Evolution of Genetic Mechanisms Regulating Reproductive Development in Plants

Characterisation of MADS-Box Genes Active during Cone Development in Norway Spruce

BY

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ABSTRACT

The reproductive organs of conifers and angiosperms differ in morphology in several fundamental respects. The conifer Norway spruce (Picea abies) form pollen and seed cones from separate meristems whereas angiosperms bear bipartite flowers with sepals and petals surrounding two inner whorls of stamens and carpels. Despite these differences in morphology this thesis present data to suggest that reproductive development in conifers and angiosperms is regulated by a similar molecular mechanism. This implies an evolutionary conservation of the major mechanism for reproductive development since the origin of seed plants.

Flower organ identity in angiosperms is determined by regulatory genes belonging to the MADS-box gene family of transcription factors. This thesis presents the cloning and characterisation of four novel MADS-box genes from Norway spruce. Three of these genes DAL11, DAL12 and DAL13 are most closely related to angiosperm B function genes i.e. genes required for petal and stamen development. DAL11, 12 and 13 all are specifically active in developing pollen cones, with different temporal and spatial expression pattern. Functional analysis in transgenic Arabidopsis and yeast suggest that the reproductive aspect of the B-function is conserved between conifers and angiosperms. The results also suggest the B-function in conifers to be separated into one shoot identity and one organ identity determinant.

A fourth gene presented; DAL10, is specifically expressed in vegetative parts of pollen- and seed cones. Phylogenetically DAL10 is not closely related to any of the known angiosperm clades, but rather forms a separate clade with other gymnosperm genes, suggesting a gymnosperm specific function. We suggest that the DAL10 activity reflects a function in the determination of the reproductive shoot.

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ABBREVIATIONS

aa       amino acids
AGI      Arabidopsis Genome Initiative
bp       base pairs
mya      million years ago
RACE     rapid amplification of cDNA ends
RT-PCR   reverse transcriptase polymerase chain reaction

The following nomenclature will be used in this thesis:
Names of genes are written in italicised upper-case letters, e.g. *AGAMOUS*.
Names of proteins are written in non-italicised upper-case letters, e.g. AGAMOUS
Names of mutants are written in italicised lower-case letters, e.g. *agamous*
The field of evolutionary developmental biology

Perhaps the most intriguing question that has engaged biologist over the years is: How did the diversity of life on earth evolve? Darwin (1859) provided an ecological explanation where natural selection within a changing environment favoured individuals that could adapt and allowed its traits to be spread to the next generation. He argued that natural variation of traits, he did not know of genes, within a population provided the base upon which selective pressure could act to evolve new features.

The Neo-Darwinian synthesis explained how the mechanisms of mutation, selection, and genetic drift worked on the genetic level to produce the directional changes in gene frequencies that underlie the evolution of novel traits (Huxley 1963). In the late 1960s protein sequences became available to the scientific community and for the first time it was possible to compare differences in gene frequencies between different species. Kimura (1977) postulated that mutations likely to be preserved to the next generation are neutral with respect to protein function. In agreement with this Doebley and Lukens (1998) argued that mutations that cause morphological changes in plants are likely to occur in cis-regulatory elements of genes and not in the protein coding sequence. Moreover, mutations that are preserved to the next generation are likely only to have mild pleiotropic effects on plant development. They found that most genes in this category encoded genes which regulate different developmental pathways by affecting the transcriptional activity of down-stream target genes, i.e. working as transcription factors.

Transcription factors are often grouped, based on sequence similarities, in families with representatives from distantly related species. This notion has led to the emergence of a whole new discipline in the interface between traditional evolutionary biology and developmental biology. The ‘evo-devo’ research field, as it popularly has been called, integrates phylogeny and evolutionary theories with molecular developmental methods and concepts.
In plants, many of the genes involved in the regulation of body plan architecture have been shown to encode transcription factors that belong to the MADS-box gene family, see Theissen and Saedler (1999) and references therein. The work done in our group focuses on the evolutionary history of the MADS-box gene family. We have tried to trace the duplication history of this gene family, to see if morphological novelties of importance for the evolution of vascular plants have coincided with an increase in the complexity of the gene family. We have also done comparative analyses of orthologous MADS-box genes from distantly related species within the seed plant lineage. In this thesis I have focused on genes involved in reproductive development in seed plants.

According to fossil data, the first seed plants appeared in the late Devonian about 350 mya (Stewart and Rothwell 1993.). Extant seed plants comprise five different groups; angiosperms, conifers, cycads, ginkgo and the gnetales. Molecular data suggest that they had a common ancestor ca 285 mya, in the Late Carboniferous (Savard et al. 1994). Recent analyses of molecular data suggest that gymnosperms i.e. conifers, cycads, ginkgo and gnetales form a monophyletic group with the angiosperms as a sister group (for a recent review see Donoghue and Doyle (2000). Although morphologically distinct, angiosperms and gymnosperms share characters, such as the ovule and tracheids. Both groups are also characterised by being heterosporous; seed plants form micro- and mega-spores from separate organs. Gymnosperms have exposed seeds, and the reproductive organs are organised in separate micro- and megastrobili. The reproductive organs in angiosperms are organised in flowers; a sterile perianth surrounding male units (stamens) with female structures (carpels) in the centre.

By comparing the molecular mechanisms by which conifers regulate cone development with well-established mechanisms of angiosperm flower development, I hope to be able to address questions regarding the extent to which these mechanisms are conserved and if differences between angiosperms and gymnosperms may be related to the evolution of the flower.
Reproductive development in conifers

Coniferophyta that comprise some 50 genera with ca. 550 species (Hart 1987) is the most numerous and widely spread of the extant gymnosperm divisions. The conifer Norway spruce, *Picea abies* (Karst) is an abundant tree in temperate forests of the Eurasian continent. Norway spruce has a long generation time, in natural stands the first cones appear after about 15 years. The genome size of Norway spruce is very large; approximately 100 times that of Arabidopsis. Phylogenetic analyses of cloned genes and analyses of their expression pattern are methods readily available for studies of spruce development. Due to the long generation time, genetic analyses using mutants defective in different aspects of cone development is not accessible and even though Norway spruce is possible to transform, (Clapham et al. 1995; Wenck et al. 1999) these methods are of limited use when studying reproductive development.

Although Norway spruce is technically difficult to work with, the scientific value of comparing conifer and eudicot angiosperm reproductive development is significant. By comparing to what extent the genetic basis for reproductive development is conserved between Norway spruce and other species we hope to be able to determine the evolutionary relationship between reproductive development in conifers and angiosperms.

