



A comparative study of early leaf development in the *Viola albida* complex

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ABSTRACT: Early leaves within the *Viola albida* complex were investigated by scanning electron microscopy in order to determine the morphological segments during morphogenesis. The early leaf development of *V. albida* var. *albida* could be morphologically divided into the eight stages in the following order: I, the initiation of shoot germination; II, the conical growth directionally of the leaf; III, the adaxial and abaxial formation of the leaf; IV, the initiation of the stipule; V, the formation of a transitional zone between the leaf blade and petiole; VI, the expansion of the upper part of the leaf blade; VII, the formation of almost all parts of the early leaf; VIII, the early simple leaf. *Viola albida* var. *takahashii* differs from *V. albida* var. *albida* by additional stages, i.e., V-1, the initiation of the first lateral lobe at the both lateral parts of the leaf after the stage V and an early lobed leaf. *Viola albida* var. *chaerophylloides* is also distinguished from two taxa by two developmental features, V-2, the initiation of a second lateral lobe below of the first lateral lobe, and an early palmately compound leaf. These findings suggest that the *Viola albida* complex would be in the process of peramorphosis, showing developmental changes in a chain of events, leading to a different leaf shape. These data would also be useful for isolating genes that give rise to different leaf morphogenesis outcomes among the taxa in the *Viola albida* complex.

Keywords: *Viola albida* complex, early leaf development, peramorphosis

Taxonomic research required taxa, having morphological variations caused by frequent hybrid and/or vegetative reproduction in nature, have been called by several categories; microspecies (Davis and Heywood, 1963), superspecies (Mayr, 1969), semispecies (Grant, 1957; Baum, 1972), multispecies (Van Valen, 1976), species complex (Grant, 1981) etc. The *Viola albida* complex, consists of three taxa, *V. albida* var. *albida*, *V. albida* var. *takahashii*, and *V. albida* var. *chaerophylloides*, and these are sympatric (Kim et al., 1991). The frequent hybridization among taxa in this complex takes places, and there are almost absence of differences in taxonomic features for dividing species except for various leaves shape (Russell, 1960; Kim, 1986; Kim et al., 1991).

It has generally known that *V. albida* var. *takahashii* might be originated by the hybridization between *V. albida* var. *albida* and *V. albida* var. *chaerophylloides* (e.g., Kim, 1986). There is, however, no direct evidences regarding this so far. This complex shows numerous kinds of leaf forms in-between simple to palmately compound leaf, so that it is taxonomically difficult to

explain (Kim and Lee, 1988; Kim et al., 1991; Jang et al., 2006; Whang, 2006). The results of morphological researches about this complex should give rise to debate in both the selection of scientific name (Regel, 1861; Maximowicz, 1877; Becker, 1902; Makino, 1912; Nakai, 1922a, 1922b; Ishidoya, 1929; Maekawa, 1954; Chung, 1959; Ito, 1962; Hashimoto, 1967; Lee, 1969, 1980; Park, 1974; Kim et al., 1991; Lee, 2003) and their classification system (Becker, 1925; Takenouchi, 1955; Maekawa and Hashimoto, 1963; Fu and Teng, 1977; Kim, 1986; Jang, 2012). Recent researches based on molecular data like DNA sequences, RAPD, ISSR, etc have supported the design adequacy of this complex (Yoo et al., 2004, 2005; Whang, 2006; Yoo and Kim, 2006; Koo et al., 2010). Further researches, however, are required to have better understand at the speciation and/or evolution in this complex. We have been conducting four different experiments on this complex; developmental investigation on early leaf morphogenesis (Choi, 2011), cross test between *V. albida* var. *albida* and *V. albida* var. *chaerophylloides* in both laboratory and wildness (An, 2015),

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and both cloning and expression of several key genes responsible for leaf morphogenesis (Srikanth, 2014; Srikanth et al., 2019).

This study aims to determine morphological segments appearing in the early leaf development of *Viola albida* complex in order to give developmental clue for distinguishing taxa in the complex. The new data would be also useful as a basic reference for genes isolation responsible for different leaf morphogenesis if there are some successful results.

