1 # Ectopic expression of *Arabidopsis* H^+-pyrophosphatase *AVP1* enhances
2 drought resistance in bottle gourd (*Lagenaria siceraria* Standl.)

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6 **Mehea Park · Jeungsul Han · Yeulkyun Ahn · Jeongho Kim · Hyeeun Lee**

7 **Yoonah Jang Roberto Gaxiola · Kendal D. Hirschi · Sunghun Park**

8

9 M. Park
10 Postharvest Team, National Institute of Horticultural & Herbal Science, Rural
11 Development Administration, Suwon, 440-706, Korea
12

13 Y. Ahn · J. Kim · H. Lee Y. Jang

14 Vegetable Division, National Institute of Horticultural & Herbal Science, Rural
15 Development Administration, Suwon, 440-706, Korea
16

17 J. Han
18 Department of Ecological Environmental Conservation, Kyungpook National University,
19 Sangju, 742-711, Korea
20

21 R. Gaxiola
22 School of Life Sciences (SOLS), Arizona State University, Tempe, AZ 85287-1501
23

24 K D. Hirschi
25 United States Department of Agriculture/Agriculture Research Service, Children's
26 Nutrition Research Center, Baylor College of Medicine, Houston, Texas 77030
27

28 S. Park
29 Department of Horticulture, Forestry and Recreation Resources, Kansas State University,
30 Manhattan, KS 66506, USA
31 Email: shpark@ksu.edu

Tel: 785 532 4412
Fax: 785 532 6949

Abstract

Bottle gourd (*Lagenaria siceraria* Standl.) has been used as a source of rootstock for grafting watermelon to improve its fruit quality. We report here the development of a bottle gourd with resistance to drought by ectopic expression of the *Arabidopsis AVP1* gene that encodes a vacuolar H^+-pyrophosphatase (H^+-PPase). The drought resistance of *AVP1*-expressing and wild-type plants was assessed by growing plants under drought conditions. After 12 days of water deprivation, both *AVP1*-expressing and wild-type plants demonstrated reduced growth. After 10 days of re-watering, wild-type plants showed minimal growth while the *AVP1*-expressing plants resumed rapid growth. Further, *AVP1*-expressing plants displayed longer primary roots and more robust root systems than wild-type plants.

Keywords: Bottle gourd · Drought stress · H^+-pyrophosphatase· Rootstock· Watermelon

Introduction

Various abiotic stresses adversely affect the growth and development of crop species. Among these abiotic stresses, drought is one of the major abiotic stresses affecting vegetables, such as watermelon. Bottle gourd (*Lagenaria siceraria* Standl.) has been used as a rootstock for watermelon to improve its performance in unfavorable soil conditions. Grafting of watermelon onto bottle gourd generally improves fruit quality and resistance to soil-borne diseases of watermelon (Lee and Oda 2003; Han et al. 2009), and the use of this technique is increasing in Europe and the U.S. (Kubota et al. 2008). Thus, it is desirable to improve the performance of bottle gourd rootstocks to enhance water use-efficiency. One strategy to produce improved drought stress in bottle gourd is through transgenic approaches. Bottle gourd transformation is currently inefficient; however, an *Agrobacterium*-mediated transformation system was established for introducing foreign genes into bottle gourd (Han et al. 2004, 2005).

Genetic manipulation of the vacuolar proton (H^+) pumps is a promising approach for generating drought-resistant crops (Gaxiola et al. 2002, 2012). A vacuolar H^+-pyrophosphatase (H^+-PPase) from *Arabidopsis*, AVP1, has been identified as a yield enhancing gene (Gonzalez et al. 2009, 2010) and has shown that overexpression of *AVP1* or other plant *H^+-PPases* in *Arabidopsis*, maize, and cotton improves their drought resistance (Li et al. 2008; Lv et al. 2009; Pasapula et al. 2011). Furthermore, increased root biomass is a consistent phenotype triggered by the overexpression of *H^+-PPases* (Li et al. 2005, 2008; Park et al. 2005a; Yang et al. 2007; Lv et al. 2008, 2009). Although it is still not clear if altered auxin homeostasis is a cause or an effect of the enhanced growth phenotype, *avp1-1* null mutants display severely disrupted root and shoot development and reduced auxin transport (Li et al. 2005), and a recent comparative study of transgenic *Arabidopsis* lines that produce enlarged leaves showed that auxin levels were increased 50% in *AVP1*-overexpressing plants (Gonzalez et al. 2010). Further, *Arabidopsis* overexpressing *AVP1* showed increased auxin polar transport from shoots to roots in seedlings, suggesting that the robust root systems developed by *AVP1*-overexpressing plants are a consequence from an enhanced auxin transport capacity (Li et al. 2005) and therefore could be instrumental to enhance plant drought resistance (Park et al. 2005a). For example, transgenic tomato plants expressing *AVP1* show increased root biomass and enhanced recovery after water deprivation (Park et al. 2005a).

A robust root system is also an essential attribute for bottle gourd used as rootstock for grafting with watermelon or other cucurbits, and ectopic expression of *AVP1* is a promising strategy to increase the vigor of the root system of rootstocks under poor soil conditions. In this study, we aimed to develop a drought resistant bottle gourd by ectopic expression of *AVP1*. We tested the drought resistance of transgenic bottle gourds expressing *AVP1*. We also investigated whether *AVP1*-expressing plants display more robust root systems than wild-type plants. The results demonstrate that transgenic bottle gourd containing *AVP1* is able to quickly recover from drought stress conditions.

1 **Materials and methods**
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3 Plant material and transformation
4
5 The bottle gourd (*Lagenaria siceraria* Standl) inbred line G5 (National Institute of Horticultural and Herbal
6 Science, Korea) was used for transformation. We used the *Agrobacterium tumefaciens* strains GV3101 or
7 LBA4404 carrying p35S::AVP1D (Fig. 1a). The plasmid contains the *AVP1D* gene driven by a tandem
8 repeat of the *35S* promoter of *Cauliflower mosaic virus* (Zhen et al. 1997; Park et al. 2005a). The *AVP1D*
9 gene is an intragenic E229D gain-of-function mutant of the *AVP1* gene, which shows a coordinated increase
10 in both pyrophosphates (PPi) hydrolytic activity and PPi-dependent H^+ translocation (Zhen et al. 1997). The
11 vector also contains the *bar* gene that encodes phosphinothricin acetyltransferase (PAT) and confers
12 tolerance to the herbicide phosphinothricin (PPT: Duchefa Biochemie, Haarlem, the Netherlands).
13 p35S::AVP1D was introduced into cotyledon explants of bottle gourd by *Agrobacterium*-mediated
14 transformation methods as described previously (Han et al. 2004, 2005). T_0 *AVP1D* plants were selected on
15 Murashige and Skoog inorganic salt medium (Murashige and Skoog, 1962) containing 1 mg/L PPT, and
16 then transferred to soil. Segregation pattern analysis on T_1 *AVP1D* plants were determined by selection on
17 0.3% (v/v) Basta™ (18% glufosinate ammonium; Kyungnoog, Korea) and polymerase chain reaction
18 (PCR) analysis with *AVP1D*-specific primers. Segregation analysis on T_2 plants from self-pollinated T_1
19 *AVP1D* plants were carried out by spraying plants with 0.3% Basta™, and homozygous T_2 *AVP1D* lines
20 were selected for use in all experiments reported in this study.
21
22 Nucleic acid analyses of transgenic bottle gourd
23
24 *PCR, Southern and northern blotting* For PCR analysis, genomic DNA was extracted from leaf tissue using
25 a DNeasy plant mini kit (Qiagen, Valencia, CA, USA). The *AVP1D*-specific primers were as follows:
26 forward (F) 5-TGCTGGTGGTATTGCTGAAATGGC-3; reverse (R), 5-
27 TGGCGATCTGAACACCGGATACAA-3. PCR products were visualized by electrophoresis on a 0.8%
28 agarose gel stained with ethidium bromide. For Southern blot analysis, genomic DNA was extracted from
29 leaf tissue of PCR-positive lines using CTAB methods (Doyle and Dickson 1987). Then, DNA (30 μg) from
30 each sample was digested with *Bam*HI, separated by electrophoresis on a 1% agarose gel, and blotted onto a
31 nylon Hybond^{TM}-N^+ membrane (Amersham Life Sciences, Little Chalfont, UK). For northern blotting
32 analysis, total RNA was isolated from root tissues of 10-day-old seedlings grown vertically on half-strength
33 Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) with 3% (w/v) sucrose and from leaves
34 and flowers of 60-day-old plants grown in soil using Trizol reagent (Invitrogen, Carlsbad, CA, USA)
35 according to the manufacturer's instructions. Then, total RNA (20 μg) from each sample was analyzed using
36 a standard blotting technique (Sambrook et al. 1989). The probe for the *AVP1D* gene was isolated from a
37 *Sma*I (2.2 kb) restriction fragment of the pRG521 plasmid (Park et al. 2005a), labeled with the Rediprime
38 II^{TM} random prime labeling system (Amersham Life Sciences), and used as a hybridization probe.

