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Characterization of a new leaf-compound radish mutant (*Raphanus sativus* L.)

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Abstract

A compound-leaf mutant of radish was induced by treatment of ethyl-methane sulfonate. We analyzed the photosynthetic, agronomic, microstructural, and quality traits of the mutant and compared them with those of wild-type. Net photosynthetic rate was approximately 30 % higher, and total chlorophyll content was approximately 36 % higher in mutant than in wild-type. However, the root weight of the mutant was only half of the wild-type. Compared with wild-type, the mutant showed 75 % higher vitamin C content, 39 % higher total soluble solids content, and 12 % lower soluble sugar content. The stomatal density was higher in compound leaves than in simple leaves. Compound leaves contained six chloroplasts per guard cell while only five in simple leaves. The degree of stomatal opening was greater in compound leaves. Compared with simple leaves, compound leaves showed thinner and looser vascular bundles and phloem cells, smaller petiole diameter, and higher density of parenchyma cells. A sequence-related amplified polymorphism analysis showed that ethyl-methane sulfonate induced DNA mutations at several loci.

Key words: Radish, mutant, compound leaf, microstructure, SRAP

Introduction

Leaves of seed plants can be classified as either simple or compound, depending on their degree of complexity (Sattler and Rutishauser, 1992). Although significant progress has been made in understanding the mechanisms that regulate simple leaf development, those that regulate compound leaf development are poorly understood (Brand *et al.*, 2007). Research on the molecular mechanisms of compound leaf development began in the late 1990s. Expression of *KNOX* in tomato, *Arabidopsis*, tobacco, cotton, poplar, and dandelion resulted in leaf variations, some even changed leaves from simple to compound (Barth *et al.*, 2009). Results of Peng *et al*. (2011) showed that KNOX protein took part in leaflet development of *Medicago Truncatula*. Shani proposed that the role of *KNOX* was to delay leaf maturity (Shani *et al.*, 2009). *ARP*, another important gene responsible for development of compound leaf was expressed in compound phyllopodia, and participated in the morphogenesis of compound leaves (Kim *et al.*, 2003). *KNOX* and *ARP* genes function antagonistically with each other in the development of simple leaf, however they co-express in compound leaves. Overlapping expression of ARP and *KNOX*1 in phyllopodium was an important character for formation of compound leaf (Nishii *et al.*, 2010). Besides *KNOX* and *ARP, NAM/CUC*3 gene family was also important in development of compound leaf (Blein *et al.*, 2008). The separation of edge of leaf blade was associated with expression of these genes. The further studies demonstrated that number of leaflets decreased when these genes expressions were minimized (Blein *et al.*, 2008). Wang pointed out that CUC2-like gene took part in partition of leaf blade of *Lotus japonicus*, and multiple components were integrated to determine the complexity of leaf in *Lotus japonicus* (Wang *et al.*, 2013). Recent research indicated that Trifoliate (Tf) gene encoded an MYB transcription factor that modulated leaf and shoot architecture in tomato (Naz *et al.*, 2013). Brassinosteroid (BR) is one of the auxins. The recent research showed that BR influenced the development of compound leaf. BR was important for formation of leaf blade boundary. Activation of BR signaling repressed *CUP-SHAPED COTYLEDON(CUC)* gene expression and caused organ fusion phenotypes (Gendron *et al.*, 2012). Caño-Delgado indicated that BR played an important role in formation of blade boundary and was down regulated by lateral organ boundaries (LOB). The activated BR could inhibit expression of genes responsible for lateral organ fusion 1 (LOF1) and CUC in phyllopodium (Caño-Delgado and Blázquez, 2013). Besides above genes, hormones, including cytokinins and auxins, also played important roles in development of compound leaf through regulating expressions of *KNOX*, *STIP,* and *WU* (Anna and Wu, 2011; Bartrina *et al.,* 2011; Skylar and Wu, 2011).

