Introduction

Gene family is a set of homologous genes that are likely to share similar functions. Changes in family size due to lineage-specific gene duplication and gene loss might provide insights into the evolutionary forces that have shaped eukaryotic genomes (Dumuth et al. 2006). Thus, inferring an evolutionary scenario for a gene family is essential for understanding the phenotypic diversification of organisms (Doyon and Liu 2007).

The K⁺ transport systems are multifunctional proteins encoded by a large gene family found in nearly all plant tissues. The critical K⁺ requirement for plants and the complex composition of plant environments make the number of K⁺ transport systems so large to enable the uptake of K⁺ from the soil and its redistribution throughout diverse plant tissues (Gierth et al. 2005; Ashley et al. 2006; Amrutha et al. 2007). In plants, potassium transporters have been classified into four major families: the KT/HAK/KUP family, Trk/HKT family, KEA (K⁺ efflux anti-porter) family, and CHX (cation/hydrogen exchanger) family (Gupta et al. 2008). Among them, KT/HAK/KUP family is the largest and it plays an important role...
in plant growth and development (Ahn et al. 2004). The first plant KT/HAK/KUP gene was identified in Arabidopsis by sequence similarity with bacteria KUP and fungi HAKs (Quintero and Blatt 1997; Santa-Maria et al. 1997). To date, KT/HAK/KUP genes have been cloned from many plant species such as pepper (Martinez-Cordero et al. 2004), tomato (Wang et al. 2002; Nieves-Cordones et al. 2007), lotus (Desbrosses et al. 2004), and seagrass (Garcia-eblas et al. 2002). Twenty-seven and 13 KT/HAK/KUP family genes have been identified based on genome-wide sequence analysis in rice (Oryza sativa; Yang et al. 2009) and Arabidopsis thaliana (Maser et al. 2001), respectively. Lineage-specific expansion of this HAK family in rice after the split of monocots and dicots has been found. However, limited information is available on KT/HAK/KUP genes in perennial species. Such long-lived species are often exposed to repeated episodes of abiotic and biotic stresses during their long life span (Brosche et al. 2005). These features might have favored the expansion of KT/HAK/KUP gene family in perennial species.

The complex history of genome duplications and chromosomal rearrangements in Populus, and the recently available genome sequences provides an opportunity to study the patterns of gene family expansion in the course of genome evolution (Tuskan et al. 2006). In this study, we first identified the KT/HAK/KUP transporters gene family members in Poplar and then integrated analysis of phylogeny, exon structure, protein domain, and paralogon were applied to elucidate the evolutionary relationships of Poplar KT/HAK/KUP transporters genes. In addition, modes of selection were measured for the KT/HAK/KUP duplicate pairs, and a relative rate test was also conducted to examine whether one of the duplicates has evolved at an accelerated rate following the duplication. With such in-depth investigation, it might provide more insights into the underlying evolution mechanisms of trees acclimating to their natural habitat.

**Methods**

**Genomic data mining, KT/HAK/KUP gene identification and nomenclature**

To identify KT/HAK/KUP genes in Populus trichocarpa, 13 full-length KT/HAK/KUP protein sequences of Arabidopsis thaliana (Maser et al. 2001; Very and Sentenac 2003) (Table S1) were used as the query sequences in TBLASTN searches (E < 1e-10) against P. trichocarpa genome database (JGI v2.0). Each matching sequences were used reiteratively to search the respective sequence databases, until no new sequences were found. Then, the tool of Pfam (Sonnhammer et al. 1997) was used to predict the potassium (K+) transporter domain (PF02705) of all the candidate proteins. The deduced nucleotide and protein sequences of KT/HAK/KUP genes in P. trichocarpa were downloaded from the 31st release of the Gramene database (http://www.gramene.org/). The EST searches for KT/HAK/KUP genes in P. trichocarpa were performed using BLASTN tool against the EST database in PopulusDB. The nomenclature for Populus KT/HAK/KUPs followed the study of Yang et al. (2009).

**Phylogenetic analysis**

To gain more insight into evolution of the KT/HAK/KUP gene family in P. trichocarpa, we downloaded the members of this family in green alga (Chlamydomonas reinhardtii) and rice (Oryza sativa L.). Multiple amino acid sequence alignments of KT/HAK/KUPs were generated using muscle3.6 (Edgar 2004) with the default setting. The bootstrap consensus phylogenetic tree inferred from 1000 replicates was constructed using the neighbor-joining method (Saitou and Nei 1987) with MEGA4 (Tamura et al. 2007).

