EVIDENCE FOR PARTIAL HOMOLOGY: EXAMINING THE MORPHOLOGICAL AND MOLECULAR RELATIONSHIPS BETWEEN SHOOT AND LEAF DEVELOPMENT IN WATERMILFOIL *(Myriophyllum aquaticum)*

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Abstract

There is evidence from both morphological and molecular studies that many shoots and leaves have analogous developmental characteristics which transcend their classical categorization. An example of this can be found in the expression patterns of the KNOX1 family of homeobox genes which function in maintenance of indeterminate growth and are expressed in both dissected leaves and shoots. Shoot and leaf morphological parallels were characterized in the aquatic angiosperm *Myriophyllum aquaticum* (Vel.) using a combination of scanning electron microscopy and standard light microscopy. A KNOX1 gene fragment was also sequenced from *M. aquaticum* and its pattern of expression was mapped at the shoot tip using RNA *in situ* hybridization as the primary technique. Leaves were found to develop lobes in an alternating basipetal fashion and originated from distinct generative centers at the leaf base. Within the tissues of the developing shoot, KNOX1 expression was found to be localized to the developing stem, provascular strands, dermal tissues of internodes, and between developing leaf bases. KNOX1 expression was also found within developing leaves where patterns varied depending on the age of the primordium. In leaves between plastochrones 1 and 3, KNOX1 is evenly expressed throughout the primordium. In older plastochrones, expression becomes localized the more recently developed lobes. By plastochrone 9, expression signal is no longer visible. The presence of distinct lobe forming centers at the base of the leaf and the corresponding KNOX1 expression during leaf and shoot development is indicative of developmental parallels between traditionally non homologous structures.

Acknowledgments

I would never have succeeded in completing my degree had it not been for the excellent support that was given me during my research. I would firstly like to thank my supervisor, Christian Lacroix. His mentorship and encouragement was invaluable throughout my time of study and I am very grateful for it. As Dean of science, his schedule was hectic to say the least, but he always made time to answer his student's questions, or to fix ageing and sometimes recalcitrant instruments. I will never forget when the thermal printer broke down. Chris enjoys proficient use of the sometimes very colorful language inherent to the French tongue. He was particularly articulate while fixing the printer. Xiaofeng (my fellow lab mate) and I made an appreciative audience. Of course I must also thank Xiaofeng (aka Frank). Although we worked on very different projects, his company during late nights in the lab was excellent and made all of that endless sectioning more bearable.

The methods portion of the project was long and intimidating. It involved many hours of trouble shooting which I would never have got through without the excellent molecular expertise of Natacha Hogan (a highly accomplished Post Doctoral student of the UPEI biology department), and the Sinha lab at UC Davis who were kind enough to let me visit and learn how to do *in situs*. Thanks also to ACCBR at the Atlantic Veterinary College for the use of their image analysis system, and to MORPH (Molecular and Organismal Research of Plant History) for their generous funding of my trip to UC Davis. My committee members, Denis Barabé and Robert Hurta, were wonderfully supportive and gave me excellent advice during the writing of this thesis. Thank you every one!

LIST OF FIGURES

TABLE OF CONTENTS

Chapter 1: Introduction and Literature review

There are a myriad of morphologies represented within the plant kingdom. These morphologies are typically divided into three different types which are describes as leaves, shoots, and roots (Goethe, 1790; De Candolle, 1813; Troll, 1937). Analyses at both the morphological and molecular levels, however, have demonstrated that plant organs are not easily divided into one of these classical categories. Quite frequently morphologies will exhibit structural and developmental overlaps between categories reminiscent of a morphological continuum (Sattler and Jeune, 1991; Janssen 1998b; Lacroix et al., 2002; Brand et al., 2007).

An example of this can be seen in comparisons between shoots and compound leaves. Structurally, compound leaves are reminiscent of distichous shoots that lack axillary meristems (Lacroix et al., 2002). At the developmental level, the compound leaf primordial produce leaflets at discrete regions in much the same fashion that leaves are produced around a meristem (Lacroix, 1995). The expression domains of the KNOX1 family of transcription offer an example of categorical overlap between shoots and compound leaves at the molecular level. KNOX1 gene are implicated in the regulation of indeterminate cell fate in shoot systems and have been found to be expressed during both shoot and compound leaf development (Leyser and Day, 2003). In contrast to this, KNOX1 genes are not expressed during the development of simple leaves (Long et al., 1996).

These developmental similarities suggest a fundamental relationship between the developmental patterns of shoots and compound leaves that transcends their traditional classifications. To further elaborate on this, it would be beneficial to examine KNOX1 in a species of plant that exhibits a leaf shape intermediate between simple and compound leaves. Such a species of interest could be found in *Myriophyllum aquaticum* (Vel.).

M. aquaticum is an invasive aquatic angiosperm, found all over North America in freshwater streams, and exhibits mature leaves that are simple with an intensely lobed lamina (Fassett, 1972; Rutishauser, 1999). There have been no previous molecular studies conducted on the leaf development of *M. aquaticum.* Considering that most of the previous work published on KNOX1 gene expression has focused mainly on model species including *Arabidopsis*, *Maize*, and *Lycopersicon*, *M. aquaticum* represents an excellent opportunity to explore KNOX1 expression in a non-model species. The nature of its leaves and the lack of molecular data therefore make *M. aquaticum* an excellent candidate for exploring developmental similarities between leaves and shoots (Jeune, 1975; Hake et al., 2005)

1.1 Literature Review

1.1.01 Characteristics of vegetative organs

A fundamental difference between plants and animal body types lies in the respective time frames of their organ development. The majority of the organs in a characteristic animal are formed during embryonic development with comparatively little organogenesis occurring after birth (Fosket, 1994). Organ development in a typical seed bearing plant is quite different. The seed embryo contains only a rudimentary stem like axis referred to as the hypocotyl, a radicle which will develop into the primary root axis, and one or two cotyledons which function as the initial nutrient source for the seedling (Raghavan, 2000; Bowes, 1996). All other structures identified with the mature plant body (i.e. stem, leaves, flowers, roots, fruit) develop after germination from two populations of undifferentiated cells at the terminal ends of the hypocotyl referred to as the apical meristems (Jurgens, 2003; Dickinson, 2000). Meristems give plants their characteristic indeterminate pattern of growth which allows for continued organogenesis throughout the plant's life cycle (Cronk, 2001).

The aerial organs of a plant are typically divided into two categories described as leaves and shoots (Eames, 1961; Guédès, 1979; Sattler and Jeune, 1991). Each category has a series of morphological traits that may be used to broadly group all observable aerial structures into either category. Leaves may be classified as dorsiventral structures that are located at a lateral position on the stem, exhibit determinate growth, and subtend an axillary meristem (Dengler and Tsukaya, 2001; Rutishauser, 1999; Tsukaya, 1995). Shoots (or stems) may be classified as

structures which are subtended by a leaf or leaf scar, exhibit relatively indeterminate growth, and are usually radial in symmetry (Sattler, 1994). The shoot axis may be divided into a series of repetitive segments referred to as phytomers (Raghavan, 2000; Tsukaya, 1995). Each phytomer develops as a unit and consists of a node and an internode (Lyndon, 1990). The internode is composed of a portion of stem which divides one nodal region from the next. The node is composed of one or more leaves connected to of a thin portion of the stem via a petiole(s) which subtends an axial meristem (Lyndon, 1990).

1.1.02 The shoot apical meristem

All vegetative organs of the plant are patterned and produced at the shoot apical meristem (SAM) located at the tip of the stem (Sussex and Steeves, 1989; Lyndon, 1998). In higher plants (gymnosperms and angiosperms), the SAM is generally protected within a bud of older tissues including vegetative leaves or scales (Raghavan, 1992). To observe the SAM, it is necessary to dissect successively younger leaves from the shoot tip until the meristem is revealed. Meristems demonstrate considerable structural variability between species, sometimes appearing as flat structures with apical diameters of 50 μm while others, such as some species of cacti, have large dome like meristems with diameters as wide as 2500 μm (Steeves and Sussex, 1989; Mauseth, 2004). Despite this variability, most SAM's share a fundamental internal cellular framework.

The shoot apical meristem (SAM) includes all tissues of the shoot apex

immediately distal to the most recently initiated leaf primordia and are composed of small, undifferentiated cells that lack chloroplasts and large central vacuoles (Sinnott, 1960; Fleming, 2006; Beck, 2005). The SAM of most angiosperms is stratified and can be divided into two main collections of cells based on patterns of cell division (Steeves and Sussex, 1989; Sinnott, 1960). Depending on the species, the outer two or three layers (L1, L2, and L3) of the SAM undergo anticlinial cell division and are collectively referred to as the tunica (Figure 1.1) (Gemmell, 1969). All epidermal tissues of the plant are derived from the L1 layer, whereas the mesophyll of the leaf is derived from the L2 and L3 layers (Dickinson, 2000). Cell layers located below the tunica are collectively referred to as the corpus and are characterized by periclinial or indiscriminate cell division with respect to the outer surface of the SAM (Raghavan, 2000). The basal regions of the corpus are referred to as the rib meristem, and give rise to nodal and internodal tissue of the stem. Surrounding the rib meristem is a doughnut shaped region referred to as the peripheral zone and is the region where leaf primordia are initiated (Gemmell, 1969; Lenhard et al., 2002).

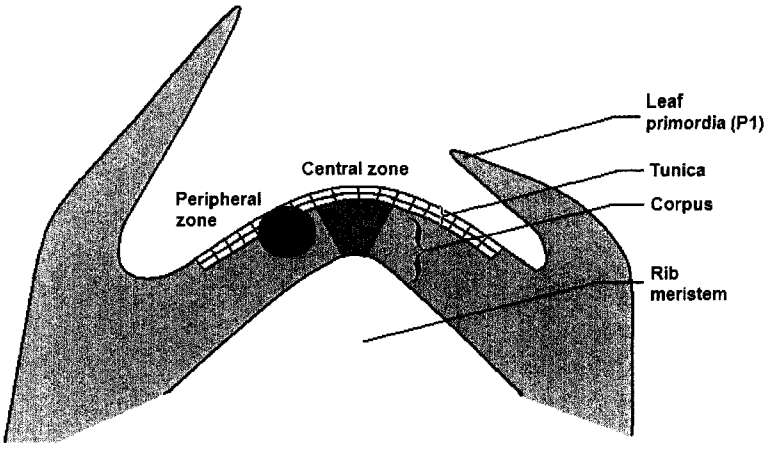

Figure 1.1 A schematic representation of the histological zonation of the shoot apical meristem (personal illustration).

The shoot apical meristem may be divided into another set of zones based on differing rates of cell division, and which incorporate cells from both the tunica and the corpus (Steeves and Sussex, 1989). The very tip of the apex is comprised of a group of comparatively large, slow dividing cells referred to as the central zone (Shani et al., 2006). These slowly dividing cells are referred to as the apical initials because of their terminal position and because of their multipotent stem cell nature (Lyndon, 1998). Providing that environmental conditions are adequate, these cells continue to divide throughout the life of the plant and allow the meristem to be self perpetuating (Jurgens, 2003; Lenhard et al., 2002).

1.1.03 Indeterminate Growth

In plants the undifferentiated self proliferation of the meristem is referred to as indeterminate growth; a state which is controlled by a carefully maintained network of phytohormones and transcription factors (Shani, 2006). Phytohormones are signaling molecules which are transported throughout the

plant to specific target tissues (Fosket, 1994; Griffiths and Gelbart, 2002). There are several families of phytohormones present in higher order plants including cytokinins, gibberellins, and auxins (Fleming, 2006). Plants that exhibit loss of function mutations within the pathways responsible for controlling indeterminate growth may either develop disorganized meristems or fail to produce a meristem at all (Long et al., 1996; Laux et al., 1996).

To maintain indeterminacy in the meristem, it is necessary to simultaneously promote and inhibit cellular proliferation. Cytokinins (CK) function as a principal component in the maintenance of this delicate balance by orchestrating a series of negative feedback loops. Cytokinins accumulate in the tissues of the central zone which promotes cell division (Shai, 2006). Accumulation of endogenous CK levels then triggers the expression of Arabidopsis Response Regulators (ARR), which then suppresses CK biosynthesis in a negative feed back loop (Leibfried et al., 2005). ARR suppression is then accomplished by a negative feed back loop between WUSCHEL (WUS), a transcription factor, and CLAVATA (CLV), a family of receptor ligands that work together to maintain meristem size (Clark et al., 1997; Mayer et al., 1998). WUS is expressed in a region of cells below the central zone and negatively regulates ARR which induces indeterminate cell fate in the apical initials (Leibfried et al., 2005). Accumulation of WUS transcripts then induces the production CLAVATA proteins within the central zone which leads to the suppression of WUS. These regulatory interactions effectively hold apical initial proliferation in check (Shani et al., 2006; Fleming, 2004).

1.1.04 Organogenesis

Not all of the cells in the SAM are functionally competent for organogenesis. Cellular proliferation in the central zone proceeds from its core toward the periphery of the SAM, generating a field of cells which are competent for leaf generation (Fleming, 2004). The initial step in leaf initiation involves isolating a select group of these peripheral cells from their neighbors for determination and subsequent leaf generation. The dynamic flux of auxin, a phytohormone produced at the shoot tip, functions as the key component in controlling the sites of leaf initiation and phyllotaxy (Reinhardt et al., 2000; Reinhardt et al., 2003). The flux of auxin is naturally directed towards the region of the apical dome expressing the lowest levels of auxin (Fleming, 2004). Once auxin begins to accumulate in regions of low expression, it subsequently becomes depleted in adjacent regions of the apical dome which then becomes the next sink site for auxin accumulation and primordia initiation (Hay et al., 2004; Teale et al., 2006). The accumulation of auxin at sites of leaf inception results in a developmental cascade where hormones and proteins such as gibberellins and expansins work together to loosen the tensile forces in the tunica and allow cells to divide in a periclinial direction resulting in leaf initiation (Fleming et al., 1999; Kessler and Sinha, 2004)

1.1.05 Leaf development and morphology

The morphology of the initial stages of leaf development varies between species. In dicotyledons, early leaf primordia appear as peg like protrusions in the

peripheral zone of the meristem, whereas in monocotyledons, they completely encircle the meristem and form a hood over the dome (Bell, 1991). Direction of growth of primordia also varies between species in that elongation may occur primarily from the base (basipetal), the tip (acropetal), or in both directions (divergent) (Romberger et al., 1993; Esau, 1965). Apical growth is followed by differential proliferation and expansion in several key sectors of the primordium. Although these sectors are typically referred to as "meristems", they are not referred to as such in the sense of apical meristems because they are not discrete and do not contain recognizable initial cells (Romberger et al., 1993).

To avoid confusion, the proliferative regions within the leaf will be referred to as blastozones as suggested by Hagemann and Gleissberg (1996). Laminar expansion is largely contributed to by cell proliferation in the marginal adaxial blastozones and is correspondingly suppressed in the region that will become the petiole (Rudall, 1992). This marginal expansion is then superseded by less specific expansion in non marginal internal regions termed the plate blastozone (Romberger et al., 1993). Subsequent leaf elongation is achieved through the intercalary blastozone which is located at the base of the leaf primordia (Bell, 1991). Differential patterns of growth between these blastozones dictate final leaf morphology. A primordium which exhibits prolonged activity in the plate meristem as opposed to the intercalary meristem will be broad and horizontally flattened as in the case of many deciduous trees. The long narrow leaves of grasses exhibit the opposite ratio where intercalary meristems experience prolonged activity and plate meristems exhibit relatively little (Bell, 1991).

Although leaves demonstrate a vast array of shapes and sizes, they may be broadly divided into the two main categories of simple and compound (Figure 1.2) (Hareven et al., 1996; Bharathan and Sinha, 2001). Simple leaves have a stalk like petiole which connects the single, continuous lamina, to the stem. Compound leaf laminas are dissected into individual leaflets which are connected via a petiolule to a branch like extension of the petiole termed the rachis (Chen et al., 1997).

Pinnate compound leaves are reminiscent of distichous axial shoots with leaflets sequentially arranged along the rachis. Compound leaves which have leaflets originating from a single point at the distal end of the petiole and lack a rachis are referred to as palmate. Palmate leaves may then be classified as non-peltate and peltate based upon, respectively, the presence or absence of an adaxial layer within the rachis (Kim et al., 2003). The level of dissection of compound leaves varies. It is possible for individual leaflets to be replaced by another series of smaller leaflets (referred to as bipinnate compounding). These leaflets may then in turn be divided into a subsequent series of leaflets, referred to as tripinnate compounding. Some developmental mutations, as observed in *Solanum lycopersicum*, will produce compound leaves with as many as 2000 leaflets per leaf (Hareven et al., 1996).

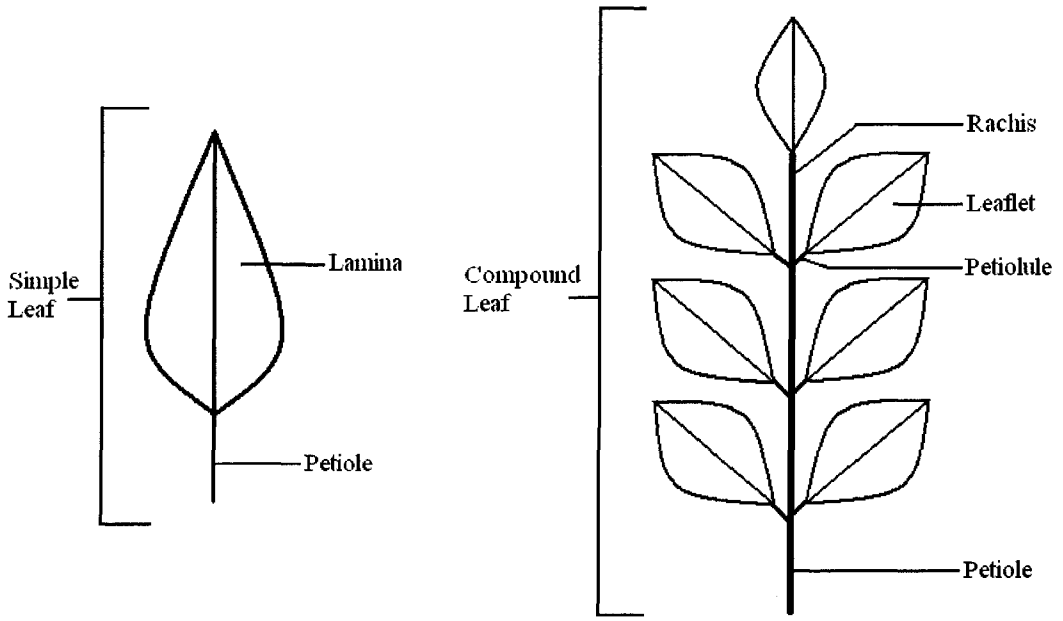

Fig.1.2 A comparison between a typical simple leaf and a typical compound leaf (personal illustration).

1.1.06 Theory in plant morphology

An essential issue of plant morphology is the delimitation of organ categories. For the past two centuries, the main paradigm of plant morphology studies has consisted of a typological paradigm in which a few categorical terms are used to describe and identify all possible structures observable in the plant kingdom (Rutishauser and Isler, 2001). This technical framework is referred to as Classical Morphology and was postulated by Goethe and De Candolle in the late 18th and the early 19th centuries. As with all scientific disciplines, founding theories must be regularly re-evaluated to ensure that they are supported by newly acquired information. If theoretical paradigms are found to be inaccurate, they must be discarded in favor of a theory which will accommodate the new data (Sattler and Rutishauser, 1997).

1.1.07 Classic morphology

The classical paradigm in plant morphology involves the detailed comparison of mature plant structures which are divided into three mutually exclusive categories: roots, stems and stem homologues (caulomes), and leaves and leaf homologues (phyllomes) (Goethe, 1790; Guédès, 1979; Esau, 1965). These terms influence our ability to comprehend and articulate plant morphology and, to a certain extent, they may even affect what is observed (Sattler and Rutishauser, 1997).

Each classical category has strict mutually exclusive definitions concerning the functional, structural, and positional characteristics of the particular organ it describes. Classical morphology uses the following three criteria to determine organ homology: relative position, special qualities, and continuum criterion (De Candolle, 1868; Sattler, 1994).

Relative position dictates that homologous organs will appear in identical positions on the organism (Rutishauser and Isler, 2001). For example, all axial shoots arise in the axils of leaves. Special qualities indicate that homologous organs will look structurally similar and share common functions (Rutishauser and Moline, 2005). In this respect, all leaves are said to exhibit dorsiventral symmetry and are the primary photosynthetic organs of the plant. Continuum criterion indicates that although they appear dissimilar, organs may still be regarded as homologous if intermediate or transitional forms are evident (Sattler, 1994). An example of this can be seen in floral organs where petals can be seen as a developmental transition between photosynthetic leaves and the reproductive

structures of the flower (petals, stamens, etc.). Therefore, leaves, petals, and stamens are considered to be homologous (Tsukaya, 1995).

Based on the above criteria, if we refer to a plant organ as a leaf or a shoot, we make certain assumptions concerning its fundamental nature. However, the mutually exclusive relationship between leaves and shoots becomes somewhat difficult to determine when comparing their early development. If a leaf primordium is dissected from the SAM at a sufficiently early stage and cultured on the appropriate medium, it will differentiate into a shoot and a subsequent plantlet (Tsukaya, 1995; Steeves and Sussex, 1989). This indicates that leaves and shoots contain the potential for similar fates during early stages of development.

