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Transport of mRNA molecules coding NAC domain protein in grafted pear and transgenic tobacco

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Abstract

Grafting is an important cultivation method and recent research on the mechanism of interactions between rootstock and scion is focused on the long-distance transport of mRNA and small RNAs in the phloem. Among these transportable molecules, *NACP* gene coding NAM, ATAF1/2, CUC2 (NAC) domain protein might be involved in apical meristem development. Here, we report the transport of *NACP* mRNA between Chinese pear (*Pyrus bretschneideri*) cv. Yali (scion) and the wild *Pyrus betulaefolia* Bunge (rootstock). Our results indicated that *NACP* mRNA can be transported in both directions from the 3rd to 10th day after micro-grafting. It can also be transported to the shoot apex 30 to 70 cm away from graft-union in 2-year-old grafted trees. For further investigation, transgenic tobaccos with 35S: *P. betulaefolia-NACP* construct were grafted on wild-type tobaccos (*Nicotiana tabacum* L. cv. Samsun). The sustainable transport of *Pyrus-NACP* mRNA through the graft-union occurred from the 15th day after grafting.

Additional key words: *Pyrus betulaefolia*, *Pyrus bretschneideri, NACP* mRNA, *Nicotiana tabacum*, rootstock, RT-PCR, scion.

Introduction

Grafting is important method for growing perennial fruit trees. Rootstocks confer support and adaptability for scion cultivars in different soil and climatic environments, it also endow advantageous properties for the scions, such as dwarfing, desired physiological characteristics, and early fertility, so as to improve fruit quality and increase economic benefits (Soumelidou *et al*. 1994, Kamboj *et al*. 1999a,b, Jensen *et al*. 2003, 2010). Despite the long history of grafting, the detailed mechanisms of controlling the growth and development of the scion by the rootstock remain to be determined.

In recent years, some specific mRNAs and proteins have shown capability of long-distance transport *via* phloem (Lucas 1995, Ruiz-Medrano *et al*. 1999, Xoconostle-Cázares *et al*. 1999, Kim *et al*. 2001, Haywood *et al*. 2005, Banerjee *et al*. 2006, 2009, Kudo and Harada 2007, Huang and Yu 2009). Among these transportable mRNAs, RNA designated as *NACP* (containing a conserved NAC domain for NAM, ATAF1/2, and CUC2; Aida *et al*. 1997) belongs to a gene family potentially involved in apical meristem development. *NACP* transcripts had been detected in companion cells and sieve elements of leaf, stem, and root, and its long-distance transport was shown using grafting experiments (Ruiz-Medrano *et al*. 1999).

So far, the only studies of the long-distance transport of mRNA in woody fruit trees are those by Kanehira *et al*. (2009), Xu *et al*. (2010), and Zhang *et al*. (2011) which reported that *AUX/IAA14* and *GAI* transcripts of apple and pear can be transported between rootstock and scion *via* the graft-union. To select transportable mRNAs, *Pyrus-NACP* (member of NAC domain protein) has been cloned. Here we investigate transport of *Pyrus-NACP* transcripts in tissue cultured *Pyrus bretschneideri* - *Pyrus*

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Abbreviations: CAPS - cleaved amplified polymorphic sequences; GAI - gibberellic acid insensitive; ISH - *in situ* hybridization; NACP - NAM,ATAF1/2,CUC2 domain protein; NCAP - non-cell autonomous pathway; RT-PCR - reverse trascriptase - polymerase chain reaction.
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betulaefolia grafts and two-year-old grafted trees. Moreover, 35S:*P. betulaefolia-NACP* cDNA transgenic tobacco was grafted on wild-type tobacco and transport of *Pyrus-NACP* mRNA was followed. It was expected that long-distance transport of mRNA through graft-union will create an innovative approach for fruit trees breeding in the near future.