Generally, leaf of radish is characterized with either pinnate or entire leaf edge, and both types of leaves are simple (http: //www.shucaiyuan.com/Technology/43/15592.shtml). Radish mutants with compound leaves were obtained by ethyl-methane sulfonate (EMS) induction during breeding. The mutants showed compound and simple leaves on the same plant (Fig. 1). This trait is easy to identify at the seedling stage, it may be a good marker for hybrid seed production in radish. Furthermore, the presence of compound and simple leaves on the same plant makes these lines interesting materials for research on development of compound leaf. We analyzed the physiological and microstructural characteristics of one of the mutants, and examined the mutation by sequence-related amplified polymorphism (SRAP) marker.

Materials and methods

Plant materials: Mutants that produced compound leaves as well as simple leaves were obtained by treatment of EMS (Fig. 1). Seeds of wild-type (control, simple leaves) and mutants were selected and sown in the field at Henan University of Science and Technology, China, in 2010 and 2011. Plants were irrigated every

2 days and fertilized (N : P_2O_5 : K_2O = 1 : 0.6 : 0.6) at a rate of approximately 50 kg N ha^{-1}.

Determination of net P_n and Chl content: We used the mutant with two leaflets (mutant 1) in this study. Net P_n was determined with a LI-6400 portable photosynthesis system (LI-COR Biosciences, Lincoln, NE, USA). Chl content was measured by following method. Approximately 0.2 g leaf material was homogenized in 80 % acetone at 4°C. The homogenate was centrifuged and fluorescence was measured at 652, 663 and 645 nm with a fluorescence spectrophotometer (Model 751, Shanghai Precision Scientific Instrument Co. Ltd, China).

Measurement of agronomic characters of the radishes: Plant height, plant width, leaf area, leaf number, root length, root diameter, and root weight were measured when plants were three months old.

Measurement of quality traits of the radishes: Fresh radishes were sampled to measure quality traits. VC content was determined by 2, 6-dichloro-indophenol titration. Soluble protein content was determined spectrophotometrically using BSA as the standard. The soluble sugar content was determined by anthracenone method. All experiments were carried out according to the methods of Zhang *et al.* (2007). TSS was determined on juice using a hand held refractometer (ATC-1 Atago, Tokyo, Japan) with automatic temperature compensation.

Observation of microstructure of the radishes: The epidermis was carefully peeled from the leaf, placed on a slide, stained with 1 % I_2-KI for 20 min, and then observed under an Olympus BX-51 microscope. Stomatal density was expressed as number mm^{-2}.

Samples were fixed in 10 % formalin for 24 h at 25 °C. Then, samples were dehydrated in 70 % ethanol for 1 h (3 times); 80 % ethanol for 1 h; 95 % ethanol for 1 h; a mixed solution (100 % ethanol : xylene = 1 : 1) for 1 h; and then in xylene for 1 h. The dehydrated samples were embedded in paraffin wax and cut into 5 µm sections with a microtome. The sections were placed in paraffin ribbon in a water bath at 40-45 °C. Sections were mounted on slides, dried for 10 min, and then stained with 1 % safranin. The slides were covered with a cover slip, mounted with Canada gum, and dried at 40 °C. The slides were examined under an Olympus BX-51 microscope.

SRAP analysis of the radishes: Total DNA was extracted as described by Wang *et al.* (2008). Bulked DNA pools from mutant and wild-type radish were constructed with DNA from 15 individual plants mixed in equimolar quantities.

Sequences of SRAP primers were designed according to Li and Quiros (2001) and synthesized by Shanghai CASB Biotechnology Co., Ltd (Shanghai, China). The PCR protocols were adopted from Li and Quiros (2001). Amplified PCR products were separated by 3 % (w/v) agarose gel electrophoresis and visualized by EB staining.

Statistical analysis: All data were statistically analyzed by one-way ANOVA, followed by Tukey's test, using SPSS 10 statistical software. Differences were considered significant at $P \leq 0.05$.

Results

Characters of mutants: Mutants with compound leaf were induced with EMS. The compound leaf has two leaflets on one petiole. The wild-type had only simple leaves, with one leaflet per petiole (Fig. 1). The mutants had only one compound leaf, the rest of the leaves on the plant was simple, like those of wild-type. In this study, we analyzed the characteristics of mutant.