There are numerous examples of organs which appear to exhibit intermediate characters between leaf and shoot categories (Sattler, 1991). For example, the phylloclades of *Asparagus* develop in the axil of a leaf and are bilaterally symmetrical which makes them partially homologous with both shoots and leaves respectively (Sattler and Rutishauser, 1997; Hirayama et al., 2007). As well, species of *Welwitchia* demonstrate indeterminate growth within their leaves. Each plant develops two leaves which continue to grow throughout the lifetime of the plant from meristematic regions located at the tips of leaves (Esau, 1965; Rutishauser, 2001). Such structures as those in the above examples could be considered either shoots or leaves based on the classical definitions.

Although classical morphology does not adequately describe all possible plant structures, it is still a useful paradigm for efficient identification of mature plant

forms which demonstrate typical morphologies. It is both convenient and necessary to think about plants in this categorical fashion for ease and speed of every day communication. In 1979, Guédès said that "It should be observed that there is no language, so no thought, and no science, without typology" (Lacroix et al., 2005).

Atypical plant structures do not necessarily falsify the classical theory. Rather they only limit the field of application in which classical categories may be used. It must be remembered however, that categories are artifacts of the human thought process and that no such divisions occur in nature. This inevitably leads to questions such as "what is a leaf?" and "what is a stem?" By using the classical theory to interpret atypical forms botanists are forced to either formulate ad hoc hypotheses which can not be readily confirmed, or purposely exclude deviant structures from the literature (Sattler, 1986).

1.1.08 Continuum morphology

Instead of forcing atypical structures into artificial categories, it may be more accurate to consider them as developmental mosaics of shared processes. A diversity of structures would therefore be due to a diversity of process combinations indicating that morphological evolution is the result of changes of process combinations (Sattler and Rutishauser, 1997). Therefore all structures are partially related to each other to varying degrees through a common morphological continuum where typical plant structures (stem, leaf, and root) represent concentrations of frequently occurring process combinations (Lacroix et al., 2003).

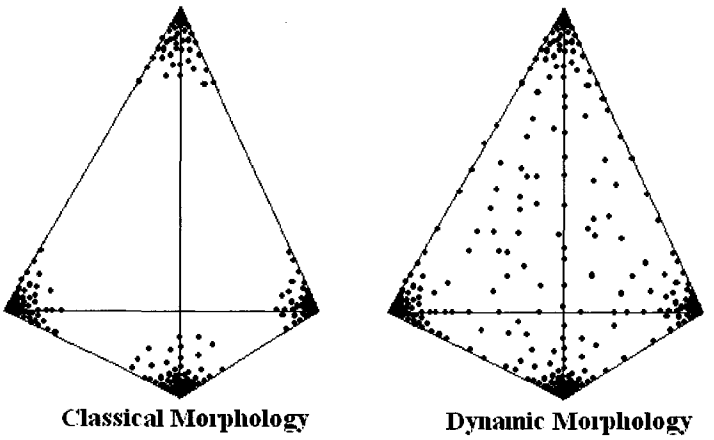

Fig. 1.3 Hypothetical illustration of the three dimensional morphospaces represented in classical and dynamic morphology (Sattler, 1986).

The morphological continuum was originally conceptualized as a three dimensional tetrahedron (Fig. 1.3) (Sattler, 1986). According to Classical Morphology, all plant forms would be localized to one of the four quadrants with no obvious intermediate distribution. From a more dynamic perspective, structures which have numerous developmental processes in common will appear closer to each other in the tetrahedron, and farther away from those structures with which they do not have as many processes in common. Therefore, it is easy to see that typical structures which have the least in common (roots, stems, leaves, and trichomes) will congregate at the four corners of the tetrahedron. (Sattler, 1986; Lacroix et al., 2003). All intermediate forms which appear to share partial homology with the four classical categories would lie somewhere between the four corners.

This new morphological model which accounts for partial homology may be referred to as dynamic morphology, although it has variously been known as process morphology, continuum morphology, and fuzzy arberian morphology

(Arber, 1950; Sattler, 1990; Lacroix et al., 2005). Within the continuum, all plant structures are partially related to each other based on similarities in process combinations. This implies, unlike the mutually exclusive categories of the classical model, that there is partial homology between all plant structures. Intermediate structures therefore cease to be anomalous, and the contradictions they present for classical theory are due to process hybridization between categories (Sattler, 1994). That being said, it should be made clear that dynamic morphology is not meant to replace but to complement classical morphology where it fails to correctly identify plant structures (Lacroix et al., 2005).

An example of dynamic morphology can be visualized in the indeterminate leaves of the genus *Guarea* (Fukuda et al., 2003; Jeune et al, 2006). The lateral position of these organs implies a homology with leaves. However their indeterminate pattern of growth indicates a homology with shoots. They can not be satisfactorily referred to as either "leaves" or "shoots". The terms "leaf" and "shoot" therefore have no useful meaning, and only serve to cloud the true nature of the structure. Under the dynamic paradigm however, the lateral organs of *Guarea* become partially homologous to both leaves and shoots and need not be categorized as either (Lacroix et al., 2005).

Within this dynamic context, the entire relationship between different structures of the plant body changes. Rather than the plant consisting of a number of mutually exclusive morphological units, the essential unit of the plant is actually the entire plant itself. The characteristic forms of shoots, leaves, and leaflets represent an encasement of structures with similar processes reiterated at

different hierarchical levels of development. This indicates that the whole plant is represented in its component parts (Arber, 1950; Lacroix et al., 2005).

Morphological studies have shown that shoots and compound leaves share certain processes of development during their ontogeny which makes it difficult to delimit classical boundaries and supports dynamic morphology as a working paradigm. Sattler and Jeune (1991) used multivariate analysis to determine if there was a structural continuum between typical representatives of classical categories (shoots, leaves, and roots) and atypical structures such as the phylloclades of *Asparagus pulmosus* or the lateral appendages of *Utricularia foliosa*. It was shown that developmental processes of typical representatives of shoots, phyllomes, caulomes, leaves and roots formed the corners of a double tetrahedron, while controversial structures occupied intermediate positions in a continuum between the corners.

Compound leaves can be thought of as an intermediate stage in a morphological continuum between simple leaves and shoots (Sinha, 1997; Lacroix et al., 2003). Deviations in early development between structures tend to generate greater disparities in homology as development proceeds. Therefore, if there is no partial homology between shoots and compound leaves, it would be expected that there would be little similarity between their early developmental stages. Developmental analysis however, has proven that this is not the case and has shown numerous similarities during the early development of compound leaves and shoots.

Compound leaves demonstrate shoot like qualities such as lateral elements flattened in a plane perpendicular to the long axis of the leaf, a dome shaped apex, and transient generative centers (Sattler and Rutishauser, 1992; Lacroix, 1995). However, as compound leaf development progresses, these similarities are lost. Leaflets of compound leaves become concave and eventually unfold in a plane parallel to the dorsiventral plane of the leaf, and leaf development become determinate as the domed apex turns into a terminal leaflet (Lacroix, 1995).

These "leaf like" characteristics are not without precedent in shoots. Many shoots eventually become determinate, and dorsiventral symmetry may be observed in the shoot systems of numerous taxa (Dengler, 1992; Lacroix and Sattler, 1994). Compound pinnate leaves could therefore be thought of as determinate, partial shoots demonstrating distichous phyllotaxy and empty leaf axils (Sattler and Rutishauser, 1992).

Lacroix et al. (2003) conducted a quantitative study to compliment a previous qualitative study relating compound leaves and shoots (Sattler and Jeune, 1991). Phyllotactic parameters such as angle of insertion and plastochron ratios were compared during the early developmental stages in 16 eudicot species exhibiting compound leaves. Growth parameters were statistically compared using multivariate analysis which allows individuals to be grouped together based on their respective distance of similarity. As in the study conducted by Sattler and Jeune (1991), shoots, compound leaves, and leaflets were not restricted to distinct clusters but appeared to form a continuum based on their phyllotactic parameters. This indicates that while the mature structures of these organs exhibit different

characteristics, there is no qualitative difference in the measured parameters of the typical categories. This suggests that pinnate leaves are arranged in a morphological continuum between simple leaves and shoots, and that these categories are not mutually exclusive as classical morphology would indicate (Sattler and Rutishauser, 1997).

A comparative developmental study conducted by Lacroix et al. (1995) also supports the view of a continuum between highly lobed simple leaves, compound leaves, and shoots. Highly lobed leaves examined in *Achillea millefolium* demonstrated developmental similarities to both compound leaves and shoots. The lobes initiated on the simple leaf appeared as those lateral elements initiated on a compound leaf with a large number of leaflets. Unlike compound leaves however, the lobes demonstrate a perpendicular orientation to the leaf axis which was maintained throughout the ontogeny of the leaf (a characteristic of shoots). These observations indicate a morphological continuum between highly lobed simple leaves, compound leaves, and shoots.

1.1.09 Transcription factors in plant development

Since it has been demonstrated quantitatively that there is an observable structural continuum between compound leaves and shoots, it is now necessary to compare and complement that with the genetics behind the developmental processes. The study of genetics examines the relationship between genotype and phenotype. If there is truly a reiteration of developmental processes between shoots and leaves, it would be expected that these structures demonstrate

similarities in the expression of core developmental genes as well (Champagne and Sinha, 2004). In fact, such an overlap is observable in the KNOX1 family of plant homeobox transcription factors.

Transcription factors relatively small proteins that bind to specific nucleotide sequences and, once bound, either suppress or induce expression of their target genes (Heldt, 1997). Changes in gene regulation lead to differences in protein expression which subsequently alters phenotype. Differences in morphology between species are therefore likely to lie not within the actual nucleotide content of genes, but in how genes are expressed (Fosket, 1994; Kramer, 2005). Since transcription factors control gene expression, they are likely candidates for phenotypic diversification and developmental study (Cronk et al., 2002).

Homeobox genes are an ancient group of developmentally important transcription factors. They were originally identified in the homeotic *Antennapedia* mutants of *Drosophila* where the antennae were replaced by legs (McGinnis et al., 1984; Duboule, 1994). Homeobox genes are expressed during early stages of development, and contain a highly conserved DNA sequence of 180 nucleotides (Duboule, 1994; Chan et al., 1998). The encoded protein is folded into three α helices with the site of DNA recognition being located in helix III, and binding to the core nucleotide sequence of ATTA (Hake et al., 2004; Chan, 1998). Homeobox genes have been identified in evolutionarily distant organisms including animals, fungi, and plants which suggest fundamental similarities in the development of all organisms (Cronk, 2001).

Mutational analysis has lead to the discovery of many homeobox containing genes in plants (Jackson et al., 1994; Mayer et al., 1998, Tsiantis and Hay, 2003). Null and dominant mutations of homeobox genes result in dramatic alterations in phenotype which researchers have used to determine the exact function of these genes during development (Hake et al., 2005). The first plant homeobox gene was identified in *Zea maize* mutants demonstrating "knot like" outgrowths along leaf vasculature (Volbertch et al., 1991). The knots were found to adopt meristem-like fates by incorporating cells which continued to divide after the surrounding cells of the leaf became differentiated. It was discovered that these "knots" were ectopically expressing a homeobox gene which later became known as KNOTTED1 (KN1) (Vollbrecht et al., 1991). Degenerate oligonucleotide probes were designed from the KN1 sequence, which lead to the discovery of many other homeobox genes of developmental significance in plants (Chan, 1998).

Almost all homeobox genes which have been identified have been found to be implicated in organ initiation and development (Scofield and Murray, 2006). The different plant homeobox families may be divided up based on the level of conservation within the homeodomain (HD), and sequence similarities in the protein structure (Chan, 1998).

The PHD-finger family of homeobox genes encodes a zinc-finger domain, and is characterized as having a cystine rich sequence N-terminal to the HD. These genes are primarily expressed in the SAM, and mutations in their sequence result in dwarfism and homeotic alterations of floral organs (Ito et al., 2004). HD-Zip

proteins contain a characteristic leucine zipper located C-terminal to the homeodomain and are typically implicated in light perception and leaf enlargement (Chan, 1998).

The homeodomains of some plant transcription factors are atypical in that they contain extra amino acid residues in the loop or the turn of the homeodomain (Kamiya et al., 2003). These atypical homeodomains have been found to be particularly important in meristematic maintenance (Kamiya et al., 2003). WUSCHEL (WUS) transcription factors, which were mentioned previously with respect to indeterminate growth, contain two extra amino acid residues in the turn, and four extra residues in the loop of the HD. Loss of function to these genes results in severe defects in SAM size (Laux et al.,1996).

Other atypical homeodomains are grouped within the three amino acid loop extension (TALE) superclass which, as the name suggests, contains three extra amino acids within the loop. The third helix in most TALE homeodomains contains a WFXN residue sequence, with the X position being particularly important in DNA binding specificity (Hake et al., 2004). There are two known families of TALE homeodomains in plants which include the BELL1 and KNOX families (Chan et al., 1998). BELL1 transcription factors contain no other distinctive sequence features outside their HD and are expressed in a wide variety of plant tissues including leaves, flowers, and roots (Kanrar et al., 2006; Chan et al., 1998). BELL1 genes have been shown to be key regulators in patterns of leaf initiation and in stem cell maintenance (Byrne et al., 2003).

1.1.10 KNOX Genes

The KNOX family of homeobox transcription factors, including the KNOTTED1 gene mentioned earlier, can be divided into two monophyletic groups, Class 1 and Class 2, based on differences within the HD, intron position, and expression patterns (Hake et al., 2004; Kerstetter et al., 1994). Members of each class have been identified in all lineages of the plant kingdom, including angiosperms, gymnosperms, ferns, and bryophytes. Unique KNOX genes which exhibit characteristics of both class 1 and 2 proteins, have been sequenced from the green algae *Acetabularia acetabulum* and *Chlamydomonas*, indicating that the divergence of class 1 and 2 took place after the colonization of land by plants 500 million years ago (Hake et al., 2004).

KNOX proteins consist of approximately 400 amino acids encoding a number of well conserved, characteristic domains N-terminal to the HD which is located at the extreme C-terminus of the protein (Figure 1.4).

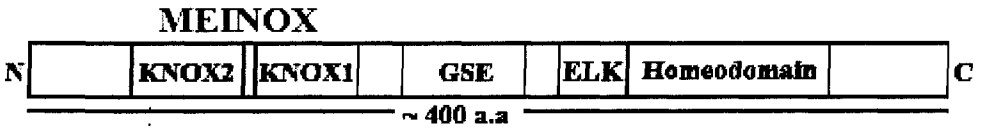

Figure 1.4 Illustration of the amino acid domains of a typical KNOX protein.

Directly N-terminal to the HD is the ELK domain (named for the first three amino acids in its sequence), the function of which is not clear but is thought to be responsible for nuclear localization of the protein (Scofield and Murray, 2006). After the ELK domain, proceeding consecutively towards the N-terminus, are the

GSE and MEINOX domains. The GSE domain is enriched in proline, glutamine, serine, and threonine residues which make up the PEST sequence. PEST domains are commonly attributed to protein degradation, which indicates that the GSE region controls KNOXI protein stability. The MEINOX domain shares significant sequence similarity with the MEIS (myeloid ecotropic viral integration site) protein found in humans (Burglin, 1997). MEIS is also a TALE homeodomain, which indicates that this particular subclass of TALE proteins predates the divergence of plants and Opisthokonts (ancestor of animals and fungi) (Hake et al., 2004). The MEINOX domain is divided into KNOX1 and KNOX2 domains, which are responsible for target gene repression and dimer formation respectively (Scofield and Murray, 2006). It has been observed that the MEINOX domain of KNOX1 proteins interacts with a MEINOX interactive domain in BELL proteins to form dimers with increased DNA binding specificity (Hay et al., 2005).

Expression patterns of Class 1 and 2 proteins are significantly different. Class 2 expression is found throughout the plant (Kerstetter et al., 1994). Class I protein expression is much more restricted than class II, and is expressed only in the SAM and in leaf anlagen of compound leaf species. Expression patterns of KNOX1 genes have been extensively studied through mutational analysis (Hareven et al., 1996; Long et al., 1996; Jasinski et al., 2007). There is a high level of redundancy in the KNOX1 gene family, with four different KNOX1 genes appearing in *Arabidopsis*, and nine in Rice (Jouannic et al., 2007). This makes it difficult to identify functions through KNOX1 mutations because many

of them demonstrate no obvious phenotype. However, two patterns of expression have been identified in KNOX1 genes which include total SAM expression, or narrow strips at the base of leaf primordia (Reiser et al., 2001). Mutations in KNOX1 genes expressed at either of these sites result in failure to produce or maintain a SAM, and irregularly shortened internodes respectively (Long et al., 1996). It is therefore possible that the redundant KNOX1 genes control different developmental pathways since they are expressed in different locations, and give rise to different mutant phenotypes.

1.1.11 KNOX function during development

The primary function of KNOX1 genes is believed to be the maintenance of indeterminate growth through the suppression of cellular differentiation in the meristem (Bharathan and Sinha, 2001; Long and Benfey, 2004; Kellogg, 2006). The domain of the meristem has been delimited by the presence of STM-like KNOX1 expression (Fleming, 2006). Severe null mutations in KNOX1 genes can result in fusion of cotyledons and the failure to form a SAM in the embryo which results in the seedling's subsequent termination (Long et al., 1996).

If KNOX1 genes are ectopically expressed in the simple leaves of *Arabidopsis* and *Z. maize*, isolated pockets of cells on the adaxial surface will return to their initial undifferentiated state and initiate shoot apical meristems (Chuck et al., 1996; Sinha et al., 1993). If left on the lamina, these ectopic meristems will develop like wild type SAMs and produce leaf primordia. If removed from the lamina and cultured on the appropriate media, ectopic meristems may develop

into mature shoots (Chuck et al., 1996). These ectopic meristems express KNOX1 genes and consistently form near vascular strands of the leaf which also ectopically express kn1 (Champagne and Sinha, 2004)). Leaf cells expressing KNOX1 fail to gain the polarity characteristic of differentiation, and remain isodiametric (Kessler and Sinha, 2004). Therefore, the ectopic presence of KNOX1 in leaves appears to endow them with shoot-like characteristics (Chuck et al., 1994; Sinha et al., 1993).

1.1.12 KNOX1 expression in leaves

From a molecular point of view, compound and simple leaves represent a dichotomy in leaf development; the branching point of which centers upon the presence or absence of KNOXI expression. Regardless of leaf complexity, KNOX1 expression is down regulated during the initial stages of leaf inception (Smith et al., 1992). In simple leaves, KNOX1 expression remains suppressed, while in compound leaves KNOX1 expression reappears between plastochrons two and seven (Bharathan et al., 2002; Hay and Tsiantis, 2006; Hay et al., 2003).

KNOX1 expression is suppressed at the site of leaf initiation by a group of homologous MYB transcription factors that includes PHANTASTICA (PHAN) in tomato, ROUGHSHEATH1 in *Maize*, and ASYMMETRICLEAVES1 in *Arabidopsis* (Hake et al., 2004; Hay et al., 2004). Together, these homologs are known as the ARP proteins (Hay and Tsiantis, 2006). Null mutations of ARP genes in simple leaves allow expansion of the KNOX1 domain into the site of leaf initiation resulting in the transformation from simple to lobed leaf shape (Müller et al., 2006). Constitutive ectopic expression of KNOX1 in compound leaves

intensifies the dissection pathway already in place, resulting in highly compound leaves which have as many as 1000 leaflets per leaf (Hareven et al., 1996).

There are numerous examples where KNOX1 genes are not expressed in the typical pattern, of which the Fabales are a typical example. Although many species in this family have compound leaves, none of them exhibit KNOX1 expression during their development. Instead they express another gene, UNIFOLIATA (UNI), which appears to take the place of KNOX1 in regulating compound leaf development (Champagne and Sinha, 2004). Conversely, some species with mature simple leaves such as the sunflower (*Helianthus annus)* exhibit KNOX1 expression during development (Tioni et al., 2003). *Lepidium oleraceum* (Brassicaceae) which has complex leaf primordia that undergo secondary morphogenesis to become simple at maturity, also demonstrate KNOX1 gene expression during their development. These examples suggest that KNOX1 expression in leaves is not as straightforward as the simple-compound paradigm and show that further analysis of KNOX1 expression in non-model species is warranted (Bharathan et al., 2002).

1.3 Research proposal and objectives

The conceptual transition from classical morphology to dynamic morphology allows researchers to explain the occurrence of morphologies which exhibit characteristics from both leaf and shoot categories. It also reveals a fundamental relationship between the developments of leaves and shoots in general. In this respect, compound leaves demonstrate shoot like features where each leaflet is

comparable to simple leaves arranged in a distichous fashion along a branch (Tioni et al., 2003).

The purpose of the proposed research is to test dynamic morphology as a relevant theory from a molecular stand point. As indicated in the first section of this chapter, evolutionary and molecular analyses of model species demonstrate a correlation between KNOX1 expression and leaf complexity. However, KNOX1 gene expression is almost always down regulated in the primordia of classically simple leaves. This indicates that processes in a compound leaf are more closely related to a shoot than those of the simple leaf. If the partial shoot theory is applicable, then a classically simple, highly lobed leaf should fall between a compound leaf and a simple leaf in a morphological continuum. Based on this theory, it could be proposed that a highly lobed simple leaf may also express KNOX1 genes during its development.

