Comparison of the Interaction Specificity of Angiosperm B- and C-genes and their Gymnosperm Orthologs

Jan Naef

Degree project in biology, 2007
Examensarbete i biologi, 10 p, 2007
Biology Education Centre and Department of Physiological Botany, Uppsala University
Supervisors: Peter Engström and Erika Groth
Summary
Little is known about the molecular mechanisms involved reproductive organ development in seed plants and about the evolution of the different reproductive strategies of angiosperms (flowering plants, or “vessel seed” plants) and gymnosperms (“naked seed” plants). The development of the angiosperm flower has been extensively studied at the molecular level. Four classes of genes, the A-, B-, C- and E-genes, specify the identity of the floral organs in a combinatorial manner. These homeotic (= identity conferring) genes contain a conserved sequence motif, the MADS-box, which encodes the DNA-binding MADS domain, and their protein products operate as heterotetrameric transcriptional regulators. Here, I compared the Bgenes APETALA 3 and PISTILLATA and C-gene AGAMOUS from the angiosperm thale cress (Arabidopsis thaliana) with their orthologs in the gymnosperm Norway spruce (Picea abies) (DEFICIENS AGAMOUS LIKE 13-I (DAL13-I) and DAL2, respectively) by analyzing which of 107 other Arabidopsis and spruce MADS domain proteins their protein products can interact. The results for the Arabidopsis and spruce genes are in general highly similar. Interestingly, DAL2 interacted with the Arabidopsis proteins SEPALATA1, of which no ortholog has hitherto been described in spruce, and SUPPRESS OR OF CONSTANS 1 (SOC1) which is an important regulator of flowering time in Arabidopsis.

The MADS-box genes, and the SEPALATA-genes in particular, are believed to be in part responsible for the appearance and radiation of angiosperms. The identification of gymnosperm orthologs of the angiosperm homeotic genes and their functional characterization could thus lead to a model of reproductive organ formation in gymnosperms and shed light on the evolution of the different reproductive strategies of seed plants.
Abbreviations
AG  AGAMOUS
PI  PISTILLATA
AGL  AGAMOUS LIKE
DAL  DEFICIENS AGAMOUS LIKE
YTH  yeast-two-hybrid
Introduction

MADS-box genes and floral organ identity in angiosperms

The MADS-box genes, named so after the first representatives of the group (MCM1, AG, DEFA and SRF) (Schwarz-Sommer et al. 1990), encode transcriptional regulators and have been found in all eukaryotic kingdoms (Alvarez-Buylla et al. 2000). These genes all share a conserved sequence motive, the MADS-box, which encodes the DNA-binding MADS domain. They play roles in many developmental processes and have been shown to constitute part of the regulatory network that controls reproductive organ development in plants (Ma 1994). The reproductive organs of angiosperms (flowering plants, or “vessel seed” plants), the flowers, are composed of four concentric whorls of extensively modified and highly specialized leaves: the sepals (the outermost whorl of only slightly modified leaves) the petals (the showy, colored leaves), the stamen (the male reproductive organs) and the carpels (the female reproductive organs) (figure 1). MADS-box proteins play a major role in floral development, and they have in particular been shown to be the molecular basis for the ABC model. This model has first been established by the analysis of homeotic floral mutants (mutants in which the identity of some floral organs is changed) and proposes that three classes of genes (A-, B- and C-genes) determine in a combinatorial manner the identity of the organs in the four whorls of the angiosperm flower. Expression of A alone specifies sepals, A together with B specifies petals, B together with C stamens and C alone specifies carpels. The model further predicts inhibition of A by C and vice versa (figure 1) (Coen and Meyerowitz 1991, Bowman et al. 1991). In Arabidopsis there are 2 A-genes, APETALA 1 (AP1, Gustafson-Brown et al. 1994, Irish and Sussex 1990, Mandel et al. 1992) and AP2 (Jofuku et al. 1994), 2 B-genes, AP3 and PISTILLATA (PI, Goto and Meyerowitz 1994, Jack et al. 1992, Jack et al. 1994, Krizek and Meyerowitz 1996) and one C-gene, AGAMOUS (AG, Mizukami and Ma 1992, Mizukami and Ma 1995, Yanofsky et al. 1990), all of which except AP2 are MADS-box genes (Jofuku et al. 1994). A-mutants have carpels in the first and stamens in the second whorl, B-mutants have sepals in the second and carpels in the third whorl while C-mutants have petals in the third and sepals in the fourth whorl. However, constitutive expression of A-, B- and C-genes is not enough to convert vegetative leaves into each of the distinct floral organs (Mizukami and Ma 1992, Krizek and Meyerowitz 1996). The activity of the B- and C-genes is dependent on a fourth factor, encoded by the E-genes (The MADS-box genes SEP1, SEP2 and SEP3 in Arabidopsis) (Pelaz et al. 2000). E-mutants have carpels in whorls two, three and four and plants constitutively expressing A-, B-, C- and E-genes have vegetative leaves transformed into floral organs. This has led to the floral quartet model, which suggests that the products of the A-, B-, C- and E- genes function as heterotetrameric transcriptional regulators (Honma and Goto 2001, Theissen and Saedler 2001).

