ΦYTON
REVISTA INTERNACIONAL DE BOTÁNICA EXPERIMENTAL
INTERNATIONAL JOURNAL OF EXPERIMENTAL BOTANY
FUNDACION ROMULO RAGGIO
Gaspar Campos 861, 1638 Vicente López (BA), Argentina
www.revistaphyton.fund-romuloraggio.org.ar

Ectopic expression of the *PttKN1* gene in *Cardamine hirsuta* mediated via the floral dip method

Expresión del gen *PttKN1* utilizando el método de inmersión floral en *Cardamine hirsuta*

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Abstract. *PttKN1* gene (*Populus tremula* × *P. tremuloides KNOTTED1*) was isolated from the vascular cambium of a hybrid aspen. Previous studies on transformed plants with the *PttKN1* gene suggested that it plays roles in plant development (typically in meristem initiation), maintenance and organogenesis in simple-leaved species. To investigate the gene functions further, sequence analysis of the deduced amino acid was conducted. The results suggested that the gene belongs to the class I *KNOX* gene (*KNOTTED1*-like homeobox genes) family and might play important roles in plant development by coding a transcription factor. The gene was introduced into *Cardamine hirsuta* using the floral dip method mediated via *Agrobacterium tumefaciens*. The primary transformed plants were obtained via kanamycin selection. Compared to the wild type, the kanamycin resistant plants demonstrated several morphological alterations, such as abnormal cotyledons, abnormal shoot meristem, flattened stem, and lobed and cup-shaped leaves. RT-PCR results showed that the above five types of kanamycin resistant plants expressed the same specific *PttKN1* gene band. This suggested that the morphological alterations were caused by the insertion and expression of the gene. However, these phenotypes were similar to other *PttKN1* transformed plants, despite the fact that *C. hirsuta* is a species with compound leaves and the other species have simple leaves. Therefore, the functions of the *PttKN1* gene on compound-leaf species have yet to be investigated via the comparison between related species such as *Arabidopsis thaliana* and *C. hirsuta*.

Keywords: *Cardamine hirsuta*; *PttKN1* gene; Floral dip.

Resumen. Se aisló el gen *PttKN1* (*Populus tremula* × *P. tremuloides KNOTTED1*) del cambium vascular de un álamo híbrido. Estudios previos en plantas transformadas con el gen *PttKN1* han sugerido que el mismo cumple roles en el desarrollo vegetal (habitualmente en la iniciación de meristemas), y en el mantenimiento y organogénesis de especies con hojas simples. Se condujo un análisis de la secuencia de aminoácidos deducidos para investigar aún más las funciones del gen. Los resultados sugirieron que el gen pertenece a la familia gen*KNOX* de la clase I (similares al grupo de genes *KNOTTED1*) y puede jugar roles importantes en el desarrollo vegetal codificando por un factor de transcripción. El gen fue introducido en *Cardamine hirsuta* usando el método de inmersión foliar utilizando *Agrobacterium tumefaciens*. Las plantas que se transformaron originalmente se obtuvieron por selección de kanamicina, un antibiótico aminoglucósido con propiedades antimicrobiales. Las plantas resistentes a la kanamicina mostraron varias alteraciones morfológicas, tales como cotiledones y tallos anormales, tallos achatados, y hojas lobuladas y con forma de copa. Los resultados de RT-PCR mostraron que los cinco tipos de plantas resistentes a la kanamicina expresaron la misma banda específica del gen *PttKN1*. Esto sugirió que las alteraciones morfológicas fueron causadas por la inserción y expresión del gen. Sin embargo, estos fenotipos fueron similares a otras plantas transformadas conteniendo el gen *PttKN1*, a pesar que *C. hirsuta* es una especie con hojas compuestas y las otras especies tienen hojas simples. Todavía se tienen que investigar las funciones del gen *PttKN1* en especies de hojas compuestas comparando especies relacionadas tales como *Arabidopsis thaliana* y *C. hirsuta*.

Palabras clave: *Cardamine hirsuta*; Gen *PttKN1*; Inmersión floral.

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Recibido / Received 14.XI.2013. Aceptado / Accepted 13.X.2014.