From a molecular point of view, studying patterns of gene expression in non-model species is vital to determining whether or not developmental regulation has been conserved across the plant kingdom (Nardmann and Werr, 2007; Champagne and Sinha, 2004). The benefit of the proposed study is therefore twofold in that it will serve to further elucidate the role KNOX1 played during the evolution of angiosperm leaf morphology, and it will facilitate the establishment of an alternate morphological paradigm in dynamic morphology.

The particular non-model species of interest for the proposed research is *Myriophyllum aquaticum* (Vell.). *M. aquaticum*, commonly known as Parrot-feather, is an aquatic angiosperm native to South America, and present in North

America, Europe, Japan, and Australia as an invasive species (Sutton, 1985; Fasset, 1972). Its common name is derived from the feather like appearance of its simple, yet highly lobed leaves. Leaves are produced in whorls of four to six by fleshy stems and may contain upwards of 20 lobes (9-10 pairs), depending on the environmental conditions (Sutton, 1985). The leaves are heterophyllous and demonstrate varying morphologies depending on whether they develop above or below water. The aerial leaves are fleshy and photosynthetic with fewer lobes compared to immersed leaves which tend to have a rotted, filamentous appearance (Fasset, 1972).

The highly lobed yet simple nature of *M. aquaticum* leaves offers an intermediate morphology between compound and simple leaves which makes it ideal for this study. In the past, developmental analyses were performed on several species of *Myriophyllum* with regards to the regions of lobe production at the base of the leaf (Jeune, 1975; Jeune, 1976; Jeune, 1977). It was discovered that these regions act as potential growth centers for lobes in much the same way that leaves are produced from specific locations around the meristem.

It is possible that *M. aquaticum* leaves may have developmental processes reminiscent of compound leaves. An alternate hypothesis is that each lobe may function as a transient meristem devoid of lateral elements. Since KNOX1 expression has been attributed to meristematic growth and increased leaf complexity, it is hypothesized that *M. aquaticum* leaf primordia will express a KNOX1 genes during their development. Therefore, the following research objectives were addressed:

1. Examination of leaf and shoot development in aerial shoot tips of *M. aquaticum* and characterize morphological similarities.

2. Sequencing and isolation a KNOX1 gene from *M. aquaticum.*

3. Characterization of KNOX1 gene expression in shoot tips of *M. aquaticum.*

1.4 Proposed methods

Preliminary research on KNOX1 expression in the tissues of *Myriophyllum aquaticum* was conducted during the summer of 2006. Four forward, and one reverse degenerate, oligonucleotide primers were designed from the kn1 peptide sequences of 21 species of plants. These primers were then used to probe for the presence of kn1 in DNA extracts from the mature leaves of *M. aquaticum* using polymerase chain reaction (PCR). The presence of a putative KNOX1 gene was detected using gel electrophoresis, and the products from the PCR reactions representing the successful primers were cloned and sent to McGill University for sequence analysis. It was discovered that two of the eight colonies sent for sequencing contained a gene sequence with high sequence specificity to kn1-like nucleotide sequences which successfully identified part of a kn1-like gene in *M. aquaticum.*

The 2006 study laid the foundation for the proposed research. It is known that *M. aquaticum* contains a putative KNOX1 gene similar to knotted1 gene found in *Maize*. The next step is to determine whether there is expression of this gene in the developing leaves of *M. aquaticum.* RNA *in situ* hybridization (ISH) is proposed as a method for localizing gene expression of kn1 within sections of

shoot tips. This technique hybridizes synthetic RNA probes complementary to gene transcripts in sections of paraffin embedded tissue. RNA probes will bind complementary mRNA and produce a subsequent chemiluminescence or coloured dye thereby pinpointing the exact location of gene expression in the tissue. The protocol to be used for RNA ISH is derived from Kramer (2005) and is specifically designed for plant tissue. *Myriophyllum aquaticum* tissue will be grown and maintained using the UPEI departmental conviron growth chambers.

Chapter 2: Materials and Methods

2.1 Gene isolation and sequencing

2.1.01 Growth conditions for *Myriophyllum aquaticum*:

Plants were grown in Conviron growth chambers at an ambient temperature of 22 °C and a light dark cycle of 16 and 8 hours. Plants were grown in trays of Miracle Grow potting mix and placed in large pans of water to simulate aquatic conditions.

2.1.02 Tissue collection for RNA extraction:

Prior to tissue collection, harvesting equipment including mortar and pestle, spatula, and 2 ml tubes were placed on dry ice and allowed to cool for 10 minutes. Tissue was harvested from *M. aquaticum* corresponding to specific tissue types (i.e. leaf, stem, and shoot tip tissue). For leaves and stems, tissue was taken only from leaf tips and internodal regions of the stem to avoid inadvertently collecting axial mersitems. Each type of fresh tissue was weighed out in 120-130 mg quantities and deposited in the pre-cooled mortar and pestle on dry ice. Once tissue became frozen and brittle (taking approximately 30 seconds depending on tissue type), it was ground using the pre-cooled mortar and pestle until a very fine powder was attained. The resulting powder was scraped into an appropriately labeled pre-cooled 2 ml tube and maintained on dry ice until use, or placed at -80°C for long term storage.

2.1.03 Total RNA Isolation:

Prior to RNA extraction, all surfaces, utensils, and solutions were made RNAse free. All glass and metal utensils were wrapped in tin foil and baked in a muffle furnace at 450°C for four hours. All plastics, including media bottle caps and stir rods, were soaked in 0.1M NaOH for 24 hours and subsequently rinsed in diethylpyrocarbonate (DEPC) (Biobasic) water (0.1%). Solutions were prepared using baked glassware and DEPC water, and then autoclaved for 30 min.

The CTAB extraction buffer (see appendix) was made the day before the planned RNA isolation. On the day of the planned extraction, tissue tubes were removed from -80°C and held on dry ice until required. Before starting the extraction, CTAB buffer was incubated at 65 °C for 10 minutes to dissolve SDS (Sigma) precipitate. CTAB buffer was then divided into 800 µl aliquots, and combined with 23.5 mg of polyvinylpolypyrollidone (PVPP) (Sigma) and 1.56 µl of β-mercaptoethanol (Sigma) immediately prior to addition to frozen tissue. Under a fume hood, each 800 µl aliquot of extraction buffer was quickly added to a tube of frozen meristematic tissue, and then thoroughly vortexed. Tissue tubes were then placed in a 65 °C water bath for 45 min, with gentle agitation every 10 minutes. Approximately one volume of chloroform:isoamyl alcohol (Sigma) was added to each tube, which were then gently inverted for ten minutes. Tubes were then centrifuged at 17 000g for 10 minutes at room temperature, followed by transfer of the supernatant (avoiding interface contamination) into a new set of tubes. Another one volume aliquot of chloroform:isoamyl was added to each tube of supernatant, followed by gentle inversion for 10 minutes and centrifugation at

17000g (4°C) for 10 minutes. The resultant supernatant was transferred into a new set of tubes and combined with approximately 2.5 volumes of 95% ethanol and 1/10 volume of 3M sodium acetate (pH 5.2). Tubes were inverted several times to ensure sufficient mixing, and then placed at –20°C for 2 hours for nucleic acid precipitation. Tubes were then centrifuged for 30 minutes at 17000g (4°C), and the resultant ethanol supernatant was decanted while being careful to avoid disturbing the pellet. The pellet was then resuspended in 400 µl of 4M LiCl and 400 µl of nuclease free water, and placed at -20°C for 4 hours. Tubes were then spun for 30 minutes at 17000g (4°C), and the supernatant was pipetted off while being careful not to disturb the pellet. The pellet was then washed with 500 µl of 70% ethanol, and dislodged from the side of the tube by gently flicking the tube with a finger. Tubes were then centrifuged at 17000g (4°C) for 10 minutes, and the resultant supernatant was carefully pipetted off. Pellets were then air dried for10 minutes, and resuspended in 30 to 50 µl of nuclease free water depending on size of pellet. If the pellet proved difficult to dissolve, tubes were placed in a 65 °C water bath for 10 minutes to aide in pellet elution. Once the pellet was completely dissolved, RNA extraction was aliquoted out into working volumes to prevent frequent freeze thawing of RNA. Aliquots were stored at -80°C until further use.

2.1.04 RNA quantification and gel electrophoresis:

RNA concentration was determined by diluting 2.5 µl of concentrated extraction in 47.5 µl of TE buffer (pH 7.5) (see appendix) resulting in a 20 x dilution. Concentrations and 260/280 ratios were determined through

spectrophotometry on a Beckman Coulter DUS30 spectrophotometer. RNA which had 260/280 ratios between 1.9 and 2.0 was considered pure and free of DNA and protein contamination. Once concentration was established, RNA integrity was determined via eletrophoresis on a horizontal gel box (Biorad). Agarose was dissolved in 0.5x TBE running buffer (see appendix) to a concentration of 1% and then microwaved for 1.5 minutes until all agarose crystals had disappeared. Agarose was allowed to cool to approximately 40 °C, at which point ethidium bromide was added to a concentration of 0.05%. Molten agar was then poured into a mould and allowed to set at room temperature for 30 minutes. Once completely solidified, the agar gel was loaded into the accompanying gel box and immersed in a solution of 0.5x TBE running buffer. Approximately 2 μg of RNA was loaded into gel lanes with 3 μl of Trackit dye (Invitrogen), and then completed with ddH_2O to a final volume of 20 μl. Samples were electrophoresed at 80V (5V/cm) for approximately 1 hour, or until yellow trackit dye had traveled ¾ of gel length. All gels were visualized under UV illumination on an Eagle Eye. RNA integrity was established based on the presence of 28s and 18s rRNA bands. If smearing was absent, and band intensity was present in a 2:1 ratio between 28s and 18s bands, RNA was considered intact and non-degraded.

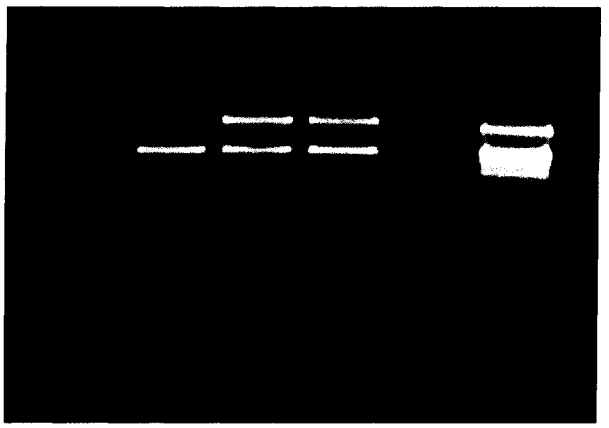

Figure 2.1 Typical RNA agar gel run at 100 V for 30 minutes. DNA ladder can be seen in the far left lane. The rest of the lanes contain RNA from shoot tips. Two crisp bands are visible for the 28 (top) and 18 (bottom) rRNA subunits are visible indicating intact RNA.

2.1.05 DNase treatment and phenol chloroform extraction:

RNA extractions were treated with RQ1 RNase-free DNase (Promega) at a concentration of 1 μg of RNA per unit of DNase. RNase free water and 10X reaction buffer were then added to RNA to generate a final concentration of 1X reaction buffer in total reaction volume. The reaction was incubated at 37°C for 30 minutes. To terminate the reaction, 1 μl of RQ1 DNase stop solution was added for every 10 μl of the total reaction volume, and the subsequent volume heated to 65 °C for 10 minutes. An equal volume of phenol:chloroform:isoamyl (ratio 24:25:1) was added and then centrifuged at 12000g and 4°C for 10 minutes. The aqueous supernatant was transferred to a new tube and an equal volume of chloroform added to remove trace amounts of phenol. Tubes were centrifuged again at 12000g and 4 °C for 10 minutes and the aqueous phase was removed to a new set of tubes. RNA was precipitated by adding 1/10 volume of 3M sodium acetate (pH 5.2) and 2.5 volumes of 100% ethanol, and then incubated at -20 °C

for 2 hours or overnight. RNA was then spun at 12000g and 4 °C for 10 minutes, and the supernatant removed without disturbing the pellet. The RNA pellet was then washed in 80% ethanol and centrifuged at 7500g and 4 °C for 5 minutes. The ethanol supernatant was then decanted and the pellet air dried for 10 minutes, or until all visual traces of ethanol had evaporated. Once dry, RNA was eluted in the appropriate amount of nuclease free water depending on pellet size and desired concentration. Generally speaking, RNA concentration ranged between 0.5 and 1µg/µl. RNA integrity was established by spetrophotometry and gel electrophoresis as described above.

2.1.06 cDNA synthesis:

All equipment was sterilized under UV light for 15 minutes before use. Complementary DNA (cDNA) was generated from RNA extracts by combining the following constituents:

Random Primers (100ng/µl) (Invitrogen)	1 µl
1 µg RNA template	_ µl
dNTP mix (10 mM each)	1 µl
Nuclease free H_2O	_ µl
Total volume	**12 µl**

The above reaction was then heated in an Eppendorf thermo cycler at 65 °C for 5 minutes. Tube contents were collected by brief centrifugation at room temperature and combined with 4 µl of 5x first strand buffer and 2 µl of 0.1M DTT. Reaction was incubated at 25 °C for 2 minutes and then combined with 1 µl SuperScript II Reverse Transcriptase (Invitrogen). The reaction was mixed by

gentle pipetting up and down and then incubated at 25 °C for 10 minutes, and then at 42 °C for 50 minutes. The reaction was terminated by incubating at 70 °C for 15 minutes.

2.1.07 Primer design and gene sequencing:

Previous research by E. Weidhaas (2006) conducted PCR analyses on DNA from mature *M. aquaticum* leaves using the following degenerate primers derived from KNOX1 sequences (see appendix): 5' AAR AAR AAR GGN AAR YTN CC 3' (forward) and 5' ACG TTT ACG TTG ATT AAT AAA CCA ATT ATT AAT TTG 3' (reverse). The cloned DNA was sequenced by Genome Quebec through McGill University, and then analyzed in the present study for KNOX gene homology using BLASTn (Genbank) optimized for somewhat similar nucleotide sequences. The resultant *M. aquaticum* KNOX gene fragment was then used in conjunction with the primer design program Primer3 (available on the Internet) to produce the following gene specific primers: 5' AGT TCA TGC GAA GGA TCG AA 3' (forward) and 5' TAT AAT GCA AGTCCC ACC AA 3' (reverse). The gene specific primers were then used in PCR analysis to probe for KNOX expression in cDNA from *M. aquaticum* shoot tips.

2.1.08 PCR:

All utensils were sterilized under UV light for 15 minutes prior to use. KNOX gene amplification was achieved by combining the following constituents:

GoTaq Green Master Mix 2X (Promega)	25 µl
KN1 Forward primer (10 µM)	2 µl
KN1 Reverse primer (10 µM)	2 µl
cDNA	4 µl
Nuclease free H_2O	17 µl
Total volume	**50 µl**

The reaction was then incubated in an Eppendorf thermo cycler according to the schematic depicted in figure 2.2. The thermal cycler was programmed to incubate at an initial denaturation temperature of 94 °C for 3 minutes, and then to commence cycling temperatures and times for primer annealing, DNA elongation, and denaturation. The optimal temperature for primer annealing was determined over a gradient of 2 °C increments between 56 °C and 64 °C. DNA elongation was carried out at a temperature of 72 °C for 45 seconds, and then denatured at 94 °C for 45 seconds. This cycle was then repeated a total of 35 times, after which amplified DNA was incubated at 72 °C for 3 minutes and subsequently held at 4 °C until retrieval. PCR products were stored at -20°C until required for further analysis. Integrity and specificity of PCR products were established by running 10 µl of amplified DNA and 2 µl of Trackit dye on an 1% ethidium bromide agarose gel at 100 volts for 30 minutes. PCR products which demonstrated a single crisp band of the correct molecular weight (corresponding to the length in

nucleotides of the desired gene fragment) were used in down stream cloning applications.

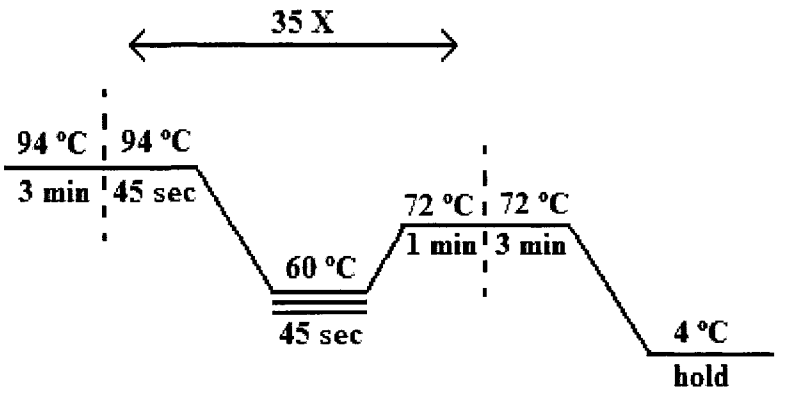

Figure 2.2 Schematic representation of PCR cycle.

2.1.09 PCR product purification:

After integrity was established through gel electrophoresis, PCR products were purified using EZ-10 spin columns (Biobasic). PCR products were removed from -20°C and combined with 3 volumes of binding buffer I (Biobasic), mixed by inverting, and added to an EZ-10 spin column. Column and PCR product was incubated at room temperature for 2 minutes, and then spun in a desktop centrifuge at 10 000 rpm for another 2 minutes. Flow through was removed from the collection tube, and the column membrane was washed with 500 ul of wash solution (Biobasic) and subsequently centrifuged at 10 000 rpm for 2 minutes. The flow through was removed, and the washing procedure repeated with an additional one minute spin to remove residual wash solution. Columns were then transferred to a new 1.5 ml tube, and column membranes were incubated in 30 µl

of elution buffer (appendix) at 37°C for 2 minutes. Columns were then spun at 10 000 rpm for 2 minutes, and resultant DNA solution was stored at -20°C until further use.

2.1.10 Cloning KNOX gene fragment:

Fresh Luria Bertani (LB) plates (see appendix) were prepared containing 1% agar and 50 μg/ml ampicillin were prepared 24 hours prior to commencement of each cloning experiment. Before beginning the cloning reaction, two selective LB plates were incubated at 37°C for 30 minutes, S.O.C medium (Invitrogen) was warmed to room temperature, and desktop water bath was equilibrated to 42°C. After 30 minutes incubation, LB plates were subcultured with 40 ul of X-gal stock solution (appendix), and maintained at 37°C until use. The *M. aquaticum* KNOX gene fragment was cloned into *E. coli* using a Dual Promoter TOPO TA cloning kit (Invitrogen). PCR products were inserted into a PCRII TOPO vector (Invitrogen) (see appendix for vector map) using the following reaction:

PCR product	3 ul
Salt Solution (appendix)	1 ul
dH_2O	1 ul
PCR II TOPO vector	1 ul
Total volume	6 ul

The above reaction was mixed by gently flicking the tube and then incubated at room temperature for 5 minutes. The reaction was then placed immediately on ice and held there until *E. coli* transformation. One tube of TOPO 10 *E. coli* was removed from -80°C and allowed to thaw on ice. Once completely thawed, 2 ul

of the plasmid preparation was added to the *E. coli* and then incubated on ice for 15 minutes. Cellular transformation was completed by heat shocking tubes of cells in a 42°C water bath for 30 seconds. Cells were then immediately transferred to ice, and supplemented with 250 µl of S.O.C. medium. Tubes of *E. coli* were then placed in sealed 15ml tubes and submerged in a water bath-shaker previously equilibrated to 37°C. Tubes were shaken for 1 hour at approximately 100 rpm. Transformed cells were then subcultured on selective plates and allowed to incubate at 37°C for 16 hours. The resultant colonies were either white or blue depending on whether they contained a vector with or without a gene insert (respectively).

2.1.11 PCR and sequence analysis of transformation:

One half of each of 9 white colonies and 1 dark blue colony were collected from selective plates inoculated the previous day. Colonies were resuspended separately in 50 ul of nuclease free water, and then added to the following reaction:

Gotaq green master mix (Promega)	12.5 ul
Forward M13 primer	1 ul
Reverse M13 primer	1 ul
DNA plasmid suspension	3 ul
H_2O	7.5 ul
Total volume/ tube	25 ul

The above reaction was incubated in a thermal cycler (Eppendorf) according to the same incubation and cycle times as used during the previous PCR protocol found in figure 2.2. PCR products were analyzed on a 1%, ethidium bromide

stained, gel run at 100 volts for 30 minutes. PCR product gels were visualized using an Eagleye fluorescence imager. Band sizes were compared to a DNA marker to determine presence of the gene insert in the plasmid. Once presence of insert was determined, colonies were inoculated into LB broth using a sterile pipette tip. The resultant cultures were processed into plasmid minipreps and sent to Genome Quebec for sequencing. Sequences were then blasted on Genbank (internet) for KNOX homology, and subsequent protein alignments were performed using the program Multalgn (Internet). Amino acid sequences of KNOX homologs from other species used in protein alignments were obtained from Genbank (see appendix).

