

Full Length Research Paper

# Cloning and expression analysis of potassium channel gene *NKT3* from *Nicotiana tabacum*

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Potassium (K<sup>+</sup>) is the predominant inorganic ion of plant cells. K<sup>+</sup> channels in higher plant cells play an important role in regulating the influx and efflux of K<sup>+</sup> from cells, and activity of these channels might be involved in plant stress resistance. A completely new K<sup>+</sup> channel gene of *Nicotiana tabacum* was obtained through homologous cloning strategy. The complete cDNA sequence was submitted to the National Center for Biotechnology Information (NCBI) GenBank, designated as *NKT3* and the accession number is FJ230956. The phylogenetic analysis indicated that *NKT3* is located at the branch of weak-inwardly rectifying K<sup>+</sup> channels and might be a member of the Shaker family. The spatial and temporal expression of the gene was also investigated. *NKT3* is expressed abundantly in the roots, while little in the leaves of *N. tabacum*. It might be involved in the process of K<sup>+</sup> acquirement and release in tobacco roots.

**Key words:** Potassium channel gene, *NKT3*, RACE, *Nicotiana tabacum*.

## INTRODUCTION

Potassium (K<sup>+</sup>) is the predominant inorganic ion of plant cells, and is recognized as a rate-limiting factor for crop yield and quality. During the past decade, a variety of K<sup>+</sup> transport systems, differing in transport affinity, voltage sensitivity, energy coupling or ionic selectivity, have been identified in plant cell membranes by physiological analysis. K<sup>+</sup> transporters and K<sup>+</sup> channels form the basis of these transport systems. Generally, transporters contribute to K<sup>+</sup> uptake of the high affinity, while K<sup>+</sup> channels play a key role in the low affinity K<sup>+</sup> uptake. But this is not a strict rule, channels may also contribute to the high affinity K<sup>+</sup> uptake (Dreyer and Uozumi, 2011; Hedrich et al., 2011).

The first K<sup>+</sup> channel gene was identified from Shaker mutants of *Drosophila* in 1987, and the cloning of the Shaker locus from *Drosophila melanogaster* provided a

starting point for molecular studies of K<sup>+</sup> channels (Kamb et al., 1987; Papazian et al., 1987; Tempel et al., 1987). Subsequently, many K<sup>+</sup> channels were identified from plants and animals (Tempel et al., 1988; Anderson et al., 1992). The K<sup>+</sup> channel *Arabidopsis thaliana* 1 (KAT1) and the *Arabidopsis* K<sup>+</sup> transporter 1 (AKT1) were the first K<sup>+</sup> channel cloned from plants, sharing structural homology to *Drosophila* Shaker channel (Schachtman et al., 1992; Sentenac et al., 1992; Bertl et al., 1995). After that, plant potassium channels are found in all plant cells and tissues, suggesting a housekeeping role in the whole life of plants (Müller-Röber et al., 1995). The Shaker family is the best-characterized family of plant K<sup>+</sup> channels (Véry and Sentenac, 2003). The family shows a common structure with six trans-membrane domains and one pore loop (6TM1P), and belongs to the voltage-dependent, K<sup>+</sup> selective ion channels. The proteins subdivide into four functional subgroups: inward-rectifying channels, outward-rectifying channels, silent channels and weakly rectifying channels, and gating are controlled by the membrane potential (Dreyer and Uozumi, 2011). Sensing

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changes in membrane potential relies on a cluster of positively charged residues on the fourth trans-membrane segment (Hedrich et al., 2011). Shaker-type channels are thought to be the major pathway for  $K^+$  uptake or secretion in different tissues and cell types in plants. In *Arabidopsis*, several inward shakers function in  $K^+$  uptake over a wide range of external  $K^+$  concentrations (Hirsch et al., 1998; Mouline et al., 2002). For instance, the inward rectifying  $K^+$  channel KAT1 expressed in *Arabidopsis* guard cells can absorb  $K^+$  when the external  $K^+$  concentration is as little as 10  $\mu$ M (Brüggenmann et al., 1999).

