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Mitochondrial Porin Isoform AtVDAC1 Regulates the Competence of *Arabidopsis thaliana* to *Agrobacterium*-Mediated Genetic Transformation

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The efficiency of *Agrobacterium*-mediated transformation in plants depends on the virulence of *Agrobacterium* strains, the plant tissue culture conditions, and the susceptibility of host plants. Understanding the molecular interactions between *Agrobacterium* and host plant cells is crucial when manipulating the susceptibility of recalcitrant crop plants and protecting orchard trees from crown gall disease. It was discovered that *Arabidopsis voltage-dependent anion channel 1 (atvdac1)* mutant has drastic effects on *Agrobacterium*-mediated tumorigenesis and growth developmental phenotypes, and that these effects are dependent on a Ws-0 genetic background. Genetic complementation of *Arabidopsis vdac1* mutants and yeast *porin1*-deficient strain with members of the *AtVDAC* gene family revealed that AtVDAC1 is required for *Agrobacterium*-mediated transformation, and there is weak functional redundancy between AtVDAC1 and AtVDAC3, which is independent of porin activity. Furthermore, *atvdac1* mutants were deficient in transient and stable transformation by *Agrobacterium*, suggesting that AtVDAC1 is involved in the early stages of *Agrobacterium* infection prior to transferred-DNA (T-DNA) integration. Transgenic plants overexpressing *AtVDAC1* not only complemented the phenotypes of the *atvdac1* mutant, but also showed high efficiency of transient T-DNA gene expression; however, the efficiency of stable transformation was not affected. Moreover, the effect of phytohormone treatment on competence to *Agrobacterium* was compromised in *atvdac1* mutants. These data indicate that AtVDAC1 regulates the competence of *Arabidopsis* to *Agrobacterium* infection.

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INTRODUCTION

Agrobacterium-mediated genetic transformation is a well-established method most widely used to generate transgenic plants in numerous dicotyledonous, and some monocotyledonous plant species. Its host range for genetic transformation has been extended to include other eukaryotic species, such as yeast, mushrooms, filamentous fungi, and human cells (Bundock et al., 1995; Gouka et al., 1999; Kunik et al., 2001; Rho et al., 2001). The course of *Agrobacterium* infection involves complex molecular and, cellular reactions involving the interaction of many bacterial and host plant factors. *Agrobacterium*-mediated plant transformation can be divided into several steps; (i) induction of *Agrobacterium* virulence (Vir) proteins by specific host signals (phenolic compounds and sugar molecules) that are released from wounded plants, (ii) processing of the transferred-DNA (T-DNA) region of the Ti-plasmid by the VirD2 protein, which nicks both borders at the bottom strand of the T-DNA, (iii) bacterial attachment to the plant surface, (iv) export of the T-strand (the processed single-stranded T-DNA covalently linked to VirD2) and Vir proteins from the bacterium to plant cells via a type IV secretion system, (v) maturation of the T-complex by interaction of T-DNA and VirE2 in the plant cytoplasm, and nuclear targeting by the nuclear import machinery of host cells, (vi) integration of T-DNA into the plant genome mediated by VirD2 and/or VirE2 and host factors regulating plant genome structures, (vii) expression of T-DNA encoded genes (reviewed by Gelvin, 2010; 2012; Lacroix and Citovsky, 2013; Tzfira and Citovsky, 2001).

Transgenic plants generated by *Agrobacterium*-mediated transformation carry a high number of single-copy transgene insertions that are stably expressed, compared to those carried by plant transformed by other techniques, such as a biolistic gene gun, which is crucial for the development of safe genetically modified crops (Shou et al., 2004). However, the frequency of transformation of crop plants remains considerably low. Similar to the disease triangle visualizing the interactions of the three components of disease; virulence of pathogens, environment, and susceptibility of host plants and predicting the level of disease caused by plant pathogens, the efficiency of plant genetic transformation by *Agrobacterium* depends largely on the virulence of *Agrobacterium* strains, the plant tissue culture conditions, and the susceptibility of host plants. The biological functions of most bacterial virulence proteins involved in the induction of *Agrobacterium* virulence have been well characterized,

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leading to the development of super virulent *Agrobacterium* strains (Liu et al., 1992). *Agrobacterium* effectors that translocate into host plant cells via a type IV secretion system can be used to abuse plant systems and evade defense responses of the plant, and to modify the genetic material of the host plants (Djamei et al., 2007). Of note, the effector functions of VirE2 are controversial. *Agrobacterium* exploits the defense responses of host plants in order to deliver T-DNA into nucleus by interacting VirE2 wrapping the T-DNA with defense-related transcription factor, VIP1. Subsequently, the VirE2-VIP1 complex is degraded by the ubiquitin-mediated proteasome via the Skp1/ Cullin/F-box protein VBF pathway prior to T-DNA integration into the host genome (Zaltsman et al., 2010; 2013). In contrast, Shi et al. (2014) postulated that VirE2 promotes tumorigenesis by sequestering the low-abundance VIP1 in the cytoplasm and suppressing host defense responses during *Agrobacterium* infection. Tissue culture techniques for recalcitrant plants are also improving through the use of explants that are competent to *Agrobacterium* infection, appropriate methods of selecting transformed cells, and efficient regeneration into mature plants, which are vital factors for efficient genetic transformation of plants by *Agrobacterium*. However, the characterization and application of host plant proteins, which might play pivotal roles in *Agrobacterium* infection, are in the early stages. It has been estimated that more than 200 *Arabidopsis* genes are directly or indirectly involved in *Agrobacterium*-mediated plant transformation (Zhu et al., 2003). The discovery that VIP1 and RAT5 are involved in nuclear translocation of the T-complex and the integration of T-DNA into the plant genome, respectively, and their physical interaction, will provide new insights that are useful for manipulating the susceptibility of host plants to *Agrobacterium* infection (Lacroix et al., 2008, Mysore et al., 2009).

