Genome-Wide Survey and Expression Analysis of Amino Acid Transporter Gene Family in Rice (*Oryza sativa* L.)

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**Abstract**

**Background:** Amino acid transporters (AATs) that transport amino acids across cellular membranes are essential for plant growth and development. To date, a genome-wide overview of the AAT gene family in rice is not yet available.

**Methodology/Principal Findings:** In this study, a total of 85 AAT genes were identified in rice genome and were classified into eleven distinct subfamilies based upon their sequence composition and phylogenetic relationship. A large number of *OsAAT* genes were expanded via gene duplication, 23 and 24 *OsAAT* genes were tandemly and segmentally duplicated, respectively. Comprehensive analyses were performed to investigate the expression profiles of *OsAAT* genes in various stages of vegetative and reproductive development by using data from EST, Microarrays, MPSS and Real-time PCR. Many *OsAAT* genes exhibited abundant and tissue-specific expression patterns. Moreover, 21 *OsAAT* genes were found to be differentially expressed under the treatments of abiotic stresses. Comparative analysis indicates that 26 AAT genes with close evolutionary relationships between rice and *Arabidopsis* exhibited similar expression patterns.

**Conclusions/Significance:** This study will facilitate further studies on *OsAAT* family and provide useful clues for functional validation of *OsAATs*.

**Introduction**

Amino acid transporters (AATs) are the integral membrane proteins which mediate the transport of amino acids across cellular membranes in higher plants, and play an indispensable role in various processes of plant growth and development, including long distance amino acid transport, response to pathogen and abiotic stresses [1–3]. By heterologous expression systems and database screening with known transporters, more than 60 distinct AAT genes have been identified in *Arabidopsis* [4], as well as several genes in other plant species. Plant AAT family includes two main families that belong to the amino acid-polyamine-choline (APC) transporter superfamily: the amino acid/auxin permease (AAAP) family and the APC family [5,6]. There are at least six subfamilies in the AAAP family, including amino acid permeases (AAPs), lysine and histidine transporters (LHTs), proline transporters (ProTs), γ-aminobutyric acid transporters (GATs), auxin transporters (AUXs), and aromatic and neutral amino acid transporters (ANTs). The APC family in plants is grouped into three subfamilies: cationic amino acid transporters (CATs), amino acid/choline transporters (ACTs) and polyamine H+symporters (PHSs) [7–9].

Previously, molecular functions of many AATs were fully characterized in *Arabidopsis*. The eight members (*AtAAP1*, *AtAAP6*, *AtAAP8*) were found in the *AtAAP* subfamily, and six AtAAPs were demonstrated to transport neutral and charged amino acids with varying specificities and affinities [10–12]. *AtAAP1* was highly expressed in the cotyledons and the endosperm and regulated the import of amino acid into root cells or developing embryo [13–15]. *AtAAP5* might transport amino acids into the developing embryo, and be the most important component in the root amino acid uptake system [16]. The analysis of *aap6* mutant demonstrated the physiological role of AtAAP6 in regulating sieve element composition [9]. *AtAAP8* might be required for amino acid uptake into the endosperm and developing embryos at early stages [17]. In addition, the functions of AAT gene members from other plant species had also been studied, such as *StAAP1* [18], *VfAAP1* and *VfAAP3* [19], *PvAAP1* [20]. Recently, it was proposed that *PvAAP1* played a major role in xylogenesis by providing proline in poplar [12].

*AtLHT1*, a specific transporter for lysine and histidine, was a key mediator of root uptake of amino acids and supply of leaf mesophyll with xylem-derived amino acids in *Arabidopsis* [21,22]. *AtLHT1* and *AtAAP5* together played critical and separate roles in root uptake of cationic amino acids at concentrations typically possessed in soils [23,24]. AtProTs were responsible for transporting proline, glycine betaine and GABA, and their expression patterns were complementary [25]. Although intracellular localization and substrate specificity were very similar, AtProTs fulfilled different roles in planta [26,27]. It was reported that the rice ProT
protein (OsProT) has 68.8% and 59.6% similarity to AtProT1 and LeProT1, respectively, and specifically transported L-Pro in a transport assay by using Xenopus laevis oocytes [28,29]. In addition, heterologous expression analysis of HisProT indicated that it might play a crucial role in the transport of proline to root tip region and involve in organ development [30]. AtGAT1 encoded high affinity γ-amino butyric acid (GABA) transporter and was highly expressed in flowers and under the condition of high GABA concentrations such as wounding and senescence [31]. AtIAAT1 was specifically expressed in flowers and cauline leaves, and transported aromatic and neutral amino acids as well as arginine and indole-3-acetic acid [32]. AtAUX1 was known as an auxin influx carrier and mediated the import of IAA which was a tryptophan-like signaling molecular [33]. AtAUX1 regulated root gravitropism and promoted lateral root formation by facilitating IAA distribution between sinks and source tissues in the Arabidopsis seedling [34,35]. PaLAX3, a homologue of AtAUX1, was likely to influence the rate of lateral root emergence by regulating the auxin inducible expression of cell-wall-remodeling genes [36,37]. In the wild cherry Prunus avium, PaLAX1 promoted the uptake of auxin into cells and affected the content and distribution of free endogenous auxin [38]. In the AtCAT subfamily, nine members were identified based on their sequence similarity. AtCAT5 functioned as a high-affinity, basic amino acid transporter in an amino acid transport in yeast [39]. AtCAT3, AtCAT6 and AtCAT8 preferentially transported neutral or acidic amino acids [40]. Meanwhile, AtBAT1, similar to a yeast GABA transporter (UGA4), was isolated as a bi-directional amino acid transporter [41]. Recently, this gene was characterized to be expressed in the mitochondrion membrane and mediate the transport of GABA from the cytosol into mitochondria [42].

Although the roles of many AATs were revealed in extensive studies of Arabidopsis, none of OsAATs was functionally characterized. Therefore, there is an urgent need for a thorough bioinformatics analysis of the OsAAT gene family. This study will provide comprehensive analyses of OsAATs, including the identification of all OsAAT family members, the phylogenetic relationships between OsAATs and AtAATs, as well as their expression profiling in different organs/tissues and under the treatments of abiotic stresses. These results will to a great extent benefit functional validation of the rice AAT genes and broaden our understandings of the roles of plant AATs.

Results
Identification of AAT Genes in Rice Genome

To explore the entire gene family of AATs in rice, through two domain searches (PF01490 and PF00324) at MSU-RGAP (MSU-Rice Genome Annotation Project) database (http://rice.plantbiology.msu.edu/analyses_search_domain.shtml), we obtained 84 and 47 sequences, respectively; while by the InterPro of European Bioinformatics Institute database, 308 protein sequences of OsAATs from japonica were deposited. In the Rice Genome Express Database, using keyword searches of “amino acid transporter” and “amino acid permease”, 45 and 31 genes were identified, respectively. Sequences from these hits were used as queries employing BLAST algorithms to search against rice genome in the databases of both MSU-RGAP and NCBI. By removal of the sequence redundancies and alternative splice forms of the same gene, we initially identified 87 putative AAT genes in rice. All candidate AAT sequences were subjected to InterProScan for searching the presence of AAT domains. Four candidates were excluded from further analysis because they only contained short and incomplete AAT domains. Taken together, a total of 85 AATs were identified in rice. Meanwhile, similar methods were used to identify family members of AATs in Arabidopsis, and a total of 63 AtAATs were subsequently discovered. The detailed information about gene locus, FL-cDNA, ORF length for each OsAAT gene and characteristics of corresponding proteins are listed in Table 1, and those of AtAATs are showed in Table S1.

The numbers and positions of exons and introns were determined through the comparison of full-length cDNA sequences and the corresponding genomic DNA sequences of each OsAAT gene by using GSDS (http://gsds.cbi.pku.edu.cn/). Introns are absent in nine coding sequences of OsAAT genes, and the number of introns in other coding sequences range from one to thirteen (Figure S1). In the same subfamily, most members share similar intron/exon structures and gene lengths. For example, the three members of the OsProT subfamily have six introns and seven exons, and are nearly 4000 bp in length. The putative transmembrane (TM) regions in OsAATs were predicted by TMHMM Server v2.0 (http://www.cbs.dtu.dk/services/TMHMM/). The number of TM regions in most OsAATs ranges from eight to thirteen (Figure S2), and OsAATs of the same subfamily have similar number of TM regions, such as 11 TMs in LHT, 14 TMs in CAT, and 13 TMs in ACT. These observations revealed that members of the same subfamily were very conservative in structure.

Chromosomal Localization and Gene Duplication

To investigate the relationship between the genetic divergence and gene duplication within the AAT family in rice, the chromosomal locations of OsAATs were determined based on the coordinates of RGAP loci (http://rice.plantbiology.msu.edu/cgi-bin/gbrowse/rice/#search). OsAAT genes are distributed in all of rice 12 chromosomes. The densities of OsAAT genes are relatively higher in specific chromosomal regions, such as the bottom of chromosome 1 and 4, and the top and bottom of chromosome 2 and 12. In contrast, several large chromosomal regions are devoid of OsAAT genes, such as in the top parts of chromosomes 4 and 9, and in the bottom of chromosomes 7 and 11. The maximum members (19) of OsAAT genes are located in chromosome 1, followed by 11 genes in chromosome 12 and 10 genes in chromosome 4. In addition, nine genes each are located on chromosome 2, 3, and 6; four genes each on chromosome 5 and 11, three genes each on chromosome 7, 8, and 10. Nevertheless, a unique gene is localized on chromosome 9 (Figure 1). Similarly, the analysis of chromosomal location and gene duplication of 63 AtAATs was also performed in Arabidopsis (Figure S3).

Among the rice 85 OsAAT genes, 55.29% (47 of 85) come from the duplication events, including 24 gene segmental duplication and 23 gene tandem duplication. The 24 (12 pairs) OsAAT genes could be assigned to rice segmental duplication blocks based upon the analysis of the MSU-RGAP segmental duplication database. Four pairs of OsGATs, two pairs each of OsAATs and OsATs, and one pair each of OsGATs and OsAUXs are located on the duplicated segmental regions of rice chromosomes mapped by TIGR when the maximal length between collinear gene pairs is 500 kb. All of these duplicated genes exhibit high sequence similarity in both transmembrane regions and other domains. According to the criterion of separation by less than 5 intervening genes and ≥50% similarity at protein level, a total of 23 genes were found to be tandemly duplicated, falling into nine groups. Six groups comprised 2 genes and two groups comprised 3 genes in each group, and one group comprised 5 genes. The tandemly duplicated genes are only localized in five chromosomes, 3 groups on chromosome 1, 2 groups each on chromosome 6 and 12, and
Table 1. The general information and sequence characterization of 85 OsAAT genes.

| S.N. | Gene | Accession Number | ORF<sup>b</sup> (bp) | Protein<sup>c</sup> | Size (aa) | MW(D) | pI | Expression<sup>d</sup> |
|------|------|------------------|----------------------|------------------|-----------|-------|----|---------------------|
|      |      | RGAP Locus<sup>e</sup> | KOME<sup>c</sup>     |                  |           |       |    |                     |
|      |      |                   |                      |                  |           |       |    |                     |
| AAP group |
| 1 | OsAAP1 | LOC_Os07g04180 AK120257 | 1464 | 487 | 52864 | 8.66 | 9 | A B C D |
| 2 | OsAAP2 | LOC_Os06g12330 AK106762 | 1455 | 484 | 51898.8 | 8.32 | 11 | A B C D |
| 3 | OsAAP3 | LOC_Os06g36180 AK102763 | 1464 | 487 | 52783.8 | 7.93 | 11 | A B C D |
| 4 | OsAAP4 | LOC_Os12g09300 AK069508 | 1407 | 468 | 50891.2 | 8.27 | 9 | A B C D |
| 5 | OsAAP5 | LOC_Os01g65660 AK073884 | 1398 | 465 | 50002.2 | 8.77 | 11 | A B C D |
| 6 | OsAAP6 | LOC_Os01g65670 AK121636 | 1401 | 466 | 50085.5 | 8.68 | 11 | A B C D |
| 7 | OsAAP7 | LOC_Os05g34980 AK065891 | 1491 | 496 | 53513.2 | 8.02 | 9 | A B C D |
| 8 | OsAAP8 | LOC_Os01g66010 AK059325 | 1467 | 488 | 52871.4 | 8.28 | 9 | A B C D |
| 9 | OsAAP9 | LOC_Os02g01210 AK060685 | 1557 | 518 | 52706.4 | 8.63 | 9 | A B C D |
| 10 | OsAAP10 | LOC_Os12g09320 NA | 1407 | 466 | 50746.9 | 8.15 | 9 | A B C D |
| 11 | OsAAP11 | LOC_Os12g09320 NA | 1407 | 466 | 50746.9 | 8.15 | 9 | A B C D |
| 12 | OsAAP12 | LOC_Os06g12350 AK106814 | 1524 | 507 | 53441.6 | 9.55 | 9 | A B C D |
| 13 | OsAAP13 | LOC_Os04g39489 AK071045 | 1401 | 466 | 50746.9 | 8.15 | 9 | A B C D |
| 14 | OsAAP14 | LOC_Os04g56470 NA | 1401 | 466 | 51377.8 | 8.64 | 9 | A B C D |
| 15 | OsAAP15 | LOC_Os06g36210 AK071510 | 1401 | 466 | 50746.9 | 8.15 | 9 | A B C D |
| 16 | OsAAP16 | LOC_Os06g36210 AK071510 | 1401 | 466 | 50746.9 | 8.15 | 9 | A B C D |
| 17 | OsAAP17 | LOC_Os04g39489 AK071045 | 1401 | 466 | 51377.8 | 8.64 | 9 | A B C D |
| 18 | OsAAP18 | LOC_Os04g39489 AK071045 | 1401 | 466 | 51377.8 | 8.64 | 9 | A B C D |
| 19 | OsAAP19 | LOC_Os04g41350 NA | 1236 | 411 | 43154.1 | 8.74 | 9 | C D |
| LHT group |
| 20 | OsLHT1 | LOC_Os08g03350 AK120257 | 1464 | 487 | 52864 | 8.66 | 9 | A B C D |
| 21 | OsLHT2 | LOC_Os12g14100 AK070297 | 1341 | 446 | 48941.5 | 8.77 | 11 | A B C D |
| 22 | OsLHT3 | LOC_Os05g14820 NA | 1371 | 456 | 49939.7 | 9.12 | 11 | C D |
| 23 | OsLHT4 | LOC_Os04g38860 NA | 1335 | 444 | 47795.9 | 9.00 | 7 | B C D |
| 24 | OsLHT5 | LOC_Os04g47420 AK065098 | 1539 | 512 | 55030.7 | 9.11 | 11 | A B C D |
| 25 | OsLHT6 | LOC_Os12g30040 AK065891 | 1491 | 496 | 53513.2 | 8.02 | 9 | A B C D |
| GAT group |
| 26 | OsGAT1 | LOC_Os05g50920 AK106883 | 1446 | 481 | 51041.1 | 9.43 | 10 | A B C D |
| 27 | OsGAT2 | LOC_Os01g43320 NA | 1365 | 454 | 48525.1 | 9.02 | 11 | B C D |
| 28 | OsGAT3 | LOC_Os01g63854 AK103684 | 1374 | 457 | 48769.2 | 8.73 | 9 | A B C D |
| 29 | OsGAT4 | LOC_Os10g27980 AK119782 | 1329 | 442 | 47984.5 | 8.92 | 10 | A B C D |
| ProT group |
| 30 | OsProT1 | LOC_Os01g68050 NA | 1344 | 447 | 49024.9 | 9.40 | 10 | B C D |
| 31 | OsProT2 | LOC_Os03g44230 NA | 1344 | 447 | 49024.9 | 9.40 | 10 | B C D |
| 32 | OsProT3 | LOC_Os07g01090 AK066298 | 1305 | 434 | 47663.8 | 9.51 | 11 | A B C D |
| AUX group |
| 33 | OsAUX1 | LOC_Os01g63770 AK100090 | 1479 | 492 | 54762 | 8.15 | 10 | A B C D |
| 34 | OsAUX2 | LOC_Os05g37470 AK111849 | 1512 | 503 | 55662.9 | 8.65 | 11 | A B C D |
| 35 | OsAUX3 | LOC_Os03g14080 AK103524 | 1575 | 524 | 58083.4 | 9.21 | 10 | A B C D |
| 36 | OsAUX4 | LOC_Os10g05690 AK102295 | 1644 | 547 | 59846.2 | 8.64 | 10 | A B C D |
| 37 | OsAUX5 | LOC_Os11g6820 NA | 1443 | 480 | 52956.5 | 9.33 | 9 | B C D |
| ANT group |
| 38 | OsANT1 | LOC_Os07g12770 AK105808 | 1275 | 424 | 45712.3 | 7.01 | 11 | A B C D |
| 39 | OsANT2 | LOC_Os03g60260 AK121940 | 1257 | 418 | 43571.8 | 7.79 | 9 | A B C D |
| 40 | OsANT3 | LOC_Os02g44980 AK100650 | 1269 | 422 | 44846.9 | 7.53 | 11 | A B C D |
| 41 | OsANT4 | LOC_Os04g47780 AK058888 | 1278 | 425 | 44788.8 | 7.84 | 9 | A B C D |

ATLa group

Analysis of Rice Amino Acid Transporters
Table 1. Cont.

| S.N. | Genea | Accession Number | ORF(bp) | Proteine | TMf | Expressiong |
|------|-------|------------------|---------|----------|-----|-------------|
|      |       | RGAP Locusb | KOMEc  | Size (aa) | MW(D) | pI |
| 42   | OsATL1 | LOC_Os06g43700 | AK070101 | 1461 | 486 | 51755.2 | 7.55 | 10 | A B C D |
| 43   | OsATL2 | LOC_Os09g26290 | NA | 927 | 308 | 32736.3 | 8.61 | 4 | C D |
| 44   | OsATL3 | LOC_Os02g49510 | AK069154 | 1347 | 448 | 48108.2 | 7.18 | 11 | A B C D |
| 45   | OsATL4 | LOC_Os06g16420 | AK120497 | 1347 | 448 | 48121.5 | 7.30 | 11 | A B C D |
| 46   | OsATL5 | LOC_Os06g42720 | AK101315 | 1377 | 458 | 49977.6 | 6.42 | 10 | A B C D |
| 47   | OsATL6 | LOC_Os02g09810 | AK069748 | 1380 | 459 | 50149.7 | 6.45 | 10 | A B C D |
| 48   | OsATL7 | LOC_Os01g61044 | AK102220 | 1380 | 459 | 47870.2 | 9.80 | 11 | A B C D |
|      |       |       |       |       |       |       |       |     |     |     |
| 49   | OsATL8 | LOC_Os11g19240 | NA | 1452 | 483 | 50225.9 | 9.08 | 11 | C D |
| 50   | OsATL9 | LOC_Os02g34730 | AK066747 | 1647 | 548 | 59332.3 | 5.07 | 10 | A B C D |
| 51   | OsATL10 | LOC_Os12g38570 | AK069006 | 1785 | 594 | 63407.2 | 4.63 | 10 | A B C D |
| 52   | OsATL11 | LOC_Os02g01100 | AK069423 | 1725 | 574 | 62911.4 | 9.29 | 9 | A B C D |
| 53   | OsATL12 | LOC_Os06g12320 | AK102220 | 1380 | 459 | 47870.2 | 9.80 | 11 | A B C D |
| 54   | OsATL13 | LOC_Os01g61044 | AK102220 | 1380 | 459 | 47870.2 | 9.80 | 11 | A B C D |
|      |       |       |       |       |       |       |       |     |     |     |
| 55   | OsATL14 | LOC_Os04g38660 | NA | 690 | 229 | 24225.6 | 9.39 | 6 | C |
| 56   | OsATL15 | LOC_Os01g41420 | NA | 1896 | 631 | 67357.3 | 9.70 | 10 | B C D |
| 57   | OsATL16 | LOC_Os01g41400 | NA | 1332 | 443 | 47386.9 | 8.75 | 10 | C D |
| 58   | OsATL17 | LOC_Os01g40410 | NA | 1383 | 460 | 49420.1 | 5.89 | 10 | C |
|      |       |       |       |       |       |       |       |     |     |     |
| 59   | OsCAT1 | LOC_Os01g11160 | AK099094 | 1851 | 616 | 65510.9 | 8.13 | 14 | A B C D |
| 60   | OsCAT2 | LOC_Os02g43860 | AK101068 | 1818 | 605 | 64436.6 | 7.57 | 13 | A B C D |
| 61   | OsCAT3 | LOC_Os03g43970 | NA | 1329 | 442 | 46938 | 8.54 | 12 | B |
| 62   | OsCAT4 | LOC_Os03g45170 | AK066436 | 1920 | 639 | 68202.9 | 5.74 | 14 | A B C D |
| 63   | OsCAT5 | LOC_Os04g45950 | AK064289 | 1686 | 561 | 60780.4 | 8.3299 | 13 | A B C D |
| 64   | OsCAT6 | LOC_Os06g34830 | AK102220 | 1380 | 459 | 47870.2 | 9.80 | 11 | A B C D |
| 65   | OsCAT7 | LOC_Os10g0090 | AK100205 | 1869 | 622 | 66053.4 | 6.73 | 14 | A B C D |
| 66   | OsCAT8 | LOC_Os11g05690 | NA | 1413 | 470 | 49758.9 | 5.96 | 12 | B D |
| 67   | OsCAT9 | LOC_Os12g06060 | NA | 1608 | 535 | 56371.8 | 7.26 | 13 | C D |
| 68   | OsCAT10 | LOC_Os12g41890 | NA | 1806 | 601 | 62597.4 | 8.44 | 14 | D |
| 69   | OsCAT11 | LOC_Os12g42850 | AK064822 | 1866 | 621 | 66108 | 5.80 | 14 | A B C D |
|      |       |       |       |       |       |       |       |     |     |     |
| 70   | OsBAT1 | LOC_Os01g42234 | AK071623 | 1599 | 532 | 57024.1 | 8.51 | 12 | A B C D |
| 71   | OsBAT2 | LOC_Os01g71700 | NA | 1089 | 521 | 38637.4 | 9.055 | 12 | B C D |
| 72   | OsBAT3 | LOC_Os01g71710 | NA | 1566 | 362 | 55915.2 | 8.8742 | 9 | C D |
| 73   | OsBAT4 | LOC_Os01g71720 | AK065371 | 1578 | 525 | 55991.8 | 7.76 | 13 | A B C D |
| 74   | OsBAT5 | LOC_Os01g71740 | NA | 1554 | 517 | 55683.9 | 8.70 | 12 | B C D |
| 75   | OsBAT6 | LOC_Os01g71760 | NA | 1527 | 508 | 54464.5 | 9.00 | 12 | C D |
| 76   | OsBAT7 | LOC_Os04g35540 | AK072850 | 1593 | 530 | 57764.3 | 8.08 | 11 | A B C D |
|      |       |       |       |       |       |       |       |     |     |     |
| 77   | OsLAT1 | LOC_Os02g47210 | AK068055 | 1695 | 564 | 60174.1 | 6.18 | 12 | A B C D |
| 78   | OsLAT2 | LOC_Os03g25840 | NA | 993 | 330 | 35288.6 | 8.98 | 3 | D |
| 79   | OsLAT3 | LOC_Os03g25869 | AK072316 | 1644 | 547 | 59972.5 | 12.42 | 5 | A B C D |
| 80   | OsLAT4 | LOC_Os03g25920 | AK107064 | 1500 | 499 | 51811.2 | 8.96 | 10 | A B C D |
| 81   | OsLAT5 | LOC_Os03g37984 | AK071314 | 1653 | 550 | 60375 | 7.27 | 10 | A B C D |
| 82   | OsLAT6 | LOC_Os08g41370 | NA | 579 | 192 | 21285.3 | 8.97 | 5 | / |
| 83   | OsLAT7 | LOC_Os12g39080 | AK099986 | 1344 | 447 | 48989.6 | 12.27 | 5 | A B C D |
| 84   | OsLAT8 | LOC_Os01g19850 | NA | 2391 | 796 | 88296.8 | 7.9546 | 7 | B C D |
one group each on chromosome 3 and 4 (Figure 1). Moreover, all of the proteins of the duplicated genes have relatively high sequence similarity. For example, OsAUX1 and OsAUX2 from segmental duplication are 82% similarity, and OsAAP3 and OsAAP18 from tandem duplication are 70% similarity. These results suggested that large-scale segmental and tandem duplication events played a significant role in the expansion of the OsAAT family.

