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Gene Note

Analyses of expression and phenotypes of knockout lines for *Arabidopsis* ABCF subfamily members

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Abstract ATP-binding cassette (ABC) proteins are one of the largest protein families found in all organisms. More than 120 genes encoding ABC proteins have been identified in *Arabidopsis* and rice. Various functions of plant ABC proteins have been characterized so far, and most of these proteins contain transmembrane domains. However, at present there has been no report on one soluble ABC protein group, the ABCF subfamily. We analyzed expression of five members of the *Arabidopsis* ABCF subfamily using promoter-GUS fusion constructs, and identified that these members are expressed in various organs of *Arabidopsis* at different stages. We also isolated knockout lines of four members of the ABCF subfamily, and showed that AtABCF3 is involved in root growth and development. These results suggest that the *Arabidopsis* ABCF subfamily functions throughout the plant and that it has important roles in development.

Key words: ABC protein, *Arabidopsis*, GCN, T-DNA.

ATP-binding cassette (ABC) proteins constitute one of the largest families in plants, and they have various functions including the transport of diverse substrates, channel regulation and pathogen resistance. ABC proteins share conserved amino acid sequence domains designated nucleotide binding domains (NBDs). Each NBD possess three characteristic motifs: a Walker A box [$GX_4GK(ST)$], a Walker B box [$(RK)X_3GX_3L$(hydrophobic)$_3$] (Walker et al. 1982) and an ABC signature [(LIVMFY)S(SG)GX_3(RKA)(LIVMYA)X-(LIVFM)(AG)] (Bairoch 1992) located between the two Walker boxes. Most ABC proteins include one or two transmembrane domains (TMDs) which contain 4–6 transmembrane α-helices. Several other ABC proteins which lack TMDs are thought to be soluble proteins. More than 120 members of the ABC family have been identified in the *Arabidopsis* and rice genomes (Sanchez-Fernandez et al. 2001; Garcia et al. 2004; Verrier et al. 2008), and 91 putative ABC proteins have been found in *Lotus japonicus* (Sugiyama et al. 2006). Several plant ABC proteins were recently characterized with respect to auxin transport (Geisler et al. 2005; Lin and Wang 2005; Terasaka et al. 2005), pathogen resistance (Kobae et al. 2006; Stein et al. 2006; Krattinger et al. 2009), heavy metal detoxification (Lee et al. 2005; Kim et al. 2006; Chen et al. 2007; Gaillard et al. 2008), and calcium channel regulation (Klein et al. 2003; Suh et al. 2007). These ABC proteins contain NBDs and TMDs (two NBDs and two TMDs constitute a full size ABC protein, and one NBD and one TMD constitute a half-size ABC protein). The ABCE subfamily of soluble ABC proteins is known as RNase L inhibitors, and these are implicated in the suppression of RNA silencing (Braz et al. 2004; Sarmiento et al. 2006). However, the function of another group of soluble ABC proteins, the ABCF subfamily, has not been analyzed in plants to date. The ABCF subfamily has two NBDs and no TMD and is also called the GCN (general control non-derepressible) subfamily. Yeast GCN20, which belongs to the ABCF subfamily, is involved in mediating activation of the eIF-2α kinase in amino acid-starved cells (Vazquez de Aldana et al. 1995).

To investigate the expression patterns of the ABCF subfamily in plants, we constructed promoter-GUS fusion genes for *Arabidopsis* ABCF subfamily genes. In *Arabidopsis*, the ABCF subfamily consists of five members, AtABCF1 (At1g60790), AtABCF2 (At5g09930), AtABCF3 (At1g64550), AtABCF4 (At3g54540) and AtABCF5 (At5g64840). The primer sequences used for promoter amplification were as follows: AtABCF1_Pro-f; GATCTCTAGATGCCAA-CCCATGACATCAATGC, AtABCF1_Pro-r; AATTCC-CGGGGACACCATCTTCAAATTATCTCC, AtABCF2_Pro-f; GATCGTCGACTGCTTCTGTTGCAACGCTA-

Abbreviations: ABC, ATP-binding cassette; GCN, general control non-derepressible; GUS, β-glucuronidase; NBD, nucleotide binding domain; TMD, transmembrane domain; X-Gluc, 5-bromo-4-chloro-3-indolyl β-D-glucuronide

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GAG, AtABCF2_Pro-r; GATCGGATCCTACCATTG-TTGGTATAAAATG, AtABCF3_Pro-f; GATCGTCG-ACAAGGTCTTCTATCTCCATGCCACTC, AtABCF3_Pro-r; GATCGGATCCAGTCATAGCCAACACAGAA-GCGACG, AtABCF4_Pro-f; GATCGTCGACATATTG-TCCAGACTCGTGAGGTTGC, AtABCF4_Pro-r; GAT-CGGATCCACCCATTTAAAACTGTACCTGACAT, AtABCF5_Pro-f; GATCGTCGACTCTCTACAACTTC-GAGAGGATG, AtABCF5_Pro-r; GATCGGATCCAC-CCATGGTTATATGTAGCGATAG. Genomic fragments of about 3.7 kb of the five ABCF subfamily members, including putative promoter regions, 5′ untranslated regions and the first two codons (*AtABCF1* promoter: 3736 bp, *AtABCF2* promoter: 3741 bp, *AtABCF3* promoter: 3716 bp, *AtABCF4* promoter: 3866 bp, *AtABCF5* promoter: 3746 bp) were fused to the GUS reporter gene of the vector pBI101, and these constructs were introduced into wild type *Arabidopsis*. The T2 generations of transgenic lines were germinated on 0.8% agar plates containing 0.5X B5 medium (pH 5.7), 1% (w/v) sucrose and kanamycin (25 μg ml^{-1}), and GUS activity was analyzed in 20-day-old seedlings as previously reported (Kato et al. 2007). After three more weeks in the growth chamber, GUS activity in flowers and siliques was also analyzed. In seedlings, GUS activity was high in leaves of *AtABCF1* promoter lines, *AtABCF4* promoter lines and *AtABCF5* promoter lines (Figure 1A, I, M), and was detected in young leaves of *AtABCF3* promoter lines (Figure 1E). GUS staining was observed in roots of *AtABCF1* promoter lines and *AtABCF4* promoter lines (Figure 1B, J), and GUS activity was observed in several root tips of *AtABCF3* promoter lines (Figure 1F) and in root epidermal cells of *AtABCF5* promoter lines (Figure 1N). In flowers, GUS staining was seen in sepals of *AtABCF1* promoter lines, *AtABCF4* promoter lines and *AtABCF5* promoter lines (Figure 1C, K, O), and in anthers of *AtABCF1* promoter lines, *AtABCF2* promoter lines, *AtABCF3* promoter lines and *AtABCF4* promoter lines (Figure 1C, G, K, Q). In *AtABCF2* promoter lines, GUS activity was detected only in anthers (Figure 1Q). *AtABCF1* promoter lines, *AtABCF4* promoter lines and *AtABCF5* promoter lines exhibited GUS staining in the upper part of carpels (Figure 1C, K, O), and *AtABCF3* promoter lines exhibited GUS staining in ovules (Figure 1G). In siliques, GUS activity was detected in valves of *AtABCF1* promoter lines, *AtABCF4* promoter lines and *AtABCF5* promoter lines (Figure 1D, L, P), and GUS activity was detected in seeds of *AtABCF3* promoter lines (Figure 1H). *AtABCF4* promoter lines exhibited GUS staining in replums (Figure 1L), and *AtABCF5* promoter lines exhibited GUS staining in septums (Figure 1P). The data obtained in this assay was summarized in Table 1. These results demonstrate that ABCF subfamily members are expressed in various organs of *Arabidopsis* at different developmental stages.