Photosynthetic characteristics of the mutant: Net photosynthesis of the compound leaf radish was approximately 30 % higher than that of a simple leaf radish. The total Chl content was about 36 % higher in radish with compound leaf than in with a simple leaf. There were significant differences in Chl *a* content, but no differences in Chl *b* content, between compound leaf mutant and wide type (Table 1). These results suggested that increase in photosynthesis in this mutant relied mainly on Chl *a*.

Agronomic characteristics of the mutant: There were no differences in leaf number, leaf area, plant width, root length, and root diameter between the wild-type and the mutant. However, there was a significant difference in fresh root weight, with that of the mutant being only half that of the wild-type (Table 2).

Quality characteristics of the mutant: The VC content was approximately 75 % higher in the mutant than in the wild-type, suggesting that this mutant was a suitable resource for improvement of VC content in radish. The mutant showed 39 % higher in TSS content, 12 % lower in soluble sugars content than the wild type (Table 3).

Characteristics of guard cells of the mutant: There were no significant differences in the size of guard cells between compound and simple leaves. The stomatal density of compound leaf was (512±15 mm^{-2}), while it was (451±22 mm^{-2}) in simple leaf (Fig. 2A, B). The former is about 13.53 % higher than the latter. The number of chloroplasts per guard cell also differed. These were six per cell on average in compound leaves while only five per cell in simple leaves. The former is about 20 % higher than that of the latter. The stomatal aperture was significantly different between compound leaf and simple leaf (Fig. 2C, D), the former was 2.13±0.11 µm, while the latter was 1.52±0.13 µm on the average.

Table 1. Photosynthetic characteristics of wild and mutant radish

Radish	Leaf	Pn µmol. (m^{-2} s^{-1})	Chla content (mg g^{-1})	Chlb content (mg g^{-1})	Chla+b (mg g^{-1})	Carotenoids (mg g^{-1})
Wild type	Simple leaf	14.53±1.43a	1.81±0.12a	2.33±0.42a	4.14±0.23a	0.55±0.01a
Mutant	Compound leaf	20.67±1.22b	3.54±0.21b	2.41±0.31a	5.95±0.31b	0.51±0.02a

Table 2. Agronomic characters of wild and mutant radish

Radish	Leaf number	Leaf area (cm^2)	Plant height (cm)	Plant width (cm)	Root length (cm)	Root diameter (cm)	Root weight (g)
Wild type	8.58±1.01a	323.35±2.3 5a	14.32±1.21a	21.67±1.35a	19.43±2.32a	6.05±0.29a	850.98±23.36b
Mutant	8.26±1.12a	345.52±3.25ab	15.19±1.30a	22.95±1.86a	15.64±2.21a	5.42±0.58a	426.23±31.68a

Table 3. Quality characters of the radishes

Radish	Vitamin C content (mg g^{-1})	Soluble sugar content (mg g^{-1})	Soluble protein content (mg g^{-1})	TSS %
Wild type	47.56±2.34a	9.20±0.12b	3.57±0.08a	8.90±0.22a
Mutant	83.23±3.87b	8.13±0.16a	3.65±0.11a	12.36±0.35b