Figure 1: Organs of he angiosperm flower and their regulation as suggested by the ABC model.
**MADS-box genes and the evolution of seed plants**

Close orthologs of *Arabidopsis* A-, B- or C-genomes have been found in all seed plants, that is in all major angiosperm divisions and in gymnosperms (“naked seed” plants) (Johansen et al. 2002, Tandre et al. 1995). Phylogenetic analysis reveals that the orthologous genes from different angiosperm and gymnosperm species are more closely related to each other than floral MADS-box genes of one species and that the major lineages of MADS-box genes arose from precursors in the common ancestors of extant seed plants (Tandre et al. 1995). However, no orthologs of the *Arabidopsis* E-genomes, *SEP1*, *SEP2* and *SEP3* have been found in gymnosperms and their evolution has been proposed to be in part responsible for the emergence and subsequent radiation of angiosperms (Zahn et al, 2005).

**Reproductive organ morphology**

The shaping of their reproductive organs is of high significance for plants. Since they have roots and hardly ever get to touch another individual of the same species, sexual reproduction is a difficult issue, the difficulty being to get their gametes to meet gametes of the opposite sex. The male gametes have to be carried by something motile to the female gametes. The only way for a plant to achieve this efficiently is to shape its reproductive organs accordingly. Gymnosperm pollen are dispersed by the wind and the female cones are aerodynamically shaped so as to lead spores specifically of the own species in the air to the ovules (Niklas and U 1983). Angiosperms, through the morphology of the flower, manage to employ animals to distribute the pollen to the ovules. Animals are less random at this task, they show selective motility, are bribeable and, most importantly, they can evolve symbiotic relationships with the plant.

**Interactions between MADS domain proteins**

The nature of the regulatory networks that control reproductive organ development in plants and what role they played during the radiation of angiosperms is not clear. The MADS domain proteins provide a good substrate for functional and phylogenetical studies to elucidate these questions. Since the MADS domain proteins are known to bind DNA as hetero- or homomultimers, both selective DNA binding and the magnitude of transcriptional activation exerted by the multimers are determined in a combinatorial manner (Favaro et al. 2003, Fan et al. 1997), each building block having its functional share (Honma and Goto 2001, Theissen and Saedler 2001). Protein-protein interactions among the MADS domain proteins thus determine to a large extent the functionality of the regulatory network, and the concept of the composite transcription factor seems a good substrate for evolution through duplication and modification of existing building blocks (Zahn et al. 2005). In a screen of the *Arabidopsis* genome for MADS-box genes, Parenicová et al (2003) identified 107 members of that family which they grouped into five major clades, MIKC, Mα, Mβ, Mγ and Mδ. All genes that have been functionally analyzed belong to the MIKC group. This group, which is the biggest of the five, is characterized by a conserved domain structure (figure 2): All its members are composed of a MADS-box (M) domain, a Intervening (I) domain, a Keratin-like (K) domain and a C-terminal (C) domain (For the domain structure of the other clades see (Parenicová et al. 2003)). The N-terminal part of the 58 amino acid long MADS-box domain is composed of multiple β-sheets and has DNA binding activity (Riechmann et al. 1996a, Hayes et al. 1988) while a short C-terminal part of this domain is involved in dimerization. The less conserved I domain contributes to the specification of dimerization. The K region it is composed of three supercoiled α-helixes, resembles the protein Keratin (hence the name) and mediates dimerisation of MADS proteins (Davies et al. 1996, Fan et al. 1997). The C-terminal domain, which is very variable and sometimes lacking, is involved in
higher-order complex interactions as in the ternary floral quartet tetramers (Egea-Cortines et al. 1999, Honma and Goto 2001).