To date, *Arabidopsis* is the only plant species in which the complete set of shaker channels has been identified and almost entirely characterized. There are nine genes coding shaker-like  $K^+$  channels, and their physiological role in nutrition, growth, and stress resistance, as well as their regulation pathway are becoming the focus of many research groups (Hedrich et al., 2011). Although shakers in other plants have a close relationship with the *Arabidopsis* shaker family, few of them have been thoroughly studied. Whether these proteins have functional differences from its *Arabidopsis* counterpart is still not determined (Véry and Sentenac, 2003).  $K^+$  channel is one of the important ways to acquire  $K^+$  in *Nicotiana tabacum* (Liu et al., 2006), and the potassium content in flue-cured tobacco leaves (the main type of *N. tabacum*) is important for the quality of tobacco leaves. Thus, it is necessary and valuable to clone and identify  $K^+$  channel gene for theoretical research and agriculture production. Sano et al. (2007) reported four  $K^+$  channel genes in tobacco by the method of homology cloning, and they are *NKT1*, *NTORK1*, *NKT2* and *NtKC1*, respectively. *NKT1* plays the main role in  $K^+$  uptake.

In this experiment, homologous cloning strategy was conducted, and a new *N. tabacum*  $K^+$  channel gene was obtained. Spatial and temporal expression analysis and phylogenetic analysis indicated that *NKT3* is located at the branch of the weak inwardly rectifying  $K^+$  channels of the shaker family. *NKT3* is mainly expressed in the roots, while little in other tissues in *N. tabacum*. It might function in the process of  $K^+$  acquirement and release in tobacco roots.

## MATERIALS AND METHODS

### Plant materials and growing conditions

Tobacco seeds of special warne were provided by the germplasm resources pool of Tobacco Research Institute, Chinese Academy of Agricultural Sciences. The seeds were sown in nutrient soil, 25 to 28°C temperature and 70% relative humidity in the greenhouse.

### Low- $K^+$ treatment

Solution for potassium treatment was prepared as follows: 215

mmol/L  $NaNO_3$ , 110 mmol/L  $Ca(NO_3)_2$ , 2 mmol/L  $MgSO_4$ , 100  $\mu$ mol/L  $FeNaEDTA$ , 200  $\mu$ mol/L  $NaH_2PO_4$ , 50  $\mu$ mol/L  $H_3BO_3$ , 0.15  $\mu$ mol/L  $CuSO_4$ , 2  $\mu$ mol/L  $MnSO_4$ , 0.15  $\mu$ mol/L  $Na_2MoO_4$ , 0.11  $\mu$ mol/L  $CoCl_2$ , 2  $\mu$ mol/L  $ZnSO_4$ , 1  $\mu$ mol/L  $KI$ . The tobacco plants, about eight-leaf stage, were replanted in the sandy soil for one week, while MS culture was used to sustain the growth of the plants. Then the solution for low- $K^+$  treatment was added to the soil. The leaves and roots of tobacco plants were collected after low- $K^+$  treatment for 0, 12, 24, 48, 72 and 96 h, respectively and the total RNA was isolated for analysis of semi-quantitative reverse transcriptase polymerase chain reaction (RT-PCR).

### Drought treatment

The tobacco plants were grown hydroponically in the MS culture for seven days, and were drought-treated by 200 mg/L polyethylene glycol (PEG) for 0, 1, 3, 6, 12 and 24 h. The total RNA was extracted for analysis of semi-quantitative RT-PCR.

### Total RNA extraction and the first-strand cDNA synthesis

Seedling roots of 60 growth days were collected and washed gently with water, and root growth point was used as experimental material. Total RNA from tobacco roots was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. After treated with DNase, the first-strand cDNA synthesis for the obtainment of whole-length *NKT3* was carried out with SMARTScribe™ reverse transcriptase (Clontech, Mountain View, CA, USA). Total RNA from tobacco different tissues (the main roots, lateral roots, stems, leaves and flowers) was extracted using TRIzol reagent. The first-strand cDNA synthesis for semi-quantitative RT-PCR of different treatment was obtained using Moloney-Murine Leukemia Virus (M-MLV) reverse transcriptase (Promega, Madison, WI, USA).