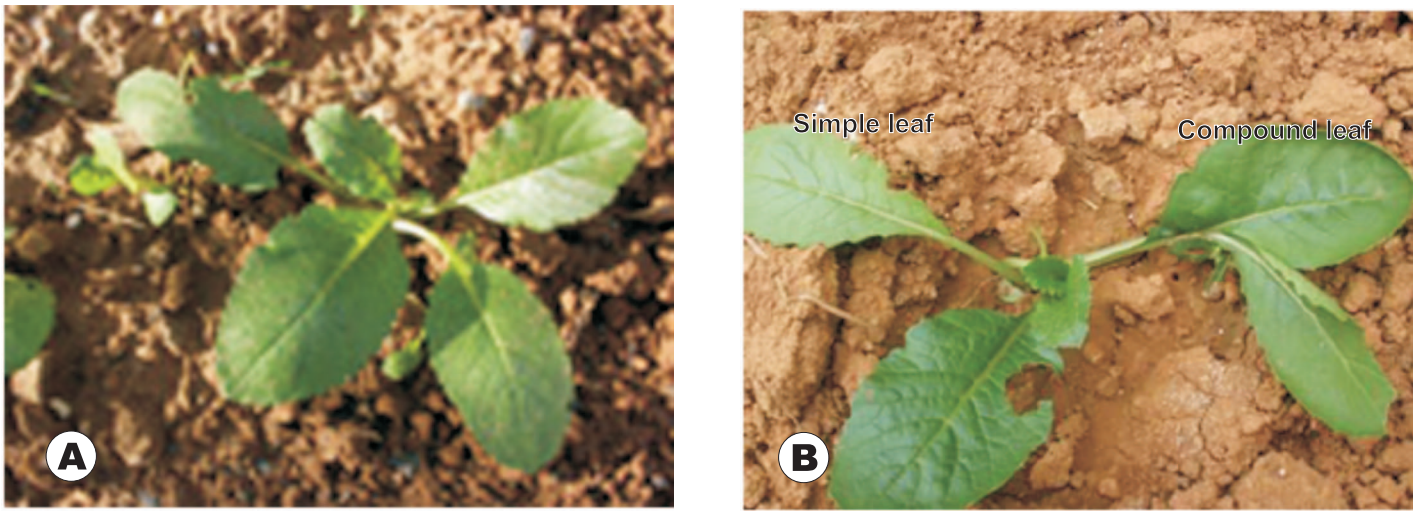

Fig. 1. Mutant and wild-type radish plants (after 30 days of sowing). A: Wild-type; B: Mutant