![MADS-I-K-C](image)

**Figure 2:** Domain structure of the MIKC MADS-box genes

**The Yeast-Two-Hybrid system**

A very convenient way to study interactions between proteins is the Yeast-Two-Hybrid (YTH) system. This method takes advantage of a transcriptional activator in yeast (*Saccharomyces cervisiae*), the GAL4 protein. This protein is composed of two domains, a DNA-binding domain (BD) which binds to the GAL4 promoter, and a transcriptional activation domain (AD). Yeast strains for YTH analysis lack the GAL4 gene but contain one or several reporter genes under the control of the GAL4 promoter. To assay whether two proteins P1 and P2 interact they are transcriptionally fused to the separated domains of GAL4, giving two hybrid proteins AD-P1 and BD-P2, which can be introduced into yeast cells on plasmids. If P1 can bind to P2 the AD-P1 construct will also bind to BD-P2 and thus give a active transcriptional activator which will bind to the GAL4 promoters and activate transcription of the reporter gene(s).

The yeast strain used in this study contains three reporter genes under control of GAL4 promoters. Two of them confer the ability to synthesize adenine (A) and histidine (H), respectively, making the strain able to grow on plates lacking A, H or both only if the proteins interact. The third reporter gene encodes a β-galactosidase. β-galactosidase activity in this strain can be measured by means of a chromogenic substrate and is a measure of the strength of the interaction between the two proteins.

The YTH system has been extensively used to study interactions of *Arabidopsis* floral MADS-box genes (Fan et al. 1997, Honma and Goto 2001). The usual incubation temperature for yeast-two-hybrids is 30°C but some groups have reported temperature-specific interactions at 20-22°C (de Folter et al. 2005). I tested both temperatures for all combinations and some interactions were indeed only detected at 22°C.

**Aim**

The aim of this study was to elucidate the relationship between the angiosperm homeotic genes and their gymnosperm orthologs and thus to shed light on the evolution of these two reproductive strategies. In particular, I have used a YTH system to study the interactions of *Arabidopsis* and gymnosperm B- and C-genes with 107 *Arabidopsis* and Spruce MADS-box genes.
Results
I screened the Arabidopsis B-class genes PISTILLATA (PI) and APETALA 3 (AP3), the Arabidopsis C-class gene AGAMOUS (AG), the conifer B-class gene DEFICIENS AGAMOUS LIKE 13-I (DAL13-I) and the conifer C-class gene DAL2 for interactions against 99 Arabidopsis and five conifer MADS-box genes (table 1) in a yeast-two-hybrid (YTH) system. I obtained the PI, AP3, AG, DAL13-I and DAL2 genes cloned into binding domain (BD) vectors and the other genes cloned into activation domain (AD) vectors in yeast. The BD and AD vectors confer leucine (L) and tryptophan (T) prototrophy, respectively, and strains containing the plasmids were thus maintained on plates lacking the corresponding amino acids. To assay whether two proteins interact, the yeast strains containing plasmids with the corresponding genes were allowed to mate, yielding strains containing both plasmids. Expression of the reporter genes conferring histidine (H) and adenine (A) prototrophy in these strains was then assayed by streaking the yeast cells on plates lacking one of these compounds and judging their ability to grow at both 22°C and 30°C. Growth indicated an interaction. I assayed each strain on four plates, two at each temperature of which one contained no H and one no A.

I tested if cells containing either the AD or the BD vector had activated reporter genes (autoactivation) by streaking them on media containing no T or L, respectively (to maintain the plasmids) and no A or H. No growth was detected on any plate after five days.