To extend understanding of efficient *Agrobacterium*-mediated transformation of plants, T-DNA-tagged *Arabidopsis* mutant libraries were examined using root transformation method and several untransformed root by *Agrobacterium* (*ura*) mutants that are resistant to *Agrobacterium* infection were identified. Here it was shown that the resistance phenotype of the *ura1* mutant results from the disruption of *AtVDAC1* gene, leading to significant decreased transient T-DNA gene expression. Furthermore, transgenic plants overexpressing *AtVDAC1* showed highly efficient transient T-DNA gene expression. However, the efficiency of stable transformation was not significantly enhanced. These data strongly indicate that the AtVDAC1 regulates the competence of *Arabidopsis* to *Agrobacterium* infection.

MATERIALS AND METHODS

Growth of *Arabidopsis thaliana* and *Agrobacterium tumefaciens*

For the *in vitro* root transformation assay, *Arabidopsis* seeds were sterilized with a solution composed of 50% commercial bleach and 0.1% SDS for 5 min, and then rinsed five times with sterile distilled water. The seeds were germinated in Petri dishes containing Gamborg's B5 medium solidified with 0.8% Bactoagar. Following incubation at 4°C for 2 days, we incubated the plates for 7 days under a light (16 h) / dark (8 h) photoperiod and 120-150 μmol/m^2 sec light intensity at 22°C. Seedlings were individually transferred into baby food jars containing solidified B5 medium and cultured for 2-3 weeks for root culture. All *Agrobacterium* strains were cultured in YEP medium supplemented with the appropriate antibiotics (rifampicin, 10 μg/ml) at 29°C. Overnight bacterial cultures were washed with 0.9% NaCl and suspended in 0.9% NaCl at OD_{600} = 1.0 for *in vitro* root transformation.

In vitro Arabidopsis root transformation assay

Roots grown on the agar surface were excised, cut into small segments (~0.5 cm) in a small amount of sterile water, and the root segments were then blotted on sterile filter paper to remove excess water. The blotted bundles of root segments were transferred to MS basal medium containing 0.8% Bactoagar, on which 2-3 drops of the bacterial suspension were placed. After 10 min, most of the bacterial solution was removed using pipette, and the bacteria and root segments were co-cultivated at 22°C for 2 days. To quantify tumorigenesis, root bundles were infected with wild-type *Agrobacterium* strain A208, which leads to the development of large, green teratoma-type tumors on the roots of *A. thaliana* ecotype Ws-0. After 2 days, the root bundles were rubbed on the agar surface to remove excess bacteria, and the bundles of root segments were then transferred to MS basal medium containing carbenicillin (100 μg/ml) to prevent bacterial growth. The plates were incubated under a light (16 h) / dark (8 h) photoperiod and 120-150 μmol/m^2 sec light intensity at 22°C for 4 weeks to induce crown gall tumors. For transient transformation assays, we infected the root bundles with *A. tumefaciens* GV3101, which contains the binary vector pBISN1, as described previously (Nam et al., 1997; 1999). After 2 days co-cultivation, the root segments were rinsed with water, and stained with X-gluc staining solution (50 mM Na_2HPO_4, 10 mM Na_2EDTA, 300 mM mannitol, and 2 mM X-gluc, pH 7.0) for 1 day at 37°C.

Flower bolt transformation assay

When the primary flower bolts of *Arabidopsis* plants grown in soil under a light (8 h) / dark (16 h) photoperiod and 120-150 μmol/m^2 sec light intensity at 22°C reached a height of 4-5 cm, wounded with a needle. Plant exudates were removed from the wound by briefly touching the sites with an Accuwipe. Then, 3 μl of a highly concentrated *A. tumefaciens* A208 suspension (2×10^{11} cfu/ml) was inoculated into the wounding sites. The plants were covered with a plastic lid for 3 days to retain the humidity and to allow infiltration of the bacterial suspension. Plants were incubated for 4 weeks under the same growth conditions to induce crown gall tumors.

Plasmid rescue

Genomic DNA (5 μg) of *ura1* mutant plants was digested with BamHI and cleaned with the use of Gel and PCR clean-up kit (Promega). The DNA was self-ligated in a final volume of 500 μl in 1X ligation buffer containing 3 units of T4 DNA ligase at 15°C for 16 h. The ligation mixture was cleaned up and dissolved in 20 μl H_2O, transformed into electrocompetent *E. coli* DH10B cells by electroporation and plated on LB medium containing ampicillin (100 μg/ml). Colonies were lifted onto a nylon membrane, the bacteria were lysed, and DNA was denatured *in situ*. A P^{32}-labeled left border sequence (4.0 kb *Eco*RI/*Bam*HI fragment of binary vector pE1829 used for T-DNA insertion mutant pools, which was a gift from Gelvin, Purdue University) was used as a hybridization probe to identify a plasmid containing the left border. Using restriction fragment analysis, plasmids containing both the left border and the plant junction DNA were identified. The plant junction fragment was sequenced and the corresponding plant sequence was confirmed by searching an *Arabidopsis* genomic database.

RNA extraction and RNA blot analysis

Total RNAs was isolated from specific tissues samples with

TRIzol reagent (Invitrogen). RNA gel blots were generated by separating RNA (10 μg) on a formaldehyde agarose gel (1.2%, w/v) followed by blotting onto a nylon transfer membrane (Amersham). Membranes were hybridized with a labelled probe for 16 h at 65°C in 20% (w/v) SDS, 20 × SSPE, 100 g/l PEG (8,000 mwt), 250 mg/l heparin, and 10 mg/ml HS DNA. Each probe was labeled with [$^{32}\alpha$-P]dATP (Amersham) using the random primer labeling kit (Stratagene). After hybridization, the membrane was washed for 30 min at 65°C in 2 × SSC and 0.1% (w/v) SDS, then for 30 min at 65°C in 1 × SSC and 0.1% (w/v) SDS, followed by 30 min at 65°C in 0.1 × SSC and 0.1% (w/v) SDS. The blots were exposed to x-ray film with an intensifying screen to detect *AtVDAC1* expression in wild-type and *ura1* mutant plants.

