学位論文　（要約）

Comparative analysis of cell division pattern and key gene expression patterns in unifacial leaves

（単面葉における形態形成鍵遺伝子の発現パターンと細胞分裂パターンとの比較解析）

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"…… *Ende dat steekt in het glas te slypen, ende in het ontdekken van de saaken, die voor onse oogen verborgen syn, niet. En het staat ook by my vast, dat van duysent menschen geen een bequaam is om sig over te geven tot zoodanige studie; om dat 'er veel tydts toe vereyst wert, veel gelt gespilt wert; ende men geduyrig met syne gedagten moet besig wesen, sal men wat uytvoeren. Ende daar en boven syn de meeste menschen niet weet gierig; ja eenigen, daar men het niet van behoorde te wagten, seggen, wat is 'er aangelegen of wy het weten? ……*"

"…… *But in lens-grinding, and discovering things hidden from our sight, these count for nought. And I'm satisfied too that not one man in a thousand is capable of such study, because it needs much time, and spending much money; and you must always keep on thinking about these things, if you are to get any results. And over and above all, most men are not curious to know: nay, some even make no bones about saying. What does it matter whether we know this or not? ……*"

Antoni van Leeuwenhoek, Sept. 28th, 1715, letter to Gottfried Leibniz

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Abstract

Unifacial leaves are unique among angiosperm leaves in that the adaxial/abaxial (ad/abaxial) polarity along the longitudinal axis of leaves is different. The proximal leaf sheath is bifacial, including both the adaxial and the abaxial polarities. The distal leaf blade, however, is unifacial, including only the abaxial polarity. The evolutionary origin and developmental processes of unifacial leaves, and the degree of abaxilization in the distal leaf blade, were much debated in the history. The "adaxial meristem hypothesis" states that unifacial leaves are formed postgenitally by the activity of the adaxial meristem in certain part of the leaf blade. The "sympodial hypothesis" states that unifacial leaves are formed by the leaf meristem succession from the bifacial leaf apex to the unifacial leaf apex. The "subunifacial hypothesis" states that unifacial leaves are formed postgenitally by marginal fusion and a small adaxial sector is retained in the distal blade. These hypotheses were proposed more than 40 years ago, based on comparative morphological, anatomical, developmental, and histogenetic analyses. These approaches are quite outdated and modern ones such as examining DNA synthesis activity and gene expression should be applied instead. In addition, in the past there is no efficient method to analyze the direction of cell division, making it hard to evaluate these hypotheses. Therefore, in my dissertation, I aim to develop an efficient method to quantify the direction of cell division, and use this method and another modern approach (*in situ* hybridization) to check these hypotheses regarding unifacial leaves.

In Chapter I, I reviewed historical and recent molecular studies regarding unifacial leaves and leaf ad/abaxial polarity. In Chapter II, I developed a pulse-chase 5-ethynyl-2'-deoxyuridine (EdU) method and demonstrated its efficiency and usefulness in the model plant Arabidopsis (*Arabidopsis thaliana*). In Chapter III, I applied this method to an ensiform unifacial leaf species *Juncus prismatocarpus* (Juncaceae) and analyzed the location and direction of cell division to evaluate the "adaxial meristem hypothesis" and the "sympodial hypothesis". I also compared cell division pattern with expression patterns of various genes known to be important in its leaf development. In Chapter IV, I cloned and checked the expression pattern of *KNOTTED1* (*KN1*) ortholog in *J. torreyi*, to examine the likely cause of "subunifacial hypothesis". I found that while the "sympodial hypothesis" should be rejected, the "adaxial meristem hypothesis" and the "subunifacial hypothesis" should be modified substantially.

List of Abbreviations

ANOVA: analysis of variation

ARF3/4: *AUXIN RESPONSE FACTOR3/4*

AS1: *ASSYMETRIC LEAVES1*

BCIP: 5-bromo-4-chloro-3-indolyl phosphate

BP: *BREVIPEDICELLUS*

BrdU: 5-bromo-2'-deoxyuridine

CRC: CRABS CLAW

DAPI: 4',6-diamidino-2-phenylindole

DAS: days after sowing

DIG: digoxigenin

DL: *DROOPING LEAF*

EdU: 5-ethynyl-2'-deoxyuridine

FAA: formalin-ethyl alcohol-glacial acetic acid

GUS: β-glucuronidase

HD-ZIPIII: class III HOMEODOMAIN LEUCINE ZIPPER

KAN: *KANADI*

KN1: *KNOTTED1*

KNAT1: *KNOTTED-LIKE FROM ARABIDOPSIS THALIANA1*

KNOX: *KNOTTED1*-LIKE HOMEOBOX

KNOX1: class I *KNOTTED1*-LIKE HOMEOBOX

miR: microRNA

NBT: nitro blue tetrazolium

NPA: 1-N-naphthylphthalamic acid

PHB: *PHABULOSA*

PHV: *PHAVOLUTA*

PRS: *PRESSED FLOWER*

RACE: rapid amplification of cDNA ends

REV: *REVOLUTA*

RS2: *ROUGH SHEATH2*

SAM: shoot apical meristem

SSC: saline sodium citrate

SSPE: saline sodium phosphate EDTA

tasiR: trans-acting short interfering RNA

WOX: *WUSCHEL*-RELATED HOMEOBOX

Chapter I: General Introduction

1. Historical studies on unifacial leaves

Angiosperm leaves have a wide range of morphological diversity (Tsukaya, 2014). The so called "unifacial leaf" is the most controversial one among various angiosperm leaf forms (Kaplan, 1975). Its proximal sheath has distinct adaxial and abaxial surfaces. As it moves toward the distal leaf blade, morphologically it appears that the two margins roll inward adaxially and ultimately fused together along the midline and the adaxial surface is progressively lost, hence being "unifacial". This adaxially marginal fusion is termed "cross zone" (Hagemann and Gleissberg, 1996) and could be diagnostic of unifacial leaves from gross morphology (Gleissberg *et al*., 2000). Thus, in general, unifacial leaves are only partially unifacial along the proximal-distal leaf axis (Hagemann, 1970; Kaplan, 1975; Gleissberg *et al*., 2000; Eberwein, 2007). In the bifacial leaf sheath, vascular bundles are arranged with their xylem poles toward the adaxial surface and their phloem poles toward the abaxial surface (collateral); whereas in the unifacial leaf blade, vascular bundles are arranged like a ring structure with their xylem poles toward inside and phloem poles toward outside (amphicribral) (Fig. 1.1. *cf*. Kaplan, 1975. Text-Fig. 1, p3). In addition to the form where the leaf sheath is relatively short and the leaf blade is relatively long, the opposite form also exists. In this form, the relatively short leaf blade is often termed "vorläusferspitze" ("precursor tip" or "forerunner tip") (Troll, 1939) (Fig. 1.1. *cf*. Kaplan, 1975. Text-Fig. 1, p3). Goebel (1928) used the term "exotrophie" to describe leaves with an expanded abaxial surface. Troll (1939) used the term "unifacial" to describe leaves that their distal part is derived from only one surface, the abaxial surface. In the classical literatures concerning unifacial leaves, the terms "dorsal" and "ventral" are commonly and interchangeably used for "abaxial" and "adaxial", respectively (*e.g.*, Kaplan, 1970a, 1970b, 1973a, 1973b, 1975), making considerable confusion. In my dissertation, I will only use the terms "abaxial" and "adaxial", to refer to the surface away from the shoot apical meristem (SAM) and the surface facing to the SAM, respectively.

Because the shape of unifacial leaves is typically oblong and terete, historically efforts have been made to compare various parts of unifacial leaves with morphologically similar eudicotyledonous

leaves and to attempt to find homology with clearly defined leaf parts such as leaf petiole and leaf lamina, from morphological (de Candolle, 1827), anatomical (Henslow, 1911; Arber, 1918, 1925) and developmental and histogenetic approaches (Troll 1939; Troll and Meyer, 1955; Hagemann, 1970; Kaplan, 1970a, 1970b, 1973a, 1973b, 1975). de Candolle (1827) proposed the "phyllode hypothesis", based on the morphological similarity between unifacial leaves and petiolar phyllodes of certain species of *Acacia* (Fabaceae) and *Oxalis* (Oxalidaceae). The "phyllode hypothesis" was later supported by Henslow (1911) and Arber (1918, 1925), based on the then popular concept of leaf zonal division of "unterblatt" ("lower zone") and "oberblatt" ("upper zone") (Eichler, 1861). Arber (1918, 1925), for example, considered unifacial leaves are derived from "unterblatt" of leaves and unifacial leaves are "debladed, transformed, and expanded" petioles or phyllodes. She emphasized the homology between unifacial leaves and petioles of eudicotyledonous leaves.

Goebel (1884, 1905) proposed the "sympodial hypothesis", in reference to the sympodial mode of stem growth. This hypothesis was supported by Thielke (1948), Roth (1949, 1957, 1961), and Ravololomaniraka (1972). These authors believed that development of unifacial leaves has two phases. In the first phase, leaf primordia arch over (acrovergent curvature) the SAM by the activity of the "primary leaf apex". In the second phase, the "primary leaf apex" ceases activity and the "secondary leaf apex" is activated on the abaxial surface and makes leaf primordia to grow in a different direction, the longitudinal direction. Because there is a succession from the "primary leaf apex" to the "secondary leaf apex", hence the term "sympodial" is applied. According to these authors, it is the activity of the "secondary leaf apex" that gives rise to the elongated form of ensiform unifacial leaves such as *Iris* (Iridaceae) (Fig. 1.2. *cf*. Kaplan, 1975. Text-Fig. 9, p51). In addition, Ravololomaniraka (1972) further recognized a "tertiary leaf apex" at the adaxial side of the leaf sheath in certain plants such as *Juncus* species (Juncaceae).

The "phyllode hypothesis" and the "sympodial hypothesis" were rejected by Troll (1932, 1939), Troll and Meyer (1955), Hagemann (1970), and Kaplan (1970a, 1970b, 1973a, 1973b, 1975), based on comparative leaf development and histogenetic features such as leaf meristem activity. However, disagreement exists as whether the development of unifacial leaves is postgenital (Hagemann, 1970; Kaplan, 1970a, 1970b, 1973a, 1973b, 1975) or congenital (Troll, 1932, 1939; Troll and Meyer, 1955).

According to Hagemann (1970) and Kaplan (1970a, 1970b, 1973a, 1973b, 1975), unifacial leaf primordia are bifacial in origin and "becoming" unifacial postgenitally as a result of the activity of "adaxial meristem". This process was also documented in phyllodes of *Acacia longifolia* and *Acacia melanoxylon* (Boke, 1940). Kaplan (1970a, 1975) demonstrated that the development of phyllodes of *Acacia cyclops*; bipinnate leaf form, transitional form, and phyllodic form of *Acacia melanoxylon*; rachis leaves of *Oxypolis greenmanii* (Apiaceae); and unifacial leaves of *Acorus calamus* (Araceae), *Allium cepa* (Amaryllidaceae), *Dracaena fragrans* (Agavaceae), *Ornithogalum caudatum* (Liliaceae), *Sansevieria suffruticosa* (Agavaceae), and *Sansevieria trifasciata* are all due to the activity of adaxial meristem, thus inferring homology among these organs. However, it should be noted that this homology is between above unifacial organs and blades of eudicotyledonous leaves. Kaplan (1975) considered the leaf sheath is derived from "unterblatt" and the part which has the "adaxial meristem" is derived from "oberblatt". Therefore, while Arber (1918, 1925) inferred homology from "suppressed leaf blade"; Kaplan (1975), on the other hand, inferred homology from "alternative course of leaf blade morphogenesis" (Rudall and Buzgo, 2002).

As pointed out by Kaplan (1975), the central problem (historically) in interpretation of unifacial leaves is the boundaries of longitudinal subunits. Contrary to the historical "unterblatt" and "oberblatt" division of leaves (Eichler, 1861), a transitional zone, characterized by adaxial meristem activity, is recognized by some authors (Troll, 1939; Roth, 1949) (Fig. 1.3. *cf*. Rudall and Buzgo, 2002. Fig. 23.10, p450). According to Kaplan (1970a, 1970b, 1973a, 1973b, 1975), the unifacial part of the leaf is made by postgenital growth of adaxial meristem. Leaf primordia are initiated as a dorsiventrally flattened, bifacial structure. Where adaxial meristem is active (the transition zone), the flat leaf primordia would grow in the direction that is perpendicular to the original flat plane and thus become rounded or elongated. The vascular bundles are first differentiated in the abaxial side as a manner typical to bifacial leaves (collateral); but later on, vascular bundles at the adaxial side differentiate from adaxial meristem. As a result they face oppositely to those first differentiated, thus forming the overall ring structure of vascular bundles (amphicribral) and complete the postgenital conversion from bifacial to unifacial (Fig. 1.4. *cf*. Kaplan, 1975. Text-Fig. 14, p98). Where adaxial meristem is not active, such as the tip of unifacial leaves, it "never completely loses its dorsiventrality". Although he recognized the ring arrangement of vascular bundles in the tip of unifacial leaves, he failed to give a

plausible explanation of such arrangement. In addition, Roth (1957) and Kaplan (1970a, 1975) also recognized “abaxial meristem” in certain species such as *Acorus calamus*, *Sansevieria cylindrica* and *Sansevieria suffruticosa*. Contrary to Hagemann’s (1970) and Kaplan’s (1970a, 1970b, 1973a, 1973b, 1975) postgenital view, according to Troll (1932, 1939) and Troll and Meyer (1955), the development of unifacial leaves is congenital. Although they also recognized “adaxial growth activity” in very early leaf development, they considered it as a special type and different from the adaxial meristem *sensu* Kaplan (1970a, 1970b, 1973a, 1973b, 1975). In their view, since the unifacial part of leaves has only abaxial surface, the term “adaxial meristem” is invalid. Instead, they used the term “rundungsmeristem” (“rounding meristem”) to describe such early “adaxial” growth activity. In addition, Kaplan (1970a, 1975) demonstrated that the presumed “leaf primordia arch over the SAM” of the “sympodial hypothesis” is merely strong “adaxial meristem” activity to give such an “arch over” impression in *Acorus calamus*, *Sansevieria trifasciata* and *D. fragrans* and rejected the “sympodial hypothesis”.

Hagemann’s (1970) postgenital view is different from Kaplan’s (1970a, 1970b, 1973a, 1973b, 1975) and he proposed the “subunifacial hypothesis”. He argued that the unifacial part is formed by postgenital fusion of leaf margins along the leaf proximal-distal axis. In bifacial leaves, this fusion is only seen at the leaf apex, thus separating the abaxial and adaxial surfaces. In unifacial leaves, however, this fusion gradually occurs at the adaxial side as two marginal meristems gradually “grow” toward each other and ultimately become fused adaxially, forming the so called “cross zone” (Hagemann and Gleissberg, 1996). Morphologically it appears that along the proximal-distal axis the adaxial surface does not grow and being furrowed in between the much expanded abaxial surface. Because the adaxial surface still accounts for a small portion of the morphologically radial leaf, hence it is not truly unifacial but “subunifacial”. It must be pointed out that Troll and Meyer (1955) argued that only the bifacial part has marginal meristems and marginal meristems are lost in the unifacial part. Hagemann (1970), in contrast, believed that marginal meristems are extended from the bifacial part and ultimately fused adaxially in the “subunifacial” part. While Kaplan (1975) agreed that a leaf is always dorsiventral more or less, he rejected the idea that fusion of marginal meristems being the reason of this.

2. Molecular studies on leaf development and leaf adaxial/abaxial polarities

All angiosperm leaves are initiated from the SAM, a group of self-renewing pluripotent cells. The pluripotency of cells in the SAM is maintained by class I *KNOTTED1*-LIKE HOMEOBOX (KNOX1) genes (Hake *et al*., 2004; Hay and Tsiantis, 2009, 2010). *KNOTTED1* (*KN1*) gene was the first homeobox gene found from plants (Vollbrecht *et al*., 1991). Subsequently, other related genes, *KNOTTED1*-LIKE HOMEOBOX (KNOX) genes, were identified as transcription factors that regulate various aspects in plant development (Hake *et al*., 2004; Hay and Tsiantis, 2009, 2010). KNOX1 genes are most similar to *KN1* among KNOX genes on the basis of sequence similarity of the homeodomain and expression patterns (Kerstetter *et al*., 1994). In plants with simple leaves such as maize, KNOX1 genes expression is confined to the SAM (except leaf founder cells) and some parts of the stem (Jackson *et al*., 1994); whereas in plants with compound leaves, KNOX1 genes expression is also seen at where leaflets will form (Bharathan *et al*., 2002; Hake *et al*., 2004; Hay and Tsiantis, 2006, 2009, 2010), with the exception of garden pea (*Pisum sativum*, Hofer *et al*., 1997) and palm (*Chamaedorea elegans*, Nowak *et al*., 2011). To initiate leaf primordia, KNOX1 genes must be downregulated in the leaf founder cells. This is achieved by a MYB transcription factor *ASSYMETRIC LEAVES1* (*AS1*) in Arabidopsis (*Arabidopsis thaliana*, Byrne *et al*., 2000); *ROUGH SHEATH2* (*RS2*) in maize (Timmermans *et al*., 1999; Tsiantis *et al*., 1999); and *PHANTASTICA* (*PHAN*) in snapdragon (*Antirrhinum majus*, Waites *et al*., 1998) (collectively, ARP genes). Consistently, ARP genes are expressed in leaf primordia, restricting KNOX1 genes (Byrne *et al*., 2000; Timmermans *et al*., 1999; Tsiantis *et al*., 1999; Waites *et al*., 1998).