To gain more insight into the function of the ABCF subfamily, we screened for knockout lines of the ABCF genes from our *Arabidopsis* T-DNA-tagged lines (Kato et al. 2007). We made primers for each gene to screen for knockout lines (AtABCF1-f; AGATCTACAGATCTC-CCGAATC, AtABCF1-r; CTTAGCATGTGACCCCAA-TCTG, AtABCF2-f; ACCAACAATGGTATTAACG-ACG, AtABCF2-r; AGCTATGTGTGAACTGTGAAGC, AtABCF3-f; TTGGTAAGTAACCATTCGCAGC, AtABCE3-r; TGCTCGATTCGAAGATTTGGG, AtABCF4-f; ATGGGTAAGAAGAAGTCAGACG, AtABCF4-r; AACTTCACTCATCAACTTCTGC, AtABCF5-f; TCGAGGGTTTCTTACTCTGCTG, AtABCF5-r; TGTTCAGTTCCATCTCTTGGAG). PCR reactions were carried out with one of the above primers and a T-DNA vector primer (LB; AAGAAAATGCC-GATACTTCATTGGC, or RB; CTACAGGACGTAACA-TAAGGGACTG) using DNA pools containing genomic DNAs derived from the T-DNA-tagged lines. We isolated knockout lines for four out of the five ABCF subfamily members (Figure 2). The T2 generations of the knockout lines were germinated on 0.8% agar plates containing 0.5X B5 medium (pH 5.7), 1% (w/v) sucrose and hygromycin B (10 μg ml^{-1}), and the phenotypes of 20-day-old seedlings were analyzed. The plants were grown for five more weeks, and their phenotypes were again analyzed. One knockout mutant, in which the *AtABCF3* gene was disrupted, exhibited a visible phenotype. We obtained four different mutant lines of *AtABCF3* (KE3890, KG10570, KG15581, KG37852). T3 seeds with these mutant lines were germinated on 0.8% agar plates containing 0.5X B5 medium (pH 5.7) and 1% (w/v) sucrose, and the root growth of the seedlings was compared to that of wild type plants. The root growth rate of all mutant lines was retarded compared to that of wild type plants (Figure 3). Elongated cells were seen in longitudinal section of the mutant roots (Figure 4), and regions composed of small and dense cells in the mutant roots were shorter than those in wild type roots (Figure 4). These phenotypes suggest that the shorter roots could be a result of fewer cell divisions. The growth rates of whole plants with the mutant lines were also reduced relative to that of wild type plants, although the mutants eventually grew to the same height as wild type plants. The mutant lines were almost fertile. Our T-DNA-tagged lines are gene and enhancer trap lines (Kato et al. 2007), and we detected GUS activity in root tips of one mutant (KG10570, Figure 5 and Table 1). Although *AtABCF3* promoter lines showed GUS activity in flowers and siliques (Figure 1G, H), GUS staining was only seen in root tips in KG10570. We do not know why *AtABCF3* promoter lines and gene trap lines having AtABCF-GUS fusion proteins have different expression patterns. One possible explanation is that the AtABCF3 proteins might

