

Protein Subcellular Relocalization of Duplicated Genes in *Arabidopsis*

Shao-Lun Liu^{1,2}, An Qi Pan^{1,3}, and Keith L. Adams^{1,*}

¹Department of Botany, University of British Columbia, Vancouver, British Columbia, Canada

²Present address: Department of Life Science, Tunghai University, Taichung, Taiwan

³Present address: Mintec Inc., Vancouver, BC, Canada

*Corresponding author: E-mail: keitha@mail.ubc.ca.

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Abstract

Gene duplications during eukaryotic evolution, by successive rounds of polyploidy and by smaller scale duplications, have provided an enormous reservoir of new genes for the evolution of new functions. Preservation of many duplicated genes can be ascribed to changes in sequences, expression patterns, and functions. Protein subcellular relocalization (protein targeting to a new location within the cell) is another way that duplicated genes can diverge. We studied subcellular relocalization of gene pairs duplicated during the evolution of the Brassicaceae including gene pairs from the alpha whole genome duplication that occurred at the base of the family. We analyzed experimental localization data from green fluorescent protein experiments for 128 duplicate pairs in *Arabidopsis thaliana*, revealing 19 pairs with subcellular relocalization. Many more of the duplicate pairs with relocalization than with the same localization showed an accelerated rate of amino acid sequence evolution in one duplicate, and one gene showed evidence for positive selection. We studied six duplicate gene pairs in more detail. We used gene family analysis with several pairs to infer which gene shows relocalization. We identified potential sequence mutations through comparative analysis that likely result in relocalization of two duplicated gene products. We show that four cases of relocalization have new expression patterns, compared with orthologs in outgroup species, including two with novel expression in pollen. This study provides insights into subcellular relocalization of evolutionarily recent gene duplicates and features of genes whose products have been relocalized.

Key words: gene duplication, whole genome duplication, subcellular localization.

Introduction

Gene duplication has been an important genetic process for producing morphological and physiological innovations during eukaryotic evolution (reviewed in Flagel and Wendel 2009; Fawcett and Van de Peer 2010). Duplicated genes are formed by several molecular mechanisms, including whole genome (WG) duplication, segmental duplication of various sized regions of chromosomes, tandem duplication, and dispersed duplications generated by RNA or DNA-based mechanisms. Many WG duplication events have occurred during angiosperm evolution (e.g., Jiao et al. 2011; Schnable et al. 2012). About 2,500 pairs of duplicated genes were derived from the most recent WG duplication event in the *Arabidopsis* lineage, referred to as the alpha WG (α -WG) duplication event, that occurred at or near the base of the Brassicaceae family (Blanc et al. 2003; Barker et al. 2009). There are many

examples of other types of duplicates, such as tandem duplicates, that are specific to the Brassicaceae family (Haberer et al. 2004; Audemard et al. 2012).

A considerable number of duplicated genes have been retained in most flowering plants. Genes that are not dosage balanced may show neofunctionalization or subfunctionalization. In subfunctionalization, ancestral functions and/or expression patterns are divided between the two duplicates (Force et al. 1999; Lynch and Force 2000). In contrast, neofunctionalization occurred when one copy acquired a new function or expression pattern and the other copy retained the ancestral expression pattern or function. In addition to neofunctionalization and subfunctionalization of expression patterns and functions, protein subcellular relocalization after gene duplication recently began to receive more attention (Byun-McKay and Geeta 2008; Marques et al. 2008;

Byun-McKay et al. 2009). Subcellular relocalization has been suggested to be an important type of molecular mechanism contributing to the preservation and functional divergence of duplicated genes in eukaryotic genomes (Byun and Singh 2013). Similar to the concepts of neofunctionalization and subfunctionalization, the protein product of a duplicated gene can acquire a new subcellular localization (referred to as neolocalization) or show dividing of ancestral subcellular localizations if there were two or more (referred to as sublocalization). After neolocalization or sublocalization, expression patterns and functions could also diverge.

A few cases of protein subcellular relocalization after gene duplication have been shown in plants. For example, a pair of coproporphyrinogen III oxidases, *CPX1* and *CPX2*, in maize shows differential targeting ability to chloroplasts and mitochondria (Williams et al. 2006). A more ancient case of subcellular relocalization is a pair of nucleus-encoded organellar ribosomal protein genes (*Rps13*) in rosids where one is localized to the chloroplast and the other has been relocalized to the mitochondria (Adams et al. 2002; Mollier et al. 2002). In addition, genes within a family with different subcellular localization have been identified (e.g., Schultz and Coruzzi 1995; Devoto et al. 1999; Heilmann et al. 2004; Kawashima et al. 2005; Murcha et al. 2007; Dixon et al. 2009; Chong et al. 2010; Lan et al. 2010), but in most cases the duplication history and sequence evolution were not studied. A large-scale evolutionary study of subcellular localization of many duplicate gene pairs, using experimental data, has not been conducted in plants.

In this study, we used experimental green fluorescent protein (GFP) data to assemble a set of duplicated genes with subcellular localization data available for both copies. That allowed us to identify cases of subcellular relocalization after gene duplication during the evolution of the Brassicaceae family and determine if genes whose products have been relocalized more often show accelerated and asymmetric amino acid sequence evolution compared with those pairs that do not show relocalization. In addition, we studied six cases in more detail to infer which gene in a duplicate pair has been relocalized, to further characterize sequence rate evolution in the relocalized genes, to determine whether the relocalized genes show changes in expression patterns, and to infer sequence changes that may have led to relocalization in some cases with sufficient available localization data.

Materials and Methods

Subcellular Localization Analysis

To examine the subcellular localization of *PRX36* (At3g50990) and *PRX72* (At5g55390), full length cDNA products were amplified by reverse transcription PCR (RT-PCR) using gene-specific primers that include the following underlined restriction enzyme site: At3g50990F-KpnI (5'-CGGGTACCATGAA

TACAAAAACGGTGAAG-3'), At3g50990R-BamHI (5'-CGCGG
ATCCAAACATCATGGTTAACCTCC-3'), At5g66390F-KpnI (5'-CGGGGTACCATGCCAAGTCATTGAACATC-3'), and At5g66390R-BamHI (5'-CGCGGATCCATAAGCATGGTTACCCTCC-3'), with the RT-PCR conditions described in Liu and Adams (2008). All PCR products were cloned in frame into modified pCambia1300 vectors with the CaMV 35S promoter located 5' upstream and GFP located immediately downstream. The inserted nucleotide sequence in the resultant plasmid was checked by DNA sequencing. The pCambia1300-At3g50990-GFP and pCambia1300-At5g66390-GFP then were transformed into *Arabidopsis thaliana* ecotype Columbia. Agrobacterium-mediated transformation was conducted using the floral dip method described in Clough and Bent (1998). A small piece of transgenic plant tissue was placed on the slide and immersed in a few drops of sterile Milli-Q water for visualizing the GFP fluorescence by using a confocal laser scanning microscope.

Plant Materials, Nucleic Acid Extraction, and RT-PCR

Different organ types (e.g., roots, rosettes, shoots, leaves, flowers, or siliques/seeds) of *Gossypium hirsutum* cultivar TM1, *Carica papaya* cultivar Sun-Up, and *A. thaliana* ecotype Columbia were harvested and then stored in a -80°C freezer for the subsequent nucleic acid extraction and RT-PCR assays. Nucleic acid extraction and RT-PCR followed the procedures described in Liu and Adams (2008). Gene-specific primers for RT-PCR are listed in the supplementary table S1, Supplementary Material online. Gene-specific primers for gene annotation of *CPK* in *C. papaya* are listed in the supplementary table S2, Supplementary Material online. The partial sequence *CPK* from *C. papaya* determined in this study was submitted to GenBank with the accession number KC692920.

Identification of Brassicaceae-Specific Duplicated Genes

Three different types of duplicates in *A. thaliana* were included: α -WG duplicates, tandem duplicates, and "other duplicates" that arose from the other types of gene duplication events. The α -WG duplicates were obtained from the study of Blanc et al. (2003). Tandem duplicates were obtained from Haberer et al. (2004), in which only clusters of two genes were selected to simplify further analyses. The "other duplicates" were identified using the following analytical procedure. First, we obtained *A. thaliana* gene families from PLAZA 2.5 (Proost et al. 2009) and then a consensus gene family tree for each gene family was generated from 100 bootstrapped maximum-likelihood (ML) trees constructed using the software RAxML (Stamatakis 2006). Second, each monophyletic group in the 50% majority consensus tree that only consists of two members was selected. Finally, the pairs of α -WG and tandem duplicates were filtered out, resulting in the list of other duplicates. In addition, we further filtered out older duplicates that were potentially derived from the duplication

events shared with other species outside the family Brassicaceae because we aimed to include the Brassicaceae-specific duplicates. As synonymous substitution values (dS) between duplicate genes can be used as proxy of the age of duplication (e.g., Blanc et al. 2003), we used the dS value to filter out pairs that were older than the average age of the alpha duplicates. A dS of one was used as the cut off because the mean dS value of all α -WG duplicates is approximately 1 (Blanc et al. 2003). The dS values were computed with an ML method using the software Codeml in PAML 4 (Yang 2007). The final list of duplicates used in this study is provided in [supplementary table S1, Supplementary Material](#) online.

Identification of Gene Pairs with GFP Localization Data Available for Both Duplicates

We first retrieved the subcellular localization data of *Arabidopsis* genes from the SUB-cellular location database for *Arabidopsis* proteins (SUBA database) (Heazlewood et al. 2007). The SUBA database integrated the subcellular localization data in *A. thaliana* from previously published papers. The subcellular localization data from the GFP approach have been shown to be more accurate than other computational prediction-based approaches (Heazlewood et al. 2005). Thus, only the GFP data were used in our study. We also performed a literature search to find duplicate genes with GFP data that were published after the last update of SUBA. To minimize false positives, we then performed several filtration steps to exclude some pairs for subsequent analyses. Several filtration steps were applied to minimize the false positives or exclude uncertain cases (see [supplementary fig. S1, Supplementary Material](#) online, for details). Protein subcellular localization has been shown to change under different environmental conditions (e.g., Alinsug et al. 2009), vary by organ type (e.g., Székely et al. 2008), and vary in an age-dependent manner (e.g., Teng et al. 2012). Thus, we excluded gene pairs where the GFP data come from different studies using different experimental conditions.

Identification of Orthologs in Outgroup Species

Prior to the asymmetric sequence rate analyses, we first identified the orthologs for 128 pairs of duplicates with localization data. To this end, reciprocal best hits of BLASTP (RBH-BLASTP) between *Arabidopsis* and other eudicots as outgroups were performed. The outgroup protein sequences of *C. papaya*, *Fragaria vesca*, *Glycine max*, *Lotus japonica*, *Malus domestica*, *Manihot esculenta*, *Medicago truncatula*, *Populus trichocarpa*, *Ricinus communis*, *Theobroma cacao*, and *Vitis vinifera* were downloaded from PLAZA 2.5 (Proost et al. 2009). Then, the results of RBH-BLASTP were filtered according to the following criteria: 1) The e value was less than or equal to 1×10^{-5} ; and 2) the alignment length for the amino acid of one protein must be at least 60% of the shorter protein. If no orthologs were identified for a duplicated gene pair through

RBH-BLASTP, putative orthologs identified by one way best hit were used. To avoid the identification of false positives, we then applied the second filtration step to ensure the identification of orthologous sequences from outgroup species. The divergence time between two duplicated genes should be younger than those between duplicates and their preduplication orthologs. To this end, the pairwise dS values were calculated to examine whether an appropriate orthologous sequence was chosen. First, coding sequences were aligned by codons using the software MUSCLE (Edgar 2004). Second, the dS value of two duplicated genes (dS_1), the duplicate gene 1 and the ortholog (dS_2), and the duplicate gene 2 and the ortholog (dS_3) were computed using the software Codeml in PAML 4 (Yang 2007). Third, only pairs with $dS_2 > dS_1$ and $dS_3 > dS_1$ were kept for the asymmetric sequence rate analysis based on our assumption for the relationship between the duplicated genes and their orthologs.

To retrieve the orthologs from outgroup species for each case study prior to asymmetric rate analysis, we first retrieved orthologs of duplicated genes from the PLAZA 2.5 orthologs list (Proost et al. 2009), then reconstructed an ML gene family phylogenetic tree using MEGA5 with assessment of the statistical node support by 500 bootstrapping replicates of ML analysis. When the gene phylogeny was not consistent with the species phylogeny (reviewed in Soltis et al. [2005]), we performed a tree topology test to see whether they are significantly different by using the Kishino–Hasegawa test (Kishino and Hasegawa 1989) and the Shimodaira–Hasegawa test (Shimodaira and Hasegawa 2001) implemented in the software TREE-PUZZLE (Schmidt et al. 2002). Taxa with multiple ancient WG duplication events in their lineage were not chosen to minimize the effects of gene duplication events on the topology test.

Analysis of Asymmetric Sequence Rate Evolution and Positive Selection

After the identification of orthologs, the rate of sequence evolution was computed using the software Codeml in PAML 4 (Yang 2007). We followed the analytical procedure described in Blanc and Wolfe (2004). Two hypotheses, unconstrained rate of evolution (i.e., asymmetric sequence evolution) and clock-like rate of evolution (i.e., symmetric sequence evolution), were tested using an ML approach with the JTT (Jones, Taylor, and Thornton) matrix for the correction of multiple substitutions. To detect which model fits better with our data, a likelihood ratio test (LRT) was applied. The twice likelihood ratio ($2\Delta L$) was compared against a chi-square distribution with $df = 1$ following the equation: $2\Delta L = -2(Ln1 - Ln2)$, where $Ln1$ is the likelihood estimates from the first test, and $Ln2$ is the likelihood estimates from the second test. Adjusted P values (i.e., Q value < 0.05) were applied to correct for multiple testing using the false discovery rate (FDR) method (Storey and Tibshirani 2003). To test whether duplicated

pairs with subcellular relocalization are often associated with asymmetric sequence rate evolution, the Fisher's exact test and Monte Carlo randomization tests were applied. For the Monte Carlo randomization test, we specified a test statistic (DIF) as a measure of the absolute difference in the frequency of asymmetric sequence evolution of duplicated pairs with different subcellular localization and those with same subcellular localization. We then compared the DIF value from the observed data (DIF_{obs}) against the null distribution of the simulated DIF value from 10,000 randomized data (DIF_{sim} ; as null hypothesis). If the null hypothesis is rejected, the frequency of asymmetric sequence evolution is significantly higher in duplicated pairs with different subcellular localization than those with same subcellular localization.

A positive selection test was performed on duplicated pairs that showed asymmetric sequence rate evolution using the software Codeml in PAML 4 (Yang 2007). A branch-site model and LRT were applied to test whether there is any positive selection acting on individual codons. Two LRTs were performed (Model A_{test1} and Model A_{test2}) on each triplet (duplicate 1, duplicate 2, and their ortholog). The copy with accelerated sequence evolution was assigned as the foreground lineage, whereas the others were designated as the background lineage. To reduce false positives, only positively selected sites with >0.95 Bayesian posterior probability were considered using the Bayes Empirical Bayes (BEB) analysis.

To better quantify asymmetric sequence rate evolution in terms of nonsynonymous (dN) substitution rate and dN/dS ratio in the case studies, we implemented the LRT to examine the presence and absence of asymmetric dN and dN/dS between duplicates using the software HyPhy (Pond et al. 2004) and software PAML 4 (Yang 2007). In contrast to the PAML package, HyPhy allows users to examine the relative nonsynonymous (dN) substitution rate between any given two duplicated genes using ML (Pond et al. 2004). After determining the orthologs and tree topology, we followed the analytical procedures described in the HyPhy manual (Pond et al. 2004) and the PAML 4 manual (Yang 2007) to examine the absence and presence of asymmetric dN and ω ($=dN/dS$) between duplicates. Briefly, when testing the evolutionary scenario that there has been asymmetric dN and dN/dS evolution between duplicated genes (e.g., dN_1 and ω_1 in gene 1 vs. dN_2 and ω_2 in gene 2), the null hypothesis of dN_1 (or ω_1) = dN_2 (or ω_2) versus the alternative hypothesis of dN_1 (or ω_1) \neq dN_2 (or ω_2) was evaluated using the LRT. If the log-likelihood value of the alternative hypothesis is significantly higher than that of the null hypothesis, it suggests that there has been asymmetric dN evolution between gene 1 and gene 2.

Analysis of Protein Isoelectric Points

We examined whether there is any significant difference in protein isoelectric point (pl) between duplicate pairs with and pairs without subcellular relocalization. The pl values of the

Arabidopsis peptide sequences were obtained using the Protein Isoelectric Point of the Sequence Manipulation Suite database with pK values from EMBOSS. A t-test was used to examine whether there is any difference in pl values between duplicates with and without subcellular relocalization. All statistical analyses were performed using the statistical package R.