2.1.12 Plasmid miniprep preparation:

The remaining half of *E. coli* colonies determined to contain the gene insert were plucked from LB plates and inoculated in 5 ml of LB liquid medium containing 50ug/ml ampicillin. Starter cultures were grown in 50 ml centrifuge tubes in a 37°C water-bath shaker for 8 hours, and then diluted 1:500 times in 12.5 ml of fresh media. Secondary cultures were grown for 12 hours under the same conditions as starter cultures. A 1.4 ml aliquot of each culture was removed and combined with 0.6 ml (30%, v/v) of sterile glycerol for long term storage at -80°C. A 1.5 ml aliquot was centrifuged at 13.5k rpm for 2 minutes, and the resultant cell pellet was processed using a EZ-10 spin column plasmid DNA miniprep kit (Biobasic). The cell pellet was suspended and lysed in 100 µl of solution I, thoroughly vortexed, and incubated at room temperature for 1 minute. Cell suspension was combined with 200 µl of solution II, gently mixed by

inverting, and incubated at room temperature for 1 minute. 350 µl of solution III was added to cell solution, incubated at room temperature for 1 minute and spun at 13.5k rpm for 5 minutes. The resultant supernatant was transferred to an EZ-10 spin column and centrifuged at 12k rpm for 2 minutes The flow through was discarded, and the membrane was then washed with 500 µl of wash solution and spun at 10k rpm for 2 minutes. The previous wash step was repeated, the flow through removed, and the column spun an extra minute at max speed to remove any residual ethanol. Plasmid DNA was eluted from column membrane with 30 µl of elution buffer previously incubated at 37 °C, and stored at -20°C.

2.2 Probe synthesis and Tissue preparation

2.2.1 Tissue fixation and paraffin embedding:

Shoot tips were dissected from plants and placed immediately in fixative (see appendix) on ice. After 30 minutes of dissection, all tissue was collected in a metal basket and placed in ice-cold fixative (see appendix) in a vacuum desiccator. Tissue was then vacuum infiltrated for 30 minutes to aid in tissue penetration of the fixative. During this period of infiltration, another collection of apices was dissected and placed in ice cold fixative. Once infiltration was complete, the secondary batch of tissue was combined with the initial batch in the vacuum desiccator, and another 30 minute period of infiltration was commenced. This process was repeated until sufficient tissue was amassed, at which point tissue was infiltrated for a final 30 minutes and placed at 4°C for 12-14 hours.

Tissue was then removed from fixative and placed in two successive 30 minute washes of 1X PBS (see appendix) at 4°C. After washing, the tissue was dehydrated through a series of washes of increasing ethanol concentration corresponding to 30%, 50%, 70%, 85%, and 95% respectively. Each wash was carried out for 30 minutes at room temperature. Once tissue was equilibrated at 95% ethanol, tissue was placed at 4°C over night. The next morning, tissue was placed in 100% ethanol for two sequential 30 minute washes at room temperature. Tissue was transferred to a 1:1 HemoDe (Scientific Safety Solvents) and ethanol solution for 30 minutes, and then to 100% HemoDe for another 30 minutes at room temperature. Tissue was then transferred to a fresh 100% HemoDe solution and placed at 55°C. For the next week, handfuls of paraplast chips (Fisher) were added twice a day to the tissue, with excess liquid poured off as required. After five days, tissue was removed from metal basket and transferred to a new vial of pure molten paraffin which was refreshed daily for the next five days. At this point, tissue was embedded in labeled paraffin rings and stored at 4°C.

2.2.2 Plasmid midiprep of antisense and negative control plasmids:

Using a sterile pipette tip, ice shavings were scraped from sense and antisense (determined from sequence analysis at McGill University) *E. coli* glycerol stocks and subcultured on LB plates (see appendix) culture media containing 1% agar and 50ug/ml ampicillin. Plates were incubated for 16 hours at 37°C until well dispersed bacterial colonies were visible. A single bacterial colony from both sense and antisense plates were transferred, using a sterile pipette tip, to separate

50 ml falcon tubes containing 10 ml of liquid LB media and 50 mg/ml ampicillin. The tops of the falcon tubes were then sealed using parafilm, and tubes were placed in a water bath shaker at 37°C for 8 hours. The starter cultures were then diluted 1:500 in 50 ml falcon tubes containing 12.5 ml of fresh LB media and 50 mg/ml ampicillin. Culture tubes were sealed and incubated in a water bath-shaker at 37 °C for 16 hours, and then combined to generate 25 ml culture volumes which were used in plasmid purification. Plasmid purification was carried out according to the Qiagen Compactprep Plasmid Purification Handbook. Bacterial cells were harvested from culture broth through centrifugation in a Beckman Coulter Allegra X-12R centrifuge at 3500 x g at 4°C for 30 minutes. The resultant pellet was resuspended in 2 ml of buffer P1 and thoroughly vortexed until all the clumps of pellet had been homogenized. Cells were then lysed by adding 2 ml of P2 buffer, mixed thoroughly by inverting, and allowed to incubate at room temperature for 3 minutes. DNA, proteins, and cellular constituents were precipitated from solution by the addition of 2 ml of S3 buffer followed by thorough inversion. Lysate was then decanted into the barrel of a Qiafilter cartridge and allowed to incubate at room temperature for 3 minutes. The cellular precipitate was then filtered out, and the resultant lysate was combined with 2 ml of buffer BB followed by thorough inversion. The adjusted lysate was then decanted into the tube extender of a compactprep midi column attached to a vacuum manifold. A vacuum was then applied to the manifold until all lysate had been drawn through the column, at which point the vacuum was turned off. Tube extenders were removed and columns were placed in 2 ml collection tubes.

Column membranes were then washed with 0.7 ml of PE buffer and spun on a microcentrifuge for 1 min at 4°C. Flow-through was decanted from collection tubes, and columns were spun for an additional minute to remove residual buffer. DNA was eluted in 100 ul of buffer EB (appendix), and stored at - 20°C. Integrity of plasmids was established by running 0.5 ul of plasmid prep on a 1% gel stained with ethidium bromide at 60 volts for 1.5 hours. Concentration was established by diluting concentrated plasmid 1:20 in TBE and running on a Beckman Coulter DU 530 spectrophotometer.

2.2.3 Plasmid restriction digestion:

Plasmids were linearized with the restriction enzyme HIND III (Invitrogen) in the following reaction:

Plasmid midiprep [1µg/µl]	20µl
HIND III (10 U/µl)	8 µl
10 x reaction buffer	20 µl
Nuclease free H2O	152µl
Total volume	200 µl

The reaction was gently mixed in at 1.5 ml tube and incubated at 37 °C for overnight. To check for digestion completion, 5 µl of the reaction was run on a 1% gel along side 200 ng of undigested plasmid (Figure 2.3). If digestion was complete, then one volume of phenol:chloroform:isoamyl was added to the reaction, vortexed vigorously, and spun on a desktop microcentrifuge for 10 minutes at 4°C. The supernatant was pipetted off and washed with one volume of chloroform:isoamyl to remove traces of phenol. The mix was vortexed vigorously and centrifuged at 13,500 rpm for 10 minutes at 4°C. The supernatant was removed to a fresh 1.5 ml tube, and precipitated for 30 minutes with 0.1

volumes of 3M sodium acetate and 2.5 volumes of 100% ethanol at -80°C. The precipitate was spun for 20 minutes at 4°C, and the resultant pellet washed with 500 ul of 70% ethanol and spun at max speed for 15 minutes at 4°C. The pellet was eluted in 13.5 ul of nuclease free H2O and stored at -20°C. Linearized plasmid concentration was checked using spectrophotometry as mentioned previously. Midipreps were made for antisense, sense, and negative control probes (sense and a 350 bp insert of lobster genomic DNA).

Figure 2.3 Representative gel of HinDIII restriction digestion of plasmid. Beginning from far left, lanes contain DNA ladder, undigested plasmids (lane 2 and 3), and digested plasmids (lanes 4 and 5). Difference in banding and run speeds indicated complete digestion.

2.2.4 RNA *in vitro* transcription and labeling:

RNA probes were transcribed from linearized plasmids using T7 RNA polymerase in the following reaction mix which was incubated at 37°C for 2 hours:

5x Transcription Buffer	5 µl
DTT	2.5µl
RNAsin (Promega)	1 µl
10x DIG RNA labeling mix (Roche)	2.5 µl
Linearized plasmid	1 µg
T7 RNA Polymerase (Promega)	2 µl
Nuclease free H2O	_ µl
Total volume	25 µl

After incubation, 1 ul was run on a 1% agar gel to assess the quantity and quality of the probe produced (Figure 2.4).

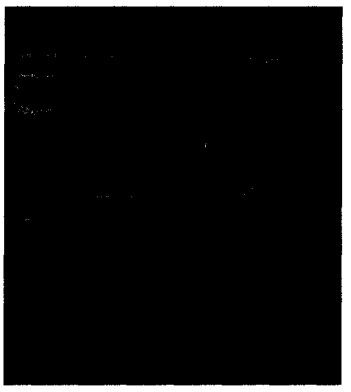

Figure 2.4 Standard gel of *in vitro* transcription before plasmid digestion with DNAse. Beginning from the far left, lanes contain DNA ladder and sense and antisense RNA probes (lane 2 and 3 respectively). Large probe bands represent plasmid templates and smaller bands represent the probe.

The plasmid template was then digested with RQ1 DNAse (Promega) in the following reaction mix which was incubated at 37°C for 1 hour:

Transcription reaction	24 µl
Nuclease free H2O	66 µl
DNAse	10 µl
Total volume	100 µl

To ensure complete digestion of plasmid template, 10 µl of the DNAse reaction was run on a 1% agar gel. If plasmid band had not disappeared, then the reaction was allowed to incubate at 37°C for another hour. Once template digestion was completed, the reaction was combined with 0.1 volumes of 3M sodium acetate and 2.5 volumes of 100% ethanol and allowed to precipitate at -80°C for 30 minutes. The precipitate was then centrifuged for 20 minutes at max speed at 4°C. The subsequent pellet washed with 500 µl of 70% ethanol and centrifuged at max speed at 4°C for 15 minutes. The pellet was allowed to air dry, and was eluted in 15 µl of H2O and stored at -80°C. Template integrity and concentration was estimated by running 1 µl on a 1% agarose gel. Each transcription reaction performed with 1 µg of plasmid template can be expected to produce approximately 10 µg of RNA template (Roche website). This concentration was used for calculating probe concentrations during subsequent *in situ* analysis.

2.3 RNA *in situ* hybridization

2.3.1 *In situ* prep work

Prior to beginning *in situ* hybridizations, all metal and glassware was baked at 450°C for four hours, and plastic components such as stirring rods and media bottle tops were soaked overnight in 0.1M NaOH. Any water used for preparing solutions was treated overnight with 0.1% DEPC and subsequently autoclaved for 30 minutes. Sectioning and slide preparation was performed the night before the planed *in situ*. Approximately 2 ml of DEPC treated H2O was pooled on glass Probe on Plus (Fisher) slides which were allowed to warm on a 42°C hot plate for 10 minutes prior to sectioning. Slides were always kept covered to prevent accumulation of dust and RNAse contamination. Paraffin blocks were sectioned to a thickness of 7 μm on a manual rotary microtome and sections were floated on preheated slides for 10 minutes. Once sections appeared translucent and fully expanded, excess water was drained and sections were allowed to adhere to slides overnight at 42°C.

2.3.2 Day 1: Probe hybridization

All wash solutions were prepared beforehand with DEPC treated water RNAse free reagents, and held in 200 ml pyrex glass beakers. Prior to beginning the series of slide washes, 150 ml of 1X protein kinase K buffer was incubated for at least 1 hour. Recipes for all the solutions mentioned below may be found in the appendix. Slides were handled with baked metal forceps and gloves were worn at all times to prevent RNAse contamination. Once set up was complete, slides

were removed from the slide warmer, placed in an up right metal slide holder, and moved in sequence from one solution to the next for the given period of time and temperature listed below for each wash:

Solution	Incubation Time (min)
Citrisolve (Fisher)	10
Citrisolve	10
100 % EtOH	2
100 % EtOH	2
95 % EtOH	2
90 % EtOH	2
80 % EtOH	2
60 % EtOH	2
30 % EtOH	2
DEPC H2O	2
2X SSC	20
0.2M HCl	20
DEPC H2O	2
DEPC H2O	2

*During DEPC H2O incubations, 10 ul of PK (Sigma) stock (100mg/ml) was added to 150 ml of preheated 1X PK buffer.

Proteinkinase K (37°C)	30
0.2% glycine in 1X PBS	2
1X PBS	2
1X PBS	2
4% PFA (In fume hood)	10
1X PBS	5
1X PBS	5

*During 1X PBS incubations, 560 ul of acetic anhydrate (Sigma) was added to 0.1M triethanolamine (Sigma). The TEA solution was stirred constantly in the fume hood during slide incubation.

TEA + acetic anhydrate (Fume hood)	10
1X PBS	5
1X PBS	5
30% EtOH	0.5
60% EtOH	0.5
80% EtOH	0.5

90% EtOH	0.5
95% EtOH	0.5
100% EtOH	0.5
100% EtOH	0.5

Slides were then held at 100% EtOH until ready for prehybridization incubation. The hybridization solution (see appendix) was generally prepared during the PK incubation and maintained at 80°C throughout the experiment. Once ready, slides were removed from 100% EtOH and allowed to air dry. A glass casserole dish was used as a hybridization chamber, the bottom of which was lined with damp paper towels to provide a humid environment for hybridization. Glass pipettes were placed in the bottom of the hybridization chamber, and once completely dry, slides were arranged face up along adjacent pipettes. To prevent contamination, antisense slides and negative control slides were placed in separate hybridization chambers. Slides were coated with 200 ul of hybridization solution (with out Riboprobes), the chamber sealed with plastic wrap and incubated at 55°C for 1 hour.

During the prehybridization incubation, probes were removed from -80°C and allowed to thaw on ice. Three different kinds of probes were used during hybridization: antisense, sense, and a fragment of lobster genomic DNA. The antisense served as the experimental probe, while the sense and the lobster gene functioned as negative controls. Once thawed, the correct proportion of probe and 50% formamide were mixed together to produce 300 ng (approximately) of probe per 40 ul formamide per slide. The formamide + probe solution was then incubated at 85°C for 5 minutes and transferred immediately to ice. Each 40 ul

aliquot was combined with 160 ul of hybridization solution to generate a total of 200 ul hybridization solution per slide. Hybridization solution was briefly vortexed, spun on a desktop microcentrifuge to remove bubbles, and returned to 80°C heat block.

After one hour of prehybridization, slides were removed from the hybridization chamber and gently blotted on kim wipes to remove excess hybridization solution. Slides were then replaced in the hybridization chamber, and coated with 200 ul of probe solution per slide. The slides were carefully covered with Parafilm and incubated overnight at 55°C.

2.3.3 Day 2: Immunological detection

Parafilm was removed from slides by gently agitating them in petri dishes filled with 0.2X SSC prewarmed to 55°C. Slides were held in coplin jars and moved through the following series of washes for the indicated times and temperatures. 0.2X SSC solutions were incubated at 55°C for 1 hour prior to slide washes, and 150 ml of NTE was allowed to incubate at 37°C until RNAse treatment. Antisense and control probe slides were kept separate for the first 0.2X SSC wash to prevent cross contamination between slides.

Solution	Time	Temperature(°C)
0.2X SSC	1 hr	55
0.2X SSC	1 hr	55
NTE	5 min	RT
NTE	5 min	RT

*During the NTE incubations, RNAse A (Sigma) was added to NTE solution prewarmed at 37°C to a concentration of 0.2mg/ml.

RNAse + NTE	30 min	37
NTE	5 min	RT
NTE	5 min	RT
0.2X SSC	1 hr	55
1X PBS	10 min	RT
BM block	45 min	RT
BM block	30 min	RT
Block 2	45 min	RT

Slides were moved from Block 2 solution and gently blotted on kim wipes to remove excess solution. Anti digoxigenin AP fab fragments were mixed in Block 2 solution to a ratio of 1:1250. Slides were coated with 400 ul of antibody solution and allowed to incubate in a dark, humid container for 2 hours at room temperature. Slides were then blotted on kim wipes and washed in three consecutive 20 minutes washes of block 2 solution. The final wash was allowed to incubate at 4°C overnight.

2.3.4 Day 3: Slide development and analysis

Slides were moved to a fresh block 2 for 20 minutes at room temperature, and through two subsequent washes in Buffer C of 20 minutes each. During buffer C washes, alkaline phosphatase substrate was prepared by adding 8 µl of 1M levamisol (Sigma) to 10 ml Western blue NBT/BCIP solution (Promega). After buffer washes were complete, substrate was carefully spread over the slides with a sterile pipette tip in 200 µl aliquots. Slides and substrate were then incubated in the dark at room temperature, and monitored on an hourly basis for color development for up to 24 hours. Once slides were judged to be fully developed (usually when pigment was strong enough to see with the naked eye), they were

rinsed for 2 minutes in TE buffer and then dehydrated through the following ethanol series:

Solution	Time (minutes)
30% EtOH	0.5
60% EtOH	0.5
80% EtOH	0.5
90% EtOH	0.5
95% EtOH	0.5
100% EtOH	0.5
100% EtOH	0.5
Citrisolve: EtOH (1:1)	1
Citrisolve	1
Citrisolve	1

Slides were then removed from citrisolve and permanently mounted with glass coverslips using Histoprep as a mounting medium. Slides were then observed using a Zeiss light microscope and pictures were captured using the accompanying digital imaging program.

2.4 SEM tissue preparation and observation:

Shoot tips were quickly dissected from plants and placed in ice cold FAA fixative (see appendix) overnight. Tissue was then washed twice in 50% ethanol for 30 minutes each, and then sequentially moved to 70% and 80% ethanol washes at room temperature of one hour each, and finally stained overnight in a 0.5% alcoholic solution of acid fuchsin. The next day, tissue was washed twice in 100% ethanol for one hour each, and dissected in 100% ethanol under a stereoscope until desired level of leaf development was revealed. Tissue was then placed in microporous specimen capsules (Marivac) and dried using a Ladd

model #28000 critical point dryer. Specimens were then mounted on pin type aluminum specimen stubs using Avery adhesive tabs and silver paint for grounding, and then coated with 600 angstroms of gold-palladium using a Denton vacuum desk II sputter-coater. Specimens were viewed using a Cambridge Stereoscan 604 scanning electron microscope (SEM) equipped with SEMICAPS digital imaging system. Hard copy images of specimens were printed on thermal paper using a Mitsubishi P67U video copy processor. Specimens were stored in pin SEM stub boxes, and placed under desiccating conditions.

Chapter 3: Results

3.1 KNOX gene isolation and sequence analysis

A fragment of DNA of approximately 300 bp was cloned (Fig. 3.1) using gene specific primers designed from a fragment of *Myriophyllum aquaticum* DNA previously isolated using degenerate primers (see appendix). Once sequenced, the fragment was blasted on Genbank and was revealed to have up to 80% nucleotide similarity to KNOX1 genes found in other species of plants. When translated into an amino acid sequence and aligned with other KNOX1 proteins (Fig.3.2), the *M. aquaticum* putative KNOX gene exhibited the presence of highly conserved domains typical of all KNOX genes. When proceeding from the N terminus to the C terminus in figure 3.2, the putative protein sequence codes for 8 amino acids of the Meinox domain, 24 non conserved amino acids, the GSE and ELK domains, and 24 amino acids corresponding to the beginning of the Homeobox. The colors in the alignment sequences represent the percent similarity of the amino acid residues in each column. Blue color corresponds to amino acids which are 50 to 90% conserved between species, and red corresponds to amino acids which are greater than 90% conserved. Black corresponds to amino acids which show less than 50% similarity between species. According to common practice of naming genes with the first letters from the genus and species, the *M. aquaticum* gene fragment was dubbed *Ma*KN1 and placed on Genbank under the accession number EU203679.

