction between OsMADS18, OsMADS6 and OsNF-YB1. OsMADS6 cloned in pBD vector was able to auto-activate the reporter genes, in order to avoid this 80 amino acids where deleted from the C-terminal part of the protein (OsMADSΔ6). cDNAs cloned in pBD are fused in frame with the DNA binding domain of GAL4, cDNAs cloned in pAD are fused to the GAL4 activation domain while the cDNAs cloned in TFT are fused to a nuclear localization signal.

(1) pADNFY-B1 and pBD18 (positive control); (2) pADΔ6 and pBD18; (3) pADNFY-B1, pBDΔ6 and pTFT (empty vector); (4) pADNFY-B1, pBD (empty vector) and pTFT-18; (5) pAD (empty vector), pBDΔ6 and pTFT-18; (6) pADNFY-B1, pBD (empty vector) and pTFT (empty vector); (7) pADNFY-B1, pBDΔ6 and pTFT-18. Panel A: the yeast strains were plated on selective medium (without histidine and supplied with 2 mM 3AT). Panel B: the same yeast strains plated on a non-selective medium (with histidine).
Fig. 6 *In vitro* interactions between OsMADS18 and the mammalian NF-Y factors.

Western blot results of the co-immunoprecipitation between OsMADS18 and the NF-Y trimer (upper), and NF-YB, NF-YC dimer (lower) complexes using antibodies against NF-YA and NF-YC, respectively. On the right: western blots showing a negative control of the co-immunoprecipitation between OsMADS18 and the NF-Y trimer (upper), and NF-YB, NF-YC dimer (lower) complexes using an unrelated antibody (CTL). The Western blots were probed with OsMADS18 antibodies. L: the fraction loaded on the column; B: fraction that binds to the column; FT, flow-through fraction.

Fig. 7 OsNF-YB1 subunits interactions. A, A high affinity CCAAT box oligonucleotide from the HSP40 promoter was used with the indicated amounts of mammalian recombinant NF-Y (lanes 1-3), with mammalian NF-YA and NF-YC and OsNF-YB1 (Lanes 4-6) or with mammalian NF-YA and NF-YC and the mutated OsNF-YB1D99S (lanes 7-9). B, Nickel NTA-Agarose purification of His-tagged OsNF-YB1 and tagless YC5, a mutant of mammalian NF-YC containing only the yeast homology domain (49). Proteins are revealed by Western blot. Load (L), flow through (FT, washes W and Imidazole-eluted (E) fractions are indicated. C, Immunoprecipitations with anti-NF-YA Mab 7 and control anti-Gata1 antibodies of NF-YA, NF-YC and OsNF-YB1 or OsNF-YB1D99S proteins. The different proteins are revealed by Western blots, probing with YC5 and TRX antibodies. Load (L) is the starting material, FT is the flow through; B is bound material.
Figure 1

A

<table>
<thead>
<tr>
<th>r</th>
<th>l</th>
<th>i</th>
<th>dk</th>
</tr>
</thead>
</table>

*OsMADS18*

*OsNF-YB1*

B

| e | g | en | s | c+ | c- |

*OsMADS18*

*OsNF-YB1*
<table>
<thead>
<tr>
<th>Bait</th>
<th>M</th>
<th>I</th>
<th>K</th>
<th>C</th>
<th>Interaction with AD-OsNF-YB1</th>
</tr>
</thead>
<tbody>
<tr>
<td>18-BD</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>yes</td>
</tr>
<tr>
<td>18M-BD</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>no</td>
</tr>
<tr>
<td>18MI-BD</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>yes</td>
</tr>
<tr>
<td>18IKC-BD</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>no</td>
</tr>
<tr>
<td>18KC-BD</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>no</td>
</tr>
<tr>
<td>18C-BD</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>no</td>
</tr>
</tbody>
</table>
### Figure 4

**A**

<table>
<thead>
<tr>
<th>MADS-BOX</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
</tr>
<tr>
<td>57</td>
</tr>
</tbody>
</table>

| OsMADS18  | MGRGVQLRRKINRQVTFSKRRNGLKAHEISVLCDADVALIFSTKGGKLYEF |
| OsMADS14  | MGRGVQLRRKINRQVTFSKRRNGLKAHEISVLCDADVALIFSTKGGKLYEF |
| OsMADS6   | MGRGVQLRRKINRQVTFSKRRNGLKAHEISVLCDADVALIFSTKGGKLYEF |
| OsMADS13  | MGRGVQLRRKINRQVTFSKRRNGLKAHEISVLCDADVALIFSTKGGKLYEF |

**B**

<table>
<thead>
<tr>
<th>I-REGION</th>
</tr>
</thead>
</table>

| OsMADS18  | SSSHSMEGILERYQSYSDERAVLEPNTED.QENWGD |
| OsMADS14  | ATSSMMFLERYQSYSDERAVLEPNTED.QENWGD |
| OsMADS6   | GSSMMFLERYQSYSDERAVLEPNTED.QENWGD |
| OsMADS13  | SSSMMFLERYQSYSDERAVLEPNTED.QENWGD |
Figure 5
Figure 6

NF-Y trimer

α-YA

CTL

α-YC

CTL

NF-YBC dimer

L B FT

B FT

L B FT

B FT

Masiero et al.
Figure 7

A

<table>
<thead>
<tr>
<th>NF-YB</th>
<th>OsNF-YB1</th>
<th>OsNF-YB1D99S</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ng 0.4 1 20</td>
<td>0.2 0.5 20</td>
<td>0.2 0.5 20</td>
</tr>
</tbody>
</table>

1 2 3 4 5 6 7 8 9

B

NTA-Agarose

<table>
<thead>
<tr>
<th>L</th>
<th>FT</th>
<th>W1</th>
<th>W2</th>
<th>W3</th>
<th>E1</th>
<th>E2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>OsNF-YB1</td>
<td>YC5</td>
</tr>
</tbody>
</table>

C

<table>
<thead>
<tr>
<th>α-YA</th>
<th>CTL</th>
</tr>
</thead>
<tbody>
<tr>
<td>L</td>
<td>FT</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>OsNF-YB1</th>
<th>NF-YA</th>
<th>NF-YC</th>
<th>OsNF-YB1</th>
</tr>
</thead>
</table>

<table>
<thead>
<tr>
<th>OsNF-YB1D99S</th>
<th>NF-YA</th>
<th>NF-YC</th>
<th>OsNF-YB1</th>
</tr>
</thead>
</table>
Ternary complex formation between MADS-box transcription factors and the histone fold protein NF-YB
Simona Masiero, Carol Imbriano, Federica Ravasio, Rebecca Favaro, Nilla Pelucchi, Mirella Sari Gorla, Roberto Mantovani, Lucia Colombo and Martin M. Kater

J. Biol. Chem. published online April 23, 2002

Access the most updated version of this article at doi: 10.1074/jbc.M202546200

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts