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Early stages of leaf development in *has* mutant of *Arabidopsis thaliana*

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Abstract

The elucidation of molecular mechanisms underlying the leaf development can be facilitated by the detailed anatomical study of leaf development mutants. We present an analysis of leaf anatomy and morphogenesis during early developmental stages in *has* mutant of *Arabidopsis thaliana*. The recessive *has* mutation affects a number of aspects in plant development, including the shape and size of both cotyledons and leaves. The earliest developmental observations suggest almost synchronous growth of the first two leaf primordia of *has* mutant. No significant disruption of the cell division pattern in the internal tissue is observed at the earliest stages of development, with the major anatomical difference compared to wild type primordia being the untimely maturation of mesophyll tissue cells in *has* mutant. At the stage of leaf blade formation, structure disruption becomes clearly evident, by irregular arrangement of the cell layers and the lack of polarity in juvenile *has* leaves. One distinguishing feature of the mutant leaf anatomy is the absence of mesophyll tissue differentiation. Altered *has* mutant leaf morphology could be at least partially accounted for by the ectopic *STM* activity that was found at the base of leaf primordia during early stages of leaf development in *has* plants.

Additional key words: *in situ* hybridization, insertional mutagenesis, leaf morphogenesis.

Introduction

Leaves are determinate, bilaterally symmetrical, lateral organs which are produced from the cells recruited from the shoot apical meristem (SAM). Leaf primordium initiation involves the formation of an outgrowth on the surface of the meristem by periclinal cell divisions in the inner layers of peripheral zone of SAM.

A number of genes have been associated with processes necessary for organ formation (Clark 1997, Barton 2001, Traas and Doonan 2001). A very early event in organ initiation is the inactivation of *SHOOT MERISTEMLESS* (*STM*) gene, a member of *KNOX* family of homeobox genes in *Arabidopsis* (Jackson *et. al*. 1994, Long *et al*. 1996, Reiser *et al*. 2000, Barton 2001). These meristematic genes are necessary for maintaining the pool of meristematic cells at the shoot apex and are expressed in the SAM but not in the presumptive leaf primordium, the initiating leaf primordium and developing leaf (Kerstetter *et al*. 1994, Long *et al*. 1996). Downregulation of *KNOX* genes in lateral organ primordia is a critical event in organ patterning, as the ectopic expression of these genes disrupts normal leaf development (Byrne *et al*. 2000, Chuck *et al*. 1996, Reiser *et al*. 2000). Set of genes other than these are also associated with the first steps in organ initiation, which include the attribution of cells to primordia, organ outgrowth, organ separation, and determination of organ identity (Traas and Doonan 2001).

In the course of development, the three-dimensional form of leaf is specified. Leaf develops and differentiates along proximodistal (base-tip), dorsoventral (top-bottom) and mediolateral (middle-to-margin) planes (Steeves and Sussex 1989, Kim and Cho 2006). After the leaf polarity

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Abbreviations: MS - Murashige and Skoog; SAM - shoot apical meristem; SEM - scanning electron microscopy; *STM* - *SHOOT MERISTEMLESS* gene.
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is set, continued cell division, expansion and differentiation of specific cells and tissue types are established. Because growth and division are tightly coupled, the coordination of cell division and expansion plays an important role in establishing leaf morphology (Arkebuer and Norman 1995). The rates of cell division and expansion at each stage of the leaf formation contribute to the final leaf shape (Steeves and Sussex 1989, Tsukaya 2002, 2003) and play an important role throughout leaf development.

To investigate the mechanisms that regulate leaf morphogenesis, we have used *has* (*handshake*) mutant of *Arabidopsis thaliana* as a model system. This is a recessive T-DNA insertion mutant, displaying perturbed apical meristem function and dwarfism (Janošević and Budimir 2006). Both cotyledons and leaves exhibit disrupted tissue organization. We have previously shown that reduced size of cotyledons, embryonic leaves, in *has* mutant is due to substantial reduction in cell number, but that this defect is partially compensated by an increase in cell size (Janošević *et al*. 2007). In this study we have shown that the *has* mutation, displaying already during primordium development, affects the factors that influence cell differentiation later during leaf development.