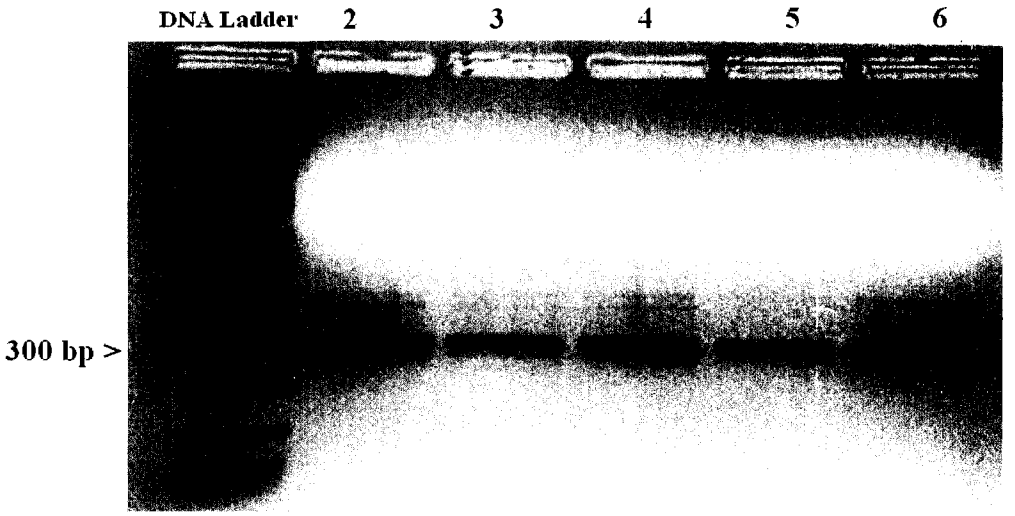

Figure 3.1 **Gel electrophoresis of PCR products cloned from cDNA from shoot tips. Beginning from the far left, lanes contain DNA ladder and PCR products amplified from five replicates of shoot tip cDNA (lanes 2–5). In all replicates, a single clear band appears at the 300 bp marker.**

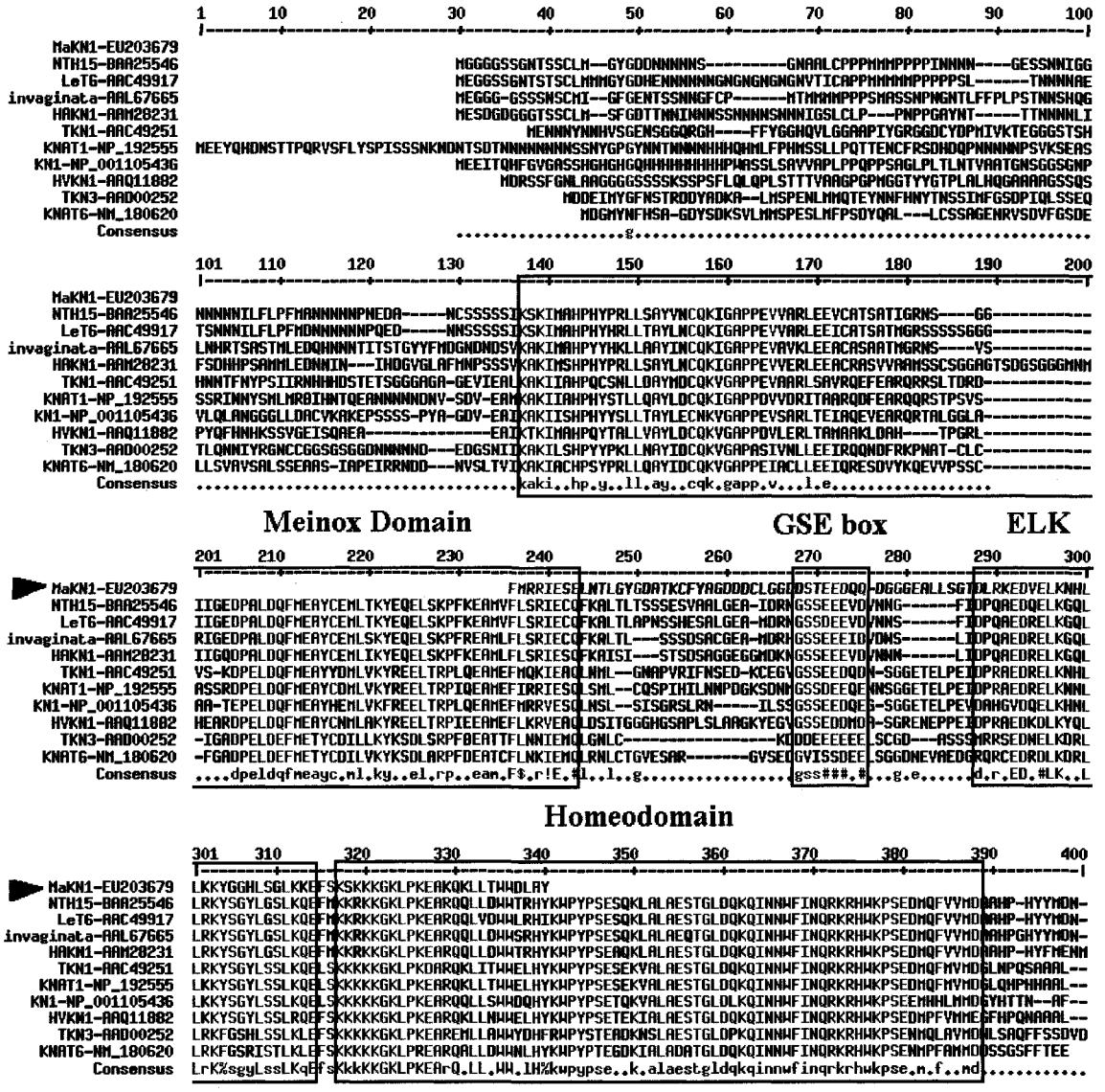

Figure 3.2 Protein alignments of MaKN1 and 10 other KNOX homologs. Percent similarities of sequences are show in red and blue which correspond to >90% and >50% respectively. Black corresponds to <50% similarity between amino acid residues. Sequence similarities can be seen which correspond to the Meinox, GSE, ELK, and Homeobox domains. Arrowheads correspond to lines containing MaKN1 putative protein.

3.2 Shoot tip morphology – microscopic analysis

3.2.1 Scanning electron microscopy – shoot tip morphology

Figure 3.3 shows scanning electron micrographs depicting the three dimensional morphology of the shoot tip. Leaf primordia appear in pentamerous alternating whorls which originate from the peripheral zone of the meristem (Fig 3.3A, B), although the number of primordia produced per whorl actually may vary between five and six. At the P1 stage, primordia appear as simple bulges in the peripheral zone of the meristem (Fig 3.3A). At stage P2, primordia have expanded into a conical leaf buttress lacking any lobes (Fig 3.3C). The first lobes appear at plastochrone 3, and are initiated at two well defined regions at the base of the leaf (Fig 3.3D). By plastochrone 4 (Fig 3.3E), primordia have developed several orders of lobes in a basipetal fashion where proximal lobes are younger (more recently developed) than those towards the distal portion of the leaf. It is also apparent at this stage that the lobes in each lobe pair develop in an alternating rhythm with respect to each other. This phenomenon becomes more apparent in older primordia (Fig 3.3F) where lobe insertions are not at the same level on the leaf axis. Throughout leaf development, lobes retain their initial orientation and symmetry (Fig 3.3F) and do not become concave as leaflets do in compound leaves. Between plastochrones five and six, small dome like protuberances appear in the axils of lobes (Fig 3.3G), and by plastochrone seven almost all the lobes of the leaf have been formed (Fig 3.3H). At P7, cellular differentiation becomes evident at the tip of the leaf as bulb like cells form on the epidermis of the distal most lobes (Fig. 3.3I). This differentiation progresses towards the base

of the leaf in subsequent primordia until the whole surface of the leaf is covered in spherical cells (used for trapping air around the leaf).

3.2.2 Light microscopy – shoot tip anatomy

Shoot tip developmental morphology of *M. aquaticum* was described using SEM and light microscopy. In median section the meristem appears dome like and is approximately 100 um in diameter at the peripheral zone (Fig 3.4A, B). Under higher magnification (Fig. 3.4D, the meristem has an internal corpus region enclosed within a two layer tunica. Provascular strands (PV) in the developing stem are apparent by plastochrone three (Fig. 3.4D) and become flanked by large empty spaces of aerenchyma (Ar) in more mature stem tissue (Fig. 3.4B, F). In median sections (Fig. 3.3A), leaf primordia may appear as either long unbroken protuberances corresponding to the central midrib portion of the leaf, or as a fragmented series of circular sections corresponding to cross sections through leaf lobes. The meristem is not apparent in marginal sections (Fig. 3.4B, C), which contain only stem, leaf, and lobe tissue. In cross section (Fig. 3.4E, F), the meristem appears as a central circle of undifferentiated cells surrounded by alternating whorls of leaf tips corresponding to primordia older than plastochrone three. Cross sections of leaf primordia appear as a central midrib flanked on either side by sections through progressively younger lobe primordial (Figure 3.4 E). Lobe sections which are closest to the mid rib are from more distal (older) lobes and are closer to the base of the each lobe. Lobe sections farther away from the mid rib are from more proximal (younger) lobes and are closer to the tip of each lobe.

Figure 3.3 Scanning electron micrographs of *M. aquaticum* shoot tips. Primordia are represented by (P) followed by their plastochrone number. A) and B) frontal and overhead views of the meristem (M) and primordia (P) [scale bars = 30 μm]. C) close up view of P2 primordium [scale bar = 15 μm]. D) Close up view of P3 primordium [scale bar = 15 μm]. E) P4 primordia, arrows indicate alternating lobe development [scale bar = 30 μm]. F) Close up view of lobes of a P6 primordia. Arrowheads indicate alternating lobe insertions [scale bar = 30 μm]. G) P5 and P6 primordia showing trichome development in lobe axils (arrows) [scale bar = 30 μm]. H) P7 primordia showing complete lobe morphogenesis [scale bar = 150 μm]. I) Close up view of P7 leaf tip. Arrowhead indicates epidermal differentiation [scale bar = 75 μm].

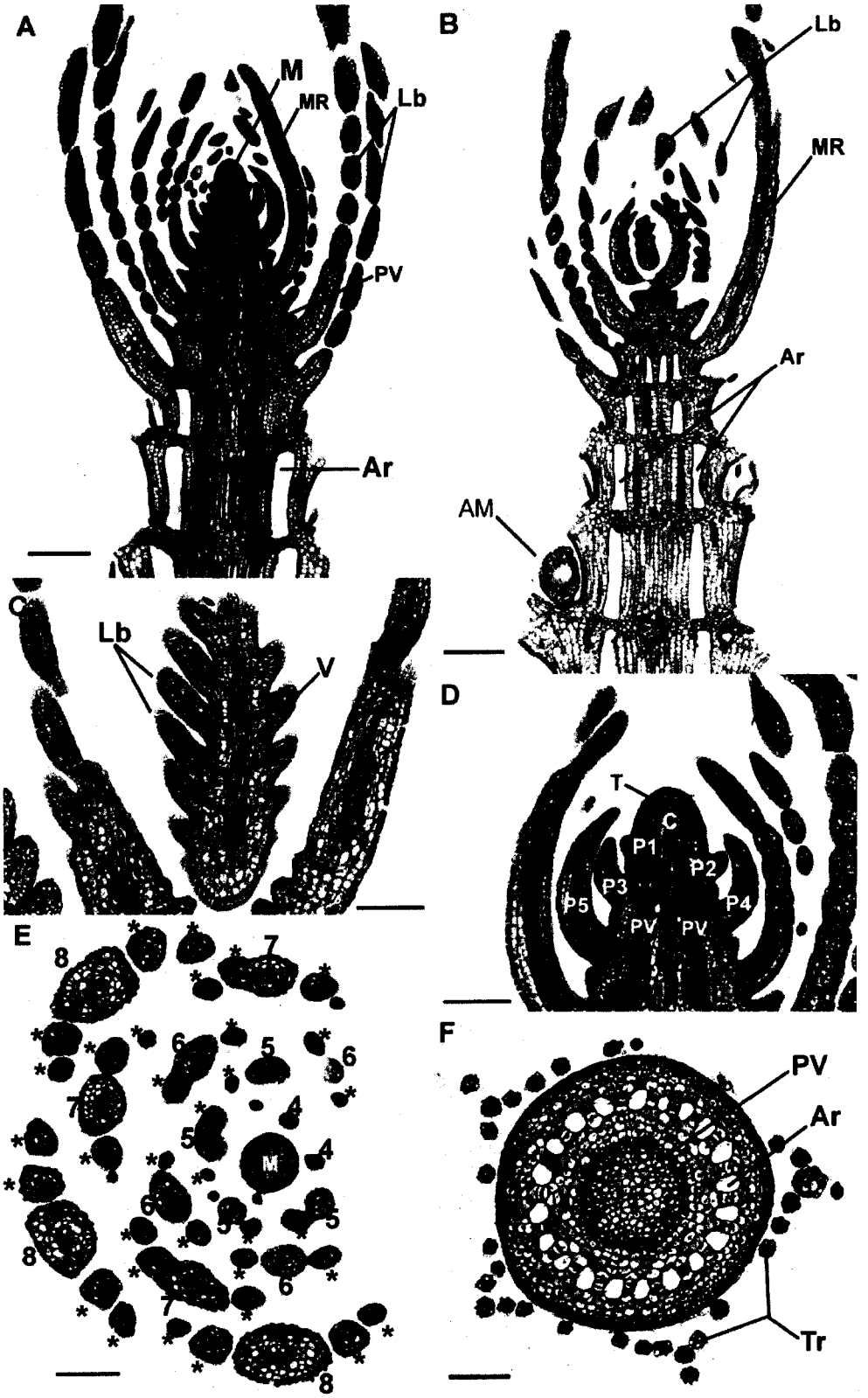

Figure 3.4 (Legend on the following page)

Figure 3.4 (Image on previous page) Longitudinal (A-D) and cross (E and F) sections of *M. aquaticum* shoot tips stained with toluidine blue. A) Longitudinal section through meristem (M), leaf midribs (MR), and lobes (Lb) [scale bar = 200 µm]. Provascular strands (PV) and aerenchyma (Ar) are evident in more mature stem tissue. B) Depicts marginal sections through the stem [scale bar = 200 µm], and C) shows a section through a young leaf. Veins are depicted by (v) [scale bar = 50 µm]. D) Section through meristem showing corpus (C) and tunica (T) organization [scale bar = 200 µm]. E) Cross section through the meristem. Sequential plastochrones are represented by their corresponding numbers, and leaf lobes are represented by (*) [scale bar = 100 µm]. F) Cross section through mature portion of the stem [scale bar = 50 µm]. Trichomes found in leaf axils are represented by (Tr).

3.3 RNA *in situ* hybridization

3.3.1 Negative controls and probe specificity

Several types of negative controls were used during *in situ* experiments to ensure specificity of probe hybridization. Sense probes were initially used as a negative control during hybridization experiments, however they proved to hybridize in much the same pattern as antisense probes. To determine if sense probe binding was due to non specific binding of digoxigenin antibodies, no probe control slides were run along antisense and sense control slides. No probe slides failed to develop any hybridization signal, indicating that signal from sense negative controls arose during probe hybridization (Fig 3.5A). Other sources have reported similar difficulties with sense probe hybridization due to faulty T7 polymerase binding during transcription (Tioni et al., 2003; Gilmartin and Bowler, 2002; Muller et al, 2001). To determine if this was the case during this particular experiment, a 350 bp probe from a lobster gene fragment was used as an alternate negative control. When hybridized under the same conditions as antisense probes, the lobster probe failed to show any hybridization signal

indicating that observed signal specifically represents KNOX1 expression (Fig 3.5B). Using a 40x objective, hybridization of antisense probes can be localized at a cellular level. In figure 3.5C, it can be seen that hybridization signal is located in the cytoplasm (Cy) of the cell indicating that probes are binding specifically to mRNA as opposed to other cellular components such as cell walls (CW) or nuclei (N).

3.3.2 KNOX *in situ* localization – Longitudinal Sections

Shoot tips of *M. aquaticum* were examined in both longitudinal and cross sections for KNOX gene expression using RNA *in situ* localization. Sites of probe hybridization are observed as a blue stain. Figure 3.6 shows serial longitudinal sections through the shoot apical meristem and leaf primordia. As sections progress through the meristem, overall patterns of putative KNOX expression are elucidated. In every section, the meristem shows hybridization signal. Provascular strands also exhibit hybridization in every section. Plastochrones 1 to 3 (P1 to P3) exhibit probe hybridization evenly throughout their respective primordia. However, the location of gene expression changes as development proceeds. Expression at plastochrone 4 (P4) appears to be located primarily in the tip and on the adaxial side of the midrib (Fig. 3.6A). As sections progress towards the outer lobed margins of the primordia, expression becomes more intensely and evenly expressed throughout the tissue. By plastochrones 5 and 6, hybridization signal has moved out of the central mid rib region of the leaf and appears primarily in the lateral lobes. In figures 3.6A and B, regions of the P5 lobe primordia can be seen appearing in the section and exhibit intense

hybridization signal. In figure 3.6 C, P5 tissues appear to exhibit less hybridization signal. As sections pass through the marginal lobes and into the central portion of the primordia, signal fades until it is completely absent (fig. 3.6D, E, and F).

Expression in plastochrones higher than P6 is more readily observed in sections through the marginal region of the shoot tip. In figure 3.7, patterns of expression can be observed in primordia between plastochrones 6 and 9. In figure 3.7A, the outer most region of the stem is in section with subsequent serial sections proceeding towards the center of the shoot apical meristem. In the central region of the P7 primordia, very little expression is evident (Fig. 3.7A). As serial sections proceed into the leaf in figures 3.7B and C, hybridization signal appears in the marginal lobes and at the leaf base. In figure C, all lobes are present in section, and it is apparent that hybridization signal is absent from older lobes at the tip of the leaf and is more strongly expressed in the tips of the younger lobes at the base of the leaf (arrows). Expression patterns appear very similar in P8 primordia, where hybridization signal is absent in the central midrib and the basal portion of lobes (Fig. 3.7A-D), but are visible in the outermost regions of marginal lobes (Fig. 3.7E-F). Hybridization signal appears to be less intense in P8 compared to P7. Similar patterns can be observed in P6 primordia in sections D through F. Sections are taken only from the central region of the P9 primordia in Figure 3.7 making it difficult to determine the presence or absence of hybridization signal.

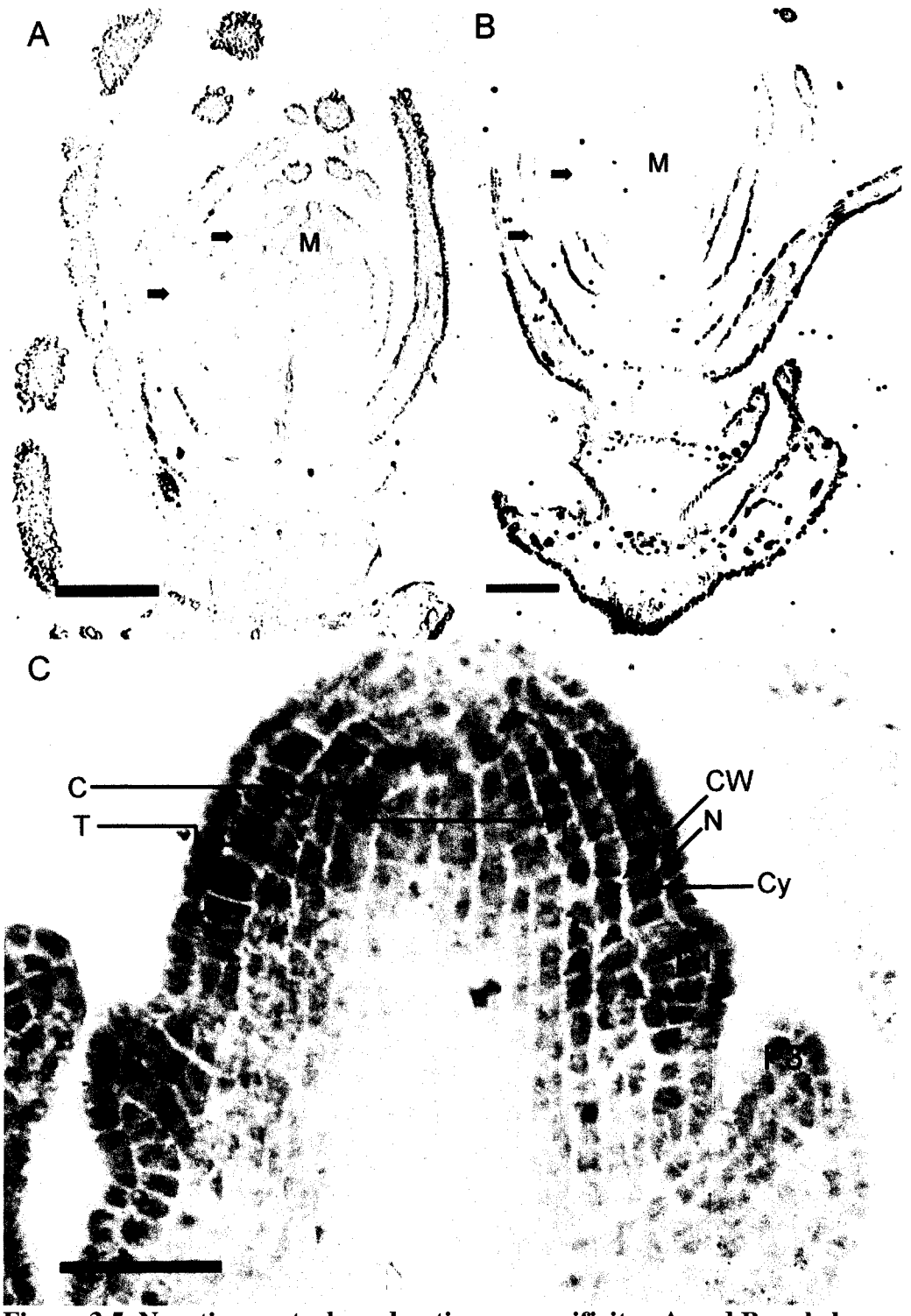

Figure 3.5 Negative controls and antisense specificity. A and B scale bars are 200 µm and C is 25 µm. A) Negative control section using lobster DNA as a probe. Hybridization signal is absent from both lobes (arrows) and meristem (M). B) No probe control slide. Hybridization signal is absent from both lobes and meristem. C) Meristem at 40x showing corpus (C) and tunica (T). Pigment is localized to cytoplasm (Cy) absent from cell walls (cw)

