Short Communication

Cellular and developmental function of ACAP type ARF-GAP proteins are diverged in plant cells

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Abstract   Vesicle transport is crucial for various cellular functions and development of multicellular organisms. ARF-GAP is one of the key regulators of vesicle transport and is diverse family of proteins. *Arabidopsis* has 15 ARF-GAP proteins and four members are classified as ACAP type ARF-GAP proteins. Our previous study identified that VASCULAR NETWORK DEFECTIVE3 (VAN3), an ACAP ARF-GAP, played crucial roles in leaf vascular formation. However, it remains question how other members of plant ACAP ARF-GAPs function in cellular and developmental processes. To characterize these, we analyzed spatial expression pattern and subcellular localization of VAN3 and three other ACAPs, so called VAN3-like proteins (VALs). Expression pattern analysis revealed that they were expressed in distinctive developmental processes. Subcellular localization analysis in protoplast cells indicated that in contrast to VAN3, which localizes on *trans-*Golgi networks/early endosomes (TGNs/EEs), VAL1 and VAL2 were localized on ARA6-labelled endosomes, and VAL3 resided mainly in the cytoplasm. These results indicated that VAN3 and VALs are differently expressed in a tissue level and function in different intracellular compartments, in spite of their significant sequence similarities. These findings suggested functional divergence among plant ACAPs. Cellular localizations of all members of animal ACAP proteins are identical. Therefore our findings also suggested that plant evolved ACAP proteins in plant specific manner.

Key words: ACAP, ARF-GAP, VAN3, VAL.

The trafficking of proteins by vesicle transport is essential for all eukaryotic cells. Membrane bound transport vesicles carry cargo proteins from one compartment to another, and discharge the cargos into a specific compartment by fusing with the target membrane. Formation of transport vesicles and selection of cargos from donor membrane are regulated by the adenosine diphosphate (ADP)-ribosylation factors (ARFs) (Donaldson and Klausner, 1994; Moss and Vaughan, 1995, 1998). ARF proteins function through a cycle of GTP-binding and GTP-hydrolysis, leading to the GTP-bound active form and GDP-bound inactive form of the proteins, respectively. Consistent with the cyclical nature of ARF action, ARF locked in either GTP- or GDP-bound forms blocks membrane traffic (Dascher and Balch 1994; Peters et al. 1995; Zhang et al. 1994). Because the GTPase cycle is critical for ARF function, a regulatory mechanism for ensuring the exchange between GTP-binding and GTP-hydrolysis of ARF protein appropriately is required for constitutive membrane traffic. These regulations are achieved through the ADP-ribosylation factor guanine nucleotide exchange

factors (ARF-GEFs) which promote the formation of ARF-GTP and ADP-ribosylation factor GTPse activating proteins (ARF-GAPs) which recognize ARF-GTP and induce hydrolysis of GTP (Naramoto et al. 2010, 2014; Nie et al. 2003a).

The ARF-GAPs are a large family of proteins named for the ability to induce the hydrolysis of GTP bound to ARF to GDP. In mammals, sixteen ARF-GAPs have been identified and the ARF-GAP proteins have been categorized into three groups; ARF-GAP1 type, Git type, and AZAP type (Randazzo and Hirsch, 2004). The ARF-GAP1 type has the ARF-GAP domain at the immediate N terminus, while Git type, in addition to N-terminal GAP domain, has a unique C-terminal targeting domain that was composed of three ankyrin (ANK) repeats followed by the Spa-homology domain (SHD) and paxillin binding site (PBS). The third group, the AZAP type, contains Bin-Amphiphysin-Rvs (BAR) domains and pleckstrin homology (PH) domains at the immediate N-terminal of the ARF-GAP domain and ANK repeats at the immediate C-terminal. Each of these is further subdivided based on additional domains.

