



## Analysis of ROP signaling in the leaf epidermis of mutant tomato with low-energy ion beam

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**ABSTRACT.** The importance of the ROP small GTPase signaling pathway in the regulation of cellular polarity growth in eukaryotes has been thoroughly studied. In this study, we examined the LeROP small GTPase (related to *Arabidopsis thaliana* genome LeROP GTPase in tomato) signaling of cell polarity growth in the mutant (M-1) tomato. Interestingly, we detected expansive growth of epidermis cells in M-1, in which the leaves appeared slightly lobed shaped. However, we observed jigsaw puzzle shaped and deeply lobed shaped leaves in wild-type leaf epidermis cells. The *t*-test showed significant difference ( $P < 0.05$ ). Based on previous studies of the AtROP gene in *Arabidopsis* leaf epidermis cells, we hypothesized that the growth of mutant M-1 tomato leaf epidermis cell is related to AtROP gene signal transmission. The results of reverse transcription-polymerase chain reaction showed the expression of LeROP2, LeROP4, and LeROP7 in M-1 mutants

were stronger than in wild-type cells. At the flowering stage, LeROP2 GTPase showed no expression in wild-type cells, but was expressed in mutant cells. This study revealed a link between the low-energy ion beam and the ROP GTPase signaling pathway in tomato. In addition, the ROP gene changes analyzed suggest a new mechanism for mutations following low-energy ion beam implantation.

**Key words:** Mutant; ROP signaling

## INTRODUCTION

Tomato (*Lycopersicon* Mill.) can be an annual or perennial herb plant and is a model plant for studying plant biology. In this study, LeROP small GTPase signaling was examined in a mutant tomato induced using a low-energy ion beam. Low-energy ions exist ubiquitously in the natural world. In the mid-1980s, a low-energy ion beam was used to improve agricultural crop varieties because of the ability of ion implantation to modify material surfaces. Low-energy ion beam implantation has been applied to the breeding of crops and microbes (Wu et al., 1990; Yu et al., 1998; Yamaguchi et al., 2003; Liang et al., 2008). Etching and damage of cells in organisms is known to occur with the use of low-energy ions implantation. The bio-effects and the preliminary mechanisms of low-energy ion beam irradiation on *Medicago truncatula* had been investigated, including dismutase, catalase, and peroxidase, which affect reactive oxygen species (Chen et al., 2010). Qian et al. (2010) and Yu et al. (2011) observed changes in DNA methylation for mutations induced by low-energy ion implantation. Phanchaisri et al. (2012) reported that changes of OsSPY and 14-3-3 expression could affect internode elongation and cause the phenotypic changes of semidwarf and spindly rice mutants by ion beam induced. Little is known regarding the mechanism of the cell polarity signaling pathway and the effect of low-energy ion beam implantation on living organisms. ROP GTPase is a plant-specific ROP subfamily which regulates cell morphology, plant development, and responses to the environment (Zheng and Yang, 2000; Fu et al., 2001; Yang, 2002). In this study, we examined the effect of low-energy ion beam implantation on LeROP GTPase signaling in a tomato mutant (M-1).

## MATERIAL AND METHODS

### Materials and plant conditions

M-1 tomato seeds were selected from the progeny generation by the low-energy ion beam implantation tomato seeds. These seeds self-pollinate and have stable genetic characteristics [slender cotyledon and deeply lobed true leaves with thin petals and fruits such as lemon with thick flesh (Liang et al., 2008)] over many generations. These seeds were sowed on a seedbed with humus, followed by transplantation of the seedlings to the growing field at the Zhengzhou city vegetable institute experimental field. The true leaves of different growth periods were collected for subsequent experiments. The wild-type was Henan No.4 tomato.

### Analysis of cell biology and data statistics

When 4 true leaves had developed in the experimental field, we picked up the leaves

and cleared the soil of their surface. Photos were acquired using a Nikon digital camera (S620, Tokyo, Japan). Cell morphology was observed using a confocal laser scanning microscope with water sealing and the ImageJ software (NIH, Bethesda, MD, USA) was used for statistical analysis.

