

Full Length Research Paper

# Nucleotide variation in *ATHK1* region of *Arabidopsis thaliana* and its association study with drought tolerance

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Accepted 2 July, 2007

The *ATHK1* gene in *Arabidopsis* encodes a putative histidine kinase that is transcriptionally up-regulated in response to changes in external osmolarity. In this work, we investigated the nucleotide variability of the *ATHK1* gene in a sample of 32 core *Arabidopsis* accessions originating from different ecoclimatic regions and their drought tolerance. The results showed that different accessions had quite difference in adaptation to drought stress. Thirty-two *Arabidopsis* accessions were clustered into four groups according to their drought tolerance capacity. Relative water content of the leaves (RWC) combined anyone of membrane permeability of leaves (MP) and water retain capacity of detached leaves (WRC) were selected as two representative physiological indexes for evaluation of comprehensive drought tolerance. Sequencing 5 515 bp encompassing *ATHK1* coding region in 32 core accessions revealed 39 polymorphisms, which formed 24 haplotypes. The polymorphism (including single nucleotide polymorphism (SNP) and insertion/deletion (Indel)) frequency was 1 SNP per 131.2 bp. In coding region of *ATHK1*, the ratio of average number of nucleotide difference  $\pi_n/\pi_s$  ratio was 0.727, suggesting that the *ATHK1* protein is not constrained against amino acid changes within the species and this gene belonged to the middle evolution rate gene. Using ANOVA analysis, it showed that the 1199 site amino acid (Ser $\leftrightarrow$ stop) variation of the eleventh haplotype (257 and 266av) was associated with not only RWC but also WRC, indicating that the change of Ser $\leftrightarrow$ stop is associated with comprehensive drought tolerance of 257 and 266av. This amino acid change may cause 257 and 266 av accessions originating from moist ecoclimatic region to be sensitive to dry climate, and likely be the evidence of adaptive evolution.

**Key words:** *Arabidopsis*, *ATHK1*, nucleotide variation, drought tolerance, association study.

## INTRODUCTION

Drought is a major agronomic problem, resulting in yield reduction of crops (Boyer, 1982). Therefore, this type of abiotic stress has been the focus of considerable attention, revealing physiological mechanisms of adaptation (Bohnert and Sheveleva, 1998). With the occurrence of water deficit, many of the physiological processes asso-

ciated with growth are affected, and under severe deficit plants may die. The effect of water stress may vary with the variety, degree and duration of water stress and the growth stage of plants.

Plant drought tolerance is not only related to biochemical and physiological processes, but also related to the variety, genotype, degree and duration of drought stress and the developmental stage (Lanceras et al., 2004). Drought tolerance is a very complex quantitative trait and affected by not only difference of plants but also

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the phase and level of drought. Plant drought tolerance is a quantitative trait controlled by multiple genes, most of which are minor genes, and there is probably one major gene playing important role in this complex genetic background. So the polymorphism of any gene can play a role in plant drought tolerance (Hao et al., 2004). We can dissect complex quantitative trait as a set of single locus trait by analyzing QTLs marker. Historically, linkage analysis is used to measure the genetic proximity of loci to each other, to map quantitative trait. However, linkage analysis has some limitation. First, the limited number of recombination events results in poor resolution for quantitative trait. Second, only two alleles at any given locus can be studied simultaneously (Flint-Garcia, 2003).

Recently, association analysis emerges as a powerful tool to identify QTL in plants. It has been suggested that association analysis could be more powerful than linkage analysis for identification of genes that control quantitative trait (Risch and Merikangas, 1996). The principle of association study is that the joint relationship of genotypes and phenotypes in population samples are studied (Long and Langley, 1999). It is a population-based survey used to identify trait-marker relationships based on linkage disequilibrium (LD). Genetic diversity is evaluated across nature population to identify polymorphism that correlate with phenotypic variation. With the advent of efficient molecular marker technologies and specific statistical methods, association study is developed and extensively used in dissection of quantitative trait. Alternatively, association studies could take advantage of the recent progress made in genomics and use the available set of candidate genes (Long and Langley, 1999).

Drought tolerance is a complex quantitative trait being understood further at the molecular genetic level. Genetic analyses have identified several candidate loci demonstrated to affect drought tolerance of plants (Gutterson and Zhang, 2004). Most of these candidate genes have already been cloned. This provides the opportunity to test naturally occurring molecular variation at the candidate genes for an association with the phenotype of drought tolerance.

Our interest focuses on regulatory genes response to environmental stress. In this context, *ATHK1* gene is a good working model. *ATHK1* was cloned and studied by Urao et al. (1999). The predicted *ATHK1* protein in *Arabidopsis* has two putative transmembrane regions in the N-terminal, and has similar structure to the yeast osmosensor synthetic lethal of N-end rule 1 (SLN1). Introduction of the *ATHK1* cDNA into the yeast double mutant *sln1Δ sho1Δ*, which lacks two osmosensors, suppresses lethality in high-salinity media and activate the high-osmolarity glycerol response 1 (HOG1) and nitrogen-activated protein kinase (MAPK) (Urao et al., 1999). The *ATHK1* transcript is more abundant in roots than other tissues under normal growth conditions and accumulates under conditions of high or low osmolarity (Urao et al., 1999). Histochemical analysis of  $\beta$ -glucuro-

nidase activities driven by the *ATHK1* promoter further indicates that the *ATHK1* is transcriptionally upregulated in response to changes in external osmolarity (Urao et al., 1999). These results suggest that *ATHK1* functions as an osmosensor and transmits the stress signal to a downstream MAPK cascade.