Whole genome sequences revealed that *Arabidopsis* genome encodes fifteen ARF-GAPs, which can be classified into two groups; ARF-GAP1 type and AZAP type based on the similarity to mammalian ARF-GAP proteins. There are no Git type ARF-GAP proteins in *Arabidopsis*. From the literature of animals, AZAP type ARF-GAP proteins were divided into four subgroups, ACAPs, ASAPs, AGAPs, and ARAPs. The acronym refers to the domains within the proteins; AZAP for ARF GAP with ANK repeats and PH domains. The "Z" stands for domain that characterizes each subclass. ACAPs (coiled-coil domains) are localized on plasma membrane (PM)/endosome (Jackson et al. 2000), ASAPs (SH3 domain) also on PM/endosome (Randazzo et al. 2000), AGAPs (GLD domain) on lysosome/endosome (Nie et al. 2002, 2003b), and ARAPs (Rho GAP domain) on trans Golgi/TGN/PM (Miura et al. 2002) respectively, while all of the ARF-GAP1 type was localized are *cis* Golgi. Because single ARF protein functions at multiple organelles, intracellular site of ARF-GAP protein action plays a pivotal role in conferring the site specificity of ARF function. Based on these results, AZAP type ARF-GAP proteins were believed to be key players of regulating the membrane traffic of post Golgi transport pathway. Interestingly, however, there are no AZAP type ARF-GAP proteins other than ACAPs in plants. In *Arabidopsis*, there are four members of ACAPs, and one of them has been characterized as VAN3 that is involved in leaf vascular continuity (Aihara et al. 2014; Koizumi et al. 2005; Naramoto et al. 2009; Sieburth et al. 2006). VAN3 was localized on *trans* Golgi networks/early endosomes (TGNs/EEs), unknown organelles, and the plasma membrane (PM) in *Arabidopsis* cells, which presumably function together with ARF-GEF GNOM that is localized at Golgi apparatus and PMs (Koizumi et al. 2005; Naramoto et al. 2009, 2010, 2014). The *van3* single mutant caused discontinuity of venation without affecting the continuity of primary veins, whereas quadruple mutation in *VAN3* (At5g13300) and its homologus *VAN3-like protein1* (*VAL1*) (At5g61980), *VAL2* (At1g60860) and *VAL3* (At1g10870) caused discontinuity of primary veins (Naramoto et al. 2010; Sieburth et al. 2006). These findings suggest the existence of functional redundancy in vascular formation among ACAPs in *Arabidopsis*. Although these previous works gave some conceptual framework of cellular and developmental function of plant ACAP proteins, subcellular localization of VAL proteins as well as the spatial expression patterns of *VALs in planta* are not reported yet.

To clarify these, we initially analyzed the spatial expression pattern of plant ACAPs. We studied the expression patterns of *VAL* genes in shoots and roots to further confirm the involvement of VALs in the vascular formation. As an index of root primodium development,

we observed the developmental stage of lateral roots. For these purposes, we produced transgenic plants harboring GUS gene fused to a 2 kb upstream promoter region of each *Arabidopsis* ACAP type ARF-GAP gene. Ten independent transgenic lines were analyzed for each promoter::GUS fusion, and the expression patterns observed in the majority of the lines were shown. Detailed information of materials and methods in these experiments are shown in Supplemental data.

As we reported previously *pVAN3::GUS* expression was broadly detected throughout the plant body with stronger expression in vascular cells and its staining was gradually restricted to vascular cells in mature shoots and roots (Figure 1A, E, I, L, and Figure 2A, E) (Naramoto et al. 2009). In contrast to the ubiquitous expression of the *VAN3*, *pVAL1::GUS* expression was detected in the extremely restricted tissues of plants (Figure 1D). *pVAL1::GUS* expression was confined to stipules (Figure 1D, H) in aerial parts, and to QC cells and columella root cap cells in the underground (Figure 2H). The gradient in GUS activity with its maximum at the primordium root tip was gradually established during its development (Figure 2D, H). GUS staining patterns of transgenic *Arabidopsis* harboring the *pVAL2::GUS* construct showed preferential staining patterns in vascular cells throughout the plant body (Figure 1B). *pVAL2::GUS* was expressed ubiquitously in the leaf at the early stage of its development with a feature of strong GUS activity in the vasculature (Figure 1M). As the leaf developed, GUS activity was preferentially restricted to the vascular tissues (Figure 1B, F, J, M). GUS staining was also observed in developing trichomes and guard cells (Figure 1J, M). In the lateral root, throughout its development, strong GUS activity was observed in stele cells, whereas weak GUS activity was observed in the root cap (Figure 2B, F). Finally, we analyzed the expression pattern of *pVAL3::GUS*. *pVAL3::GUS*, similarly to *pVAN3::GUS*, was broadly expressed throughout the plant (Figure 1C). In leaves, *pVAL3::GUS* was ubiquitously expressed throughout its developmental process with a slight increase of GUS activity in trichomes and guard cells (Figure 1C, G, K, N). In the lateral root, GUS activity was detected from the earliest stages. Later, its activity was detected exclusively in primordium margins (Figure 2C). After the lateral root emerged, GUS activity was broadly detected in the regions other than meristematic cells (Figure 2G). These analyses identified that expression patterns of *VAN3*, *VAL2*, and *VAL3* were overlapped in leaf vascular cells although their spatial expression patterns were in overall differently regulated in tissue specific manner.