### Bioinformatic analysis

According to the *Arabidopsis* Information Resource (TAIR), 11 ROP GTPase genes have been reported and the relevant nucleotide sequences of ROP GTPase genes in the tomato genome were identified using BLAST from the National Center for Biotechnology Information. The sequence alignment and orthology and phylogenetic trees were analyzed using ClustalW and DNAMAN. Forward and reverse primers were designed for reverse transcription-polymerase chain reaction (RT-PCR) using Primer Premier 5.0 (Palo Alto, CA, USA).

### Gene expression analysis

#### *Extraction of total RNA and synthesis first-strand cDNA*

Total RNA was isolated from different tomato tissues with TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer protocol. First-strand cDNAs were synthesized using TaKaRa Reverse Transcription M-MLV (Rnase H-) reagents. The 10- $\mu$ L reaction contained 5  $\mu$ M oligo-dT primers, 1  $\mu$ g total RNA, 1 U/ $\mu$ L RNase inhibitor, 0.5 mM dNTP mixture, and 5 U/ $\mu$ L RTase M-MLV (RNase H-). Reverse-transcription reactions were carried out at 42°C for 60 min and were terminated by heating to 72°C for 15 min and cooling to 10°C for 5 min.

#### *RT-PCR*

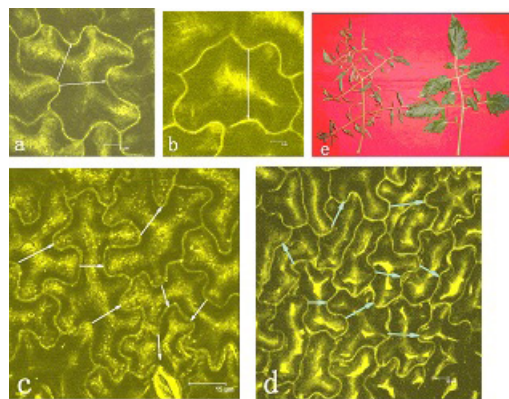
RT-PCR analysis was carried out in Biometra TGradient PCR (Goettingen, Germany). The 25- $\mu$ L reaction contained 2 mM MgCl<sub>2</sub>, 0.25 U *Taq* polymerase (TaKaRa, Shiga, Japan), and 0.5  $\mu$ M gene-specific primers. The reaction conditions were as follows: initial denaturation at 94°C for 4 min for 35 cycles; denaturation at 94°C for 30 s, annealing at 45-65°C for 30 s, extension at 72°C for 45 s, and final extension at 72°C for 5 min. LeROP primers were designed according to the 11 ROP genes in the *Arabidopsis* genome. The  $\beta$ -actin primers were used as internal reference primers to evaluate the quality of first-strand cDNA. The following primers were used for RT-PCR:  $\beta$ -actin (F: 5'-ATGGCAGACGGAGAGGATATTCA-3' and R: 5'-GCCTTTGCAATCCACATCTGCTG-3'), ROP1 (F: 5'-TGGTTGTCAATGGAGC-3' and R: 5'-TCTTTGTCTTGGGAGG-3'), ROP2 (F: 5'-CGTGTCTCTTGATTTCTTAC-3' and R: 5'-CTTTTCTTCTGCTTGGGTG-3'), ROP3 (F: 5'-ACCAGCAACACTTTTCCC-3' and R: 5'-TAAGCAGCAGCACAATC-3'), ROP4 (F: 5'-CGTGTCTCTTGATTTCTTAC-3' and R: 5'-CTTTTCTTCTGCTTGGGTG-3'), ROP5 (F: 5'-ATGTGGTTGTCAATGGAGC-3' and R: 5'-TTTCTTGTCTTGGGAGG-3'), ROP6 (F: 5'-ACCAGCAACACTTTTCCC-3' and R: 5'-TAAGCAGCAGCACAATC-3'), ROP7 (F: 5'-TTGTCAATGGGGCTACTGTC-3' and R: 5'-CTTTTCTTTCGCTTGGGTG-3'), ROP8 (F: 5'-GTCTTCTCATCTCCTACAC-3' and R: 5'-GCACTCAACATAAGCCACT-3'), ROP9 (F: 5'-TTCCCTACTGACTATGTTCC-3' and R: 5'-GTGCTTCTTGCCTTTTCTT-3'), ROP10 (F: 5'-TGTTACTGTTGGTGATGG-3'

and R: 5'-TTCTGCTGAGTCTTGGAG-3'), ROP11 (F: 5'-CTGTAGGCGATGGTG-3' and R: 5'-TTGGGCAGGTGTTAT-3'). RT-PCR products were electrophoresed on a 1% agarose gel at 120 V and stained with ethidium bromide (10 mg/mL). Bands were visualized and photographed using a Shanghai Tianneng GEL imaging system (Shanghai, China).

## RESULTS

### Cytological analysis

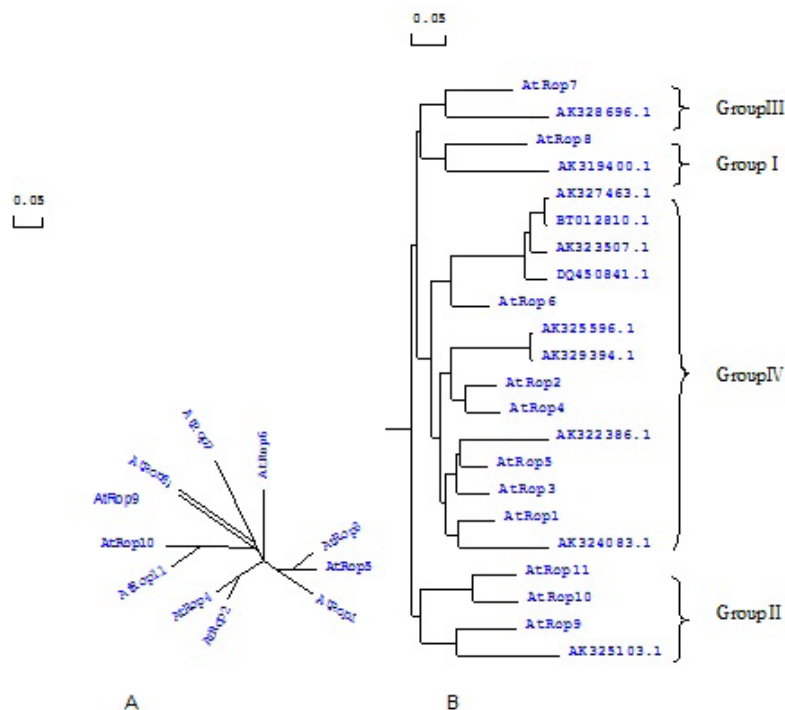
At the development stage with 4 true leaves, the morphology of the leaf epidermal cells were observed. The edges of the cell in the M-1 mutant expressed blunt, lobed shape edges, in contrast with the lobe shaped cells in wild-type plants. Statistical analysis revealed the average diameter of the cell lobe position in M-1 mutant leaves was much longer than that of wild-type ( $P < 0.05$ ). These differences were very significant (Figure 1).



**Figure 1.** Morphologic observation of the true leaf epidermal cell (a, c: wild-type; b, d: mutant; e: the shape of the true leaf in M-1 mutant (left) and wild type (right); the arrows show the position of ear crack produced).

### Bioinformatic analysis

Based on AtROP GTPase genes in the *Arabidopsis* database (<http://www.tigr.org/tdb/e2kl/ath1>), the relevance of the nucleotide sequence was searched in the tomato genome. Additionally, we constructed phylogenetic trees using the DNAMAN software (Figure 2A). Phylogenetic analysis revealed that the consistency of 11 ROP genetic sequences was 49.06% in the *Arabidopsis thaliana* genome, and we identified highly repetitive base sequences, including TGTGT, GATGG, GATTATGT, AGTGC, AATGT, TGGGAT ACTGC, GATGT, AAGTGGA, and GATCT, among others. AtROP GTPase genes can be divided into 4 sub-groups based on overall sequence homology and sequence divergence in the *Arabidopsis* genome, including group I AtROP8 (AT2G44690.1), group II were AtROP9-11 (AT4G28950.1, AT3G48040.1, AT5G62880.1), group III was AtROP7 (AT5G45970.1), group IV were AtROP1-6 (AT3G51300.1, AT1G20090.1, AT2G17800.1, AT1G75840.1, AT4G35950.1, AT4G35020.1) (Figure 2A).



**Figure 2.** Phylogenetic trees of Rop genes in *Arabidopsis* and tomato. **A.** Phylogenetic trees of AtRop GTPase gene in *Arabidopsis* genome. **B.** Phylogenetic tree of LeRop GTPase gene in tomato and *Arabidopsis* genome.

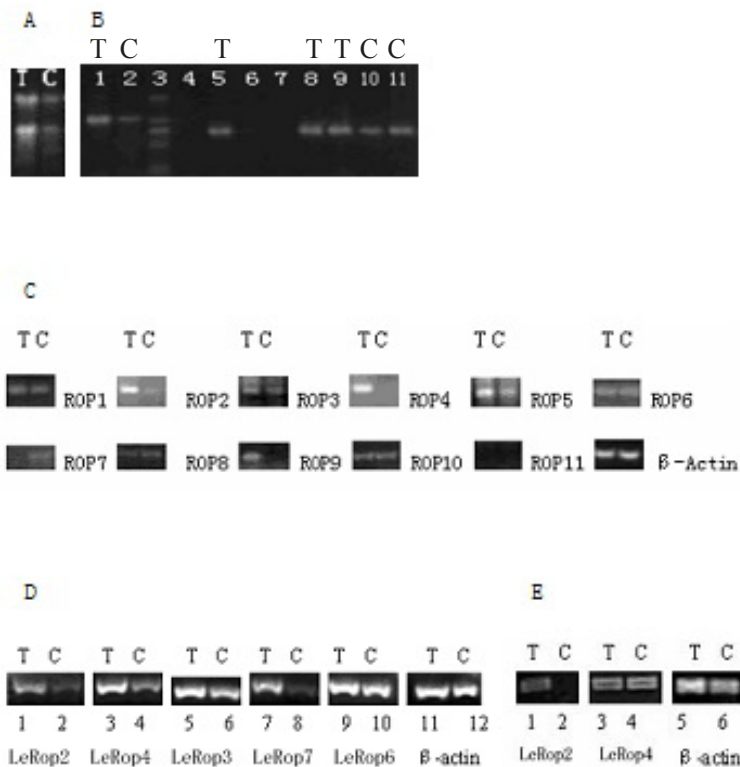
Analysis of the nucleotide sequences of ROP genes indicated that the Rac/ROP gene showed higher homology in all types of plant genera, reaching higher than 79%. In this study, the relevant homology base sequences of 11 ROP genes were acquired for the tomato genome according to sequence homology and structure domain from GenBank using the BLAST program for searching. Sequence alignment of ROP genes showed that the nucleotide sequences of most genes examined were homologous to the ROP gene. The similarity was at least 72%. Based on these results, we suggest that ROPs in the tomato genome should be referred to as LeROP GTPases. We aligned the ROP gene of *Arabidopsis* with the tomato genome and constructed phylogenetic trees (Figure 2B) using the DNAMAN software. In the tomato genome, there were 3 genes, AK319400.1, AK325103.1, and AK328696.1, related to AtROP8, 9, and 7, respectively, which belong to *Arabidopsis* groups I, II, and III. There were 2 genes (AK329394.1 and AK325596.1) related to AtROP1, 4 genes (AK327463.1, BT012810.1, AK323507.1, and DQ450841.1) related to AtROP6, 1 gene (AK324083.1) related to AtROP1 and AK322386, and 1 gene related to AtROP5 and AtROP3. These results revealed that ROP signaling is ubiquitously present in vegetables, which is very important for studying plant development.

We aligned nucleotide sequences from the tomato genome with those from the *Arabidopsis* genome and found 43.32% similarity. Some nucleotide sequence were the same in both organisms (AATGT, TGGGA, AAGTGG, and TTTGA). The tomato genome showed some similar nucleotide sequences (TGTGT, GATGG, TGTGT, AGTGC, ATGG, TGGGATACT-

GC, CCTGA, AGAA, and TTTGA) that were not present in the *Arabidopsis* genome. Thus, we inferred different proteins were encoded by the tomato and *Arabidopsis* genomes. ROP signaling widely exists in the plant kingdom and shows high species diversity. These results are important for studying species evolution.

## RT-PCR

In this study, we conducted RT-PCR to analyze the response of LeROP signaling in mutant M-1 and wild-type tomato. When 2 leaves were present, the expression levels of the LeROP1, LeROP3, LeROP5, LeROP6, LeROP10 genes were the same, while the expression levels of LeROP2, LeROP4, LeROP7, and LeROP9 differed. The expression level of LeROP2 and LeROP9 were lower in wild-type. The LeROP4 and LeROP7 genes were not expressed in the true leaves of the M-1 mutant. The LeROP11 gene was not expressed in wild-type and M-1 mutant true leaves. LeROP2, LeROP4, and LeROP7 gene expression levels were higher in M-1 than in wild-type when 5-6 true leaves were present. LeROP2 was not expressed in wild-type true leaves during flowering (Figure 3).



**Figure 3.** Analysis of RT-PCR of the LeRop gene in Mutant M-1 and wild-type tomato. **A.** Agarose gel electrophoresis of the extracted total RNA. Lane 3 = marker 2000; lanes 4, 6, and 7 = blank control. **B.** Amplified picture of agarose gel electrophoresis of the first-cDNA. **C.** Expression level of the LeRop GTPase gene at the time of two true leaves. **D.** Expression level of the LeRop GTPase gene at five to six true leaf time. **E.** Expression level of the LeRop GTPase gene at plant flowering time.  $\beta$ -actin was the reference primer. T is mutant M-1. C is wild-type.



## DISCUSSION

Our results indicate that the ROP family (Rho-related protein from plants) is homologous and belongs to the Rho family of small GTPases, which regulate cellular morphogenesis in fungal, insect, and mammalian cells (Miller and Johnson, 1994; Nakano et al., 2002; Park and Bi, 2007; Georgiou and Baum, 2010). ROP small GTPases are cell signaling proteins in plants that regulate cell growth, development, trafficking, auxin transduction, and the stress response system (Li et al., 1998; Zheng and Yang, 2000; Yang, 2002). Cytoplasmic RAC/ROPs are recruited to the cell membrane and activated in response to extracellular signals perceived and mediated by cell surface-located signaling assemblies, followed by transduction of the signals to regulate cellular processes (Zou et al., 2011). Murphy and Peer (2012) showed that PIN endocytosis is controlled by ROP-RIC activity in leaf pavement cells and root cells. Additionally, various studies examined the mechanism mediating cell polarity growth in plant. ROP2 inhibits PIN1 endocytosis via accumulation of cortical actin microfilaments induced by the ROP2 effector protein RIC4 (Nagawa et al., 2012). Chen et al. (2012) reported that the signaling module auxin-ABP1-ROP6/RIC1-clathrin-PIN1/PIN2 is common to the feedback regulation of auxin transport during both root and aerial development. Lin et al. (2013) identified the conserved microtubule-severing protein katanin as a downstream component of the ROP6-RIC1 signaling pathway, which facilitates the well-ordered arrangement of cortical microtubules.

In this study, we observed epidermis cell morphological heteromorphosis in the true leaves of the M-1 mutant tomato induced by low-energy ion beam implantation. Low-energy ion beams interrupt intracellular contents through cell wall or cell membrane modifications and cause a series of internal or external reactions. Various studies have implemented ion implantation and bombardment, and this technique is typically applied in breeding studies, including rice, wheat, flowers, and microbes (Wu et al., 1990; Morishita et al., 2003; Okamura et al., 2003; Yamaguchi et al., 2003; Gu et al., 2006; Liang et al., 2008). Most of these previous studies only observed variations in the external shape of organisms for improving biomass yield. There have also been some reports examining retrotransposons, mitochondria (Chen et al., 2008; Ya et al., 2010), breaking of dormancy and seed germination (Šerá et al., 2009, 2010), and peroxidase expression and activities (Li et al., 2009). Similarly, studies revealed the quantitative and temporal aspects of radiation-induced bystander mutagenesis in WTK1 human lymphoblast cells and suggested low levels of ionizing radiation-induced adaptive response were effective for reducing bystander mutagenesis through subsequent gamma-ray exposures (Zhang et al., 2009). Schettino et al. (2010) reported radiation microbeam (e.g., X-rays, charged particles, and electrons) as highly spatial and temporal probes in subcellular and tissue responses.