Materials and methods

Pears (*Pyrus betulaefolia* Bunce and *P. bretschneideri* Yali) were cultured in Murashige and Skoog (1962; MS) medium containing 0.5 mg dm^{-3} benzylaminopurine (BA) and 0.2 mg dm^{-3} indoleacetic acid (IAA). Tobacco (*Nicotiana tabacum* L.) was cultured in MS medium without hormones. The culture conditions were following: day/night temperature of 25/23 °C, a 14-h photoperiod, irradiance of 80 - 100 μmol m^{-2} s^{-1}, and relative humidity of 85 %.

Micro-grafting *P. bretschneideri* (scion) on *Pyrus betulaefolia* (rootstock) was conducted according to Zhang *et al*. (2011). Tissue samples were collected from ten individual plants at each time point from the 1st to 10th day after grafting (Fig. 1*A*,*B*). Ten individual samples were mixed for analysis. Ungrafted *P. betulaefolia* and *P. bretschneideri* were used as negative control, the mixture of *P. betulaefolia* and *P. bretschneideri NACP* cDNA were used as positive control. The heights of three two-year-old grafted trees were 70, 70, and 40 cm, respectively. Tissue from scion containing phloem and leaves was sampled every 10 cm along the scion stem (apart from the graft-union) (Fig. 1*E*).

Tobacco micro-grafting experiment was performed exactly as described by Mallory *et al*. (2001). New leaves appeared on the 5th day after grafting in the scion apex (Fig. 1*C*,*D*), the samples from ten grafts per line were harvested on the 5th, 15th, 25th, and 35th day after grafting. Grafts combinations were 35s:*P. betulaefolia NACP* - wild type (WT) and WT - 35s:*P. betulaefolia NACP*, and control grafts (including 35s:pBI 121-35s: pBI 121,

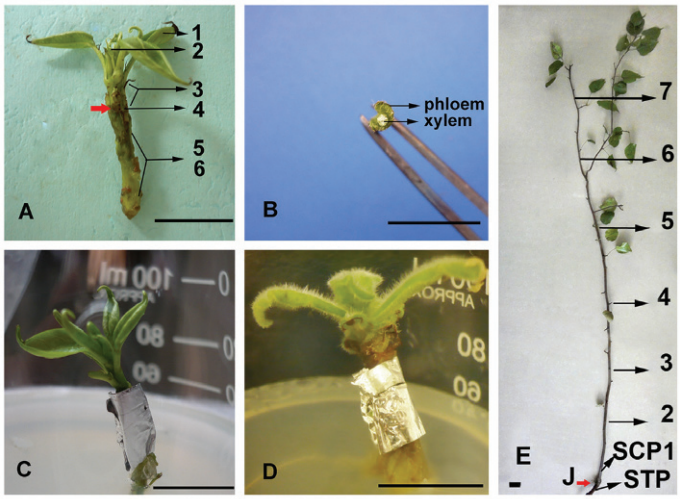

Fig. 1. Heterografting of *Pyrus* and tobacco. *A* - *Pyrus bretschneideri* scion on *Pyrus betulaefolia* rootstock and sampled tissues: 1 - leaf of scion, 2 - shoot tip of scion, 3 - phloem tissue from scion stem, 4 - graft-union, 5 - phloem tissue from rootstock stem, 6 - xylem tissue from rootstock stem. *B* - Separation of inner tissue (xylem) and outer tissue (phloem) of the scion stem. *C* - Micro-grafted *Pyrus*. *D* - Micro-grafted tobacco. *Scale bars* = 1 cm. *E* - Two-year-old grafted tree (*P. betulaefolia*.as the rootstock and *P. bretschneideri* as the scion) was sampled every 10 cm apart from the graft-union (1 - 7): SCP - phloem tissue from scion stem, J - graft-union, STP - phloem tissue from rootstock. *Scale bar* = 1 cm. *Red arrows* in panel *A* and *E* indicate the graft-union.

Table 1. Primer sequences used in the current study.