INTRODUCTION

The *PttKN1* gene (*Populus tremula* × *P. tremuloides KNOTTED1*) was isolated from the vascular cambium of a hybrid aspen (Hu et al., 2005; Meng et al., 2009a). To investigate the gene function, it was introduced into *Begonia maculata* (Xu et al., 2011), carnation (Meng et al., 2009a), cockscomb (Meng et al., 2009b), coleus (Xu et al., 2013), *Petunia hybrida* (Hu et al., 2005) and tobacco (Ding et al., 2008; Xu et al., 2015) plants via *Agrobacterium tumefaciens* transformation. Generally, the *PttKN1* gene transformed plants exhibit a series of morphological alterations like dwarfish and fascicular plants, lobed leaves, altered leaf surfaces, flattened stems, ectopic meristems, etc. (Hu et al., 2005; Ding et al., 2008; Meng et al., 2009a, 2009b; Xu et al., 2011; Xu et al., 2013; Xu et al., 2015). These morphological alterations were very similar to those described for the class I *KNOX* (*KNOTTED1*-like homeobox genes) gene transformed plants (Lincoln et al., 1994; Chuck et al., 1996). Hence, it was speculated that the gene belongs to the class I *KNOX* gene family (Hu et al., 2005), and plays roles in plant development (typically in meristem initiation), maintenance and organogenesis (Xu et al., 2011). However, the sequence alignment of the *PttKN1* gene was never conducted to make sure it's a close homolog. The relationship between the *PttKN1* gene and the other class I *KNOX* genes was therefore mainly deduced from comparative morphology studies of the transgenic plants, and it needs further confirmation. Moreover, the above all *PttKN1* gene transformed plants came from simple-leaf species. The understanding of the role of the *PttKN1* gene in compound-leaf species is complex.

Cardamine hirsuta is an annual plant native to Europe and Asia. The plant, with compound leaves, is a small crucifer related to the simple-leaf model species *Arabidopsis thaliana*. Generally, *C. hirsuta* is considered a weed and used to study the ecological distribution and evolution in biological invasion (Yatsu et al., 2003; Kudoh et al., 2007); the herbicidal activity and weed control (Dixon & Clay, 2004; Wehtje et al., 2006); the effect of global change (especially elevated atmospheric CO_2) on plant developmental processes and plant diversity (Leishman et al., 1999; Hartley et al., 2000; Springer & Ward, 2007), and the evolution of diversity in some fungi (Ploch et al., 2010). Interestingly, the mechanism for explosive seed dispersal in *C. hirsuta* was investigated, which clarified the reasons to make the plant an aggressive weed in many situations (Vaughn et al., 2011). Recently, *C. hirsuta* is emerging as a powerful system for comparative development studies because of its distinct advantages of being a diploid, self-compatible plant that can be used for genetic analyses and transformation (Hay & Tsiantis, 2006; Canales et al., 2010). Hay and Tsiantis (2006) compared the genetic differences in leaf form between *A. thaliana* and *C. hirsuta.* Thereafter, compound leaf development in *C. hirsuta* was analyzed (Barkoulas et al., 2008). The results showed that the auxin maxima promote leaflet development partly by down regulating the KNOX expression in developing leaflets (Barkoulas et al., 2008). These studies showed that KNOX proteins might direct leaflet formation in *C. hirsuta* (Hay & Tsiantis, 2006; Canales et al., 2010), and that this species could be a new model system for studying compound leaf development (Barkoulas et al., 2008; Canales et al., 2010).

In the present study, conserved domain was identified in the PttKN1 protein sequence, and then they were aligned with their homologs. Furthermore, the *PttKN1* gene was introduced into the genome of *C. hirsuta* using the floral dip method mediated via *A. tumefaciens*. The results of kanamycin selection, morphological comparison and RT-PCR analysis suggested that the foreign gene had successfully ectopic expressed in *C. hirsuta*. These analyses would enhance our understanding on the functions of the *PttKN1* gene in compound-leaf development.