The seed cone

Norway spruce is monoecious; the microsporangia and the megasporangia of Norway spruce are born on cones on the same tree (Figure 1). Norway spruce seed cones are formed in the upper one third of the tree, in apical positions of lateral branches. The seed cone consists of spirally arranged ovuliferous scales, each subtended by a sterile bract. Comparisons between vegetative shoots and seed cones (Figure 1a and c) demonstrate the similarity in arrangement of needle primordia and ovuliferous scale/bract units along the axis of the shoot. In the spruce mutant *acrocona* intermediate structures of vegetative shoots and seed cones are produced, in these structures ovuliferous scales subtended by needles can be found. The intermediate structures found in the acrocona mutant is in agreement with fossil data that suggest the seed cone to be a
Figure 1. Reproductive and vegetative shoots of Norway spruce (Picea abies). The figure show scanning electron micrographs of a seed cone collected on the 7th of August (a), pollen cone collected on the 30th of July (b) and vegetative shoot collected on the 13th of September (c).
modified long shoot homologous to the vegetative shoot and the ovuliferous scales to be highly reduced fertile short shoots (Florin 1951.).

Seed cone development in Norway spruce extends over two growth-seasons. Cone initiation occurs in early summer and is followed by a period of ovuliferous scale/bract differentiation. Pollination occurs in the following spring and the seed develops during the following summer and autumn and is released the second winter after initiation. In natural stands cones are abundantly produced approximately every third year (Tirén 1935; Wennström 1994). Climate and temperature influence the number of cones produced (Lindgren et al. 1977). During years of high cone production, a large number of available shoots, in the upper part of the tree, produce cones instead of vegetative shoots. By studying the branching pattern of Norway spruce, (Tirén 1935) showed that a branch needs ca. two years of vegetative growth to reconstitute its morphology after the setting of a seed cone. This suggests that there are morphological constraints on cone production, which serves to reduce the number of buds consumed by cones during years of low cone production and that these constraints most likely explain the periodicity in cone production.

**The pollen cone**

Norway spruce pollen cones (Figure 1b) are most commonly produced in the lower part of the tree at the base of annual shoots, but can also be positioned in apical or lateral positions of lateral branches. Pollen cone development extends over approximately one year. Initiation occurs in the spring or during early summer. At mid-summer the first microsporophyll primordium becomes visible halfway up the cone axis. Subsequently microsporophylls are initiated along the bud axis in a spiral arrangement. Differentiation of microsporophylls occurs during approximately one month [I] and as the microsporophyll closest to the apex of the cone is initiated the apical meristem of the pollen cone is lost. In each microsporophyll two microsporangia harbouring pollen mother cells develop. Meiosis and the subsequent shedding of pollen occur after winter dormancy in late spring. (For a description of the reproductive development in American *Picea* species see (Fraser 1965; Harrison and Owens 1982; Owens and Moulder 1977; Tompsett 1977)) Pollen cones are considered to be simple structures that
differ in their branching order as compared to the compound structure of seed cones, such that the entire pollen cone corresponds to one ovuliferous scale. This is in agreement with fossil data that suggest the conifer pollen cone to be a reproductive short shoot (Banks 1972).

**Reproductive development in angiosperms**

Angiosperms, flowering plants, have ovules enclosed by a carpel (*angiosperm* meaning vessel seed) and reproductive organs arranged in flowers. According to fossil data angiosperms as a group diverged from a common ancestor seed plant during the early Cretaceous, 130-90 mya (Crane *et al.* 1995). The angiosperm phylogeny have recently been resolved by combined analyses of multiple data sets. (Mathews and Donoghue 1999; Parkinson *et al.* 1999; Qiu *et al.* 1999; Soltis *et al.* 1999). These analyses all place Amborellaceae, which consist of the single species Amborella trichopoda as a sister to all flowering plants, with Nymphaeales (water lilies) as the closest relatives. The remaining angiosperms fall into a basal assemblage of Magnoliid dicots, were two monophyletic groups can be recognised, the monocots and the eudicots (APG 1998).

**The ABC model**

Much of the recent research on flower development has been concentrated to a few eudicot species. The flower of eudicots is typically composed of a bipartite perianth with sepals in the outermost first whorl of organs and petals in the second whorl, followed by stamens in the third and carpels in the fourth whorl (Figure 2). A model proposed by (Coen and Meyerowitz 1991) suggests that three gene functions; A, B, and C act in a combinatorial manner to specify the identity of the organs in the different whorls. A alone specifies sepals, A in combination with B specify petals, B together with C stamens, and C alone specifies carpel identity. In addition to acting as organ identity determinants the model predicts that A and C function regulate each other negatively and that the C function is involved in termination of the floral meristem (Figure 3). This model was originally based on analysis of mutants from two eudicot species, Arabidopsis thaliana and Antirrhinum majus (Bowman *et al.* 1991; Meyerowitz 1992; Schwarz-Sommer *et al.* 1990; Sommer *et al.* 1990).
Figure 2. Schematic organisation of flower organs in Arabidopsis, Amborella and a grass flower. Left: The Arabidopsis flower consists of four types of organs organised in four separate whorls, a central carpel is surrounded by six stamens followed by four petals and four sepals. Middle: The female flower of Amborella consists of spirally arranged organs, five central carpels followed by two staminoides, eight tepals (perianth organs that are not differentiated as sepals or petals) and three subtending bracts. Male flowers have a similar organisation although stamens in place of outer carpels (not shown). Right: A typical grass flower consists of a central carpel surrounded by three stamens. The perianth is built up by two lodicules (abaxial), a palea (abaxial) and a lemma (adaxial).