Materials and Methods

Plant materials

More than 1,000 individuals in *Viola albida* complex were collected from 2005 to 2015 at Naejangsan and Jeoksangsan Mountains for this study. They were transplanted at laboratory of about 66 m² that has been regulated in both temperature and intensity of illumination, and their growth and development were observed more than seven years. The facilities locate in The 1st Science Building of Chonbuk National University. The individuals showing constant in shape during the several generations were selected for developmental studies.

Seed germination and collection of young shoots

The matured seeds were collected from both chasmogamous and cleistogamous flowers, and then stored at 4°C refrigerator. They were washed in tap water with liquid detergent for five minutes, and then sterilized using 70% ethanol and a mixture of distilled water (250 µL), lax (250 µL), and Triton X-100 (25 µL). Sterilization was done by dipping the seeds in ethanol 70% for 1 min and transferring them to sodium hypochloride solution at 2% for 20 min. Sterilized seeds were inoculated into 150 mL glass flasks containing 50 mL of MS medium (Murashige and Skoog, 1962) supplemented with 0.4 mg/L thiamine, 1 mg/L pyridoxine, 0.5 mg/L nicotinic acid, 100 mg/L mio-inositol and 0.5 g/L hydrolyzed casein, diluted for the concentration defined in each treatment. The inoculation process was done under aseptic incubator in a sterile environment. The flask inoculated with sterilized seed were capped with plastic lids and transferred to a growth room. They were remained for six months at 23°C with 16 h lighting at an intensity of eight foot candles. Seeds were monitored daily and were evaluated for contamination and seed germination.

Scanning electron microscopy

Young shoots serially collected were fixed by 4% glutaraldehyde solution for the purpose of scanning electron microscopy (SEM) observation. After full fixation, young shoots were washed with distilled water and dehydrated with

a graded ethanol series (30, 50, 70, 80, 90, 95, and 100%) consisting of 10 min steps for each ethanol concentration followed by a graded ethanol-acetone series with three steps. Young shoots were subsequently freeze dried (VFD-21S, Hidachi, Tokyo, Japan), mounted on SEM stubs with double-sided adhesive tape, coated with gold in 15 nm thickness, and examined with a Hidachi ISI ABT (SR-50) SEM operated at 10 kV. All the stubs prepared are housed in the Botany Laboratory of Chonbuk National University.

Results

General features in early leaf development

The shape of shoot apical meristem (SAM) before germination is long elliptical in all taxa of *Viola albida* complex (Fig. 1A, B). The SAM, constituted with about 200 cells, of *V. albida* var. *albida* is a little bit smaller than those of *V. albida* var. *takahashii* and *V. albida* var. *chaerophylloides*, about 250 cells. After germination, the SAM showed gradual changes to develop young leaf. The morphogenesis of young leaves then were rapidly made up within a week, and their size reached up to around 1,000 µm in width and 3,000 µm in length (Figs. 1–3). The morphological segments of early leaf development, appeared in the taxa of the complex, could be distinguished in comparing the features of both mature leaves as well as general shapes in very early stages of germination. Morphological segments of early leaves development detected in this study were different among taxa in the complex (Figs. 1–3).

Morphological segments of early leaves in *Viola albida* complex

The features of early leaf development in *V. albida* var. *albida* was showed in Fig. 1 in detail. There are the SAM before germination (Fig. 1A), the initiation of germination (Fig. 1B), the initiation of conical growth directionally (Fig. 1C), ended up conical growth (Fig. 1D), the formation of adaxial and abaxial side of leaf (Fig. 1E), the initiation of stipule (Fig. 1F), the formation of transitional zone between leaf and petiole (Fig. 1G), the expansion of upper part of leaf (Fig. 1H), the formation of almost all parts of early leaf (Fig. 1I), and the adaxial side of early simple leaf (Fig. 1J). The features of early leaf development in *V. albida* var. *takahashii* was showed in Figs 1–2 in detail. Its morphological segments during early leaf development are the same as those of *V. albida* var. *albida* till the formation of transitional zone between leaf and petiole. The different features are, however, the first initiation of lateral lobe in both parts of leaf (Fig. 2A), the expansion of leaf blade (Fig. 2B), the formation of almost all of early leaf (Fig. 2C),