Radioactive signals were detected with a BAS-1800II Bio-Imaging analyzer (Fujifilm, Tokyo, Japan).

qRT-PCR Total RNA was prepared as described in nothern blotting analysis.. Then, 10 μg RNA was treated with DNaseI (Promega, Madison, WI, USA) and used to synthesize cDNA with the PrimeScript RT reagent kit (TaKaRa, Kyoto, Japan). Each reaction contained 2 μl cDNA template, 10 μl 2× SYBR Premix Ex Taq II (TaKaRa), 2 □l primer (final concentration of 500 nM) in a total volume of 20 μl. The following primers were used for the reactions: *AsAVP1* F-3'-TGCTTTCGTGCTTGGTGCTGTTAC-5'and *AsAVP1* R-3'-ACCAATAGACCACTCGCTGCAAGA-5' for *AVP1D*; and *CsActin* F 3'-TCGAGACTGCAAAGAGCAGTTCCT-5', *CsActin* R 3' TGGCTGGAATAGAACTTCTGGGCA-5' for *Actin*. Quantitative Real-time PCR (qRT-PCR) amplifications were performed with a CFX96 system (BioRad, Hercules, CA, USA). Amplications were performed with the following program: 95℃ for 10 sec and 44 cycles of 95℃ for 5sec, 60℃ for 30sec and 95 ℃ for 10sec. Melt curve analysis have done from 60 ℃ to 95℃ and have no abnormal curve. Actin RNA served as the internal control. Relative changes in gene expression detected by qRT-PCR were analyzed using the $2^{-\Delta\Delta C(T)}$ method (Livak and Schmittgen. 2001)

Soil water deficit experiment and root growth assay

Seeds of homozygous T_2 *AVP1D*-expressing and non-transformed lines were germinated in Petri plates containing distilled water and transferred into pots filled with soil. The temperature of the greenhouse was maintained within a range of 25 °C to 27 °C under a 16-h light/8-h dark photoperiod. Two-week-old seedlings were watered regularly to full field capacity for 21 days, and then water was withheld for 12 days until plants showed severe wilting symptoms. After 12 days of water deprivation, plants were re-watered for 10 days, and then growth performance of above-ground parts [plant height, number of internodes, and chlorophyll content (determined by using a leaf chlorophyll meter; SPAD 502 Minolta, Osaka, Japan)] were collected. Mean values were analyzed by Duncan's multiple-range test using SAS software (SAS Institute, Cary, NC, USA). Three independent experiments were performed with *AVP1D*-expressing and non-transformed lines. For root growth assay, *AVP1D*-expressing and non-transformed plants were removed from pots after soil water deficit experiment as described above. Intact roots of the plants were washed with distilled water and the primary root length was then measured. Statistical significance of differences between wild type and transgenic lines was analyzed by t-test at $P<0.05$.

Results and discussion

To obtain transgenic bottle gourds, 2,463 cotyledon explants were co-cultivated with *Agrobacterium* and transferred to selection medium. After herbicide selection, of the 479 regenerated and elongated shoots, 159 rooted plantlets were acclimated in soil pots. To determine whether these 159 plants contained *AVP1D* genes, PCR analysis was conducted to amplify the *AVP1D* gene sequence and three plants were confirmed

1 as potential transformants (Fig. 1b, parts of the data are shown). Based on PCR analysis, the transformation
2 efficiency was 1.8% and remained comparable to that of other reports (Han et al. 2005, 2009), and our
3 results are consistent with previous studies that *Cucurbitaceae* including bottle gourd cannot be efficiently
4 transformed using *Agrobacterium*-mediated transformation (Park et al. 2005b, Han et al. 2009). For
5 example, *Agrobacterium*-mediated transformation frequency of watermelon rootstock (*Citrullus lanatus*)
6 was 0.1~0.3% (Park et al. 2005b). Of the three PCR-positive lines, two lines (lines AVP1D-28 and -31)
7 were subjected to Southern blot analysis (one PCR-positive plantlet failed to survive). Genomic DNA was
8 digested with *Bam*HI (yielding border fragments which include a portion of the inserted T-DNA and
9 genomic DNA) and hybridized with the *AVP1D* probe. Digestion of the bottle gourd genomic DNA with the
10 restriction enzyme revealed the transgene copy number from the number of hybridizing bands and
11 independent transformation events from the two different hybridization patterns. AVP1D-28 and -31 lines
12 contain a single-copy insertion event (Fig. 1c). The endogenous *AVP1* homolog was not detected in the
13 wild-type lines by Southern analysis (Fig. 1c). These transgenic lines were resistant to PPT (Fig. 2a,b) and
14 were subjected to further phenotypic characterization. Homozygous T_2 transgenic lines were obtained
15 through segregation analysis (a segregation pattern of 3:1 on 0.3% Basta™) and PCR analysis of T_2 seeds
16 from self-pollinated T_1 plants (data not shown).
17 *AVP1D* expression analyses were also conducted with T_2 transgenic plants (lines AVP1D-28 and -
18 31) and controls. qRT-PCR analysis showed that *AVP1D* transcripts were present in all tested tissues,
19 including flower, leaf, and root tissues, while no *AVP1D* transcripts were detected in controls (Fig. 3a).
20 Interestingly, relatively higher levels of *AVP1D* transcripts were detected in root tissues of *AVP1D*-
21 expressing plants (Fig. 3a), although the constitutively expressed *CaMV 35S* promoter is not a tissue-
22 specific promoter. Previous studies also demonstrated that different levels of gene expression were observed
23 in different tissue/organ types, plant species and development stages (Sunilkumar et al. 2002, Samac et al.
24 2004). Therefore, it will be interesting to further examine whether relatively higher levels of *AVP1D*
25 transcripts in root tissues are observed in other *AVP1D*-expressing plant species driven by the *CaMV 35S*
26 promoter. We confirmed this result with a northern blotting analysis. The RNA gel blot result also showed
27 that *AVP1D* was expressed in all tissues but at particularly high levels in roots of line AVP1D-28, which is
28 consistent with the results of the qRT-PCR analysis (Fig. 3b). However, no significant differences were
29 observed in resistance/growth performance between line AVP1D-28 and line AVP1D-31 under drought
30 conditions (Table 1, Fig. 4a-d, and Fig. 5).
31 For growth performance under non-stress conditions, plant height, number of internodes, leaf size,
32 and leaf chlorophyll of 3-week-old seedlings of *AVP1D*-expressing transgenic and control lines were
33 initially measured before water deprivation, and in this conditions there were no significant differences in
34 growth performance between control and transgenic lines (Table 1). For the drought resistance experiments,
35 the seedlings of *AVP1D*-expressing transgenic and control lines were grown in soil for 21 days, and then
36 water was withheld for 12 days. After 12 days of water deprivation, both control and transgenic plants
37 showed water-deficit wilting symptoms (Fig. 4a). Upon re-watering, both transgenic lines AVP1D-28 and -
38 31 showed better recovery and resumed growth more rapidly than control plants at 10 days after re-watering

(Fig. 4b). In addition, transgenic plants had larger leaves than those of controls (Fig. 4c and Table 1), and the average plant height, number of internodes, and leaf chlorophyll content in transgenic lines were significantly greater than those in control plants (Table 1), indicating transgenic bottle gourd expressing *AVP1D* had significant advantages over control plants in their ability to recover after a water-deficit stress. Our results are consistent with previous findings that enhanced *AVP1* expression can led to increased drought resistance in transgenic plants (Park et al. 2005a; Qin et al. 2012; Pasapula et al. 2011).

In our previous studies, the more extensive root system of transgenic tomato plants expressing *AVP1D* allowed greater uptake of water during water deficit, enhancing resistance to drought stress (Park et al. 2005a). Therefore, we hypothesized that enhancing *AVP1D* expression in bottle gourd could improve root development, which may then enhance resistance to drought stress and facilitate improved water deficit recovery. To test this idea, we phenotyped the *AVP1D*-expressing transgenic bottle gourd. Indeed, *AVP1D*-expressing bottle gourd showed a more vigorous and bigger root system than that of control plants under water deficit condition (Fig. 4d), and the average primary root length in transgenic plants was ~20% longer than that in control plants after water deprivation/re-watering (Fig. 5), suggesting a more robust and deeper root system in the *AVP1D*-expressing bottle gourd can be associated with increased drought resistance.

Salinization of soil is a common problem in continuous cultivation of vegetables, such as greenhouse-grown watermelon. The vascular H^+-pump gene *AVP1* and its homologs in other species have been transformed into major crop species, and have enhanced the salt tolerance of transgenic crops including rice, Chinese cabbage, cotton, and peanut (Zhao et al. 2006; Pasapula et al. 2011; Park et al. 2012; Qin et al. 2012). Recently, it was reported that plants over-expressing *AVP1* outperformed wild-type controls under nutrient-deficient conditions. Lettuce plants over-expressing *AVP1D* showed a better performance than controls under NO_3-limited conditions (Paez-Valencia et al. 2013). Yang et al. (2007) also reported that expression of *AVP1* is increased under phosphorus (P) deficiency and that tomato, *Arabidopsis* and rice plants transfoprmed with a 35S::AVP1 expression cassette outperformed controls under limiting P, suggesting that genetic manipulation of AVP1 could help to reduce agricultural losses caused by P limitation in low-P soils and allow optimal use of non-renewable P fertilizer. In the future, it will be useful to determine whether watermelon grafted onto transgenic bottle gourd expressing *AVP1D* displays increased yield in saline and nutrient-deficient soils.