### Middle fragment amplification

The conserved amino acid sequence was obtained through the  $K^+$  channel protein sequence alignments between *Arabidopsis*, carrot, tomato and potato. The conserved amino acid region was selected to design degenerate primers for middle fragment amplification. Degenerate primers were designed as follows: Forward primer FP1: 5'-TA(C/T)(C/T)T(A/G/C/T)AT(A/C/T)GG (A/G/C/T)AA(C/T)ATGAC-3'; Reverse primer RP1: 5'-CA(A/G/C/T)A(G/A)(A/G/C/T)AC(A/G/C/T)CC(T/G/A)AT(C/T)TC(A/G/C/T)CC-3'. The middle fragment of *NKT3* was amplified from the first-strand cDNA obtained in the reverse transcription reaction with high-fidelity Ex Taq Hot Start DNA polymerase (Takara, Dalian, China).

### 5' and 3' terminal cDNA amplification

5' terminal cDNA amplification primers 5'GSP (5'-CTGCC TTTATCTCTGAG ACCAACTGGAC-3') was designed based on the middle fragment template that had higher homology with tomato *LKT1* and potato *SKT1*. In order to reduce non-specific amplification, the length of designed primers GSP was 28 nt, GC content was more than 50% and  $T_m$  was above 65°C. According to Clontech SMARTer™ rapid amplification of cDNA ends (RACE) amplification kit instruction, using reverse transcription-dilution as a template with universal primer mix (UPM, provided in the kit) and 5'GSP as primers, 5' terminal fragment was amplified. 3' terminal cDNA amplification primers 3'GSP (5'-GTCCAGTTGGTCTCAG

AGATAAAGGCAG-3') was designed based on the sequences of tomato *LKT1* and potato *SKT1*. According to the kit instruction, 3' terminal fragment was amplified using primers UPM and 3'GSP.

### Full-length cDNA amplification

After splicing the 5' RACE sequence, middle fragment and 3' RACE sequence, a full-length sequence was acquired. Specific primers were designed as follows: forward primer FP2: 5'-AACCTCAGCCAACAAAAT-3'; reverse primer RP2: 5'-ATCCGTTTCTGTTTCCTC-3'. The integrated encoding frame sequence was amplified from the cDNA by using high-fidelity Ex Taq Hot Start DNA polymerase (Takara, Dalian, China) with the primer pair FP2 and RP2.

### Spatial and temporal expression analysis of *NKT3*

The primers for RT-PCR analysis were designed as follows: forward specific primer FP3: 5'-TTGTTCCGCGGCATT-3'; reverse specific primer RP3: 5'-CTGCTCGAAACTGAGGCTATGTT-3'. The pair primers of FP3 and RP3 were used for expression analysis of *NKT3* in different tissues. *Actin* was used as the reference gene, and its primers were designed as follows: actin1: 5'-GTATTGTGTTGGACTCTTG-3', actin2: 5'-ATTTCTTTGCTCATTCTATC-3'. The predicted size of amplicons from *NKT3* and *actin* are 56 and 76 bp, respectively. Total RNA samples of the tobacco main roots, lateral roots, stems, leaves and flowers were used as templates, and the cDNA obtained was used for RT-PCR analysis. Total RNA of tobacco roots under normal and low-K<sup>+</sup> or drought condition was used as templates for real-time fluorescence quantitative PCR. The RNA transcript obtained was diluted to 5<sup>0</sup>, 5<sup>-1</sup>, 5<sup>-2</sup>, 5<sup>-3</sup>, 5<sup>-4</sup>, 5<sup>-5</sup> concentration. Real-time fluorescence quantitative PCR was performed using SYBR Premix Ex Taq<sup>TM</sup> (Takara, Dalian, China) with *actin* primers (actin1 and actin2) and *NKT3* primers (FP3 and RP3), and the machine used is 7300 real-time PCR System (Applied Biosystems).