Results and Discussion

Diverged Localization of Two Duplicated Peroxidases, Accelerated Sequence Evolution, and Regulatory Neofunctionalization

During a study of duplicated gene expression in *A. thaliana* (Liu et al. 2011), we identified two class III peroxidase genes with organ-specific complementary expression patterns. The genes, *PRX36* (At3g50990) and *PRX72* (At5g66390), were derived from the alpha polyploidy event at the base of the Brassicaceae family (Blanc et al. 2003; Bowers et al. 2003). They code for proteins with divergent N-termini (supplementary fig. S2A, Supplementary Material online). The divergent N-terminus is the result of the abolition of the original start codon and gain of a new start codon (supplementary fig. S2B, Supplementary Material online). As localization signals are often located at the N-terminus, we hypothesized that they might have different subcellular localizations. *PRX36* (also known as *PER36*) recently was shown to localize to the cell wall (Kunieda et al. 2013). We concurrently performed a GFP subcellular localization assay to determine whether the products of both *PRX36* and *PRX72* are targeted to different subcellular locations (see Materials and Methods for details). We found that *PRX36* is localized to the cell wall (fig. 1A) whereas *PRX72* is located in the cytosol (fig. 1B). It has been shown that the cell wall localization signal peptide can be located at the N-terminus (e.g., Chen et al. 2004) and thus the divergent N-terminus of *PRX36* (supplementary fig. S2, Supplementary Material online) may function as a cell wall localization signal peptide. Our data indicate that there has been a change in localization after gene duplication, and suggest that *PRX36* was relocalized.

PRX36 and *PRX72* have contrasting expression patterns. From both microarray expression data (from Schmid et al. 2005) and our RT-PCR assays, *PRX36* and *PRX72* showed a complementary and reciprocal expression pattern, where only *PRX36* is expressed in siliques and only *PRX72* is expressed in roots (fig. 1C and supplementary fig. S3A, Supplementary Material online). To gain insights into the ancestral expression pattern, we examined expression of three peroxidase genes that are related to *PRX36* and *PRX72* by the beta-WG duplication event in the Brassicaceae lineage: *PRX49* (At4g36430) and the tandem duplicate pair of *PRX14* (At2g18140) and *PRX15* (At2g18150); tree topology testing indicated that all three genes share a common ancestor with *PRX36* and *PRX72*.

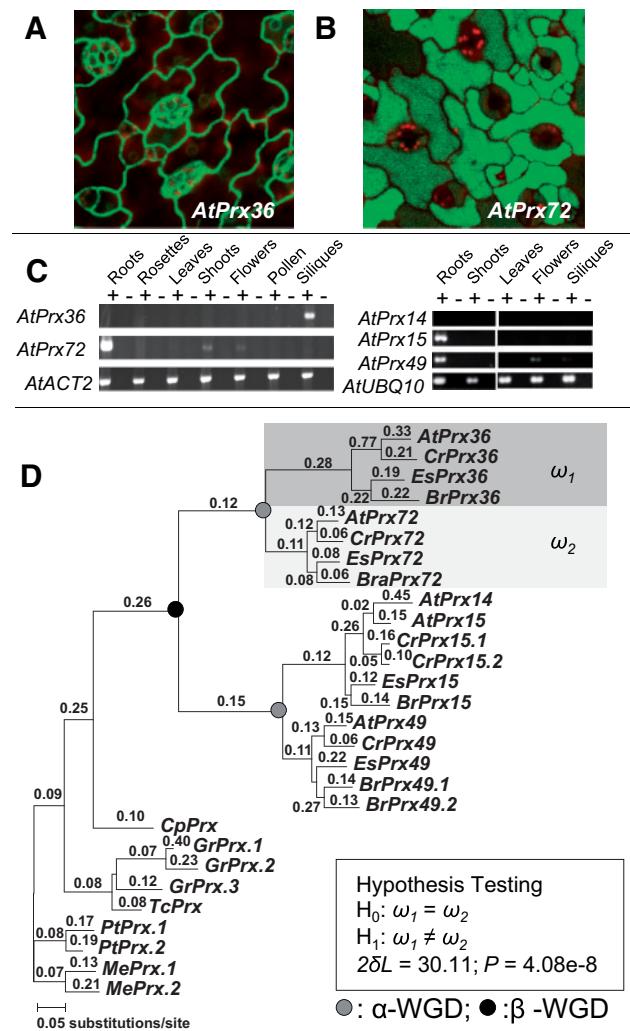


Fig. 1.—Subcellular relocalization, asymmetric sequence evolution, and gene expression divergence in a pair of peroxidases. (A) GFP subcellular localization of PRX36. (B) GFP subcellular localization of PRX72. (C) RT-PCR expression assays of PRX36 and PRX72. Plus signs (+) indicate reactions with reverse transcriptase and minus signs (−) indicate reactions without reverse transcriptase. ACT2 and UBIQ10 were positive controls. (D) PAML analysis of PRX36, PRX72, and their orthologs in other species. Numbers above the branches indicate the dN/dS ratios. dN analysis is shown in supplementary figure S4, Supplementary Material online. Species include: At, *A. thaliana*; Cr, *Capsella rubella*; Es, *Eutrema salsugineum*; Br, *Brassica rapa*; Cp, *C. papaya*; Gr, *G. raimondii*; Tc, *T. cacao*; Pt, *P. trichocarpa*; and Me, *M. esculenta*. See supplementary figures S2 and S4, Supplementary Material online, for locus numbers of each gene.

after the divergence of the Brassicaceae from the Caricaceae (fig. 1D and supplementary fig. S4A, Supplementary Material online). Our RT-PCR assays showed that PRX49 and PRX15 are highly expressed in roots with low or no expression in most other organ types (fig. 1C), largely consistent with the microarray results (supplementary fig. S3A, Supplementary Material online). In contrast, PRX14

was not expressed in any of the assayed organ types (fig. 1C), although the microarray results showed expression in roots (supplementary fig. S3A, Supplementary Material online). None of the three genes are expressed in siliques, as is PRX36. Their expression patterns are much more similar to PRX72 than PRX36, suggesting that PRX36 has a derived expression pattern of high expression in siliques and no expression in roots which is indicative of regulatory neofunctionalization (a new expression pattern) of PRX36 after its formation by gene duplication.

Duplicated genes sometimes show an asymmetric rate of amino acid sequence evolution with one copy evolving more rapidly than the other, which has been associated with functional divergence and neofunctionalization (e.g., Blanc and Wolfe 2004; Liu and Adams 2010; Panchin et al. 2010; Owens et al. 2013). To determine whether PRX36 shows accelerated and asymmetric sequence rate evolution, we performed a detailed sequence rate analysis by incorporating orthologous sequences from outgroup species in a phylogenetic framework using analyses of dN/dS and dN (see Materials and Methods for more details). The results showed that PRX36 evolved much faster than PRX72 after gene duplication (fig. 1D and supplementary fig. S4B, Supplementary Material online).

Identification of Brassicaceae-Specific Duplicates with Divergent Localization

After characterizing a case of subcellular relocalization of a pair of duplicated peroxidase genes, we assembled a set of gene pairs in *A. thaliana* that were formed by duplication during the evolution of the Brassicaceae family, for which experimental localization data are available for both duplicates. We used experimental localization data because results from localization prediction programs often show inconsistency among programs and high error rates (e.g., Heazlewood et al. 2005; Millar et al. 2006). We used the GFP data from the SUB-cellular location database for *Arabidopsis* proteins (SUBA) database (Heazlewood et al. 2005) plus results from a literature search for papers on fluorescent protein localization experiments published since the last update of SUBA (see Materials and Methods for details). For a set of evolutionarily recent duplicates, we included genes duplicated by the α -WGD duplication event (α -WGD) at the base of the Brassicaceae family (Blanc et al. 2003; Bowers et al. 2003) plus tandem duplicates (Haberer et al. 2004) and other types of duplicates that originated during the evolution of the Brassicaceae family (see Materials and Methods for details). We obtained a final set of 128 gene pairs (supplementary table S3, Supplementary Material online).

We found that 19 of 128 duplicate pairs (15%) showed divergent localizations (table 1 and supplementary table S3, Supplementary Material online). Fourteen of them showed complete changes of protein subcellular localization, whereas

Table 1

Subcellular Localization, Type of Gene Duplication, Sequence Rate Evolution, and Putative Gene Function of 19 Relocalized Duplicated Pairs

Gene 1	SCL	Gene 2	SCL	Duplicate Type	Putative Function	Reference
At3g49560 (<i>TIM</i>)	cp	At5g24650 (<i>TICM</i>)	mt; cp	α-WGD	Inner membrane translocase	Murtha et al. 2007
At2g23800 (<i>GGPS2</i>)	ER	At4g36810 (<i>GGPS1</i>)	cp	α-WGD	Geranylgeranyl pyrophosphate synthase	Okada et al. 2000
At1g14450 (<i>NADH</i>)	ER	At2g02510 (<i>NADH</i>)	mt; per; cp	α-WGD	NADH dehydrogenase	Abu-Abied et al. 2009
At1g17050 (<i>SPS2</i>)	cp	At1g78510 (<i>SPS1</i>)	ER	α-WGD	Solanoyl diphosphate synthase	Jun et al. 2004; Hirooka et al. 2005
At1g02510 (<i>TPK4</i>)	pm	At4g01840 (<i>TPK5</i>)	Va	α-WGD	K ⁺ channel protein	Becker et al. 2004; Voelker et al. 2006
At3g05790 (<i>LON4</i>)	mt; cp	At5g26860 (<i>LON1</i>)	mt	α-WGD	Lon protease-like protein	Ostersetzer et al. 2007
At1g55920 (<i>SAT1</i>)	cp	At3g13110 (<i>SAT3</i>)	mt	α-WGD	Serine O-acetyltransferase	Noji et al. 1998
At3g01330 (<i>DEL3</i>)	cy; nu	At5g14960 (<i>DEL2</i>)	nu	α-WGD	E2F-like transcription factor	Kosugi and Ohashi 2002
At1g13270 (<i>MAP1C</i>)	cp	At3g25740 (<i>MAP1B</i>)	mt; cp	α-WGD	Methionine aminopeptidase	Giglione et al. 2000
At1g13460 (<i>PP2A B' θ</i>)	per	At3g26020 (<i>PP2A B' η</i>)	nu; cy	α-WGD	The B subunit of protein phosphatase 2A	Matre et al. 2009
At3g50990 (<i>PRX36</i>)	cw	At5g66390 (<i>PRX72</i>)	cy	α-WGD	Class III peroxidase	This study
At5g04870 (<i>CPK1</i>)	per; lb	At3g10660 (<i>CPK2</i>)	ER	α-WGD	Calcium-dependent protein kinase	Lu and Hrabak 2002; Dammann et al. 2003; Coca and San Segundo 2010
At2g39800 (<i>P5CS1</i>)	cyb	At3g55610 (<i>P5CS2</i>)	cy	α-WGD	Δ-1-pyrroline-5-carboxylate synthetase	Székely et al. 2008
At3g10550 (<i>MTM1</i>)	cyb	At5g04540 (<i>MTM2</i>)	cy	α-WGD	Myotubularin-like phosphatases	Ding et al. 2012
At1g31630 (<i>AGL86</i>)	cy	At1g31640 (<i>AGL92</i>)	nu	Tandem	MADS-box transcription factor	Carrie et al. 2009
At2g33110 (<i>VAMP723</i>)	ER	At2g33120 (<i>VAMP722</i>)	pm; en	Tandem	SNARE binding protein	Uemura et al. 2004
At3g08720 (<i>S6K2</i>)	nu	At3g08730 (<i>S6K1</i>)	cy	Tandem	Serine/threonine protein kinase	Mahfouz et al. 2006
At5g39510 (<i>VTI11</i>)	TGN/PVC	At5g39630 (<i>VTI14</i>)	ER	Tandem	SNARE binding protein	Uemura et al. 2004
At4g15415 (<i>PP2A B' γ</i>)	nu; cy	At3g21650 (<i>PP2A B' ζ</i>)	mt; cy	Other	The B subunit of protein phosphatase 2A	Matre et al. 2009

NOTE.—cp, chloroplast; cpl, cell plate; cy, cytosol; cyb, cytosolic subcellular body; ck, cytoskeleton; en, endosome; lb, lipid bodies; mt, mitochondrion; nu, nucleus; per, peroxisome; pm, plasma membrane; SCL, subcellular localization; TGN/PVC, trans-Golgi network/prevacuolar compartment; va, vacuole. Boldface indicates the statistically significant accelerated copy after correction for multiple testing.

five of them showed expansion or contraction of protein subcellular localization. Use of GFP experimental data resulted in a relatively small set of duplicated gene pairs (128), from a genome-wide perspective, which makes it more difficult to infer a percentage of relocalized duplicates, but provided an increased confidence in localization inferences compared with localization prediction programs. Although there is no evidence to suggest that the gene pairs in our study are a biased set in terms of conserved versus diverged subcellular localization, such a bias is possible from a relatively small sample of duplicated genes that could result in an

underestimate or overestimate of the percentage with subcellular relocalization. Nonetheless our results suggest that a considerable number of duplicates may have evolved different subcellular localizations during the evolution of the Brassicaceae family. A recent study using a prediction program to infer subcellular relocalization of duplicated genes in *Arabidopsis* and several other eukaryotes inferred that 28% of duplicates in *A. thaliana* have been relocalized (Byun and Singh 2013). Our estimation of 15% may be lower because we used experimental data rather than a subcellular localization prediction program, we only analyzed genes formed by

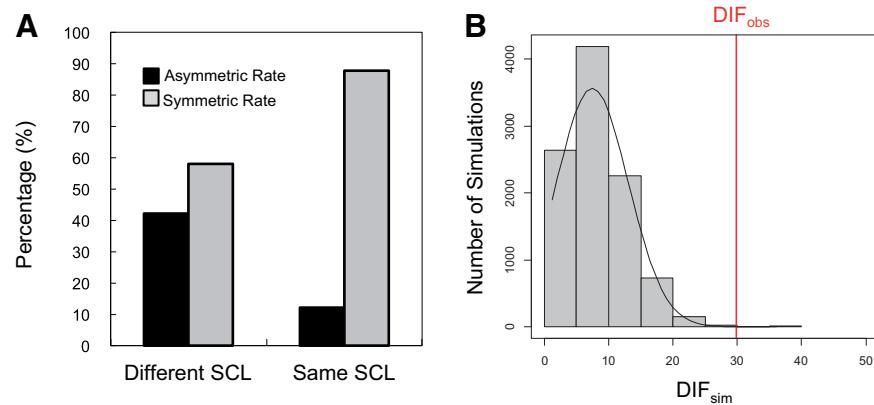


FIG. 2.—Asymmetric sequence evolution in duplicated gene pairs. (A) Diagram showing the frequency of asymmetrically evolved duplicated pairs in duplicated pairs with different subcellular localization (SCL) and duplicated pairs with the same SCL. (B) Histogram showing the distribution of the difference in frequency of asymmetric sequence evolution between duplicated pairs with different SCL and those with the same SCL (DIF_{sim}) from 10,000 Monte Carlo randomization tests. Red line indicates the observed value (DIF_{obs}).

duplication during the evolution of the Brassicaceae, and our sample size was smaller. A study of subcellular relocalization in *Saccharomyces cerevisiae*, using GFP data, showed that 88 of 238 duplicate protein pairs (37%) showed different localization. Thus subcellular relocalization of duplicated genes may be relatively common in other organisms too.

Duplicates with Divergent Localization Often Have Asymmetric Sequence Evolution

After relocalization, a duplicated gene could perform a similar function, or there could be functional diversification compared with the ancestral function. Asymmetric sequence rate evolution, where one copy has experienced a significantly elevated rate of amino acid changes relative to the other copy, can be used as an indicator for possible functional divergence (e.g., Dermitzakis and Clark 2001; Blanc and Wolfe 2004; Kim and Yi 2006; Byrne and Wolfe 2007). To evaluate whether there has been any significant asymmetric sequence evolution for the 19 duplicate pairs with divergent localization, and to test the hypothesis that relocalized duplicates show more cases of asymmetric sequence rate evolution than those with the same subcellular localization, we identified orthologs of each duplicate from outgroup species and performed asymmetric sequence rate analysis (e.g., Blanc and Wolfe 2004; Liu et al. 2011; see Materials and Methods for details). Based on our analysis, 8 of 19 relocalized duplicates (42%) and 13 of 109 nonrelocalized duplicates (12%) showed significant asymmetric sequence evolution (table 1; LRT: $Q < 0.05$ in supplementary table S3, Supplementary Material online; false discovery rate-corrected for multiple tests). The protein sequences of duplicated pairs with subcellular relocalization evolve asymmetrically more frequently than those without subcellular relocalization (Fisher's exact test: One-tailed $P = 4 \times 10^{-3}$; Monte

Carlo randomization test: Two-tailed $P = 9 \times 10^{-4}$; fig. 2). We also compared the frequency of relocalized duplicate gene pairs with asymmetric sequence evolution obtained here with gene pairs from Blanc and Wolfe (2004), who analyzed 833 duplicated gene pairs from the α -WGD using the same method to what we used. A randomization test using results from Blanc and Wolfe (2004) showed that the percentage of gene pairs with asymmetric rate evolution was significantly lower than for the relocalized gene duplicates studied here ($P = 0.038$).