In this study, nucleotide variation at the *ATHK1* locus was analyzed in worldwide samples of 32 core accessions of *Arabidopsis thaliana*. Drought tolerance evaluation in the same set of *A. thaliana* accessions was used to search for an association with nucleotide variation at the *ATHK1* locus. Our objectives, firstly, to compare the level and pattern of nucleotide sequence variation in the *ATHK1* gene with that of other previously studied genes, secondly, to examine the relationship between the polymorphism in the *ATHK1* genes and drought tolerance.

## MATERIALS AND METHODS

### Plant materials

Thirty-two accessions of *A. thaliana* were used in this study (Table 1). They originated from different ecoclimatic regions, and all were obtained from Versailles stock center (<http://dbsgap.versailles.inra.fr/vnat/>). Table 1 shows list of 32 *Arabidopsis* core ecotypes

### Measurement of relative water content of the leaves (RWC)

*Arabidopsis* seeds were germinated on MS agar medium, and then 1-week-old seedlings were transferred to 7 cm pots filled with gravel and nutritive soil (1:1) and cultured at 23/19°C day/night temperature, a day/night cycle of 16/8 h. After growing under well-watered conditions for 4 weeks, the plants were subjected to drought treatment without further watering. Plant leaves were sampled after 7 days of drought treatment. Relative water content of the leaves (RWC) was assayed according to the method of Mata and Lamattina (2001). Three independent experiments were carried out and for each experiment two plants were used from each accession, 12 leaves were sampled per plant.

### Measurement of membrane permeability (MP)

Drought treatment was done according to method of RWC measurement. One gram of *Arabidopsis* leaves was used for analysis of the membrane permeability according to the method of Mata and Lamattina (2001). Mean values were calculated from three independent experiments.

### Measurement of water usage efficiency (WUE)

Drought treatment was done according to method of RWC measurement. WUE was determined with the equation: WUE = net photosynthetic rate / transpiration rate. Net photosynthetic rate and transpiration rate were measured with the LI-6400 Portable Photosynthesis System every 3 days for two times, and for each experiment two plants were selected from each ecotype.

**Table 1.** List of 32 *Arabidopsis* core accessions.

Number	Versailles number*	Location/country	Latitude	Longitude	Mean monthly number of rain days
1	163av	Canary Island/Spain	N 28°00'	W 15°30'	0.67d
2	94av	Martuba/cyrenaika(Libya)	N 31°34'	E 22°46'	3.6d
3	76av	Blanes/Spain	N 41°41'	E 2°48'	4.1d
4	178av	Madrid/Spain	N 40°29'	W 3°22'	4.6d
5	157av	Ibel Tazekka/Morocco	N 34°04'	W 4°12'	5.6d
6	162av	Catania/Italy	N 37°30'	E 15°06'	6.0d
7	50av	Palemo/Italy	N 38°07'	E 13°22'	6.2d
8	62av	Stockholm/Sweden	N 59°19'	E 18°03'	6.2d
9	25av	St Jean Cap Ferrat/France	N 43°41'	E 7°20'	6.3d
10	236av	Shakdara river/ Tadjikistan	N 37°29'	E 71°30'	7.5d
11	203av	Tadjikistan/ Tadjikistan	N 38°55'	E 68°47'	7.6d
12	91av	Tsu/Japan	N 34°19'	E 129°19'	7.8d
13	8av	Le Pyla/France	N 46°12'	E 6°10'	7.9d
14	101av	Geneve/Switzerland	N 46°12'	E 6°10'	8.0d
15	42av	Bologna/Italy	N 44°29'	E 11°20'	8.1d
16	186av	Warthe /Poland	N 52°44'	E 15°15'	10.0d
17	234av	Slapy/Czechoslovakia	N 49°49'	E 14°24'	11.5d
18	200av	Greenville/USA	N 43°11'	E 85°15'	11.7d
19	209av	Kindalville/ USA	N 43°00'	E 85°00'	11.8d
20	180av	Bulhary/Czechoslovakia	N48°49'	E 16°45'	12.1d
21	252av	Akita pref/Japan	N 39°43'	E 140°06'	12.7d
22	92av	Stobowa/Russia	N 52°57'	E 36°04'	13.7d
23	68av	Tenela/Finland	N 60°04'	E 23°18'	13.8d
24	244av	Wassilewskija/Russia	N 52°13'	E 30°38'	14.0d
25	83av	Edinburgh/UK	N 50°57'	E 3°13'	14.1d
26	70av	Kaunas/Lithuania	N 54°54'	E 23°54'	14.4d
27	257av	Sakata, Yamagata pref/Japan	N 33°55'	E 139°50'	14.2d
28	266av	Konchezero/ Russia	N 60°07'	E 34°01'	15.8d
29	224av	Oystese/Norway	N 60°23'	E 6°13'	17.2d
30	172av	Burren/Eire(Europe)	N 57°07'	E 9°04'	18.6d
31	166av	Cape Verdi Islands/ Cape Verde Islands	N 16°00'	W 24°00'	No data
32	215av	Ostpr/Poland	N 53°31'	E 20°12'	No data

\*The number name is from Versailles stock centre (<http://dbsgap.versailles.inra.fr/vnat/>).