Complementation of yeast *por1* mutant with *Arabidopsis* VDAC isoforms

The VDAC-deficient yeast strain *S. cerevisiae* Y17374 (*por1Δ his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 MATα*; purchased from EUROSCARF, Germany) and wild-type strain Y10000 were used in functional complementation assays. Yeast cells were transformed via the modified lithium acetate yeast transformation method or by electroporation. Each member of the *AtVDAC* gene family was designed by PCR amplification, and then it cloned into the yeast constitutive expression vector pVT-U, which is a URA3-based high-copy-number plasmid that contains the strong constitutive alcohol dehydrogenase (ADH) promoter to achieve high levels of expression (Blachly-Dyson et al., 1997). Yeast strain Y17374 was transformed with constructs expressing each *AtVDAC* gene, and the transformants were selected on synthetic complete medium minus uracil. Several colonies per construct were streaked onto YPG plates containing glycerol as the carbon source and grown at 30°C and 37°C.

Genetic complementation of the *ura1* mutant plant

The coding regions of *AtVDAC* genes were PCR amplified, subcloned into pBluescript and sequenced. To generate transgenic plants overexpressing each *VDAC*, the coding regions of *AtVDAC* were cloned downstream of the cauliflower mosaic virus (CaMV) 35S promoter in the sense orientation in the binary vector pCAMBIA1300-35S. The resulting constructs were introduced into *A. tumefaciens* GV3101 using the tri-mating method. The resulting *A. tumefaciens* strains were used to transform homozygous *ura1* mutant plants via a vacuum infiltration procedure (Bent and Clough, 1998). Transgenic plants were selected on B5 medium containing carbenicillin (100 μg/ml) and hygromycin (20 μg/ml), then transferred to soil and allowed to set seed. Tumorigenesis assays were performed with the selected homozygous lines.

Phylogenetic analysis

A phylogenetic tree was generated using PROTPARS, a maximum-parsimony algorithm that is included in the PHYLIP version 3.5 software package. Topological robustness was assessed by bootstrap analysis with 100 replicates using simple taxon addition. Sequences used for alignments were identified by BLAST searches of DDBJ/EMBL/GenBank database. Sequences conserved in VDAC, homologs of VDAC, plant VDACs, and animal VDACs were aligned with the FCLUSTAL_ W program, and alignments were refined manually. Several short sequences within the N-terminal region that could not be unambiguously aligned were excluded from the analysis.

RESULTS

Identification of *Arabidopsis* mutants resistant to transformation by *Agrobacterium*

To screen for *Arabidopsis* mutant plants showing altered tumor formation after infection with *Agrobacterium*, an *in vitro* root transformation assay that maintains the most frequently infected tissue condition by soil-borne *Agrobacterium* in nature was used. Small root segments of individual 3-4 week old T-DNA insertion mutagenized plants were inoculated with oncogenic *A. tumefaciens* A208. This bacterial strain induced the formation of large green teratoma-type tumors on *Arabidopsis* ecotype Ws-0 that was the parental ecotype used to generate the T-DNA insertion mutant library. The remaining shoots of each tested plant were placed into solidified culture medium to allow root regeneration. After observing the results of the root inoculation, the re-rooted plants that showed resistance were transferred into soil, and allowed to set seeds for progeny recovery. Out of approximately 3000 plants investigated, several plants were selected as putative mutants resistant to infection by *Agrobacterium tumefaciens*. These mutants termed *ura* (untransformed root by *Agrobacterium*), for resistance to *Agrobacterium* transformation. As shown in Fig. 1A, the *ura1* mutant incited few small and yellow tumors on root bundles, compared to the numerous large green teratoma-type tumors incited by *A. tumefaciens* A208 on root bundles of wild-type plants. The *ura1* mutant also exhibited the morphological leaf variegation phenotype which co-segregated with the resistance phenotype (Fig. 1B).

ura1 mutant was disrupted in the mitochondrial voltage dependent anion channel, *AtVDAC1* gene

To determine the genetic characteristics of the *ura1* mutant, homozygous mutant plants were backcrossed, which are resistant to phosphinothricin, as the pollen donor to the wild-type, Ws-0, plants and selected F1 hybrids by germinating seeds from each cross on Gamborg's B5 medium containing phosphinothricin (10 μg/ml). All F1 plants showed a susceptible phenotype, indicating that the resistance phenotype of the *ura1* mutant is recessive. Genetic analysis of the F2 population showed that phosphinothricin resistance is segregated in a 3:1 ratio, indicating that a single linkage group was disrupted by T-DNA insertion in the *ura1* mutant. Furthermore, to examine the co-segregation of the T-DNA insertion with the *Agrobacterium* resistance phenotype, individual F2 plants were grown on solidified Gamborg's B5 medium without phosphinothricin. Root bundles were infected with *A. tumefaciens* A208 and transferred onto MS basal medium without hormones to induce tumor formation. The phenotypes that were susceptible and resistant to *Agrobacterium* infection were segregated in a 3:1 ratio in the F2 population (Supplementary Table S1). More importantly, all of the F2 progeny tested displaying *ura1* mutant phenotypes were homozygous for phosphinothricin resistance. These results indicate that the *ura1* mutant phenotype is tightly linked to the locus into which the T-DNA has integrated.