Protein Subcellular Relocalization Is Not Associated with Changes in Protein Isoelectric Point

After relocalization, there can be a change in the protein isoelectric point (pl), for adapting to the pH of the new cellular compartment, as shown in yeast (Marques et al. 2008). Thus, we might expect to find a greater pl difference for relocalized duplicated genes than nonrelocalized duplicated genes because relocalized duplicated genes would evolve a distinct pl through subcellular pH adaptation. To test the hypothesis that pl changes occur in relocalized duplicates in plants, we estimated the pl difference (Δpl) between duplicated pairs. Our analysis showed no significant difference between duplicated pairs with subcellular localization and those without subcellular localization (fig. 3; t -test: two-tailed $P = 1.0000$). Thus it appears that yeast and plants differ in this regard, although we cannot eliminate the possibility that our smaller sample size (128 vs. 238) might have an effect.

Case Studies of Relocalized Genes

We next present sequence and expression analyses to further characterize five cases of subcellular relocalization. The goals were to infer which gene in a duplicate pair has been

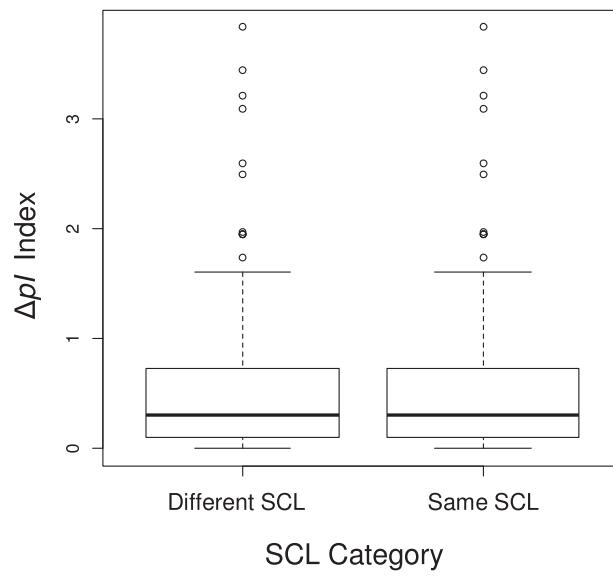


Fig. 3.—Analysis of the pI difference (ΔpI) between duplicated pairs. Box plots of ΔpI in gene pairs with different subcellular localization (SCL) and those with the same subcellular localization. Higher ΔpI values indicate a greater difference in pI between the duplicates.

relocated, further characterize sequence rate evolution in the relocalized genes, determine whether the relocalized genes show changes in expression patterns, and infer sequence changes that may have led to relocalization in some cases with sufficient available localization data. The five duplicated gene pairs selected for analyses were members of families for which there were enough localization data available so that we could make an inference about which gene was relocalized.

Neolocalization and Positive Selection in VAMP723

One gene pair showing accelerated sequence rate evolution of one copy is the tandemly duplicated gene pair for vesicle-associated membrane proteins VAMP723 (At2g33110) and VAMP722 (At2g33120) that contain SNARE (soluble N-ethyl-maleimide sensitive factor receptors) domains (Uemura et al. 2004; Sanderfoot 2007). VAMP722 has two major functions: Involvement in secretory trafficking to the plasma membrane and cell plate formation (Zhang et al. 2011), and contributing to the plant immune response upon the infection with powdery mildew fungi by participating in the formation of an SDS-SNARE complex with the plasma membrane proteins *PEN1* and *SNAP33* (Kwon et al. 2008); in contrast the function of VAMP723 has not been characterized. We used a gene family approach and GFP subcellular localization data from a study of 54 genes with SNARE domains (Uemura et al. 2004) to infer the ancestral, preduplication, state of localization. We found that there have been multiple duplications of the VAMP72 genes to create five genes in *A. thaliana* (fig. 4 and

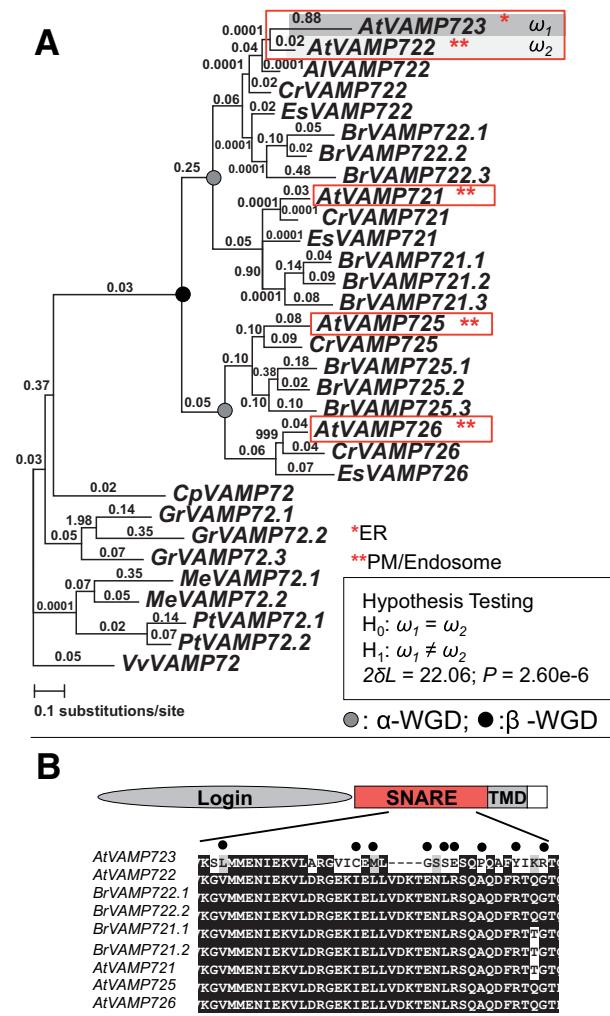


Fig. 4.—Neolocalization, asymmetric sequence evolution, and gene expression divergence in a pair of VAMP proteins. (A) PAML analysis of VAMP genes. Numbers above the branches indicate the dN/dS ratios. dN analysis is shown in supplementary figure S5, Supplementary Material online. Subcellular localization for the proteins in *A. thaliana* is highlighted in red. VAMP722 is relocalized from PM/endosome to ER. Species include: At, *A. thaliana*; Al, *Arabidopsis lyrata*; Cr, *Capsella rubella*; Es, *Eutrema salsugineum*; Br, *Brassica rapa*; Cp, *C. papaya*; Gr, *G. raimondii*; Tc, *T. cacao*; Pt, *P. trichocarpa*; Me, *M. esculenta*; and Vv, *V. vinifera*. See supplementary figure S5, Supplementary Material online, for locus numbers of each gene. (B) Amino acid alignment showing the position of positively selected sites in the SNARE domain of VAMP723 inferred using the empirical Bayes approach.

supplementary fig. S5, Supplementary Material online). Only VAMP723 is localized to the endoplasmic reticulum (ER), whereas the other four gene products are localized to the plasma membrane and endosome. These results strongly suggest that the ancestral state of localization for the VAMP722/VAMP723 gene pair was to the plasma membrane and endosome, and that VAMP723 has been relocalized to the ER.

Table 2

Parameter Estimates and LRT Statistics for the Gene AtVAMP723

Branch-Site Model	P	L	Estimates of Parameters	Positively Selected Sites
Model A test1	64	-6,306.97	$p_0=0.733, p_1=0.036, (p_2+p_3=0.231)$ $\omega_0=0.065, \omega_1=1.000, \omega_2=6.229$	144L (0.960) 159C (1.000) 161M (0.995) 163G (0.996) 165S (0.999) 166E (1.000) 169P (0.954) 173Y (1.000) 176R (0.951)
Model A test2	63	-6,311.76	$p_0=0.391, p_1=0.019, (p_2+p_3=0.590)$ $\omega_0=0.064, \omega_1=1.000, \omega_2=1.000$	Not allowed.

NOTE.—P, number of free parameters for the estimation of ω ratios; p_{0-4} , four different site classes in the branch-site model; ω_{0-2} , three different ω ratios in four different site classes; L, the estimation of log-likelihood value. Boldface indicates parameters for positive selection.

^aValue indicates the posterior probability based on the BEB analysis.

The relocalization appears to be a relatively recent evolutionary event. Results from our phylogenetic analysis showed that VAMP723 and VAMP722 phylogenetically group together, to the exclusion of the VAMP722 genes from *Brassica* and *Eutrema* (fig. 4A), indicating that they are duplicates that formed during evolution of the *Arabidopsis* lineage after it diverged from the *Eutrema* lineage.

We investigated the sequence evolution of VAMP723 and VAMP722 in more detail and determined whether VAMP723 showed any evidence of positive selection. We estimated the nonsynonymous substitution rate (dN) and the dN/dS (omega) ratio (ω) for the branch leading to members of the VAMP72 subfamily in *A. thaliana*, as well as orthologs from *Brassica rapa*, *C. papaya*, *Gossypium ramondii*, *T. cacao*, *M. esculenta*, *P. trichocarpa*, and *V. vinifera*. The results indicated that VAMP723 has a much higher dN and ω than its duplicated partner, VAMP722 (fig. 3A and supplementary fig. S5, Supplementary Material online). From the positive selection analysis, several codons in VAMP723 were detected as positively selected and the majority of those sites are located within the SNARE domain (table 2; fig. 4B).

To determine whether VAMP723 evolved a different expression pattern compared with its duplicated partner, VAMP722, and their closely related paralog VAMP721, we performed gene-specific expression assays using RT-PCR. Results from RT-PCR showed that all three genes were expressed in all examined organ types, but the expression of VAMP723 was very weak in pollen (supplementary fig. S5C, Supplementary Material online). Thus, there has been only limited expression divergence of VAMP723 after duplication from VAMP722.

Relocalization, Novel Expression Pattern, and Accelerated Sequence Evolution of *VTI14*

The *VTI14* (At5g39630) gene is a member of the SNARE family, like the *VAMP* genes in the previous section.

Based on both phylogenetic tree and syntenic block analyses, *VTI14* was formed by tandem duplication of *VTI11* (At5g39510), and the *VTI14/VTI11* precursor was derived by the α -WGD along with *VTI13* (supplementary fig. S6, Supplementary Material online). *VTI11* and *VTI13* (At3g2900), as well as the more distant paralog *VTI12*, are localized in the prevacuolar compartments and the trans-Golgi network (Uemura et al. 2004). *VTI14*, in contrast, is localized to the endosome (Uemura et al. 2004). Our gene family phylogenetic analysis suggested that the most recent common ancestral subcellular localization of the *VTI1* subfamily is localized in the prevacuolar compartments and the trans-Golgi network (fig. 5A), which would indicate that *VTI14* has gained a new targeting ability to the endosome since formation by tandem duplication.

To further examine the asymmetric sequence evolution in *VTI14*, we conducted a more detailed asymmetric rate analysis between *VTI11* and *VTI14* by examining both the omega ratio and the dN rate with additional taxon sampling. The ML analysis showed that *VTI14* evolved much faster than its duplicated partner, *VTI11* (fig. 5A and supplementary fig. S6, Supplementary Material online), indicative of asymmetric rate evolution between *VTI14* and *VTI11*. No evidence of positively selected sites was detected in *VTI14*, suggesting that the rapidly evolving sequence was the result of relaxation of purifying selection.

We examined expression patterns of *VTI14* to compare with *VTI11*, the other related paralog *VTI13*, and the orthologs from *C. papaya* and *G. hirsutum*. Gene-specific gene expression was conducted using RT-PCR. *VTI14* expression was restricted to flowers, pollen, and siliques (fig. 5B). In contrast, orthologs from *Carica* and *Gossypium*, as well as both closely related paralogs, *VTI11* and *VTI13*, showed a broad expression pattern across multiple organ types (fig. 5B). Thus, *VTI14* appears to have acquired a very restricted expression pattern after duplication from the common ancestor with *VTI11*.

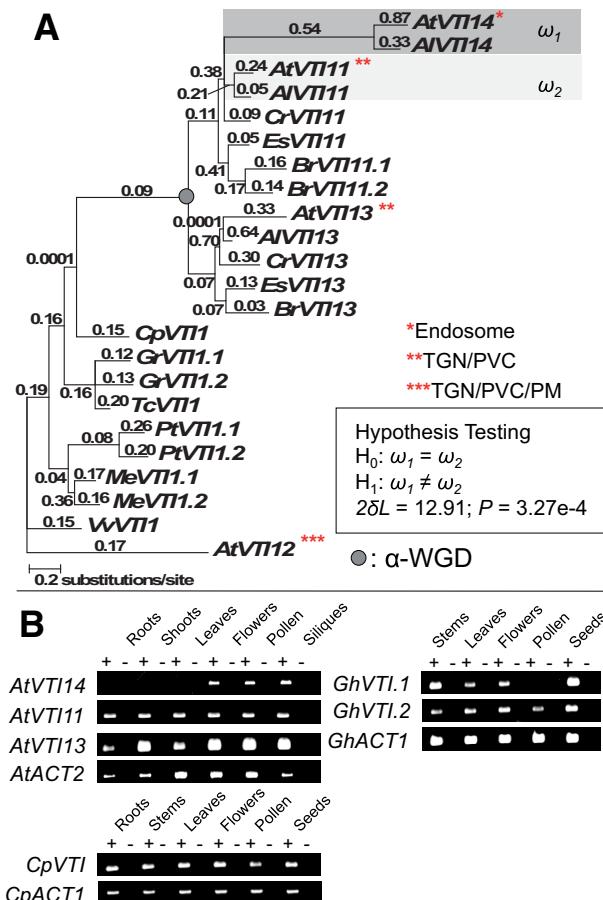


Fig. 5.—Neolocalization, asymmetric sequence evolution, and gene expression divergence in a pair of *VTI* proteins. (A) PAML analysis of *VTI* genes. Numbers above the branches indicate the dN/dS ratios. dN analysis is shown in supplementary figure S6, Supplementary Material online. Subcellular localization for the proteins in *A. thaliana* is highlighted in red. *VTI14* was relocalized from TGN/PVC (trans-Golgi network/prevacuolar compartments) to endosome. Species include: At, *A. thaliana*; Al, *Arabidopsis lyrata*; Cr, *Capsella rubella*; Es, *Eutrema salsugineum*; Br, *Brassica rapa*; Cp, *C. papaya*; Gr, *G. raimondii*; Tc, *T. cacao*; Pt, *P. trichocarpa*; Me, *M. esculenta*; and Vv, *V. vinifera*. See supplementary figure S6, Supplementary Material online, for locus numbers of each gene. (B) RT-PCR expression assays of *VTI14*, *VTI11*, and *VTI13* in *A. thaliana*, and their orthologs in *C. papaya* and *G. hirsutum*. Plus signs (+) indicate reactions with reverse transcriptase and minus signs (−) indicate reactions without reverse transcriptase. *ACT2* was a positive control in *A. thaliana*, whereas *ACT1* was a positive control in *C. papaya* and *G. hirsutum*.

Relocalization and Regulatory Neofunctionalization in Pollen of *TPK4*

A pair of tandem-pore potassium ion (K^+) channel proteins, *TPK4* (At1g02510) and *TPK5* (At4g01840), which are α -WG duplicates, shows different subcellular localizations. *TPK4* plays a role in potassium homeostasis and membrane voltage control in the growing pollen tube (Becker et al. 2004), but the function of *TPK5* remains uncharacterized. *TPK5* and four

other members of this gene family are localized to vacuoles (Becker et al. 2004). In contrast, *TPK4* is localized to the plasma membrane (Becker et al. 2004) and ER (Dunkel et al. 2008). The localization data for the TPK family strongly suggest that the plasma membrane and ER are the derived subcellular location for *TPK4*.

Our sequence rate analyses of dN/dS and dN showed that *TPK4* evolved faster than its duplicate *TPK5* (fig. 6A and supplementary fig. S7, Supplementary Material online). The asymmetric rate evolution in both ω and dN indicated that *TPK4* has accumulated many more amino acid changes than *TPK5* since gene duplication.

TPK4 is predominately expressed in pollen (Becker et al. 2004). Our RT-PCR assay indicated that *TPK5* is broadly expressed, in contrast to *TPK4* (fig. 6). Thus, the expression profile between *TPK5* and *TPK4* has diverged in a complementary organ-specific manner. The complementary organ-specific expression pattern between *TPK4* and *TPK5* can result from one of two possible evolutionary fates, regulatory subfunctionalization and regulatory neofunctionalization. We therefore conducted expression assays of their orthologs from closely related outgroup species in Eurosids II (i.e., *C. papaya* and *G. hirsutum*) by using RT-PCR. Orthologs from both *C. papaya* and *G. hirsutum* were not expressed in pollen (fig. 6B), suggesting that *TPK4* acquired its novel regulatory context in pollen (i.e., regulatory neofunctionalization). In contrast, *TPK5* still reflects its ancestral expression pattern with a broad expression profile except for pollen.