## RESULTS

### Middle fragment amplification and sequence analysis of potassium channel gene

The degenerate primers FP1 and FP2 used for middle fragment amplification was designed according to the K<sup>+</sup> channel protein sequence alignments between Arabidopsis, carrot, tomato and potato. The template cDNA was synthesized using the RNA extracted from the roots of tobacco plants under normal growth condition. A fragment with a length of 490 bp was acquired and sequenced (Figure 1A).

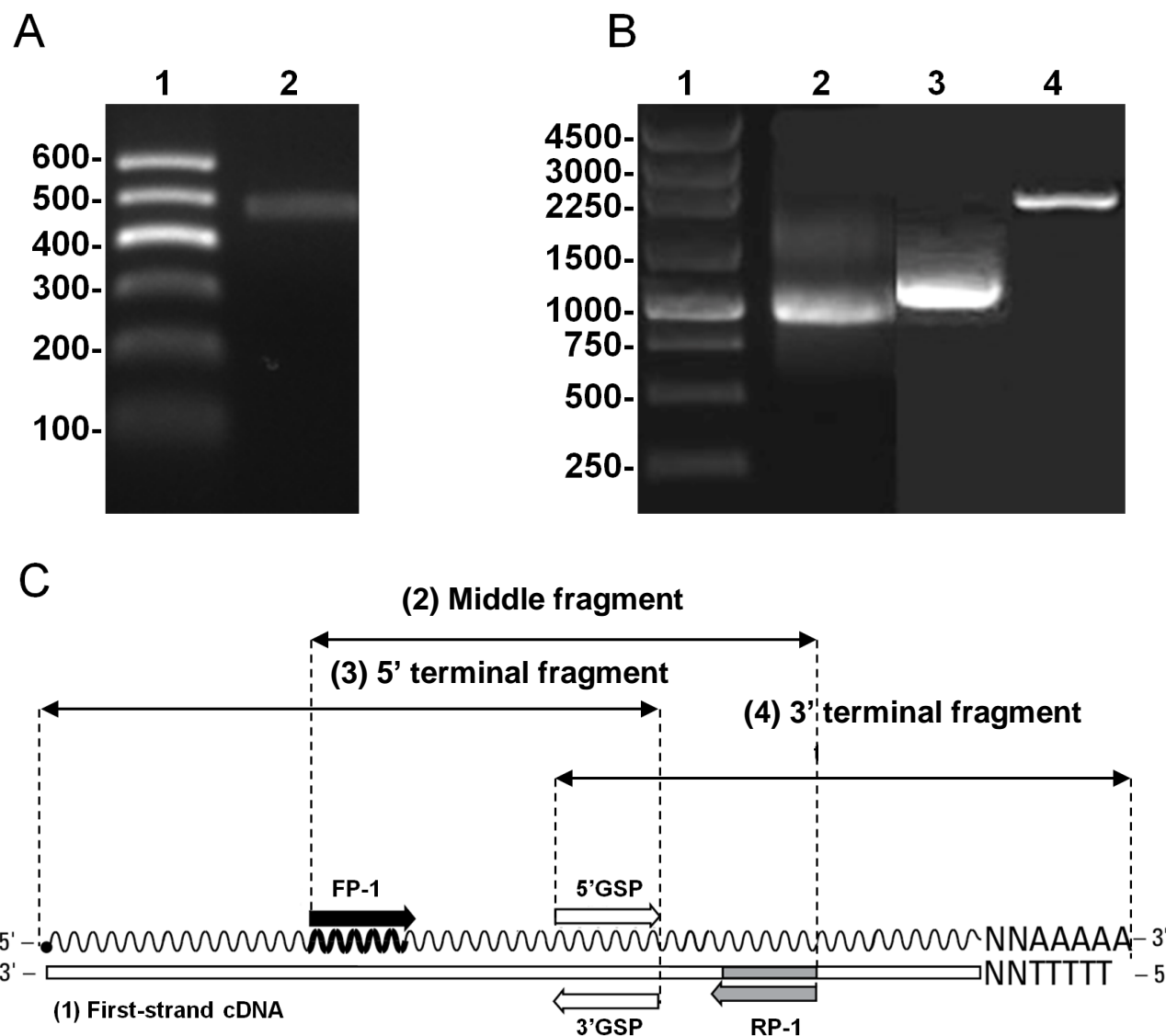
Basis Local Alignment Search Tool (BLAST) researches showed that the fragment had a high homology with other K<sup>+</sup> channel proteins. It shared 94% identity with tomato *LKT1* (CAA65254), 94% with potato *SKT1* (CAA60016) and shared 89% identity with tomato *NKT5* (ACJ05642). The result indicates that a fragment of a K<sup>+</sup> channel protein was obtained through the homologous cloning strategy.