A plasmid rescue technique was used to isolate the T-DNA/plant DNA junction fragments of the *ura1* mutant plants. DNA sequence analysis of the junction regions revealed that a T-DNA was inserted into the 6th exon of the *AtVDAC1* (At3g01280) gene, which encodes voltage-dependent anion channel 1 (Fig. 1C). These channels are reported localize to the mitochondrial outer membrane and regulate the permeability of the mitochondria to metabolites (Tateda et al., 2011). RNA blot hybridization showed that the *AtVDAC1* transcript in *ura1* mutant was undetectable (Fig. 1D), suggesting that the re-

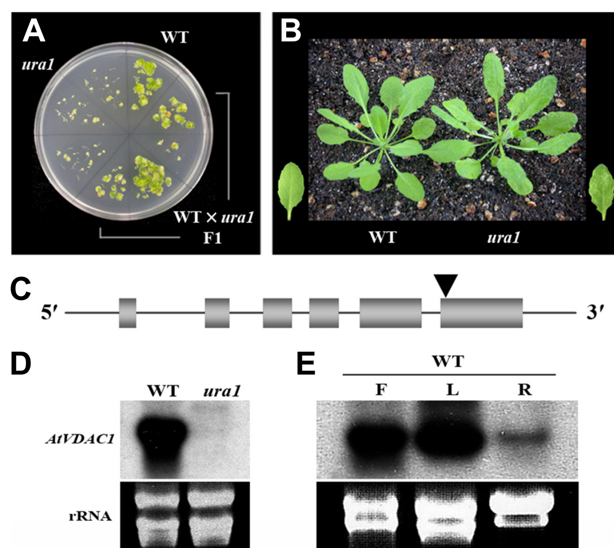

Fig. 1. Characterization of *ura1* mutants resistant to oncogenic *Agrobacterium tumefaciens* A208. (A) Sterile root segments of wild-type (WT), *ura1* mutant, and F1 progeny plants were infected with *A. tumefaciens* A208 (OD_{600} = 1.0). After 2 days co-cultivation, tumors were induced on MS basal medium containing carbenicillin (100 μg/ml) for 4 weeks. (B) Phenotypes of 4-week-old WT and *ura1* mutant plants. Leaf variegation showing pale green area is associated with the *ura1*mutation. (C) Genomic organization of the *AtVDAC1* locus composed of six-exons (grey boxes) and a T-DNA (black triangle) insertion site at the 6th exon of the *AtVDAC1* gene leading to the *ura1*mutant. (D) Expression of *AtVDAC1* in WT and *ura1* mutant plants. (E) Organ-specific expression of *AtVDAC1*. rRNA was used as a loading control. F, flowers; L, leaves; R, root tissue.

sistance phenotype of the *ura1* mutant to *Agrobacterium* infection results from lack of AtVDAC1protein located in the outer membrane of mitochondria. In wild-type plants, the *AtVDAC1* transcript was highly accumulated in flowers and leaves compared to roots (Fig. 1E).

Complementation of *por1*-deficient yeast strain with the *AtVDAC* gene family

Although multiple isoforms of VDAC have been identified in multicellular organisms, unicellular yeast, *Saccharomyces cerevisiae,* contains only two *VDAC* genes, designated *POR1* and *POR2*. Of note, a yeast strain lacking the *POR1* gene (Δ*por1*) is able to grow on yeast media containing glycerol as the sole carbon source at 30°C but not at 37°C (Blachly-Dyson et al., 1997). This temperature-dependent phenotype is commonly used to investigate the activity of other VDACs (Sampson et al., 1997). As described in MATERIALS AND METHODS, four distinct *Arabidopsis VDAC* genes were constitutively expressed in the *por1*-deficient yeast strain Y17374. The transformed yeast strains expressing *AtVDAC1*, *AtVDAC2*, and *AtVDAC4* grew even at the restrictive temperature of 37°C on YPG medium, indicating that they functionally complement the yeast *VDAC* gene. However, *AtVDAC3* could not restore the yeast Δ*por1* mutant, although it has high similarity with *AtVDAC1* (Fig. 2). These results suggest that four *AtVDAC* isoforms may have functionally distinct roles in plants.

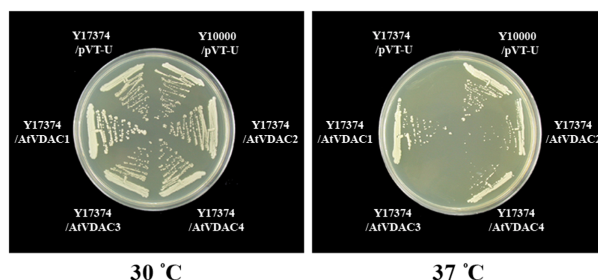

Fig. 2. Functional complementation of the *porin1*-deficient yeast strain (Y17374) by the *Arabidopsis* VDAC gene family. The WT yeast strain Y1000 and the *porin1*-deficient yeast strain Y17374 were transformed with plasmids lacking an insert (pVT-U) or harboring an *AtVDAC1*, *AtVDAC2*, *AtVDAC3*, or *AtVDAC4* insert (VDACs/pVT-U). The transformants were streaked on media containing 2% glycerol as the sole carbon source and incubated at 30°C and 37°C for 5 days.

Complementation of the *ura1* mutant with *AtVDAC* gene family

To confirm that the resistance phenotype of the *ura1* mutant to *Agrobacterium* infection resulted from disruption of the *AtVDAC1* gene and functional redundancy among *AtVDAC* genes, genetic complementation test was performed by overexpressing individual genes of the *AtVDAC* family in the *ura1* mutant. Although the mutant is resistant to *Agrobacterium*-mediated somatic cell transformation via roots and shoots, It was able to introduce these transgenes into homozygous *ura1* mutant plants using *Agrobacterium*-mediated germ-line transformation via flowers, consistent with the findings reported for *rat* mutants (Mysore et al., 2000b). Transgenic plant lines overexpressing individual genes of the *AtVDAC* family were selected for further experiments. Two tumorigenesis assays, *in vitro* root transformation and flower bolt transformation, were conducted using *A. tumefaciens* A208 in wild-type, *ura1* mutant, and selected transgenic plants. Overexpression of *AtVDAC1* complemented the tumorigenesis-resistant phenotype of the *ura1* mutant and also restored morphological defects including leaf variegation, which is caused by a loss of chlorophyll, but not by cell death, and the short roots compared to those of the wild-type (Figs. 3 and 4A). These results demonstrate that disruption of the *AtVDAC1* gene is responsible for resistance against *Agrobacterium*-mediated tumorigenesis and developmental defects in the *ura1* mutant. The *ura1* phenotype also attempted to reconstitute by expressing *AtVDAC1* homologous genes *AtVDAC2, AtVDAC3*, and *AtVDAC4* under the control of a CaMV 35S promoter in *ura1* mutant plants. Only the *AtVDAC3* gene partially complemented the tumorigenesis-resistant phenotype of the *ura1* mutant, indicating that *AtVDAC1* and *AtVDAC3* are functionally redundant in response to *Agrobacterium*-infection (Fig. 4B). However, developmental defects were not recovered in any of the transgenic plants compared with the wild-type, suggesting that *AtVDAC1* may have a distinct role during plant development.