In conclusion, when bottle gourd is used as a rootstock, root proliferation is an important factor in grafting with a scion. Transgenic bottle gourd expressing *AVP1D* showed an improved root system and greater water use-efficiency when compared with controls.

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1
2 **Table 1.** Growth performance of *AVP1D*-transgenic bottle gourd in soil under water
3 deficit conditions

Days	Lines	Height (cm)	No. Internode	Leaf[b] (cm)		Chlrophyll content(SPAD)
				Width	Length	
Initial[a]	WT	13.4±0.4a	2.2±0.1a	6.6±0.5a	6.7±0.2a	37.7 ± 1.1a
	Vector only	15.2±0.4a	2.0±0.a1	8.1±0.5a	7.6±0.2a	37.6 ± 1.1a
	AVP1D 28	13.7±0.3a	1.9±0.1a	6.4±0.4a	7.2±0.1a	38.4 ± 0.4a
	AVP1D 31	14.1±0.9a	2.1±0.2a	7.7±0.8a	7.6±0.4a	35. 5 ± 2.0a
12 days after water deficit	WT	21.5±2.4a	2.8±0.3a	7.0±0.6b	7.0±0.2a	32.3±1.6a
	Vector only	22.3±0.7a	3.5±0.1a	8.8±0.2ab	7.3±0.1a	32.3 ± 1.2a
	AVP1D 28	27.5±0.9a	3.7±0.1a	9.0±0.2ab	7.4±0.2a	38.1 ± 0.8a
	AVP1D 31	28.5±1.3a	3.4±0.1a	9.4±0.2a	8.0±0.1a	36.5 ± 0.9a
10 days after re-watering	WT	31.7±0.7a	5.2±0.1b	5.9±0.4b	6.5±0.4a	29.7 ± 1.1c
	Vector only	31.5±2.2a	5.0±0.1b	5.8±0.2b	5.2±0.2a	30.2 ± 0.3bc
	AVP1D 28	62.3±2.0b	6.8±0.1a	7.7±0.3a	6.3±0.2a	33.0 ± 0.5ab
	AVP1D 31	56.8±2.0b	6.5±0.1a	7.2±0.2a	5.5±0.2a	35.0 ± 0.5a

4 [a] Initial: 3-week-old seedlings of controls (wild-type: WT, vector only transformant; Vector
5 only) and homozygous transgenic bottle gourd plants (AVP1D-28 and AVP1D-31) at
6 beginning of drought stress treatment.
7 [b] Data represents SPAD values measured at three different positions on the largest leaf of
8 each seedling. Values are means (n = 20 transgenic plants from each transgenic line, 20
9 control plants from each wild-type and vector only transformant). Different letters in the
10 same row indicate significant difference at $P<0.05$.
11

Figure legends

Fig. 1. Molecular characterization of two *AVP1*-expressing transgenic bottle gourd lines. **a** T-DNA regions of p35S::AVP1D. Abbreviations: RB, right border; LB, left border; Nos-pro, nopaline synthase promoter; Bar, bialaphos resistance; Nos-ter, nopaline synthase terminator; AVP1D, the E229D gain-of-function mutant of the *Arabidopsis* H^+-PPase AVP1; N-ter, nopaline synthase terminator; 2x35S, tandem repeat of the cauliflower mosaic virus (CaMV) 35S promoter. **b** PCR analysis of transgenic bottle gourd plants expressing *AVP1D*. Lanes: WT (wild-type bottle gourd G5); AVP1D-28 and AVP1D-31, transgenic bottle gourd lines; PC, positive control (plasmid). *AVP1*-specific primers were used for PCR analysis. **c** Southern blot analysis of transgenic bottle gourd. Genomic DNA (30 μg) from PCR-positive bottle gourd plants was digested with *Bam* HI, and hybridized with *AVP1D* probe. Lanes: M, 1kb plus ladder marker; WT (wild-type G5); AVP1D-28 and AVP1D-31, transgenic bottle gourd lines expressing *AVP1D*; PC, positive control (plasmid).