### Phylogenetic Analysis and Multiple Sequence Alignment

In order to evaluate the evolutionary relationship among the 85 members of OsAATs, phylogenetic analysis was performed based on the alignment of full-length amino acid sequences of the 85 AAT proteins. Eleven distinct clades supported with high bootstrap values by the neighbor-joining method were identified. Additionally, Bayesian inference was also used to construct the phylogeny by using MrBayes program (Figure S4). Based on the domain composition and phylogenetic relationship, the OsAATs can be divided into two main families similar to that in Arabidopsis, including the AAP and APC family (Figure 2A). The AAP family consists of 58 OsAATs, including eight distinct subfamilies: amino acid permeases (AAPs), lysine, histidine transporters (LHTs), proline transporters (ProTs), GABA transporters (GATs), auxin transporters (AUXs), aromatic and neutral amino acid transporters (ANTs) and amino acid transporter-like (ATL) subfamilies. So far, the characterization of these genes in the Arabidopsis family consists of 58 OsAATs, including eight distinct subfamilies: amino acid permeases (AAPs), lysine, histidine transporters (LHTs), proline transporters (ProTs), GABA transporters (GATs), auxin transporters (AUXs), aromatic and neutral amino acid transporters (ANTs) and amino acid transporter-like (ATL) subfamilies. So far, the characterization of these genes in the Arabidopsis subfamilies. ATL subfamilies consist of two phylogenetic clades that are named as ATLα and ATLβ, respectively. The five auxin transporters in OsAAPs subfamily were initially described as auxin influx-like carriers (OsLAX1-5) [43,44]. Since the abbreviation LAX in rice was also used for the lax panicle and OsLAX1 and OsLAX2 were functionally characterized to regulate the formation of axillary meristems [45,46], the five auxin transporters in rice were renamed to OsAAP1-5. The APC family is comprised of 27 OsAAPs and subdivided into three distinct subfamilies, including the cationic amino acid transporters (CATs), the amino acid/choline transporters (ACTs) and the polyamine H^+/-symporters (PHSs).

In order to identify orthologous genes between rice and Arabidopsis, a combined phylogenetic tree with OsAATs and AtAATs was also established by using the neighbor-joining method (Figure S5). Similar subfamilies were formed as compared to the tree of OsAATs. Each clade of distinct subfamily consists of AATs from both rice and Arabidopsis, indicating the main characteristics formation of AAT family in rice and Arabidopsis before the split of monocotyledonous and dicotyledonous plants. Moreover, the difference in the total number of OsAATs and AtAATs is mainly due to the variation in the number of genes in AAP subfamily and APC family; there were 19 AAPs in rice and 8 in Arabidopsis; 27 APCs in rice and 15 in Arabidopsis. Moreover, the MEME motif search tool was employed to identify the motifs shared in the OsAATs. Subsequently, 20 motif sequences were identified and shown in Table S2. Besides, the distributions of these motifs in OsAATs were shown in Figure 2B. Several motifs were widespread among OsAATs in AAP family (e.g. motif 2 and 6). In contrast, other motifs were specific to only one or two subfamilies. For example, motifs 1 and 7 were specific to subfamily AAPs, and motifs 8 and 5 were specific to AUXs and ACTs, respectively, while motif 12 and 17 were found in subfamily CAT and ATLα, respectively. However, motif 15 was present in the AAP, GAT, ATLβ and ANT subfamilies. In addition, motif 11 exclusively appeared in the ATLα, CAT and PHS subfamilies.

The alignments of the OsAATs' amino acid sequences illustrated that most of TM regions in the same subfamily were very conserved. In addition, several TM regions of different members varied insignificantly both in length and amino acid composition. The alignment of the OsLHT members was shown in Figure 3 as an example. The overall identities of the protein sequences of these genes are 55.73%. There are five conserved motifs in OsLHTs, including motif 2, 6, 10, 4 and 18. Motif 2 was found to be located in the first and second TM region, and motif 6 comprised the fourth and fifth TM region. Motif 10 consisted in the sixth TM region. Motif 4 was located at the eighth TM region and extended into the following sequences before the ninth TM region. Motif 18 was located in the tenth and eleventh TM region.