Materials and methods

The *has*-mutant of *Arabidopsis thaliana* L. ecotype Wassilevskija was identified in a collection of T-DNA tagged lines made in INRA, Versailles, France (Bechtold *et al*. 1993, Bouchez *et al*. 1993). The *has*-phenotype is tightly linked to the T-DNA kanamycin resistance marker. Wild-type and *has*-mutant plants of *A. thaliana* were grown *in vitro* on Murashige and Skoog (1962; MS) half-strength mineral medium or on medium adapted from Estelle and Somerville (1987). The irradiance was 47 μmol(PAR) m^{-2} s^{-1} with a 16-h photoperiod, the temperature was 25 ± 2 ºC.

For scanning electron microscopy (SEM), plant material was fixed in 3 % glutaraldehyde in phosphate buffer at pH 7.2 for 24 h. After fixation, samples were rinsed with phosphate buffer, dehydrated in ethanol and stored at 4 ºC. Tissues were subsequently dried using liquid carbon dioxide. Dried samples were covered with a carbon-gold-palladium mixture and examined using a *Philips 525M*° scanning electron microscope operated at 10 kV (*Philips*, Einthoven, The Netherlands).

Specimens for light microscopy were fixed in 3 % glutaraldehyde in phosphate buffer (pH 7.2) under vacuum for 1 h, and left in fixative for 3 d at 4 ºC. After a short wash in buffer, material was postfixed in 1 % osmium tetroxide in phosphate buffer at 4 ºC, during 24 h. Fixed material was washed, dehydrated and embedded in *Araldite* resin. Sections (1 - 1.5 μm) were cut on *LKB III* (Vienna, Austria) ultramicrotome and stained with methylene blue. Sections were photographed with a *Jenamed* photomicroscope (*Carl Zeiss*, Jena, Germany). For histological analyses, at least three plants were used.

In situ hybridization was carried out as described (Janošević and Budimir 2006) by using DIG-labeled RNA probes for a full length *STM* according to the manufacturer's instructions.

Results

The recessive *has* mutation affects both shape and size of *Arabidopsis* leaves. Wild type plants produce different types of leaves (Fig. 1), while the *has* mutant plants do not show apparent heteroblastic characteristics (Fig. 2). At the vegetative phase of development, in contrast to flattened, round to oval leaves of the wild type (Fig. 3), leaves of the *has* mutant are narrow and finger-like (Fig. 4), producing small lobed blades later, during reproductive phase of development (Fig. 2). At this phase of development mutant plants are much smaller than the wild type, with average height of 1.11 ± 0.29 cm compared to the wild type plants with average height of 14.42 ± 0.56 cm.

The earliest developmental observations of a juvenile 4-day-old *has* seedling suggest almost synchronous growth of the first pair of true leaves. They arise as small round outgrowths at the opposite sides of the apical meristem. The majority of mesophyll cells of a 4-d-old *has* seedlings are meristematic, with prominent nuclei and mostly lacking vacuoles (Fig. 5). Wild-type leaf primordia of the same age exhibit dorsoventral asymmetry in that the regions of abaxial epidermis and adjacent mesophyll contain enlarged, highly vacuolated cells, whilst no vacuolisation is observed in the region of adaxial epidermis and majority of subjacent mesophyll cells (Fig. 6). In *has* mutant dorsoventral asymmetry becomes evident only later. In the 8-d-old *has* primordium, apart from dividing meristematic cells, vacuolated cells were also present in its inner tissue (Fig. 7). Therefore, at these early stages of development, the major anatomical difference compared to wild type primordia is the untimely maturation of mesophyll tissue cells in *has* mutant (Figs. 7, 8). However, no significant disruption of the cell division pattern in the internal tissue is observed until the stage of leaf blade formation, so that it becomes clearly evident only in a 12-d-old *has* mutant

(Fig. 9). Another distinguishing feature of the mutant leaf anatomy is the absence of mesophyll tissue differentiation. Mesophyll tissue failed to differentiate into palisade and spongy tissue, so that all mesophyll