#### Measurement of water retain capacity of detached leaves (WRC)

Drought treatment was done according to method of RWC measurement. Two plants were selected from each ecotype and we used average water retain capacity of detached leaves in 12 h (accumulative water retain capacity/ 12 h). Detached leaves were weighed in the morning as fresh weight (FW). Instant weight (IW) was determined by subjecting detached leaves in the container with constant temperature (20°C) and constant humidity (30%) every one hour until 12 h. Dry weight (DW) was obtained after drying the samples at 80°C for at least 24 h. WRC measurements were calculated according to the formula:  $WRC (\%) = [(FW-IW)/(FW-DW)] \times 100$ . Mean values were calculated from three independent experiments.

#### Measurement of ABA content

Drought treatment was done according to method of RWC mea-

surement. One gram of the tissue was suspended in 15 mL of extraction solution containing 80% methanol, 100 mg/L butylated hydroxytoluene, and 0.5 g/L citric acid monohydrate. The suspension was stirred overnight at 4°C and centrifuged at 1000 g for 20 min. The supernatant was transferred to a new tube and frozenly dried under vacuum. The dry residue was dissolved with 100 µL of methanol plus 900 µL of Tris-buffered saline (50 mM Tris, 0.1 mM MgCl<sub>2</sub>·6H<sub>2</sub>O, and 0.15 M NaCl, pH 7.8). ABA concentration in the solution then was determined using the Phytodetek ABA immunoassay kit (Idetek, Inc., Sunnyvale, CA).

#### Comprehensive drought tolerance analysis

K-means cluster program of SPSS was used to analyze above five physiological index of drought tolerance (RWC, MP, WUE, WRC and ABA content) of 32 *Arabidopsis* accessions, then clustered with comprehensive drought tolerance.

**Table 2.** Primer pairs of *ATHK1* gene for SNP analysis.

Number of primer pairs	Upper primer	Lower primer	Product length (bp)
P1	5'-TCCTTCATGGGTTTCTGATTTGT-3'	5'-CTTGTGAGATTCCTGCGATGTT-3'	1269
P2	5'-TGGGCTCTGTTTGCAGT-3'	5'-CTGTTGCGTATTGCTCATTG-3'	1274
P3	5'-CAGAACTGATAAGGCAACTGGATG-3'	5'-GCGTTCTTACAAGACTTCTGCAA-3'	1097
P4	5'-GGATGGTGCAGAACATAAAT-3'	5'-ACTTCCGTTTCTCTGTTTCTTA-3'	1298
P5	5'-ACCTCGACAGATTCTCCAACA-3'	5'-CTGCGAAACTTTGTCTTGTC-3'	1285

PCR amplification fragment of *ATHK1* is located between nucleotide positions from 421 to 6001 of GenBank No.AC003952.

### Selection of representative index of comprehensive drought tolerance

Representative index of comprehensive drought tolerance was selected with R cluster program of SPSS.

### DNA extraction

The total DNA was isolated using a modified CTAB method (Doyle and Doyle, 1987). 2 g fresh young leaves were harvested from 4 weeks *Arabidopsis* plants, it was then grind with a mortar and pestle in liquid nitrogen, and later transfer to 50 ml centrifuge tube. Add activated 10 mL CTAB DNA extraction solution whose temperature is 65°C (2% CTAB, 1.4 M NaCl, 100 mM Tris-HCl, pH 8.0, 20 mM EDTA, 0.2%  $\beta$ -mercaptoethanol), incubate at 60°C for 50 min, swirl one times per 5 - 10 min. Add 10 mL chloroform:isoamyl alcohol (24:1 v/v), mix gently but thoroughly. After RNase treatment and a second chloroform/isoamyl alcohol extraction, the DNA was pelleted and dissolved in 200  $\mu$ L TE (10 mM Tris-HCl and 1 mM EDTA, pH 8.0) to a final concentration of 100 - 200 ng/ $\mu$ L DNA.

### Primer design and testing of *ATHK1*

*ATHK1* genome sequence was selected from *Arabidopsis* genome of Columbia (Genebank No. AC003952). Five primer pairs were designed from *ATHK1* genomic sequence using Oligo 6.0 software. Two PCR products are overlapping (Table 2).