In Arabidopsis, genes belonging to the MADS-box gene family of transcription factors carry out all, or part of, the A, B and C functions. APETALA1 (AP1) is part of the A function (Gustafson-Brown et al. 1994; Mandel et al. 1992), APETALA3 (AP3) and PISTILLATA (PI) constitute the B function (Goto and Meyerowitz 1994; Jack et al. 1992) and the C function is mediated by the AGAMOUS (AG) gene (Bowman et al. 1991; Mizukami and Ma 1992; Yanofsky et al. 1990). Recently, Pelaz and co-workers have shown that B and C functions in Arabidopsis require the activity of three closely related and functionally redundant MADS-box genes, SEPALLATA1, 2 and 3 (SEP1, 2 and 3) (Pelaz et al. 2000). The sep1, 2 and 3 triple mutant develop sepaloid organs in all four whorls. Similarly the C function in Gerbera hybrida has been shown to be dependent on the expression of GRCD1, an SEPI-like MADS-box gene (Kotilainen et al. 2000). In addition to the A, B, and C functions some authors (Angenent et al. 1995; Colombo et al. 1995) have proposed the existence of a D-function, which is to specify ovules. This notion is based on studies in Petunia hybrida where the two MADS-box genes FBP7 and FBP11 have been shown to be necessary and sufficient for ovule development. The Arabidopsis ortholog of FBP7 and FBP11 is AGL11, which is
expressed specifically in developing ovules and associated placental tissue (Rounsley et al. 1995).

**The MADS-box gene family**

**The MIKC-organisation**

As noted above, many of the genes involved in floral development encode transcription factors belonging to the MADS-box gene family. MADS is an acronym for the four founder proteins; MCM1 from *Saccharomyces cerevisiae*, AGAMOUS from Arabidopsis, DEFICIENS from *Antirrhinum majus* and SRF from *Homo sapiens* (Schwarz-Sommer et al. 1990). These genes all contain a highly conserved motif, the MADS-box, which encodes a DNA-binding domain (Passmore et al. 1988). The MADS-domain (M), in plant proteins, is located in the N-terminus of the protein. It is followed by a less conserved intervening region (I), which sometimes also is called the linker, a moderately conserved K-domain (K) which is involved in protein-protein interactions (Ma et al. 1991) and a variable C-terminal extension (C) (Ma et al. 1991; Pnueli et al. 1991). More than 80 MADS-box genes have been identified in the newly sequenced genome of the angiosperm Arabidopsis (Riechmann and Ratcliffe 2000) and about 40 of these genes encode proteins that have the MIKC organisation described above, whereas most of the others encode proteins that lack the K-domain (Alvarez-Buylla et al. 2000b; Svenson 2000). MADS-box genes of MIKC type have been cloned from a wide range of plant species and different genes have been suggested to be active in almost all phases of plant development from root development to specification of reproductive structures (for a recent review see Theissen et al. (2000)).

**Interactions between MADS-box genes**

MADS-box genes have been shown to regulate the activity of other genes by binding to their promoter regions as dimers. The proteins encoded by the Arabidopsis A and C function genes, *API* and *AG*, form homodimers. AP3 and PI, and their orthologs in *Antirrhinum*, DEFICIENS (DEF) and GLOBOSA (GLO), however, form heterodimers in vitro and compelling genetic data suggest that they also act as heterodimers in vivo (Davies et al. 1996; Krizek and Meyerowitz 1996a; McGonigle et al. 1996; Riechmann et al. 1996a; Riechmann et al. 1996b). The ABC model predicts an interaction between
Figure 3. The ABC-model as presented by Coen and Meyerowitz 1991. Organ identity in the angiosperm flower is determined by three gene functions A, B and C. The genes that mediate these gene functions are expressed in an overlapping manner already in the floral meristem (left) and continuous expression throughout organ development are needed to maintain organ identity (right). A alone specifies sepals. Expression of A in combination with B specifies petals. B together with C stamens and C alone specify the carpel.

B-proteins and A-proteins in whorl two and between B-proteins and C-proteins in whorl three. How this interaction is mediated is yet not known. It might be a direct physical interaction or an indirect interaction mediated by other, still unknown secondary factors. The DEF/GLO complex in Antirrhinum can interact with the meristem identity protein SQUAMOSA and form a ternary complex in yeast (Egea-Cortines et al. 1999). This interaction adds to the complexity of the regulatory function mediated by different MADS-box genes and might be the molecular basis for the establishment of whorled phyllotaxis and combinatorial interactions of floral organ identity genes.

The Phylogeny of the MADS-box gene family

The phylogeny of the MADS-box gene family is characterised by gene duplications followed by structural divergence of the duplicated genes (Doyle 1994; Purugganan et al. 1995; Tandre et al. 1995; Theissen et al. 1996). Previous analyses have shown that many MADS-box genes from different seed plants fall into functionally related clades (Tandre et al. 1995; Theissen et al. 1996). For example such that A and C function genes from different species form separate clades. The B- function genes form two separate clades, defined by AP3 and PI respectively, but the entire B-class clade is monophyletic. The C-class clade, defined by AGAMOUS, also includes the Arabidopsis paralogs AGL1 and AGL5, supposedly due to a recent duplication of the C-class genes.
within the eudicot lineage. The *agl1* and *agl5* double mutant is defective in the
development of the carpel dehiscence zone and the genes have consequently been
renamed *SHATTERPROOF1* and 2 (Liljegren et al. 2000). Thus, even though it is
possible to assign functional conservation between major clades of MADS-box genes,
like the floral homeotic A, B and C class genes, recent duplications within a clade may
be a cause for deviation from this general statement. Recent analysis of genes active
during vegetative development in Arabidopsis suggests that the functional conservation
found among A, B and C class genes holds true also for other aspects of plant
development (Alvarez-Buylla et al. 2000a).

The recent completion of the Arabidopsis genome makes it possible to include all
MADS-box genes from a single plant in a phylogenetic analysis for the first time and it
will, thus, be possible to define all major groups present within Arabidopsis. If these
clades represent the whole diversity of angiosperm MADS-box genes remains an open
question. However, it is likely, given the rapid divergence of angiosperms, that at least
the core eudicots possess similar sets of MADS-box genes. To resolve whether or not
this hypothesis is valid also for monocots and basal angiosperms a broader sampling
from such species is needed.

MIKC type MADS-box genes have been cloned from a number of species outside of the
seed plant lineage including ferns, mosses and lycopsids (Hasebe et al. 1998; Munster et
al. 1997; Svensson et al. 2000). None of the genes cloned from plants outside of the
seed plant lineage appears as an ortholog to any of the seed plant genes. However the
non-seed plant genes cloned do not form a monophyletic clade basal to all seed plants
either, but rather appear as separate groups dispersed among the seed plant genes. This
suggests that part of the duplication history of the MIKC type MADS-box genes is
confined to seed plants and possibly coincided with the evolution of novel features such

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**Figure 4.** Phylogenetic interrelationship of plant MADS-box genes. Gene sequences were
compiled from GenBank except for DAL11, DAL12, and DAL13 that derive from this study [II].
The phylogeny was established with maximum parsimony using an alignment of nucleotide
sequences of the MADS- and K boxes. The tree presented is a 50% majority rule consensus
tree of eight equally parsimonious trees. Bootstrap support is indicated on major branches.
Subfamilies are labelled after an Arabidopsis representative of the respective clade. Genus
names for the species from which the genes were isolated are given next to the gene names.
as heterospory, the ovule, the seed coat or the organisation of the perianth (Tandre et al. 1995; Theissen et al. 1996). The phylogeny of the family also indicates that parts of the duplication history of the MADS-box gene family have preceded the evolution of seed plants and that a set of MADS-box genes was present in the last common ancestor of all seed plants. However, given the rapid divergence and complicated duplication history of the MADS-box gene family the deep branches of the phylogenetic tree (Purugganan et al. 1995; Tandre et al. 1995) to this date are difficult to resolve.

In the following sections I will first go through the present knowledge regarding to what extent the ABC-model applies to angiosperms outside of the core eudicots, in basal angiosperms and monocots, and then present our own data regarding MADS-box genes active during cone development in Norway spruce and discuss how these data relate to the ABC model.

**Is the ABC model valid outside of the core eudicots?**

The flowers of *Amborella* consist of spirally arranged organs and are functionally unisexual, but they have a bisexual organisation as the female flowers usually have one or two staminodes outside of the gynoecium (Figure 2). Both female and male flowers are surrounded by tepals, which from centre and out gradually become bract-like (Endress and Igersheim 2000). The structure of the *Amborella* flower, provided that it reflects the structure of the first primitive flower, suggests that the ancestral angiosperm flower only consisted of stamens and a carpel surrounded by bracts, whereas the perianth may be a derived character appearing in later angiosperm lineages (Irish 2000). Provided that all angiosperm flowers are defined by the ABC system this implies that at least the C function is preserved within the angiosperm lineage. MADS-box genes orthologous to the Arabidopsis C-function gene *AGAMOUS* have been cloned from a number of angiosperm species including both core eudicots (Tsuchimoto *et al.* 1993; Yanofsky *et al.* 1990); and monocots (Schmidt *et al.* 1993; Theissen *et al.* 1995). A-class genes have been cloned both from eudicots and monocots. The function of the A-class genes however, differs even between different eudicot species. floral meristem identity (Huijser *et al.* 1992) and it has been hypothesised that this might reflect the
ancestral A-function (Theissen *et al.* 2000). This also implies that the A-function *i.e.*
sepal and petal identity determinant, is a derived character. The B-function as defined
by the core eudicot model species is dual; B-genes are involved in specification of both
petals and stamens. Two recent reports regarding B-class gene The A-class gene
*SQUAMOSA* from *Antirrhinum* is involved in the determination of the activity in basal
angiosperms and in monocots have provided new information regarding to what extent
the B function is conserved in these groups of angiosperms (Ambrose *et al.* 2000;

**Expression of B-class genes in basal angiosperms**

Analyses of expression patterns of *AP3* and *PI* orthologs in the lower eudicot subclass
Ranunculidae show that these genes are active in stamens throughout floral
development (Kramer, E. M. and Irish 1999). This is a conserved expression pattern as
compared to higher eudicot species and indicates that the reproductive part of the B-
function is conserved. The *AP3* and *PI* orthologs are also expressed in petal primordia
in the Ranunculidae examined, but as the flower develops, expression is reduced in
these floral organs. In higher eudicot taxa such as Arabidopsis and *Antirrhinum*
continuos expression of the B-class genes are required for normal petal development.
These data suggest that in Ranunculidae *AP3* and *PI* representatives have been recruited
to new tissue- or cell-type specific roles in the petals and that other genes may have
been recruited to perform the role of specifying petal identity either alone or in concert
with the *AP3* and *PI* orthologs (Kramer, E. M. and Irish 1999). In Ranunculidae the
petals are considered to be derived from stamens (Takhtajan 1991) and the expression
pattern of the *AP3* and *PI* orthologs in petal primordia are consistent with that idea.
However, in the magnoliid dicot *Michelia figo* the perianth organs, the tepals, are
thought to be derived from bracts. The *AP3* and *PI* orthologs are expressed in tepal
primordia in *M. figo*. If this is due to an independent recruitment of B-genes to these
organs or if the tepals of *M. figo* are indeed derived from stamens is still elusive
(Kramer, E.M. and Irish 2000).
**B genes in monocots**

Grasses have flowers that contain a central carpel and stamens surrounded by sterile organs known as paleas, lemmas and lodicules (Figure 2). The lemma and palea are considered to be bract-like structures. Lodicules have been differently interpreted as modified petals or as novel structures of the grass flower (Clifford 1988; Cocucci and Anton 1988). The maize *silky* mutant has stamens transformed into carpels and lodicules replaced with structures, which resemble paleas or lemmas. This is in agreement with the idea that the *silky* mutant is a B-type mutant. The *SILKY* gene is orthologous to the Arabidopsis *AP3* gene and the expression of the *SILKY* gene is restricted to stamens and lodicules. These results provide molecular evidence for recognising lodicules as modified petals (Ambrose *et al.* 2000). This notion is based on the assumption that it is possible to assign homology by comparing the activity of orthologous genes (for a detailed discussion about homology see Svensson 2000). However, this may also be a case of parallel evolution where the AP3 orthologs have been independently recruited in the core eudicot lineage and the monocot lineage to specify petals and lodicules respectively (Irish 2000).

These results suggest that the reproductive part of the B function is shared among angiosperms and most probably separated from the vegetative B-function, which may have evolved differently within the angiosperm lineage.
RESULTS AND DISCUSSION

Is the ABC model applicable to seed plants outside of the angiosperm lineage?

In the previous section I have discussed data that suggest that the ABC model might, at least in its reproductive BC sense, be valid for all angiosperms. This implies that the B and C function was present in the last common ancestor of all angiosperms. The question then arises whether or not the B and C functions regulate reproductive development in the closest sister group to the angiosperms, the gymnosperms.

If the B and C functions of the ABC model were conserved between angiosperms and conifers one would expect genes homologous to B and C type genes to be present also in conifers.