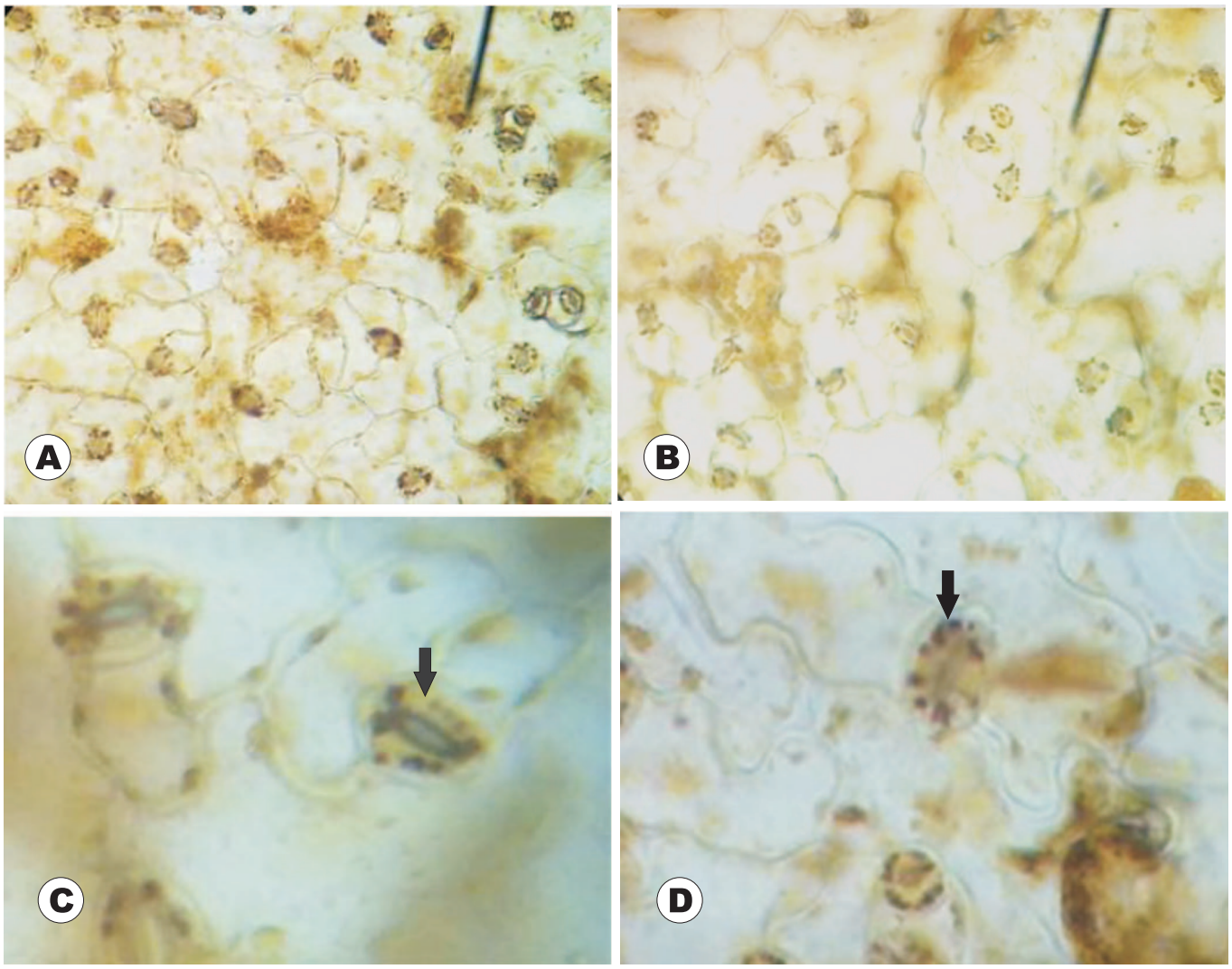

Fig. 2. Stomatal characteristics of compound and simple leaves. A: Stomatal density (compound leaf); B: Stomatal density (simple leaf); C: Stomatal aperture (compound leaf); D: Stomatal aperture (simple leaf).