For the B-class genes, no interactions were detected after five days of incubation at either temperature, which confirms previous reports that full length B-genes reveal no interactions in a YTH system (Yang et al. 2003).

For the C-class, the usual incubation time of five days, as suggested by the protocol followed, was increased to 20 days since many interactions were too weak to be detected after five days. An interaction was counted as positive if single colonies were detected on at least one of the four plates. However, the longer incubation time for these interactions as compared to the controls containing only BD or AD vectors increased the possibility of false positives due to autoactivation which was not detected on the fifth day control plates. The results for the C-genes are shown in table 1 and summarized in table 2, which also shows the results of previous studies.
Table 1: The genes tested in this yeast-two-hybrid screen and the result for *AGAMOUS* (*AG*) and *DEFICIENS AGAMOUS LIKE 2* (*DAL2*) at 22°C and 30°C.

<table>
<thead>
<tr>
<th>name 1</th>
<th>name 2</th>
<th>DAL2</th>
<th>AG</th>
<th>DAL2</th>
<th>AG</th>
<th>DAL2</th>
<th>AG</th>
<th>DAL2</th>
<th>AG</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>30°C</td>
<td>22°C</td>
<td>30°C</td>
<td>22°C</td>
<td>30°C</td>
<td>22°C</td>
<td>30°C</td>
<td>22°C</td>
</tr>
<tr>
<td>P1</td>
<td></td>
<td>AGL34</td>
<td>x</td>
<td>x</td>
<td>AGL73</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AP3</td>
<td></td>
<td>AGL35</td>
<td>x</td>
<td>x</td>
<td>AGL74N</td>
<td>x</td>
<td>x</td>
<td>AGL77</td>
<td></td>
</tr>
<tr>
<td>AG</td>
<td></td>
<td>AGL36</td>
<td>AGL74-I</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AGL1</td>
<td>SHP1</td>
<td>AGL75</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AGL2</td>
<td>SEP1</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>AGL76</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AGL3-I</td>
<td>SEP4-I</td>
<td>AGL77</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AGL4</td>
<td>SEP2</td>
<td>x</td>
<td>x</td>
<td>AGL78</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AGL5</td>
<td>SHP2</td>
<td>AGL79</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AGL6</td>
<td></td>
<td>AGL80</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AGL7</td>
<td>AP1</td>
<td>AGL81</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AGL8</td>
<td>FUL</td>
<td>AGL82</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AGL9</td>
<td>SEP3</td>
<td>x</td>
<td>x</td>
<td>AGL83</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AGL10</td>
<td>CAL1</td>
<td>ND</td>
<td>ND</td>
<td>AGL84</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AGL11</td>
<td>STK</td>
<td>x</td>
<td>AGL85</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AGL12</td>
<td></td>
<td>AGL86</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AGL13</td>
<td></td>
<td>AGL87</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AGL14</td>
<td></td>
<td>AGL88</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AGL15</td>
<td></td>
<td>AGL89</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AGL16</td>
<td></td>
<td>AGL90</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AGL17</td>
<td></td>
<td>AGL91</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AGL18</td>
<td></td>
<td>AGL92</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AGL19</td>
<td></td>
<td>AGL93</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AGL20</td>
<td>SOC1</td>
<td>x</td>
<td>AGL94</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AGL21</td>
<td></td>
<td>AGL95</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AGL22</td>
<td>SVP</td>
<td>x</td>
<td>AGL96</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AGL23</td>
<td></td>
<td>AGL97</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AGL24</td>
<td></td>
<td>x</td>
<td>AGL98</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AGL25</td>
<td>FLC/FLF</td>
<td>x</td>
<td>x</td>
<td>AGL99</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AGL26</td>
<td></td>
<td>x</td>
<td>AGL100</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AGL27</td>
<td>MAF1/FLM</td>
<td>x</td>
<td></td>
<td>AGL101</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AGL28</td>
<td></td>
<td>x</td>
<td>AGL102</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AGL29</td>
<td></td>
<td>x</td>
<td>AGL103</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AGL30</td>
<td></td>
<td>x</td>
<td>AGL104</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AGL31</td>
<td>MAF2</td>
<td>x</td>
<td>AGL105</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AGL32-I</td>
<td>ABS/TT16</td>
<td>x</td>
<td></td>
<td>AGL106</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AGL32-II</td>
<td>ABS/TT16</td>
<td>x</td>
<td></td>
<td>AGL107</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*AG* and *DAL2* were cloned into binding domain vectors, the genes in the rows into activation domain vectors (these genes were given a standardized name when first discovered (name 1) and a more descriptive name if later their function was elucidated (name 2)). An x denotes that yeast cells containing both vectors grew on plates lacking histidine, plates lacking adenine, or both, when incubated at the corresponding temperature. The last row shows the number of interactions of *AG* and *DAL2* detected at each temperature.
Table 2: Interactions of AG and DAL2