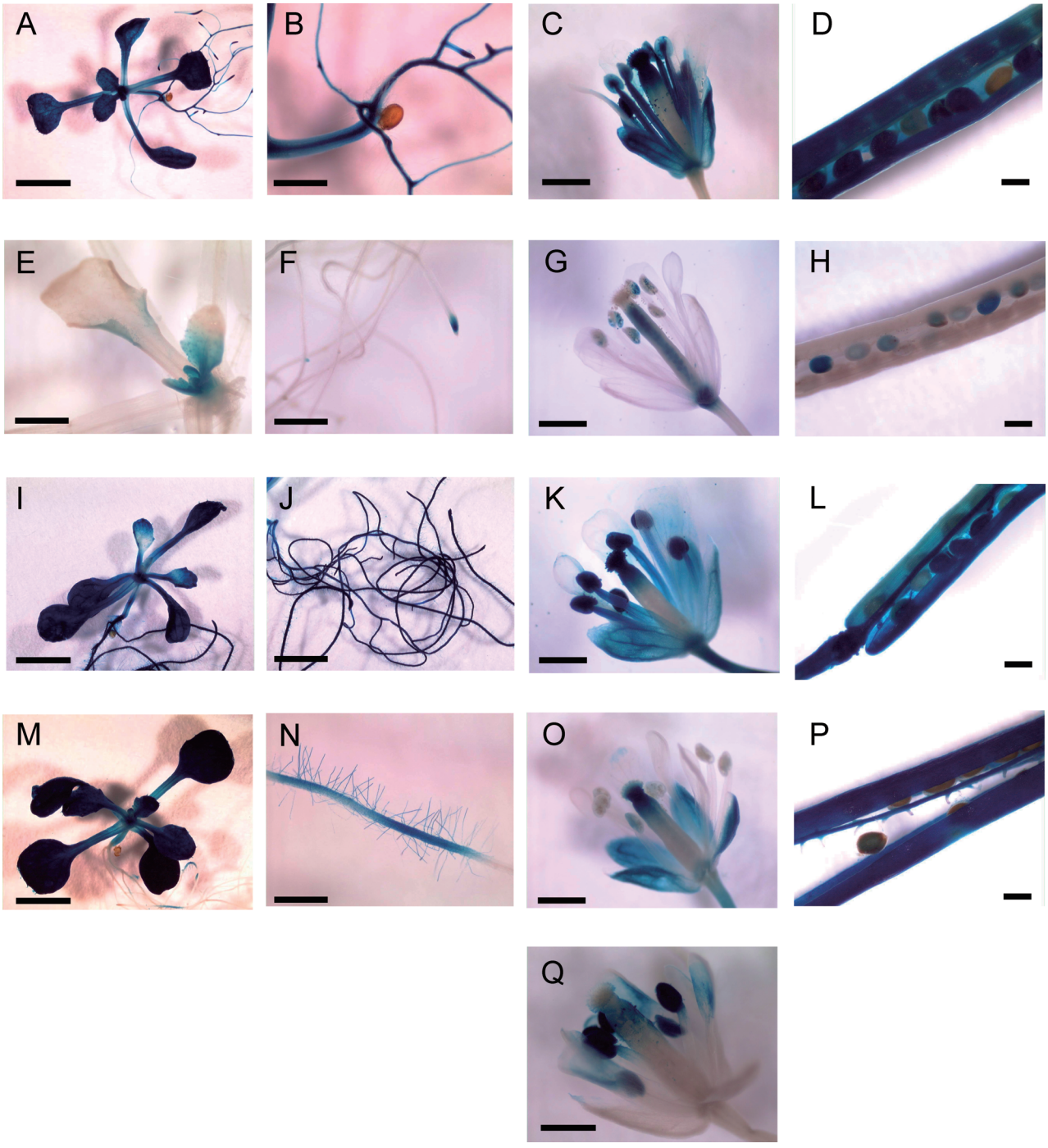

Figure 1. GUS expression patterns of the promoter-GUS transgenic lines. (A–D) *AtABCF1* promoter lines. (E–H) *AtABCF3* promoter lines. (I–L) *AtABCF4* promoter lines. (M–P) *AtABCF5* promoter lines. (Q) *AtABCF2* promoter lines. T2 seeds were germinated on agar plates containing kanamycin, and leaves (A, E, I, M) and roots (B, F, J, N) of 20-day-old seedlings were stained with X-Gluc. The plants were grown for three more weeks, and flowers (C, G, K, O, Q) and siliques (D, H, L, P) were stained with X-Gluc. The staining patterns of the plants were examined by microscopy. Scale bars = 10 mm (A, I, J, M), 3 mm (B, E, F), 2 mm (N), 1 mm (C, G, K, O, Q), 200 μm (D, H, L, P).