### PCR amplification

PCR amplification fragment of *ATHK1* is located between nucleotide position from 421 to 6001 of GenBank No.AC003952. PCR amplification of *ATHK1* was performed in a 50  $\mu$ L volume with PTC-100 thermocycler. Fifty microlitre reaction solution contained 1  $\mu$ L genomic DNA (20ng/ $\mu$ L), 5  $\mu$ L 10 $\times$  PCR buffer, 4  $\mu$ L 2.5 mM dNTP, 2  $\mu$ L 10 mM upper primer, 2  $\mu$ L 10 mM lower primer, 0.4  $\mu$ L Taq enzyme (5U/ $\mu$ L), double distilled water 35.6  $\mu$ L. The thermocycler was programmed as follows: one cycle at 94°C for 4 min, followed by 30 cycles of 94°C for 1 min, 53°C for 1 min, and 72°C for 2 min, then one cycle at 72°C for 7 min.

### DNA sequencing

PCR products were purified with gel extract kit of Omega and sequenced directly on CEQ2000 DNA analysis system (Beckman). Sequencing reaction was performed in 10  $\mu$ L volume using the Beckman CEQ2000XL sequencing Kit with following PCR condition: 30 cycles of 96°C for 20 s, 50°C for 1min, and 60°C for 4 min. Any ambiguous sequences were resolved by repeated sequencing of

the PCR product.

### Sequencing analysis

Sequence contigs and sequence alignment were analyzed with Vector NTI Suite 7.0 and DNASTar softwares. The analyzed region is located between nucleotide position from 724 to 5840 (Genebank No. AC003952). Each SNP was confirmed by repeated sequencing of the PCR products from both ends. Sequence polymorphisms, Tajima'S D and Fu-Li'S D\* test were analyzed using the DnaSP 4.0 (Rozas and Rozas, 1999), and nucleotide variation was estimated as nucleotide diversity ( $\pi$ ( $\pi$ ),  $\theta$ ), and  $4N\mu$ ( $\theta$ ).

### Association analysis

Phylogenetic reconstruction was carried out with the MEGA2 software. ANOVA analysis was carried out with the SPSS software package. To evaluate the statistical significance of differences in comprehensive drought tolerance among *A. thaliana* accessions, we used representative physiological index of comprehensive drought tolerance per accession for a one-way ANOVA analysis. To account for the correlation of molecular variation, we tested for a significant association between *ATHK1* amino acid sequence variation and representative index variation of comprehensive drought tolerance by analyzing significant differentiation between haplotypes with one-way ANOVA analysis (Tables 7 - 8 and Figure 2). To determine the significance level for representative index change of comprehensive drought tolerance relative to reference accession Columbia, we evaluated the significance of the interaction between haplotypes ( haplotype including Columbia vs. the focal haplotype) using ANOVA analysis.

## RESULTS

### Comprehensive drought tolerance analysis

Drought tolerance is a complex response to soil water deficit, high temperature, low temperature and salt stress. Drought tolerance may include a range of morphological, physiological and biochemical adaptations that enable plant to survive period of unfavourable growth conditions. Plant drought tolerance is a quantitative trait affected by many factors, most of which are minor factors. So, it is difficult to evaluate plant comprehensive drought tolerance with only one index. There is no comprehensive standard system for evaluating drought tolerance. Many researchers have suggested appraising plant drought

**Table 3.** Primal data for assessment of comprehensive drought resistance.

Versailles number	RWC (%)	MP (%)	WUE	ABA (ng/g FW)	WRC (%)
8av	86.90±0.03	4.700±0.132	1.997±0.776	153.996±1.810	4.887±0.517
25av	91.00±0.08	4.380±0.321	3.610±0.564	132.377±5.660	4.798±1.169
42av	81.20±0.02	5.300±0.381	1.973±1.082	102.290±6.210	5.441±0.435
50av	83.12±0.02	8.150±0.231	2.664±0.476	137.988±6.660	4.348±0.493
62av	78.60±0.02	7.230±0.424	2.171±0.726	68.865±4.780	5.018±0.675
68av	84.05±0.02	3.900±0.465	2.565±0.283	227.000±20.88	4.674±0.911
70av	86.80±0.03	6.200±0.196	3.543±0.881	474.322±16.84	3.434±0.765
76av	83.60±0.08	3.150±1.016	2.404±0.669	119.552±11.58	5.490±0.432
83av	84.20±0.02	10.70±0.123	3.531±0.215	148.882±2.070	5.368±0.276
91av	80.39±0.05	6.900±4.132	2.078±0.171	128.759±5.340	5.649±0.245
92av	84.97±0.03	3.750±0.324	2.345±0.267	103.002±5.150	5.708±0.632
94av	84.09±0.07	5.430±2.336	2.510±0.521	139.065±6.010	4.570±0.321
101av	85.34±0.02	3.780±0.445	2.340±0.509	156.255±11.29	2.859±0.616
157av	95.34±0.01	7.000±0.432	3.670±0.737	454.890±19.94	3.424±0.178
162av	82.18±0.02	7.900±0.298	0.567±0.317	327.401±4.020	5.889±0.212
163av	86.53±0.03	3.650±0.728	2.671±0.522	649.657±33.19	3.958±0.342
166av	85.25±0.04	4.890±0.141	2.774±0.399	167.016±1.940	5.373±0.278
172av	82.63±0.03	18.78±0.876	2.982±1.344	147.396±23.12	5.889±0.341
178av	78.24±0.03	2.350±0.289	3.326±0.194	438.490±10.67	3.565±0.287
180av	90.00±0.03	9.500±0.212	2.500±0.445	63.599±1.520	4.661±0.376
186av	82.80±0.02	5.550±0.201	0.675±0.272	109.597±2.660	3.008±0.276
200av	75.44±0.01	4.560±0.090	2.530±0.487	59.730±6.640	5.405±0.024
203av	91.21±0.04	3.000±0.112	2.767±0.090	277.734±13.67	3.127±0.267
209av	88.46±0.07	3.890±0.278	1.845±0.987	257.630±4.990	3.150±0.222
215av	87.16±0.08	6.500±0.265	1.832±1.160	117.721±1.410	3.152±0.510
224av	81.53±0.03	2.150±0.325	1.007±0.348	42.240±2.030	5.714±0.380
234av	78.43±0.03	4.900±0.178	2.191±0.312	80.789±1.050	4.283±0.643
236av	79.42±0.02	5.000±0.367	3.335±0.336	40.476±1.330	4.601±0.189
244av	87.85±0.02	4.200±0.078	2.780±0.366	103.561±3.360	4.340±0.453
252av	85.43±0.03	12.310±0.116	1.757±0.906	153.996±1.810	6.076±0.564
257av	76.24±0.10	11.250±0.378	0.488±0.556	89.138±7.750	6.073±0.648
266av	82.71±0.87	15.430±0.110	2.132±0.171	103.561±3.360	5.717±1.166