<table>
<thead>
<tr>
<th>Interactions with Reference</th>
<th>PI</th>
<th>AP3</th>
<th>AG</th>
<th>AGL2(SEP1)</th>
<th>AGL4(SEP2)</th>
<th>AGL6</th>
<th>AGL7(AP1)</th>
<th>AGL9(SEP3)</th>
<th>AGL15</th>
<th>AGL16</th>
<th>AGL20(SOC1)</th>
<th>AGL21</th>
<th>AGL24</th>
<th>AGL25(FLC/FLF)</th>
<th>AGL31(MAF2)</th>
<th>AGL32-II(ABS/TT16)</th>
<th>AGL34</th>
<th>AGL35</th>
<th>AGL39</th>
<th>AGL44(ANR1)</th>
<th>AGL44(AnR1)</th>
<th>AGL62</th>
<th>AGL66</th>
<th>AGL74N</th>
<th>AGL79</th>
<th>AGL97</th>
<th>AGL99</th>
<th>DAL1</th>
</tr>
</thead>
<tbody>
<tr>
<td>AG</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>/</td>
<td>/</td>
<td>/</td>
<td>/</td>
<td>/</td>
<td>x</td>
<td>x</td>
<td>/</td>
<td>/</td>
<td>/</td>
<td>/</td>
<td>/</td>
<td>/</td>
<td>/</td>
<td>/</td>
<td>/</td>
<td>/</td>
<td>/</td>
<td>/</td>
<td>/</td>
<td>/</td>
<td>DAL2</td>
</tr>
<tr>
<td>AG</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>/</td>
<td>/</td>
<td>/</td>
<td>/</td>
<td>/</td>
<td>x</td>
<td>x</td>
<td>/</td>
<td>/</td>
<td>/</td>
<td>/</td>
<td>/</td>
<td>/</td>
<td>/</td>
<td>/</td>
<td>/</td>
<td>/</td>
<td>/</td>
<td>/</td>
<td>/</td>
<td>/</td>
<td>DAL2</td>
</tr>
<tr>
<td>AG</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>/</td>
<td>/</td>
<td>/</td>
<td>/</td>
<td>/</td>
<td>x</td>
<td>x</td>
<td>/</td>
<td>/</td>
<td>/</td>
<td>/</td>
<td>/</td>
<td>/</td>
<td>/</td>
<td>/</td>
<td>/</td>
<td>/</td>
<td>/</td>
<td>/</td>
<td>/</td>
<td>/</td>
<td>DAL2</td>
</tr>
<tr>
<td>AG</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>/</td>
<td>/</td>
<td>/</td>
<td>/</td>
<td>/</td>
<td>x</td>
<td>x</td>
<td>/</td>
<td>/</td>
<td>/</td>
<td>/</td>
<td>/</td>
<td>/</td>
<td>/</td>
<td>/</td>
<td>/</td>
<td>/</td>
<td>/</td>
<td>/</td>
<td>/</td>
<td>/</td>
<td>DAL2</td>
</tr>
<tr>
<td>AG</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>/</td>
<td>/</td>
<td>/</td>
<td>/</td>
<td>/</td>
<td>x</td>
<td>x</td>
<td>/</td>
<td>/</td>
<td>/</td>
<td>/</td>
<td>/</td>
<td>/</td>
<td>/</td>
<td>/</td>
<td>/</td>
<td>/</td>
<td>/</td>
<td>/</td>
<td>/</td>
<td>/</td>
<td>DAL2</td>
</tr>
<tr>
<td>AG</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>/</td>
<td>/</td>
<td>/</td>
<td>/</td>
<td>/</td>
<td>x</td>
<td>x</td>
<td>/</td>
<td>/</td>
<td>/</td>
<td>/</td>
<td>/</td>
<td>/</td>
<td>/</td>
<td>/</td>
<td>/</td>
<td>/</td>
<td>/</td>
<td>/</td>
<td>/</td>
<td>/</td>
<td>DAL2</td>
</tr>
</tbody>
</table>

