AtVAM3 is Required for Normal Specification of Idioblasts, Myrosin Cells

Haruko Ueda 1, Chiaki Nishiyama 1, Tomoo Shimada 1, Yasuko Koumoto 1, Yasuko Hayashi 2, Maki Kondo 3, Taku Takahashi 4,5, Ichiro Ohtomo 1, Mikio Nishimura 3 and Ikuko Hara-Nishimura 1, *  

1 Department of Botany, Graduate School of Science, Kyoto University, Kyoto, 606-8502 Japan  
2 Faculty of Science, Niigata University, Niigata, 950-2181 Japan  
3 Department of Cell Biology, National Institute for Basic Biology, Okazaki, 444-8585 Japan  
4 Division of Biological Sciences, Graduate School of Science, Hokkaido University, Sapporo, 060-0810 Japan

Myrosin cells in Capparales plants are idioblasts that accumulate thioglucoside glucosidase (TGG, also called myrosinase), which hydrolyzes glucosinolates to produce toxic compounds for repelling pests. Here, we show that AtVAM3 is involved in development of myrosin cells. It has been shown that yeast VAM3 is a Q-SNARE that is involved in vesicle transport of vacuolar proteins and vacuolar assembly. We found that two Arabidopsis atvam3 alleles, atvam3-3 and atvam3-4/ssm, accumulate large amounts of TGG1 and TGG2 that are enzymatically active. An immunogold analysis revealed that TGGs were specifically localized in the vacuole of myrosin cells in atvam3 mutants. This result indicates that TGGs are normally transported to vacuoles in these mutants and that AtVAM3 is not essential for vacuolar transport of the proteins. We developed a staining method with Coomassie brilliant blue that detects myrosin cells in whole leaves by their high TGG content. This method showed that atvam3 leaves have a larger number of myrosin cells than do wild-type leaves. Myrosin cells were scattered along leaf veins in wild-type leaves, while they were abnormally distributed in atvam3 leaves. The mutants developed a network of myrosin cells throughout the leaves: myrosin cells were not only distributed continuously along leaf veins, but were also observed independent of leaf veins. The excess of myrosin cells in atvam3 mutants might be responsible for the abnormal abundance of TGGs and the reduction of elongation of inflorescence stems and leaves in these mutants. Our results suggest that AtVAM3 has a plant-specific function in development of myrosin cells.

Keywords: AtVAM3 — Idioblast — Myrosin cell — Myrosinase — SNARE — TGG.

Abbreviations: At12S, Arabidopsis 12S globulin; At2S, Arabidopsis 2S albumin; AtAleu, Arabidopsis aleurain; BSA, bovine serum albumin; CBB, Coomassie brilliant blue; Col-0, Columbia; EndoH, endoglycosidase H; GC/MS, gas chromatography and mass spectrometry; GFP, green fluorescent protein; mRFP, monomeric red fluorescent protein; PBS, phosphate-buffered saline; PNGase, N-glycosidase F; RT–PCR, reverse transcription–PCR; SNARE, soluble N-ethylmaleimide-sensitive factor attachment protein receptor; TGG, thioglucoside glucosidase.

Introduction

The idioblast is a special type of cell and differs both in size and morphology from the adjacent cells. A myrosin cell is an idioblast specific to Capparales plants, which have a unique defense mechanism called the ‘myrosinase–glucosinolate system’ (Rask et al. 2000). Myrosin cells are considered to be involved in plant defense, because they accumulate thioglucoside glucosidase (TGG, also called myrosinase). TGG (EC 3.2.1.147) hydrolyzes glucosinolates into a glucose molecule and an unstable aglucone, which is converted into a final compound such as a toxic isothiocyanate or nitrile (Wittstock and Halkier 2002). Glucosinolates are enriched in S-cells (Koroleva et al. 2000). Some S-cells are found in Arabidopsis flower stalks and occur close to myrosin cells in a plant (Andreasson et al. 2001). Damage to plant tissue by insects or microorganisms releases glucosinolates that then come in contact with myrosinases. This mechanism is effective against generalists but not against some herbivores that have acquired a system to overcome it (Ratzka et al. 2002, Wittstock et al. 2004).

Myrosinases are multiply expressed in various Brassicaceae species (Rask et al. 2000). So far, three myrosinase genes have been reported in Arabidopsis: TGG1 and TGG2 are functional genes, and TGG3 is a pseudogene (Xue et al. 1995, Zhang et al. 2002). In recent years, the expression and the distribution of transcripts of TGG1 and TGG2 and their products in various organs have been examined. Using in situ hybridization, the TGG1 and TGG2 transcripts have been detected in leaves, sepals, petals and gynoeciums (Xue et al. 1995). Using reverse transcription–PCR (RT–PCR), both TGG1 and TGG2 transcripts have been detected in leaves, cotyledons, flowers and stems, and only TGG2 has been weakly detected in siliques (Zhang et al. 2002). Analysis of the TGG1 promoter showed that it is active in idioblasts of the phloem and guard cells, but inactive in roots and seeds (Husebye et al. 2002, Thangstad et al. 2004). Immunocytochemical analysis indicated that TGGs are localized exclusively in myrosin cells in the phloem parenchyma (Andreasson et al. 2001). The phenotypes of Arabidopsis mutant ssm (short stems and midribs) include wavy leaves, delayed growth and semi-dwarfism (Ohtomo et al. 2005). The ssm mutant is a single
AtVAM3 is involved in specification of idioblasts