Primers	Sequence(5'-3') - forward	Sequence(5'-3') - reverse
NACP	ATGGAAAATACTTCTGGGTTT	GAGATCAATAATTCCAGAGGC
NACPS	ATAAGGAGATTTACAAGGCA	TTGAGATCAATAATTCCAGA
Actin1	CATACATGGCAGGCACATTG	ATTGGAATGGAAGCTGCTGG
XBANACP	GCTCTAGAATGGAAAATACTTCTG	GTCATCGCTCACTGAGAGCTCG
NPTII	CGGCTATGACTGGGCACAACAGACAAT	AGCGGCGATACCGTAAAGCACGAGGAA
NACPprobe	CGAAGCTTAATGAGTGGGTGATTTGTAG	CGTCTAGATCCTGTGGGAACTCTATTTT

WT-WT, and 35s:*P. betulaefolia NACP*-35s:*P. betulaefolia NACP*).

Total RNA was extracted from tissues of grafts using the cetyltrimethylammonium bromide (CTAB) method as described by Chang *et al*. (1993) and then treated with RNase-free DNase I (*Takara*, Dalian, China). cDNA synthesis was performed with *M-MLV* (*Promega*, Madison, WI, USA).

Cleaved amplified polymorphic sequences (CAPS) of reverse trascriptase - polymerase chain reaction (RT-PCR) products were amplified with the forward and reverse primers NACPSF and NACPSR (Table 1) under the follow condition: initial denaturation at 94 °C for 1 min followed by 35 cycles of 94 °C for 30 s, 60 °C for 30 s and 72 °C for 1 min, and a final extension at 72 °C for 7 min, then they were digested with *ScrF*I (*Takara*) and separated on 3 % (m/v) agarose gel.

Specific forward and reverse primers (NACPF and NACPR), designed according to *BLASTx* analysis, were used for *Pyrus-NACP* gene cloning and primers XBANACPF and SNANACPR were used for 35s: *P. betulaefolia-NACP* cDNA construction. PCR conditions were as follows: initial denaturation at 94 °C for 1 min followed by 35 cycles of 94 °C for 30 s, 56 °C for 30 s and 72 °C for 2 min, and a final extension at 72 °C for 10 min. Amplified *P. betulaefolia-NACP* cDNA sequences were purified and subcloned into the PMD18-T simple vector (*Takara*, Dalian, China). T vector containing the *P. betulaefolia-NACP* cDNA was digested with *Xba*I and *SnaB*I and then inserted into the same sites of transformation vector pBI 121 (Genbank ID: AF485783) which contained 35S CaMV promoter, resistant tag *neomycin phosphotransferase* II (*NPT*II), and a *nopaline synthase (NOS)* 3' transcriptional terminator sequence without *β-glucuronidase* (*GUS*) gene.

For tobacco transformation, disks of plant tissue cut from tobacco leaves were inoculated with *Agrobacterium tumefaciens* L. strain EHA105 containing the plasmid 35S: *P.betulaefolia*-NACP on MS medium with 500 mg dm^{-3} cefalotin, 0.5 mg dm^{-3} IAA, and 2.0 mg dm^{-3} zeatin. After 48 h in darkness, the explants were transferred to screening MS medium containing 500 mg dm^{-3} cefalotin and 100 mg dm^{-3} kanamycin, and further transferred to MS medium containing 2.0 mg dm^{-3} indolebutyric acid to promote root development.

For *in situ* hybridization (ISH), digoxigenin-labeled antisense and sense RNA probes were made using a digoxygenin RNA labeling kit according to the manufacturer's instructions (*Roche Applied Science*, Penzberg, Germany). The 492-bp fragment containing a specific sequence region was amplified using RT-PCR with the primers NACPprobeF and NACPprobeR (Table 1) under the following conditions: initial denaturation at 94 °C for 1 min followed by 35 cycles of 94 °C for 30 s, 55 °C for 30 s, 72 °C for 1 min, and a final extension at 72 °C for 7 min. Hybridization and immunological detection of the hybridized probes were conducted according to Banerjee *et al*. (2006). The hybridization signals were observed and recorded by light microscopy (*BX51*, *Olympus*, Tokyo, Japan). The tissue cultured *P. betulaefolia* stem (5-cm long) was fixed in 4 % (m/v) paraformaldehyde at 4 °C for 15 h and then dehydrated using ethanol gradient series containing 30, 50, 70, 85 and 95 % ethanol. The materials were embedded in *Paraplast Plus* (*Sherwood Medical*, St. Louis, MO, USA). Ten-micrometer slices were cut with microtome and mounted on glass slides.