RWC, Relative water content of leaves; MP, Membrane permeability of leaves; WUE, Water usage efficiency; WRC, Water retain capacity of detached leaves.

tolerance with comprehensive method for more physiological indexes. We scored the drought tolerance with five physiological indexes (RWC, MP, WUE, WRC and ABA content) of 32 *Arabidopsis* accessions (Table 3) with K-means cluster program of SPSS, and then clustered them into four groups: most tolerant groups, tolerant group, middle tolerant group and drought sensitive group:

Most tolerant group: 163av; Tolerant group: 203av, 209av, 70av, 162av, 178av, 157av.

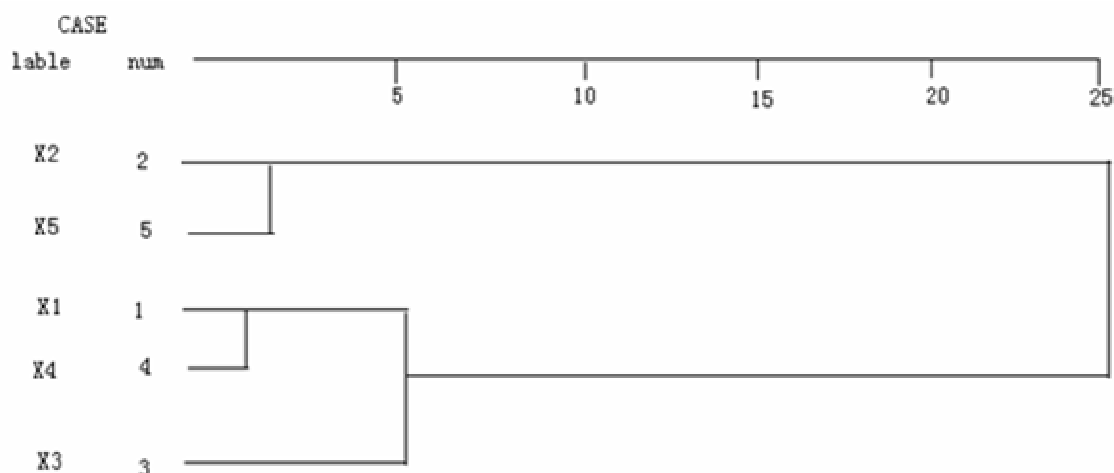
Middle tolerant group: 8av, 166av, 215av, 172av, 68av, 50av, 94av, 91av, 83av, 25av, 101av.

Sensitive to drought group: 252av, 244av, 236av, 224av, 92av, 62av, 186av, 234av, 180av, 266av, 76av, 42av,

200av, 257av.

In order to carry out association analysis between *ATHK1* sequence variation and representative index of comprehensive drought tolerance variation, representative physiological index were selected by R cluster program of SPSS (Figure 1). The result showed that MP and WRC were clustered into one group; other three indexes (RWC, WUE and ABA content) were clustered into another group.

We calculated the relative exponent  $R^2$  among values (X1, X3 and X4) in group 2, and then selected maximum value ( $R_{X1}^2=0.132$ ) RWC as one representative physiological index. Comprehensive drought tolerance could be evaluated by RWC combining anyone of MP and WRC.