Results

TGG1 and TGG2 are significantly accumulated in atvam3-4/ssm

The ssm mutant expressed abnormal AtVAM3 with the insertion of a 19 amino acid peptide between the SNARE motif and the transmembrane domain of AtVAM3 (Ohtomo et al. 2005). We designated ssm as atvam3-4 in this study. To clarify whether the peptide insertion into AtVAM3 affects the transport of various proteins to the vacuole, we examined the differences of various protein profiles between atvam3-4 and Col-0 (wild type). Total homogenates from Col-0 and atvam3-4 leaves were subjected to SDS-PAGE with CBB staining. Fig. 1a shows that two protein bands corresponding to 75 and 65 kDa were more abundant in atvam3-4 than in Col-0. The N-terminal sequence of these proteins revealed that the 75 kDa protein is TGG1 (At5g26000), and the 65 kDa protein is TGG2 (At5g25980) (Supplementary Fig. S1). We raised antibodies against two peptides that were specific for TGG1 and TGG2, respectively (Jahn et al. 2003). AtVAM3 was reported to be expressed in leaves, roots, inflorescence stems, flower buds and young siliques (Sato et al. 1997). AtVAM3 is localized on the vacuolar membranes in the shoot apical meristems in seedlings (Sato et al. 1997) and the pre-vacuolar compartment in the roots (Sanderfoot et al. 1999).

In this study, we found abnormal abundances of TGGs in two atvam3 mutants; one expresses a form of AtVAM3 in which an additional peptide is inserted and the other is a knockout mutant of the AtVAM3 gene. The expression and localization of TGGs in the mutants indicate that the abnormally abundant accumulation of TGGs is caused by an excess of myrosin cells. These results suggest that AtVAM3 is involved in development of myrosin cells.

Fig. 1 Identification of abnormally accumulated proteins in atvam3-4 leaves. (a) Protein profiles of wild-type (Col-0) and atvam3-4 leaves. The leaf homogenates were subjected to SDS-PAGE and subsequent CBB staining. Proteins of 75 and 65 kDa were accumulated in atvam3-4 but not in Col-0. (b) Immunoblot of Col-0 and atvam3-4 leaves with anti-TGG1 and anti-TGG2 antibodies. Extracts of 37-day-old rosette leaves were incubated in the absence (−) (lanes 1, 5, 9, and 13) or presence (+) of either Endo H (0.033 U ml⁻¹) at pH 5.5 (lanes 2, 6, 10 and 14) or PNGase F (6.7 U ml⁻¹) at pH 7.0 (lanes 4, 8, 12 and 16). Alternatively, the extract was treated with Endo H digestion followed by PNGase F (lanes 3, 7, 11 and 15). Asterisks (*) and ** indicate deglycosylated forms of TGG1 and TGG2, respectively. Molecular masses are indicated on the left in kDa.

accumulation level between atvam3-4 and Col-0 (data not shown). The immunoblot analysis revealed that the accumulation level of Arabidopsis aleurain (AtAleur), a vacuolar marker protein, was almost the same between Col-0 and atvam3-4 (Fig. 1c). Previously, we reported that atvam3-4, which has a defect in vacuolar sorting, mis-sorted the storage proteins and reduced the accumulation levels of mature storage proteins in seeds (Shimada et al. 2003a). To clarify whether a similar reduction occurs in atvam3-4, we examined the accumulation levels of mature storage proteins, 2S albumin and 12S globulin, in seeds. There were no differences in the accumulation levels between Col-0 and atvam3-4 (Fig. 1c). Recently, we found that
AtVAM3 is involved in specification of idioblasts

The accumulation levels of endomembrane proteins, RD21, γTIP, BiP and PDI (protein disulfide isomerase), in atvam3-4 are the same as in Col-0 (Ohtomo et al. 2005). These results suggest that AtVAM3 is involved in accumulation of only TGG1 and TGG2 among endomembrane proteins we examined.

TGG1 has nine potential N-glycosylation sites, whereas TGG2 has four sites (Supplementary Fig. S1). Deglycosylated polypeptides of both TGG1 and TGG2 were about 60 kDa (Fig. 1d), which is consistent with the molecular mass estimated by the amino acid sequences. The slight differences in size between endoglycosidase H (Endo H) and N-glycosidase F (PNGase) digests are due to the specificity of glycosidases, i.e. an N-acetylglucosamine unit remains linked to their asparagine residues after the Endo H treatment but not after the PNGase treatment. This result suggests that both TGG1 and TGG2 are glycoproteins with high-mannose glycans, and that the difference in molecular size between TGG1 and TGG2 is due to the difference in the number of N-glycans.

The phenotype of AtVAM3 knockout mutant atvam3-3 is similar to that of atvam3-4

We established an AtVAM3 null mutant (T-DNA-tagged knockout mutant atvam3-3), which expressed no AtVAM3 protein (Fig. 2a, c). Previously, it was reported that a T-DNA-tagged knockout mutant of AtVAM3 was lethal to the male gametophyte in the ecotype Wassilewskija (Sanderfoot et al. 2001b). However, we found that atvam3-3 in the ecotype Col-0 is viable (Fig. 2b). We compared the phenotype of atvam3-3 with that of atvam3-4. The morphological phenotypes of the atvam3-3 mutant are similar to, but weaker than, those of atvam3-4 (wavy leaves, delayed growth and semi-dwarfism) (Fig. 2b). Both TGG1 and TGG2 were more abundant in atvam3-3 than in Col-0 but less abundant than in atvam3-4 (Fig. 2c). These phenotypes were not remarkable in heterozygous plants (data not shown), indicating that the phenotypes of atvam3-3, like those of atvam3-4, are recessive (Ohtomo et al. 2005).

atvam3-4 has a 34 bp deletion in the sixth intron of AtVAM3, which causes mis-splicing of the gene (Ohtomo et al. 2005). The mis-splicing leads to the insertion of a 19 amino acid peptide between the SNARE motif and the transmembrane domain of AtVAM3 (Fig. 2c). To study the effect of the 19 amino acid insertion into AtVAM3 on its localization, we compared intracellular localizations between AtVAM3 fused to green fluorescent protein (GFP–AtVAM3) and 19 amino acid peptide-inserted AtVAM3 fused to monomeric red fluorescent protein (mRFP–AtVAM3-4) by transient expression assay with leaves. The image of GFP–AtVAM3 showed that