Gain-of-function mutants for knox1 result from the ectopic expression of KNOX1 genes outside the SAM, i.e., in leaf primordia. In maize, there are five known gain-of-function mutants for knox1: *KN1* (Vollbrecht *et al*., 1991), *RS1* (Schneeberger *et al*., 1995), *LIGULELESS3* (*LG3*) (Fowler and Freeling, 1996; Muehlbauer *et al*., 1999), *LG4* (Fowler and Freeling, 1996), and *GNARLEY1* (*GN1*) (Foster *et al*., 1999). All these mutants have disrupted organization along the proximal-distal axis of the leaf, having distal tissues to adopt proximal identities (Hake *et al*., 2004). For example, leaves of *kn1*, the first characterized knox1 mutant, have their blade adopting sheath, auricle, and ligule tissues, all of which are proximal to the blade in the wild type (Vollbrecht *et al*., 1991). Initially, roles of KNOX1

genes had been discussed mainly on the proximal-distal organization of leaves (Freeling and Hake, 1985; Vollbrecht *et al*., 1991; Freeling, 1992; Sinha and Hake, 1994; Schneeberger *et al*., 1995; Fowler and Freeling, 1996; Foster *et al*., 1999; Muehlbauer *et al*., 1999). However, the focus of the research has moved to their roles on maintaining the meristematic activity and downstream developmental pathways in this decade (Hake *et al*., 2004; Hay and Tsiantis, 2009, 2010), although the role on proximal-distal organization of leaves has been recently revisited (Ramirez *et al*., 2009).

As leaf primordia are being initiated, adaxial/abaxial (ad/abaxial) polarities are established. *PHAN* is the first polarity gene recognized (Waites and Hudson, 1995). In *phan* mutants, the leaf is rod-shaped and has only abaxial characters and adaxial characters are lost (Waites and Hudson, 1995). In addition, it was noticed that novel axes of growth were formed at ectopic boundaries between the adaxial cell fate and the abaxial cell fate. It was therefore concluded that the adaxial cell fate and the abaxial cell fate are mutually exclusive and the juxtaposition of both is required for leaf blade lateral growth (Waites and Hudson, 1995). Besides *PHAN*, the class III homeodomain leucine zipper (HD-ZIPIII) transcription factor subfamily, including *PHABULOSA* (*PHB*), *PHAVOLUTA* (*PHV*), and *REVOLUTA* (*REV*), also plays an important role in specifying the adaxial cell fate (McConnell *et al*., 2001; Emery *et al*., 2003; Juarez *et al*., 2004; Itoh *et al*., 2008a). Complementary to *PHAN* and HD-ZIPIII, other transcription factors, including *AUXIN RESPONSE FACTOR3/4* (*ARF3/4*) and *KANADI* (*KAN*), play important roles in specifying the abaxial cell fate (Kerstetter *et al*., 2001; Eshed *et al*., 2001, 2004; Pekker *et al*., 2005; Candela *et al*., 2008; Itoh *et al*., 2008b; Zhang *et al*., 2009). In addition, small RNAs are also of great importance in regulating leaf ad/abaxial polarities. microRNA390/trans-acting small interfering RNA-ARF (miR390/tasiR-ARF) is produced at the adaxial side of leaf primordia and then travels to the abaxial side and regulates the expression of *ARF3/4* (Nogueira *et al*., 2007; Chitwood *et al*., 2009); whereas miR166 is expressed in a gradient with its maximum at the abaxial side of leaf primordia and regulates the expression of HD-ZIPIII (Juarez *et al*., 2004). In addition, the marginal outgrowth is regulated by *PRESSED FLOWER* (*PRS*) in the *WUSCHEL*-RELATED HOMEOBOX (WOX) transcription factor family (Matsumoto and Okada, 2001).

The study by Yamaguchi *et al*. (2010) was the first and only study of the development of ensiform unifacial leaves of *J. prismatocarpus* using molecular and genetical approaches. In this study, it was

