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Functional analysis of MADS-box genes controlling ovule development in *Arabidopsis* using the ethanol-inducible *alc* gene-expression system

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Abstract

In Arabidopsis, different combinations of ABC organ identity proteins interact in the presence of SEPALLATA (SEP) proteins to regulate floral organ differentiation. Ectopic expression of *SEP3* in combination with class A and B or B and C genes is sufficient to homeotically convert vegetative leaves into petal-like organs and bracts into stamen-like structures, respectively. Recently, it has been shown that the three MADS-box genes *SEEDSTICK* (*STK*), *SHATTERPROOF1* (*SHP1*) and *SHP2* act redundantly to control ovule identity. Protein interaction assays performed in yeast in combination with genetic studies demonstrated that these MADS-box factors only interact in the presence of SEP proteins to form complexes that determine ovule differentiation. Here, we address the question whether the ectopic co-expression of ovule identity proteins is sufficient to induce the homeotic conversion of vegetative leaves into carpel-like structures bearing ovules. We present the phenotypic characterization of *Arabidopsis* plants that ectopically express ovule identity factors under the regulation of the ethanol inducible gene expression system. These experiments indicate that the ectopic co-expression of SEP3 and SHP1 and/or STK is probably not sufficient to homeotically transform vegetative tissues into carpels with ovules. However, comparing the phenotypes obtained by ectopic expression of STK and/or SHP1 with or without SEP3 shows that co-expression of factors that are able to form complexes in yeast cause more extreme homeotic transformations, confirming the functional role of these complexes in vivo.

Keywords: Arabidopsis; Ethanol induction; MADS-box; Ovule; Protein complex

1. Introduction

Homeotic genes are responsible for the activation of pathways that determine the identity of organs. In plants, mechanisms underlying floral organ identity are described since early 1990s when the ABC model was proposed (reviewed by Coen and Meyerowitz, 1991). This model was based on phenotypic observations of homeotic flower mutants in *Antirrhinum* and *Arabidopsis* and demonstrated the existence of a genetic relation between three classes of genes, indicated as A, B and C genes. The action of class A genes was proposed to be responsible for sepal identity while the combination of class A and B genes is necessary for petal formation. Class B and C genes together regulate the identity of stamen and class C genes alone determine the identity of carpels (Carpenter and Coen, 1990; Bowman et al., 1991; Coen and Meyerowitz, 1991; Weigel and Meyerowitz, 1994). Subsequent cloning of these floral identity genes showed that they encode transcription factors, most of them belonging to the MADS-box family (Sommer et al., 1990; Yanofsky et al., 1990; Mandel et al., 1992; Jack et al., 1992; Goto and Meyerowitz, 1994).

The ABC model has been extended with an additional class indicated as class E genes or *SEPALLATA* (*SEP*) genes. This class consists of four members, *SEP1*, *SEP2*, *SEP3* and *SEP4*, encoding MADS-box factors that show partial redundant functions in floral organ identity determination. The triple knock-out *sep1 sep2 sep3* has indeterminate flowers with petals, stamens and carpels homeotically transformed into sepals (Pelaz et al., 2000). Recently, a *sep1 sep2 sep3 sep4* quadruple mutant was described in which all floral organs were

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transformed into organs similar to leaves (Ditta et al., 2004). These results show that the *SEP* genes are necessary for the function of class A, B and C genes since the quadruple *sep1 sep2 sep3 sep4* mutant phenocopies the *abc* mutant.

The molecular basis for the genetic relation among floral organ identity genes has been explained by experiments of Honma and Goto (2001) and Pelaz et al. (2001). They addressed the question whether the expression of combinations of class A, B, C and *SEP* genes would be sufficient to convert leaves into floral organs. They generated transgenic *Arabidopsis* lines that ectopically expressed a combination of A, B and *SEP3* genes or B, C and *SEP3* genes. These experiments showed that these factors were indeed sufficient to affect leaf identity. Transgenic plants expressing A, B and SEP3 genes had vegetative leaves converted into petal-like organs and those expressing B, C and *SEP3* genes had cauline leaves and all floral organs converted into stamen-like structures. Interaction studies in yeast showed that SEP3 proteins interact with A, B and C MADS-box factors forming multimeric complexes. These interaction data explained the molecular nature of the genetic interactions among A, B, C and SEP factors.