Early stage of T-DNA gene expression are defected in *ura1* mutant

To determine what step of the *Agrobacterium*-mediated transformation process is defective in the *ura1* mutant, the efficiency of transient T-DNA gene expression determined by inoculating

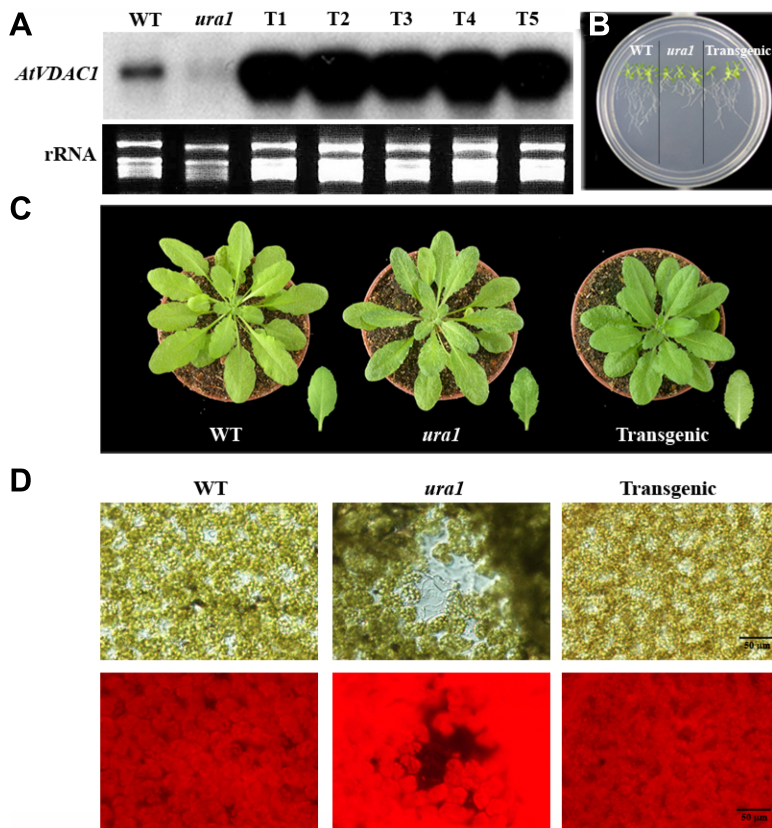

Fig. 3. Functional complementation of the *ura1* mutant with the *AtVDAC1* gene. (A) RNA blot analysis of *AtVDAC1* expression in WT, *ura1* mutants, and transgenic plants overexpressing *AtVDAC* in the *ura1* mutant. rRNA was used as a loading control. (B, C) Developmental phenotypes, leaf variegation (B), and root growth retardation (C), of *ura1* mutants were restored in the transgenic lines. Plants were grown in soil for 4 weeks under an 8-h light/16-h dark photoperiod at 22°C. Seedlings germinated under sterile conditions were placed on Gamborg's B5 agar medium and cultured vertically for 2 weeks in a growth chamber (22°C, 8-h light/16-h dark). (D) Fluorescence in the epidermal cells of WT, *ura1* mutant, and transgenic lines under visible (top row) or ultra-violet light (bottom row) microscopy. No Fluorescence was detected in epidermal cells in the variegated area of *ura1* mutants. Bar = 50 μm.

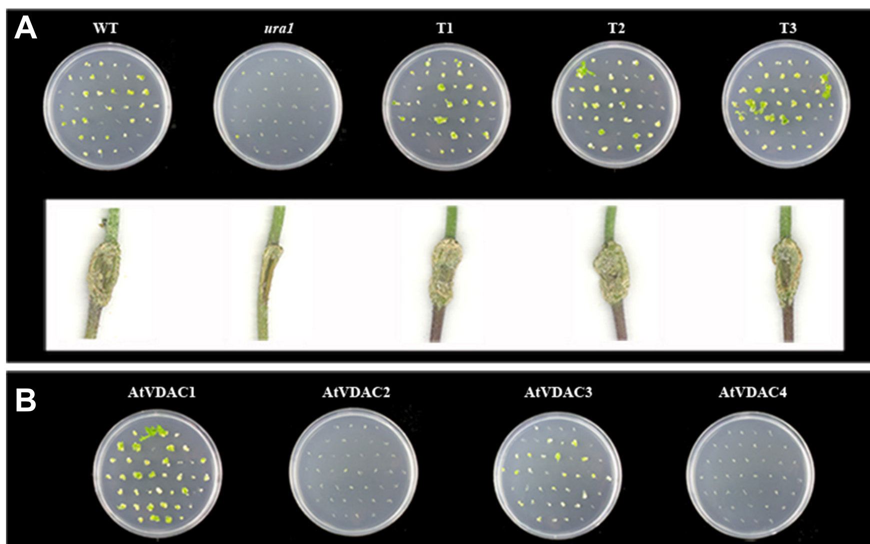

Fig. 4. Functional complementation of *ura1* mutant plants with *Arabidopsis VDAC* genes. (A) WT, *ura1* mutant, and three independent transgenic plants overexpressing *AtVDAC1* in the *ura1* mutant were tested with two different tumorigenesis assays: *in vitro* root transformation assay (top) and flower blot transformation assay (bottom). (B) Transgenic plants overexpressing each member of the *Arabidopsis VDAC* gene family in the *ura1* mutant were tested with *in vitro* root transformation assay.