demonstrated that the leaf sheath has *PHB* expression at the adaxial side and *ARF3* expression at the abaxial side. In addition, *J. prismatocarpus* has two subclasses of *PRS*, *PRSa* and *PRSb*. *PRSa* is expressed at the margins of the leaf sheath. The expression patterns of these genes indicate the leaf sheath of *J. prismatocarpus* is the same as bifacial leaves. However, in the leaf blade, only *ARF3* is expressed and there is no *PHB* expression (except in xylem cells). This indicates that the leaf blade of *J. prismatocarpus* is abaxial at the molecular level. It was further demonstrated that *DROOPING LEAF* (*DL*), a transcription factor in the CRABS CLAW (CRC)/DL subfamily of the YABBY (YAB) transcription factor family, is expressed at the central adaxial region of the leaf blade from P1 to P2 stages but only at vascular bundles from the P3 stage. In *J. wallichianus*, a closely related terete unifacial leaf species, *DL* is expressed only at vascular bundles throughout. Interspecific hybridization study between these two species indicated *DL* from *J. prismatocarpus* is responsible for the ensiform unifacial leaf development. This was further confirmed by mutant analyses. In *J. prismatocarpus* mutants which develop terete leaf shape, the expression of *DL* in the central adaxial region is lost. In addition, it was found that *PRSb* is expressed at reorganized leaf margins (or pseudo margins) of the unifacial leaf blade from the P3 stage in *J. prismatocarpus*. Since true leaf margins are absent in the unifacial leaf blade, this reveals an alternative mode of leaf flatness growth. In bifacial leaves of rice (*Oryza sativa*), *DL* is expressed in the central part of leaf primordia and promotes cell proliferation and thickening of the midrib (Yamaguchi *et al*., 2004). Based on above findings, a model of ensiform unifacial leaf blade development in *J. prismatocarpus* was proposed (Fig. 1.5): initially the leaf blade is terete due to abaxialization, as in the bifacial leaf mutants which lack adaxial regulators; then *DL* promotes cell proliferation towards the SAM and the shape of the leaf blade becomes ensiform; finally, after the leaf blade growth direction is changed and leaf margins are reorganized, the leaf blade is further modified to assume its final shape by the activities of *DL* in vascular bundles and *PRSb* in reorganized leaf margins (Yamaguchi *et al*., 2010).

3. Questions to be addressed and the outline of the dissertation

From two preceding sections, it is very obvious to conclude that there exist some major discrepancies between historical studies on unifacial leaves and more recent molecular studies on leaf ad/abaxial polarities in terms of the understanding of developmental processes of unifacial leaves. For example, it was demonstrated that the leaf blade of *J. prismatocarpus* is abaxial at the molecular level (Yamaguchi *et al*., 2010). This finding is clearly inconsistent with Hagemann's (1970) "subunifacial hypothesis" and Kaplan's (1970a, 1970b, 1973a, 1973b, 1975) "adaxial meristem hypothesis". Another example is that it was demonstrated that only the leaf sheath of *J. prismatocarpus* has true leaf margins (defined as the presence of juxtaposition of both adaxial and abaxial surfaces) whereas in the leaf blade they are absent (Yamaguchi *et al*., 2010). This is also inconsistent with Hagemann's (1970) "subunifacial hypothesis" that leaf margins are gradually fused adaxially in the upper part of unifacial leaves. In addition, Kaplan's (1970a, 1970b, 1973a, 1973b, 1975) analyses of "adaxial meristem" in various species is purely based on examining the density of nuclear staining, size of cells, and the degree of vacuolation, criteria used to define "meristem" in the past and are already outdated. It lacks direct evidence of whether meristem activity is indeed present in the modern sense.

In my opinion, the terms "adaxial meristem" and "abaxial meristem" are vague and should be clarified for the following reasons. First, these two terms only imply positional instead of molecular information. According to Kaplan (1970a, 1970b, 1973a, 1973b, 1975), "adaxial meristem" is located at the most adaxial region to the major vascular bundle and "abaxial meristem" is located at the most abaxial region to the major vascular bundle (Fig. 1.3). However, molecularly, as demonstrated by Yamaguchi *et al*. (2010), both parts are abaxial in *J. prismatocarpus*. In this sense, "rounding meristem" proposed by Troll and Meyer (1955) is more appropriate. Second, these two terms do not imply the direction of growth. Examining the density of nuclear staining, size of cells, and the degree of vacuolation are neither accurate nor sufficient to judge the direction of growth. Although examining the mitotic figures (Kaplan, 1970a) can provide limited clue, it is far from enough to draw a firm conclusion about the direction of growth. This is largely due to in the past the lack of an efficient method to analyze the direction of cell division. Therefore, I aim to develop such a method and apply it to *J. prismatocarpus* to analyze both the location and direction of cell division to evaluate

the validity of "adaxial meristem" (to be more appropriate, "rounding meristem") and compare with known genes' expression patterns.