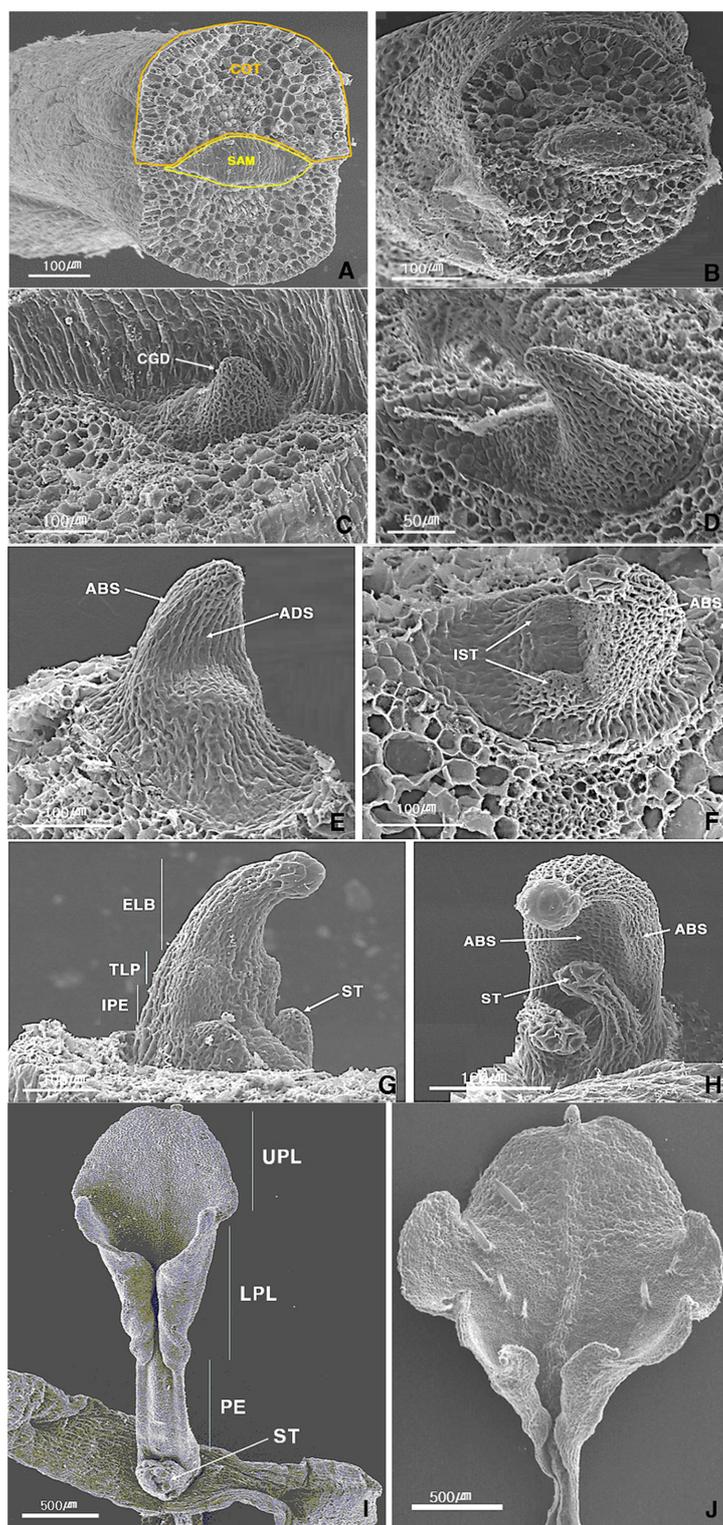


Fig. 1. Scanning electron micrographs of early leaf development in *Viola albida* var. *albida*. **A.** Shoot apical meristem before germination. **B.** Initiation of germination. **C.** Initiation of conical growth directionally. **D.** Ended up conical growth. **E.** Formation of adaxial and abaxial side of leaf. **F.** Initiation of stipule. **G.** Transitional zone between leaf and petiole. **H.** Expand of upper part of leaf. **I.** Formation of almost all parts of early leaf. **J.** Adaxial side of early simple leaf. ABS, abaxial side of leaf; ADS, adaxial side of leaf; CGD, conical growth directionally; COT, the site of tear off a cotyledon; ELB, early leaf blade; IPE, initiation of petiole; IST, initiation of stipule; LPL, lower part of leaf blade; PE, petiole; SAM, shoot apical meristem; ST, stipule; TLP, transitional zone between leaf and petiole; UPL, upper part of leaf blade.