**DAL2 is a conifer C-class gene**

Evidence for the existence of plant MADS-box genes outside of the angiosperm lineage was first presented by Tandre et al. (1995) who showed that one gene from Norway spruce, **DAL2 (DEFICIENS AGAMOUS LIKE-2)** was phylogenetically closely related to the angiosperm C-class genes (Figure 4). **DAL2** is specifically expressed in pollen- and seed cones, as schematically illustrated in Figure 5. The expression pattern in pollen cones is restricted to developing microsporophylls [II] and in seed cones to the ovuliferous scale [IV](Tandre et al. 1998). Since then, **DAL2** orthologs with expression patterns similar to that of **DAL2** has also been reported from *Picea mariana* and *Gnetum gnemon* (Rutledge et al. 1998; Winter et al. 1999).

However, these data do not prove that there has been a functional conservation between spruce and angiosperm C genes, only that related genes are present and that they are active in structures that have similar function. Since no mutant data is available in conifers, an alternative method for assessing functional conservation was used. Transgenic Arabidopsis plants which constitutively expressed the spruce C-class gene, **DAL2** were produced (Tandre et al. 1998) and shown to
Figure 5. Schematic picture of expression patterns of MADS-box genes active during early pollen and seed cone development. Roman numbers indicate growth phases; apical dome formation, (I); lateral organ differentiation, (II); cell specific differentiation, (III); Shown are drawings of longitudinal sections of pollen cones (to the left) and seed cones (to the right) with the expression pattern of DAL11, DAL12, DAL13, DAL2 and DAL10. (Tandre et al. 1998) [II;III;IV]

phenocopy ectopic expression of the endogenous C-class gene AGAMOUS. Transgenic Arabidopsis flowers expressing DAL2 had carpelloid sepals and stamenoid petals demonstrating that the spruce gene could functionally substitute for AGAMOUS expression under ectopic expression conditions. Similar results were also obtained with the DAL2 ortholog from Black spruce (Rutledge et al. 1998). These experiments suggest that, even though conifers do not form flowers but rather pollen and seed cones, the C-function is conserved between conifers and angiosperms and thus likely among all seed plants. The question is then what the ancestral C-function is, since it is not obvious that carpels of angiosperm flowers, and ovuliferous scales of seed cones, are homologous organs. As DAL2 is expressed in cells of the seed and pollen cones that are
going to form mega- and micro-spores respectively, it is possible that the ancestral C-function was to specify the identity of reproductive organs (Tandre et al. 1998)[II]. Ectopic expression of AGL1 and AGL5 cause phenotypic alterations in whorl one and two as seen in plants constitutively expressing AG or DAL2. Apart from the phenotypic effects found in whorl one and two, ectopic expression of AGL1 and AGL5 also affects carpel development, an effect not found in either AG or DAL2 ectopic expressors. This suggests that the function of AG and DAL2 indeed may be conserved, whereas the AGL1 and AGL5 function may be derived later during evolution and thus represents a novelty in the angiosperm lineage.

**DAL11, DAL12 and DAL13 are conifer B-type MADS-box genes [II,III]**

The presence of C genes in conifers suggest other parts of the regulatory system to be conserved, most notably the B- function, since interaction between B and C functions are required for stamen development in Arabidopsis (Baum 1998; Tandre et al. 1995). In support of this hypothesis we have presented the cloning of three B-like MADS-box genes DAL11, DAL12, and DAL13 [II] from Norway spruce. In parallel with our work, two other groups also cloned gymnosperm B-like MADS-box genes, one from Pinus radiata (Mouradov et al. 1999) and one from Gnetum gnemon (Winter et al. 1999).

The notion that DAL11, DAL12, and DAL13 are phylogenetically related to the angiosperm B-class genes is based on a phylogenetic analysis of conserved parts of the nucleotide sequence (Figure 4) and supported by the presence of C-terminal motifs previously found specifically in angiosperm B-proteins (Kramer, E. M. et al. 1998). Angiosperm B-class genes form two separates clades and even though the phylogenetic analysis clearly positions DAL11, 12, and 13 most closely to the angiosperm B genes, the exact position of the spruce genes in relation to the two B-class clades, represented by PI and AP3, can not be clarified. We distinguish two possible hypotheses (Figure 6). The gene duplication leading to the PI and AP3 lineages occurred in the last common ancestor of conifers and angiosperms. According to this hypothesis DAL11 and DAL13 are most closely related to the PI clade and DAL12 to the AP3 clade. Alternatively, the
Figure 6. Two hypotheses regarding the position of the conifer B-like genes DAL11, DAL12 and DAL13 in relation to the angiosperm B-class genes here represented by AP3 and PI. a) The split between the angiosperm AP3 and PI clades occurred before the divergence between angiosperms and conifers, thus positioning DAL11 and DAL13 as sisters to the PI-clade and DAL12 as a sister to the AP3-clade. b) The divergence of AP3 and PI clades occurred within the angiosperm lineage, which suggest a basal position of the conifer genes in relation to the angiosperm B-class clades.

*AP3* and *PI* gene duplication is angiosperm specific and occurred after the split between angiosperms and gymnosperms, which would suggest the conifer genes to be placed basal to the whole B-class clade. Structural data within the C-terminus supports the first hypothesis; *DAL12* shares a paleo-AP3 motif with AP3 orthologs from basal angiosperms whereas *DAL11* and *DAL13* share a motif in common for all PI orthologs. The second hypothesis is supported by intron positions. None of the conifer genes have the derived intron pattern shared among angiosperm B-class genes. Instead *DAL11* and *DAL13* conform to the *AGAMOUS* intron pattern which is likely to be ancestral among MIKC-type MADS-box genes (Svenson 2000). *DAL12* has a derived intron pattern that does not resemble that of either the angiosperm B-class genes or the ancestral *AGAMOUS* pattern.
Expression patterns of conifer B-genes

DAL11, DAL12, and DAL13 are all specifically expressed in the developing pollen cone but with different temporal and spatial distributions (Figure 5), suggesting specific activities during pollen cone development [II].

DAL11 and DAL12 are expressed throughout the newly initiated pollen cone. But, as growth continues, DAL12 is only expressed until winter dormancy, whereas DAL11 is continuously expressed throughout the cone ontogeny [II, III]. DAL13 has a more restricted spatial expression pattern and is exclusively expressed in cells that are going to form microsporophylls and later in the developing microsporophylls [II, III]. These results suggest that DAL11 and DAL12 may act as shoot identity determinants, whereas DAL13 may act as an organ identity determinant. This implies that the decision to make male reproductive organs in conifers is more complex than in angiosperms and involves both a decision of shoot identity as well as a decision of organ identity [II].