MATERIALS AND METHODS

Plant material. *Cardamine hirsuta* plants with three to four true leaves were collected from the experimental field of Lanzhou University, Gansu Province, China. Then, they were planted in pots containing a mixture of vermiculite, perlite and sphagnum moss (1:1:1) to complete their life cycle and obtain seeds. The harvested seeds were sowed later under the same conditions to get more plantlets for transformation. The plantlets, at the inflorescence developmental stage, were used as experimental materials. All plants were cultured in a greenhouse under a 16 h light photoperiod with cool white fluorescent light of 24 μmol/m^2/s at 23 °C.

Plant transformation, screening and observation. *Cardamine hirsuta* plants were transformed using the floral dip method as previously described by Xu et al. (2013). The *A. tumefaciens* GV3101 strain, carrying the *PttKN1* gene and the pPCV702 plasmid, was used for the transformation (Hu et al., 2005). The *A. tumefaciens* cells were harvested and resuspended to a final OD_{600} of approximately 0.3 using 1/2 MS medium with 3% sucrose, 5 mg/L BA and 0.01% Silwet L-77. Plants with developed inflorescences and evident floral buds were dipped into the above solution for 3 min. The dipped inflorescences were covered with a black plastic bag for 24h to maintain humidity and keep them out of direct sunlight. Then, plants were grown under normal conditions, and seeds were harvested and stored.

Seeds from the wild type and dipped plants were sterilized and cultured on 1/2 MS medium with 100 mg/L kanamycin to allow germination and further selection. After 30 d, the kanamycin resistant (Km^R) seedlings were transferred to pots and grown to maturity. The morphological variations of the Km^R plants were carefully observed and photographed.

RT-PCR assay. RNA was extracted from the wild type and Km^R *C. hirsuta* plants by Trizol reagent (Invitrogen, America). RT-PCR was performed with one step RNA PCR kit (TaKaRa Biotechnology, Dalian, China) according to the manufacturer's instructions. The primers of forward 5'-CTGCTCGTCAAGAGTTTGG-3' and reverse 5'-TCTCAGGTAGTTCAGTCTCCC-3' were used to yield a 297 bp fragment. Amplification was conducted under the following conditions: one cycle of 50 °C for 30 min; one cycle of 94 °C for 2 min; 30 cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 1 min; and finally elongated at 72 °C for 5 min. RT-PCR products were electrophoresis-separated on 1% agarose gel and photographed with an Alpha Imager™ 2000 Documentation Analysis System.

Sequence alignment and phylogenetic tree construction. Sequence similarities of the protein encoded by the *PttKN1* gene were studied using the BLASTX. Alignments of amino acid sequences were performed using the DNAMAN program version 8.0. Phylogenetic trees were constructed with the neighbor-joining (NJ) method using the MEGA software version 4.0. Physical and chemical parameters of the deduced protein were determined using a ProtParam tool. Secondary structure was predicted by GOR4. Protein Localization Sites were predicted by PSORT 6.4.

Accession numbers of the sequences utilized here were as follows: KNAT1 (NP_192555), KNAT2 (NP177208), KNAT3 (X92392), KNAT4 (X92393), KNAT5 (NM119356), KNAT6 (NP173752), KNAT7 (AF308451), STM (NP_176426.1), KNATM-B (NM_001160868.1), PTS/TKD1 (EU352653.1), FCL1 (HQ695002), NTH1 (BAA76750.1), NTH9 (BAA76903.1), NTH15 (BAA25546.1), NTH20 (BAA76904.1), NTH22 (BAA76905.1), KN1 (CAA43605.1), RS1 (AAA86287), ARBORKNOX1 (AAV28488.1).

RESULTS AND DISCUSSION

***PttKN1* sequence and phylogenetic analysis.** Nucleotide sequence of the *PttKN1* gene and the deduced amino acid sequence have been reported (Meng et al., 2009a). The PttKN1 protein was also inferred to belong to the KNOX family according to the morphological comparisons between *PttKN1* and class I *KNOX* transformed plants (Hu et al., 2005; Meng et al., 2009a). Then, it was thought that the possible functional domains of the PttKN1 protein were the same as in the other KNOX proteins (Meng et al., 2009a). However, it lacks mapping to establish the association between the sequence and the conserved domain. Moreover, further analysis is still necessary on the PttKN1 sequence to make sure of its homologs for a better understanding on its functions.