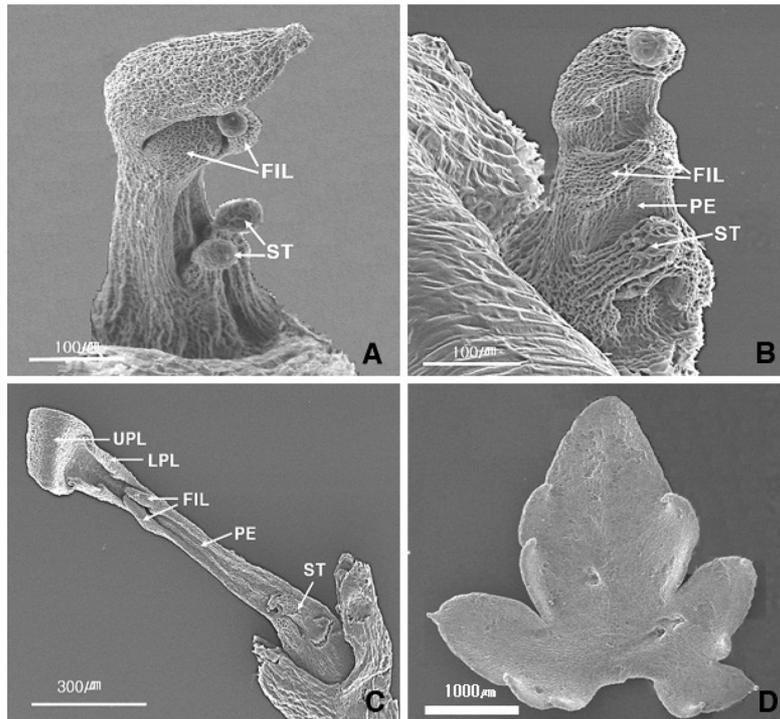


Fig. 2. Scanning electron micrographs of some early leaf development of *Viola albida* var. *takahashii*. **A.** First initial of lateral lobe at both parts of leaf. **B.** Expand of leaf blade. **C.** Formation of almost all of early leaf. **D.** Adaxial side of early lobed simple leaf. See Fig. 1. to know before Fig. 2A. FIL, first lobe of leaf; LPL, lower part of leaf; PE, petiole; ST, stipule; UPL, upper part of leaf.