Our expression analyses of the spruce genes DAL11, DAL12 and DAL13 provide independent support for the functional association of the spruce genes to the angiosperm B-class genes. These analyses are performed at the RNA level. We have performed RNA-blot experiments to examine gene expression in different tissues and in situ hybridisation for localisation of transcripts in different cell types within the pollen cone. Angiosperm MADS-box genes that are active in regulating floral development have expression patterns that rather accurately reflect their different roles in the process (Rounsley et al. 1995). In Arabidopsis, the AP3 protein has been shown to have both autonomous and non-autonomous effects on different cell layers of petals and stamens. However, the non-autonomous effect does not depend on transport of the AP3 transcript but rather on activation of different sets of downstream genes involved in cell signalling (Jenik and Irish 2001). This suggests that in situ hybridisation experiments in most cases are sufficient when analysing expression pattern of MADS-box genes and that our results reflect the activity of the spruce genes during pollen cone development.
Spruce B-like genes affect floral development when expressed in transgenic Arabidopsis

Constitutive expression of DAL11, DAL12, or DAL13 in transgenic Arabidopsis result in phenotypic alterations which in all three cases can be related to the B-function. DAL11 and DAL12 cause homeotic changes of sepals into petals. Expression of DAL13 in transgenic Arabidopsis causes a phenotype, which differs from wild type plants and plants expressing DAL11 or DAL12 in both vegetative and floral morphology. The plants were dwarfed, with pointed dark green leaves, and had flowers that opened prematurely. Flower organs were often bent and abnormally shaped due to ectopic anther tissue on sepals, petals and carpels [III].

The phenotype found in plants expressing DAL11 or DAL12 is similar to what has been found in transgenic Arabidopsis plants constitutively expressing the endogenous B-class gene PI (Krizek and Meyerowitz 1996b). However, complete conversion of first whorl sepals into petals requires ectopic expression of both AP3 and PI (Krizek and Meyerowitz 1996a). AP3 is expressed only at early stages of sepal development in wild type flowers (Jack et al. 1992). The complete conversion of sepals into petals found in flowers expressing DAL11 or DAL12 suggests that the spruce proteins may interact, either as homodimers, or as heterodimers together with AP3, with down-stream targets of the AP3/PI complex.

Apart from the homeotic conversion of first whorl organs into petals, flowers expressing DAL11 or DAL12 occasionally have altered floral organs in whorls three and four. In these flowers, stamens fail to elongate and carpels are often unfused which suggest a negative interference with the endogenous Arabidopsis B-function. The apparent contradiction with a gain of function phenotype similar to that resulting from ectopic expression of endogenous B-genes in whorl one, and loss of B-function in the third and fourth whorls, suggests that the spruce genes have different abilities to interact with the separate regulatory factors of the different whorls. Such regulatory factors could be A or C-class genes, the SEP1, 2, 3 genes, or different down stream targets of the AP3/PI complex. We suggest that some of these elements may allow interaction with the
conifer genes, causing a gain of function phenotype such as ectopic petals in whorl one, and some may not, causing a loss of function phenotype.

Arabidopsis plants that constitutively express \textit{DAL13} has ectopic anther tissue in the first, the third, and the fourth whorls of organs. Anther development requires simultaneous expression of both B and C class genes. This suggests that \textit{DAL13} may act as a B gene and also have the ability to activate endogenous C-class genes. Alternatively \textit{DAL13} may activate genes involved in anther formation independently of the C-function, thus in a manner that is not in agreement with the ABC paradigm. The idea that \textit{DAL13} would act to specify anther tissue or more broadly microsporangium-bearing organs when expressed in Arabidopsis, is in agreement with the specific expression pattern of this gene in developing pollen cone microsporophylls [II, III]. Provided that this reflects the \textit{DAL13} function in spruce these data suggest an ancestral B-function as being a determinant of microsporangium-bearing organs, by a mechanism that is independent of C-function genes.

\textbf{Interactions between spruce B-like proteins and Arabidopsis B-class proteins}

\textit{In vitro} translated AP3 and PI proteins have been shown to preferentially interact with each other and not with other MADS-box genes (Riechmann \textit{et al.} 1996a). Reichman (1996) also assayed DNA binding capacity and dimerisation specificity by electrophoreotic mobility-shift assays (EMSA) combined with domain swapping between \textit{AP3/PI} and the C-class gene \textit{AGAMOUS}. These experiments showed that the region responsible for dimerisation specificity in the AP3/PI heterodimer is the I region and the K-domain. In a transient transformation assay AP3 and PI were shown to be dependent on each other for nuclear localisation (McGonigle \textit{et al.} 1996). This suggests that the interaction of \textit{AP3} and \textit{PI} results in the formation of a protein complex that generates or exposes a co-localisation signal required to translocate the resulting complex to the nucleus. The interactions between the B-proteins in other angiosperm species have also been shown to be specific. In \textit{Antirrhinum}, DEF and GLO form heterodimers in both yeast two-hybrid experiments and gel-shift assays (Davies \textit{et al.} 1996). A large survey of interactions between Petunia MADS-box proteins indicate that the B proteins
specifically interact with each other but not with other classes of MADS-box genes (Angenent et al. 2000).

We have used the yeast two-hybrid system to assay protein-protein interactions between the conifer B-like proteins and their orthologs in Arabidopsis. There are several reasons for doing such an experiment. Firstly, if interactions between the spruce B-like proteins and the Arabidopsis B-class gene products could be established, this would serve as independent support for their position as putative B-genes and also provide functional information about how they relate to the two B-class clades. Secondly, results that clarify how the spruce B-proteins interact would also facilitate the interpretation of the phenotypes found in the transgenic Arabidopsis lines. Thirdly, information on interactions between the spruce proteins themselves might also be informative in relation to how the genes are expressed in pollen cones and their activity during pollen cone development. Protein-protein interactions assayed with the yeast two-hybrid system indicate that all three spruce proteins form homodimers, but also that DAL11 and DAL13 may form heterodimers with each other and AP3 [III]. Non of the spruce genes formed heterodimers with PI.

Provided that the dimerisation is so specific that it can be used as a criterion for assigning a gene to a specific AP3 or PI clade, the ability of DAL11 and DAL13 to form heterodimers with AP3 suggest DAL11 and DAL13 to be phylogenetically closely related to PI but not to AP3. DAL12 may have lost its ability to form heterodimers with PI, or is less are closely related to the angiosperm B-genes than suggested by the phylogenetic analysis. Our results do not clarify whether or not the spruce proteins, when transformed into Arabidopsis act as homodimers or as heterodimers, but the complete conversion of sepals into petals found in transgenic Arabidopsis plants expressing both DAL11 and DAL12 suggests that they may function as homodimers. Similarly the DAL13 phenotype provide evidence to suggest that the DAL13 protein may act as a homodimer in the transgenic Arabidopsis plants.