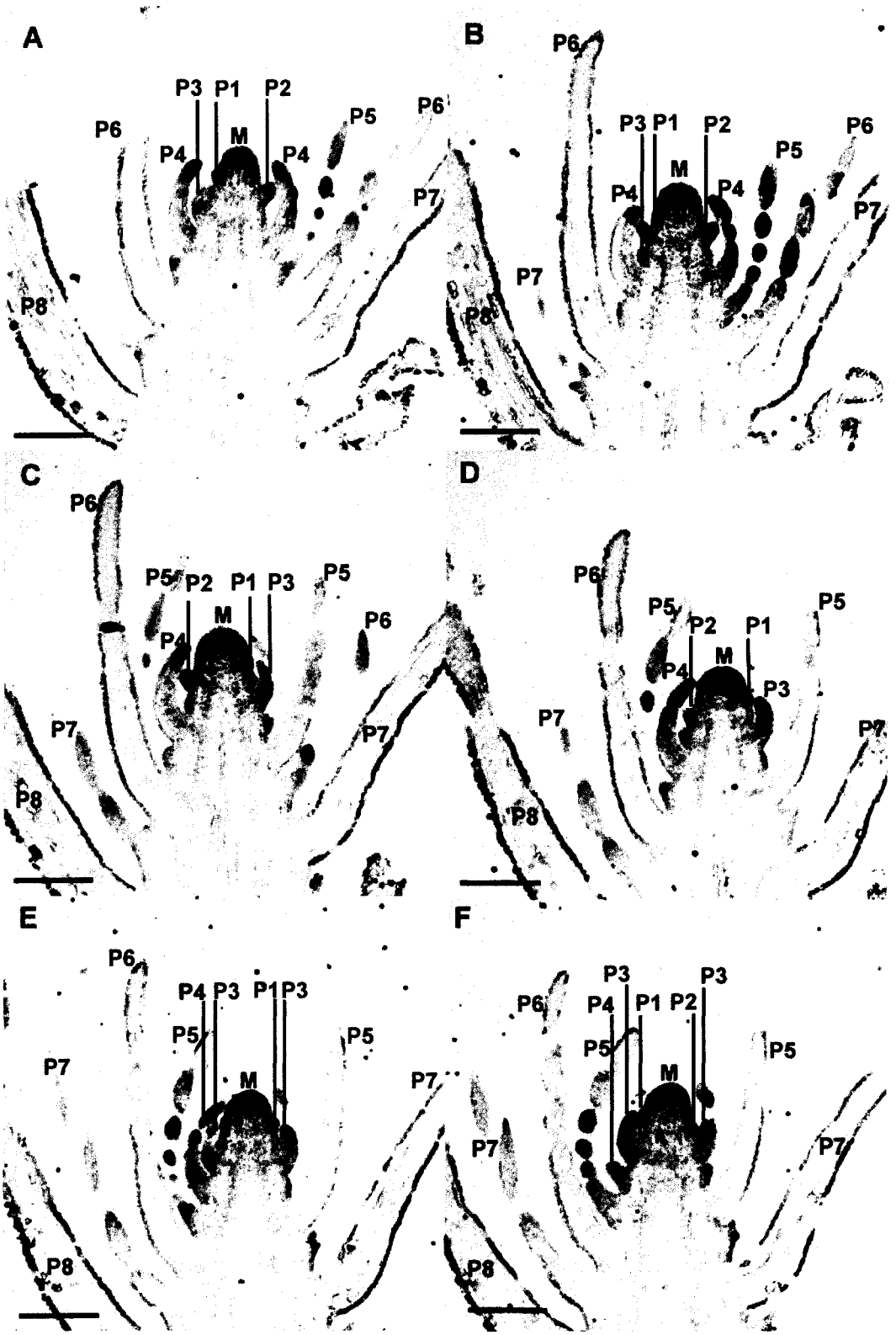

Figure 3.6 Serial longitudinal sections (A-F) through a single shoot apical meristem (M) exhibiting putative KNOX gene expression. Primordia are represented by (P) followed by their respective plastochrone number. Scale bars = 200 µm.

Figure 3.7 **Serial longitudinal sections through the marginal region of the shoot tip and primordia exhibiting putative KNOX1 expression. Arrows point to regions of putative expression. Primordia are represented by (P) and corresponding plastochrone number. Scale bar = 100 μm.**

3.3.3 KNOX *in situ* localization – cross sections

Serial cross sections offer a complementary perspective of KNOX1 expression in the shoot tip of *Myriophyllum aquaticum* (Figs. 3.8 and 3.9). Regions of the shoot more distal to the meristem can be observed in figure 3.8, whereas sections closer to and at the level of the meristem may be observed in figure 3.9. In Figure 3.8 A), sections begin at the P8 node and proceed up the stem until the P7 internode (Fig. 3.8F). By examining serial sections of the mature stem, it can be seen that gene expression varies between nodal and internodal regions of the stem. At the P8 node in figure 3.8A, there appears to be very little hybridization signal in either the lower lobes of P9 or at the leaf bases of P8. As sections proceed into the P7 internode in figures 3.8B and C however, expression is evident in the outer regions of the stem as well as in the provascular cylinder. At this point, expression is also apparent in the lobes of the P8 primordia. Expression remains absent in all sections of P9 primordia. In figures 3.7D and E, the location of the expression pattern in the outer tissues of the stem becomes isolated to pockets of tissue between the leaf bases and in the lobes of P7 primordia. At this point lobes of P8 still exhibit expression, however as serial sections proceed up the primordia, expression becomes weaker until it is completely absent at the level of the nodal region of plastochrone 6 in figure 3.7 F).

Serial sections in Figure 3.9 begin at the P3 node (3.9A) and progress towards the meristem (3.9F). In sections close to the meristem, the difference in pattern of expression between nodes and internodes is less obvious as the hybridization

signal becomes more evenly expressed in the developing pith. In sections at the level of the meristem (Fig. 3.9E, F) gene expression appears equally in all cells. Cross sections show similar patterns of expression in leaves as seen in longitudinal sections where plastochrones 1 and 3 exhibit hybridization signal evenly throughout the primordia and plastochrones P4 or older show signal primarily in lobes.

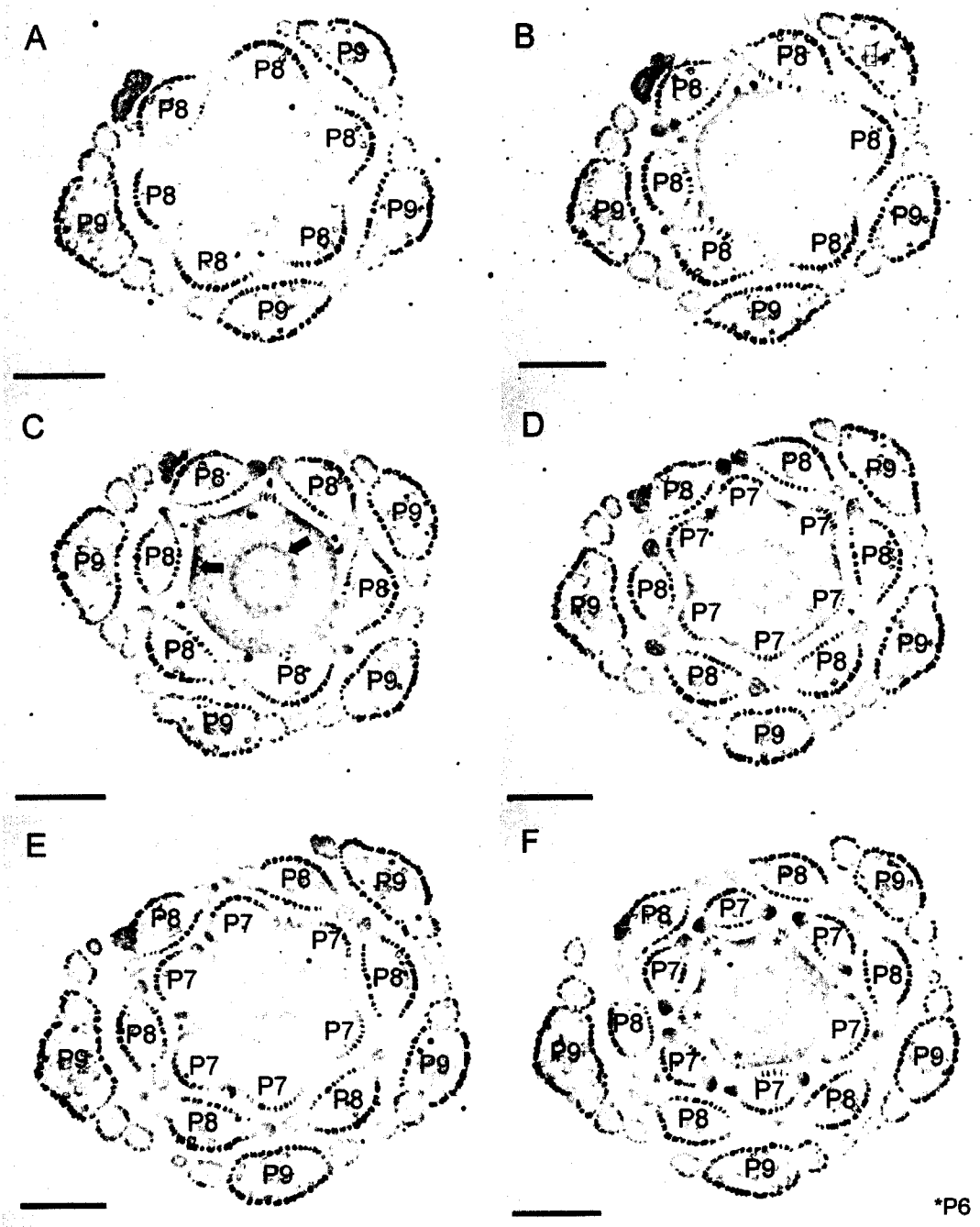

Figure 3.8 **Serial cross sections (A-F) beginning at plastochrone 8 (A) proceeding towards the shoot apical meristem and finishing at plastochrone 6 (F). Primordia are represented by (P) followed by their respective plastochrone number. In figure F), (*) represents the leaf bases of P6. Scale bar = 200 µm.**

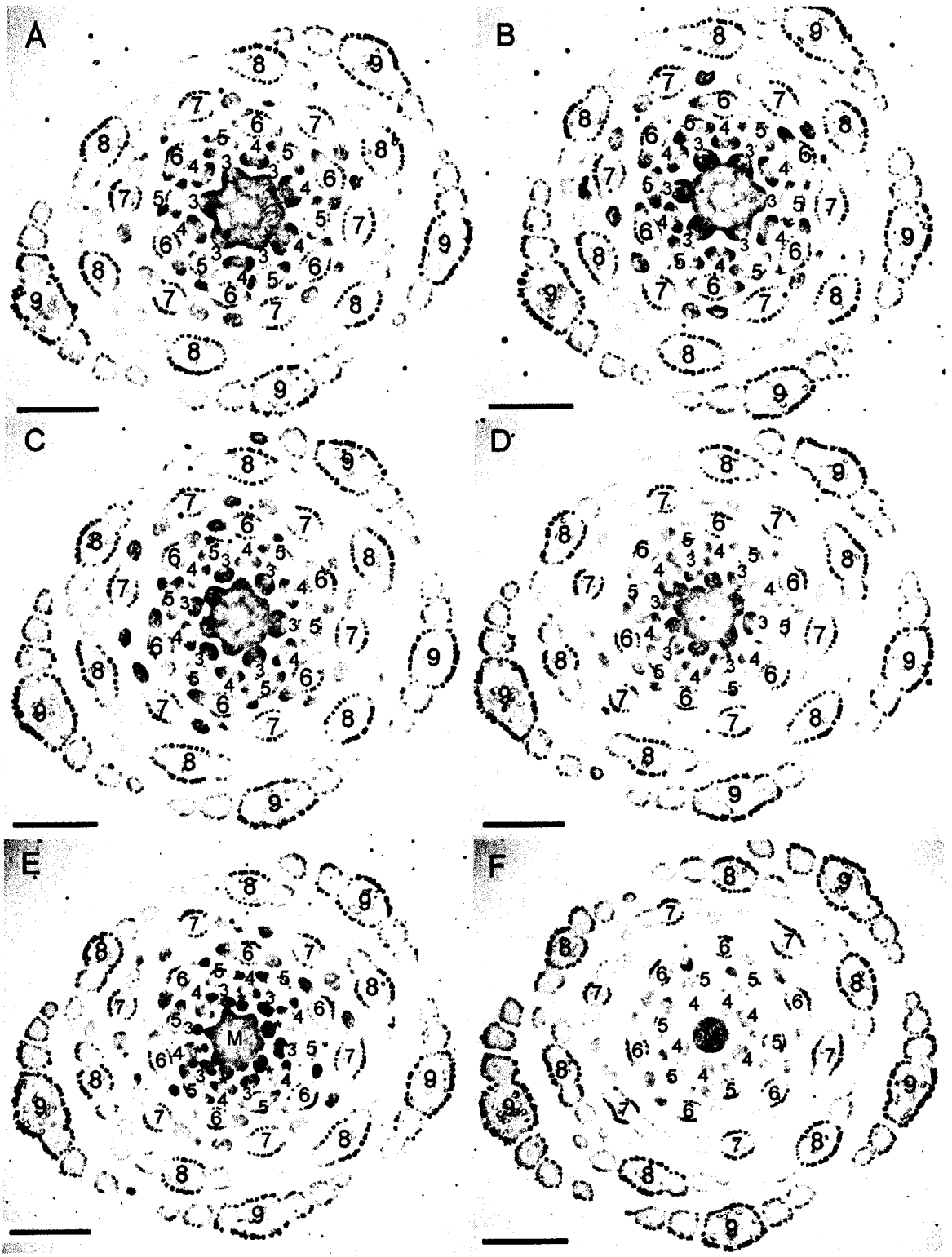

Figure 3.9 **Serial cross sections (A-F) of the shoot tip proceeding towards the meristem. Sections begin at the P3 node (A) and end at the level of the meristem meristem (F). Primordia are represented by the number of their respective plastochrones. In figure (C–E), P2 is represented by a (*). Scale bars = 200 μm.**

Chapter 4: Discussion

There has been considerable debate concerning the nature of relationships of plant organs both within and between species (Sattler 1994; Kaplan 2001; Bharathan and Sinha, 2001). In the past, plant morphology has touted the use of three mutually exclusive archetypes (leaves, roots, and shoots) for delineating plant organs and understanding their developmental relationships (Kirchoff et al., 2008). Leaves are said to occupy a lateral position on the plant body, be bilaterally symmetrical, and have a determinate growth pattern. Roots and shoots may be radially symmetrical, have indeterminate patterns of growth, but occupy opposite poles of the plant axis (Bell, 1991). Shoots must occupy either a terminal position, or be subtended by a leaf, but they may not themselves subtend of lateral organ. The opposite consideration applies to leaves in that they may subtend an axillary organ, but never occupy the axil themselves (Sattler, 1994). For the vast majority of situations, this paradigm is perfectly efficient at correctly identifying plant organs. However, situations exist where plant organs appear to exhibit characteristics from more than one structural category making correct identification of organ homology difficult (Rutishauser and Isler, 2001).

This situation is exemplified at the molecular level by the expression of KNOX genes during both shoot and leaf development (Sinha, 1997; Hofer, 2001a). In species with simple leaves, KNOX1 expression is isolated to the meristem, while in compound leaves KNOX1 expression may be found in both the meristem and compound leaf primordia (Champagne and Sinha, 2004; Goliber et al., 1999). The overlap in KNOX1 expression during leaf and shoot

development is well documented in many species of angiosperms, and has been used to support the idea that there are shoot-like processes at work during the early stages of development in compound leaves (Brand et al., 2007; Lacroix et al., 2005; Hofer, 2001a; Sinha et al., 1997). This is evidence that developmental parallels exist between classical organ categories and suggests the presence of a morphological continuum of plant form (Sattler, 1986; Rutishauser and Moline, 2005).

The goal of this thesis was to characterize morphological and molecular similarities between shoot and early stages of leaf development using the aquatic angiosperm *Myriophyllum aquaticum* as a model. This species of plant was desirable for this purpose because of its highly lobed leaves which were previously suggested to exhibit shoot like characteristics during development (Jeune, 1975; Jeune, 1976; Jeune, 1977). Morphological similarities were ascertained by examining the three dimensional morphology of the shoot tip through SEM analysis. This was then complimented with molecular analysis of KNOX1 expression patterns during shoot and leaf development. It was discovered that developmental parallels are in fact evident during leaf and shoot development in *M. aquaticum* which suggests that these organs may not be mutually exclusive from each other but are partially homologous. The following sections analyze the results of this study in further detail and elaborate on how they may be used to support a more dynamic paradigm of plant morphology.

4.1 General developmental morphology

M. aquaticum does not exhibit an overtly atypical shoot or leaf development, and so does not contradict classical categorization as some plant species do. The meristem is radial and demonstrates a two cell layer tunica overlaying an inner corpus as is common in most angiosperms (Steeves and Sussex, 1989; Raghavan, 1999). Mature leaves are easily identified using classical morphological analysis as determinate, lateral structures which subtend axillary buds. SEM analyses on *M. aquaticum* leaf development show that leaf primordia are produced from the peripheral zone of the meristem in alternating whorls.

Although the leaves of *M. aquaticum* may at first appear similar to compound leaves during development, they are more correctly identified as highly lobed simple leaves. *M. aquaticum* produce lobes from specific locations along the leaf axis in a manner that is reminiscent of leaflet development in compound leaves (Lacroix et al., 2002). However, compound and lobed morphologies deviate from each other as lateral elements develops. As leaflets develop, they undergo a change in orientation from being flattened in a perpendicular plane to a parallel plane with regards to the leaf axis. As well, once change in orientation takes place, leaflets become concave on their adaxial surface (Lacroix and Sattler, 1994; Lacroix, 1995). In highly lobed simple leaves, as seen in *M. aquaticum*, lobes do not undergo a change in orientation and do not become concave. This indicates that the leaves of *M. aquaticum* represent a different structural leaf class as compared to compound leaves.

The leaves and shoots of *M. aquaticum* appear to exhibit similar morphological characteristics which point towards developmental parallels between the two structures. Leaf-shoot parallels may be observed during lobe development. The lobes of *M. aquaticum* are produced basipetally and appear to originate from two very distinct centers at the base of the leaf in an alternating fashion. This alternating production of lobes could be thought of as reminiscent of the production of leaf primordia on a distichous shoot where each nodal region contains a single leaf and successive leaves are arranged 180° from each other (Jean, 1994). In other words, the same processes which result in distichous leaf patterning may also be functioning during lobe development. This highlights the importance of considering the plant as a collection of developmental processes that can be mixed and matched, or shared between partially homologous structural categories in a dynamic morphological continuum.

A possible area of future research could be found in examining the role of auxin during leaf development of *M. aquaticum*. Where auxin has been studied in model species, it has been found to be closely tied to controlling the position of lateral organs around the shoot (Reinhardt et al., 2003; Quint and Gray, 2006). Points of accumulation of auxin in the peripheral zone of the meristem will correspond to the next site of primordia initiation (Benkova et al., 2003; Reinhardt et al., 2004). In *Arabisopsis*, auxin typically works in concert with the transcription factor ASYMMETRIC LEAVES1 to suppress KNOX1 expression in the simple leaf primordia (Hay et al., 2006). However, it has been discovered in the compound leaved relative of *Arabidopsis, Cardamine hirsuta*, that KNOX1

will actually co-accumulate with auxin at site of growth foci to promote leaflet production which further illuminates differences in KNOX1 expression between simple and compound leaves (Barkoulas et al., 2008). If this process is repeated in *M. aquaticum*, it may be that the growth centers at the base of the leaf primordia could also experience fluctuating levels of auxin which control the pattern of lobe production. Techniques used to explore this theory could include the affects of auxin on lobe production via microinduction analyses, or it could involve immunolocalization analyses of the auxin transport protein PIN during leaf development (Hay et al., 2004).

Another potential future area of research would be to explore the organogenetic capacity of the lobe producing centers at the base of the leaf. If the two regions of lobe production act as ephemeral meristematic centers for lobe production, it might be expected that if one or both of these centers were destroyed it would affect lobe development. Possible outcomes of destruction of these generative centers could be the production of mature leaves which lack lobes, or with lobes on only one side of the leaf axis. This experiment was previously attempted on a rudimentary level by mechanically destroying the centers with fine needles (Jeune, 1976). A more refined experiment using microsurgical laser ablation may produce more reliable results (Reinhardt et al., 2004).

It could be suggested that the lobe like trichomes in lobe axils of leaves at plastochrone six also represent another structural parallel between leaves and shoots. It could not be established if these trichomes were produced in a basipetal or acropetal direction, or if they formed simultaneously in all axils. It was

observed however that some leaves produced axillary trichomes and some did not. This could be thought of as reminiscent of axillary meristems which may either develop in leaf-shoot axils or remain dormant (Romberger et al., 1993; Lyndon, 1990). These trichomes are not presumed to be homologous to axillary meristems, but their placement and overall patterning is very reminiscent of the leaf-shoot system as a whole and could represent an encasement of a common developmental framework between leaves and shoots (Lacroix et al., 2005).

4.2 Molecular analysis – KNOX expression

The founding concept behind continuum morphology involves the principle of shared developmental processes between organ categories (Arber, 1950; Sattler, 1986). Supporting evidence for the presence of shared developmental processes may be observed in mature plant structures that appear to have similar developmental patterns or mature forms (Rutishauser, 1999; Dengler, 1999; Fukuda, 2003). Such structural similarities are apparent in *M. aquaticum* as was described in the previous section. However, although it is possible to visually observe the physical manifestation of these shared processes, their actual molecular nature remains enigmatic. By examining the expression patterns of KNOX1 in *M. aquaticum*, it was possible to complement the observed structural similarities with molecular identification and characterization of one of the key master control genes that function during plant development (Leyser and Day, 2003; Hofer, 2001a).