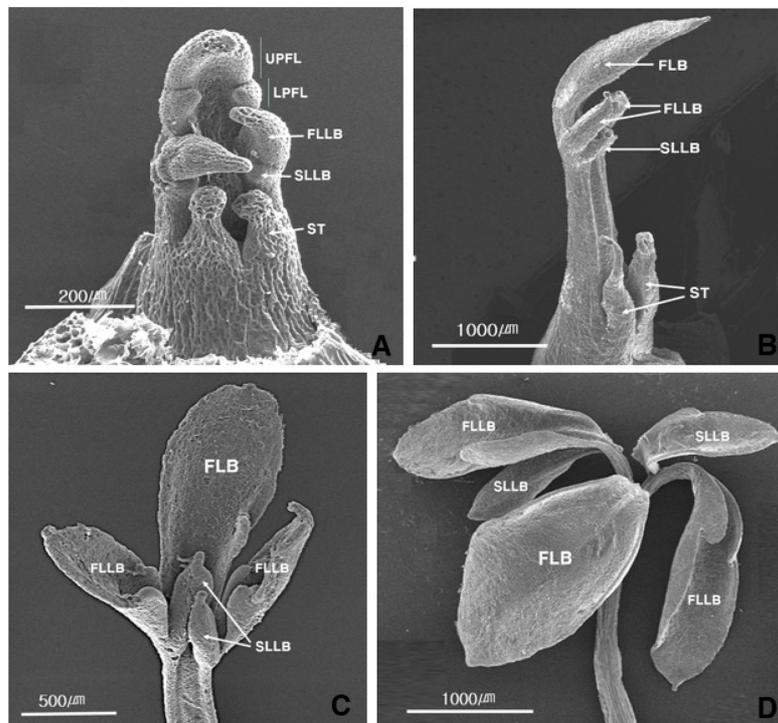


Fig. 3. Scanning electron micrographs of some early leaf development of *Viola albida* var. *chaerophylloides*. **A.** First initial of second lateral lobe at both parts of leaf. **B.** Formation of almost all of early leaf. **C.** Expand of leaf blade. **D.** Early palmate compound leaf. See Fig. 1. to know before Fig. 3A. FLB, first leaf blade; FLLB, first lateral leaf blade; LPFL, lower part of first leaf; SLLB, second lateral leaf blade; ST, stipule; UPFL, upper part of first leaf.

and the adaxial side of early lobed simple leaf (Fig. 2D). The early leaf development of *V. albida* var. *chaerophylloides* was showed in Figs 1–3 in detail. Its morphological segments during early leaf development are the same as those of both *V. albida* var. *albida* till the formation of transitional zone between leaf and petiole and *V. albida* var. *takahashii* till the first initiation of lateral lobe of the leaf. The different features are, however, the first initiation of second lateral lobe in both parts of leaf (Fig. 3A), the formation of almost all of early leaf (Fig. 3B), the expansion of leaf blade (Fig. 3C), and the early palmately compound leaf (Fig. 3C).

In conclusion, taxa within the complex could be distinguished based on the features described above. The morphological segments of early leaf development in *V. albida* var. *albida* were categorized into eight stages: I, the initiation of shoot germination; II, the conical growth directionally of leaf; III, the adaxial and abaxial formation of leaf; IV, the initiation of stipule; V, the formation of transitional zone between leaf blade and petiole; VI, the expansion of upper part of leaf blade; VII, the formation of almost all part of early leaf; VIII, the early palmate compound leaf. *Viola albida* var. *takahashii* is clearly separated from *V. albida* var. *albida* by the features of both V-1, the initiation of first lateral lobe at both parts of early leaf, and VIII, the early lobed simple leaf. *Viola albida* var. *chaerophylloides* is also separated from *V. albida* var. *albida* and *V. albida* var. *takahashii* by three features, V-1, the initiation of first lateral lobe at both parts of early leaf, V-2, the second lateral lobe at the below of first lateral lobe, and VIII, the early palmate compound leaf.

Discussion

Viola albida var. *albida* distributes widely on the Korean Peninsula, and its speciation course is of interest. This species complex consists of three taxa, *V. albida* var. *albida*, *V. albida* var. *takahashii*, and *V. albida* var. *chaerophylloides* (Kim et al., 1991). Interestingly, it is not difficult to find the spots where all taxa in this complex are sympatric distribution or neighborhood areas across many of mountains (Whang, 2006). Taxa in this complex hardly find differences in taxonomic characters except for various leaves shapes. There are, however, numerous leaf shapes from simple to palmately compound forms together with intermediate types in-between, so that the establishment of both species limitation and classification have long been debating among researchers (e.g., Kim et al., 1991; Whang, 2006; Jang, 2012). Many researches on this complex have been conducted because of this exciting attention to taxonomists (e.g., Kim et al., 1991; Jang, 2012). The authors have been also studying for

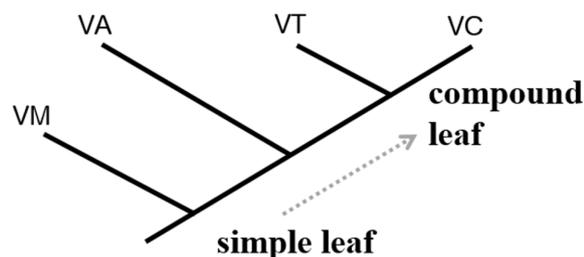


Fig. 4. A suggested dendrogram of the *Viola albida* complex based on Table 1 data. VA, *V. albida* var. *albida*; VC, *V. albida* var. *chaerophylloides*; VM, *V. mandshurica*; VT, *V. albida* var. *takahashii*.

several decades to this complex in order to find the courses of speciation and evolution (Whang and Kim, 1985; Kim et al., 1991; Whang, 2006; Koo et al., 2010).