Results

P. bretschneideri and *P. betulaefolia NACP* genes contained 1 259 bp with two introns (111 bp and 96 bp) and encoded 350 amino acids. By *BLAST* (http://www.ncbi.nlm.nih.gov), both amino acid sequences displayed 80 % homology with *CmNACP* (Genbank ID:ACI01723.1) and 98 % with *MdNACP* (Genbank ID: ADL36806.1), and exhibited obvious characteristics of five conserved sequence domains of given NAC family. Phylogenetic tree of the *Pyrus-NACP* transcripts with other 16 NAC family members drawn with *DNAMAN* (*Lynnon Biosoft*, Vaudreuil, Canada), displayed the high levels of homology with *CmNACP* (Genbank ID:ACI01723.1), *MdNACP* (Genbank ID: ADL36806.1), *CUC1/2* (GenBank ID: ACB31149.1, AB002560), and *PhNAM* (GenBank ID: AAM47025.1). Therefore, it was confirmed that *P. betulaefolia* and *P. bretschneideri NACP* genes belonged to NAC domain protein gene familiy, so they were named as *DL-NACP* (GenBank ID: JF803738) and *YL-NACP* (GenBank ID: JF803737), respectively. They share 98 % identity except for ten single nucleotide polymorphisms (SNPs) in cDNA sequence.

A CAPS marker was developed to discriminate DL-*NACP* and YL-*NACP* using the restriction enzyme *ScrF*I. As predicted in sequence analysis, a 752 bp RT-PCR product amplified from DL-*NACP (P. betulaefolia)* was cleaved into three fragments of 41, 302 and 409 bp whereas that from YL-*NACP* (*P. bretschneideri*) was cleaved into two fragments of 41 and 711 bp. All tissues of rootstock and scion exhibited unique restriction band patterns before the 2nd day after grafting. Nevertheless, from the 3rd to 10th day, both rootstock and scion had incorporated fragments from its grafting partner except for the xylem (Fig. 2). This result indicated that in micro-grafts of tissue cultured *P. betulaefolia* and *P. bretschneideri*, the *Pyrus-NACP* was endogenous mRNA which can be simultaneously transported from rootstock to scion and from scion to

rootstock in 3rd - 10th day after grafting. For grafted pear trees, in the first 70 cm of two-year-old tree (Fig. 1*E*), DL-*NACP* (*P. betulaefolia*) mRNA was able to transport to phloem and leaves of the scion (there were no leaves present in 20 cm) in the distance of 50 - 70 cm away from the graft-union. Likewise, YL-*NACP* (*P. bretschneideri*) mRNA was also able to transport to the rootstocks. In the second tree with the same height, DL-*NACP* mRNA was able to transport to phloem 30 - 70 cm away and to leaves of the scion 30 - 50 cm away from the graft-union (there were no leaves present in 20 cm) but YL-*NACP* mRNA could not transport to the rootstock. In the third 40 cm one, DL-*NACP* mRNA was able to transport to phloem 10 - 40 cm and to leaves 20 - 40 cm away from the graft-union (there were no leaves present in 10 cm), however, YL-*NACP* mRNA cannot transport to the rootstock (Fig. 3).