### Amplification of the 5' and 3'-terminal sequences and acquirement of the full-length gene

According to the sequences of tomato *LKT1* and potato *SKT1*, two terminal specific primers, 5'GSP and 3'GSP, were designed, for the target gene had higher identity with these two genes. Two pairs of primers, 5'GSP/UPM and 3'GSP/UPM were used for the amplification of the 5' and 3'-terminal sequences, respectively. Two fragments were obtained in 5' and 3' RACE, with a length of 1280 and 863 bp, respectively (Figure 1B, lane 2 and 3). Additionally, the 863 bp fragment had a poly (A) tail of 29 nucleotides, which indicated that the C-terminus of the coding region was obtained. Subsequently, the full-length gene was amplified using a pair of primers of FP2 and RP2. A PCR product with a length of 1984 bp was acquired (Figure 1B, lane 4). The process to obtain the full-length gene is shown as Figure 1C. Sequencing analysis indicated that the full-length gene was consistent with the 1280, 490 and 863 bp fragments obtained in the previous experiments, which confirmed that the gene was transcribed and expressed in tobacco. The 1280, 490 and 863 bp sequence were spliced by ContigExpress software, and a 2112 nt gene sequence was obtained. The gene was designate as *NKT3*, and the sequence was submitted to Genbank, with accession number FJ230956. The 5'-UTR was 54 nt in length and the 3'-UTR 132 nt. The open reading frame (ORF) began with an AUG at 55 to 57 nt and encoded a putative protein with 641 amino acids.

### Protein sequence and phylogenetic analysis of *NKT3* protein

The putative protein sequence analysis was then performed. BLAST research indicated that the whole protein showed a greater homology with tobacco *NtKC1* (BAD81035) (95%), carrot *KDC1* (CAB62555) (70%) and Arabidopsis *KAT3* (AEE86097) (58%), which indicated that a new K<sup>+</sup> channel gene was acquired from *N. tabacum*. Further analysis showed that there were three conserved domains harboured in the protein, they are: (I) the conserved domain of ion transport proteins, (II) cyclic nucleotide-monophosphate binding domain or effector domain of catabolite gene activator protein family (CAP family) of transcription factor, and (III) the conserved domain of voltage-dependent potassium channels (Figure 2A). These are the structural characteristics of the Shaker family. The protein sequence analysis indicated that *NKT3* might be a member of the Shaker family. A phylogenetic analysis was performed between *NKT3* and K<sup>+</sup> channel proteins from tobacco, Arabidopsis and other plants. Cluatalx 1.83 was used for sequence alignment and MEGA4 for mapping analysis. The phylogenetic tree was divided into six primary branches, among which



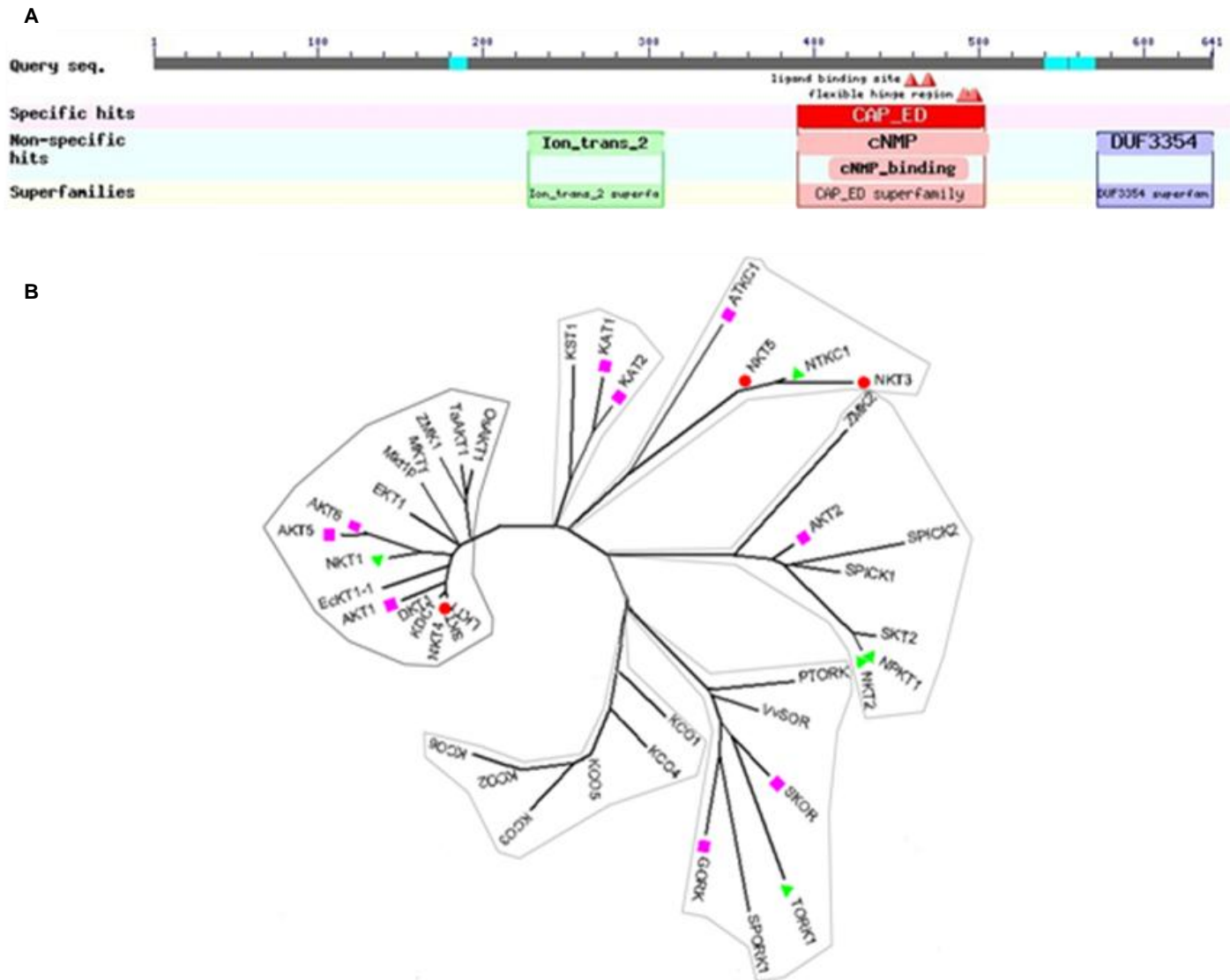
**Figure 1.** Electrophoresis analysis of the middle, 5' and 3'-terminal fragments of potassium channel gene. (A) The amplification of the middle fragment of potassium channel gene; (1) DNA marker; (2) The 490 bp PCR product. (B) The amplification of the 5' and 3' terminal fragments of potassium channel gene; (1) DNA marker; (2) the 863 bp PCR product; (3) the 1280 bp PCR product; (4) the 2000 bp full-length gene. (C) The diagram of the process for obtaining the full-length potassium channel gene.

branch I, II, III, IV and V are Shaker family  $K^+$  channel proteins, while branch VI is KCO family  $K^+$  channel protein. *NKT3* is located in branch III (Figure 2B).