Recently, it has been shown in yeast that MADS-box proteins important for ovule identity in *Arabidopsis* can assemble into higher order complexes (Favaro et al., 2003). These complexes contain combinations of the MADS-box proteins AGAMOUS (AG), SEEDSTICK (STK), SHATTERPROOF1 (SHP1), SHP2 and the SEP1, SEP2 and SEP3 proteins. Pinyopich et al. (2003) showed that *STK*, *SHP1* and *SHP2* play a redundant role in ovule identity determination since the *stk shp1 shp2* triple mutants develop flowers in which most of the ovules are converted into carpel-like or leaf-like structures. The importance of *AGAMOUS* during ovule development was studied by Western and Haughn (1999) in the *ap2-6* background. In this mutant, the number of ectopic ovules that developed on the sepals was six times higher than those developing on the sepals of the *ap2-6 ag-1* double mutant. Moreover, most of the ectopic ovules in the double mutant were abnormal in structure. These data indicate that *AG* plays a role in ovule identity determination but besides that they indicate that an *AG*-independent pathway is responsible for carpel and ovules development on the sepals of the *ap2 ag* double mutant. Remarkably, carpels and ovules are completely absent in the first whorl organs of the *ap2 ag shp1 shp2* quadruple mutant (Pinyopich et al., 2003), indicating that *SHP* genes can promote carpel and ovule development in the absence of *AG*.

The role of *SEP* genes in ovule development came from genetic titration experiments in which *SEP* gene activity was reduced (Favaro et al., 2003). These experiments showed that the *SEP1/sep1 sep2 sep3* mutant is a phenocopy of the *stk shp1 shp2* triple mutant demonstrating that *SEP* genes are indeed necessary for ovule development. The role of SEP proteins in ovule identity determination was confirmed by protein interaction experiments demonstrating that STK, SHP1, SHP2 and AG do not interact directly but multimeric complexes can be formed when SEP3 is added in yeast three-hybrid experiment (Favaro et al., 2003). These results suggest that ovule development in *Arabidopsis* is controlled by MADS-box protein complexes that are composed of SEP factors and different combinations of ovule identity proteins.

Here, we investigate whether the ectopic co-expression of ovule identity genes is sufficient to convert vegetative leaves into carpel-like structures bearing ovules. We created transgenic plants, which ectopically expressed different combinations of *SEP3*, *STK* and *SHP1*. As previously shown, plants transformed with *35S*::*STK*, *35S*::*SHP1* or *35S*::*SHP2* showed homeotic transformation of sepals into carpel-like structures bearing stigmatic tissue and ectopic ovules (Liljegren et al., 2000; Favaro et al., 2003; Pinyopich et al., 2003). Unfortunately, the ectopic expression of ovule identity genes has pleiotropic effects on plant development. Most of the transgenic plants die immediately after germination. Few plants grow further, they remain very small in size and flower after producing two or four curled leaves. In all the cases these plants show a very short life-span. To avoid these kinds of effects in transgenic plants expressing a combination of ovule identity genes we used the ethanol-regulated *alc* gene expression system to induce ovule identity genes later in development. This system consists of two components, a transcription factor ALCR, which activity depends on the presence of ethanol and the promoter p*AlcA* which is activated by ALCR (Caddick et al., 1998; Salter et al., 1998; Roslan et al., 2001).

Transformation was performed with multiple vectors with each T-DNA encoding a visible selectable marker (EYFP, DsRed and ECFP) under the control of the strong napin seed-specific promoter (Stuitje et al., 2003). Phenotypic analysis of transgenic *Arabidopsis* plants ectopically expressing various combinations of ovule identity genes revealed that co-expression of these genes is not sufficient to homeotically transform leaves into carpel and/or ovule structures. However, the obtained data clearly show that the co-expression of ovule identity genes affects the identity of all the floral organs. The severe floral phenotype of *SEP3–STK* and *SEP3–STK–SHP1* plants support the hypothesis that these proteins act in a combinatorial manner in the regulation of ovule identity through the formation of protein complexes necessary for the transcriptional regulation of target genes.

2. Results

2.1. Inducible expression of ovule identity genes

Previous experiments showed that ectopic expression of the ovule identity genes *STK*, *SHP1* and *SHP2* in *Arabidopsis* induced the homeotic transformation of sepals into carpelloid structures (Liljegren et al., 2000; Favaro et al., 2003). Furthermore, biochemical studies in yeast demonstrated that these transcription factors form multimeric protein complexes for which they require SEP proteins (Favaro et al., 2003). In this study we investigate whether the co-expression of ovule identity genes can induce carpel and ovule formation on vegetative leaves as has been reported by Honma and Goto