DAL11 and DAL13 are expressed in overlapping domains in the developing pollen cone. Thus, it is possible that the interaction found in the yeast two hybrid experiments
reflects their activity as heterodimers during pollen cone development. Given the recent results regarding ternary complex formation between the DEF/GLO complex and SQUAMOSA in *Antirrhinum* (Egea-Cortines et al. 1999), it is possible to envision interactions between the spruce B-genes and other MADS-box genes active during pollen cone development.

The B-function in conifers

Constitutive expression of *DAL11, DAL12* or *DAL13* in transgenic Arabidopsis alters floral morphology in a manner that suggests an interference or interaction with the angiosperm B-function. Transgenic Arabidopsis plants expressing *DAL11* or *DAL12* show a phenotypic change that is in agreement with the existing ABC model provided that *DAL11* and *DAL12* act as B function genes. The expression of *DAL13* seems to affect floral development independently of C function. The difference in phenotypic effects found in plants expressing *DAL11/DAL12* and plants expressing *DAL13* suggests that the activity of *DAL11* and *DAL12* differ, with respect to the B-function, from that of *DAL13*. Provided that the activity of *DAL11/DAL12* and *DAL13* in transgenic Arabidopsis reflects their activity in spruce we suggest that *DAL11* and *DAL12* act in the definition of shoot identity and that *DAL13* is active in microsporangium identity determination. This hypothesis is consistent with the expression patterns of *DAL11* and *DAL12* in the apical dome of developing pollen cones and the specific expression pattern of *DAL13* in microsporophylls (Figure 5). Thus, B-function in conifers according to this hypothesis is dual and involves both shoot identity determination and organ identity determination. If this division reflects an ancestral feature of all seed plants remains to be resolved. Our data suggest that B-function, in the sense of being a microspore bearing organ determinant, is conserved between gymnosperms and angiosperms and thus possibly among all seed plants.
Is there an A function in conifers? [IV]

Recent refinements of the ABC-model suggests the ancestral A-function within angiosperms, mainly to be involved in the determination of floral meristem identity, whereas the organ identity determination function may be a derived feature of Arabidopsis. Conifer cones can be described as reproductive shoots. The main difference between an angiosperm flower and a conifer cone is not necessarily the difference between flowers being bisexual and cones unisexual, but rather that cones have an additional level of hierarchy in their branching order as compared to flowers. The additional level of hierarchy found in conifers is presumably lost within the angiosperm lineage, a loss that may have coincided with the evolution of the compact bisexual flower. Thus, a strict interpretation of the angiosperm A function, even in its modified form, as a determinant of a floral meristem identity, is not applicable to conifers. An analogous function, however, would be to define the developing shoot as a reproductive shoot.

We have cloned a MADS-box gene, $DAL10$, which is a candidate for a function as determinant of reproductive shoot identity. It is specifically expressed in seed and pollen cones throughout cone development [IV]. In situ hybridisation experiments on differentiating seed cones show that this gene is specifically expressed in cells that is going to form or that is building the sterile bract that subtends the ovuliferous scale (Figure 5). Expression at later stages is expanded into the central parts of the cone. Expression in pollen cones is restricted to the central region of the cone. Thus $DAL10$ is expressed in vegetative parts of reproductive shoots e.g. pollen and seed cones. $DAL10$ is phylogenetically not related to any of the known angiosperm clades, but forms a separate clade with other gymnosperm genes from Gnetum (Becker et al. 2000; Shindo et al. 1999). Our analysis has included all MADS-box genes of MIKC- type present within the recently sequenced Arabidopsis genome. The phylogenetic analysis suggests that the angiosperm sister to this clade is either lost or highly divergent. Given the poor sampling of MADS-box genes from angiosperms outside of the eudicot clade it is possible that a sister of $DAL10$ might be present in these species. The apparent monophyly of the $DAL10$ clade implies that the genes within this clade are active in processes specific to gymnosperms. The genes from Gnetum present in the clade are
also expressed in reproductive structures, but with a specific expression pattern that differs from that of \textit{DAL10}, suggesting a divergence of function between genes in the group. We suggest \textit{DAL10} to be a determinant for reproductive shoots, a function that is lost during the evolution of angiosperm flower. If this hypothesis is correct, this is the first example that describes, how a gene loss within the MADS-box gene family has contributed to the differences in morphology found within the seed plant lineage

Constitutive expression of \textit{DAL10} in transgenic Arabidopsis plants causes phenotypic alterations of plant development. Plants expressing \textit{DAL10} are dwarfed, have curled leaves and form terminal flowers. The \textit{DAL10} phenotype shows similarities to what has been found in Arabidopsis plants constitutively expressing \textit{DAL2} or the endogenous C-class gene \textit{AGAMOUS}. The curled leaf phenotype is much more severe in \textit{DAL10} plants and homeotic conversions of flower organs are only found in conjunction with terminal flowers. This suggested that part of the \textit{DAL10} phenotype could be explained by ectopic expression of \textit{AGAMOUS}. The ability of DAL10 to activate \textit{AGAMOUS} was confirmed by RT-PCR [IV] which suggests that \textit{DAL10} have the ability to activate at least the C-class genes. The phenotype of Arabidopsis plants expressing \textit{DAL10} might be consistent with a role in the establishment of reproductive identity. Apart from the activation of \textit{AGAMOUS}, plants expressing \textit{DAL10} forms terminal flowers, a phenotype that is found also in plants which ectopically express floral meristem identity genes. Thus, even though conifers and angiosperms are fundamentally different in their organisation of their reproductive organs, analogous processes in defining a meristem as a reproductive meristem exist. We suggest \textit{DAL10} to be part of this function in conifers, and that, as apart of this function, \textit{DAL10} may interact with genes that have conserved functions in angiosperms and conifers, which is reflected in the phenotype found in \textit{DAL10} expressors.
A model for early cone development in Norway spruce

What can we tell about reproductive development in Norway spruce from the results presented in this thesis? The genes presented in this thesis are all active during different stages of cone development. They are expressed in a manner that suggests them to be involved in pattern formation, and they are phylogenetically related to angiosperm genes required for proper establishment of organ identity. Under the assumption that the expression pattern and relation to the angiosperm floral homeotic genes actually reflects the function of these genes in Norway spruce, the following model can be envisioned.