Although Hagemann's (1970) "subunifacial hypothesis" is not supported by molecular studies in *J. prismatocarpus*, there do exist some exceptions that morphologically support the "subunifacial hypothesis", such as seen in *Luisia teres* (Orchidaceae) and *Senecio radicans* (Asteraceae) (personal observation). In addition, Ozerova and Timonin (2009) provided anatomical and developmental evidences of *Senecio acaulis*, *Senecio crassissimus*, *Senecio hallianus*, *Senecio herreianus*, and *Senecio rowleyanus* being subunifacial. This strongly suggests that both unifacial and subunifacial forms exist and they may represent two different types of leaf. In truly unifacial leaves such as *J. prismatocarpus*, the proximal leaf sheath has adaxial and abaxial surfaces; whereas the distal leaf blade has only abaxial surface. In subunifacial leaves, the proximal leaf sheath has adaxial and abaxial surfaces, same as truly unifacial leaves; whereas the distal leaf blade also has adaxial and abaxial surfaces, different from truly unifacial leaves. Therefore, it appears that subunifacial leaves have a small portion of adaxial tissues from the proximal sheath extends into the distal blade. Superficially, this resembles gain-of-function mutants for knox1 (Vollbrecht *et al*., 1991; Freeling, 1992; Schneeberger *et al*., 1995; Fowler and Freeling, 1996; Foster *et al*., 1999; Muehlbauer *et al*., 1999). Gain-of-function mutants for knox1 have disrupted organization along the proximal-distal axis, having distal tissues that adopt proximal identities (Hake *et al*., 2004). It is therefore interesting and bold to hypothesize that the symptom seen in subunifacial leaves is due to the ectopic expression of *KNOX1* in leaves. To check this hypothesis, I aim to study the expression pattern of *KN1* ortholog in *J. torreyi*, a species in a unifacial clade but morphologically appears to be subunifacial.

In Chapter II, I developed a pulse-chase 5-ethynyl-2'-deoxyuridine (EdU) method that can rapidly quantify the direction of cell division and demonstrated its efficiency and usefulness in the model plant Arabidopsis (Yin and Tsukaya, 2016). In Chapter III, I applied this method to *J. prismatocarpus* and analyze not only the location but also the direction of cell division, to evaluate the validity of previously proposed hypotheses about unifacial leaves and compare with known genes' expression patterns. In Chapter IV, I cloned and checked the expression pattern of *KN1* ortholog in *J. torreyi*, to examine the hypothesis that the ectopic expression of KNOX1 in leaves results in subunifacial.

Chapter V: General Discussion and Conclusion

The morphogenesis of unifacial organs (including unifacial leaves, phyllodes of *Acacia*, and rachis leaves of *Oxypolis*) is one unresolved question in classic botany. It is also one typical example of how people's view on a particular question has been changed and advanced when newer approaches and tools become available. When only plant morphology and plant anatomy were considered, the "phyllode hypothesis" (de Candolle, 1827; Henslow, 1911; Arber, 1918, 1925) was the accepted hypothesis about unifacial organs. However, when comparative developmental approaches were available and became popular, it was soon rejected. Arber (1950), later in her career, had rejected her original "phyllode hypothesis" (Arber, 1918, 1925). "New" hypotheses such as the "sympodial hypothesis" (Thielke, 1948; Roth, 1949, 1957, 1961; Ravololomaniraka, 1972), the "adaxial meristem hypothesis" (Kaplan, 1970a, 1970b, 1973a, 1973b, 1975), and the "subunifacial hypothesis" (Hagemann, 1970) were formed based on comparative developmental and anatomical evidences. Although the details are very much different, these hypotheses have one feature in common. They all attempt to use the "meristematic activity" to explain the morphogenesis of unifacial organs. However, the criteria used to judge the "meristematic activity" include the density of nuclear staining, size of cells, the degree of vacuolation, and occasionally, mitotic figures (Kaplan, 1970a), all of which are already outdated. Lacking a direct and powerful method to judge "meristematic activity" is probably the reason why these authors could not reach a definitive conclusion on the morphogenesis of unifacial organs. Surprisingly, it seems that almost nobody has followed this unresolved classic question raised since the 19th century and it has almost been lost in history, even though nowadays we know quite a lot about leaf development and leaf ad/abaxial polarities at the molecular level. Therefore, I take one step forward, attempting to evaluate these hypotheses using modern molecular approaches and using *Juncus* (Juncaceae) as a model system.

The "sympodial hypothesis" focuses on the spatial change of leaf "meristematic activity". It asserts that there is a succession of leaf meristem from the leaf tip to the leaf abaxial side (Fig. 1.2). Kaplan (1970a, 1975) already demonstrated that the presumed "leaf primordia arch over the SAM" is merely strong "adaxial meristem" activity to give such an "arch over" impression in various species. Regardless whether "adaxial meristem" is present or not (details see next paragraph), my EdU study

showed that there is no such spatial succession between various developmental stages of the leaf blade of *J. prismatocarpus*. Therefore, the "sympodial hypothesis" should be rejected, although studies in other unifacial species are needed.