root segments from wild-type, *ura1* mutant and transgenic plants with *Agrobacterium* GV3101 harboring the T-DNA binary vector pBISN1, which contains plant active *nptII* gene, and a *gus*A-intron gene under the control of a super-promoter (Narasimhulu et al., 1996). The intron of the *gusA* gene permits expression of β-glucuronidase (GUS) activity in plant cells but not in bacteria. Two days after co-cultivation, the root segments were stained for GUS activity using the chromogenic substrate X-gluc. As shown in Fig. 5, most of the root segments of wild-type plants were stained blue at both termini. In contrast, root segments of *ura1* mutant plants showed significantly reduced levels of X-gluc staining. GUS activity detected early after

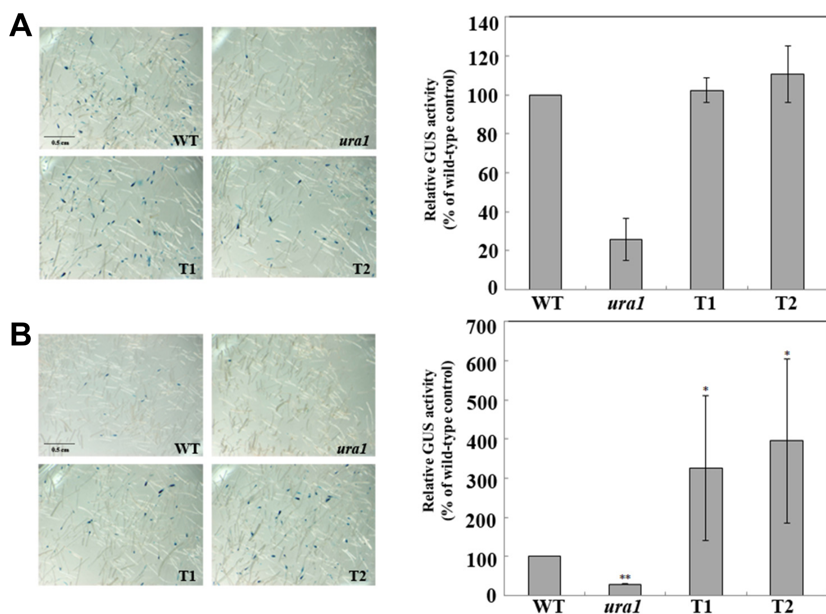

Fig. 5. Overexpression of *AtVDAC1* enhanced transient T-DNA gene expression. Sterile root segments of WT, *ura1* mutant and transgenic lines overexpressing *AtVDAC1* were infected with *Agrobacterium* GV3101 containing pBISN1. Standard (A; OD_{600} = 1.0) and diluted *Agrobacterium* suspensions (B; OD_{600} = 0.1). Two days after co-cultivation, the root segments were stained with X-gluc and the percentage of root segments showing GUS activity was determined. Asterisks indicate a significant difference from WT plants using a *t*-test (*p < 0.05, **p < 0.01). Bar = 0.5 cm.

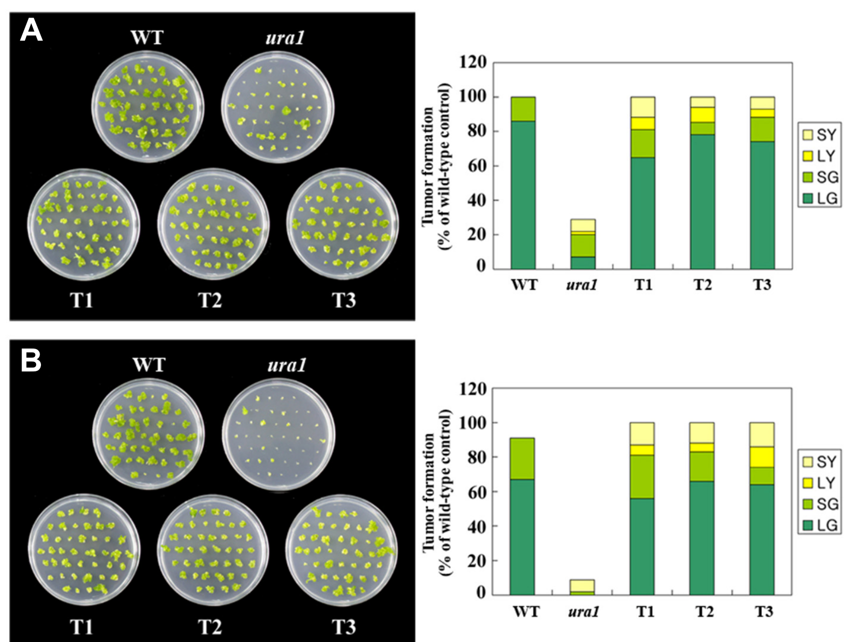

Fig. 6. The effect of *AtVDAC1* overexpression on stable tumor formation. Sterile root segments of WT, *ura1* mutant, and transgenic lines overexpressing *AtVDAC1* were infected with *A. tumefaciens* A208 (A; OD_{600} = 1.0) and diluted *Agrobacterium* suspensions (B; OD_{600} = 0.1). Tumor formation was scored 4 weeks after infection and is presented as the percentage of root segments that produced tumors. Tumors were morphologically scored as large green (LG), small green (SG), large yellow (LY), or small yellow (SW).

infection most probably represents transient T-DNA gene expression, which is not yet integrated into the plant genome (Nam et al., 1997). Thus, *ura1* mutant plants might be defective during the early stage(s) of *Agrobacterium*-mediated transformation. Defective transient T-DNA gene expression in the *ura1* mutant was completely restored in transgenic plants that overexpress *AtVDAC1* (Fig. 5A). Moreover, diluted *Agrobacteria* solution (OD_{600} = 0.1) as well as a standard bacterial solution (OD_{600} = 1.0) to distinguish the effects of *AtVDAC1* overexpression on *Agrobacterium* infection was used. The efficiency of transient T-DNA gene expression in the root segments of transgenic plants overexpressing *AtVDAC1* was 4-fold higher than that in the wild-type plants (Fig. 5B). Interestingly, tumors representing stable transformation induced on the root segments of these transgenic plants were slightly larger and teratoma-type compared to those of wild-type plants. However, the effect of *AtVDAC1* overexpression on stable tumor formation was not as drastic as that of transient gene expression (Fig. 6). These data indicate that the AtVDAC1 protein determines how competent *Arabidopsis* plants are to *Agrobacterium*-mediated transformation by enhancing T-DNA transfer and transient T-DNA expression, but not T-DNA integration into the host genome.

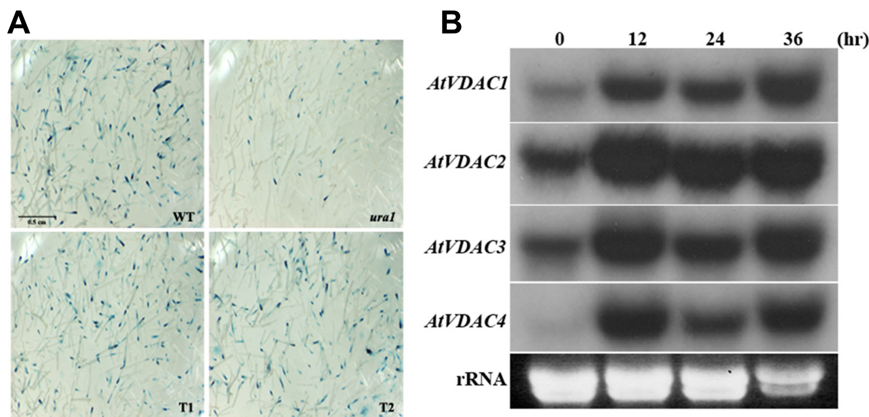

Fig. 7. Effects of phytohormone pretreatment on transient T-DNA gene expression and expression profiles of *AtVDAC* gene family. (A) Sterile root segments of WT, *ura1*mutant, and transgenic lines overexpressing *AtVDAC1* were incubated on callus inducing medium (CIM) containing phytohormones for 2 days. As described in Materials and Methods, root segments were inoculated with Agrobacterium GV3101containing pBISN1. Two days after co-cultivation, the root segments were stained with X-gluc to determine the efficiency of transient GUS expression. Bar = 0.5 cm. (B) Total RNAs were isolated from whole *Arabidopsis* WT plants cultivated in CIM for indicated periods. RNA blot analysis was conducted using each *AtVDAC* full length cDNA as a probe.

Effect of phytohormone treatment on competence to *Agrobacterium* infection is compromised in *ura1* mutant

In general, competence to *Agrobacterium*-mediated transformation can be enhanced by phytohormone treatment and wounding (Geier and Sangwan, 1996; Sangwan et al., 1991; 1992). Pre-incubation of root segments from wild-type and transgenic plants overexpressing *AtVDAC1*on callus inducing medium (CIM) for 2 days before co-cultivation with *Agrobacterium* drastically increased transient GUS gene expression (Fig. 7A). However, the effect of phytohormones treatment on the transient GUS expression of root segments of *ura1* mutant plants was relatively minor, indicating that AtVDAC1 protein is required to enhance competence to *Agrobacterium* following phytohormone treatment (Fig. 7A). Moreover, RNA blot analysis using total RNA obtained from wild-type plants and pre-incubated on CIM for various periods of time showed that the patterns of *AtVDAC* gene transcription significantly increased in a time-dependent manner in response to phytohormone treatment (Fig. 7B). Taken together, these data demonstrate a strong correlation between *AtVDAC1* gene expression and early T-DNA gene expression, suggesting that AtVDAC1 plays an important role in modulating competence to *Agrobacterium* infection.

DISCUSSION

Agrobacterium-mediated plant genetic transformation depends on both the activity of bacterial virulence proteins, and the activity of diverse host cellular proteins. Many plant factors are thought to be involved in the process of *Agrobacterium*-mediated transformation including bacterial attachment to the plant cell surface, transfer of T-strands of the bacteria to plant cells across the plant cell wall and membrane, transport of the T-complex to the plant nucleus, and stable T-DNA integration and expression in the host plant genome (Gelvin, 2012). Following the isolation of *rat* mutants using a forward genetic screening approach, many plant genes have been investigated in association with *Agrobacterium* infection using yeast two-hybrid, reverse genetics, and genome wide transcriptome analysis (Anand et al., 2007a; 2007b; Crane and Gelvin, 2007; Ditt et al., 2006; Lee et al., 2009; Nam et al., 1999; Tzfira et al., 2001; Vinna et al., 2003). In this study, several *ura* mutants from the T-DNA insertion *Arabidopsis* mutant pools that were resistant to *Agrobacterium* infection were identified. It was discovered that *AtVDAC1* gene deficiency in *ura1* mutants had drastic effects on *Agrobacterium*-mediated tumorigenesis and growth developmental phenotypes, and that these effects are dependent on the genetic background. The *ura1* mutant on the Ws-0 genetic background arose from the disruption of the *AtVDAC1* gene by the T-DNA insertion at the early of 6th exon region covering one-third of gene, and this loss of expression was confirmed by RNA blot analysis (Figs. 1C and 1D). However, an *AtVDAC1* gene knock-out mutant on a Col-0 genetic background (salk_034648) appears to be embryonic lethal, because no homozygous mutant plants exist (Tateda et al., 2011). The surviving *atvdac1* mutant alleles on the Col-0 genetic background have T-DNA insertions at the end of the gene, 3′UTR, and intron, generating normal or truncated transcripts that partially inhibit gene functions (Pan et al., 2014; Tateda et al., 2011). Differences in the genetic background could account for this discrepancy, and it is possible that the Ws-0 genetic background may suppress the lethal phenotype of the *AtVDAC1* gene knock-out mutation in some way. Consistent with this idea, when heterozygote plants of *AtVDAC1* gene knock-out mutant on a Col-0 genetic background were crossed with Ws-0 wild-type plants, homozygote plants of *AtVDAC1* gene knock-out mutant were found in F2 population (data not shown). The effects of the genetic background are a major concern during molecular breeding for improved crop plants. For instance, the effects of the major aluminum (Al)-tolerance locus, *AltSB*, in sorghum is diverse in near-isogenic lines derived from the cross between Al-tolerant plants and susceptible elite plants, emphasizing the importance of regulatory loci acting *in trans* derived from the donor's genetic background (Melo et al., 2013).

Among the *AtVDAC* gene family, *AtVDAC3* is evolutionally similar to the *AtVDAC1* (Supplementary Fig. S1) complemented *ura1* mutant phenotype, but not *porin1*-deficient yeast strain, indicating that the functional redundancy of *AtVDAC3* and *AtVDAC1* is specific for *Agrobacterium*-mediated plant transformation (Figs. 2 and 3). Interestingly, both AtVDAC1 and AtVDAC3 proteins carry the conserved mitochondrial porin signature (MPS; PROSITE Pattern ID: PS00558) motif, suggesting that they are functionally distinct from AtVDAC2

and ATVDAC4, which carry a diverse MPS motif (Tateda et al., 2011). This functional redundancy may explain why *ura1* mutant did not show a complete resistance phenotype to *Agrobacterium* infection. However, *AtVDAC3* gene knock-out plants (SAIL_238_A01) in a Col-0 genetic background showed a normal response to *Agrobacterium* infection, suggesting a minor contribution of AtVDAC3 to *Agrobacterium*-mediated plant transformation (Supplementary Fig. S2). The double mutant, *AtVDAC1* and *AtVDAC3*, is predicted to show strong resistance to *Agrobacterium* infection.

Studies have shown that high competence of explants resulting from appropriate phytohormone treatment can enhance the early stage of *Agrobacterium* attachment, T-DNA transfer into plant cells, and transient T-DNA expression, but cannot enhance the stable transformation efficiency due to other rate-limiting steps, such as T-DNA integration and stable expression (Chateau et al., 2000; De Buck et al., 2000). Pre-treatment of root bundles of the *Arabidopsis* ecotype Ws-0, which is a relatively easily transformed ecotype, with phytohormones for 2 days before co-cultivation with *Agrobacterium* significantly increased transient GUS gene expression, but not in *ura1* mutant (Fig. 7A), supporting the idea that *AtVDAC1* acts downstream of phytohormone treatment to regulate competence to *Agrobacterium* infection. Consistent with this, expression of *AtVDAC1* and *AtVDAC3* were increased following treatment with phytohormones, which is a preferential environment for *Agrobacterium* infection in nature (Fig. 7B).

Because the competence of plant cells to *Agrobacterium* infection is tightly linked to the maintenance of the metabolically active stage of cells, mitochondria need to transport a wide variety of charged solutes across membranes to generate sufficient ATP (Sangwan et al., 1992). VDACs are known to play a crucial role in the regulation of metabolic and energetic functions of mitochondria. Therefore, it is assumed that *AtVDAC1* knock-out plants would exhibit reduced mitochondrial metabolism, which is responsible for the observed phenotypes of the *ura1* mutant. Consistent with this hypothesis, the callus growth rate of root segments from *ura1* mutant plants on CIM in the absence of selection was mildly retarded, when compared to that of the wild-type and transgenic plants overexpressing *AtVDAC1* (Supplementary Fig. S3). Similarly, silencing *HcVDAC1* expression in human cells using shRNA resulted in reduced ATP production and decreased cell growth (Abu-Hamad et al., 2006). It is also possible to speculate that each VDAC isoform has distinct roles in diverse cellular reactions including metabolite flux, apoptosis, receptor binding, and cytoskeleton (Shoshan-Barmatz et al., 2010). Mitochondria play a crucial role in the apoptotic cell death of mammalian cells by releasing the apoptosis-inducing factor, cytochrome c (Cyt c), into the cytoplasm, where it activates caspases. VDACs are involved in regulating mitochondrial membrane permeability during apoptosis. Anti-apoptotic proteins close the VDAC, whereas pro-apoptotic members interact with the VDAC to generate a protein-conducting channel through which Cyt c can pass (Tsujimoto and Shimizu, 2002); however, their roles in the regulation of outer membrane permeability remain to be elucidated. Baines et al. (2007), using three single VDAC knock-out mice, demonstrated that the VDACs are dispensable for mitochondrial-dependent cell death. Furthermore, the HsVDAC2 isoform specifically inhibits mitochondrial apoptosis. Cells deficient in HsVDAC2, but not cells lacking the more abundant HsVDAC1, were more susceptible to apoptotic cell death due to the lack of protein suppressing apoptotic protein BAK activity (Cheng et al., 2003). In addition, since maize suspension cultures undergo apoptosis in response to *Agrobacterium* infection, suppression of apoptosis in maize cells expressing anti-apoptotic genes from baculovirus, *p35* and *iap,* significantly enhances the efficiency of *Agrobacterium*-mediated transformation (Hansen, 2000). Thus, the improved mitochondrial function observed following *AtVDAC1* overexpression can compensate for higher levels of ATP production which is necessary for cellular homeostasis and may protect plant cells from apoptotic cell death by avoiding the generation of ROS from organelles under stress conditions. As a result, overexpression of the *AtVDAC1* gene may enhance the competence of *Arabidopsis* roots to *Agrobacterium* infection by suppressing apoptotic stress.

Note: Supplementary information is available on the Molecules and Cells website (www.molcells.org).

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