4.2.1 Probe specificity:

Although they have been identified in many different species, there has not been any recorded data on KNOX gene expression in the order Saxifragales before this thesis. Therefore, before expression analysis could be undertaken, a KNOX1 gene from *M. aquaticum* had to be first isolated and then sequenced to ensure its homology with other KNOX1 genes. Orthologous genes (derived at the time of divergence between taxa) may be grouped together based on the presence of conserved amino acid domains in protein sequences (Granger et al., 1996; Janssen et al., 1998; Jourannic et al., 2007). It is therefore possible to determine the homology of the *M. aquaticum* putative KNOX1 probe based on the presence of these conserved amino acid domains. By using the program BlastN, the *M. aquaticum* gene fragment was analyzed with respect to all available gene sequences archived in the Genbank database and it was found to show up to 80% sequence similarity in some cases. This strongly indicates that the *M. aquaticum* gene fragment is indeed a KNOX gene.

KNOX genes may be divided into groups I and II based on sequence similarity of the homeobox and the presence and location of introns (Volbercht et al., 1991; Reiser, 2000). One of the key features of KNOXI genes is the presence of an intron within the first few residues of the homeodomain (Long et al., 1996; Harrison et al., 2005). KNOXII genes have this intron, but also exhibit the presence of a second intron within the ELK domain (Kerstetter, 1994). The initial degenerate primers were used to probe total DNA extractions, which include the presence of both introns and exons in their sequence, unlike total RNA extractions

and their subsequent cDNA counterparts. When compared to protein sequences of other KNOX genes, the *M. aquaticum* fragment exhibits a section of non-conserved base pairs within the homeodomain, but not outside it, indicating that the sequence is homologous to KNOXI rather than KNOXII genes. Taking the above data into consideration, it is reasonable to conclude that the isolated gene fragment is a putative KNOXI homolog. However, it cannot be confirmed as such until the full length cDNA sequence has been isolated and functional analysis performed on the encoded protein.

A single species of plant may have multiple KNOXI homologs which are expressed during plant development. *Arabidopsis thaliana* has 4 KNOXI homologs in its genome while *Oryza sativa* has six (Jouannic et al., 2007). Although they all appear to share functional redundancy, they exhibit specific regions of expression within the shoot tip (Reiser, 2000; Hareven et al., 1996; Groot et al., 2005). The specific regions of gene expression are typified by the expression domains seen in the two *Arabidopsis* KNOX genes STM and KNAT1, and LeT6 which is the KNAT1 homolog in *Lycopersicum esculentum* (Reiser et al., 2000). In Figure 4.1, it can be seen that STM is expressed in the meristem and in the basal ground tissue of the stem while KNAT1 is expressed in the internode regions between leaf primordia as well as the meristem. LeT6 shows expression throughout the meristem, the ground tissue, and in developing leaf primordia.

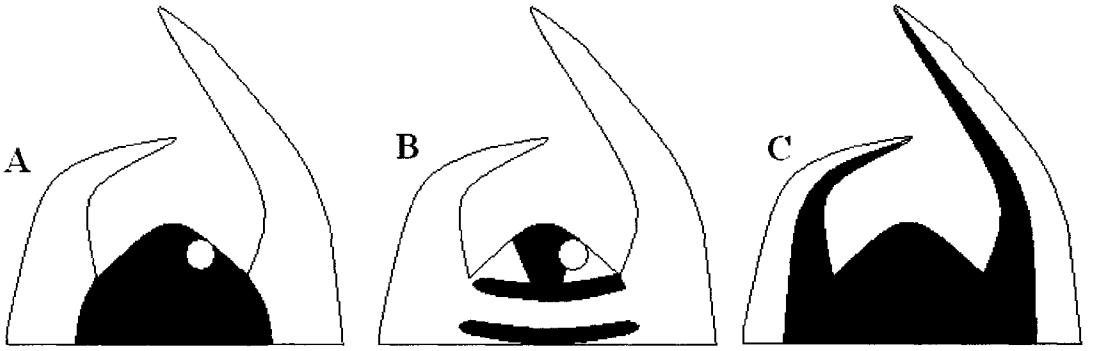

Figure 4.1. Three typical patterns of expression exhibited by KNOX1 genes. A) Represents typical binding patterns for STM like genes. B) Shows typical patterns for KNAT1 like genes. C) Shows the typical pattern of expression for LeT6 in the apex of Tomato.

When examining the patterns of expression of KNOX in *M. aquaticum*, it is evident that it appears to exhibit patterns reminiscent of all three types of expression. There is expression present at the meristem, the developing stem, and within the developing leaf primordial (Figures 3.6 and 3.7). Depending on whether the section is taken at the internode or the nodal region of the stem, the expression pattern will vary (Figure 3.8). In internodal regions, KNOX expression is evident in the provascular strands of the vascular cylinder, as well as in the outer tissue of the stem. As serial sections progress from internodal to nodal regions, the KNOX expression becomes isolated to regions of the stem between the leaf bases. These observations indicate that the *M. aquaticum* putative KNOX probe could be binding to more than one KNOXI gene. This is entirely possible when considering the region of the KNOX gene the RNA probe was cloned from. The RNA probe includes the ELK domain and a region of the homeodomain, both of which are heavily conserved between KNOX homologs. To distinguish between KNOX homologs within *M. aquaticum*, a region of the

gene should be cloned that is poorly conserved, such as the region upstream of the MEINOX domain (Figure 3.2). This would avoid non specific binding between KNOX homologs and allow for the determination of specific patterns of expression for individual genes.

4.2.2 KNOX1 expression and continuum morphology:

Although tissue sections can only represent static snapshots of the dynamic process that is plant development, the expression of KNOX1 exhibited at each plastochrone may be extrapolated to be representative of the changes in gene expression that each leaf undergoes during its entire development. In this way, it can be seen in *Myriophyllum* that a single leaf primordium expresses KNOX1 up until it reaches plastochrone 9. During this period of time, the overall pattern of expression of KNOX1 appears to change as primordia develop. When the primordium is a simple buttress and has yet to initiate lobes, KNOX1 is expressed ubiquitously throughout the primordium. With the onset of lobe development at plastochrone three, KNOX1 disappears from the central portion of the leaf and becomes localized to lobes between plastochrones 4 to 8. This pattern is reminiscent of KNOX1 expression as observed in the leaves of other plant species (Hareven et al., 1996; Bharathan et al., 2002).

KNOX1 genes were consistently found in the meristem of all *M. aquaticum* shoot tips examined which is typical of KNOX1 like expression in other species as well (Jackson et al., 1994; Lincoln, 1994; Sakomoto et al., 1999). The fidelity of this expression pattern led to the initial suggestion that KNOX genes such as STM could be used as molecular markers for determining meristem identity

during plant development (Hake, 1996). However, as shown in this study and others, KNOX1 genes are as much in evidence in developing leaves as they are in the meristem (Janssen et al., 1998a; Bharathan et al., 2002; Harrison et al., 2005). Under the paradigm of dynamic morphology, organs such as the meristem represent collections of processes that interact with the environment to generate plant form (Sattler, 1986; Sattler, 1996). It may therefore be more accurate to suggest that, rather than the physical structure of the meristem, KNOX genes could represent the developmental process of indeterminate growth that functions within both the meristem and compound leaf primordia (Janssen et al., 1998a; Hay and Tsiantis, 2006; Brand, 2007).

In *M. aquaticum*, it appears that KNOX1 expression is primarily localized to those regions of the shoot tip actively undergoing cell division; namely the meristem itself and the leaf lobes. In both meristems and leaves KNOX1 has been correlated with the presence of increased levels of the cell cycle protein Histone H4 indicating that KNOX1 promotes cell cycling in the tissues it is expressed in (Groot et al., 2005; Hay and Tsiantis, 2006b). In the meristem, KNOX transcription factors respectively repress and promote gibberellin and cytokinin biosynthesis which promotes cell cycling and prevents cell determination (Sakamoto et al., 2001; Sakamoto et al., 2006; Hay et al., 2003). Extrapolation of mutant phenotypes suggests that KNOX1 genes may promote indeterminate growth in the leaves as well as the meristem. Ectopically and over-expressed KNOX1 has been correlated with increased leaf complexity as well as the presence of ectopic meristem in lobe axils and on the surface of the leaf

lamina (Chuck et al., 1996; Hareven et al., 1996; Hay et al., 2002). These studies indicate that KNOX1 confers indeterminate cell fate in both leaves and shoots.

In the leaf, the genetic pathways which KNOX1 transcription factors act upon are less clear, so it is not possible at present to fully assess the similarity of the indeterminacy represented in leaves and in shoots. There are apparent differences in the negative regulation of KNOX1 expression between species with simple and compound leaves which indicate an inherent flexibility in its molecular interactions. In species with simple leaves, such as *Arabidopsis*, *Maize*, and *Antirrhinum*, a group of closely related Myb transcription factors known collectively as ARP (*ASYMMETRIC LEAVES1, ROUGH SHEATH2, PHANTASTICA*) negatively regulates KNOX1 genes at the sites of incipient leaf primordia (Byrne et al., 2000; Hay et al., 2006a). In compound leaved species, KNOX1 and ARP proteins are expressed simultaneously in leaves and work together to control leaflet placement (Kim et al., 2003; Hay et al., 2003b). More research on the functional role of KNOX1 in leaves is needed to gain a better understanding of the developmental parallel which the presence of KNOX1 genes in the leaves of *M. aquaticum* represents.

It is sometimes implied that KNOX1 gene expression is characterized by either simple or compound leaf patterning, but as shown in this study and others, it is known that KNOX genes frequently transcend this categorical context (Hofer et al., 2001b; Bharathan et al., 2002; Tioni et al., 2003). It is inaccurate to assume that simply because mature forms appear similar or dissimilar, does not mean that they will naturally exhibit similar or dissimilar developmental pathways

(Jaramillo and Kramer, 2007; Wilkins, 2002). This inherent ambiguity in the way we conceptualize homology underlines the importance of exploring development in non model species such as *M. aquaticum*, and establishing homology at multiple biological (molecular, structural, and functional) levels (Rutishauser and Moline, 2005; Kirchoff et al., 2008).

The fact the KNOX1 is expressed in the meristem, early leaf primordia, and then later in lobes suggests a morphological continuum between these three structures where the shared developmental process would be indeterminate growth (Sinha et al., 1993; Hofer et al., 2001b). From a dynamic point of view, consider *M. aquaticum* as an entire structure rather than a compilation of non-homologous organ types. With this perspective in mind, it becomes evident that in *M. aquaticum*, KNOX1 expression is localized to terminal tips of the plant structure (ie: meristem, young leaf primordia, and lobes) and appears to be less strongly expressed in more distal regions. All three of these structures are developmentally reminiscent of each other in that each develops as an undifferentiated, radial projection with radial symmetry where KNOX1 is likely promoting indeterminate growth to varying degrees. Therefore, if one were to forget categorical names such as shoot and leaf, one might suggest that the above three structures represent repetitions of the same basic process at different developmental levels (Arber, 1950; Lacroix et al., 2005). This of course is not the entire picture because there are many genes and developmental processes active during leaf development and differentiation that are not present during shoot development which ultimately results in different mature structures (Tsiantis and

Hay, 2003; Shani et al., 2006). However, KNOX1 is expressed during the initial stages of organogenesis before differentiation has occurred in the leaf (before plastochrone 7) which suggests that although leaves and shoots have different mature forms, their early developmental stages are more closely related. The presence of KNOX1 genes during early stages of leaf development serves to illustrate a morphological continuum between previously supposed non-homologous structures (Sattler, 1996; Hofer et al., 2001b).

4.3 Conclusion

Studies such as the one presented in this thesis demonstrate the inherent limitations in a classical morphological paradigm where all structures are divided up into a few mutually exclusive categories (Sattler, 1994; Rutishauser and Isler, 2001; Lacroix et al., 2005; Kirchoff et al., 2008). Such categories cover up structural and molecular similarities between categories which impedes science's ability to correctly interpret homologous relationships between organs (Arber, 1950). An alternative, and yet complementary paradigm to classical morphology can be found in dynamic morphology where plant form may be described as a collection of developmental processes within a three dimensional morphospace. In this framework, all plant forms are related based on the number of shared processes and their corresponding proximity within the morphospace (Sattler and Jeune, 1991; Lacroix et al., 2002; Jeune et al., 2006). From the data collected in this thesis, it can be seen that *Myriophyllum aquaticum* demonstrates developmental similarities at both the structural and molecular levels. Leaf lobes develop in a pattern reminiscent of distichous shoot development, and exhibit

KNOX1 gene expression which indicates a prolonged meristematic state within the lobes themselves. These observations point to the presence of developmental parallels in shoot and leaves and support the idea of a morphological continuum in plant form.

REFERENCES

Arber, A. 1950. The natural philosophy of plant form. Cambridge University Press, Cambridge, UK.

Barkoulas, M., Hay, A., Kougioumoutzi, E., and Tsiantis, M. 2008. A developmental framework for dissected leaf formation in the *Arabidopsis* relative *Cardamine hirsute*. Nature Genetics 40: 1136-1141.

Beck, C.B. 2005. An introduction to plant structure and development. Cambridge University Press, NY.

Bell, A.D. 1991. Plant Form: An illustrated guide to flowering plant morphology. Oxford University Press, NY.

Benkova, E., Michniewicz, M., Sauer, M., Teichmann, T., Seifertova, D., Jurgens, G., and Firml, J. 2003. Local, efflux-dependent auxin gradients as a common module for plant organ formation. Cell 115: 591-602.

Bharathan, G., and Sinha, N.R. 2001. The regulation of compound leaf development. Plant Physiology 127: 1533-1538.

Bharathan, G., Goliber, T.E., Moore, C., Kessler, S., Pham, T., and Sinha, N.R. 2002. Homologies in leaf form inferred from KNOX1 gene expression during development. Science 296: 1858-1860.

Bowes, B.G. 1996. A color atlas of plant structure. Iowa State University Press, Iowa.

Brand, A., Shirding, N., Shleizer, S., and Ori, N. 2007. Meristem maintenance and compound-leaf patterning utilize common genetic mechanisms in tomato. Planta 226: 941-951.

Byrne, M.E., Barley, R., Curtis, M., Arroya, J.M., Dunham, M., Hudson, A., and Martienssen, R.A. 2000. Asymmetric leaves1 mediates leaf patterning and stem cell function in Arabidopsis. Nature 408: 967-971.

Champagne, C., and Sinha, N. 2004. Compound leaves: equal to the sum of their parts? Development 131: 4401-4412.

Chan, R.L., Gago, G.M., Palena, C.M., and Gonzalez, D.H. 1998. Review: Homeoboxes in plant development. Biochimica et Biophysica Acta 1442: 1-19.

Chen, J., Janssen, B., Williams, A., and Sinha, N. 1997. A gene fusion at a homeobox locus: Alterations in leaf shape and implications for morphological evolution. The Plant Cell 9: 1289-1304.

Chuck, G., Lincoln, C., and Hake, S. 1996. KNAT1 induces lobed leaves with ectopic meristems when overexpressed in Arabidopsis. The Plant Cell 8: 1277-1289.

Clark, S.E., Williams, R.W., and Meyerowitz, E.M. 1997. The CLAVATA1 gene encodes a putative receptor kinase that controls shoot and floral meristem size in *Arabidopsis*. Cell 89: 575-585.

Cronk, Q. 2001. Plant evolution and development in a post-genomic context. Nature Reviews 2: 607-619.

Cronk, Q., Bateman, R.M., and Hawkins, J.A. 2002. Developmental genetics and plant evolution. Taylor and Francis, NY.

De Candolle, A.P. 1813. Théorie élémentaire de la botanique, ou exposition des principes de la classification naturelle et del l'art de décrire et d'étudier les végétaux. Déterville, Paris.

Dengler, N.G. 1992. Patterns of leaf development in anisophyllous shoots. Canadian Journal of Botany 70: 676-691.

Dengler, N.G. 1999. Anisophylly and dorsiventral shoot symmetry. International Journal of Plant Science 160: S67-S80.

Dengler, N.G. and Tsukaya, H. 2001. Leaf morphogenesis in dicotyledons: Current issues. International Journal of Plant Science 162: 459-464.

Dickinson, W. 2000. Integrative plant anatomy. Harcourt Academic Press, CA

Duboule, E. 1994. Guidebook to the homeobox genes. Oxford University Press, Oxford.

Eames, A.J. 1961. Morphology of angiosperms. McGraw-Hill, New York.

Esau. K. 1965. Plant anatomy. John Wiley & Sons, Inc. USA.

Eckardt, N. 2004. The Role of PHANTASTICA in Leaf Development. The Plant Cell 16: 1073-1075.

Fassett, N.C. 1972. A manual of aquatic plants. The University of Wisconsin press, London.

Fleming, A., Caderas, D., Wehrli, E., McQueen-Mason, S., and Kuhlemeier, C. 1999. Analysis of expansin induced morphogenesis on the shoot apical meristem of tomato. Planta 208: 166-174.

Fleming, A. 2004. The control of leaf development. New Phytologist 166: 9-20.

Fleming, A. 2006. Metabolic aspects of organogenesis in the shoot apical meristem. Journal of Experimental Botany 57: 1863-1870.

Fosket, D.E. 1994. Plant growth and development: a molecular approach Academic Press, CA.

Fukuda, T., Yokoyama, J., and Tsukaya, H. 2003. Phylogenetic relationships among species in the genera *Chisocheton* and *Guarea* that have unique indeterminate leaves as inferred from sequences of chloroplast DNA. International Journal of Plant Science 164: 13-24.

Gemmell, A.R. 1969. Developmental plant anatomy. William Clowes and Sons Ltd. London, Great Britain.

Gilmartin, P.M., and Bowler, C. 2002. Molecular plant biology: Volume 2. Oxford University Press, Oxford.

Goethe, J.W. 1790. Versuch die metamorphose der pflanzen zu erklären. Vol. 1. Erringer, Gotha, Germany.

Goliber, T., Kessler, S., Chen, J., Bharathan, G., and Sinha, N. 1999. Genetic, molecular, and morphological analysis of compound leaf development. Current Topics in Developmental Biology 43: 259-291.

Granger, C.L., Callos, J.D., and Medford, J.I. 1996. Isolation of an *Arabidopsis* homolog of the maize homeobox *Knotted-1* gene. Plant Molecular Biology 31: 373-378.

Groot, E.P., Sinha, N., and Gleissberg, S. 2005. Expression patterns of STM-like KNOX and histone H4 genes in shoot development of the dissected leaved basal eudicot plants in *Chelidonium majus* and *Eschoscholzia californica* (Papaveraceae). Plant Molecular Biology 58: 317-331.

Guédès, M. 1979. Morphology of seed plants. 326 pp. Cramer, Vaduz.

Hagemann, W., and Gleisserg, S. 1996. Organogenetic capacity of leaves: the significance of marginal blastozones in angiosperms. Plant Systems and Evolution 199: 121-152.

Hake, S., Smith, H.M.S., Holtan, H., Magnani, E., Mele, G., and Ramirez, J. 2005. The role of knox genes in plant development. Annual Review of Sell and Developmental Biology 20: 125-151.

Hareven, D., Gutfinger, T., Parnis, A., Eshed, Y., and Lifschitz, E. 1996. The Making of a Compound Leaf: Genetic Manipulation of Leaf Architecture in Tomato. Cell 84: 735-744.

Harrison, J., Moller, M., Langdale, J., Cronk, Q., and Hudson, A. 2005. The role of KNOX genes in the evolution of morphological novelty in *Streptocarpus*. The Plant Cell 17: 430-443.

Hay, A., Kaur, H., Phillips, A., Hedden, P., Hake, S., and Tsiantis, M. 2002. The gibberellin pathway mediates KNOTTED1-type homeobox function in plants with different body plans. Current Biology 12: 1557-1565.

Hay, A., Jackson, D., Ori, N., and Hake S. 2003. Analysis of the competence to respond to KNOTTED1 activity in *Arabidopsis* leaves using a steroid induction system. Plant Physiology 131: 1671-1680.

Hay, A., Barkoulas, M., Tsiantis, T. 2006. PINning down the connections: transcription factors and hormones in leaf morphogenesis. Current Opinion in Plant Biology 9: 575-581.

Hay, A., Barkoulas, M., Tsiantis, T. 2006a. ASYMMETRIC LEAVES1 and auxin activities converge to repress BREVIPEDICELLUS expression and promote leaf development in *Arabidopsis*. Development 133: 3955-3961.

Hay, A., and Tsiantis M. 2006b. The genetic basis for differentiation in leaf form between *Arabidopsis* and its wild relative *Cardamine hirsuta*. Nature Genetics 38: 942-947.

Heldt, H. 1997. Plant biochemistry and molecular biology. University of Oxford Pess. NY.

Hirayama, Y., Yamada T., Oya Y., Ita M., Kata M., and Yumika R.I. 2007 Implications of class I KNOX and YABBY genes in *Ruscus aculeate* (Asparagaceae) with implications for phylloclade homology. Development Genes and Evolution 217: 363-372.

Hofer, J., Gourlay, C.W., Ellis, T.H.N. 2001a. Genetic control of leaf morphology: A partial view. Annals of Botany 88: 1129-1139.