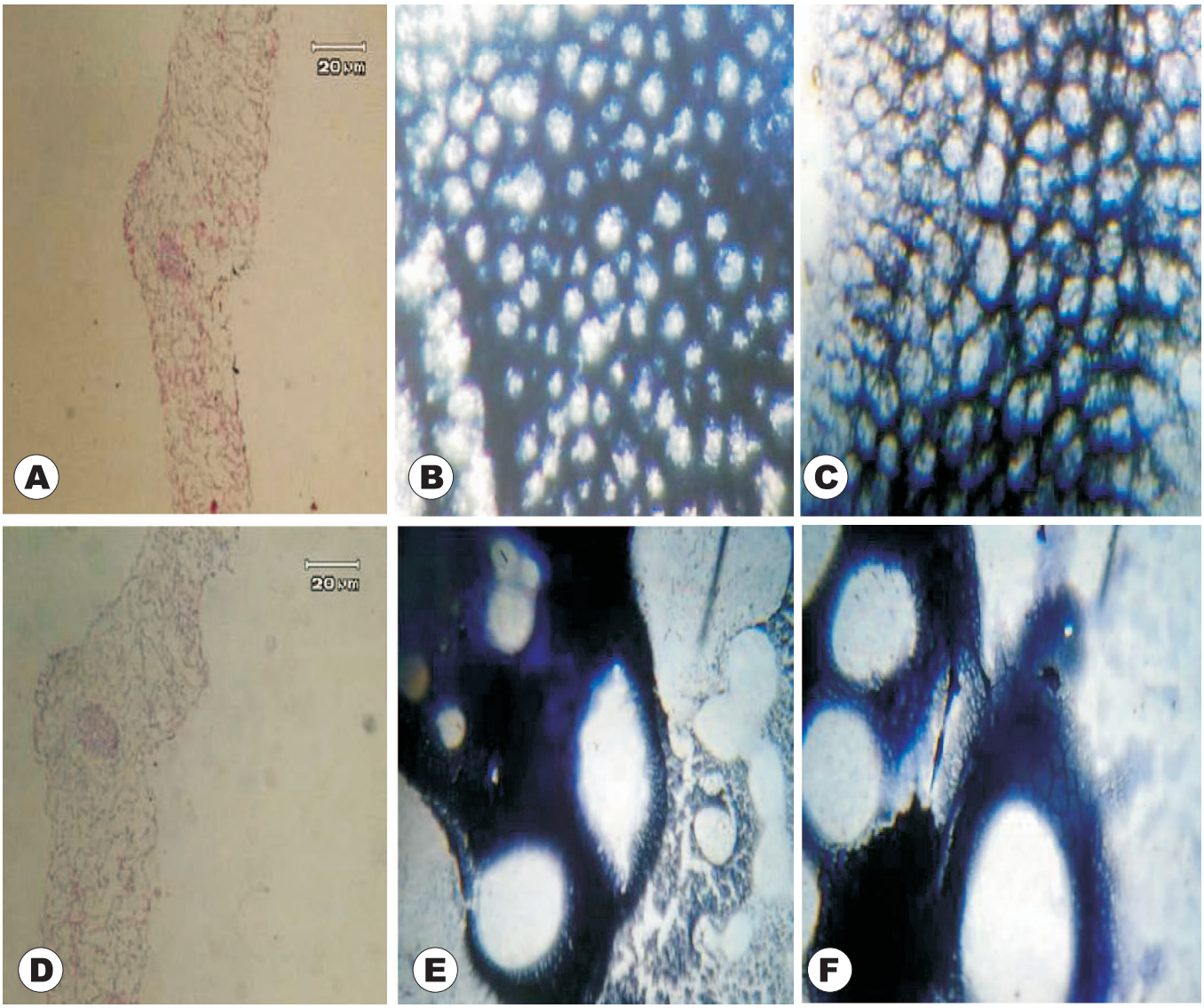

Fig. 3. Microstructural characteristics of simple and compound leaf. TS or VS of leaf and petiole. A: Vascular bundle in compound leaf; B: Phloem in simple leaf; C: Phloem in compound leaf; D: Vascular bundle in simple leaf; E: Petiole in compound leaf (cross section); F: Petiole in simple leaf (cross section)

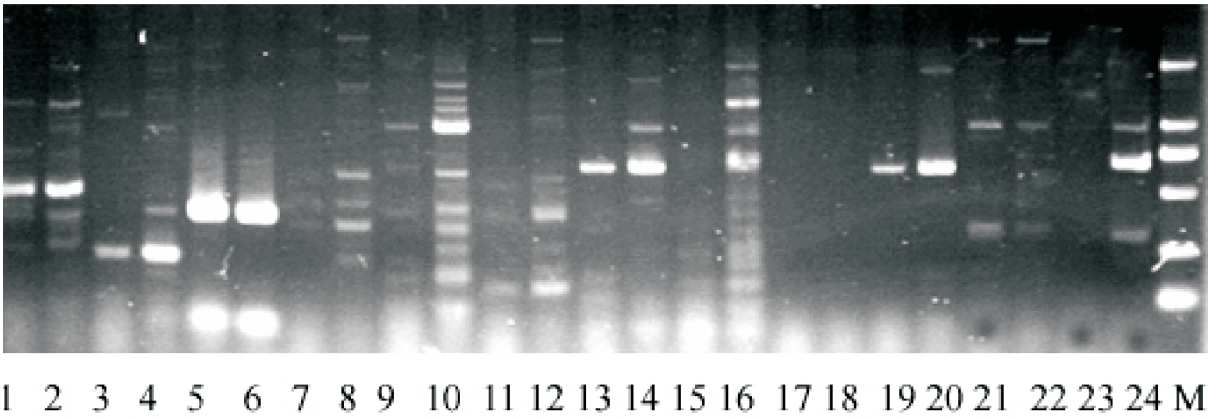

Fig 4. SRAP analysis of mutant and wild-type radish. 1.3.5.7.9.11.13.17.19.21.23: Mutant; 2.4.6.8.10.12.14.16.18.20.22.24: Wild-type; M: marker

Microstructural characteristics of the mutant: Vascular bundles and phloem cells were thinner, looser, and more regular in the simple leaf than those in compound leaf (Fig. 3A-D). The diameter of the petiole in compound leaves was about 2.23±0.15 mm, which was smaller than that in simple leaves, about 3.16±0.23 mm (Fig. 3E, F). These results suggested that compound leaves may have a lower ability to transport water, mineral nutrients, and photosynthates compared with that of simple leaves.

SRAP analysis of the mutant: Twelve pairs of SRAP primers were used to analyze polymorphisms between the mutant and

wild-type. Eight pairs of primers showed polymorphisms (Fig. 4). In total, 105 bands were produced, 34 of which were polymorphic. This suggested that there were mutations at several loci. In other words, the compound leaf character must be controlled by more than one gene. The next step is to identify and clone these genes to clarify the mechanisms of compound leaf development.