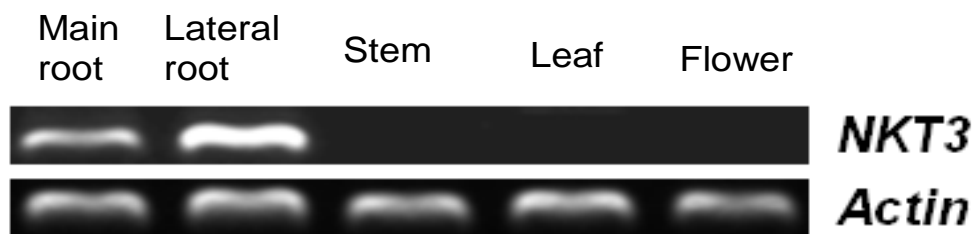
#### Analysis of spatial and temporal expression of *NKT3*

Spatial and temporal expression of *NKT3* was determined by semi-quantitative RT-PCR. Total RNA from different tissues of tobacco plants (the main roots, lateral roots, stems, leaves and flowers) under normal growth condition was isolated and the result of detection is shown in Figure 3. *NKT3* was mainly expressed in

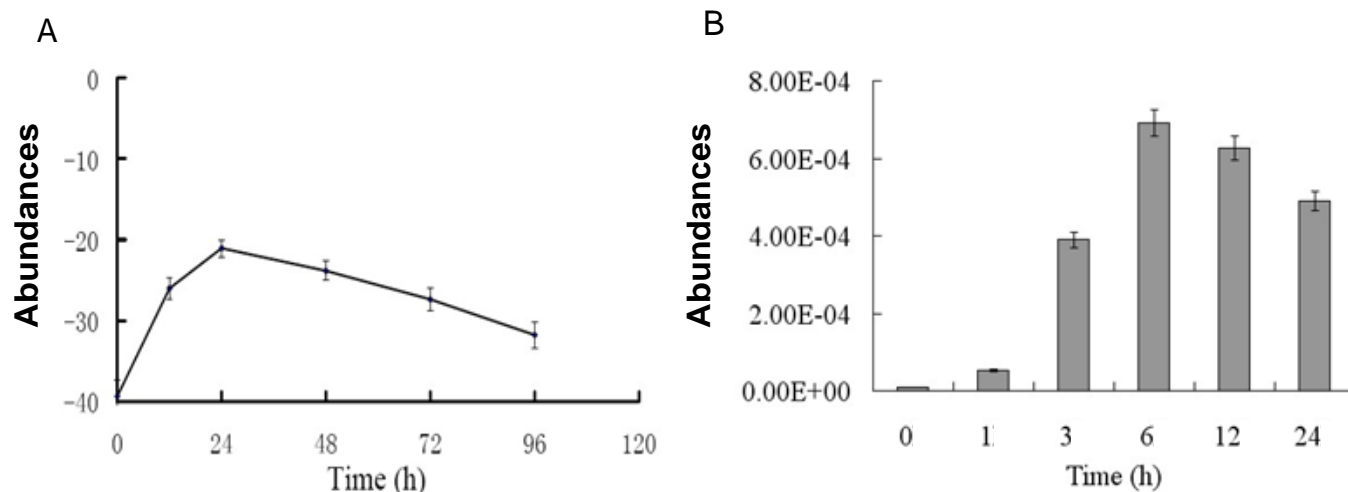
tobacco roots, and the expression level was greatly increased in lateral roots. There was no or slight expression in the leaves, stems or flowers of tobacco. The result hints that *NKT3* mRNA was mainly expressed in tobacco roots, and it might play some role in the potassium absorption of tobacco roots. Semi-quantitative RT-PCR was conducted to determine *NKT3* expression change of tobacco roots when the plants were under low- $K^+$  or drought treatment. The *NKT3* mRNA level of tobacco roots was up-regulated greatly and reached the peak at 24 h after low- $K^+$  treatment, and then declined rapidly (Figure 4A). When tobacco plants were under drought treatment, the *NKT3* mRNA level was increased



**Figure 2.** The result of protein blast and phylogenetic analysis of NKT3. (A) The three conserved domains harboured in NKT3; they are (I) the conserved domain of ion transport proteins, (II) cyclic nucleotide-monophosphate binding domain or effector domain of the CAP family, and (III) The conserved domain of voltage-dependent potassium channels, which were marked in green, red and purple, respectively. (B) The phylogenetic tree of K<sup>+</sup> channels from *N. tabacum*, Arabidopsis and related plants. *NKT3*, *NKT4* and *NKT5* from *N. tabacum* are indicated by red circles. Shaker-type K<sup>+</sup> channels from Arabidopsis are indicated by rose-red squares. The other reported K<sup>+</sup> channels from *N. tabacum* are indicated by green triangles.