**Chromosomal location and expansion patterns of Populus HAK genes**

To categorize expansion of the KT/HAK/KUP gene family, we examined the chromosomal locations of all members of this family in Poplar (JGI v2.0). Segmental (chromosomal segments) duplication, tandem duplication (duplications in a tandem pattern), and transposition events result in gene family expansion (Cannon et al. 2004). In this study, we focused on the segmental and tandem duplication. Previous analysis of the Populus genome has identified paralogous segments created by the whole-genome duplication event in the Salicaceae (salicoid duplication), 60 to 65 million years ago (Tuskan et al. 2006). A method similar to that of Maher et al. (2006) was used to identify segmental duplications. First, the distributions of KT/HAK/KUP genes relative to Tuskan’s duplicate genomic blocks (Tuskan et al. 2006) are illustrated using SyMAP (Synteny Mapping and Analysis Program) v3.3 (Soderlund et al. 2006). Next, 10 protein-coding genes upstream and downstream of each pair of paralogs were obtained from the annotated genomes of Poplar (JGI v2.0). Finally, the similarity between the genes flanking one KT/HAK/KUP gene and those flanking the other KT/HAK/KUP gene in each pair of paralogs was determined. Thus, we recognized that paralogous KT/HAK/KUP genes are originated from a duplication event if they resided within a region of conserved protein-coding genes.

**Exon structure and protein domain of HAK genes**

Gene intron/exon structure information of alga, arabi-dopsis, rice, and Poplar HAK genes was collected from the
genome annotations of Phytozone v6.0 (http://www.phytozone.net/), and a web application named Gene Structure Display Server (GSDS) (http://gsds.cbi.pku.edu.cn/) was used to draw gene structure diagram. Exon boundaries within the coding regions of each tandem or segmental duplicated Populus KT/HAK/KUP pairs were determined according to the 31st release of the Gramene database (http://www.gramene.org/). The numbers of nucleotides (nt) for each exon as well as the phase of each splicing site also were determined. The potential domains of each HAK protein was identified based on the Pfam database (Finn et al. 2008) to better understand its evolution within different species.

**Ratio of nonsynonymous to synonymous substitutions (dN/dS) and relative evolutionary rate test**

The coding sequences of the Poplar HAK genes were aligned following the amino acid alignment by CodonAlign 2.0 (http://homepage.mac.com/barryghall/CodonAlign.html). The numbers of nonsynonymous nucleotide substitutions per nonsynonymous site (dN) and the numbers of synonymous nucleotide substitutions per synonymous site (dS) were estimated with the yn00 program of PAML4 (Yang 2007). Tajima relative rate tests (Tajima 1993) were performed with amino acid sequences for the 11 Poplar KT/HAK/KUP duplicate pairs using MEGA4 (Tamura et al. 2007).

**Results**

**Identification and phylogenetic analysis of Populus KT/HAK/KUP genes**

A total of 31 full-length genes encoding putative KT/HAK/KUP proteins were identified in the P. trichocarpa genome (Table 1). The number was larger than that of KT/HAK/KUP genes present in the rice (27 HAK genes) and almost 2.5 times to that of Arabidopsis (13 HAK genes) genomes. In addition to 21 KT/HAK/KUP genes identified by Yang et al. (2009), 10 genes also encoded KT/HAK/KUP potassium transporter, which was named as PtHAK22 to PtHAK31, respectively. Two genes (PtHAK23 and PtHAK30), each with 2 JGI loci, were re-predicted using FGENESH+ 2.6 software. The length of KT/HAK/KUP transporters in Polranged from 258 aa to 860 aa, and the number of exons ranged from 1 to 11. The results of EST searches showed that all the KT/HAK/KUP genes in Polranged at least one significant EST hit (score > 400, E < 1e-10). These results indicate that all the KT/HAK/KUPs were expressed in Polr genome.