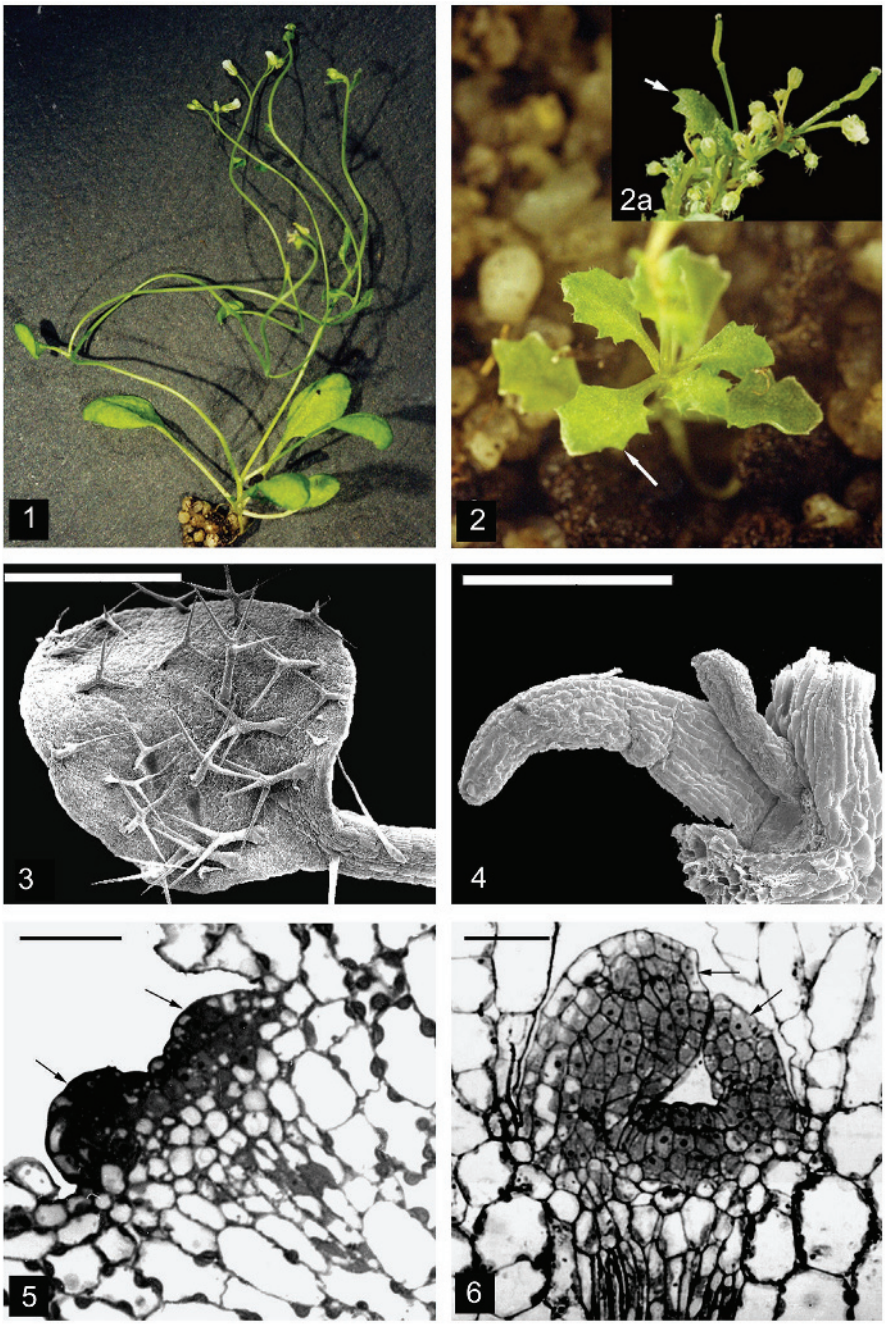

Fig. 1. Wild type plant ecotype Wassilevskija, 100-d-old.
Fig. 2. The *has* mutant plant, 100-d-old. Note lobed leaves (↑). Fig. 2a. Lobed bract of the *has* mutant inflorescence (↑).
Fig. 3. Scanning electron micrograph of leaf of 12-d-old wild type plant, *bar* = 500 μm.
Fig. 4. Scanning electron micrograph of leaf of a 20-d-old *has* mutant plant, *bar* = 500 μm.
Fig. 5. Longitudinal section of vegetative shoot apical meristem with developing leaf primordia (↑) of a 4-d-old *has* plant. Note meristematic mesophyll cells mostly lacking vacuoles, *bar* = 25 μm.
Fig. 6. Longitudinal section of vegetative shoot apical meristem with leaf primordia already exhibiting dorsoventral assymetry (↑) of a 4-d-old wild type plant, *bar* = 25 μm.