**Figure 1.** Cluster analysis of representative physiological index of comprehensive drought tolerance. X1, relative water content of leaves (RWC); X2, membrane permeability of leaves (MP); X3, water usage efficiency (WUE); X4, ABA content; and X5, water retain capacity of detached leaves (WRC).

**Table 4.** Patterns of nucleotide variation in the *ATHK1* region of *Arabidopsis thaliana*.

Region	Size (bp)	SNP	Indel	$\pi$	$\theta$	Transition/Transversion
Whole region	5117	36	3	0.00086	0.0016	1.57
Coding region	3624	19	0	0.00056	0.00137	1.71
Non-coding region	1493	17	3	0.00184	0.00221	1.42
5'-flanking	131	2	0	0.0048	0.00379	1.00
3'-flanking	105	0	0	0	0	0
Introns	1257	15	3	0.00155	0.00206	1.5

The analyzed region is located between nucleotide position from 724 to 5840 (Genebank No. AC003952). Sequence polymorphisms were analyzed using the DnaSP 4.0 (Rozas and Rozas, 1999) and nucleotide variation was estimated as nucleotide diversity ( $\pi$ ,  $\theta$ ).

### DNA polymorphisms at the *ATHK1* locus

At *ATHK1* locus, the nucleotide polymorphism (including single nucleotide polymorphism (SNP) and insertion/deletion (Indel)) frequency was 1 polymorphism per 131.2bp, on average 1 SNP per 142.1bp and 1 Indel per 1705.7bp (Table 4).

Nineteen SNPs and no Indel were found in the coding region, SNP frequency was 1 SNP per 190.7bp, and they formed 11 clades according to amino acid variation (Table 4 and Figure 2). Seventeen SNPs and 3 Indels were discovered in non-coding region, on average 1 SNP per 87.8bp and 1 Indel per 497.7 bp. Among them, two SNP were discovered in the 5'-flanking sequence, on average 1 SNP per 65.5 bp; fifteen SNPs and 3 Indels were discovered in introns, on average 1 SNP per 83.8 bp and 1 Indel per 419bp. Nucleotide polymorphism in non-coding region was 1.55 times higher than that in coding region. The nucleotide diversity ( $\pi$ ) for the whole region was 0.00086 (Table 4), which was lower than that of previously studied genes ChiA, ChiB and FRI of *Arabido-*

*opsis* (Kawabe et al., 1997, 1999; Le Corre et al., 2002). For ChiA (Kawabe et al., 1997), ChiB (Kawabe and Miyashita, 1999) and FRI (Le Corre et al., 2002), nucleotide diversity ( $\pi$ ) was 0.00214, 0.01040 and 0.0091 respectively. Similar to previously studied genes, nucleotide diversity was significantly lower in coding region than that in non-coding region. Table 4 shows patterns of nucleotide variation in the *ATHK1* region of *Arabidopsis thaliana*.

Nineteen SNPs were discovered at the *ATHK1* coding region. Five synonymous and fourteen nonsynonymous mutations were detected in this region. The ratio of average number of nucleotide difference  $\pi_n/\pi_s$  was 0.727 (Table 5), higher than that of those previously studied genes *CAL*, *AP3* and *PI* (Le Corre et al., 2002).

This result indicates that the *ATHK1* protein is not constrained against amino acid changes within the species, and this gene belonged to the middle evolution rate gene.  $\pi_n/\pi_s$  ratio in exon 1 is 5.36, this high value indicates that evolution rate in this region is very quick. Table 5 show analysis of nucleotide diversity in *ATHK1* coding region of *A. thaliana*.

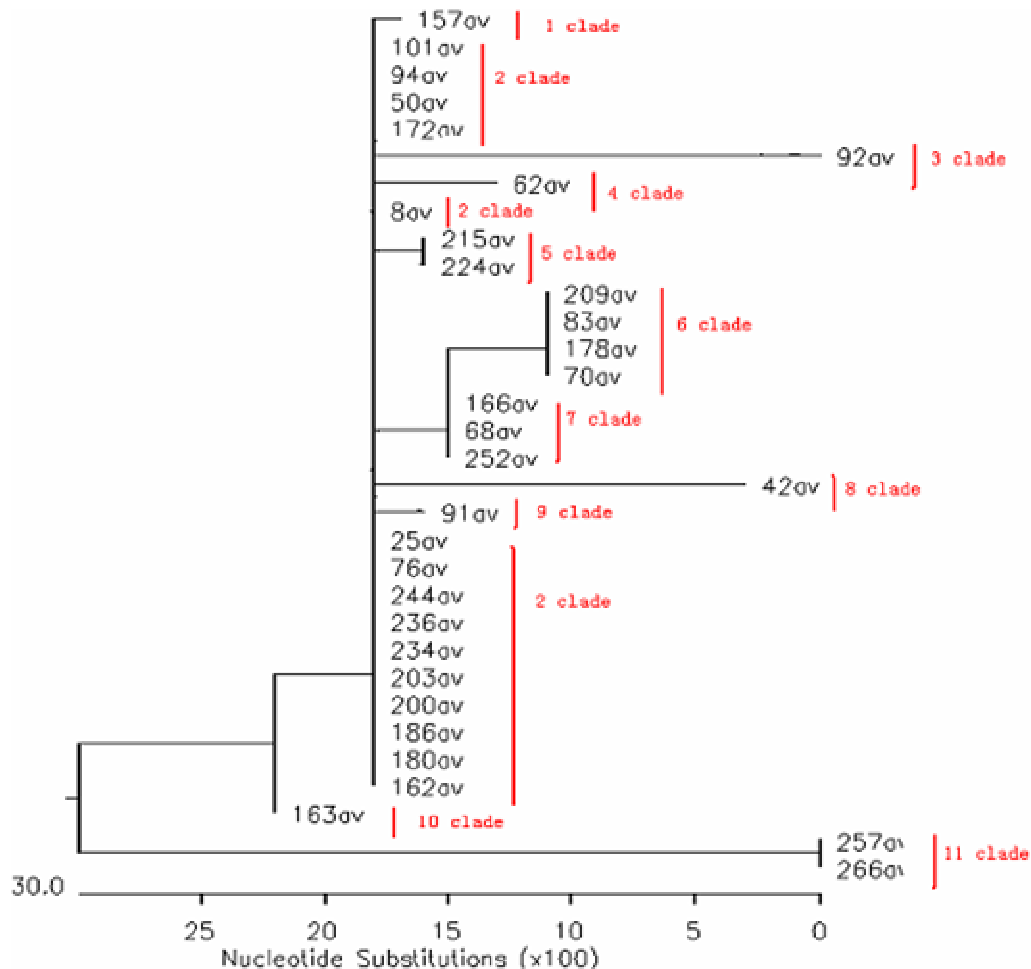


Figure 2. Neighbor-joining trees representing *ATHK1* amino acid clade relationships.

Table 5. Analysis of nucleotide diversity in *ATHK1* coding region of *Arabidopsis thaliana*.

Region	Length	Synonymous			Nonsynonymous			$\pi_n / \pi_s$
		N	S	$\pi_s$	N	S	$\pi_n$	
All coding regions	3624	823	5	0.00071	2797	14	0.00052	0.727
Exon 1	259		1	0.00125		2	0.0067	5.36
Exon 3	777		0	0		1	0.00125	-
Exon 4	245		1	0.0054		0	0	0
Exon 6	125		2	0.00125		0	0	0
Exon 9	70		1	0.00125		1	0.00125	1
Exon 12	1042		0	0		7	0.0016	-
Exon 13	234		0	0		3	0.0020	-

N = Number of sites; S = Number of polymorphic sites.

**The relationship between polymorphisms in the *ATHK1* coding region and comprehensive drought tolerance**

To find the correlation of genotypic and phenotypic variation, we tested for a significant association between

*ATHK1* sequence variation and representative index of comprehensive drought tolerance variation by analyzing significant differentiation between haplotypes with ANOVA analysis (Table 6 and Figure 2). In the coding region of *ATHK1*, the sequencing result showed that eleven different clades were detected in 32 accessions (Figure 2).

**Table 6A.** ANOVA analysis on 5 physiological indexes based on 11 clades of *ATHK1* protein.

Parameter	Relative water content of leaves (RWC)			Membrane permeability of leaves (MP)			Water usage efficiency (WUE)		
	Between groups	Within groups	Total	Between groups	Within groups	Total	Between groups	Within groups	Total
Degree of freedom	10	80	90	10	51	61	10	90	100
Sum of squares	5.606E-02	0.188	0.244	2.521E-02	6.747E-02	9.268E-02	33.252	51.818	85.070
Mean squares	5.606E-03	2.350E-03		2.521E-03	1.323E-03		3.325	0.576	
F value	2.386			1.906			5.775		
Significance	0.016			0.066			0.000		

**Table 6B.** ANOVA analysis on 5 physiological indexes based on 11 clades of *ATHK1* protein.

Parameter	ABA content			Water retain capacity of detached leaves (WRC)		
	Between groups	Within groups	Total	Between groups	Within groups	Total
Degree of freedom	10	85	95	10	76	86
Sum of squares	1425061.649	491373.212	1916434.861	31.632	74.494	106.127
Mean squares	142506.165	5780.861		3.163	0.980	
F value	24.651			3.227		
Significance	0.000			0.002		

**Table 7.** Multiple comparison of ANOVA analysis on relative water content of leaves (RWC) based on 11 clades of *ATHK1* protein. Multiple Comparisons, Dependable Variable: X, LSD

(I)Y	(J)Y	Mean Difference (I-J)	Standard Error	Significance	95% Confidence interval	
					Lower Bound	Upper Bound
2.00	1.00	-.1033	2.895E-02	.001*	-.1609	-4.5657E-02
	3.00	-5.1775E-03	2.895E-02	.858	-6.2782E-02	5.243E-02
	4.00	6.267E-02	2.895E-02	.033*	5.066E-03	.1203
	5.00	1.040E-03	2.112E-02	.961	-4.1000E-02	4.308E-02
	6.00	-1.0641E-03	1.683E-02	.948	-3.3658E-02	3.153E-02
	7.00	-5.7670E-03	1.886E-02	.758	-4.2910E-02	3.138E-02
	8.00	3.266E-02	2.895E-02	.263	-2.4944E-02	9.026E-02
	9.00	4.051E-02	2.895E-02	.165	-1.7091E-02	9.812E-02
	10.00	-1.6021E-02	2.895E-02	.581	-7.3625E-02	4.158E-02
	11.00	2.513E-02	2.290E-02	.047*	-2.0448E-02	7.071E-02

\*: The mean difference is significant at 0.05 level.

Except MP, the differentiation of other four physiological indexes between clades is highly significant (Table 6). Multiple comparisons indicated that only the 1199 amino acid site (Ser $\leftrightarrow$ stop resulted from 4984th G $\leftrightarrow$ C) variation of the eleventh clade (257av and 266av) is associated with not only RWC but also WRC (Table 7 and 8). This means that the change of Ser $\leftrightarrow$ stop is associated with comprehensive drought tolerance of 257 and 266 av. Table 6 shows ANOVA analysis on 5 physiological indexes based on 11 clades of *ATHK1* protein.

Table 7 shows multiple comparison of ANOVA analysis

on relative water content of leaves (RWC) based on 11 clades of *ATHK1* protein. Table 8 shows multiple comparison of ANOVA analysis on water retain capacity of detached leaves (WRC) based on 11 clades of *ATHK1* protein. Figure 2 shows neighbor-joining trees representing *ATHK1* amino acid clade relationships

## DISCUSSION

In the *ATHK1* gene region, the polymorphism (SNP and



**Table 8.** Multiple comparison of ANOVA analysis on water retain capacity of detached leaves (WRC) based on 11 clades of ATHK1 protein. Multiple Comparisons, Dependable Variable: X, LSD.

(I)Y	(J)Y	Mean Difference (I-J)	Standard Error	Significance	95% Confidence interval	
					Lower Bound	Upper Bound
2.00	1.00	1.2410	.7174	.088	-.1877	2.6697
	3.00	-0.9073	.5927	.130	-2.0877	.2730
	4.00	-.7707	.5927	.197	-1.9510	.4097
	5.00	.1060	.4334	.807	-.7573	.9693
	6.00	.5450	.3500	.124	-.1522	1.2422
	7.00	-.9215	.3834	.019*	-1.6852	-.1578
	8.00	-.6840	.5927	.252	-1.8644	.4964
	9.00	-1.1040	.5927	.066	-2.2844	7.637E-02
	10.00	.9527	.5927	.112	-.2277	2.1330
	11.00	-1.2123	.4334	.007*	-2.0756	-.3491

Indels) frequency was 1 SNP per 131.16 bp. Nucleotide polymorphism in non-coding region was 1.55 times higher than that in coding region. This distribution was similar to that observed in human (Wang et al., 1998; Bensen et al., 2003), maize (Ching et al., 2002; Tenaillon et al., 2001), rice (Nasu et al., 2002) and soybean (Zhu et al., 2003; Voryell et al., 1999). One explanation for this lay in the possible heterosis conferred by great selection pressure on coding region.

In *ATHK1* coding region, high density of single nucleotide polymorphism (SNP) was discovered, SNP frequency was 1 SNP per 190.7 bp. Among them, five synonymous and fourteen nonsynonymous mutations were detected in this region. The ratio of average number of nucleotide difference  $\pi_n / \pi_s$  was 0.727, indicating that the *ATHK1* protein is not constrained against amino acid changes within species and positive selection affected these region intensively (Table 5), and this gene was undergoing adaptive evolution.

The major goal of this study is to link phenotypic and genotypic variation to learn more about the function of the *ATHK1*. *ATHK1* exhibits marked sequence variability in association with the representative index of comprehensive drought tolerance. The strongest evidence for association analysis comes from the amino acid Ser $\leftrightarrow$ stop variation of the eleventh clade (257 and 266av). This polymorphism was discovered near the reaction regulation region of histone protein kinase (*ATHK1*). The change of Ser $\leftrightarrow$ stop may affect the osmosis signal output function of *ATHK1*. It is noteworthy that the branches of 257 and 266av, the two accessions grow in relative moist environment. The result of this amino acid change may be their sensitivity to dry climate. 257 and 266 av grow respectively in Sakata Yamagata pref (Japan) and Konchezero (Russia). The mean monthly number of rain days all year in these two places is respective 14.2 and 15.8 days (Table 1) (<http://dbsgap.versailles.inra.fr/vnat/>). It is highly significant than that of reference population 186av, which is 10 days. This case is likely to be the evidence of adaptive evolution.

## ACKNOWLEDGMENTS

This study is financially supported by grants from China National Key Basic Research Program (No.2003CB114302), National Natural Science Foundation of China (No.3040228), Foundation of Beijing Municipal Committee of Science and Technology (No.H012010240240113) and the Beijing Agricultural Innovative Platform - Beijing Natural Science Fund Program (YZPT02-06).

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