An x indicates that the product of a gene in the top row interacted with the product of the gene in the first column, according to the reference in the second column. The references are: 1) This study. Very weak interactions are denoted by “/” 2) Erika Groth’s studies (unpublished results), full length proteins, incubated at 30°C. 3) Favaro et al. (2003), full length proteins, incubated at 30°C. 4) de Folter et al. (2005), full length proteins, incubated at 20°C. 5) Fan et al. (1997), KC domains only, incubated at 30°C (these interactions were confirmed by immunoprecipitation experiments). 6) Riechmann et al. (1996a), immunoprecipitation experiments only.

23 interactions were detected at 22°C but not at 30°C (most pronounced in DAL2 x AGL15, AGL16 and AGL20 and AG x AGL24, AGL74N and DAL1) and no interaction was detected at 30°C only. Five proteins interacted with both DAL2 and AG only at 22°C (AGL25, AGL34, AGL44, AGL62 and DAL1, most pronounced in AGL25, AGL62 and DAL1). The interactions AG x AGL31, AGL32-II, AGL34, AGL35 and AGL66 and DAL2 x AGL11 and AGL44 were very weak as judged intuitively by colony size and number.

The interaction patterns of AG and DAL2 in general are highly similar: They behaved the same (that is both interacted or both did not interact) for 94 out of 106 tested proteins (89%). Two proteins were found to interact with DAL2 only, ten interacted with AG only and twelve with both (table 2).

Measurements β-galactosidase activity were performed for one protein that interacted with both AG and DAL2 (SEP1), one that interacted with none of them (PI), two proteins that seemed to differ in their interaction strength with AG and DAL2 (SEP3 and SOC1), and for one protein which was previously (Erika Groth 2006, unpublished results) but not in this study found to interact with AG (AGL79) (figure 3). The expression levels of the reporter gene encoding β-galactosidase depend on how strong the proteins interact, and β-galactosidase activity therefore reflects interaction strength.

The interactions that were judged negative on plates lacking H or A showed background β-galactosidase activities of 3.6-7.2. The interaction AG x SEP1 was 100 times as strong as this background and ten times as strong as DAL2 x SEP1 while DAL2 x SEP3 showed no activity.
higher than background. DAL2 x SOC1 was slightly above. In general these results confirmed the findings from the H and A assay except for the interaction DAL2 x SEP3 that had been judged positive but was here found to be very weak.

Figure 3: Quantitative assay of β-galactosidase activity in yeast-two-hybrid strains having either *AGAMOUS* (*AG*) or *DEFICIENS AGAMOUS LIKE 2* (*DAL2*) cloned into the binding domain vector and either *PISTILLATA* (*PI*), *SEPALATA 1* (*SEP1*), *SEP3*, *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1* (*SOC1*) or *AGAMOUS LIKE 79* (*AGL79*) into the activation vector. Expression of β-galactosidase in these cells reflected interaction strength between the encoded proteins. Of each strain, three independent samples were analyzed and each sample was measured in triplicate.
Discussion

Some interactions were not detected at 30°C
If an interaction between two proteins is crucial for correct flower development one would suppose it to be stable over the whole temperature range the plant is naturally exposed to, which for Arabidopsis clearly includes 22°C – 30°C. A yeast cell, however, is an abnormal environment for a plant protein and its folding might be disrupted at higher temperatures, while in planta correct folding is guaranteed over a wider temperature range.