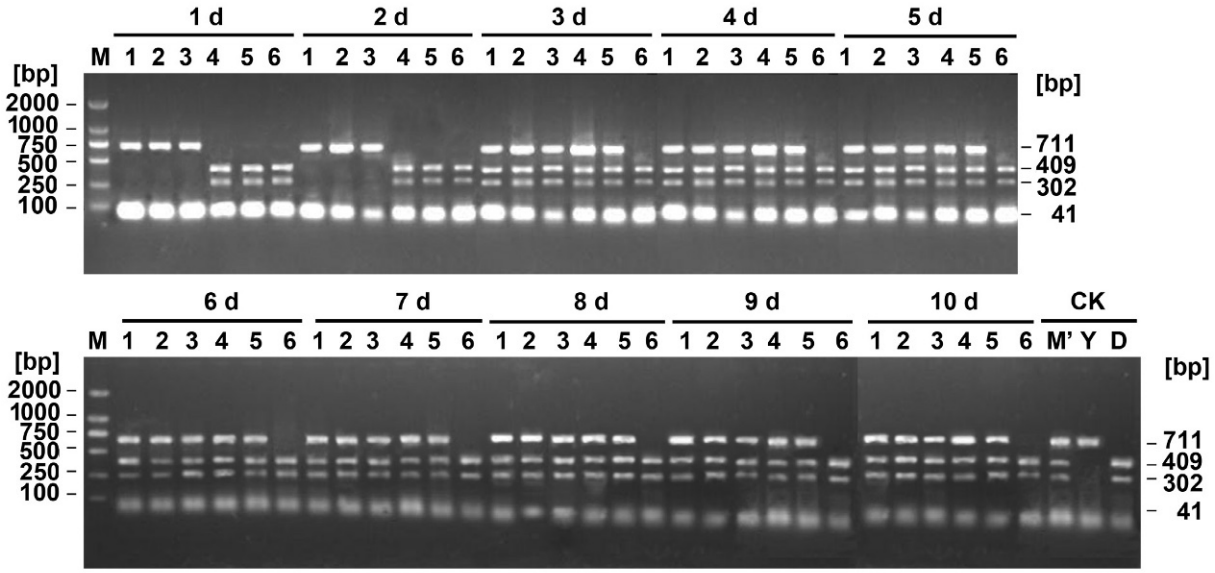

Fig. 2. Cleaved amplified polymorphic sequences (CAPS) analysis of RT-PCR products from micro-grafting of *Pyrus* sampled from the 1st to 10th day after grafting. Scion (*Pyrus bretschneideri*): 1 - leaf of scion, 2 - shoot tip of scion, 3 - phloem tissue from scion stem, 4 - graft-union. Rootstock (*Pyrus betulaefolia*): 5 - phloem tissue from rootstock stem, 6 - xylem tissue from rootstock stem. *Abbreviations*: CK - control, D - ungrafted *Pyrus betulaefolia,* M' - mixture of cDNA from *P. bretschneideri* and *P. betulaefolia,* Y - ungrafted *P. bretschneideri*. M - DL2000 DNA ladder (*Takara*).