Regulatory Neofunctionalization in Pollen and Potential Sequence Changes Causing Neolocalization of *CPK2*

To examine sequence changes that cause subcellular relocalization, and characterize expression and sequence evolution in another gene whose product has been relocalized, we studied a pair of calcium-dependent protein kinase genes, *CPK2* (At3g10660) and *CPK1* (At5g04870) that were duplicated by the α -WG duplication. GFP localization experiments have shown that they possess different subcellular localization ability: *CPK2* is localized in the ER (Lu and Hrabak 2002) and *CPK1* is localized in peroxisomes and lipid bodies (Dammann et al. 2003; Coca and San Segundo 2010). However, no comparisons of the localizations between the two genes have been made previously. Thus, we evaluated the previous localization studies to gain insights into which gene product was relocalized and what sequence changes may have allowed for relocalization. Lu and Hrabak (2002) experimentally showed that the first ten amino acids of *CPK2* are enough for its subcellular localization to the ER. Dammann et al. (2003) showed that replacing the first seven amino acids of *CPK1* resulted in loss of peroxisome targeting, indicating that the region is a type II N-terminal peroxisomal targeting signal (PTS2) (Reumann 2004). Thus, the peroxisome targeting function appears to be located in the first seven amino acids which we show are highly

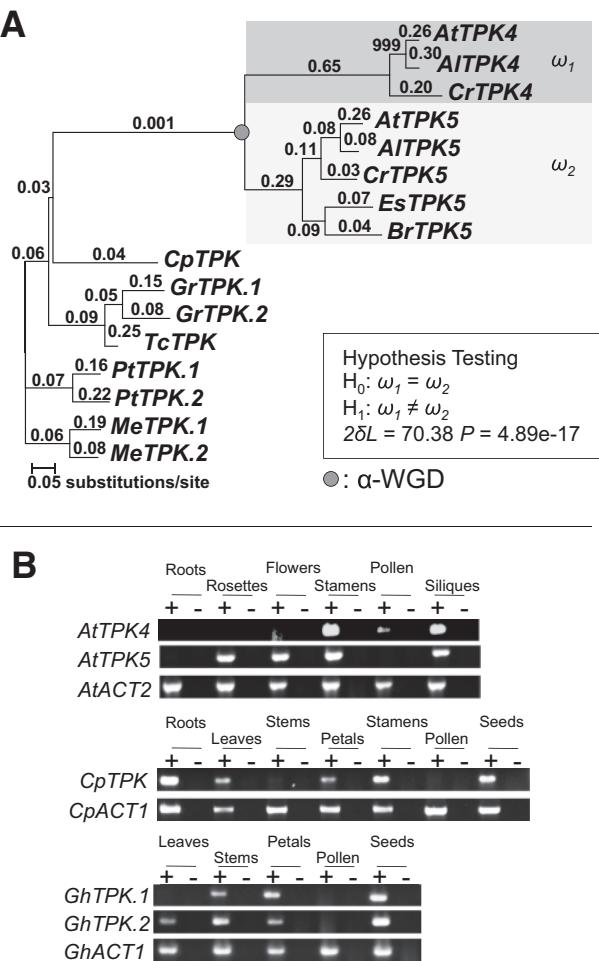


Fig. 6.—Asymmetric sequence evolution, neolocalization, and gene expression divergence in a pair of K^+ channel protein genes. (A) PAML analysis of *TPK* genes. dN analysis is shown in [supplementary figure S7](#), [Supplementary Material](#) online. Numbers above the branches indicate the dN/dS ratios. Species include: At, *A. thaliana*; Al, *Arabidopsis lyrata*; Cr, *Capsella rubella*; Es, *Eutrema salsugineum*; Br, *Brassica rapa*; Cp, *C. papaya*; Gr, *G. raimondii*; Tc, *T. cacao*; Pt, *P. trichocarpa*; and Me, *M. esculenta*. See [supplementary figure S7](#), [Supplementary Material](#) online, for locus numbers of each gene. (B) RT-PCR expression assays of *TPK4* and *TPK5* in *A. thaliana*, and their orthologs in *C. papaya* and *G. hirsutum*. Plus signs (+) indicate reactions with reverse transcriptase and minus signs (-) indicate reactions without reverse transcriptase. *ACT2* was a positive control in *A. thaliana*, whereas *ACT1* was a positive control in *C. papaya* and *G. hirsutum*.

conserved in other species (fig. 7A). This implies that *CPK1* retains the ancestral subcellular localization and *CPK2* acquired its subcellular localization to the ER after duplication. It appears that mutations in amino acids 4 and/or 9 of *CPK2* may have allowed for targeting to the ER, and the substitution of thymine to alanine or valine at position 4 abolished targeting to the peroxisome (fig. 7A).

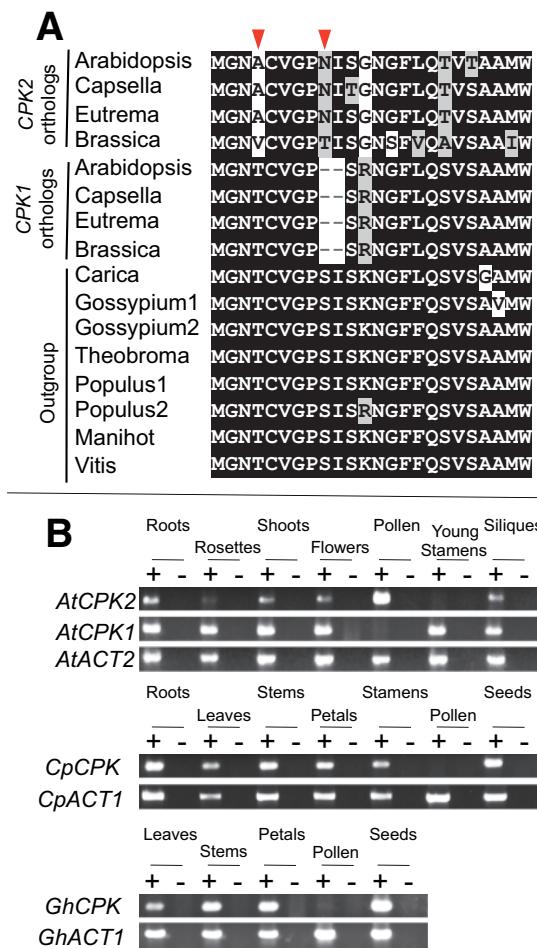


Fig. 7.—Neolocalization and gene expression divergence in *CPK2*. (A) Alignment showing the N-terminal targeting region. Arrowheads indicate *CPK2*-specific amino acid changes in the *CPK2* orthologs. Gene locus numbers are in [supplementary figure S8](#), [Supplementary Material](#) online. (B) RT-PCR expression assays of *CPK1* and *CPK2* in *A. thaliana*, and their orthologs in *C. papaya* and *G. hirsutum*. Plus signs (+) indicate reactions with reverse transcriptase and minus signs (-) indicate reactions without reverse transcriptase. *ACT2* was a positive control in *A. thaliana*, whereas *ACT1* was a positive control in *C. papaya* and *G. hirsutum*.

To determine whether there has been divergence in expression patterns of *CPK1* and *CPK2* since gene duplication, we compared their expression profiles across different organ types by analyzing microarray data from 63 different organ types and developmental stages (Schmid et al. 2005). *CPK2* is predominantly expressed in roots, stamens, and pollen, whereas *CPK1* is broadly expressed across multiple organ types but not in pollen ([supplementary fig. S3B](#), [Supplementary Material](#) online). Thus, the expression profile between *CPK1* and *CPK2* diverged in a complementary organ-specific manner. To confirm the microarray data, we performed expression assays using RT-PCR. *CPK1* was strongly expressed in every examined organ type except for pollen

where no expression was detected (fig. 7B). In contrast, *CPK2* was predominantly expressed in pollen, and showed weaker expression in roots, shoots, flowers, and siliques (fig. 7B). Thus, *CPK1* and *CPK2* show a complementary organ-specific expression pattern in pollen. We assayed expression of orthologs from *C. papaya* and *G. hirsutum* using RT-PCR with multiple organ types. Both species showed expression in various organ types but not in pollen (fig. 7B), strongly suggesting that *CPK1* reflects the ancestral expression pattern and the expression of *CPK2* in pollen was derived after gene duplication. Thus, *CPK2* has undergone regulatory neofunctionalization in pollen. Finally, asymmetric sequence evolution was not found between *CPK2* and *CPK1* (supplementary fig. S8, Supplementary Material online).