**Figure 3.** Expression of *NKT3* gene in various tobacco tissues under normal growing condition. PCR products were amplified from RNA transcripts derived from the main root, lateral root, stem, leaf and flower, and separated on a 10% agarose gel. The upper lanes indicate the *NKT3* fragments amplified from RNA transcripts. The *actin* served as a control.



**Figure 4.** Expression change of *NKT3* in tobacco roots after low-K<sup>+</sup> potassium or drought treatment. (A) Bar graphs showing relative abundance of *NKT3* gene transcripts before and after low-K<sup>+</sup> treatment. Y<sub>value</sub> = log relative expression quantity. (B) Bar graphs showing relative abundance of *NKT3* gene transcripts before and after drought treatment.

and reached a maximum at 6 h, and then declined (Figure 4B). These results demonstrate that *NKT3* might be involved in potassium absorption of the tobacco roots and stomatal movement of the tobacco leaves.

## DISCUSSION

Although many K<sup>+</sup> channel proteins have been deeply characterized in *Arabidopsis*, only several genes were identified in tobacco (Sano et al., 2007). It is necessary to determine the appropriate tobacco cultivars for cloning of K<sup>+</sup> channel genes. In this experiment, a new K<sup>+</sup> channel gene was acquired from tobacco cultivar special warne, which has strong roots and well-developed lateral roots. Special warne not only possesses a higher efficiency in K<sup>+</sup> uptake, but also in K<sup>+</sup> accumulation and utilization (Niu et al., 1996). We also tried to obtain this gene in other tobacco cultivars with a lower K<sup>+</sup> uptake efficiency but failed. Therefore, we speculated that it might be easier to obtain K<sup>+</sup> channel genes in tobacco cultivars with a higher efficiency and to develop the following functional analysis.

The semi-quantitative RT-PCR results indicated that *NKT3* was expressed more strongly in the roots than in other tissues such as leaves, stems and flowers, and much higher abundance of *NKT3* mRNA was detected in the lateral root than in the main root (Figure 3). The result is consistent with *Arabidopsis AKT1* (Cao et al., 1995), carrots *KDC1* (Downey et al., 2000) and tomato *LKT1* (Hartje et al., 2000) for that the root hair is the main expression site of the genes. It hints that the gene might be expressed in a tissue-specific mode. Many genes and

its products have been specifically expressed in tobacco roots, including putrescine N-Methyltransferase gene (PMT), nicotine demethylase gene CYP82E4 family (Suzuki et al., 1999; Chakrabarti et al., 2008). And their specific expression is concerned with their biological function. Although more future confirmation is required for the *NKT3* expression, it is considered that *NKT3* might play an important role in K<sup>+</sup> acquirement and release in tobacco roots.

As is shown in Figure 2, *NKT3* is located in branch III, closely to *NtKC1* and *AtKC1*. *AtKC1* is an alpha-subunit from the *Arabidopsis* shaker-like potassium family, which mediates K<sup>+</sup> uptake of root hair. The protein is likely to be a K<sup>+</sup>-uptake modulatory subunit needed to adjust the characteristics of plant potassium uptake channels (Reintanz et al., 2002). *NtKC1* and *NKT1* function as K<sup>+</sup> uptake channels in the assay of complementation of *Escherichia coli* strain T2455, while *NKT1* represents an inwardly rectifying K<sup>+</sup> channel (Sano et al., 2007). *NKT3* might be a homolog of *AtKC1* and *NtKC1*, and participate in the potassium inwardly rectification. The mRNA level of *NKT3* increased rapidly when tobacco plants were after low-K<sup>+</sup> or drought treatment, but the mechanism of up-regulation is still unclear. Further investigation is needed to determine the expression, the contribution and the regulatory mechanism of the gene.

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