The sequence was analyzed with the ProtParam program. The physical and chemical parameters of the PttKN1 protein were: molecular weight=42.45 kDa; theoretical isoelectric point (pI)=6.03; estimated half-life=30 h; instability index=51.22, and it was classified as an unstable protein. The protein had no transmembrane and signal peptide, and was a soluble protein (SignalP, TMHMM and ProtScale programs). The predicted protein localization sites showed that the PttKN1 protein could be a nucleus protein with 70% certainty. Further prediction of secondary structure showed that the PttKN1 protein consisted of a 41.03% alpha helix, 10.60% extended strand, and 48.37% random coil. Conserved domain search and sequence alignment revealed that this protein has all the typical features of a class I KNOX protein, namely a MEINOX domain, a GSE box, an ELK domain and a Homeodomain (Fig. 1A) (Bürglin, 1998; Nagasaki et al., 2001). The phylogenetic tree was constructed according to the neighbor-joining method. Results showed that the members of the KNOX family are grouped into three classes, namely class I, class II and class M KNOX (Kerstetter et al., 1994; Magnani & Hake, 2008). Comparison of amino acid sequences indicated that the PttKN1 protein shares a high sequence identity with the tobacco NTH20 (69.05%) and *Arabidopsis* KNAT1 (62.31%). Phylogenetic tree analysis indicated that PttKN1 is grouped with class I KNOX proteins (Fig. 1B). These data suggested that the *PttKN1* gene could play roles in plant development by coding transcription factor as other class I *KNOX* genes typically do in meristem initiation, maintenance and organogenesis (Xu et al., 2011).

Morphological alterations of the transgenic *C. hirsuta*. To investigate the gene function, *C. hirsuta* was transformed with the *PttKN1* gene using the floral dip method mediated via *A. tumefaciens*. The plants with well-developed inflorescences and evident immature floral buds were used to achieve a high-frequency transformation. These plants were inoculated for 3 min with the prepared *A. tumefaciens*. About a month after the floral dipping, seeds were harvested and selected on 1/2 MS medium containing 100 mg/L kanamycin. Morphological alterations of Km^R seedlings were compared with morphological characteristics on seedlings coming from their wild type plant.

Generally, wild type *C. hirsuta* seedlings have two symmetrical cotyledons with smooth margin (Fig. 2A) and one round hypocotyl (Fig. 2E). While some Km^R seedlings showed distorted, lobed- or multi-cotyledons (Fig. 2B-D), twin-seedlings showed two hypocotyls shared by the same root (Fig. 2F). Furthermore, Km^R plants displayed several morphological alterations compared to the wild type. On wild type plants there was one main inflorescence and compound leaves which consisted of round leaflets arranged alternately along the central leaf stem (Fig. 2G). Morphological alterations on Km^R phenotypes included plants with two apical buds (Fig. 2H) and multi-inflorescences (Fig. 2I), clustered plants (Fig. 2J), ectopic bud formation (Fig. 2K),

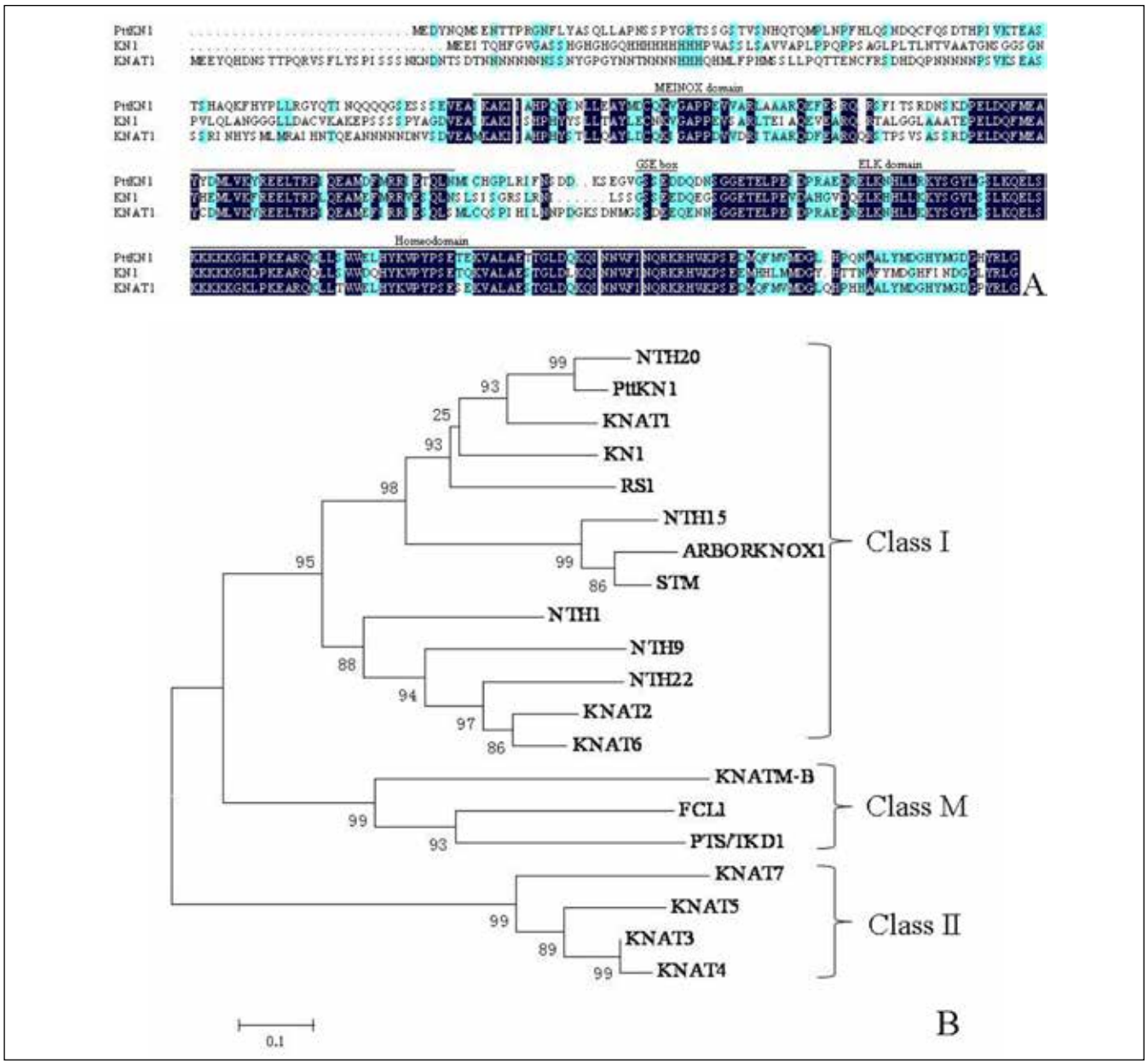

Fig. 1. Sequence alignment and phylogenetic tree of the PttKN1 protein and selected relatives from other plant species. (A) Alignment of the deduced amino acid sequences of PttKN1, KN1 and KNAT1. Identical and conserved amino acid residues are boxed. The different functional domains are indicated by black lines above the corresponding sequences. (B) Phylogenetic tree of PttKN1 and its relative KNOX family. Bar means 0.1 amino acid substitutions per residue.

Fig. 1. Secuencia de alineamiento y árbol filogenético de la proteína PttKN1 y parentescos seleccionados de otras especies vegetales. (A) Alineamiento de la secuencia de aminoácidos deducida de PttKN1, KN1 y KNAT1. Residuos de aminoácidos idénticos y conservados se muestran como cajas. Los dominios funcionales diferentes se indican como líneas negras por encima de las secuencias correspondientes. (B) Arbol filogenético de PttKN1 y su familia de parientes KNOX. La barra indica 0,1 sustituciones de aminoácidos por residuo.

flattened stem (Fig. 2L), lobed leaflets (Fig. 2M) and cup-shaped leaves (Fig. 2N). Interestingly, silique of the Km^R plants was short and wide (Fig. 2P) compared to the long and narrow silique of the wild type (Fig. 2O). Meanwhile, it displayed altered seed arrangement and a reduced number of seeds.