Potential Sequence Changes Causing Neolocalization of *PP2A B'*

A pair of genes for the beta subunit of phosphatase 2A *B'*, *PP2A B'* θ (At1g13460) and *PP2A B'* η (At3g26020), was formed by the α -WG duplication. GFP localization experiments revealed that *PP2A B'* θ localized to peroxisomes and *PP2A B'* η localized to the nucleus and cytosol (Matre et al. 2009). When the four amino acids at the C-terminus were deleted from *PP2A B'* θ , it no longer localized to the peroxisomes (Matre et al. 2009), indicating that the SSL peptide at the C-terminus, which is one of the PTS1 peroxisome localization signals (Reumann et al. 2007), is responsible for localization to the peroxisomes. The *PP2A B'* genes have not been studied in the context of their evolution after duplication. We performed comparative sequence analysis to determine which gene's product shows relocalization. We compared the C-termini of *PP2A B'* θ and *PP2A B'* η with orthologs in outgroup species to gain insights into the ancestral localization (fig. 8). Only *PP2A B'* θ contains the SSL peroxisome localization peptide, strongly suggesting that the ancestral subcellular localization is not to peroxisomes. Thus it appears that *PP2A B'* θ has been relocalized to the peroxisomes by creation of an SL peptide at the C-terminus, either by addition of the SL peptide or by mutation of two amino acids after duplication that were independently deleted in *PP2A B'* η (fig. 8 and supplementary fig. S9A, Supplementary Material online). In addition, we predicted the subcellular localization of orthologs from outgroup species using the PTS1 peroxisome predictor software (Neuberger et al. 2003). None of them showed evidence of potential peroxisomal targeting (supplementary fig. S9B, Supplementary Material online), further supporting the inference that *PP2A B'* θ relocalized to peroxisomes after gene duplication. Additional support for neolocalization comes from the inference of their ancestral subcellular localization using a gene family approach. In the *PP2A B'* gene family, two additional members, At3g21650 (*PP2A B'* ζ) and At4g15414 (*PP2A B'* γ), have been shown to target mitochondria/cytosol and nucleus/cytosol (Matre et al. 2009), suggesting that

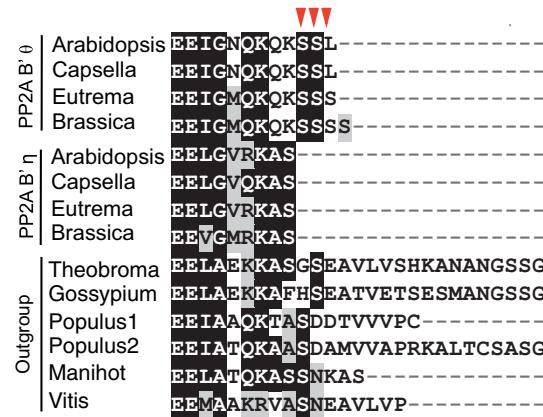


Fig. 8.—Alignment of subcellular targeting signal regions in *PP2A B'*. The C-terminal region of a pair of protein phosphatase 2A proteins, *PP2A B'* θ and *PP2A B'* η , and their orthologs in the outgroup species are shown. Signal peptides for peroxisome localization are indicated by inverted triangles. Gene locus numbers are in supplementary figure S10, Supplementary Material online.

peroxisomal targeting is not the ancestral state in the *PP2A B'* gene family.

The SSL motif is present in *PP2A B'* θ in *Arabidopsis* and *Capsella*, but not in *Brassica* or *Eutrema* that have SSS and SSSS, respectively (fig. 8). *Arabidopsis* and *Capsella* belong to the same tribe within the Brassicaceae, whereas *Brassica* and *Eutrema* are in a different clade (Bailey et al. 2006). Thus, it appears that relocalization of *PP2A B'* θ occurred in a common ancestor of *Arabidopsis* and *Capsella* after divergence from the common ancestor of *Brassica* and *Eutrema*. *PP2A B'* θ was formed by duplication during the α -WG duplication at the base of the Brassicaceae family. Thus, relocalization of *PP2A B'* θ in the *Arabidopsis*–*Capsella* lineage may have occurred long after its formation by gene duplication. An alternative possibility is that relocalization of *PP2A B'* θ occurred in a common ancestor of all four Brassicaceae species mentioned above and then *PP2A B'* θ in *Brassica* and *Eutrema* experienced mutations that abolished peroxisomal localization.

Except for marginally significant asymmetric ω evolution (supplementary fig. S10A, Supplementary Material online), asymmetric amino acid or dN sequence evolution was not found between *PP2A B'* θ and *PP2A B'* η (table 1; supplementary fig. S10B, Supplementary Material online). Finally, *PP2A B'* θ and *PP2A B'* η have very similar expression patterns, from AtGenExpress microarray data (Schmid et al. 2005).

Features of Relocalized Genes

Asymmetric protein sequence rate evolution in one member of a duplicate pair has been inferred as a potential indicator of functional divergence because one copy has experienced an accelerated rate of amino acid replacements in comparison to

its duplicated partner (e.g., Dermitzakis and Clark 2001; Blanc and Wolfe 2004; Kim and Yi 2006; Byrne and Wolfe 2007). In this study, we found that significantly more gene pairs with divergent localization of the products showed asymmetric rate evolution (42%) than those pairs whose products have the same localization location (12%). We then presented evidence for a few cases in which the more rapidly evolving gene has undergone relocalization. Some duplicated gene products that have undergone relocalization may have experienced changes in their cellular roles, or functional diversification, in the new cellular location, which were promoted by amino acid sequence changes. In duplicated genes of yeast, Marques et al. (2008) showed that gene pairs with the same subcellular localization tend to have lower amino acid sequence divergence than duplicated pairs with different subcellular localization. Our results are consistent with that study, indicating that the trend also extends to plants.

In this study, we show that duplicated genes with subcellular relocalization sometimes can show changes in expression patterns including regulatory neofunctionalization (a new expression pattern compared with the inferred ancestral state). In three cases (*CPK2*, *TPK4*, *PRX36*), we presented evidence that the gene with the relocalized product shows regulatory neofunctionalization. Another type of new expression pattern after duplication, the expression pattern becoming restricted to a small number of organ types compared with the ancestral gene, is illustrated by *VTI14*. The above examples indicate that duplicated genes whose products have been relocalized can show major changes in expression patterns and regulatory neofunctionalization. However, not all relocalized gene products show major changes in expression patterns, as shown with *VAMP723* and *PP2A B'*. Subcellular relocalization is caused by changes in amino acid sequences, whereas regulatory neofunctionalization is typically caused by changes in regulatory sequences. Some genes in this study showed both phenomena (*CPK2*, *TPK4*, *VTI14*, and *PRX36*), whereas others showed only one.

In addition to asymmetric and accelerated sequence rate evolution and regulatory neofunctionalization, a few cases show evidence for changes in cellular roles or functions. For example, *PRX36* is specifically expressed in siliques and was shown recently to be required for seed coat mucilage extrusion (Kunieda et al. 2013). *PRX36* has been shown to loosen the outer cell wall of the seed coat in facilitating the mucilage extrusion by targeting to the outer cell wall. Its paralog, *PRX72*, is localized in the cytoplasm and thus not involved in cell wall functions. Thus, *PRX36* has a different cellular role, involved in seed coat mucilage extraction, than *PRX72*, but the products of both genes may function as peroxidases. In another example, involving *VTI11* and relocalized *VTI14*, *VTI11* has been shown to form SNARE complexes with *SYP2* and *SYP5*-type syntaxins (reviewed in Surpin and Raikhel 2004), which mediate trafficking to lytic vacuoles (Sanmartín et al. 2007). However, *VTI14* does not localize trans-Golgi network

and the prevacuolar compartments but instead localizes to endosomes. Thus, *VTI14* likely has a different cellular role from *VTI11*.

Evolutionary Timing of Subcellular Localization after Gene Duplication

In this study, we examined genes duplicated during the evolution of the Brassicaceae family as a group for assessing subcellular relocalization of evolutionarily recent duplicates in plants. A large majority of the duplicated genes showing subcellular relocalization were duplicated at the base of the Brassicaceae in the α -WGD. A few cases of relocalization are more evolutionarily recent. For example, both *VAMP723* and *VTI14* were formed by duplication in the *Arabidopsis* lineage after it diverged from the *Brassica* lineage. In contrast to our findings in plants, studies of subcellular relocalization showed that relocalization after gene duplication in animals and yeasts often evolved in duplicated genes that were derived from ancient WG duplication event in yeasts (~100 Ma) and animals (~500 Ma), but it was less common in younger duplicated genes (Marques et al. 2008; Kassahn et al. 2009; Szklarczyk and Huynen 2009). Both Szklarczyk and Huynen (2009) and Wang et al. (2009), in studies of mitochondrial protein localization, showed that relocalized duplicates were ancient, before the divergence of bilateria and before the divergence of vertebrates (Szklarczyk and Huynen 2009) or roughly corresponding to the time of the two WG duplication events early in animal evolution (Wang et al. 2009). Thus, it is possible that subcellular relocalization of duplicated genes is a more common ongoing process in plants than in animals and yeasts.

Supplementary Material

Supplementary figures S1–S10 and tables S1–S4 are available at *Genome Biology and Evolution* online (<http://www.gbe.oxfordjournals.org/>).

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