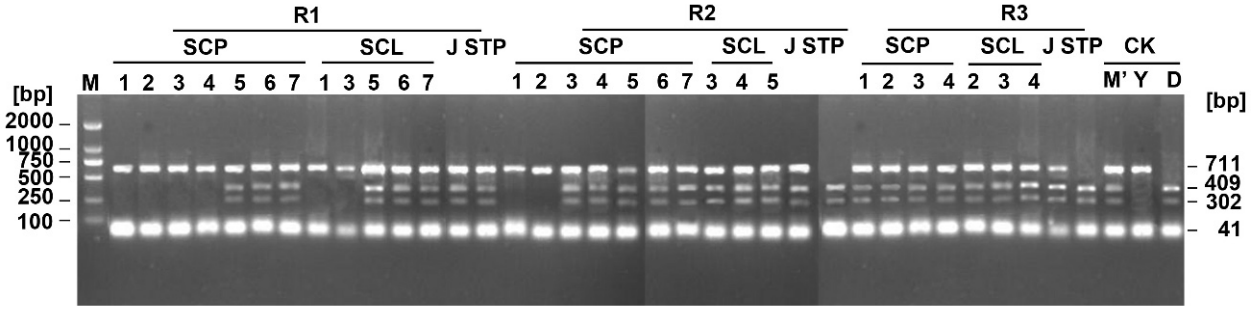

Fig. 3. CAPS analysis of RT-PCR products from heterografting of three 2-year-old trees (R1, R2, and R3) *Abbreviations*: M - DL2000 DNA ladder, CK - control, D - ungrafted *Pyrus betulaefolia,* M' - mix of cDNA from *P. bretschneideri* and *P. betulaefolia*, Y - ungrafted *P. bretschneideri*, SCL - scion leaf, SCP - phloem tissue from scion stem, J - graft-union, STP - phloem tissue from rootstock, 1 to 7 - sampled every 10 cm apart from the graft-union.

ISH was performed on transverse sections of *P. betulaefolia* to identify the exact location of DL-*NACP* transcripts. From the cell size and array pattern, it was possible to identify the phloem which was located near the stem pith and xylem (Fig. 4*A*). DL-*NACP* mRNA was detected in phloem with an antisense probe (Fig. 4*C*,*D*); however, no signal was recorded either in the xylem or when a sense probe was used (Fig. 4*B*).

Six lines of the transgenic tobaccos were identified by RT-PCR with specific primer pairs NACPF/NACPR and NPTIIF/NPTIIR (Fig. 5*A*). In order to detect DL-*NACP* mRNA transport in upward direction, DL-*NACP* cDNA transgenic tobacco lines used as the rootstock were grafted with WT. *Pyrus-NACP* RT-PCR indicated that

DL-*NACP* mRNA was able to transport from rootstock to scion on the 15th day after grafting (Fig. 5*B*). When DL-*NACP* cDNA transgenic tobaccos were used as scion, DL-*NACP* mRNA could also move from scion to rootstock at the same time (Fig. 5*B*). However, *DL-NACP* mRNA transport in one line was not detected on the 25th and 35th day. The result indicated that the transport of *Pyrus-NACP* mRNA was sustainable in both directions. Control grafts were included to demonstrate that the manipulation of plant tissues did not induce gene expression. As anticipated, the *DL-NACP* was not detected in the scion leaves of WT/WT and pBI 121/pBI 121 grafts but was amplified in the 35S:DL-NACP/35S: DL-NACP graft. All these results lead to a conclusion that *Pyrus-NACP* can transport within a transgenic graft system in both directions.

Discussion

Grafting is a cultivation method that is used for acquiring excellent characteristics (Kamboj *et al*. 1999b). Phenotype, fruit production, and qualities can be changed when the same scion is grafted onto different rootstocks (Smaka *et al*. 2010). Therefore, it is of great interest to study the complicated interactions between the rootstock and the scion of fruit trees for enhancing resistance, increasing crop yield, and improving fruit quality.

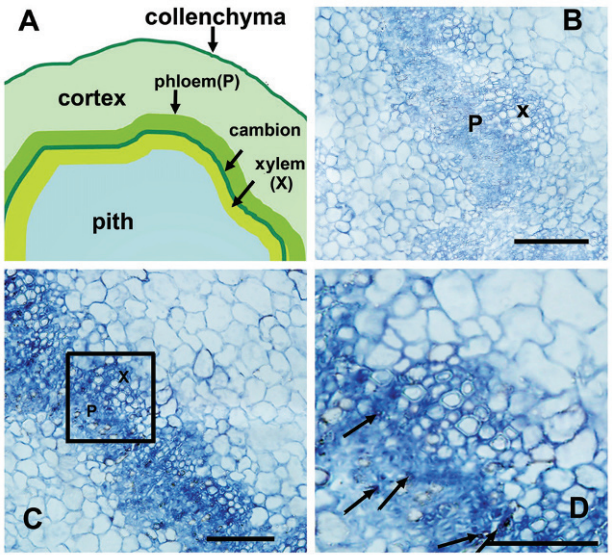

Fig. 4. *In situ* hybridization of transverse sections of *Pyrus betulaefolia* stem. Sections were hybridized with a digoxigenin-labeled 495-base RNA copy of a sequence from *DL-NACP*. *A* - Schematic transverse section of a *Pyrus* stem. Phloem was located near the stem pith and xylem. *B* - Sense probe. *C*, *D* -Antisense probe. The *boxed area* in *C* is enlarged in *D*. The *arrows* in *D* indicate a positive DL-NACP signal. *Abbreviations*: P - phloem, X - xylem. *Scale bars* = 50 μm.