### Expression Analysis of OsAAT Genes in Various Organs at Different Developmental Stages

Several approaches were employed to analyze the expression patterns of the OsAAT genes in different tissues and organs, including expressed sequence tag (EST) profiles, microarray data and massively parallel signature sequencing (MPSS) tags. In analysis of EST profiles (http://www.ncbi.nlm.nih.gov/unigene/), the availability of any corresponding full-length cDNA (FL-cDNA) and ESTs in UniGene database at NCBI was searched. Seventy-one of 85(83.53%) OsAAT genes have at least one corresponding FL-cDNA and ESTs in UniGene database at NCBI was searched. Seventy-one of 85(83.53%) OsAAT genes have at least one corresponding FL-cDNA and EST sequence (Table S5); fifty-nine OsAAT genes of them (69.41%) have both FL-cDNA and EST evidence, whereas twelve genes (14.12%) have only EST evidence (Table 1), indicating that most of these genes are expressed. Various OsAAT genes show high expression abundance in stem, root, leaf, panicle, and seed, several of which have tissue-
Figure 1. Chromosomal localization and gene duplication events of OsAAT genes. Respective chromosome numbers are indicated at the top of each bar. The scale on the left is in megabases (Mb). The cleavages on the chromosomes (vertical bars) indicate the position of centromeres. The chromosome order is arranged to bring duplicated regions in the vicinity.

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specific or abundant expression patterns: OsAAP6, OsAAP14, and OsAUX3 in callus; OsLHT2, OsATL10 and OsLAT5 in flower; OsATL15, OsLAT7 and OsCAT11 in leaf; OsAAP1, OsAAP15 and OsAAP17 in panicle; OsLHT1, OsAUX1 and OsBAT7 in root; OsBAT5 in seed and OsBAT4 in stem (Table S3).

Microarray data (http://signal.salk.edu/cgi-bin/RiceGE) of various tissues during vegetative and reproductive developmental stages of rice was used for analyzing the expression profiles of OsAAT genes, including young root (YR), mature leaf (ML), young leaf (YL), shoot apical meristem (SAM), panicles (P1–P6), and seed (S1–S5) development. At least one probe could be found on Affymetrix rice whole-genome array platform (GPL2025) for 80 of 85 OsAAT genes. A hierarchical cluster display generated from the average log signal values for the 80 OsAAT genes in selected organs indicates the differential expression patterns of these genes (Table S4). The expression patterns of 80 OsAAT genes can be divided

Figure 2. Phylogenetic relationship and protein motifs of OsAATs. (A) Phylogenetic tree of OsAATs constructed by neighbor-joining method. Bootstrap values from 1000 replicates are indicated at each node. Scale bar represents 0.2 amino acid substitution per site. The proteins on the tree can be divided into eleven distinct subfamilies. Subfamilies ATL are further divided into two groups (ATL a and ATL b). The branches of different subfamilies are marked by different colors. (B) Protein motifs of OsAATs. Each colored box represents a specific motif in the protein identified using the MEME motif search tool. The order of the motifs corresponds to their position within individual protein sequences.

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into six major groups (Figure 4). Twelve OsAAT genes in group I show high expression levels in all examined organs, expect for five genes with relatively low expression in certain organs (OsAAP16 and OsLHT11 in SAM, OsAAP15 in SAM and S5, OsATL4 in SAM and P1–P3, and OsAAP1 in YL). Group II includes 13 genes that show abundant expression level in vegetative organs. For example, four genes (OsAUX2, OsAAP4, OsProT3 and OsATL12) display specifically or predominantly expression level in YR; OsCAT2, OsAAP7 and OsCAT4 were abundantly expressed in YR, ML and YL. Group III comprises 21 genes that show relatively high expression level in specific organs (OsAUX3 in SAM, OsBAT1 in YL and ML, OsBAt1, OsGAT3, OsGAT4 and OsLHT4 in YR, OsCAT6, OsAAP18, OsLHT2, OsAAP17 and OsATL10 in P6). Conversely, group IV contains 18 genes that show relatively low expression level in all examined organs. Group 5 comprises 8 genes with preferential expression patterns in different stages of seed development, meanwhile, OsProT2 and OsCAT1 are highly expressed in ML, and OsAAP13 and OsLAT7 in YR. Group VI consists of 4 genes which show relatively high expression levels in several reproductive organs (OsAUX1 and OsLHT6 in SAM, OsATL13 in P1–P4 and S4–S5, OsANT1 in S4–S5).

In the mRNA MPSS database of rice (http://mpss.udel.edu/rice/), the expression of OsAAT genes were investigated. MPSS generates hundreds of thousands of molecules per reaction and provides a quantitative assessment of transcript abundance. MPSS signatures are available for the 80 genes in at least one of the libraries (Table S5), further indicating that most OsAAT genes are expressed. Differential expression abundances are displayed by number of tags (tpm, transcripts per million), being low in <50, moderate in between 50 tpm and 500 tpm, and strong in >500 tpm. A large percentage of OsAAT genes (47 of 80) have low expression level, 30 genes moderate level, and three genes (OsAUX1, OsATL5 and OsATL15) high level (Table S5).

To validate the results of digital expression analysis, we detected the expression levels of several representative genes by real-time PCR analysis. The results show that they are in general agreement with the data of microarray and MPSS tags. For example, OsAAP13, OsATL13, OsAUX4 and OsAAP8 are significantly expressed in stems, meanwhile, OsAAP13, OsATL15 and OsAAP8 are also expressed in roots and leaves, and OsAUX4 in root and in panicles at early stages of development (P1) (Figure 5A); OsATL9, OsCAT1, OsBAT7, OsAAP5, OsANT1, OsATL6 and OsAAP7 are predominantly or highly expressed in leaves (Figure 5B); OsLHT6, OsAUX5 and OsAAP11 are mainly expressed in panicles at early stages of development (P1), and OsAUX5 is also highly expressed in roots (Figure 5C). Otherwise, OsProT1, OsAUX1, OsAUX2, OsATL13, OsAAP4, OsANT3 and OsATL1 are predominantly or highly expressed in panicles at late stages of development (P2), and OsATL13 similarly in panicles at early stages of development (Figure 5D). OsBAT1 and OsAAP6 are highly expressed in roots and seeds, respectively. OsAUX3 is predominantly expressed in stem and P1, but was not detected in either leaf or P2 (Figure 5E).

Expression Regulation of OsAAT Genes under Abiotic Stresses

To determine the response of OsAAT genes to abiotic stress, the data of microarray (GSE6901) of 7-day-old seedlings subjected to the treatments of drought, salt and cold stresses were analyzed. It was revealed that a total of 21 genes were evidently down- or up-regulated (<0.5 or >2) in at least one of the stress conditions examined as compared with the control (Figure 6A–G). The expression of three genes (OsAAP15, OsATL6 and OsANT3) were up-regulated by all three stresses (Figure 6A), five genes (OsATL13, OsAAP6, OsAAP11, OsAAP13 and OsAAP5) were up-regulated by drought and salt stresses (Figure 6B), and two genes (OsGAT2 and OsLHT6) and three genes (OsANT4, OsBAT7 and OsATL11) were specifically up-regulated by drought and salt stress, respectively (Figure 6C and D). However, four genes (OsAUX1, OsAAP4, OsBAT4 and OsAAP8) were down-regulated by all three stresses (Figure 6E), three genes (OsProT3, OsAUX2 and OsLAT7) were down-regulated by drought and salt stresses (Figure 6F), and one gene (OsATL9) was down-regulated by drought stress (Figure 6G). By using real-time PCR analysis, we validated the expression levels of 9 representative OsAAT genes in 7-day-old seedling under three stress conditions. It was found that the results were in very well agreement with the microarray data (Figure 6 H, I, J and K), indicating that OsAAT genes might participate in abiotic stress signaling pathways and play important roles in response to these stresses.