affect GUS expression in gene trap lines, because the AtABCF-GUS fusion proteins in gene trap lines contain 676 out of 715 amino acids of the AtABCF3 protein. Another possibility is that the introns of the *AtABCF3* gene, which are present in gene trap lines but not in promoter-GUS lines, might affect expression of the *AtABCF3-GUS* fusion genes. GUS activity was observed in root tips of both *AtABCF3* promoter lines and gene trap lines, indicating that disruption of *AtABCF3* in the root tips of the mutants impaired root growth. Expression of the *AtABCF3* gene and analyses of knockout mutants suggested that AtABCF3 contributes to root growth and development.

The knockout lines of other 3 members (*AtABCF1*,

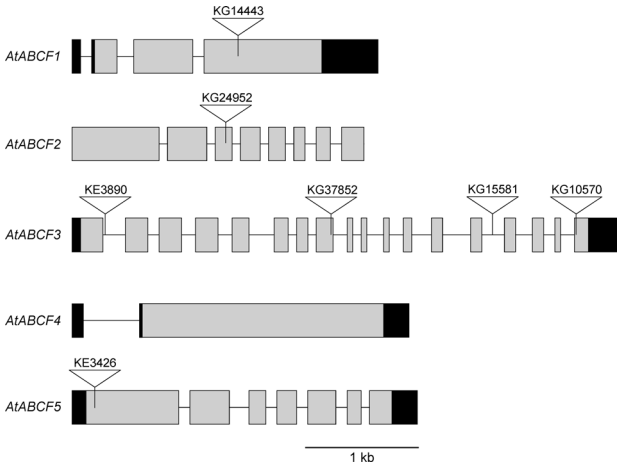

Figure 2. Gene structures of ABCF subfamily members and T-DNA insertion sites. Genomic organizations of the *AtABCF1* (At5g60790), *AtABCF2* (At5g09930), *AtABCF3* (At1g64550), *AtABCF4* (At3g54540) and *AtABCF5* (At5g64840) are shown. Exons are represented by boxes. The 5′ and 3′ untranslated regions are shown in black. Protein coding regions are shown in gray. Triangles indicate the locations of T-DNA insertions. KG lines (gene trap lines) have pGTAC-LUS vectors (Kato et al. 2007), and KE lines (enhancer trap lines) have pETAC-LUS vectors in which a 35S minimal promoter replaces the first intron of the *Arabidopsis rbcS3B* gene in the vector pGTAC-LUS.

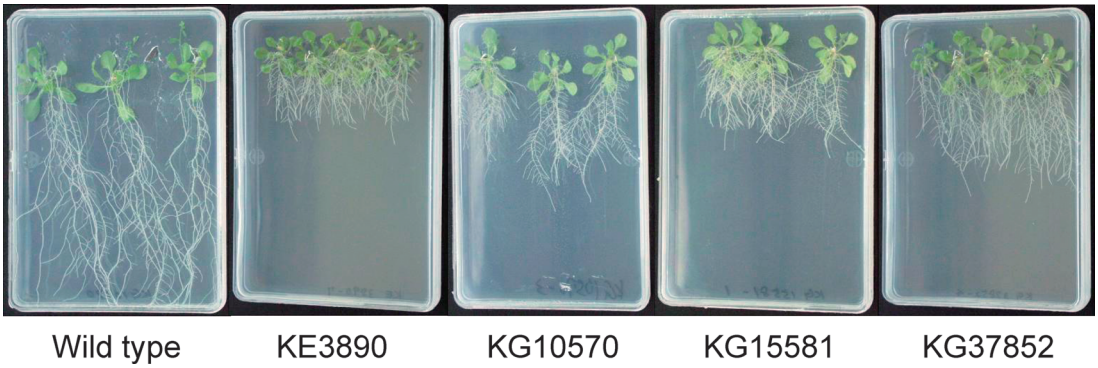

Figure 3. Root growth of wild type and T-DNA knockout lines of the *AtABCF3* gene (KE3890, KG10570, KG15581, KG37852). T3 seeds were germinated on agar plates, and the root growth of 20-day-old seedlings was photographed.