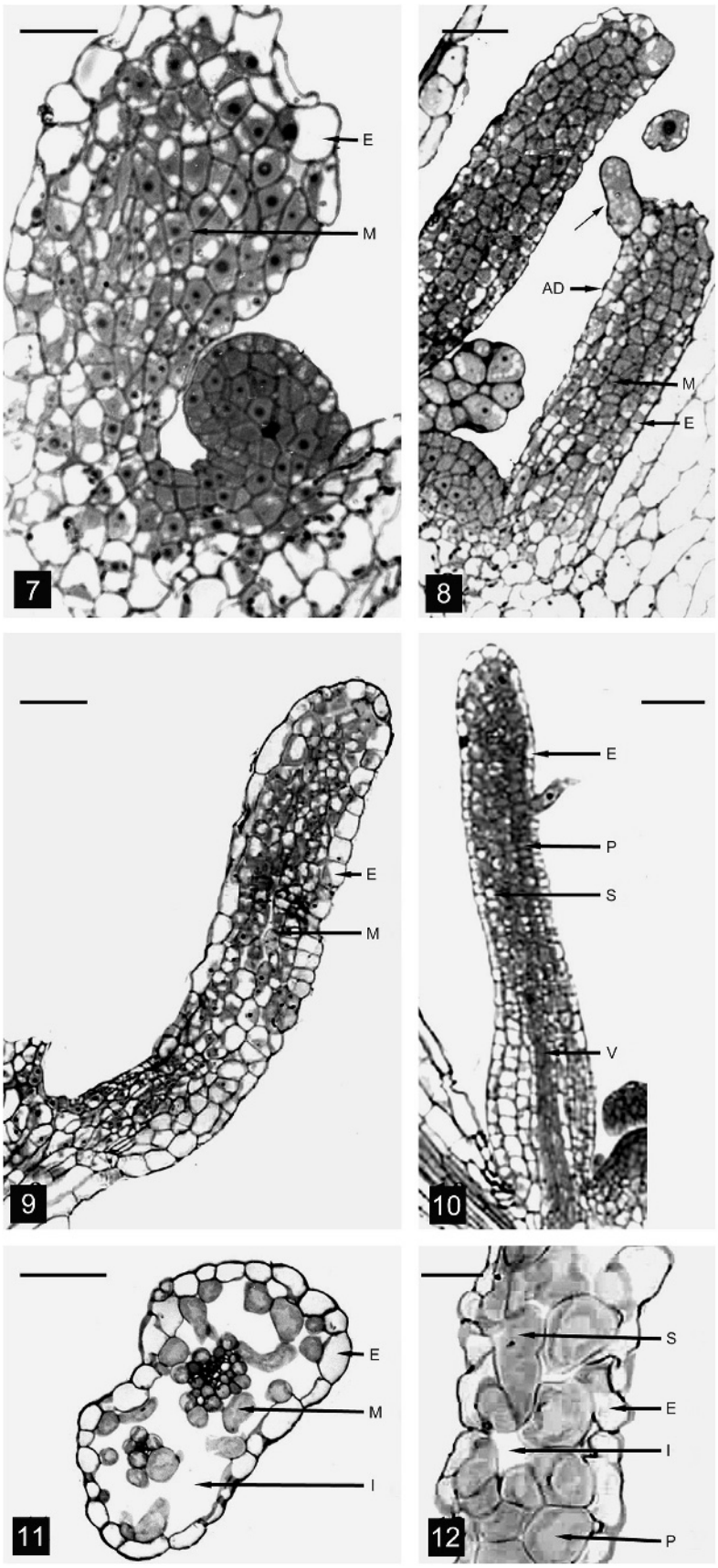

Fig. 7. Longitudinal section of leaf primordia of an 8-d-old *has* plant. Note vacuolisation of the irregularly shaped cells of the abaxial epidermis as well as in the internal tissue, *bar* = 25 μm.
Fig. 8. Longitudinal section of leaf of an 8-d-old wild type plant. Cell divisions occur mainly in anticlinal direction in developing lamina of elongated and flattened young leaves. Note trichome on adaxial side (↑), *bar* = 25 μm.
Fig. 9. Longitudinal section of leaf of a 12-d-old *has* plant showing that all mesophyll cells are similar in appearance, irregularly shaped and highly vacuolated, *bar* = 50 μm.
Fig. 10. Longitudinal section of leaf of a 12-d-old wild type plant reveals differentiation of mesophyll tissue into palisade (adaxial side) and spongy tissue (abaxial side), *bar* = 50 μm.
Fig. 11. Cross section of leaf of a 20-d-old *has* plant showing absence of mesophyll tissue differentiation, *bar* = 100 μm.
Fig. 12. Cross section of leaf of a 20-d-old wild type plant reveals differentiation of mesophyll tissue into palisade (adaxial side) and spongy tissue (abaxial side), *bar* = 25 μm.
E - epidermis, M - mesophyll tissue, P - palisade tissue, S - spongy tissue, I - intercellular spaces, AD - adaxial side.

cells were similar in appearance, in contrast to the wild type leaves of the same age (Figs. 9, 10). During further development, mesophyll cells of *has* leaves become loosely packed without distinguishable layer organization which is typical for the wild type leaves (Figs. 11, 12). Immuno-histological study performed by *in situ* hybridization on sections of the 12-day-old seedlings, revealed the activities of *STM* gene at the base of developing leaf primordia (Fig. 13), and young leaves (Fig. 14) of *has* plants.

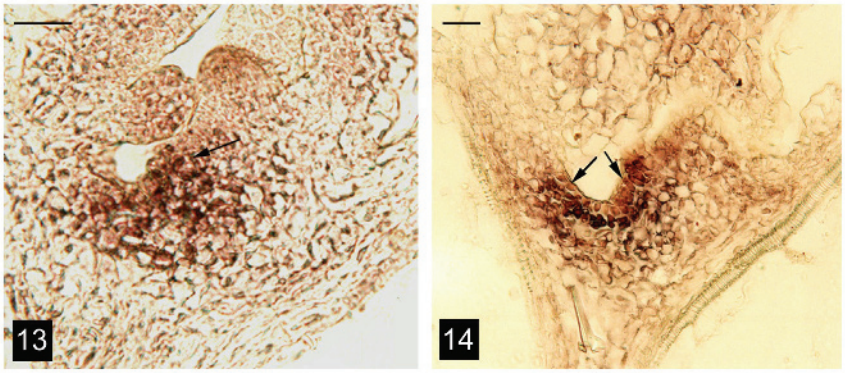

Fig. 13. STM expression at the vegetative shoot apex and developing leaf primordia (↑) of a 12-d-old *has* plant, *bar* = 100 μm.
Fig. 14. STM expression at the vegetative shoot apex and the base of leaf (↑) of a 12-d-old *has* plant, *bar* = 100 μm.

Discussion