It is too difficult to prove the evolution of organism that is a phenomenon gradually taking place for a long time (e.g., Barrick et al., 2009). It is also never easy for what the experimental demonstration as to morphological changes taking place in real time on the basis of the evolutionary point of view. In these context, we have recently done four different experiments for this complex: 1) the investigation of morphogenesis from seed germination to early leaf shape (Choi, 2011), 2) the investigation of the leaf features of second generation after the cross test in both laboratory and wildness (An, 2015), 3) cloning of genes that have directly related to the morphological segments during early leaf development (Srikanth, 2014), and 4) and the investigation of the expression pattern of their genes (Srikanth, 2014; Srikanth et al., 2019).

This article is about a comparative study of early leaf development showing the morphological segments. This data, showing developmental differences, were primary useful as some diagnostic features to distinguish the taxa in the complex. This data would be employed not only for cloning of genes that have directly related to the morphological segment during early leaf development, but also for testing their differential expression pattern (Srikanth et al., 2019).

The data obtained this study were Figs. 1–3, and their scientific interpretations summed up in Fig. 4 and Table 1. On the basis of Table 1, the crucial morphological differences among taxa could be summarized as follows. After the development of feature V (the formation of transitional zone between leaf blade and petiole) in *V. albida* var. *albida*, two features, V-1 (the initial of first lateral lobe at both parts of early leaf) and V-2 (the initial of second lateral lobe at the below of first lateral lobe), were repeatedly developed in *V. albida* var. *takahashii* and *V. albida* var. *chaerophylloides*

Table 1. Some diagnostic characters from early leaf development of the *Viola albida* complex.

Characters	Character numbering	Taxa			
		VA	VT	VC	VM ^a
Initiation of shoot germination	I	O	O	O	O
Conical growth directionally of leaf	II	O	O	O	O
Adaxial and abaxial formation of leaf	III	O	O	O	O
Initiation of stipule	IV	O	O	O	O
Formation of transitional zone between leaf blade and petiole	V	O	O	O	O
Initial of first lateral lobe at both parts of early leaf	V-1	N/A	O	O	N/A
Initial of second lateral lobe at the below of first lateral lobe	V-2	N/A	N/A	O	N/A
Expand of upper part of leaf blade	VI	O	O	O	O
Formation of almost all part of early leaf	VII	O	O	O	O
Early simple heart leaf	VIII	O	O	O	N/A
Early lobed simple leaf	VIII-1	N/A	O	O	N/A
Early palmately compound leaf	VIII-2	N/A	N/A	O	N/A

See Figs. 1–3 for the abbreviation of Arabian number.

VA, *V. albida* var. *albida*; VT, *V. albida* var. *takahashii*; VC, *V. albida* var. *chaerophylloides*; VM, *V. mandshurica*; N/A, not available.

^aOut group.

respectively. There were, therefore, absent in two features like V-1 and V-2 in *V. albida* var. *albida*, and also absent in the feature V-2 in *V. albida* var. *takahashii*, so that these two taxa were clearly distinct from *V. albida* var. *chaerophylloides*. In addition, two different types of heterochrony, like spatial and temporal changes in development in a descendant relative to its ancestor, has long been known as the mechanisms most important for evolution (Haeckel, 1875).

The meaning of above differences would be that two taxa, *V. albida* var. *takahashii* and *V. albida* var. *chaerophylloides*, are peramorphosis comparing to the ancestor, *V. albida* var. *albida*. Because these two taxa have evolved excessive stages besides all developmental stages of ancestor type (e.g., Buendía-Monreal and Gillmor, 2018). A suggested topology based on the above shows in Fig. 4. In putting *V. mandshurica* having simple leaf as an out-group, *V. albida* var. *albida* had initially originated in the Korean Peninsular and/or its neighborhood area, and then *V. albida* var. *takahashii* and *V. albida* var. *chaerophylloides* might have been originated caused by hybridization with other species and/or genetic changes.

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Conflict of Interest

The authors declare that there are no conflicts of interest.

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