Discussion

Leaves can be simple, compound, or one of numerous intermediate forms.The development of plant leaf can be roughly divided into three continuous phases: leaf initiation, organogenesis, and histogenesis (Holtan and Hake, 2003). The special attributes that characterize monocotyledonous leaves have led to morphological interpretations like the phyllode theory, leaf base theory, and the unifacial concept. All of them aimed to interpret monocotyledonous leaves in terms of dicotyledonous leaves and to establish morphological differences between them. The mutant characterized in the present study showed simple and compound leaves existed on the same plant, which may suggest that formation of simple and compound leaves occured via essentially the same developmental process, as suggested by Bharathan *et al.* (2002). Besides morphology, many researches focused on molecular mechanisms of development of compound leaf. Up to now, several key genes and genetic regions involved in the control of leaf shape/size have been identified, including microRNA-regulated genes (Usami *et al.*, 2009), ribosome-related genes (Fujikura and Horiguchi, 2009), and a chromosomal segment (Horiguchi *et al.*, 2009). In recent decades, studies on development of compound leaf have focused on two model plants, tomato and pea. There were two key gene families involved in the development of compound leaf, the *KNOX* and *ARP* gene families (Nishii *et al.*, 2010). Many studies have sought to determine the roles and regulation of these two interesting gene families (Barth *et al.*, 2009; Shani *et al.*, 2009; Nishii *et al.*, 2010; Peng *et al.*, 2011). However, many important points are still to be addressed. There may be other gene families involved in regulating development of compound leaf. The SRAP analysis in this study indicated that mutations at several genetic loci contributed to the leaf phenotype. Further research is required to determine whether these genetic loci contained *KNOX* family genes, *ARP* family genes, or both, or novel genes.

All types of Chl molecules function as light-harvesting pigments. Cells with higher Chl contents could collect and transfer more light energy (Wang *et al.*, 2008). Consequently, they showed higher photosynthetic efficiency and often, higher yield as well. However, we observed that the mutant had much higher Chl content, especially Chl *a* content. With higher Chl content, photosynthates will be higher, thus the weight will be higher, too. However the results showed that root weight of the mutant was only half of the wild-type. Why does this happen? What was the fate of the photosynthates? Further research is required to answer this question. Nevertheless, the results showed that there was not a simple, positive relationship among Chl content, photosynthesis, and economic yield. The complexity of this relationship has become a problem for plant breeding programs. Therefore, in addition to being an interesting research material for study on compound leaf development, this mutant will also be useful to study the relationships among Chl content, photosynthesis, and yield.

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References

Anna, S. and X.L Wu, 2011. Regulation of meristem size by cytokinin signaling. *J. Integr Plant Biol.,* 56: 446-454.

Barth, S., T. Geier, K. Eimert, B. Watillon, R.S. Sangwan and S. Gleissberg, 2009. *KNOX* overexpression in transgenic *Kohleria* (*Gesneriaceae*) prolongs the activity of proximal leaf blastozones and drastically alters segment fate. *Planta,* 230: 1081-1091.

Bartrina, I., E. Otto, M. Strnad, T. Werner and T. Schmulling, 2011. Cytokinin regulates the activity of reproductive meristems, flower organ size, ovule formation, and thus seed yield in *Arabidopsis thaliana*. *Plant Cell,* 23: 69-80.

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

Blein, T., A. Pulido, A. Vialette-Guiraud, K. Nikovics, H. Morin, A. Hay, I.E. Johansen, M. Tsiantis and P. Laufs, 2008. A conserved molecular framework for compound leaf development. *Science,* 322(5909): 1835-1839.

Brand, A., N. Shirding, S. Shleizer and N. Ori, 2007. Meristem maintenance and compound-leaf patterning utilize common genetic mechanisms in tomato. *Planta,* 26: 941-951.

Caño-Delgado, A.I. and M.A. Blázquez, 2013. Spatial control of plant steroid signaling. *Trends Plant Sci.,* 18(5): 235-236.

Fujikura, U.G. and M.R. Horiguchi, 2009. Coordination of cell proliferation and cell expansion mediated by ribosome-related processes in the leaves of *Arabidopsis thaliana*. *Plant J.,* 59: 499-508.

Gendron, J.M., J.S. Liu, M. Fan, M.Y. Bai, S. Wenkel, P.S. Springer, M.K. Barton and Z.Y. Wang, 2012. Brassinosteroids regulate organ boundary formation in the shoot apical meristem of Arabidopsis. *Proc. Natl. Acad. Sci. USA,* 51: 21152-2157.

Holtan, H.E. and S. Hake, 2003. Quantitative trait locus analysis of leaf dissection in tomato using *Lycopersicon pennellii* segmental introgression lines. *Genetics*, 165: 1541-1550.

Horiguchi, G., N. Gonzalez, G.T.S. Beemster, D. Inze and H. Tsukaya, 2009. Impact of segmental chromosomal duplications on leaf size in the grandifolia-D mutants of *Arabidopsis thaliana*. *Plant J.,* 60: 122-133.

Kim, M., S. McCormick, M. Timmermans and N. Sinha, 2003. The expression domain of *PHANTASTICA* determines leaflet placement in compound leaves. *Nature,* 424: 438-443.

Li, G. and C.F. Quiros, 2001. Sequence-related amplified polymorphism (SRAP), a new marker system based on a simple PCR reaction: its application to mapping and gene tagging in Brassica. *Theor. Appl. Genet.,* 103: 455-461.

Naz, A.A., S. Raman, C.C. Martinez, N.R. Sinha, G. Schmitz and K. Theres, 2013. Trifoliate encodes an MYB transcription factor that modulates leaf and shoot architecture in tomato. *Proc. Natl. Acad. Sci. USA,* 6: 2401-2406.

Nishii, K., M. Möller, C. Kidner, A. Spada, R. Mantegazza, C.N. Wang and T. Nagata, 2010. A complex case of simple leaves: indeterminate leaves co-express *ARP* and *KNOX1* genes. *Dev. Genes. Evol.,* 220: 25-40.

Peng, J.L., J.B. Yu, H.L. Wang, Y.Q. Guo, G.M. Li, G.H. Bai and R.J. Chen, 2011. Regulation of compound leaf development in *Medicago Truncatula* by fused compound leaf1, a class M KNOX gene. *Plant Cell,* 11: 3929-3943.

Sattler, R. and R. Rutishauser, 1992. Partial homology of pinnate leaves and shoots: orientation of leaflet inception. *Bot. Jahrb. Syst.*, 114: 61-79.

Shani, E., Y. Burko, L. Ben-Yaakov, Y. Berger, Z. Amsellem, A. Goldshmidt, E .Sharon and N. Ori, 2009. Stage-specific regulation of *Solanum lycopersicum* leaf maturation by class 1 *KNOTTED1-LIKE HOMEOBOX* proteins. *Plant Cell,* 21: 3078-3092.

Skylar, A. and X.L. Wu, 2011. Regulation of meristem size by cytokinin signaling. *J. Integr. Plant Biol.,* 6: 446-454.

Usami, T., G. Horiguchi, S. Yano and H. Tsukaya, 2009. The more and smaller cells mutants of *Arabidopsis thaliana* identify novel roles for *SQUAMOSA PROMOTER BINDING PROTEIN-LIKE* genes in the control of heteroblasty. *Development,* 136: 955-964.

Wang, F.H., G.X. Wang, X.Y. Li, J.L. Huang and J.K. Zheng, 2008. Heredity, physiology and mapping of a chlorophyll content gene of rice (*Oryza sativa* L.). *J Plant Physio.,* 165: 324-330.

Wang, Z., J. Chen, L. Weng, X. Li, X. Cao, X. Hu, D. Luo and J. Yang, 2013. Multiple components are integrated to determine leaf complexity in *Lotus Japonicus*. *J. Integr. Plant Biol.,* 55(5): 419-433.

Zhang, L.J. and J.J. Fan, 2007. *Experiments for Plant Physiology*. Beijing: China Agricultural University Press (in Chinese)

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