Fig. 2. Transgenic bottle gourd (T_2) and herbicide resistance test. **a** Response of T_2 transformants (AVP1D-28 and AVP1D-31) and wild-type (WT) to 3% (v/v) of a Basta solution (18% glufosinate ammonium; Kyungnoog, Korea) one week after spray. **b** Close-up picture of resistance test to Basta solution for AVP1D-28, AVP1D-31 and wild-type lines 3 days after application. The leaves of transformants and wild-type bottle gourd were thoroughly swabbed with 3% (v/v) of a Basta solution (18% glufosinate ammonium).

Fig. 3. Spatial expression of *AVP1D* gene in bottle gourd. Total RNA extracted from flower (F), leaf (L) and root (R) of control plants (wild-type: WT, vector only transformant: Vector only) and homozygous transgenic plants (T_2 AVP1D-28, AVP1D-31). **a** Quantitative real-time PCR (qRT-PCR) was performed to quantify *AVP1D* transcripts. *AVP1D* transcript level was normalized to that of *Actin* (for details, see Materials and Methods). qRT-PCR experiments were conducted in triplicate using three independent RNA extractions. The *error bars* indicates ± SD (n = 3). **b** Northern blot analysis of control and transgenic plants.

Fig. 4. Comparison of *AVP1D* expressing transgenic plants and control plants grown under drought stress. **a** Plants under water deficit stress after 12 days of water deprivation. **b** Plants recovered from drought stress. Picture was taken after 10 days of re-watering. **c** Leaf phenotypes after recovery. Picture was taken 20 days after of recovery. **d** Root phenotypes after drought stress.

1 Picture was taken 12 days after soil water deficit.

2

3 **Fig. 5.** Comparison of primary root length of *AVP1D*-expressing transgenic plants (AVP1D-28,
4 AVP1D-31) and control plants (wild-type and vector controls) after drought treatment. Primary
5 root length was measured 12 days after water deficit followed by 10 days of re-watering. The
6 *error bars* indicates ± SE (n = 20). *Single asterisk* indicate significant difference at the $P<0.05$.

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1
2 **Figure 1**
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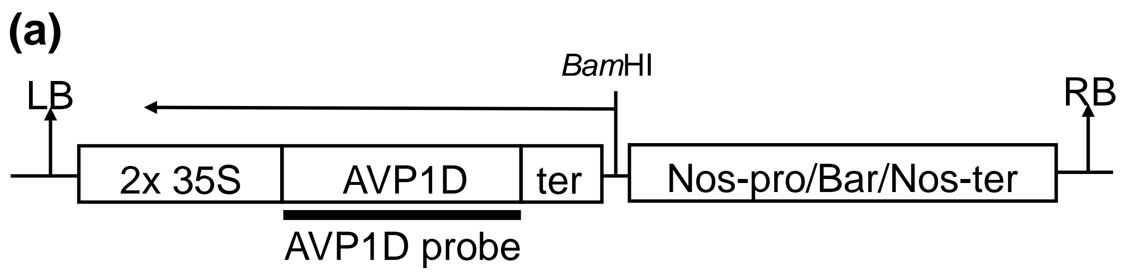