There have been no reports regarding the relationship between cell morphology and ROP GTPase signaling with low-energy ion beam irradiation. The epidermal cell morphology of M-1 tomato true leaves showed expanded growth relative to the wild-type tomato. We used RT-PCR to detect morphological changes in leaf epidermal cells caused by molecules similar to ROP small GTPase in *Arabidopsis*. Eleven ROP genes were identified in the *Arabidopsis* genome, which acted as switches in regulating various aspects of plant growth, development, and responses to the internal and external environment. Additionally, we found that ROP signaling networks regulate differences in the time required for tomato growth. There were some differences in LeROP expression levels, particularly in seedling and meristematic tissue between M-1 mutant and wild-type tomato true leaves. These results agree with those of Li et

al. (2001), who reported that ROP proteins are distributed in various tissues during vegetative growth, but preferentially accumulate in meristems and rapidly growing cells, including leaf primordial cells, columella cells, epidermis and cortex cells, mesophyll cells, and vascular tissues in all organs. Georgiou and Baum (2010) found that polarity proteins and Rho GTPases cooperate to spatially organize epithelial actin-based protrusions. In addition, ROP proteins appeared to be less abundant in epidermal and tissues of mature leaves and stems. They were also consistently found in inflorescence meristems, organ primordia, developing ovules, and anthers. In addition, we showed that ROP2 and ROP9 gene expression levels were lower in wild-type tomato true leaves than in the M-1 mutant. Moreover ROP2, ROP4, ROP7, and ROP9 gene expression levels differed at time of 2 true leaves, but ROP4 and ROP7 were absent in the M-1 mutant tomato. The results of Fu et al. (2002) suggested that expression of a dominant-negative mutant for ROP2 inhibited polar cell expansion, whereas expression of a constitutively active mutant caused isotropic expansion in the early phase. In this study, cytological observation revealed expansionary growth in epidermis cells of M-1 mutant tomato true leaves. Li et al. (1998) found 97% amino acid identity in ROP2 and ROP4 in leaves. RT-PCR experimental results of mutants showed that ROP2 expression level increased when 2 true leaves were present, but this was not observed in wild-type. ROP, ROP4, and ROP7 signaling levels were higher than in wild-type when 5-6 leaves were observed. The ROP2 gene was not expressed in the flowering stage in wild-type. In contrast, the ROP2 gene was observed in the M-1 mutant tomato. Thus, the cell morphological characteristics of M-1 true leaves were consistent with the expression of ROP GTPase signaling. Studies demonstrated that the expression of a constitutively active ROP2 mutant in *Arabidopsis* generated an even distribution of fine microfilaments throughout the cell cortex, delayed formation of well-ordered cortical microtubules associated with neck formation of stage II cells, and eliminated interdigitation by increasing expansion in the neck regions (Fu et al., 2002, 2005). A dominant-negative ROP2 mutant inhibited lobe development by preventing fine microfilament formation. In this study, we observed epidermis cell interdigitation by increasing expansion in the neck regions of the M-1 mutant tomato leaf, which was similar in the *Arabidopsis* constitutively active ROP2 mutant. Thus, a close relationship exists between ROP signaling coordination and the morphology of tomato mutant M-1 leaves. However, whether there was direct contact between ROP signaling and low-energy ion beam implantation remains unknown. Our preliminary results show that ROP signaling mainly modulates plant growth, development, proliferation, and differentiation in response to low-energy ion beam implantation stimuli. This study provides reference value for explaining ion beam mutation breeding.

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