The "adaxial meristem hypothesis" asserts that the "adaxial meristem" causes the thickening growth. This presumed "adaxial meristem" is considered to be located at the most adaxial region of the "transition zone" (Fig. 1.3). Although it is useful in explaining the development of unifacial leaves, it obviously contradicts to some molecular evidences. First, historically, the ad/abaxial polarity was not known at the molecular level. Yamaguchi *et al*. (2010) recently showed that there is no adaxial identity in the leaf blade of *J. prismatocarpus* at the molecular level. Therefore, the term "adaxial meristem" itself is incorrect. Although "rounding meristem" proposed by Troll and Meyer (1955) has no such defect, it seems that this term is only applicable to those terete species. The shape of the leaf blade of ensiform species is not "round". Second, the impression of "adaxial meristem" is based on anatomical and histogenetic evidences, as cells located at that geographical region are very small, easily stained, and have no vacuolation. EdU, instead, offers a direct visualization of cell division. Here I demonstrated that in cross sections, there are some cells already expanded and vacuolated in the central region of the leaf blade also have the EdU signal (Fig. 3.7). Therefore, those outdated criteria are not accurate. If we define cells are able to divide belong to the leaf meristem, then the leaf meristem as detected by EdU signals would include across the entire leaf blade. Third, as revealed by my pulse-chase EdU analysis, while the thickening growth is primarily contributed by the geographical "inner" region (corresponds to the "adaxial meristem" *sensu* Kaplan), other regions together do contribute to ~40% of the thickening growth. Therefore, thickening growth is not restricted to a certain region. Instead, it occurs across the entire leaf blade. Based on above reasons, the "adaxial meristem hypothesis" must be modified substantially.

The "subunifacial hypothesis" asserts that marginal meristems fusion results in a small adaxial sector being wedged by the abaxial surface. Although such presumed marginal fusion is unlikely, morphologically subunifacial species do exist. Because the ectopic expression of knox1 alters the leaf sheath and blade patterning in maize (*Z. mays*), such unique case of modified organogenesis of subunifacial leaves might be also caused by ectopic expression of *KN1* homolog in the leaf. I

demonstrated that indeed, *JtKN1*, the ortholog of *KN1* of maize, is expressed in leaf primordia from the P1 to the P4 stage. Therefore, while the "subunifacial hypothesis" should be retained, its likely cause is different from initially proposed by Hagemann (1970). Its detailed organogenesis mechanisms are to be elucidated in the future.

In addition to these hypotheses, Yamaguchi et al. (2010) proposed a model of ensiform unifacial leaf development in *J. prismatocarpus*. In this model (Fig. 1.5), *DL* promotes cell proliferation towards the SAM and the shape of the leaf blade is changed from the initially terete to ensiform; after the leaf blade growth direction is changed and leaf margins are reorganized, the leaf blade is further modified to assume its final shape by the activities of *DL* in vascular bundles and *PRSb* in reorganized leaf margins. This model is based on various genes expression patterns and genetical evidences, and uses these information to infer cell proliferation. I checked directly the cell division pattern using EdU, and compared with *DL*, *PRSa*, and *PRSb* expression patterns. I found that *DL*-expressing cells are able to influence the cell division activity of non-*DL*-expressing cells, not only spatially but also temporally, although it is also possible that thickening growth is not controlled by *DL* alone. In addition, *PRSa* has a more direct local influence on the cell division activity in leaf margins in the leaf sheath than *PRSb* does in reorganized leaf margins in the leaf blade. This comparison approach provides invaluable insights into how key genes influence the cell division activity spatially and temporally during the development of unifacial leaves.

In my dissertation, I examined various hypotheses of unifacial leaf development. I developed a pulse-chase EdU method to quantify the direction of cell division. I first demonstrated its usefulness in Arabidopsis, and then applied it to an ensiform unifacial leaf species *J. prismatocarpus*. I found there is no succession of EdU signals, thus rejecting the "sympodial hypothesis". I also found thickening growth is not restricted to the "adaxial meristem" (*sensu* Kaplan), thus providing evidences to modify the "adaxial meristem hypothesis". I found *JtKN1* is ectopically expressed in *J. torreyi* leaf primordia, thus providing evidence to the likely cause of the "subunifacial hypothesis". I also compared the cell division pattern with known genes expression patterns in *J. prismatocarpus*, making it possible to modify the existing model about ensiform unifacial leaf development. The new method I developed (pulse-chase EdU method) and the new approach I used (compare the cell

division pattern and the key genes expression patterns) could also be utilized in other plant species to answer important questions. My dissertation study is an example of how modern methods and approaches can provide new insights into old and unresolved issues.

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