Early shoot development in Norway spruce is influenced by two important decisions. The first, which is a sex determination event, involves a capacity for the shoot to develop either as a pollen- or a seed-cone. This choice is influenced by positional criteria, in that pollen and seed cones are formed in distinct positions on the annual shoot, both in relation to the branch and to the whole tree. The second decision occurs at the transition to reproductive growth and determines whether the shoot will realize its reproductive potential and develop as a seed or pollen cone, or develop as a vegetative shoot. This transition is influenced by climate conditions during the early summer of initiation and can be manipulated by external applications of the plant hormone GA until and during the shoots form apical domes prior to lateral organ differentiation (Fogal et al. 1996; Lindgren et al. 1977; Owens 1983).

The capacity to develop as either pollen or seed cones require factors that separates male from female shoot meristems. We suggest that the B gene \textit{DAL12} and possibly also \textit{DAL11}, acts as male determinant in the apical dome of pollen cones [II]. Thus, the activity of the B genes renders the shoot the capacity to develop as a pollen cone, whereas shoots that do not express \textit{DAL11} and \textit{DAL12} by default have the capacity to develop as seed cones. Apart from \textit{DAL11} and \textit{DAL12} we have also identified a third gene, \textit{DAL13}, which is active in cells that is going to form microsporophylls in the apical dome of pollen cones, and in developing microsporophylls throughout pollen cone development. \textit{DAL13} may act as an organ identity determinant to specify lateral organs as microsporophylls [II].
Whether the capacity for pollen cone development is realized or not, as outlined above, depends on the second decision event, which may lead to a transition to reproductive growth. The reproductive signal is probably regulated in a similar manner in both seed cones and pollen cones and is likely active in both types of shoots, since, during years when climate conditions are inductive with respect to cone production, both seed cones and pollen cones are produced abundantly. The transition to reproductive growth requires a factor that defines the shoot as a reproductive shoot. The activity of \textit{DAL10} in vegetative parts of pollen and seed cones during the transition to reproductive growth suggests that this gene may be part of this transition in defining the shoot as a reproductive shoot.

During the differentiation of lateral organs, pollen cones develop microsporophylls that each harbours two microsporangia. The megasporangia in seed cones develop within the ovuliferous scale. Analogous with the C-function in angiosperms being a determinant for the carpel identity i.e. female identity, the putative C-gene, \textit{DAL2}, in Norway spruce has been suggested to be involved in the definition of the seed cone as female (Tandre \textit{et al.} 1998). However, data presented in this thesis suggests that \textit{DAL2} is not active in seed cones until lateral organs have begun to differentiate and that the expression is restricted to the developing ovuliferous scale primordia [IV]. In pollen cones \textit{DAL2} is active during lateral organ differentiation in cells that are going to form the microsporangia [II]. This suggests that the C gene in conifers is not active as a determinant of reproductive identity but rather has a function related to the development of micro- and megaspores.
CONCLUDING REMARKS

The results presented in this thesis and previous results from our group (Tandre et al. 1995; Tandre et al. 1998), as well as and others (Mouradov et al. 1999; Rutledge et al. 1998) suggest that, central parts of the molecular mechanism regulating reproductive development are conserved between angiosperms and conifers, and thus among all seed plants. The activity of B and C type genes in reproductive structures of both angiosperm and conifers suggests that the evolution of these genes may have coincided with the evolution of separate micro- and mega-spores. This implies that the evolution of the ancestral B and C genes might have been a prerequisite for the evolution of seed plants.

The recent completion of the Arabidopsis genome has allowed us for the first time to include in our analysis all MIKC-type MADS-box genes present within the genome of a single species [IV]. The interesting outcome of this analysis is the identification of several groups that are potentially angiosperm or gymnosperm specific, due to either losses or functional divergence. Thus, the evolution of the MADS-box gene family is not only characterised by series of gene duplications but also by gene losses. Our characterisation of the gymnosperm specific gene DAL10 provides an example where a potential loss of a gene function may be associated with the evolution of the compact bisexual angiosperm flower from an ancestor with reproductive organs born on fertile shoots.
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REFERENCES


Errata


**Thesis**
p.7 second paragraph last sentence replace “…encoded genes which” with… encoded **proteins** which…”.
p 9 first paragraph, third row replace “…Picea abies (Karst)…” with **Picea abies (L.) Karst.**
p. 18 first paragraph, fourth row replace “… a set of MADS…” with “… a set of **MIKC type** MADS…”.

p18 and 19 last and first paragraph should read:
The function of the A-class genes however, differs even between different eudicot species. The **A-class gene SQUAMOSA from Antirrhinum is involved in the determination of the floral meristem identity (Huijser *et al.* 1992)** and it has been hypothesised that this might reflect the ancestral A-function (Theissen *et al.* 2000). This also implies that the A-function *i.e.* sepal and petal identity determinant, is a derived character. The B-function as defined by the core eudicot model species is dual; B-genes are involved in specification of both petals and stamens. Two recent reports regarding B-class **gene activity in basal angiosperms and in monocots have provided new information regarding to what extent the B function is conserved in** these groups of angiosperms (Ambrose *et al.* 2000; Kramer, E. M. 1999).

p. 21 second heading replace “DAL2” with **DAL2**.
p. 24 second sentence replace “…DAL12 shares a paleo-AP3 motif with AP3 orthologs from basal angiosperms whereas DAL11 and DAL13 share…” with “…**DAL12** shares a paleo-AP3 motif with AP3 orthologs from basal angiosperms whereas **DAL11** and **DAL13** share…”
p. 35 9th sentence replace “Marie Lindgren” with “Marie **Lindersson**”
Paper I

Paper II

Paper III

p. 6, last row replace “… primordia (Figure 1 a)” with “… primordia (Figure 1 b)”
p.7, second row replace “…pith (Figure 1b)” with “…pith (Figure 1a)"
p. 8 second paragraph last sentence add replace “… was confirmed by RT-PCR.” with “was confirmed by RT-PCR (data not shown).”
p. 17, in figure legend 3 replace “…organs (A and B), third whorl organs (C and D) and fourth whorl organs (E and F)....” with “…organs (A and D), third whorl organs (C and F) and fourth whorl organs (B and E)....

Paper IV