To gain more insight into evolution of the KT/HAK/KUP gene family in P. trichocarpa, the previously genome-wide identified amino acid sequences of 13 KT/HAK/KUP genes in dicotyledonous Arabidopsis thaliana, and 27 in monocotyledonous rice (Oryza sativa L.) (Table S1) were used in our phylogenetic analysis. Also, four genes in green alga (Chlamydomonas reinhardtii) were used as outgroup in our study. Phylogenetic analysis defined clearly the six KT/HAK/KUP groups (Fig. 1), namely I-a, I-b, II-a, II-b, III, and IV group. The classification in this analysis was consistent with the result of Banuelos et al. (2002) and Yang et al. (2009). The KT/HAK/KUP genes from each of the groups were found in all three studied species of the higher plants with the exception of members of group IV, which was absent in the Arabidopsis genome. The II-b group constituted the largest clade containing 12 Poplar KT/HAK/KUP members, and the I-a group formed the second largest clade containing six Poplar members. Additionally, the I-a and I-b groups further clustered forming a larger clade and implying that they originated from a common ancestor by subsequent gene duplication. Also, the same as II-a and II-b groups. Structural analyses can provide valuable information concerning duplication events when interpreting phylogenetic relationships within gene families. Thus, the exon/intron structure of each member of the KT/HAK/KUP family was analyzed (Fig. 1B). Most of the KT/HAK/KUP genes in high plant comprise 8–10 exons (less than that in green alga) and a last exon with the maximum length. Most members within the individual groups shared similar intron/exon structures, consistent with the phylogenetic classification. For all KT/HAK/KUP protein family members, K+ potassium transporter domain is present in all species with a high degree of similarity (Fig. 1C) except CrHAK1’s Myb-like DNA-binding domain, indicating the functional conservation features of these protein. But some truncated K+ potassium transporter domain are found in some KT/HAK/KUP proteins, indicating a functional divergence to some extent of this protein family members during the long evolution process.

**Genomic organization of the Populus KT/HAK/KUP gene family**

The physical locations of 29 KT/HAK/KUPs were dispersed to 13 of the 19 Populus chromosomes, while the other two HAK genes were localized to unassembled genomic sequence scaffolds and thus were not mapped to any particular chromosome (Table 1). The distribution of the KT/HAK/KUP genes among the chromosomes appears to be uneven: chromosomes 4, 6, 11, 16, 17, and 18 harbor no KT/HAK/KUP genes, while relatively high densities of KT/HAK/KUPs with some apparent tandem duplications were discovered in some locations on chromosomes 1 (seven genes) and 3 (five genes), and each of chromosomes 2, 5, 7, 9, 13, and 19 possess a single HAK
Two paralogs located in tandem in a chromosomal segment are likely the result of single-gene duplication (Olinski et al. 2006). According to this, six pairs of tandem duplicated paralogs (PtHAK1/PtHAK19, PtHAK20/PtHAK22, PtHAK6/PtHAK11, PtHAK13/PtHAK14, PtHAK3/PtHAK18, PtHAK28/PtHAK29) were discovered in 31 Poplar HAK genes (Fig. 2).

The identification of chromosomal homologous segments within genomes (known as paralogons) can aid in studying genome evolution, such as genome duplication and rearrangement (Murphy et al. 2005). Paralogous segments of Populus genome created by the whole-genome duplication event in the Salicaceae (salicoid duplication), 60 to 65 million years ago, have been identified (Tuskan et al. 2006). The distributions of KT/HAK/KUP genes relative to the duplicate genomic blocks are illustrated in Figure 2A. Of the 31 genes, only nine are located outside any duplicate blocks. Next, 10 protein-coding genes upstream and downstream of each pair of paralogs were obtained from the annotated genomes of Poplar. Finally, the similarity between the genes flanking one HAK gene and those flanking the other HAK gene in each pair of paralogs was determined. Eight pairs of segmental duplicated paralogs (PtHAK1[19]/PtHAK6[11], PtHAK10/PtHAK5, PtHAK2/PtHAK7, PtHAK24/PtHAK25, PtHAK21/PtHAK27, PtHAK13[14]/PtHAK18[3], PtHAK28[29]/PtHAK9, PtHAK8/ PtHAK16) were discovered in 31 Poplar KT/HAK/KUP genes, suggesting the ancestral relationship of these chromosomes or chromosome fragments.

### Exon structure and splice site analysis

Conserved exon structures including exons with the same numbers of nucleotides as well as the conserved intron.
phases indicate similarities of the studied genes (von Schantz et al. 2006). Exons colored red are conserved in length and almost the same splice phase among all Populus HAK genes, including 261-nt exon, 53-nt exon, and 255-nt exon (Fig. 3). In addition to these three exons, there are equal-length-exons with the same splice phase shared by each duplicated HAK pairs. At least five conserved exon structure was shared by the tandem or segmental duplicated HAK pairs except two shared by PtHAK5/PtHAK10 pairs (Fig. 3), supporting the common ancestral relationship of these duplicated pairs.

**Evolution of the coding sequences of the 11 duplicated KT/HAK/KUP pairs**

Modes of selection can be estimated by the ratio of the numbers of nonsynonymous substitutions per nonsynonymous site (dN) to the numbers of synonymous substitutions per synonymous site (dS), that is, dN/dS > 1 indicates positive selection; dN/dS < 1, purifying selection; and dN/dS = 1, neural selection (Yang and Nielsen 2000). The combination of phylogenetic, exon structure and splice site, and paragon analyses revealed 11 pairs of Poplar KT/HAK/KUP duplicates (Table 2). The nonsynonymous substitution rates of all these 11 duplicate pairs are markedly lower than their synonymous substitution rates and their dN/dS values are <1 (Table 2), suggesting that these duplicates likely have been subject to purifying selection. Furthermore, Tajima (Tajima 1993) relative rate tests were conducted to investigate whether one of the KT/HAK/KUP duplicates has evolved at an accelerated rate following the duplication. A statistically significant increase in evolutionary rate occurs in three segmental duplicated pairs (PtHAK1/PtHAK6, PtHAK13/
PtHAK18, PtHAK24/PtHAK25) (Table 3). These eight pairs are included in the Group I-b, II-b, and III in our phylogenetic analysis.

Discussion

Gene duplication including segmental (chromosomal segments) duplication, tandem duplication (duplications in a tandem pattern), and transposition events can result in gene family expansion (Cannon et al. 2004), and functional diversification among gene family members have been considered as an important source of evolutionary innovation in complex organisms. Lineage-specific expansion of KT/HAK/KUP gene families in rice has been reported (Yang et al. 2009). However, there is far less information about this family for woody plant species. Although 21 Poplar KT/HAK/KUP genes were mentioned in rice’s comparative genomic study (Yang et al. 2009), only part of KT/HAK/KUP genes have been identified because of the authors focus and sequenced gap of P. trichocarpa genome database (JGI v1.0), and no evolutionary information, expansion pattern, and modes of selection about this family was mentioned. In our study, through genome-wide in-depth investigation, we identified 31 members of KT/HAK/KUP potassium transporter gene family in Poplar genome, larger members than that in rice, and more than twice of the members in Arabidopsis. To investigate the expansion of KT/HAK/KUP family in Poplar, a phylogenetic tree was reconstructed using plant full-length KT/HAK/KUP proteins. The phylogenetic tree divided the plant KT/HAK/KUP genes into six distinct groups, and Populus KT/HAK/KUP genes were dispersed to all groups. Paralogous segments of Populus genome created by the whole-genome duplication event in the Salicaceae (salicoid duplication), 60 to 65 million years ago, have been identified (Tuskan et al. 2006). Of the 31 genes, only nine are located outside of any duplicate genomic blocks. Eight pairs of paralogous genes were found, suggesting that segmental duplication of genes was the major force for expansion of the KT/HAK/KUP family in Poplar. It should be noted that six pairs of tandem duplicated paralogs (PtHAK1/PtHAK19, PtHAK20/PtHAK22, PtHAK6/
PtHAK11, PtHAK13/PtHAK14, PtHAK3/PtHAK18, PtHAK28/PtHAK29) were discovered in 31 Poplar KT/HAK/KUP
genes. Of these six pairs, four pairs (PtHAK13/PtHAK14,
PtHAK3/PtHAK18, PtHAK1/PtHAK19, and PtHAK6/PtHAK11) were also found in segmental duplications. We
speculate segmental duplications might occur before tan-
dem duplications as far as these four pairs. Maybe four
HAK genes were descended from two ancestral HAK genes
through segmental duplication, and then tandem duplica-
tions occurred leading to four pairs (eight genes) present
in the current Poplar genome. Further investigation sug-
gests that segmental duplication and tandem duplication
all contributed to the expansion of this family in Poplar.
Such conclusion presented here is further supported by the
high degree of conservation of exon structures (Fig. 2) and
paralogon analysis (Fig. 3).

Figure 3. Exon structures (5'→3') and splice site analysis of each tandem or segmental duplicated Populus KT/HAK/KUP pairs. The exons that
transverse or flank the splice sites are indicated above each exon boundary. Numbers in boxes are nucleotide length. Exons colored red are
conserved in length among all Populus KT/HAK/KUP genes. Exons colored green, brown, darkblue, blue, gray, orange, lime, aqua, or rose are
conserved in length between each duplicated Populus KT/HAK/KUP pairs. The size of each exon is not drawn to scale.

Table 2. Divergence between paralogous KT/HAK/KUP gene pairs in Populus.

| No | Gene 1 | Gene 2 | dN   | dS   | dN/dS  |
|----|--------|--------|------|------|--------|
| 10 | PtHAK23 | PtHAK4 | 0.0049 ± 0.0035 | 0.0219 ± 0.0127 | 0.2248 |
| 20 | PtHAK22 | PtHAK30 | 0.1614 ± 0.0119 | 0.1767 ± 0.0216 | 0.9132 |
| 30 | PtHAK13 | PtHAK18 | 0.0907 ± 0.0156 | 0.2917 ± 0.0578 | 0.3108 |
| 45 | PtHAK10 | PtHAK5 | 0.0445 ± 0.0106 | 0.2409 ± 0.0513 | 0.1849 |
| 55 | PtHAK1 | PtHAK6 | 0.0123 ± 0.0055 | 0.1965 ± 0.0441 | 0.0627 |
| 65 | PtHAK2 | PtHAK7 | 0.0150 ± 0.0062 | 0.3348 ± 0.0628 | 0.0449 |
| 75 | PtHAK24 | PtHAK25 | 0.1157 ± 0.0181 | 0.2378 ± 0.0473 | 0.4864 |
| 85 | PtHAK28 | PtHAK9 | 0.0476 ± 0.0110 | 0.2786 ± 0.0571 | 0.1709 |
| 90 | PtHAK16 | PtHAK31 | 0.0000 ± 0.0000 | 0.0067 ± 0.0067 | 0 |
| 105 | PtHAK3 | PtHAK14 | 0.0257 ± 0.0082 | 0.2532 ± 0.0507 | 0.1015 |
| 115 | PtHAK21 | PtHAK27 | 0.0583 ± 0.0123 | 0.1857 ± 0.0420 | 0.3141 |

These gene pairs were identified at the terminal nodes of the gene tree shown in Figure 1. Gene pairs created by tandem duplication (T), segment
duplication (S), or other (O) events are indicated in the first column of the table. Synonymous (dS) and nonsynonymous substitution (dN) rates are
presented for each pair.
After gene duplication, two duplicates can undergo substantial functional divergence, and it plays a major role in the evolution of new functions (Ohta 1989). In this study, the combination of phylogenetic, exon structure and splice site, and paragon analyses revealed 11 pairs of Poplar KT/HAK/KUP duplicates, and purify selection has been found to contribute to the evolution of these pair. These results suggested that purify selection contributed to evolution of this gene family. Furthermore, Tajima relative rate tests identified accelerated evolutionary rates in three of the duplicates (Table 3), but none of the three pairs reported to have dN/dS > 1. Therefore, the rate increases might simply be at silent sites which do not contribute to functional diversification.

In summary, 31 Poplar KT/HAK/KUP transporter genes have been identified in genome-wide analysis, and segmental duplication and tandem duplication events contributed to the expansion of this family in Poplar. Purify selection and asymmetric evolutionary rates have been found to contribute to the evolution of this gene family. With such in-depth investigation, it might provide more insights into the underlying evolution mechanisms of trees acclimating to their natural habitat.

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Conflict of Interest

None declared.

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Table 3. Tajima relative rate tests of Populus KT/HAK/KUP duplicate genesa.

| Testing group                      | Mt | M1 | M2 | X²  | P     |
|-----------------------------------|----|----|----|-----|-------|
| PtHAK23/PtHAK4 with               | 458| 7  | 3  | 1.60| 0.20590|
| AThAK5                            | 295| 10 | 9  | 0.05| 0.81855|
| PtHAK22/PtHAK30 with              | 392| 1  | 0  | 1.00| 0.31731|
| AThAK5                            | 606| 10 | 28 | 8.53| 0.00350|
| PtHAK16/PtHAK31 with              | 610| 7  | 14 | 2.33| 0.12663|
| AThKUP10                          | 355| 17 | 17 | 0.00| 1.00000|
| PtHAK10/PtHAK5 with               | 613| 20 | 17 | 0.24| 0.62187|
| AThKUP7                           | 161| 7  | 27 | 11.76| 0.00060|
| PtHAK21/PtHAK25 with              | 353| 8  | 12 | 0.80| 0.37109|
| OsHAK13                           | 572| 50 | 14 | 20.25| 0.00001|
| PtHAK13/PtHAK18 with              | 152| 11 | 7  | 0.89| 0.34578|
| AtKUP12                           |    |    |    |     |       |
| PtHAK28/PtHAK9 with               |    |    |    |     |       |
| OsHAK13                           |    |    |    |     |       |

aThe Tajima relative test was used to examine the equality of evolutionary rate between Populus duplicate pairs.

bMt is the sum of the identical sites and the divergent sites in all three sequences tested.

cM1 is the number of unique differences in the first paralog.

dM2 is the number of unique differences in the second paralog.

If P < 0.05, the test rejects the equal substitution rates between the two duplicates and infers that one of the two duplicates has an accelerated evolutionary rate.
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Supporting Information

Additional Supporting Information may be found in the online version of this article:

**Table S1.** KT/HAK/KUP potassium transporter gene family genes in Arabidopsis thaliana, Oryza sativa L., and Chlamydomonas reinhardtii

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