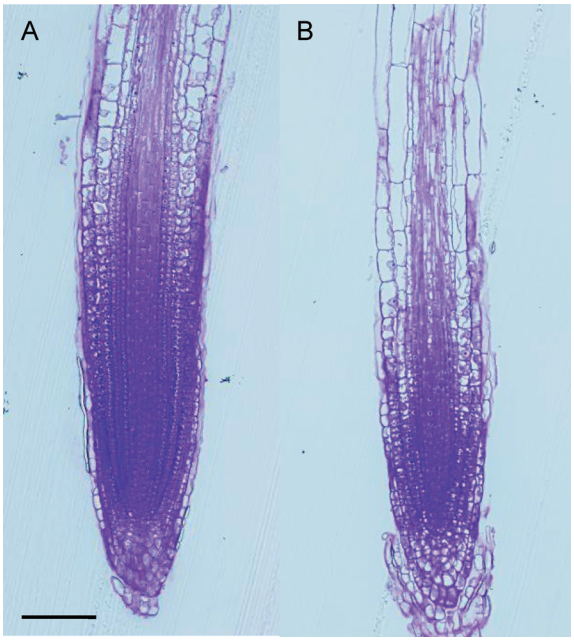

Figure 4. Longitudinal section of 14-day-old roots of wild type (A) and *AtABCF3* knockout line (B). Roots were fixed in formaldehyde acetic acid containing 37% (w/v) formaldehyde, glacial acetic acid, and 70% (v/v) ethanol (5 : 5 : 90; [v/v/v]). Samples were then dehydrated through an ethanol series of up to 100% ethanol, and embedded in Technovit 7100 resin (Kulzer). Sections (3 μm) were cut, mounted on glass slides, stained with 0.5% toluidine blue, and examined by microscopy. Scale bar = 100 μm.

Figure 5. GUS expression pattern of the gene trap line KG10570. T2 seeds were germinated on agar plates containing hygromycin B, and 20-day-old seedlings were stained with X-Gluc. The staining patterns of the plants were examined by microscopy. Scale bar = 10 mm.

AtABCF2 and *AtABCF5*) did not exhibit visible phenotypes in the present study. The *AtABCF2* and *AtABCF5* genes exist in the duplicated region of chromosome 5 in the *Arabidopsis* genome (*AtABCF2*: 3 Mb region of chromosome 5, *AtABCF5*: 25 Mb region of chromosome 5. Arabidopsis Genome Initiative 2000). Therefore these two genes may have redundant

Table 1. GUS expression of *Arabidopsis* ABCF promoter lines and gene trap line.

Organs	*AtABCF1* promoter line	*AtABCF2* promoter line	*AtABCF3* promoter line	*AtABCF3* gene trap line	*AtABCF4* promoter line	*AtABCF5* promoter line
20-day-old seedling						
root vascular tissue	+	−	−	−	+	−
root tip	+	−	+	+	+	−
root epidermal cell	−	−	−	−	+	+
leaf	+	−	+	−	+	+
Flower						
sepal	+	−	−	−	+	+
anther	+	+	+	−	+	−
cerpel	+	−	−	−	+	+
ovule	−	−	+	−	−	−
Silique						
seed	−	−	+	−	−	−
valve	+	−	−	−	+	+
replum	−	−	−	−	+	−
septum	−	−	−	−	−	+

+; GUS staining was observed., −; GUS staining was not observed.

functions. Expression patterns of the *AtABCF1* gene are similar to that of the *AtABCF4* gene, and the function of the two genes may also be redundant. Producing double knockout mutants might cause visible defects. Another possibility is that defects in the knockout lines may be visible under different conditions, for example under biotic or abiotic stress conditions. Further analyses will be needed to understand the functions of the ABCF subfamily in detail.

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