Comparative Expression Analysis of Rice and Arabidopsis AAT Genes

In order to explore valuable clues for the study of gene function, a comparative analysis of the expression patterns of rice and Arabidopsis AAT gene family was performed. With the exception of the expression data of OsAAT genes in pollens from MPSS tags, the other expression data of rice and Arabidopsis AAT genes were extracted from microarrays in root, leaf, inflorescence, pollen, seed/silique, and under abiotic stress. The ratios of the absolute values divided by the average of all microarray values were used for the analysis (Table S6).

Most of the rice and Arabidopsis AAT genes were found to be presented on at least one data set, while five genes (OsCAT3, OsLAT6, OsANT4, AtVAAT10 and AtVAAT3) were absent from the two data sets (Figure 7). By integrating the data of microarray and MPSS tags, we found that 28 AAT genes were highly expressed in at least two organs examined and 21 AAT genes acted in tissue-specific manners. The expression levels of 36 AAT genes were extremely low in all examined organs. Six members of GAT subfamily and 4 of 6 in ProT subfamily were moderate and low expressed in all examined organs. On the contrary, nine members of AUX subfamily were high and/or moderate expressed in at least one organ except OsAUX5 that were lowly expressed in all examined organs.

Meanwhile, this comparison also indicated that 26 AAT genes with close evolutionary relationships between rice and Arabidopsis showed similar expression patterns. Correlation coefficients between expression patterns ranged from 0.7 to 0.9 among most homologous genes (Table S7). For example, AtLHT6, AtLHT3 and OsLHT3 were very lowly expressed in all organs examined and evidently down-regulated in stems by drought and salt stresses (Figure 7B). OsAUX2 and AtLAX2 were mainly expressed in root, inflorescences and seeds and evidently down-regulated by salt stress (Figure 7E). OsCAT1 and AtCAT5 showed moderate or extremely low expression level except OsCAT1 in leaf and were down-regulated by drought stress (Figure 7F). OsANT2 and AtANT2 were up-regulated by salt stress (Figure 7F), and OsBAT7 and AtBAT1 were down-regulated by cold stress (Figure 7K). More-
over, OsGAT1 and AtGATL1 were moderately expressed in seeds (Figure 7C). OsUX1 and AtUX1 had high or moderate expression level in root, leaf, inflorescences and seeds (Figure 7D). OsANT3 and AtANT3 were highly expressed in all organs except OsANT3 in pollens (Figure 7G), while OsATL1 and AtT5 were very lowly expressed in root, leaf and seeds (Figure 7H). AtT3, OsATL6, OsATL5 and OsATL6 were predominantly expressed in leaf, inflorescences and seeds (Figure 7I).

In addition, the expression of OsAAT genes in rice differed from those of their Arabidopsis homologs. For example, OsLHT1 was highly expressed in root, leaf, inflorescences and seeds, while AtLHT8 was only highly expressed in pollens, and had extremely low expression level in root, leaf, and seeds (Figure 7A). In conclusion, the expression patterns of genes provide foundation for future functional studies of AAT genes in both rice and Arabidopsis.

Expression Pattern Divergence of Paralogous OsAAT Genes Involved in Duplication

The analysis on the expression pattern of OsAAT genes present in segment and in tandem duplication was performed. Out of the 12 pairs of segmentally duplicated genes, probe sets were available for 10 pairs on Affymetrix gene chip (http://signal.salk.edu/cgi-bin/RiceGE). The expression pattern was very much similar for 2 pairs of genes (OsAAP7 and OsAAP8, OsUX1 and OsUX2), indicating they were just formed due to duplication and retention of function (Figure 8A). The fate of 2 pairs (OsATL3 and OsATL4, OsGAT4 and OsGAT1), in Figure 8B could be described as nonfunctionalization, where one copy of the paralog had almost negligible expression in all organs, and which may be due to the fact that gene with low expression level would tend to lose its function in due course of evolution [47]. For the rest six pairs of paralogous genes (OsAAP11 and OsAAP16, OsGAT1 and OsGAT2, OsANT1 and OsANT2, OsANT3 and OsANT4, OsATL5 and OsATL6, and OsCAT1 and OsCAT6), the expression patterns were very divergent for one or more of the tissues detected, indicating neo-functionalization (Figure 8C).

Expression pattern analysis was also done for tandemly duplicated OsAAT genes. Two groups of genes, (OsAAP2, OsAAP17 and OsATL12, OsAAP15 and OsAAP16) had highly similar expression pattern and hence retention of expression (Figure 8D); whereas, for four groups of genes (OsAAP4 and OsAAP12, OsAAP5 and OsAAP6, OsATL13 and OsATL14, OsBAT2, OsBAT3, OsBAT4, OsBAT5 and OsBAT6), one copy of the paralog lost expression in all organs (Figure 8E). Furthermore, three pairs (OsAAP3 and OsAAP18, OsATL15 and OsATL16, OsLAT3 and OsLAT4) showed divergent expression profiles (Figure 8F).

Discussion

Organization of AAT Family Genes in Rice

In previous reports, several AAT genes in Arabidopsis were classified and functionally characterized in detail, such as genes in the AaLAP subfamily [11,12,15], the AaUX subfamily [33-37] and the AaCAT subfamily [39-42]. However, members of AAT family in rice are still unknown so far, and none of them is functionally characterized. In this study, we identified 85 OsAAT genes which were divided into nine subfamilies based on sequence similarity in amino acid, and there was obvious difference in numbers among subfamilies. The largest number found in OsAAP subfamily was nineteen, and the smallest found in OsProT subfamily was only treated as a functional subfamily.
Combination phylogenetic analysis indicated that the rice \textit{AAT} subfamilies were very good agreement with that in \textit{Arabidopsis} (Figure S5), which revealed that \textit{AAT} families in rice and \textit{Arabidopsis} were formed before the split of monocotyledonous and dicotyledonous plants [4]. With the exception of \textit{ATLa} and \textit{ATLb} subfamilies, at least one gene in other nine subfamilies was functionally identified in \textit{Arabidopsis}. In addition, due to the significant increase in the number of \textit{AAP} subfamily genes and APC family genes, the number of 85 Os\textit{AAT} members was far greater as compared to the number of 63 in \textit{Arabidopsis} (Table S1). The increase in the number of \textit{OsAAT} members reflects that expansion and rearrangement of the genome may be successfully undergone in the \textit{OsAAT} family, and these transporters increased in rice might play an important role in order to adapt to specific functions.

Chromosomal mapping of \textit{OsAAT} family genes show their variable distribution on 12 rice chromosomes, but its most members are localized on chromosome 1, 4 and 12. Meanwhile, duplicated segments are mainly presented on chromosome 1, 3, 6 and 12, and nine gene groups in tandem duplication are localized only on five chromosomes (three, one, two and two groups on chromosome 1, 3, 4, 6 and 12, respectively) (Figure 1). Gene structure analysis reveals that most members in the same \textit{OsAATs} subfamily are structurally conserved in the number of intron/exon and gene length (Figure S1), which indicates their close
Figure 7. Expression comparison between rice and Arabidopsis AAT genes in different organs and under abiotic stresses. The OsAAT and AtAAT genes are displayed according to the order in the corresponding phylogenetic tree (Figure S5). The expression data of OsAAT genes in different organs are combined from microarrays (M1) and MPSS (M2). In microarrays and MPSS data, red, green, yellow and light blue boxes indicate high (more than 2,300 tpm), moderate (between 1 and 2, between 50 and 300 tpm), low (between 0.5 and 1, the signature numbers between 0 and 50 tpm), and extremely low (less than 0.5, no signature) expression levels, respectively. The symbol ''\'6'' represents no probe or signature on microarray and MPSS. ''\'m'','', ''\'.'' and ''\'-'' represent expression values that are evidently higher (>2), lower (<0.5) and no evident difference (0.5-2) under abiotic stresses compared to the control, respectively. (A) and (B-K) Showing homologous genes with distinct and similar expression patterns, respectively. R, root; L, leaf; I, inflorescence; P, pollen; S, siliques (Arabidopsis) or seed (Rice); DSS and DSR, drought stressed shoot and root; SSS and SSR, salt stressed shoot and root; CSS and CSR, cold stressed shoot and root.
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Duplication Contributed to the Expansion and Functional Divergence of OsAATs Family

Gene duplication is thought to be an important means of gene family expansion and functional diversity during evolution, which may occur through three major pathways: chromosomal segmental duplication, tandem duplication and retroposition [48–50]. In the present study, our analysis on gene duplication reveals that 47 of 85 (55.30%) OsAAT genes are duplicated genes, 24 genes (28.24%) are involved in the segmental duplication, and 23 genes (27.06%) in tandem duplication, indicating that segmental and tandem duplications contribute almost equally to the expansion of the OsAAT gene family. Moreover, 16 genes in OsAPC family with 27 members are involved in duplication events, which may be largely responsible for the number difference of the member between OsAPC and AtAPC genes.

When gene duplication occurs, expression patterns and original functions of these genes may be retained [51]. Consistent with that, the comparison analysis for expression pattern of paralogous OsAAT genes involved in duplication indicates that OsAAP7 and OsAAP8, and OsAAP15 and OsAAP16, localized on segmental and tandem duplication, respectively, exhibited similar expression patterns in both development stages and abiotic stresses, indicating their overlapping functions (Figure 8A and 8D). However, it was well known that a great degree of expression and functional divergence might be present between two duplicated genes due to the intense selection pressure and the need for the diversification [49]. Most duplicated OsAAT genes exhibit distinct expression patterns and response to various abiotic stresses, such as OsANT3 and OsANT4, OsCAT1 and OsCAT6 (segmentally duplicated genes) (Figure 8C); OsAAP4 and OsAAP12, OsLAT3 and OsLAT4 (tandemly duplicated genes) (Figure 8E and 8F). These results suggested that chromosomal duplication events not only facilitated the expansion of the OsAAT family, and also lead to expression divergence of paralogous OsAAT genes involved in duplication. The absolute values of duplicated genes obtained from microarray data were compared in various organs under abiotic stresses. X-axis indicates representative samples and Y-axis is scales of expression level. The segmentally and tandemly duplicated genes are shown on the left and the right, respectively. (A) and (D) Showing gene pairs that are retention of expression; (B) and (E) showing gene pairs described as non-functionalized; (C) and (F) showing gene pairs described as neo-functionalization.

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differences between duplicate genes, further contributing to the establishment of gene functional diversity during their evolution.

**Expression Patterns Divergence and Putative Function of OsAATs**

The analysis on temporal and spatial expression patterns of OsAAT genes may provide useful information for establishing their putative functions [52–54]. Our microarray analysis showed that the expression patterns of the 80 OsAAT genes could be divided into six major groups. Some preferential or tissue-specific expression OsAAT genes were also identified. Only three (OsAUX3, OsAUX4 and OsLHT6) of the 80 genes were found to exhibit either preferential or tissue-specific expression in SAM, sixteen in YR and/or YL, and/or ML. In addition, ten and eleven genes were preferentially or specifically expressed during different development stages of panicles and seeds, respectively. Interestingly, several genes were most highly expressed in several specific tissues, for example, OsATL12 were preferentially expressed in YR, P5 and P6, and OsCAT1 and OsANT4 had high expression level in ML, S1 and S2.

In *Arabidopsis*, AtAUX1 and AtLAX3 were primarily expressed in root and promoted lateral root formation [55,56]. Our data showed that OsAUX1, an ortholog of AtAUX1 and AtLAX3, had high expression levels in all the organs, especially in YR and P5, and OsAUX2, OsAUX4 and OsAUX5 were almost preferentially expressed in YR. Similar expression patterns suggest that these root preferentially or specifically expressed genes might play important roles in root formation and development. The results of some representative genes from real-time PCR showed that four genes (OsAAP8, OsAAP15, OsATL15 and OsAUX4) are predominantly or specifically expressed in stems. It is known that AtAAP2 may play critical role in the long-distance transport of amino acid [7,13]; AtAAP3 with mainly expression in root vascular tissue may be involved in amino acid uptake from phloem [57]; AtAAP6 might be responsible for amino acid uptake from xylem [58]. Therefore, combining their phylogenetic relationship and role of the AtAAPs, we infer that OsAAP8 and OsAAP15 might participate in the uptake and long-distance transport of amino acid.

Moreover, we also identified one gene (OsAAP6) that was preferentially expressed in early stages of seed development. AtAAP8 had been demonstrated that may be involved in amino acid uptake into the endosperm and supplying the developing embryo with amino acids during early embryogenesis [17]. It may be conjectured that these AAT members might also play critical roles in nutrient transport during seed development.

**Roles of OsAATs in Response to Abiotic Stresses and in Reproductive Development**

The expression profile of a gene can provide a valuable clue for its functional study [53]. Our expression analysis by using qRT-PCR and/or microarray data reveal that a subset of OsAAT genes from different subfamilies shows significant and differential expression pattern under three abiotic stresses (Figure 6 and 7). It is known that amino acid transport is highly regulated by environmental signals, such as light, low temperature, high salt and/or drought [59]. The expression of AtAAP4 and AtAAP6 were reported to be down-regulated by salt stress in *Arabidopsis* [60], as well as MccAA1 in *M. crystallinum* [61]. On the contrary, AtProT2 [60], MtAAT1 [61], and HvProT [62] were found to be strongly induced by salt stress. Similarly, in our investigation, seven genes (e.g. OsAAP4, OsAAP8, OsBAT4) and 11 genes (e.g. OsAAP6, OsAAP11, OsANT3) in OsAAT family were evidently down- and up-regulated, respectively, under salt and drought stresses (Figure 6).

The investigation suggests that the OsAAT genes may play a critical role in abiotic stress signalling in rice.

Many evidences demonstrate that there is an interaction between developmental processes and stress responses, and some genes may be co-regulated by both environmental factors and developmental cues [63]. It was reported that a network of rice genes are associated with stress response and seed development [64]. By expression analysis from microarray data and qRT-PCR, we also found that several OsAAT genes (such as OsANT3, OsAAP6, OsAAP11, OsATL13 and OsAUX4), which were differentially expressed during at least one of the panicles and seed developmental stages, were significantly down- or up-regulated by one or more of the stress conditions. These genes may play important roles in plant growth and response to different abiotic stress conditions during reproductive development. This suggests that a number of OsAAT genes are likely to be involved in main developmental processes and stress responses. And their direct relationship requires further experimental validation.

**Conclusion**

In conclusion, the results of this study display the genomic framework, classification, duplication manner and conserved motifs of the 85 OsAAT members, along with their expression patterns during different developmental stages as well as under abiotic stress treatments. These data will provide an insight into further understanding of functions of AAT members and their roles in rice growth and development. Our findings would be valuable in selecting candidate genes for functional validation studies of AAT members in rice. However, future research by adopting RNAi and overexpression strategies or insertion mutagenesis is required to explore the precise role of individual OsAAT gene.

**Materials and Methods**

**Plant Materials and Abiotic Stress Treatment**

To analyze the expression pattern of representative OsAAT genes, the rice seedlings of *Nipponbare* were grown under normal conditions in Wuhan University. The tissues and organs for expression pattern analysis were: (i) 30-day-old root (R, root) and leaf (L, leaf); (ii) 60-day-old stem(St); (iii) 3–5 cm panicles (P1); (iv) 20–25 cm panicles (P2); and (v) seed at 7 DAP (day after pollination) (Sd). For stress treatments, the 7-day-old seedlings were carefully transferred onto paper at 28℃ as drought stress after which were air dried, placed in 400 mM NaCl solution at 28℃ as salt stress, and kept in sterile water for 3h at 4℃ as cold stress. Parallel control samples were prepared by keeping the seedlings in water for 3h. All materials were collected and immediately frozen in liquid nitrogen, and stored in −80℃ environment until RNA extraction.

**Database Screening and Identification of OsAATs**

Several approaches were employed for the mining of all putative AAT members in the rice genome. Firstly, BLASTP searches of AAT domains (PF01490 and PF00324) were performed on websites of MSU-RGAP (http://rice.plantbiology.msu.edu/ domain_search.shtml). Secondly, protein sequences of putative OsAAT members were downloaded from Search Interpro (http://www.ebi.ac.uk/interpro/ISearch?query = PF01490 and PF00324). Thirdly, key words “amino acid transporter” and “amino acid permease” were used as queries to search against Rice Genome Express Database (http://signal.salk.edu/cgi-bin/RiceGE). Resulting protein sequences were then used as queries to perform two database searches against MSU-RGAP (http://rice.
Analysis of Rice Amino Acid Transporters

Expression Data Analysis of OsAATs

The EST, microarrays and MPSS expression data of OsAAT genes were extracted from UniGene database at NCBI [http://www.ncbi.nlm.nih.gov/], the Rice Functional Genomic Express Database [http://signal.salk.edu/cgi-bin/RiceGE] and the MPSS project [http://mpss.udel.edu], respectively. On the other hand, the expression data of AtAATs comparable to those used for rice were also downloaded (Table S3, S4 and S5). EST and MPSS data were used to detect expression pattern of OsAATs in different organs. The data of microarrays were used to analyze expression profiles of OsAATs in organs during different development stages (GSE6093) and under abiotic stresses (GSE6901). The absolute signal values were respectively divided by the average of all absolute values. The Cluster and Treeview software [68] were used to generate hierarchical cluster displays using the logarithmic values of the ratios in previous step. In expression comparative analysis of OsAATs and AtAATs, the genes that were up- or down-regulated at least two-fold with P<0.05 were considered to be differentially expressed.

Quantitative Real-time PCR Analysis

To confirm the expression of representative OsAAT genes in rice organs, quantitative real-time PCR analysis was performed by SYBR-green fluorescence using gene-specific primers (Table S8). Primers were checked using dissociation curve analysis after the PCR reaction for their specificity. At least two or three independent biological replicates were made for real-time PCR analysis in different organs and under stress treatments, respectively. Three technical replicates were taken in each biological replicate. The first-strand cDNA was synthesized from DNaseI-treated total RNA using reverse transcriptase (ReverTra Ace, TOYOBO); 10-fold diluted cDNA samples were used for qRT-PCR. TransStart Eco Green qPCR SuperMix (TransGen, CHINA) was used to determine the expression levels for the genes in a Rotor-Gene Q real-time PCR machine (Qiagen). The following program was used: 95°C for 30 s; 40 cycles of 95°C for 10 s, 55°C for 15 s, 72°C for 10 s. Rice UBQ5 was used as an internal control gene [69]. The relative expression levels were analyzed by the standard curve method, three times diluted series of a mixed cDNA pools were selected to build a stand curve for each gene [68]. The given values of these diluted series are 1, 3, 9 and 27 (from low to high).

Supporting Information

Figure S1 Structures of OsAAT genes. Gene structure analysis for 85 OsAATs is performed by using GSDS (http://gsds.cbi.pku.edu.cn/). The untranslated-regions (UTR), exons and introns are represented by gray boxes, black boxes and lines, respectively. (TIF)

Figure S2 Prediction of the transmembrane regions of 85 OsAATs. The transmembrane regions of 85 OsAATs were predicted by using the TMHMM Server v2.0 (http://www.cbs.dtu.dk/services/TMHMM/) and displayed according to the order in Table 1. (TIF)

Figure S3 Chromosomal localization and duplication of AtAAT genes. Chromosome numbers are indicated at the top of each bar. The scale on the left is in megabases (Mb). The cleavages on the chromosomes (vertical bars) indicate the position of centromeres. (TIF)

Figure S4 Bayesian phylogenetic analysis of OsAATs using MrBayes program. Numbers at the nodes are posterior probability for MrBayes reconstructions. The numbers in AAP subfamily are marked by the letters. (TIF)

Figure S5 Phylogenetic analysis of OsAATs and AtAATs. The phylogenetic tree of all AATs from Arabidopsis and rice after multiple sequence alignment using the full-length protein sequences is constructed by neighbor-joining method. The branches of different subfamilies are marked by different colors. (TIF)
Table S1 The general information and sequence characterization of 63 AtAAT genes.

(DOC)

Table S2 The MEME motif sequences and lengths in OsAAT proteins.

(DOC)

Table S3 The EST expression profiles of OsAAT genes.

(DOC)

Table S4 The microarray analysis of OsAAT genes in various organs under abiotic stresses.

(DOC)

Table S5 The MPSS analysis of OsAAT genes.

(DOC)

Table S6 Data for expression comparison of OsAAT and AtAAT genes.

(DOC)

Table S7 Correlation coefficients between expression patterns from homologous genes.

(DOC)

Table S8 Primers used in qRT-PCR of OsAAT genes.

(DOC)

Author Contributions

Conceived and designed the experiments: HMZ, JZ. Performed the experiments: HMZ, HLM, LY, XW. Analyzed the data: HMZ. Contributed reagents/materials/analysis tools: HMZ, HLM, LY, XW. Wrote the paper: HMZ, JZ.

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