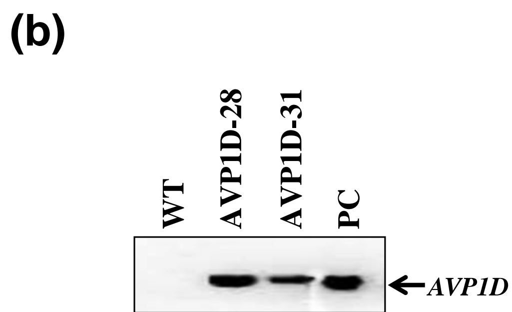

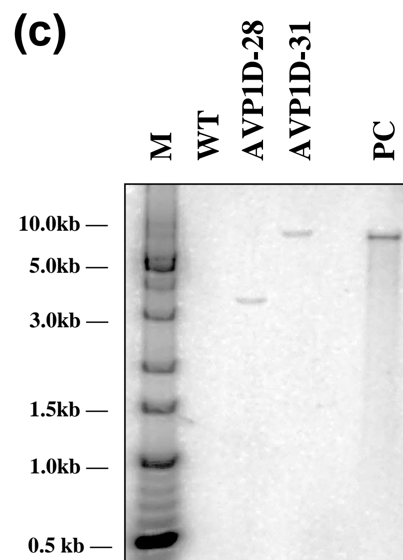

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2 **Figure 2**

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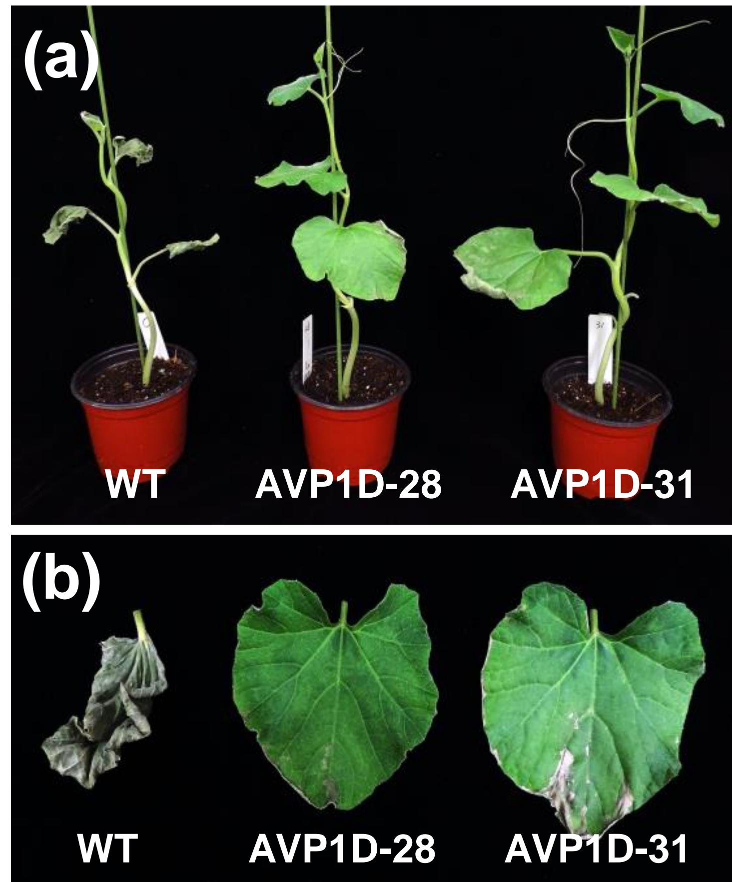

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2 **Figure 3**
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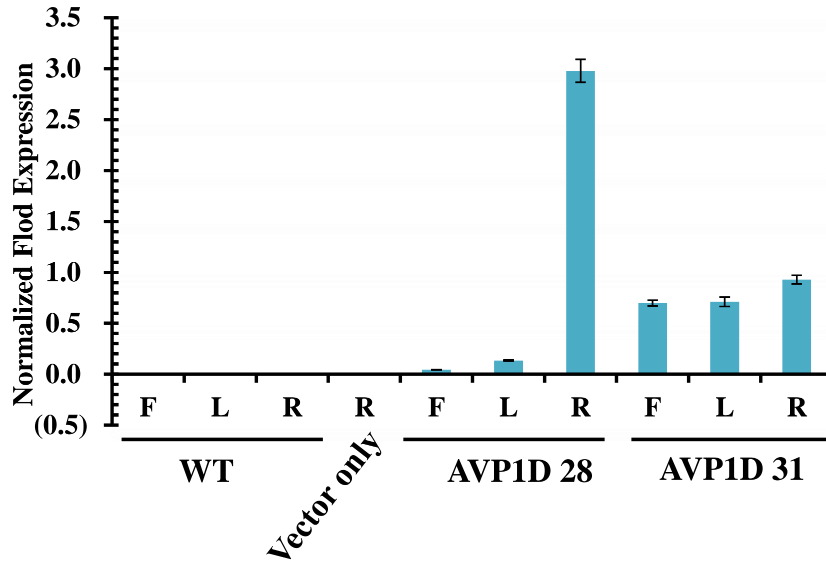
5 **(a)**



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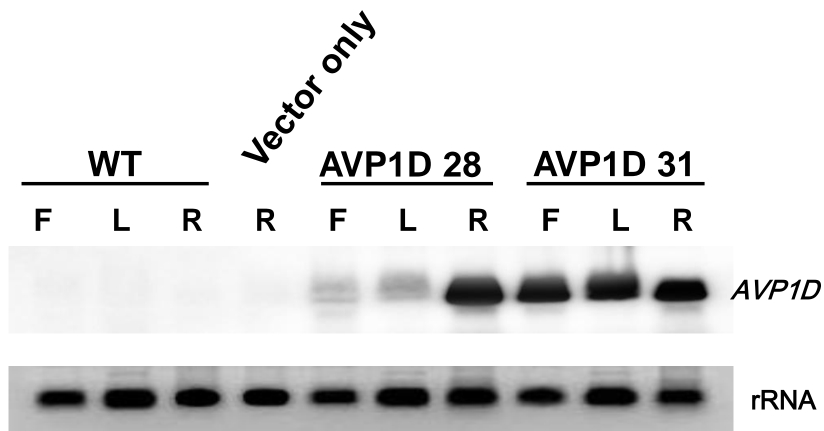
8 **(b)**