Current focus of plant developmental biology is the elucidation of molecular mechanisms underlying developmental processes. Various mutants with altered leaf morphology related to abnormal expansion along axes have been isolated, and some of the genes responsible for the mutant phenotypes have been cloned and characterized (Tsukaya 2002, Kim and Cho 2006). To investigate the factors that regulate leaf development we have focused on early stages of leaf morphogenesis in *has* mutant of *Arabidopsis thaliana*.

In this study we have shown that the early stages of *has* leaf development are characterized by the untimely maturation of the mesophyll tissue cells without any significant disruption of the cell division pattern. Structure disruption becomes clearly evident only later, at the stage of leaf blade formation, and is evinced by irregular arrangement of the cell layers and the lack of polarity. Mechanisms responsible for the establishment of proximodistal and dorsoventral polarity in leaves have been described by Ha *et al*. (2003), Waites *et al*. (1998), Lynn *et al*. (1999), Siegfried *et al*. (1999) and McConnel *et al*. (2001). According to McConnel and Barton (1998), regulation of polarity along leaf primordia is closely related to the activity of the SAM, the structure and function of which were previously shown to be perturbed in *has* mutant (Janošević and Budimir 2006). Disrupted cell differentiation and lack of polarity in *has* leaves could be attributed to the ectopic expression of *STM*, normally absent from the incipient primordia of the wild type plants. Ectopic *STM* activity that was found at the base of leaf primordia during early stages of leaf development in *has* plants could therefore at least partially account for the altered *has* mutant leaf morphology.

More detailed studies on leaf development mutants such as *has* are needed to identify temporal and spatial changes in the genetic control elements and their products implicated in basic aspects of leaf development, with the eventual aim to provide critical information about the molecular basis for the control of normal leaf development.

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