In the *PttKN1* transformed plants, morphological alterations of abnormal phyllotaxy (Xu et al., 2011; Xu et al., 2013) and ectopic shoots are well reported and discussed (Hu et al., 2005; Xu et al., 2011). They were both thought related to the reduced apical dominance caused via the strong local meristematic activity, which was one of the typical characters of

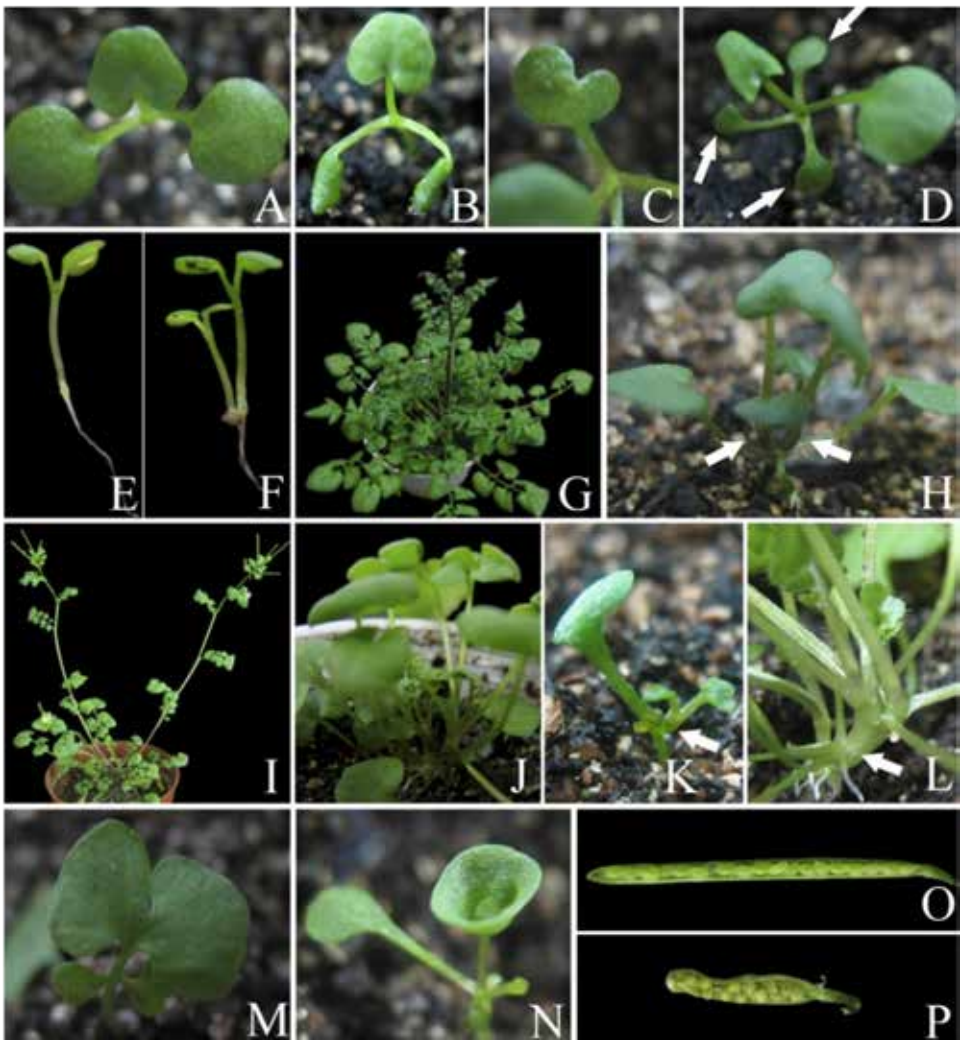

Fig 2. Effect of *PttKN1* overexpression on *C. hirsuta* development. (A) cotyledons of wild type. (B-D) cotyledons of transgenic seedlings; B: distorted cotyledons; C: lobed cotyledon; D: multi-cotyledons (arrows). (E) wild type seedling; (F) transgenic twin-seedling. (G) wild type plant; (H-N) transgenic plants; H: plant with two apical buds (arrows); I: plant with multi-inflorescences; J: clustered plant; K: ectopic bud formation (arrow); L: flattened stem (arrow); M: lobed leaf; N: cup-shaped leaf. (O) silique of wild type; (P) silique of transgenic plant.
Fig. 2. Efecto de la sobreexpresion de *PttKN1* en el desarrollo de *C. hirsuta*. (A) cotiledones del tipo silvestre. (B-D) cotiledones de plántulas transgénicas; B: cotiledones distorsionados; C: cotiledón lobulado; D: multicotiledones (flechas). (E) plántula de tipo silvestre; (F) plántula melliza transgénica. (G) planta de tipo silvestre; (H-N) plantas transgénicas; H: planta con dos yemas apicales (flechas); I: planta con multi-inflorescencias; J: planta agrupada; K: formación de yema ectópica (flecha); L: tallo achatado (flecha); M: hoja lobulada; N: hoja con forma de copa. (O) silicua de planta silvestre; (P) silicua de planta transgénica.

the *PttKN1* transformed plants. Moreover, lobed- and cup-shaped leaves were also typical in the *PttKN1* transformants (Xu et al., 2013). In transgenic *C. hirsuta*, lobes were observed on apical and lateral leaflets; and sometimes, supernumerary leaflets were also initiated, which showed a limited increase in leaf complexity. *PttKN1*/ class I *KNOX* was sufficient to reactivate the differential ability of leaflets (Hay & Tsiantis, 2006). To our knowledge, it had been confirmed that the lobes or ectopic leaf formation were involves meristematic activities in simple-leaved species (Chuck et al., 1996). However, it was thought unlikely the redeployment of a full SAM program, but modulation of cell proliferation underpins leaflet formation in *C. hirsuta* (Barkoulas et al., 2008). The reported data suggested that polar localization of PIN1 protein might facilitate lateral leaflet formation in *C. hirsuta* (Barkoulas et al., 2008). Therefore, increased leaf complexity in *PttKN1* transgenic *C. hirsuta* might be induced via the altered endogenous auxin level. Frequently, class I *KNOX* and *PttKN1* transformants displayed increased cytokinin levels (Tamaoki et al., 1997; Yanai et al., 2005; Xu et al., 2015). Nevertheless, the way that cytokinin biosynthesis promotes leaflet formation in *C. hirsuta* is unknown (Canales et al., 2010).

Cup-shaped leaves were also reported in *PttKN1* transgenic *C. hirsuta*, *B. maculata* and tobacco (Ding et al., 2008; Xu et al., 2013; Xu et al., 2015). This morphological alteration was thought as a typical *LePHAN* (PHANTASTICA) down regulation phenotype (Kim et al., 2003a), which encodes MYB transcription factor. Therefore, it might be that the antagonistic expression pattern of strong *PttKN1/* class I *KNOX* overexpression causes *PHAN* down regulation (Kim et al., 2003a), reducing the adaxial domain of the leaf primordia (Kim et al., 2003b) for determining the final morphology of the cup-shaped leaf. In fact, *PttKN1* transgenic *B. maculata* had also shown needle-like leaves (Xu et al., 2011), which may also form as the same matter. Moreover, the organ boundary gene cup-shaped cotyledon (*cuc*) that encodes NAC-domain proteins could define the distal boundary of leaflets (Aida et al., 1997) and is required for leaflet formation in compound-leaved species (Blein et al., 2008; Berger et al., 2009). Several evidences suggested that CUC activity promotes KNOX expression also functions upstream of it during SAM formation and lateral organ boundary development (Takada & Tasaka, 2002; Blein et al., 2008). Therefore, the formation of cup-shaped leaves in *PttKN1* transgenic *C. hirsuta* was the alteration of the adaxial cell fates that were established and maintained via the overexpressed *PttKN1* and other key factors.