Recent studies have suggested that certain mRNAs, small RNAs, move through the plant vascular system based on the non-cell autonomous pathway (NCAP) mechanism modulating plant development and morphogenesis (Lucas *et al*. 1993, Goodenough *et al*. 1996, Gurdon *et al*. 1998, Clark *et al*. 2001, Kragler *et al*. 2001, Gómez *et al*. 2005, Schmitt *et al*. 2008, Ham *et al*. 2009). *GAI* mRNA was transported into strong sinks and accumulated in developing organs in tomatoes and *Pyrus* (Haywood *et al*. 2005, Zhang *et al*. 2011); whereas in heterograft of pumpkin and cucumber, *CmNACP* transcript encoding NAC domain protein was present in meristems and floral organs (Xoconostle-Cázares *et al.* 1999, Ruiz-Medrano *et al.* 1999).

In our study, we demonstrated that innate *Pyrus-NACP* mRNA serving as a long-distance signal is transported from 3rd to 10th day after micro-grafting of *P. bretschneideri* on *P. betulaefolia* (Figs. 1,2,3). *In situ* hybridization experiments proved that phloem was a specialized vascular tissue facilitating transfer of *Pyrus-NACP* mRNA from source to sink tissues (Fig. 4). This results are similar as those reported by Ruiz-Medrano *et al*. (1999). In addition, *Pyrus-NACP* mRNA of rootstock was present in scion stem and leaves at 30 - 70 cm from the graft-union in two-year-old trees (Fig. 3). This suggested that *Pyrus-NACP* mRNA was predominantly transported and accumulated in vegetative and floral apices, similar to *NAM, CUC2*, and *NAP* in *Petunia* and *Arabidopsis* (Souer *et al*. 1996, Aida *et al*. 1997, Sablowski and Meyerowitz 1998). Owing to different growth status, development of tissues, and structures of single plant, *Pyrus-NACP* mRNA was able to move to different heights in three different trees. Although the studies reported that *CmNACP* transport occurred in grafted pumpkin as rootstock and cucumber as scion through SE-CC of phloem (Ruiz-Medrano *et al*. 1999), there was no study on whether *NACP* mRNA can be transported as transgenic tobacco. In our study, when the transgenic tobacco was used as rootstock grafted with WT (Fig. 5*A*), DL-*NACP* mRNA was transported from rootstock to scion. Moreover, undetected DL-*NACP* mRNA was in one line using transgenic scion grafted to WT rootstock on the 25th and 35th day from the grafting (Fig. 5*B*) implying that *DL-NACP* mRNA transport varied among individual plants.