An intriguing speculation is that the difference actually exists in planta and provides part of the temperature dependent floral induction pathway. This would imply a dual role for some genes in floral induction and floral formation pathways, as previously suggested by de Folter et al. (2005). It should also be noted that the 22°C incubator was exposed to daylight while the 30°C incubator was dark, which could in theory account for the observed difference.

To further characterize the differences in yeast, the quantitative β-galactosidase assay could be modified for 22°C and the interactions in question measured at both temperature relative to a stable control. To find out whether a difference exists in planta, the phenotype of a flower meristem that develops at a constant temperature of 30°C could be analyzed.

No interactions were detected for the products of full-length B-genes
The proteins encoded by Arabidopsis B-genes have been shown previously not to interact in full length in yeast but only when the MADS-domain had been removed (Yang et al. 2003). This has here been confirmed and it has been shown further that the product of the spruce gene DAL13-I, which is orthologous to angiosperm B-genes, does not interact with any of the tested MADS domain proteins in full length. It should be noted that this does not reflect the situation in planta, but is an artifact caused by the yeast environment in which the interactions were studied.

The interaction patterns of AG and DAL2 are highly similar
AG and DAL2 are phylogenetically closely related and cause similar phenotypes when ectopically expressed in Arabidopsis (Tandre et al. 1998). This suggests that DAL2 is functionally similar to AG and able to interact with the same Arabidopsis proteins. This study showed that the two proteins did indeed have similar interaction patterns when analyzed in yeast, yet they differed in some interesting aspects (see below). Since many interactions known to exist in planta are not revealed in a YTH system with full length proteins, further analysis is required to see which differences in the interaction patterns of AG and DAL2 really exist in planta. Such should include YTH studies with proteins lacking the MADS domain (as has been done for the B-genes (Yang et al. 2005)) or immunoprecipitation experiments. It could also be worth while to study the behavior of a hybrid protein containing, for example, the M, I and C domains of AG but the K domain of DAL2 (as has been done for AP1, AP3, AG and PI by Riechmann et al. (1996)) or to see whether DAL2 can substitute for AG if expressed in AG mutants (as is the case for CyAG, a Cycad ortholog of AG (Zhang et al. 2004)).

DAL2 interacted with SOC1, AG did not
One of the tested Arabidopsis proteins, SOC1 interacted with the spruce protein DAL2 but not with its Arabidopsis ortholog, AG. Orthologs of SOC1 have been found in spruce but they have not yet been analyzed in a YTH system. SOC1 is an important regulator of flowering time in Arabidopsis (Onouchi et al. 2000, Samach et al. 2000), and the spruce orthologs of SOC1 might

10
be a good starting point to study the regulation of reproductive versus vegetative growth in gymnosperms.

**DAL2 interacted strongly with SEP1**
The interaction between AG and the products of the SEP-genes is important for their activity in *Arabidopsis* (Honma and Goto 2001, Theissen and Saedler 2001). Although the SEP-genes are believed to be angiosperm specific (Zahn et al. 2005) and no orthologs of the SEP-genes have been found in Spruce, DAL2 interacted with SEP1. This could mean that there is a SEP-ortholog in spruce that has not yet been characterized. Alternatively, the interaction properties of DAL2 could have been conserved since the emergence of angiosperms while the SEP-lineage has been lost in gymnosperms.

**Phylogenetic relationship of the genes whose proteins interacted**
All genes whose proteins interacted strongly with AG group into the MIKC and the Mα clades of MADS-box genes which are more closely related to each other than to the other three MADS clades (Parenicova et al. 2003). Almost all the genes whose products interact with DAL2 are also found in these two clades, except *AGL34*, which is located in the Mγ clade. Since proteins in this clade lack the K-domain, which is known to be important for interactions between MIKC proteins (Yang et al. 2003), further analysis of the interaction between *AGL34* and AG and DAL2 could reveal alternative interaction mechanisms of MADS proteins.

