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Article Addendum

MADS about MOSS

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Classic *MIKC*-type MADS-box genes (*MIKC^c*) play diverse and crucial roles in angiosperm development, the most studied and best understood of which is the specification of floral organ identities. To shed light on how the flower evolved, phylogenetic and functional analyses of genes involved in its ontogeny, such as the *MIKC^c* genes, must be undertaken in as broad a selection as possible of plants with disparate ancestries. Since little is known about the functions of these genes in non-seed plants, we investigated the developmental roles of a subset of the *MIKC^c* genes present in the moss, *Physcomitrella patens*, which is positioned informatively near the base of the land plant evolutionary tree. We observed that transgenic lines possessing an antisense copy of a *MIKC^c* gene characteristically displayed knocked-down expression of the corresponding native *MIKC^c* gene as well as multiple diverse phenotypic alterations to the haploid gametophytic and diploid sporophytic generations of the life cycle.[1] In this addendum, we re-examine our findings in the light of recent pertinent literature and provide additional data concerning the effects of simultaneously knocking out multiple *MIKC^c* genes in this moss.

The moss, *Physcomitrella patens*, is the only non-seed plant that is amenable to an investigation of MADS-box gene function comparable to that achieved in angiosperms. *P. patens* possesses six *MIKC^c* genes which cluster into two distinct phylogenetic clades.[2] We recently reported a functional genetic analysis of the three genes (*PPM1*, *PPM2* and *PpMADS1*) within the *PPM2*-like clade as an initial contribution towards gaining an understanding of the role(s) of *MIKC^c* genes in this moss.[1]

By fusing the respective *MIKC^c* promoters to a *GUS* reporter gene, we found that both *PPM1* and *PpMADS1* exhibited fairly ubiquitous expression patterns in both gametophytic and sporophytic tissues. The levels of *PPM1* expression were generally higher than those of *PpMADS1*, and *PpMADS1* was not expressed in antheridia, suggesting subtle differences in the functions of these genes. The observed patterns of widespread expression resemble those characterising the majority of vascular, non-seed plant *MIKC^c* genes[3-7] and accord with RT-PCR results of Quodt and coworkers.[2] Our in situ *GUS* expression and RT-PCR results[1] showed that *PPM2* was not expressed or was expressed at levels too low to be detected by these methods. Conversely, the original isolation of *PPM2* cDNA[8] and data from more recent expression studies[2] indicated that *PPM2* is expressed (albeit inconsistently and weakly) ubiquitously with elevated levels of expression sometimes observed in gametangia, sporophytic feet and basal portions of sporophytic setae.[2] The contradictory expression data for *PPM2* may derive from differences between the *PPM2*-reporter gene constructs used by the respective research groups[1,2] or perhaps from variations in moss culture conditions.

We also employed an antisense approach designed to knock down expression of *PPM1*, and perhaps closely related *MIKC^c* genes, in order to discern MADS-box gene function in *P. patens*. Knocked-down strains displayed a complex mutant phenotype comprising delayed gametangia formation and sporophyte production, diminished sporophyte yields, and morphological abnormalities in both leaves and sporophytes, findings that are generally consistent with the ubiquitous expression pattern of *PPM1*[1] and *PPM2*'s expression as described by Quodt et al.[2]

The phenotypes of strains with single gene knockouts of *PPM1*, *PPM2* or *PpMADS1* appeared to be perfectly normal, not displaying any of the phenotypic alterations observed in *PPM1* gene knock-down mutants. While it is possible that subtle, transient or conditional phenotypic changes went unnoticed, it seems more probable that genetic redundancy is responsible for these results since the *PPM2*-like genes exhibit a very high level of sequence similarity. In an effort to circumvent the problem of functional redundancy, we generated all double knockout combinations for *PPM1*, *PPM2* and *PpMADS1*. However, the double mutants were also phenotypically unchanged. Finally we attempted to produce triple mutants by co-transforming single *PPM2* knockout lines with *PPM1* and *PpMADS1* linear knockout constructs. Of the 31 stable transformants from two transformation experiments, 55% were shown to be double mutants in which the original *PPM2* knockout was accompanied by a second gene knockout in either *PPM1* or *PpMADS1*. However, no triple knockouts were obtained. Given the knockout frequencies generally observed in batch transformation experiments in our laboratory and those of others,[9] between two and five of the transformants had been expected to be triple mutants. These preliminary data, albeit

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involving a relatively small sample of transformants, suggest that *PPM1*, *PPM2* and *PpMADS1* triple knockouts may be lethal.

We have related compelling evidence that functionally redundant *PPM2*-like *MIKC^c* genes are involved in several aspects of the moss developmental program. It has been argued that broad expression patterns like theirs represent the ancestral state of MADS-box genes in land plants, and that the sporophytic- and organ-specific expression patterns that characterise many *MIKC^c* genes in extant spermatophytes, including those that specify floral organ identity, correspond to a derived condition that evolved in the spermatophyte lineage following its separation from lineages that led to bryophytes and ferns and fern allies.[10] Nevertheless, it is the apparent participation of *PPM2*-like genes in the formation of gametangia (the differentiation of reproductive organs from non-reproductive tissues at the gametophore apex) that is particularly interesting and assumes a special significance because of its analogy to the proposed role for ancestors of seed plant C-function MADS-box genes (identifying those regions of the vegetative SAM that will become reproductive organs).[11] Furthermore, expression studies of *MIKC^c* genes in two charophycean algae, the presumed progenitors of all terrestrial plants,[12-14] suggest that they too are involved in haploid reproductive cell differentiation.[15] While these functional similarities do not infer orthology and may be coincidental, we should not discount yet the admittedly controversial hypothesis that some *MIKC^c* genes in non-seed plants, for example *PPM2*-like genes of Physcomitrella, are homologous to spermatophyte class C genes and that the ancient role proposed for ancestral class C genes[11] has been conserved, in some form, in all major terrestrial plant taxa.

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