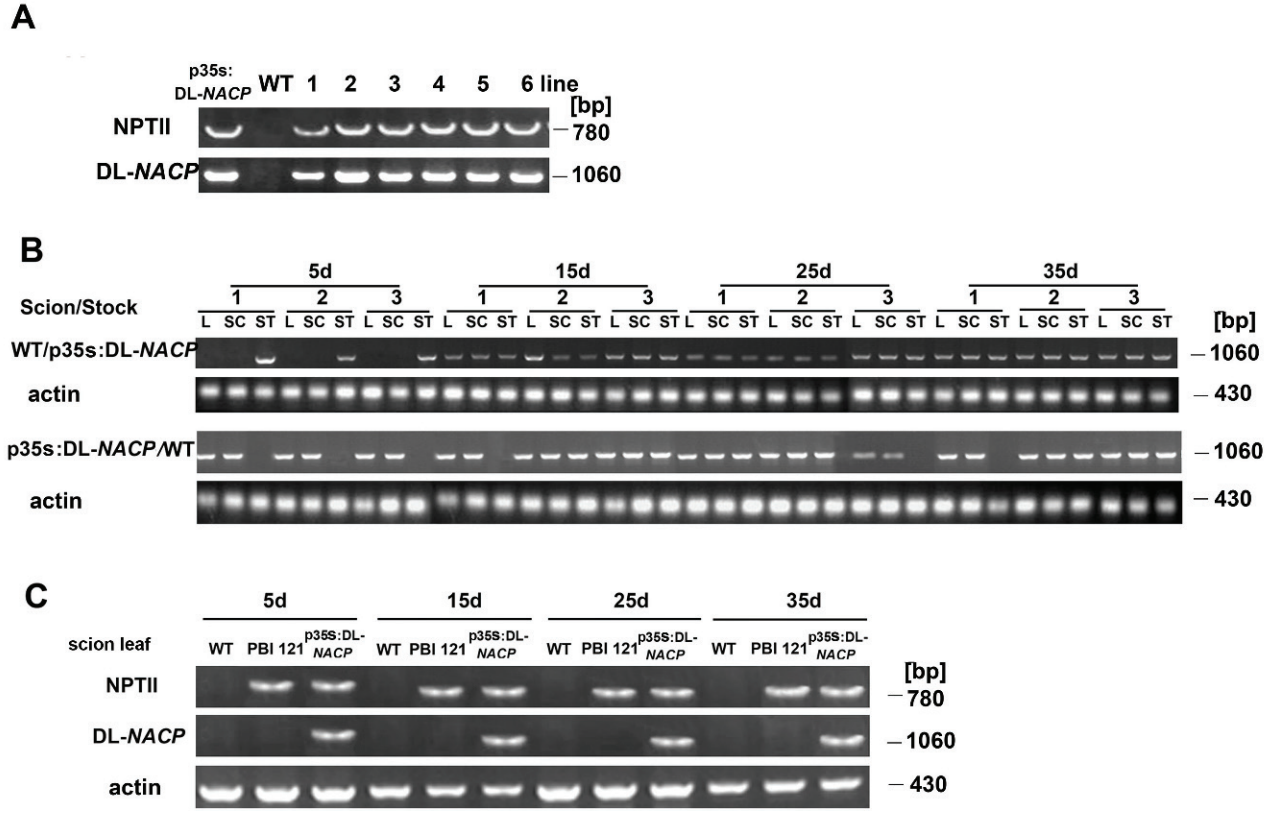

Fig. 5. RT-PCR identification of DL-*NACP* mRNA transport in transgenic grafts. *A* - The first row shows the amplification of *NPT*II (the resistant tag in pBI 121 vector) with primer pairs NPTIIF/NPTIIR from DL-*NACP* mRNA transgenic tobacco lines. The second row shows the amplification of DL-*NACP* with primer pairs NACPF/NACPR from transgenic tobacco lines (35s: DL-*NACP* - plasmid of DL-*NACP* transgenic tobacco. WT - wild type, lanes 1 to 6 - DL-*NACP* transgenic tobacco lines. *B* - WT/p 35s:DL-*NACP*; WT tobacco used as scion was grafted onto 35s: DL-*NACP* cDNA transgenic tobacco p35s: DL-*NACP*/WT, reverse grafting, 35s: DL-*NACP* cDNA transgenic tobacco used as scion was grafted onto WT. DL-*NACP* was tested by RT-PCR with primer pairs NACPF/NACPR. 1 to 3 - grafted lines. L - scion new leaves, SC - scion stem, ST - rootstock stem, C - control grafts including WT, 35s: DL-*NACP* (DL-*NACP* cDNA transgenic tobaccos), 35s: pBI 121 (pBI 121 transgenic tobaccos) grafted by themselves respectively. *NPT*II was identified in scion leaves of three transformed lines by primer pairs NPTIIF/NPTIIR, and DL-*NACP* was identified by primer pairs NACPF/NACPR on the 5th, 15th, 25th, and 35th day after grafting.

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