**Conclusions and perspectives**
I showed here that the spruce gene *DAL2*, an ortholog of angiosperm C-class genes, is functionally very similar to the *Arabidopsis* C-class gene *AG*. While the context in which AG works has been elucidated (Honma and Goto 2001), little is known about the homeotic MADS-box genes in spruce. Since DAL2 is able to bind to SEP1, the product of one of the *Arabidopsis* E-class genes, it is possible that orthologous genes exist in spruce and are involved in the regulatory network of reproductive organ development. Screens for orthologs of A-, B-, C- and E-genes in spruce and their functional characterization through interaction studies, ectopic expression in *Arabidopsis* and mutant analysis could lead to a model similar to the floral quartets in spruce.
Materials and methods
Yeast strain and plasmids
I used the yeast strain PJ69-4A (MATa trp1-901 leu2-3,112 ura3-52 his3-200 gal4Δ gal80Δ LYS::GAL1-HIS3 GAL2-ADE2 met2::GAL7-lacZ) and the plasmids pGAD-C and pGBD-C as constructed by James et al. (1996). The yeast strain contains three reporter genes (HIS3, ADE2 and lacZ) under the control of three different GAL4 inducible promoters, and a GAL4 deletion. The plasmids basically encode, under the control of the ADH1 promoter, the GAL4 activation domain (AD) or the GAL4 binding domain (BD) followed by a polylinker site to clone in the gene in question. The AD vector contains LEU2, The BD vector TRP1 and transformed yeast cells can thus be selected on media lacking leucine or tryptophan, respectively. The plant genes had been cloned into the plasmid vectors, and these transformed into yeast, by Erika Groth.

Media and solutions
I prepared the full medium YPAD and synthetic defined (SD) media lacking one to three of the compounds leucine (L), tryptophan (T), adenine (A) and histidine (H) as described in the PorQuest™ yeast-two-hybrid manual (Invitrogen). Since the his3-200 mutation is leaky, 3-Amino-1,2,4-Triazole (3AT), a histidine analog, was added to SD-H media (to a concentration of 5µM) to prevent false positives.

Yeast two-hybrid analysis
I followed the The ProQuest™ two-hybrid-system (Invitrogen) for yeast two-hybrid analysis. In short, yeast cells transformed with the BD- and AD- vectors containing the Plant genes in question were selected on SD-T and SD-L plates, respectively. From these stocks, overnight cultures were grown in the corresponding selective liquid media. The strains were systematically mated by dropping 5µl overnight culture over each other on YPAD plates and incubating over night at 30°C. The mated cells were then streaked on SD–LT plates to select for cells containing both plasmids, grown for four days at 30°C and thereafter stored at 4°C. All combinations were streaked on two plates of each SD–LTA and SD–LTH+5µM 3AT, one of each was incubated at 30°C and one at 22°C. The plates were assayed after five days for the B-class genes (AP3, PI and DAL13-I) and after 20 days for the C-class genes (AG and DAL2). Yeast cells containing only the BD-vectors were streaked on SD–LA and SD–LH plates and cells containing only the AD vectors on SD–TA and SD–TH plates to control for autoactivation.

Quantitative β-galactosidase assay
β-galactosidase activity was assayed with cells that were harvested in mid-log phase using chlorophenol red-β-D-galactopyranoside (CPRG) as a substrate, following the ProQuest™ manual (Invitrogen). In short, β-galactosidase activity was inferred by photometrically determining the amount of chlorophenol red that had been released from CPRG by β-galactosidase activity in the sample.
Acknowledgements
I want to thank Peter Engström for making it possible for me to take part in this interesting project and all the people in the institute for generously helping me when things weren’t so clear. This study was done with supervision from doctorand Erika Groth at the Department of Physiological Botany, Uppsala University, Sweden.
References


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


Krizek BA, Meyerowirz EM (1996) The *Arabidopsis* homeotic genes APETALA3 and PISTILLATA are sufficient to provide the B class organ identity function. Development 122:11-22

Ma H (1994) The unfolding drama of flower development: Recent results from genetic and molecular analyses. Genes and Development 8:745-756


Mizukami Y, Ma H (1995) Separation of AG function in floral meristem determinacy from that in reproductive organ identity by expressing antisense AG RNA. Plant Molecular Biology 28:767-784