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2 **Figure 4**

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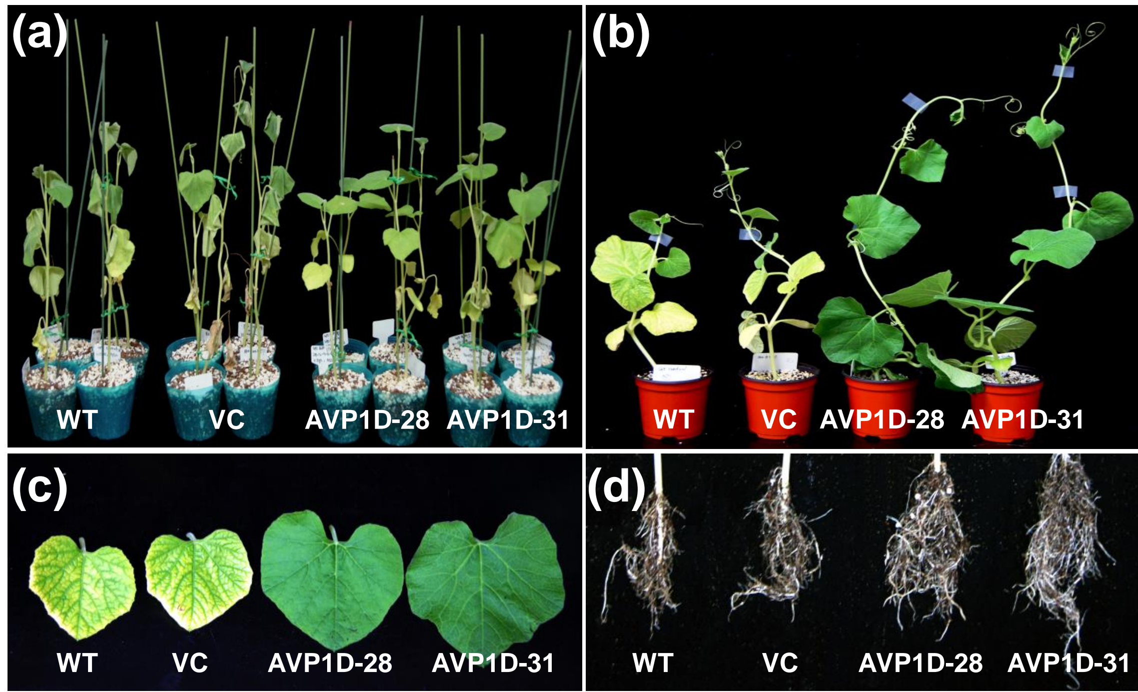

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2 **Figure 5**
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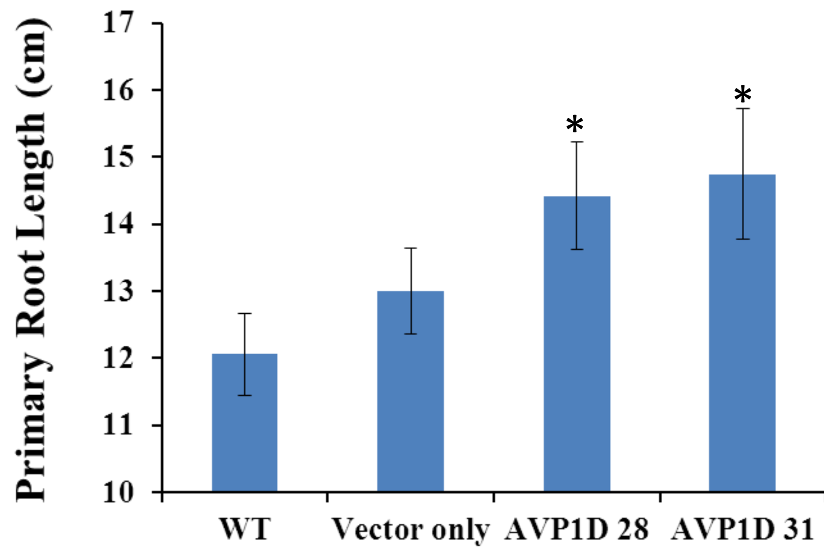

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