---

**Fig. 2** Arabidopsis AtVAM3 mutants. (a) Schematic representation of the AtVAM3 gene and the positions of mutations in the atvam3-3 and atvam3-4 alleles. The AtVAM3 gene is composed of seven exons (filled boxes) and six introns (solid lines), and AtVAM3 is composed of a SNARE motif and a transmembrane domain (TMD). atvam3-3 is a T-DNA-tagged knockout mutant. atvam3-4 lacks 34 bp in the sixth intron of the AtVAM3 gene, which is not spliced, and has AtVAM3-4 with a 19 amino acid insertion between a SNARE motif and a TMD. (b) Morphological phenotypes of 25- and 42-day-old plants of atvam3-3 and atvam3-4. (c) Immunoblot of 38-day-old leaves of Col-0 and the mutants with either anti-AtVAM3 antibody or a mixture of anti-TGG1 and anti-TGG2 antibodies. (d) Peptide-inserted AtVAM3 was fused to mRFP (mRFP–AtVAM3-4) and co-expressed with the AtVAM3 and GFP fusion protein (GFP–AtVAM3) transiently in Arabidopsis leaves. The cells were inspected with a confocal laser-scanning microscope.
AtVAM3 mutants (atvam3-4 and atvam3-3) had a large central vacuole in the leaves (Fig. 2d), although the yeast vam3 mutants accumulate numerous small vesicles instead of large vacuoles (Wada et al. 1997). The result suggests that AtVAM3 is not required for vacuolar morphology in plants. This result was also supported by electron microscopy with the mutants (Fig. 4). The image of mRFP–AtVAM3-4 was completely merged with the image of GFP–AtVAM3 in Col-0, atvam3-4 and atvam3-3. These results indicate that AtVAM3-4 is localized on the vacuoles and pre-vacuolar compartments. Images of GFP–AtVAM3 and mRFP–AtVAM3-4 also showed spherical structures (bulbs) with a double membrane within the lumen of central vacuoles as reported previously (Saito et al. 2002, Uemura et al. 2002), although green fluorescence is higher than red fluorescence. It is possible that intact AtVAM3 is more selectively concentrated on the bulb structures.

Fig. 3 Expression and accumulation of TGG1 and TGG2 in atvam3-4. (a) Organ-specific expression of TGG1, TGG2 and PYK10. Rosette leaves, roots, flower stalks, flower and siliques from 41-day-old plants, 6-day-old seedlings and dry seeds were homogenized in SDS sample buffer (5 µl mg⁻¹ FW except for 2 µl mg⁻¹ FW of roots and 50 µl per 50 grains of dry seeds). The homogenates were subjected to SDS–PAGE and subsequent immunoblot with either a mixture of anti-TGG1 and anti-TGG2 antibodies or anti-PYK10 antibody. (b) mRNA levels of the TGG1 and TGG2 genes. Total RNA from seedlings and rosette leaves of Col-0 and atvam3-4 was subjected to reverse transcription and subsequent PCR. The number of cycles of the PCR is indicated on the right. Actin was used as an internal control.

Organ-specific expression and accumulation of an abnormally large amount of TGG1 and TGG2 in atvam3-4

Fig. 3a (upper) shows the expression patterns of TGG1 and TGG2 in various organs of Col-0 and atvam3-4 on immunoblots with a mixture of anti-TGG1 and anti-TGG2 antibodies. In Col-0, TGG1 was detected strongly in rosette leaves, flowers and siliques, and slightly in flower stalks and seedlings, whereas TGG2 was detected mainly in flowers and siliques. The accumulation level of TGG2 was low in 41-day-old rosette leaves (Fig. 3a and Supplementary Fig. S2). In the atvam3-4 mutant, the organs expressing TGG1 were the same as in Col-0, but the accumulation levels were considerably higher. TGG2 accumulated in rosette leaves, flower stalks, flowers and siliques of atvam3-4, whereas it was hardly detected in rosette leaves and flower stalks in Col-0. No band was detected in roots or dry seeds of any plant. These results indicated that the aerial parts of vegetative organs in atvam3-4 accumulate abnormally large amounts of TGG1 and TGG2. In contrast, PYK10, which is a β-glucosidase possessing 40% identity to TGG1 and TGG2 at the amino acid sequence level, is expressed specifically in roots and seedlings (Matsushima et al. 2003), and the amount of accumulation of PYK10 was the same in Col-0 and atvam3-4 (Fig. 3a, lower).

The RT–PCR analysis showed that the mRNA levels of TGG1 and TGG2 were also much higher in atvam3-4 than in Col-0 (Fig. 3b). The levels of TGG1 and TGG2 mRNA in seedlings were almost the same as those in 28-day-old rosette leaves of Col-0 and atvam3-4, whereas the TGG proteins were not yet accumulated in seedlings (Fig. 3).

Developmental changes of the amounts of TGG1 and TGG2 were examined by immunoblot analysis of the extracts from rosette leaves. In Col-0, the amounts of TGG1 and TGG2 per fresh weight seemed to be constant from 10 to 31 d (Supplementary Fig. S2). In atvam3-4, the levels of TGG1 and TGG2 were constantly high from 10 to 52 d (Supplementary Fig. S2). Both mRNAs and proteins of TGGs were decreased in senescing Col-0 leaves (Fig. 3b and Supplementary Fig. S2).

Abnormal development of myrosin cells in atvam3-4

It has been shown that TGGs are localized in myrosin cells. We established a convenient way of detecting myrosin cells accumulating TGGs. Immunofluorescent analysis of thin sections of 48-day-old Col-0 rosette leaves with anti-TGG1 and anti-TGG2 antibodies identified the TGG proteins in myrosin cells. To answer
AtVAM3 is involved in specification of idioblasts

In this study, we determined the distributions of TGG1 and TGG2 in *atvam3-4* by using both immunofluorescent analysis and the CBB staining method. The fluorescent signal of anti-TGG1 antibody was significant in myrosin cells (Fig. 4h, j), which were stained with CBB (Fig. 4g, i). The CBB-positive cells had a large central vacuole, which was homogeneously filled with electron-dense material compared with the surrounding cells on the electron micrograph (Fig. 4m, n). An immunogold analysis of the serial sections showed that an electron-dense vacuole contained both TGG1 and TGG2 in *atvam3-4* (Fig. 4k, l), suggesting that TGG1 and TGG2 were normally transported to the vacuole of myrosin cells in *atvam3-4*. Cells other than myrosin cells did not accumulate TGGs in the mutant. On the other hand, the fluorescent signal of anti-TGG2 antibody was barely detected in the CBB-positive myrosin cells (data not shown) and the number of the gold particles staining for TGG2 was much lower than the number staining for TGG1 in the same cell (Fig. 4k, l). These results were inconsistent with the result from the immunoblot (Fig. 1 and 3) where almost equal levels of TGG1 and TGG2 were detected in *atvam3-4* leaves. This inconsistency might be due to the aggregation nature of TGG2, which makes the antibodies inaccessible (discussed below).

In Col-0, the myrosin cells of the vegetative organs were scattered peripherally in the phloem tissue among parenchyma cells (Fig. 4a), as previously reported (Andreason et al. 2001, Husebye et al. 2002). On the other hand, the myrosin cells seemed to be not always peripheral to leaf veins in *atvam3-4*, and the frequency of appearance of the myrosin cells was undoubtedly higher than that in Col-0 (Fig. 4a, g). We also stained whole rosette leaves with CBB, which made it possible

**Fig. 4** Localization of TGG1 and TGG2 in *atvam3-4*. Sections of rosette leaves of Col-0 (48-day-old, a–d; 26-day-old, e and f) and *atvam3-4* (48-day-old, g–n) plants were subjected to CBB staining (a, c, g, i, m), immunofluorescent analysis (b, d, h, j) and immunogold analysis (e, f, k, l) with anti-TGG1 and anti-TGG2 antibodies, and electron microscopic analysis (n). (c), (d), (i) and (j) are enlarged images of the boxed area in (a), (b), (g) and (h), respectively. Asterisks show myrosin cells. CW, cell wall; V, vacuole.
AtVAM3 is involved in specification of idioblasts to obtain a broad view of the distribution and the number of myrosin cells. At higher magnification, the CBB-positive myrosin cells were irregularly horned and/or long (Fig. 5g, j). These idioblasts were observed to be scattered at low frequency along the veins of the Col-0 leaves (Fig. 5a, d). We examined the TGG1-deficient mutant, attgg1-1, which accumulated a small amount of TGG2 in rosette leaves (Fig. 7c). Although idioblasts were found in the attgg1-1 mutant as fre-
Fig. 6  Differential distribution of TGG1 and TGG2 on subcellular fractionations. (a) Differential centrifugation of the homogenates of aerial parts of 38-day-old Col-0, attvam3-4, attgg1-1 and attgg2-1 plants. Total homogenate (T), 1,000×g pellet (P1), 8,000×g pellet (P8), 100,000×g pellet (P100) and 100,000×g supernatant (S) were subjected to SDS-PAGE followed by an immunoblot with a mixture of anti-TGG1 and anti-TGG2 antibodies. Note that the ratio of the loaded amount of each fraction of Col-0, attvam3-4, attgg1-1 and attgg2-1 was 3 : 1 : 3 : 3.  (b) A 30–60% (w/v) sucrose density gradient centrifugation of the homogenate of the aerial part of 30-day-old attvam3-4 plants. One percent (v/v) of total homogenate (T), 1% (v/v) of 1 ml fractions (1–16) and 5% (v/v) of the pellet (P) were subjected to SDS-PAGE followed by an immunoblot with a mixture of anti-TGG1 and anti-TGG2 antibodies.

Fig. 7  Thioglucosidase activities in attvam3-4. (a, b) Thioglucosidase activities in the aerial part of 22-day-old plants of Col-0, attvam3-4, attgg1-1 and attgg2-1. Thioglucosidase activity was measured using sinigrin as a substrate. The concentrations of released glucose were quantified by the glucose oxidase–peroxidase method. Symbols used are: Col-0 (filled triangles), attvam3-4 (filled circles), attgg1-1 (open circles) and attgg2-1 (open squares). (c) Immunoblot of the enzyme solution used in (a) and (b) with a mixture of anti-TGG1 and anti-TGG2 antibodies.

Differential behavior on subcellular fractionation between TGG1 and TGG2

The immunohistochemical analyses suggested that the behavior of TGG2 is different from that of TGG1 (Fig. 4). To elucidate the behavior of TGG1 and TGG2, the plant homogenate was fractionated by subcellular fractionation and then the distribution of TGG1 and TGG2 in each fraction was examined by an immunoblot analysis using a mixture of anti-TGG1 and anti-TGG2 antibodies.

In Col-0, most of the TGG1 was fractionated into the soluble fraction (S), whereas about half of the TGG2 was fractionated into the S fraction and the other half was fractionated into the P1 and P100 fractions (Fig. 6a), indicating that TGG1 and TGG2 behaved differently on subcellular fractionation. On the other hand, in attvam3-4, TGG1 behaved in the same way as it behaved in Col-0, but TGG2 did not. A large amount of the TGG2 in attvam3-4 was detected in the P1 and P8 fractions. We also investigated both attgg1 and attgg2 mutants. Interestingly, most TGG2 in attgg1-1 was detected in the P1 fraction and the rest was detected in the P8 fraction. TGG1 in attgg2-1 behaved in the same way that it behaved in Col-0 and attvam3-4. These results indicate that the behavior of TGG1 is constant in Col-0, attvam3-4, attgg1 and attgg2, whereas the behavior of TGG2 is affected by the ratio of TGG1 to TGG2.

We also performed the sucrose density gradient centrifugation with the extract from attvam3-4 (Fig. 6b). A considerable amount of TGG2 was distributed among fractions 2–16 and in the pellet. This was in agreement with the findings of differential centrifugation analysis (Fig. 6a). These results suggest two possibilities. First, TGG2 easily forms aggregates, although TGG1 does not. Secondly, TGG2 localizes in high-density organelles, although such organelles were not detected in myrosin cells and other cells by electron microscopy (Fig. 4).

attvam3-4 accumulates enzymatically active TGGS

To determine whether the abnormally abundant TGG1 and TGG2 in the mutants are enzymatically active, we measured thioglucosidase activities in extracts of Col-0, attvam3-4, attgg1-1 and attgg2-1 using sinigrin as a substrate. Fig. 7a shows that the substrates were rapidly depleted and the amount of released glucose was saturated in the reaction mixture of attvam3-4 after ~15 h incubation. On the other hand, the amount of glucose still increased linearly in the reaction mixtures of Col-0 and attgg2-1 during the 48 h incubation. The specific activity determined for attvam3-4 was about 6.6 times higher than the specific activity for Col-0 (Fig. 7b). The spe-
AtVAM3 is involved in specification of idioblasts

AtVAM3 is involved in specification of idioblasts 171 specific activity determined for *attgg1-1* was about 8% of that for Col-0, indicating that most of the thioglucosidase activity in Col-0 is responsible for TGG1. These results were in good agreement with the proportion of TGGs estimated by the immunoblot analysis (Fig. 7c), suggesting that *atvam3-4* accumulates enzymatically active TGGs.

**Estimation of endogenous substrates of TGG1 and TGG2**

We analyzed the volatile substances produced from homogenates of Col-0 and *atvam3-4* plants by gas chromatography and mass spectrometry (GC/MS) to estimate endogenous substrates for TGGs. Col-0 and *atvam3-4* gave peaks 1–6 of isothiocyanates and nitriles, which might be the products of the glucosinolate hydrolysis (Fig. 8a, b). The signals of peaks 1–4 and 6 in *atvam3-4* were slightly higher than those in Col-0, and additional peaks 7–10 were found in *atvam3-4* (Fig. 8a, b). These results were consistent with the finding that *atvam3-4* exhibited a higher TGG activity toward glucosinolates than Col-0 (Fig. 7). The major volatile product (peak 5) was estimated to be 5-[methylsulfanyl]pentanenitrile (Fig. 8), which might be derived from 4-methylthiobutyl glucosinolate called glucorucin. Glucoerucin is a major glucosinolates in rosette leaves of Col-0 (Petersen et al. 2002, Brown et al. 2003). Both 5-methylhexanenitrile (peak 1) and 1-isothiocyanato-4-methylpentane (peak 4) might be derived from 4-methylpentyl glucosinolate, while both heptanenitrile (peak 2) and 1-isothiocyanatohexane (peak 9) might be derived from hexyl glucosinolates (Fig. 8). This result suggests that these glucosinolates produce both nitrile and isothiocyanate under this condition. It was reported that which product is produced from a glucosinolate depends not only on the structure of the glucosinolate side chains, but also on the plant species and reaction conditions such as pH (Wittstock and Halkier 2002). Possible endogenous substrates for TGGs to produce volatile substances include 4-methylthiobutyl-, 4-methylpentyl-, hexyl- and 3-methylbutyl-glucosinolates.

**Discussion**

**A novel function for AtVAM3 in development of a special type of cells**

The developmental mechanism of myrosin cells is not known. In this study, we found that *atvam3* mutants have a larger number of myrosin cells than do wild-type plants. Why is the number of myrosin cells increased in *atvam3* mutants? In the mutants, the myrosin cells do not exhibit a disorganized distribution in leaves. Myrosin cells appear to form a continuous network all over the mutant leaves, while they are scattered along leaf veins in wild-type leaves (Fig. 5). This observation suggests that the increased number of myrosin cells is caused by accelerated cell differentiation or cell division in the mutants. Differentiated myrosin cells may release substances that prevent the surrounding cells from being differentiated to myrosin cells. It is likely that AtVAM3 serves as a negative regulator for the cell differentiation or the cell division of myrosin cells.

Here we show that AtVAM3 is required for normal specification of myrosin cells, which are idioblasts in *Capparales* plants. Idioblasts, which are special type of cells and differ both in size and morphology from the adjacent cells, are distributed in leaves of most dicot plants. It is possible that VAM3 is involved in differentiation of some idioblasts, as is AtVAM3. AtVAM3 will provide a clue to clarify the molecular
mechanism underlying development of a specific type of the cells.

AtVAM3 and vesicle transport of vacuolar proteins

Yeast Vam3p has been shown to be required for vacuolar assembly and vesicle transport to the vacuoles (Darsow et al. 1997; Wada et al. 1997). AtVAM3 was proposed to be involved in vacuolar assembly (Sato et al. 1997). However, atvam3 mutants had a large central vacuole in the plants (Fig. 2, 4), indicating that AtVAM3 is not essential for formation of a central vacuole. Previously, Sanderfoot et al. (1999) reported that AtVAM3 is localized in pre-vacuolar compartments and interacts with AtVTI11 (AtVTI1a, Qk-SNARE). They suggest a role for AtVAM3 in post-Golgi trafficking. We examined the transport of two vacuolar proteins, RD21 (Ohtomo et al. 2005) and AtAIEu (Fig. 1c). The vacuolar proteins are synthesized as precursors and then transported to the vacuoles, where the precursors are converted to produce their mature forms (Yamada et al. 2001, Holwerda et al. 1992). The fact that there was no accumulation of the precursor proteins in the atvam3-4 mutant shows that the vacuolar transport of the proteins is normal in the mutant. Both TGG1 and TGG2 were also transported to the atvam3-4 vacuoles (Fig. 4k, l). These results indicate that AtVAM3 is not essential for vacuolar transport of at least these four proteins. Further experiments are necessary to demonstrate the involvement of AtVAM3 in vesicle transport of vacuolar proteins in plants.

Why atvam3-3 shows weaker phenotypes than atvam3-4

The morphological phenotypes and the abnormal abundances of TGG1 and TGG2 were more conspicuous in atvam3-4 than in atvam3-3. atvam3-4 has a form of AtVAM3 that contains an additional peptide, whereas atvam3-3 is a null mutant of the AtVAM3 gene (Fig. 2c). The weak phenotypes in atvam3-3 may be due to the presence of an alternative Qk-SNARE that can function in a SNARE complex to a certain extent in place of AtVAM3. A candidate for the alternative Qk-SNARE is AtPep12, because (i) it shows a high similarity to AtVAM3 at the amino acid sequence level; (ii) it is partially co-localized with AtVAM3 (Uemura et al. 2004); and (iii) it interacts with AtVTI11 (Qk-SNARE) and SYP5 (Qk-SNARE) (Sanderfoot et al. 2001b). In yeast, it was reported that Vam3p and Pep12p can partially substitute for one another (Darsow et al. 1997). AtPEP12 might compensate for a lack of AtVAM3 in atvam3-3. On the other hand, it is possible that the modified AtVAM3 protein (AtVAM3-4) inhibits the compensation by AtPEP12, leading to conspicuous phenotypes in atvam3-4.

There are a few reports about how the peptide insertion into SNARE molecules affects their role in the vesicle fusion. The in vitro fusion assay showed that the precise distance between the SNARE motif and the transmembrane domain of yeast Vam3p (Wang et al. 2001) or mouse VAMP2 (McNew et al. 1999) was critical for vesicle fusion. In Vam3p, insertion of only three amino acid residues after the SNARE motif significantly impaired fusion, and insertion of 12 residues abolished fusion (Wang et al. 2001). The insertions did not alter the ability of yeast Vam3p to associate with other components to form a SNARE complex (Wang et al. 2001). These reports suggest that the modified AtVAM3 (AtVAM3-4) in atvam3-4 interacts with AtVTI11 and SYP5 to form a SNARE complex but has no function in vesicle fusion.

Two more atvam3 alleles (vam3-t/atvam3-1 and sgr3-1) were reported. vam3-t/atvam3-1 mutation produces AtVAM3 that has a 21 amino acid deletion of the SNARE motif of AtVAM3 (Ohtomo et al. 2005), and the sgr3-1 mutation, which has a defect of shoot gravitropism, has a single amino acid substitution (E182K) just before the SNARE motif (Yano et al. 2003). In vam3-t/atvam3-1, we observed the morphological phenotypes (Ohtomo et al. 2005) and the abnormal accumulation of TGGs because of an increased number of myrosin cells (data not shown). However, unexpectedly, sgr3-1 did not accumulate TGGs abnormally, as shown in the above atvam3 mutants (data not shown).

TGG1 and TGG2 in Arabidopsis plants

Previous studies of Arabidopsis myrosinases TGG1 and TGG2 used a monoclonal antibody (3D7) against Brassica napus myrosinase (Andreason et al. 2001, Husebye et al. 2002). However, we found that 3D7 specifically detected TGG2 (Fig. 1b). Polyclonal antibodies that we raised against each of TGG1 and TGG2 specifically detected TGG1 and TGG2, respectively. Thus, these antibodies are useful tools to clarify the difference in nature of these myrosinases. Our antibody experiments showed that (i) the N-glycosylation level affects the molecular size of TGGs (Fig. 1d); (ii) the organ specificity of TGGs is different in the wild-type Col-0 (Fig. 3a); and (iii) the intracellular behaviors of TGG1 and TGG2 are different in both wild-type plants and the atvam3 mutants (Fig. 4, 6).

TGG2 might forms aggregates with itself or with other components, unlike TGG1. Some β-glucosidases have been reported to form aggregates in maize (Blanchard et al. 2001), flax (Fieldes and Gerhardt 1994), ort (Kim et al. 2005) and Arabidopsis (Nagano et al. 2005). Some myrosinases are present in complexes together with other proteins such as myrosinase-binding proteins in extracts of oil seed rape (Ériksson et al. 2002). However, it is unlikely that myrosinase-binding proteins are co-localized with myrosinases in vacuoles, because myrosinase-binding proteins have no signal peptides at their N-termini.

Materials and Methods

Plant materials and growth conditions

Arabidopsis thaliana ecotype Col-0 was used as the wild-type plant. Seeds of knockout mutants atvam3-3 (SALK_075924), attg1-1 (SALK_130474) and attgg2-1 (SALK_038730) were donated by the Salk Institute Genome Analysis Laboratory. Seeds of Arabidopsis
were surface sterilized and then sown onto 0.5% Gellan Gum (Wako, Tokyo, Japan) that contained 1% sucrose and Murasige–Skoog’s medium. After the incubation for 3–5 d at 4°C to break seed dormancy, the plants were grown at 22°C under continuous light.

**Preparation of antibodies**

Two peptides derived from TGG1 (AQNQTIVPSDVHT) and TGG2 (AHALDFPSPEKLT) (Supplementary Fig. S1) were chemically synthesized with a peptide synthesizer (model 431A; Applied Biosystems, Tokyo, Japan). Cross-linking of each peptide with bovine serum albumin (BSA) and immunization of a rabbit were conducted as described previously (Matsushima et al. 2003). A cDNA encoding the region of AtAleu was inserted into PET32a (Novagen), and *Escherichia coli* BL-21 (DE3) cells were transformed with the plasmid. The His-tagged protein was purified with a chelating column (HiTrap, Amersham Pharmacia Biotech). The purified protein was injected into a rabbit to produce a specific antibody against AtAleu.

**SDS–PAGE and immunoblot analysis**

SDS–PAGE and immunoblot analysis were performed as described previously (Shimada et al. 2003b). Antibodies used are anti-TGG1 (diluted 5,000-fold), anti-TGG2 (diluted 5,000-fold), anti-12S globulin (anti-At12S, diluted 10,000-fold) (Sato et al. 1997), anti-AtAleu (diluted 2,000-fold), anti-PYK10 (anti-PYK10/CM, diluted 3,000-fold) (Matsushima et al. 2003), and anti-AtVAM3 (diluted 5,000-fold) (Sato et al. 1997).

**Deglycosylation with PNGase and Endo H**

Extracts from rosette leaves of 37-day-old plants were boiled in the presence of 0.1% SDS and 1% 2-mercaptoethanol for 5 min for denaturation. The extract (150 µl) was mixed with 1 U of PNGase (Roche Diagnostic GmbH, Mannheim, Germany) in 0.1 M sodium phosphate (pH 7.0) containing 0.1% SDS and 1% 2-mercaptoethanol. The extract (150 µl) was also mixed with 0.005 U of Endo H (Roche Diagnostic GmbH) in 0.1 M sodium acetate (pH 5.5) containing 0.1% SDS and 1% 2-mercaptoethanol. The mixtures were incubated for 16 h at 37°C. After Endo H digestion, the reaction mixture was divided into two fractions, and one of them was additionally treated with PNGase F. Deglycosylated proteins were subjected to SDS–PAGE followed by an immunoblot analysis with anti-TGG1 and TGG2 antibodies.

**N-terminal sequencing**

Proteins that were separated on the blots with CBB staining were subjected to automatic Edman degradation on a gas-phase sequence analyzer (model 477; Applied Biosystems).

**Proteomic analysis**

The aerial parts of six 36-day-old Col-0 and *atvam3-4* plants were homogenized in 5 ml of the reswelling buffer (7 M urea, 2 M thiourea, and 2% [w/v] 3-[3-cholamidopropyl]dimethylammonio)propanesulfonic acid) with a pestle and mortar for 1 min. The homogenates were centrifuged at 15,000 rpm for 15 min at 20°C, and the supernatants were subjected to isoelectric focusing using dry polyacrylamide gel strips (Immobiline DryStrip, pH 4–7, 13 cm; Amersham Biosciences), SDS–PAGE and mass spectrometry, as described (Matsushima et al. 2004). Protein spots obtained from the two-dimensional electrophoresis were analyzed by matrix-assisted laser-desorption ionization time of flight mass spectrometry and then were identified by searching the Mascot search engine (http://www.matrixscience.com/).

**RT–PCR**

Total RNA was isolated with an RNeasy plant mini kit (Qiagen, Valencia, CA, USA). Total RNA (1 µg) was subjected to first-strand cDNA synthesis using Ready-To-Go RT–PCR Beads (Amersham Biosciences). An aliquot (1 µl) was subjected to PCR with Ex Taq polymerase (TAKAR BIO INC., Otsu, Japan). The following gene-specific primers were used: 5′-AAGGGAGGAGGAATGAAGCCTTCTTA-3′ and 5′-ACTAGTTICATGCATCTGGACCTCTTTCCG-3′ for the At5g26000 (TGG1) gene; 5′-ACTAGTTCATGGAACGCTTCTTCATAT-3′ and 5′-AAATGGAAGGAGATCGACACAAATCACCATTAC-3′ for the At5g25980 (TGG2) gene; and 5′-AGAGATTCAGATGCCAGAAGTCGCTTGTTCGCC-3′ and 5′-AACGATCTTGAGGACCTGCTCCTACATACCT-3′ for Actin (Ratcliffe et al. 2003).

**Plasmid construction**

The chimeric gene encoding mRFP–AtVAM3-4 was produced by three rounds of PCR amplification as follows. First, the DNA fragment for an mRFP was amplified using mRFP in pRSETa (Campbell et al. 2002) as a template and a set of oligonucleotide primers, 5′-CTCGGAGGAGGAGGAATGAAGCCCTCCC-3′ and 5′-AAATGGAAGGAGATCGACACAAATCACCATTAC-3′. Second, the AtVAM3-4 DNA fragment was amplified using reverse transcripts prepared from *atvam3-4* leaves as a template and a set of oligonucleotide primers, 5′-GGCCGCCAGGAAGGAATGACGCTTTTCAGATTGTTAC-3′ and 5′-GGCGGCCTCAAGCAGATCCGTTTTTGGTGTGGACATTA-3′. Thirdly, the mRFP–AtVAM3-4 DNA fragment was amplified using both PCR fragments as templates and a set of oligonucleotide primers, 5′-CTCGAGAGATGCCCTCCCAGGACGCTTTAC-3′ and 5′-GCGGCCGCCTCAAGCAGATCCGTTTTTGGTGTGGACATTA-3′. The amplified fragment mRFP–AtVAM3-4 was inserted into the pETBlue vector (Novagen, Madison, WI, USA). The fragment mRFP–AtVAM3-4 was cut out with *Bam*HI and *Spe*I, and re-inserted into the *Bam*HI–*Spe*I site of SP-GFP-CTTP/pB1221 (Tamura et al. 2003).

**Transient expression**

Seven-day-old *Arabidopsis* plants were co-transformed with chimeric genes encoding mRFP–AtVAM3-4 and GFP–AtVAM3 by particle bombardment as described previously (Matsushima et al. 2004).

**Preparation of thin sections**

Rosette leaves of 26- and 48-day-old plants were fixed with 4% (w/v) paraformaldehyde and 1% glutaraldehyde in 0.05 M cacodylate buffer (pH 7.4) at 4°C for 3 h. After washing with 0.02 M cacodylate buffer (pH 7.4), these tissues were dehydrated with acetone and embedded in LR white resin at −20°C. Sections were cut on an ultramicrotome (Leica, Reichert Division, Vienna, Austria) for both light microscopic and electron microscopic analyses.

**Light and fluorescence microscopic analysis**

The thin sections were fixed on MAS (Matsunami adhesive silane)-coated glass slides (Matsunami Glass Ind., Osaka, Japan) and incubated in CBB solution (45% methanol, 10% acetic acid, and 0.25% CBB R250) for 10 min. After washing with water, the slides were examined with a light microscope.

For the immunofluorescence analysis, after the incubation in phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, and 1.8 mM KH₂PO₄) for 10 min, the slides were incubated in blocking buffer of PBS containing 5% BSA (Sigma, St Louis, MO, USA) for 30 min. The slides were incubated with the anti-TGG1 and anti-TGG2 antibodies (diluted 1,000-fold in the blocking buffer) for 4 h at room temperature. After washing three times with PBS for 5 min, the slides were incubated with secondary antibodies, Alexa Fluor 488 goat anti-rabbit IgG (H+L) (Molecular Probes, Eugene, OR, USA).
USA), for 1 h at room temperature. The secondary antibodies were diluted 1,000-fold. After washing three times with PBS for 5 min, the slides were examined with a fluorescence microscope.

**Immunoelectron microscopic analysis**

Ultrathin sections were mounted on Formvar-coated nickel grids. The sections were treated with blocking solution of PBS containing 2% BSA for 30 min and then incubated with anti-TGG1 or anti-TGG2 antibodies (diluted 1,000- to 2,500-fold) for 12 h at 4°C. After washing with PBS, sections were incubated with AuroProbe EM anti-rabbit IgG (H+L) (diluted 30-fold; 15 min; Amersham Biosciences) for 30 min. After washing, sections were stained with a solution of 1% (w/v) uranyl acetate and 0.4% (w/v) lead citrate. All sections were examined with a transmission electron microscope (model H-300; Hitachi, Tokyo, Japan) at 80 kV.

**CBB staining of plants**

The 17-day-old plants were boiled for 3 min in CBB solution. After the incubation for 3–5 h in the chloral hydrate solution [chloral hydrate : water : glycerol, 8 : 2 : 1 (w/v/v)], the plants were mounted on a glass slide with 60% glycerol and examined with a light microscope and a stereoscopic microscope.

**Subcellular fractionation**

Total protein homogenates from the aerial part of 38-day-old plants were subjected to differential centrifugation to obtain a 1,000× g pellet (P1), 8,000× g pellet (P8), 100,000× g pellet (P100) and 100,000× g supernatant (S), as described (Matsushima et al. 2003). Alternatively, the aerial parts of 30-day-old plants (1.2–1.5 g) were chopped with a razor blade in a Petri dish on ice in 2 vols of chopping buffer that contained 50 mM HEPES-NaOH (pH 7.5), 5 mM potassium phosphate (pH 7.0) and then 100 mM sinigrin was added. The mixture was incubated for 0–48 h at 37°C, and a linear gradient from 40 to 250°C at 5°C min⁻¹ and (iii) keeping the temperature at 250°C for 5 min. The data were analyzed by Shimadzu GCMS Solution (version 1.02) and estimation of the substances was based on comparison of the substance’s mass spectra with the GC/MS system data bank (Nist 27 and 147 Libraries).

**Supplementary material**

Supplementary material mentioned in the article is available to online subscribers at the journal website www.pcp.oupjournals.org.

**Acknowledgments**

We thank Dr. Y. Fukao (Kyoto University) for his helpful support with proteomic analysis, Mr. K. Fuji (Kyoto University) for preparation of specific antibodies against AtAlea, Dr. H. Azuma (Kyoto University) for his help with GC/MS analysis, Ms. Y. Makino (National Institute for Basic Biology) for help with peptide sequencing, Ms. T. Morí (National Institute for Basic Biology) for help with peptide synthesis, and Mr. J. Nakamura (Niigata University) for his help with the electron micrograph. We are grateful to Dr. M. Morita (Nara Institute of Science Technology) for her kind donation of seeds of the sgr3-1 mutant, to Dr. M. H. Sato (Kyoto Prefectural University) for his kind donations of anti-AtVAM3 antibodies and GFP-AtVAM3 plasmid, to Dr. R.Y. Tsein (Howard Hughes Medical Institute) for his kind donation of mRFP plasmid, and to Dr. A. M. Bones (Norwegian University of Science and Technology) for his gift of the 3D7 monoclonal antibodies against myrosinases. This work was supported by CREST of the Japan Science and Technology Corporation, Grants-in-Aid for Scientific Research (nos 15570048, 16085203, 16657013, and 17070002), 21st Century COE Research Kyoto University (A14) from the Ministry of Education, Culture, Sports, Science and Technology of Japan, and for a postdoctoral fellowship to H.U. from the Japan Society for the Promotion of Science (No. 14001013).

**References**


(Received October 22, 2005; Accepted November 15, 2005)