Identification of transgenic lines. In order to confirm the successful genetic transformation, RNA were isolated from the leaves of both the wild type and different Km^R plants (includes plants with lobed cotyledons, multi apical buds, cup-shaped leaves, lobed leaves or flattened stems). The non-transformed wild type plant was used as control. As shown in Figure 3, no signal was detected for the wild type; at the same time, one specific band about 300bp indicated that the foreign *PttKN1* gene had integrated and ectopic expressed into the genome of transgenic *C. hirsuta*. The results of kanamycin selection, phenotype observation and RT-PCR analysis identified that the foreign *PttKN1* gene had successfully integrated and expressed into the transgenic *C. hirsuta* plant.

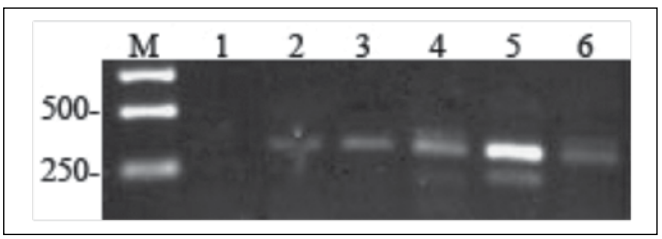

Fig. 3. RT-PCR analysis of *PttKN1* gene expression. M-marker; lane 1- wild type plant as control; lane 2- lobed cotyledon; lane 3- multi apical buds; lane 4- cup-shaped leaf; lane 5- lobed leaf; lane 6- flattened stem. The 300bp indicates *PttKN1*-specific bands.
Fig. 3. Análisis RT-PCR de la expresión del gen *PttKN1*. M-marcador; carril 1- planta silvestre (control); carril 2- cotiledón lobulado; carril 3- yemas multiapicales; carril 4- hoja con forma de copa; carril 5- hoja lobulada; carril 6- tallo achatado. Las bandas específicas de *PttKN1* se indican como 300bp.

***C. hirsuta* as a model system for studying class I KNOX functions in compound leaf development.** It was well reported that morphological alterations of the *PttKN1* transformants from simple-leaved plants were mainly focused on altered leaf arrangement and morphology (e.g., lobed-, cup-shaped and distorted leaves) (Xu et al., 2013). Notably, the transgenic *C. hirsuta* plants showed similar phenotypes with other *PttKN1* transformants despite the fact that *C. hirsuta* is a compound-leaved species while others are simple-leaved species. This demonstrated that the *PttKN1*/class I *KNOX* over-expression in *C. hirsuta* does not increase leaf complexity and indeterminacy remarkably as in tomato (Efroni et al., 2010). Generally, tomato and pea were known as a reference system for the studies of compound leaf development (Efroni et al., 2010). Recently, *C. hirsuta* was emerged as a new model system for that (Hay & Tsiantis, 2006; Barkoulas et al., 2008; Canales et al., 2010; Efroni et al., 2010) because of its close relation to *Arabidopsis*. It was thought that the comparisons between *C. hirsuta*, tomato and pea would provide an outline on compound leaves formation. Furthermore, comparisons between related species such as *A. thaliana* and *C. hirsuta* would provide valuable data.

Taken together, the analysis of nucleotide sequence of the *PttKN1* gene and its deduced amino acid sequence suggested that the gene belongs to the class I *KNOX* gene family. The gene could play roles in plant development by coding transcription factor as other class I *KNOX* genes typically in meristem initiation, maintenance, and organogenesis. Then, the *PttKN1* gene was introduced into *C. hirsuta* via floral dip method mediated by *A. tumefaciens* to investigate its function on the development of compound leaves. The transgenic plants displayed several morphological alterations like ectopic shoots, and lobed- and cup-shaped leaves. This was similar to other *PttKN1* transformants despite the fact that *C. hirsuta* is a compound-leaved species while others are simple-leaved species. These morphological alterations suggested the pleiotropic roles of the *PttKN1* gene as a morphological regulator. However, its roles on compound leaves have yet to be investigated via the comparisons between related species such as *A. thaliana* and *C. hirsuta*, and among other compound-leaved species.

ACKNOWLEDGEMENTS

This study was supported by the National Natural Science Foundation (31401910) and the Fundamental Research Funds for the Central Universities (2014YB040), P.R. China.

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