Therefore, VAL2, VAL3, and VAN3 may redundantly regulate vascular formation. However, as it was reported previously, *van3val2* and *van3val3* double mutants as well as *van3val2val3* triple mutants did not enhance
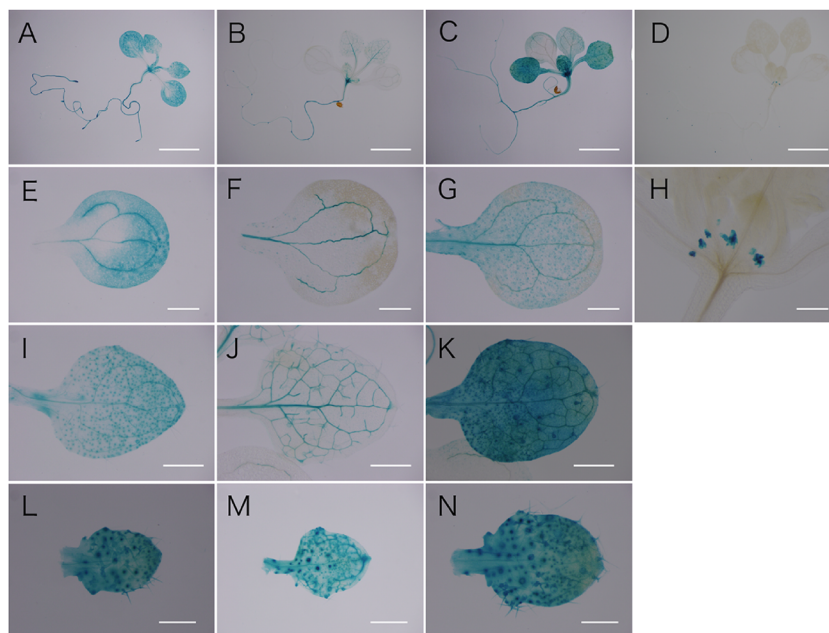
Figure 1. Expression patterns of the *VAN3* and the *VAL* genes in leaves and cotyledons of 11 day old *Arabidopsis* seedlings. Histochemical localization of *pVAN3::GUS* (A, E, I, L), *pVAL2::GUS* (B, F, J, M), *pVAL3::GUS* (C, G, K, N), and *pVAL1::GUS* (D, H) activities were examined in aerial part of 11-day-old *Arabidopsis* seedlings. Seedlings (A–D), the cotyledon (E–G), the first rosette leaf (I–K), and the third rosette leaf (L–N). Scale bars: 4 mm (A–D), 1 mm (E–G and I–K), 400 µm (H), and 250 µm (L–N).
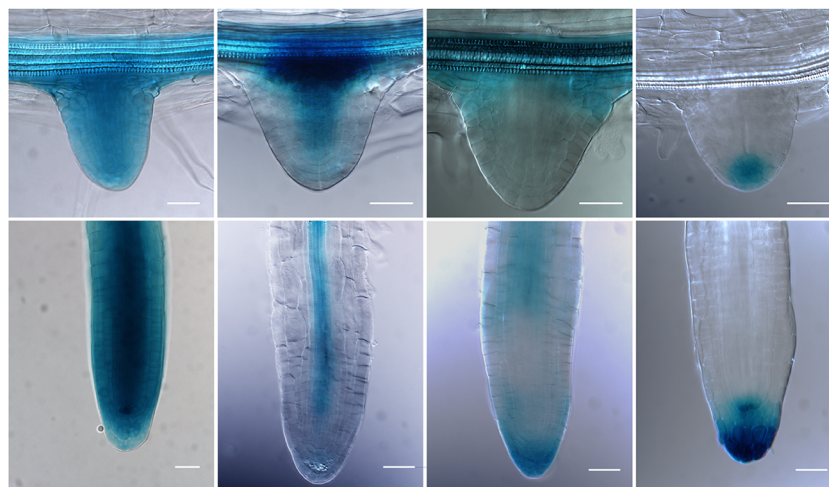

Figure 2. Expression patterns of the *VAN3* and the *VAL* genes during lateral root formation. Histochemical localization of *pVAN3::GUS* (A, E), *pVAL2::GUS* (B, F), *pVAL3::GUS* (C, G), and *pVAL1::GUS* (D, H) activities were examined in lateral roots of 11-day-old *Arabidopsis* seedlings. Lateral roots during emergence (A–D), and mature lateral roots (E–H). Scale bars: 20 µm.

the vascular discontinuity of *van3* mutants. Strikingly, additional mutation in *VAL1* to the *van3val2val3* triple mutants enhanced the leaf vascular phenotypes (Naramoto et al. 2010; Sieburth et al. 2006), whereas *VAL1* did not express in leaf vascular cells (Figure 1H). Notably, *val1val2val3* triple mutants were reported to show weak but significant leaf vascular phenotypes (Sieburth et al. 2006). These findings indicated that relationship of VAN3 and VALs were complex and also imply that VALs did not necessarily function redundantly to VAN3 in the establishment of vascular continuity.

To examine whether plant ACAPs are functionally differentiated in plant cells or not, we next analyzed the subcellular localization of VAL proteins. For this purpose, we performed double labeling experiments of VALs and organelle markers. We transiently expressed a C-terminal sGFP fusion protein of VALs and a monomeric red fluorescent protein (mRFP) (Campbell et al. 2002) fusion protein of several organelle markers under the control of the CaMV 35S promoter in protoplasts prepared from *Arabidopsis* suspension cultured cells. Fluorescence of sGFP and mRFP-
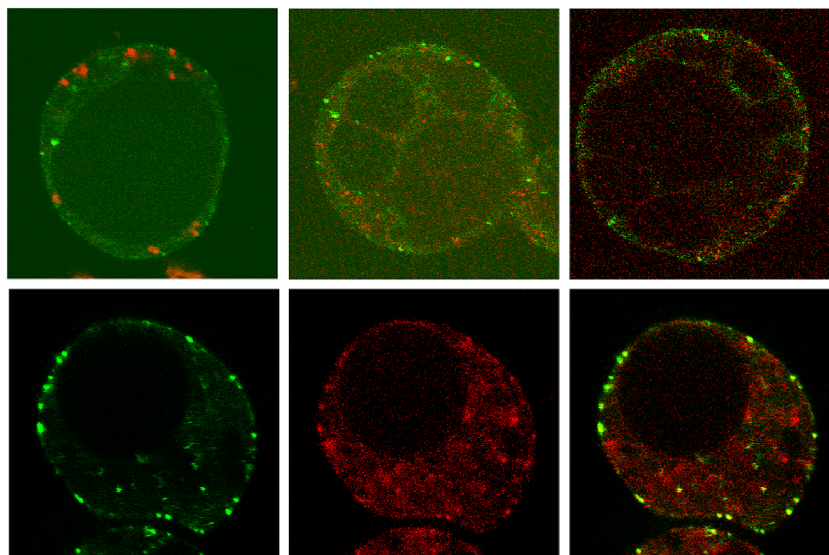
Figure 3.   Subcellular localization of sGFP-tagged VAL1 (Green) and mRFP-tagged subcellular markers (Red) in *Arabidopsis* suspension cells. (A–C) Merged image of VAL1-sGFP and the Golgi body marker ST-mRFP (A), the TGN marker mRFP-SYP41 (B), and the endosome marker mRFP-ARA7 (C). (D–F) Localization of VAL1-sGFP (D), endosome marker mRFP-ARA6 (E), and a merged image of D and E (F). Arrows indicate the VAL1-sGFP stained organelles that were not marked by the endosome marker ARA6-sGFP. Scale bars: 5 μm.
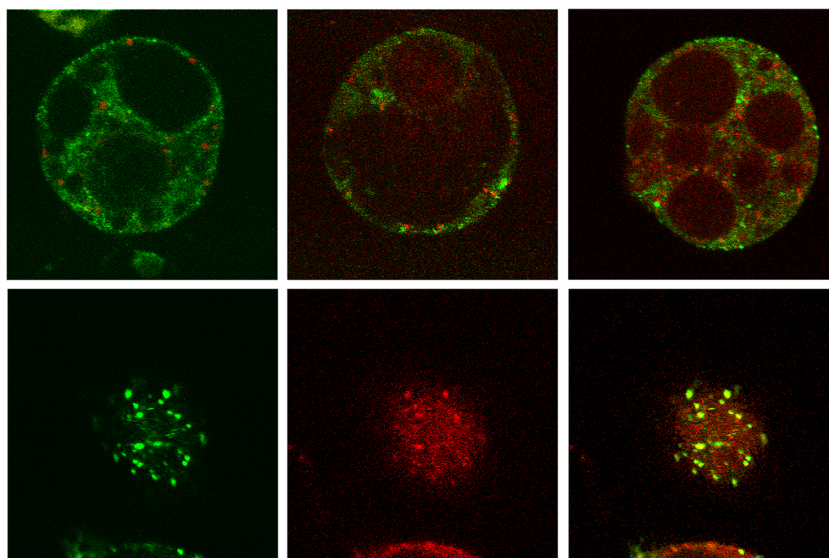

Figure 4.   Subcellular localization of sGFP-tagged VAL2 (Green) and mRFP-tagged subcellular markers (Red) in *Arabidopsis* suspension cells. (A–C) Merged image of VAL2-sGFP and the Golgi body marker ST-mRFP (A), the TGN marker mRFP-SYP41 (B), and the endosome marker mRFP-ARA7 (C). (D–F) Localization of VAL2-sGFP (D), endosome marker mRFP-ARA6 (E), and a merged image of D and E (F). Arrows indicate the VAL2-sGFP stained organelles that were not marked by the endosome marker ARA6-sGFP. Scale bars: 5 μm.
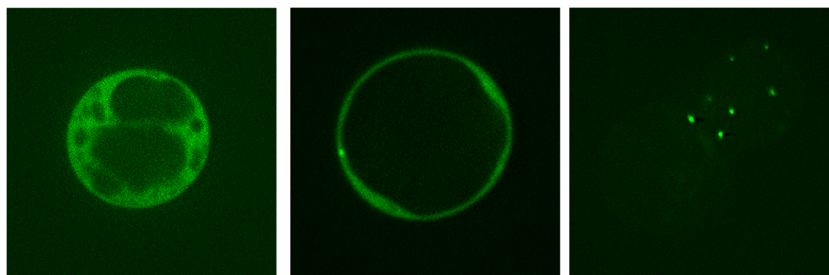

Figure 5.   Subcellular localization of sGFP-tagged VAL3 and VAN3 in *Arabidopsis* suspension cells. (A, B) VAL3-sGFP was distributed in cytosol. An arrow indicates the punctate signal of VAL3-sGFP. (C) VAN3-VENUS showed punctate structures.

fusion proteins was examined with a confocal laser scanning microscopy. Detailed information of materials and methods in these experiments are shown in Supplemental data.

First, we examined the subcellular localization of VAL1. When sGFP tagged VAL1 was expressed in *Arabidopsis* suspension cells, green fluorescent dot like structures were observed. The localization of VAL1-sGFP did not overlap with the *trans* Golgi marker ST-mRFP or the TGN/EE marker mRFP-SYP41 (Bassham et al. 2000; Jin et al. 2001; Kim et al. 2001; Wee et al. 1998; Uemura et al. 2004) (Figure 3A, B) that are consistent with previous subcellular localization analysis in tobacco epidermal cells (Yoo et al. 2008). These results indicate that VAL1 is not located on the Golgi body or TGN/EE. Next, we examined whether VAL1 was localized on the multivesicular endosomes. As endosome markers, we used mRFP-ARA7 and ARA6-mRFP (Ueda et al. 2001, 2004). We did not observe clear colocalization between VAL1-sGFP and mRFP-ARA7 (Figure 3C), whereas some population of the green fluorescent signals of VAL1-sGFP clearly overlapped with the punctate red fluorescent signals of ARA6-mRFP (Figure 3D–F). Interestingly, however, other population of the VAL1-sGFP did not overlap the red fluorescent signals of ARA6 (Figure 3F arrows). These results show that VAL1 is localized on both ARA6-mRFP stained endosomes and uncharacterized organelles.

In addition, when sGFP tagged VAL2 is expressed in *Arabidopsis* suspension cells, it was localized on ARA6-positive endosomes (Figure 4D–F), whereas it did not colocalize to the *trans* Golgi marker ST-mRFP (Figure 4A), the TGN/EE marker mRFP-SYP41 (Figure 4B), or GFP-ARA7 positive endosomes (Figure 4C). However, all population of VAL2-sGFP did not overlap with ARA6-mRFP (Figure 4F arrows). These results indicate that VAL2 is, just like VAL1, localized on both ARA6-mRFP stained endosomes and uncharacterized organelles. As it was reported previously, VAN3 is not localized on ARA6 stained endosomes but on both SYP41 stained TGNs/EEs and unknown organelles (Koizumi et al. 2005). These findings implied that the intracellular localization of VAN3 is not identical to VAL1 and VAL2 in arabidopsis suspension protoplast cells, although there still remains the possibility that some population of VAN3 colocalizes with VAL1 and/or VAL2 in uncharacterized organelles.

In contrast to the staining patterns of VAL1-sGFP and VAL2-sGFP, VAL3-sGFP showed the typical cytoplasmic distribution (Figure 5A), and sometimes dot like structures were observed (Figure 5B arrows). Because the VAL3 stained organelles are smaller than that of VAN3 (Figure 5C), VAL3 may be also localized on distinctive organelles from VAN3 stained organelles. In any case, VAL3 mainly localized at PMs, which differs from the subcellular localization of VAN3 proteins.

These results suggested that VAN3 and each of VALs regulate a distinctive intracellular membrane trafficking (Figures 3-5). Previous research on animal ACAPs indicated that both BAR domain and PH domain that recognize the membrane curvature and phospholipids, respectively can function cooperatively in its membrane targeting (Peter et al. 2004). In fact, we also showed that localization of VAN3 is regulated by BAR and PH domains (Naramoto et al. 2009). According to these reports, the difference of subcellular localization among plant ACAPs may be caused by the difference of the lipid binding specificity of PH domains or the membrane curvature sensing competence of BAR domains. The distinct subcellular localizations among plant ACAPs may contribute to divergent of developmental functions of them.

The divergent subcellular localization among plant ACAPs contrasts to the identical subcellular compartmentation of the animal ACAPs. In animals, each of the AZAP type ARF-GAP proteins, ACAPs, AGAPs, ARAPs, and ASAPs, is localized on distinct intracellular organelles and all together play indispensable roles in membrane trafficking in post Golgi transport pathway. In *Arabidopsis* genome, however, there are no AZAP type ARF-GAP proteins other than ACAPs. Therefore, plant divergent ACAPs may have evolved to compensate for the divergent functions of different AZAP type ARF-GAP subfamily members.

Now efforts are underway to further clarify the functional diversity of plant ACAPs. For this purpose, we are performing the promoter-swap experiments to VALs, in which the cDNAs of all plant ACAPs will be fused to the *VAN3* cis-regulatory sequences and introduced into the *van3* mutant, and then the complementation of the venation discontinuity will be examined. Simultaneous subcellular localization analysis of VAN3 and VAL proteins in *Arabidopsis* seedlings will be also indispensable because our transient expression system by using 35S promoter may cause their mislocalizations. Combinational analyses of these experiments will clearly indicate how molecular functions of plant ACAPs are divergent.

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