Hofer, J., Gourlay, C.W, Micheal, A., and Ellis, T.H.N. 2001b. Expression of a class 1 *knotted1*-like homeobox gene is down-regulated in pea compound leaf primordia. Plant Molecular Biology 45: 387-398.

Ilic, K., Kellogg, E.A., Jaiswal, P., Zapata, F., Stevens, P.F., Vincent, L.P., Avarham, S., Reiser, L., Pujar, A., Sachs, M.M., Whitman, N.T., McCouch, S.R., Schaeffer, M.L., Ware, S.H., Stein, L.D., and Rhee, S.Y. 2007. Plant structure ontology, a unified vocabulary of anatomy and morphology of a flowering plant. Plant Physiology 143: 587-599.

Jackson, D., Veit, B., and Hake, S. 1994. Expression of maize KNOTTED1 related homeobox genes in the shoot apical meristem predicts patterns of morphogenesis in the vegetative shoot. Development 120: 405-413.

Janssen, B., Lund, L., and Sinha, N. 1998a. Overexpression of a homeobox gene, LeT6, reveals indeterminate features in tomato compound leaf. Plant Physiology 117: 771-786.

Janssen, B., Williams, A., Chen, J., Mathern, J., Hake, S., and Sinha, N. 1998b. Isolation and characterization of two knotted-like homeobox genes from tomato. Plant Molecular Biology 36: 417-425.

Jaramillo, M. A. and Kramer, E. A. 2007. The role of developmental genetics in understanding homology and morphological evolution in plants. The International Journal of Plant Science 168: 61-72.

Jasinski, S., Kaur, H., Tattersall, A., and Tsiantis, M. 2007. Negative regulation of KNOX expression in tomato leaves. Planta 226: 1255-1263.

Jean, R.V. 1994. Phyllotaxis: A systemic study in plant morphogenesis. Cambridge University Press, NY.

Jean, R.V., and Barabe, D. 1998. Symmetry in plants. World Scientific, Singapore

Jeune, B. 1976. Experimentation microchirurgicale sur la feuille de *Myriophyllum brasiliense*. Adansonia 16: 107-117.

Jeune, B. and Sattler, R. 1992. Multivariate analysis in process morphology of plants. Journal of Theoretical Biology 156: 147-167.

Jouannic, S., Collin, M., Vidal, B., Verdeil, J., Tregear, J.W. 2007. A class I KNOX gene from the palm species *Elaeis guineensis* (Arecaceae) is associated with meristem function and a distinct mode of leaf dissection. New Phytologist 174: 551-568.

Jurgens, G. 2003. Growing up green: Cellular basis of plant development. Mechanisms of Development 120: 1395-1406.

Kaplan, D. R. 2001. The science of plant morphology: Definition, history, and place in modern biology. American Journal of Botany 88: 1711-1741.

Kellogg, E.A. 2006. The difference between simple and complex leaves. Nature Genetics, 38: 865-866.

Kerstetter, R., Vollbrecht, E., Lowe, B., Veit, B., Yamaguchi, J., and Hake, S. 1994. Sequence analysis and expression patterns divide the maize knotted1-like homeobox genes into two classes. The Plant Cell 6: 1877-1887.

Kessler, S., and Sinha, N. 2004. Shaping up: the genetic control of leaf shape. Current Opinion in Plant Biology 7: 65-72.

Kim, M., McCormick, S., Timmermans, M., and Sinha, N. 2003a. The expression domain of PHANTASTICA determines leaflet placement in compound leaves. Nature 4224: 438-443.

Kim, M., Pham, T., Hamidi, A., McCormick, S., Kuzoff, R., and Sinha, N. 2003b. Reduced leaf complexity in tomato wiry mutants suggests a role for PHAN and KNOX genes in generating compound leaves. Development 130: 4405-4415.

Kinder, C.A., Timmermans, M.C.P. 2007. Mixing and matching pathways in leaf polarity. Current Opinion in Plant Biology 10: 13-20.

Kirchoff, B., Pfeifer, E., Rutishauser, R. 2008. Plant structure ontology: How to label plant structures with doubtful or mixed identities? Zootaxa, In review.

Kramer, E.M. 2005. Methods for studying the evolution of plant reproductive structures: comparative gene expression techniques. Methods in Enzymology 395: 617-635.

Lacroix, C., and Sattler, R. 1994. Expression of shoot features in early leaf development of *Murraya paniculata* (Rutaceae). Canadian Journal of Botany 72: 678-687.

Lacroix, C. 1995. Changes in leaflet and leaf lobe form in developing compound and finely divided leaves Bot. Jahrb. Sys. 117: 317-331.

Lacroix, C., Jeune, B., and Prucell-MacDonald, S. 2003. Shoot and compound leaf comparisons in eudicots: dynamic morphology as an alternative approach. Botanical Journal of the Linnean Society 143: 219-230.

Lacroix, C., Jeune, B., and Barabé, D. 2005. Encasement in plant morphology: an integrative approach from genes to organisms. Canadian Journal of Botany 83: 1207-1221.

Laux, T., Mayer, K.F.X., Berger, J., and Jurgens, G. 2006. The WUSCHEL gene is required for shoot and floral meristem integrity in *Arabidopsis*. Development 122: 87-96.

Leyser, O., and Day S. 2003. Mechanisms in plant development. Blackwell Publishing, MA.

Long, J.A., Benfey, P.N. 2006. Transcription factors and hormones: New insights into plant cell differentiation. Current Opinion in Cell Biology 18: 710-714.

Long, J.A., Moan, E.I., Medford, J.I., and Barton M.K. 1996. A member of the knotted class of homeodomain proteins encoded by the STM gene of *Arabidopsis*. Nature 379: 66-69.

Lyndon, R.F. 1990. Plant Development: The cellular basis. Uwin Hyman Inc. London, UK.

Lyndon, R.F. 1998. The shoot apical meristem: Its growth and development. Cambridge University Press, Cambridge, UK.

Mauseth, J. D. 2004. Giant shoot apical meristems in cacti have ordinary leaf primordia but altered phyllotaxy and shoot diameter. Annals of Botany 94: 145-153.

Mayer, K.F.X., Schoof, H., Haecker, A., Lenhard, M., Jurgens, G., and Laux, T. 1998. Role of WUSCHEL in regulating stem cell fate in the *Arabidopsis* shoot meristem. Cell 95: 805-815.

Müller, K.J., He, X., Fischer, R., and Prufer, D. 2006. Constitutive knox1 expression in dandelion (*Taraxacum officinale*, Web.) changes leaf morphology from simple to compound. Planta 224: 1023-1027.

Nardmann, J., Werr, W. 2007. The evolution of plant regulatory networks: What *Arabidopsis* cannot say for itself. Current Opinion in Plant Biology 10: 653 – 659.

Pien, S., Wyrzykowska, J., McQueen-Mason, S., Cheryl, S., and Fleming, A. 2001. Local expression of expansin induces the entire process of leaf development and modifies leaf shape. Proceedings of the National Academy of Sciences 98: 11812-11817.

Quint, M., and Gray, W. M. 2006. Auxin signaling. Current Opinion in Plant Biology 9: 448-453.

Raghavan, V. 2000. Developmental biology of flowering plants. Springer, NY.

Ramage, C.M., and Williams, R.R. 2004. Cytokinin-induced abnormal shoot organogenesis is associated with elevated knotted1-type homeobox gene expression in tobacco. Plant Cell 22: 919-924.

Reinhardt, D., Mandel, T., and Kuhlemeier,C. 2000. Auxin regulates the initiation and radial position of plant lateral organs. The Plant Cell 12: 507-518.

Reinhardt, D., Pesce, E. R., Stieger, P., Mandel, T., Baltensperger, K., Bennett, M., Traas. J., Friml, J., and Kuhlemeier, C. 2003. Regulation of phyllotaxis by polar auxin transport. Nature 426: 255-260.

Reinhardt D., Frenz M., Mandel T., and Kuhlemeier C. 2004. Microsurgical and laser ablation analysis of leaf positioning and dorsoventral patterning in tomato. Development 132: 15-26.

Reiser, L., Sanchez-Baracaldo, P., Hake, S. 2001. Knots in the family tree: evolutionary relationships and function of *knox* homeobox genes. Plant Molecular Biology 42: 151-166.

Romberger, J.A., Hejnowicz, Z., and Hill, J.F. 1993. Plant structure function and development: A treatise on anatomy and vegetative development, with special reference to woody plants. Springer-Verlag, Maryland.

Rudall, P. 1992. Anatomy of flowering plants: An introduction to structure and development 2nd edition. Cambridge University Press, Cambridge.

Rutishauser, R. 1999. Polymerous leaf whorls in vascular plants: Developmental morphology and fuzziness of organ identities. International Journal of Plant Science 160: S81-S103.

Rutishauser, R., and Isler, B. 2001. Developmental genetics and morphological evolution of flowering plants, especially Bladderworts (*Utricularia*): Fuzzy arberian morphology complements classical morphology. Annals of botany 88: 1173-1202.

Rutishauser, R., and Moline, P. 2005. Evo-devo and the search for homology ("sameness") in biological systems. Theory in Biosciences 124: 213-241.

Sakamoto, T., Nishimura, A., Tamaoki, M., Kuba, M., Tanaka, H., Iwahori, S., and Matsuoka, M. 1999. The conserved KNOX domain mediates specificity of Tobacco KNOTTED1-type homeodomain proteins. The Plant Cell 11: 1419-1431.

Sakamoto, T., Kamiya, N., Ueguchi-Tanaka, M., Iwahori, S., and Matsuoka, M. 2001. KNOX homeodomain protein directly suppresses the expression of a

gibberellin biosynthetic gene in the tobacco shoot apical meristem. Genes and Development 15: 581-590.

Sakamoto, T., Sakakibara, H., Kojima, M., Yamamoto, Y., Nagasaki, H., Inukai, Y., Sato, Y., and Matsuoka, M. 2006. Ectopic expression of KNOTTED1-like homeobox protein induces expression of cytokinin biosynthesis genes in rice. Plant Physiology 142: 54-62.

Sattler, R. 1986. Biophilosophy: analytic and holistic perspectives. Springer-Verlag, Germany.

Sattler, R. 1991. Process morphology: structural dynamic in development and evolution. Canadian Journal of Botany 70: 708-714.

Sattler, R., and Jeune, B. 1991. Multivariate analysis confirms the continuum view of plant form. Annals of Botany 69: 249-262.

Sattler, R., and Rutishauser, R. 1992. Partial homology of pinnate leaves and shoots: Orientation of leaflet inception. Botanische Jahrbücher für Systematik, Pflanzengeschichte und Pflanzengeographie 114: 61-79.

Sattler, R. 1994. Homology: the hierarchical basis of comparative biology. Academic Press, Inc. USA.

Sattler, R., and Rutishauser, R. 1996. The fundamental relevance of morphology and morphogenesis to plant research. Annals of Botany 80: 571-582.

Scofield, S., and Murray, J.A.H. 2006. Knox gene function in plant stem cell niches. Plant Molecular Biology 60: 929-946.

Shani, E., Yanai, O., and Ori, N. 2006. The role of hormones in shoot apical meristem function. Current Opinion in Plant Biology 9: 484-498.

Smith, L.G., Greene, B., Veit, B., and Hake, S. 1992. A dominant mutation in the maize homeobox gene, Knotted-1, causes its ectopic expression in leaf cells with altered fates. Development 116: 21-30.

Sinha, N.R., Williams, R.E., and Hake, S. 1993. Overexpression of the maize homeobox gene knotted-1, causes a switch from determinate to indeterminate cell fates. Genes and Development 7: 787-795.

Sinnott, E.W. 1960. Plant morphogenesis. McGraw-Hill Book Company, Inc. USA.

Steeves, T.A., and Sussex, I.M. 1989. Patterns in plant development. Cambridge University Press, NY.

Sutton, D.L., 1985. Biology and Ecology of *Myriophyllum aquaticum.* Proceedings of the first international symposium on Watermilfoil (*Myriophyllum* spicatum) and related Haloragaceae species, Aquatic Plant Management Society, Vicksburg, Mississippi.

Teale, W.D., Paponov, I.A., and Palme, K. 2006. Auxin in action: signaling, transport, and the control of plant growth and development. Nature Reviews 7: 847-859.

Tioni, M., Gonzalez, D.H., and Chan, R.L. 2003. Knotted1-like genes are strongly expressed in differentiated cell types in sunflower. Journal of Experimental Botany 54:681-690

Troll, W. 1937. Morphologie der ho¨heren Pflanzen. Tome I. Gebru¨der Borntraeger. Berlin. Reprinted in 1967 by Koeltz, Koenigstein-Taurus.

Tsiantis, M., and Hay, A. 2003. Comparative plant development: the time of the leaf? Nature Reviews: Genetics 4: 169-180 .

Tsukaya, H. 1991. Developmental genetics of leaf morphogenesis in dicotyledonous plants. Journal of Plant Research 108: 407-416.

Tsukaya, H. 2005. Leaf shape: genetic controls and environmental factors. International Journal of Developmental Biology 49: 547-555.

Tsukaya, H. 2006. Mechanisms of leaf-shape determination. Annual Review of Plant Biology 57: 477-496.

Vollbrecht, E., Veit, B., Sinha, N., and Hake, S. 1991. The developmental gene Knotted-1 is a member of a *Maize* homeobox gene family. Nature 350: 241-243.

Wilkins, A.S. 2002. The evolution of developmental pathways. Sinauer, Sunderland, MA.

APPENDIX

Solution Recipes

* All reagents used in solutions were certified nuclease free, and all solutions used in recipes were autoclaved prior to use.

Solution		Reagents
CTAB extraction buffer:	5 ml	1M Tris (pH 8)
	2 ml	0.5M EDTA (pH 8)
	14 ml	5M NaCl
	0.05g	SDS
	1g	CTAB

- Complete volume to 50 ml with nuclease free water. To dissolve SDS, heat solution at 55C for 10 minutes.

TE Buffer (pH 8):	10 mM	Tris
	1 mM	EDTA

- pH to 8 using HCl, then complete to desired volume using nuclease free water

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10X TBE Buffer (pH 8):	108g	Tris base
	55g	Boric acid
	40 ml	0.5M EDTA (pH 8)

- Fill to 800 ml with nuclease water and adjust pH to 8 with HCl. Complete volume to 1 L with water. To generate 1X working solution, dilute one aliquot ten times.

In situ fixative:	10 ml	16% Paraformaldehyde
	0.4 ml	Dimethylsulfoxide (DMSO)
	4 ml	10X PBS
	25.6 ml	dH_2O

- Aliquot into 15 ml falcon tubes and keep on ice at all times. Keep under fume hood.

20X SSC:	175.3g	NaCl
	88.2 g	Sodium Citrate
	1 L	DEPC H2O

- Add nuclease free components to 800 ml of DEPC H2O, adjust pH to 7 with 1M HCl, and complete volume to 1L.

10X Proteinase K buffer:	0.1M	Tris HCl (pH 8)
	50mM	EDTA (pH 8)

10X PBS:	37g	NaCl
	4.97g	Na_2HPO_4 (MW: 141.96)
	2.07g	NaH_2PO_4 (MW: 137.99)
	500 ml	DEPC H2O

- Add components to 450 ml DEPC H2O, adjust pH to 7, and complete volume to 500 ml.

NTE:	50 ml	5M NaCl
	5 ml	1M Tris HCl (pH 7.5)
	1 ml	0.5M EDTA (pH 8)
	444 ml	DEPC H2O
	500 ml	Total volume

BM Block:	1 g	Roche Block reagent
	20 ml	1M Tris HCl (pH 7.5)
	6 ml	5M NaCl
	174 ml	DEPC H2O
	200 ml	Total volume

- Constant stirring and very low heat is required to dissolve protein into a milky solution. Allow to stir for 1 hour prior to use.

Block 2:	5 g	Bovine albumin serum (BSA)
	1.5 ml	Triton X-100
	50 ml	1M Tris HCl (pH 7.5)
	15 ml	5M NaCl
	432.5 ml	DEPC H2O

	500 ml	Total volume

- Constant stirring is required for 1 hour prior to use to ensure complete dissipation of triton.

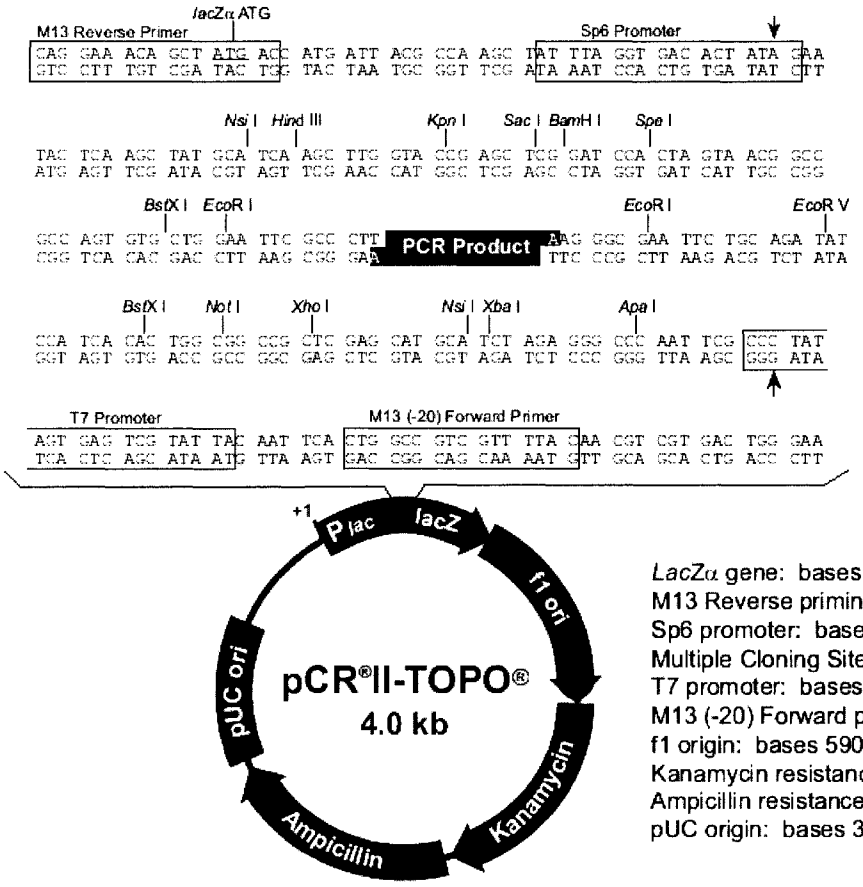
C buffer:	4 ml	5M NaCl
	20 ml	1M Tris HCl (pH 9.7)
	10 ml	1M MgCl
	166 ml	DEPC H2O
	200 ml	Total volume

10X *is situ* salts:	30 ml	5M NaCl
	5 ml	1M Tris HCl (pH6.5)
	5 ml	0.5M EDTA (pH 8)
	3.9g	NaH_2PO_4 $2H_2O$
	3.55g	Na_2HPO_4
	ml	DEPC H2O
	50 ml	Total volume

Hybridization Solution :	20 ul	10X *in situ* salt solution
	80 ul	100% Formamide
	40 ul	50% Dextran sulfate (w/v)
	4 ul	50X Denhardts solution
	18 ul	tRNA 10 mg/ml stock
(Sigma)		
	160 ul	total volume per slide

- Dextran sulfate required heating at 80°C for melting. Reagent volumes were scaled up according to the number of *in situ* slides used. Each aliquot of 160 ul was combined with 40 ul of 50% formamide + probe before slide application.

pCRII – TOPO Vector map (Invitrogen) :



*Lac*Zα gene: bases 1-589
M13 Reverse priming site: bases 205-221
Sp6 promoter: bases 239-256
Multiple Cloning Site: bases 269-383
T7 promoter: bases 406-425
M13 (-20) Forward priming site: bases 433-448
f1 origin: bases 590-1027
Kanamycin resistance ORF: bases 1361-2155
Ampicillin resistance ORF: bases 2173-3033
pUC origin: bases 3178-3851

Lobster gene fragment used as negative control:

GGTCCCGTCCTCATCTCAGCGATGGATTTCTGTGGTGCTTCATATCGGGGAGGCAGCA
CCACAGTGGGGGACTGCTCTGTACTATTAAGGGCAGATGGCGCCATACGGCTAAAAG
TTATTATAGTGTCGAGGACACCGCTGCTGTCACACGTGTTGACACGTCTGAGTTGAAC
CGCCATAAGAAATTGCCCGATTGGCATACGGGCTTTATACAAGGTCTTGCCCTTACTC
CTCCATTTAATGGAGACCCATAGTTCAGGGTCGAACATTTCCTCACGGGGCATGTGGC
AAGATCCACCCAGGTCGTAAGAACATTACACCCGCATTA

*The underlined regions correspond to the forward and reverse primers respectively.

Degenerate primers, Weidhaas, 2006:

Forward Primer

D P E L D Q F M

In code :

5' GAY CCN GAR YTN GAY CAR TTY ATG 3'

Total degeneracy : 1024
Length : 24

Reverse Primer

Q I N N W F I N Q R K R

Reverse complement in code :

5' NCK YTT NCK YTG RTT DAT RAA CCA RTT RTT DAT YTG 3'

Total degeneracy: 18 432
Length : 35

ClustalW alignment of KNOX1 sequences, Weidhaas 2006: