Basic and applied studies on long-distance transport of *GIBBERELLIC ACID INSENSITIVE* mRNA in plants

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Abbreviations

Cgb	*CoYMV pro:Atgai-bar*
CgT	*CoYMV pro:Atgai-T7*
CoYMV pro	*Commelina yellow mottle virus* promoter
GA	Gibberellic acid
GAI	Gibberellic acid insensitive
GO	Gene Ontology
IBA	Indole-3-butyric acid
MEPM	Meropenem
mRNA	Messenger RNA
PCR	Polymerase chain reaction
qRT-PCR	Quantitative real-time PCR
RFLP	Restriction fragment length polymorphisms
RNP	RNA-protein complex
RT-PCR	Reverse transcription-polymerase chain reaction
SAM	Shoot apical meristem
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SNP	Single nucleotide polymorphisms
T7-tag	T7-epitope tag
WT	Wild type
6-BA	6-Benzylaminopurine

Preface

Gibberellins (GAs) were first found from the "foolish seedling" disease in rice (*Oryza sativa* L.) by a Japanese scientist, Eiichi Kurosawa, in 1926. In 1935, Teijiro Yabuta first isolated it from fungal strains (*Gibberella fujikuroi*) provided by Kurosawa, and named the isolate as gibberellin. GAs are one group of diterpenoid acids which belongs to the five important hormones in plant, which regulate plant growth and development processes such as stem elongation, break dormacy and stimulate flowering under long-day condition (Kende and Zeevaart 1997; Sun and Gubler 2004; Ueguchi-Tanaka et al. 2007; Aya et al. 2009; Ariizumi et al. 2011; Ma et al. 2011). The classification of gibberellins is not based on function like other hormone, but on the basis of structure (the ent-gibberellane skeleton). In order of discovery, the gibberellins are named GA_1....GA_n. Gibberellic acid (GA_3) was the first gibberellin to be characterised structurally. There are also currently GAs identified from fungi (Spector and Phinney 1968) and bacteria (MacMillan et al. 2002; Bottini et al. 2004), but not all of which are bioactive for plants. GAs to plant growth regulation can be generally divided to GA biosynthesis and GA response period.

Mutants related with GAs are classified to several categories. The GA synthesis pathway has already clarified and the function of it was also exemplified by the GA-deficient mutant. *Arabidopsis ga1-3* mutant is one of them, which lacks an enzyme in the GA synthesis pathway, ent-kaurene synthetase A. GA level in these mutants were lacking compared with normal plants. The mutant shows dwarf and dark green leaf (Peng et al. 1997), and the GA-deficient phenotype can be recovered by treated with exogenous GA (Richards et al. 2001). Another GA insensitive mutant was also found in some plant varieties, *Gibberellic acid-insensitive* (*gai*, *Arabidopsis*), *D8* (maize, *Zea mays*), and *Rht-B1b*/*Rht-D1b* (wheat, *Triticum aestivum*) mutants (Koornneef et al. 1985; Harberd and Freeling 1989; Peng and Harberd 1993; Ross et al. 1994; Peng et al. 1997, 1999). But treated with exogenous GA cannot correct the dwarf phenotype (Harberd et al. 2009). The characteristic features of *GAI* and its transcript are described in Chapter I in detail.

Some cDNA clones have been found in phloem sap of plants, such as pumpkin (Ruiz-Medrano et al. 1999), barley (*Hordeum vulgare*, Doering-Saad et al. 2002), caster bean (*Richinus communis*, Doering-Saad et al. 2006) and *Arabidopsis thaliana* (Deeken et al. 2008). Some RNAs were proved to have an ability of long-distance transport to the shoot meristem.

In grafting system, there were also studies proved that many mobile elements can move between stock and scion, such as transport of alkaloids and secondary metabolites (Wilson 1952), flowering signals (An et al. 2004; Zeevaart 2006; Notaguchi et al. 2008), growth substances (Bulley et al. 2005; Foo et al. 2007), and RNAs (Tournier et al. 2006).

Among them the several genes have provided corroborative evidence for transport via graft-union (Table 1). Lucas's team in the USA obtained the first evidence a 16-kD *Cucurbita maxima* (pumpkin) phloem protein (Cmpp16) mRNA paralogs with a viral movement protein, transports through sieve element in heterografting between pumpkin and cucumber (Xoconostle-Cázares et al. 1999). Another experiment on *C. maxima* non-cell-autonomous protein (CmNACP) mRNA also showed the ability of phloem transport (Ruiz-Medrano et al. 1999). *in situ* RT-PCR on cucumber scion, which was heterografted onto transgenic pumpkin, suggested that the transcripts were present in meristems and floral organs .

Fused gene pyrophosphate-dependent phosphofructokinase (PFP)-LeT6 (KNOTTED-1-like homeobox) induces *Mouse ear* (*Me*) of leaf in tomato (*Lycopersicon esculentum* Mill.) (Chen et al. 1997). Kim et al. (2001) proved that the transcript of *PFP-LeT6* had effect on wild type (WT) which morphology was altered after grafting onto *Me*. Besides, the present of *PFP-LeT6* mRNA was detected in WT scion grafted on *Me* stock. The same result was also reported in potato (*Solanum tuberosum*) scion grafted on *Me* tomato (Kudo and Harada 2006). These results provided strong evidence for the existence and function of a supra cellular information signal that migrated between the scion and the rootstock.

On the other hand, RNA long-distance transport is not only upstream, but also downstream in phloem. In potato (*S. tuberosum* subsp. *andigena*), *StBEL5* (BEL1-like transcription factor), which related with regulating tuber formation by targeting genes that control growth, was detected the transport from leaves and petioles to root, and then caused tuber formation at the stolon tips (Banerjee et al. 2006). Moreover, *StBEL5* transcripts were detected in WT stock grafted with over-expressed *StBEL5* scion under short-day (SD) conditions. The untranslated regions of *StBEL5* mRNA are related with mediating its long-distance transport, maintaining transcript stability, and controlling translation (Banerjee et al. 2009).

Several phloem cDNAs were selected from phloem sap of pumpkin scion grafted onto melon stock. As the result of detection, six cDNA clones out of 43 examined transcripts were capable transport in a long-distance behavior from melon stock to pumpkin scion. Three of them were associated with the auxin-signal transduction, which suggested that the auxin signalings perhaps also correlate with the RNA long-distance transport in phloem. Kanehira et al. (2010) reported that one of those genes *MpSLR/IAA14* transported through graft union in

apple 2-years-old tree.

Table 1. Long-distance transport endogenous mRNAs through sieve element, which confirmed by grafting experiments.

Genes	Functional description	Plant used	References
PP16	RNA movement protein showing paralog to viral movement protein	Pumpkin	Xoconostle-Cázaresa et al. 1999
NACP	Homolog of NAC domain protein	Pumpkin	Ruiz-Medrano et al. 1999
PFP-LeT6	Phosphofructokinase (PFP) - LeT6 (KNOTTED-1-like homeobox)	Tomato, Potato	Kim et al. 2001; Kudo and Harada 2007
GAI	Negative regulator of GA response genes	Tomato, *Arabidopsis*	Haywood et al. 2005; Ham et al. 2009; Huang and Yu, 2009
BEL5	Transcription factior belonging to TALE family	Potato	Banerjee et al. 2006; Banerjee et al. 2009
AUX/IAA14	Transcriptional repressor of auxin responsive genes	Melon, Apple	Omid et al. 2006; Kanehira et al. 2010

In this study, the *Atgai* (lacks a 17-amino acid segment of *AtGAI* at DELLA domain) mRNA was accumulated in companion cell by *Commelina yellow mottle virus* promoter. I characterized the phloem transported *Atgai* mRNA in tobacco grafting system and figured out the dwarf of scion induced by transported *Atgai* transcripts. The results can let us understand much more distinctly about *Atgai* mRNA transport. Furthermore by use of this mechanism, a new semi-dwarf apple rootstock was produced, which provide a new breeding method of horticulture crops to develop dwarf rootstock.

Chapter I

Characterization of *Atgai* transport through phloem

I-1 Introduction

The importance of gibberellins (GAs) to angiosperm growth regulation has been exemplified by the phenotype of GA-deficient mutants. The GA-deficient *Arabidopsis thaliana ga1-3* mutant lacks *ent*-kaurene synthetase A, an enzyme in the GA biosynthesis pathway, exhibits a characteristic severe dwarf phenotype (Richards et al. 2001). Mutants such as *ga1-3* are GA-sensitive dwarf mutants that are known in a number of different plant species and typically carry recessive mutations that reduce the activity of GA biosynthesis enzymes (Yamaguchi 2008). Further molecular characterization of various GA response mutants led to the discovery of the GID1 (GIBBERELLIC INSNSITIVE DWARF1) and DELLA proteins, key components of the molecular GA-GID1-DELLA mechanism that enables plants to respond to GA (Fig. I-I). Genetic and molecular studies have identified the GA receptors and several positive and negative components in the GA signaling cascade (Sun and Gubler 2004; Hartweck and Olszewski 2006). Among them, three major players are the GA receptors, the DELLA repressor proteins, and the F-box proteins that control the stability of DELLA proteins. Ueguchi-Tanaka et al. (2005) demonstrated that GID1 is a soluble GA receptor in rice. Discovery of the molecular identity of the endogenous plant GA-opposable growth inhibitory factor resulted from the molecular cloning of genes encoding what are now known as the DELLA protein.

The *Arabidopsis gai* mutation confers dominant, GA-insensitive dwarfism (Koorneef et al. 1985; Peng and Harberd 1993). An insertional mutagenesis approach enabled the molecular cloning of *gai* via isolation of a *Dissociation* (*Ds*) transposon inactivated allele (Peng et al. 1997). The amino acid sequence of GAI suggested that it might also be a transcriptional regulator (Peng et al. 1997). The *gai* open reading frame carries a small in-frame deletion mutation and thus encodes an altered product, a mutant gai protein that lacks a 17-amino acid segment, now known as the DELLA domain, named after its first five amino acids. Molecular genetic analysis of GA-insensitive dwarf mutants also identified an F-box protein (SLY1) that is part of a DELLA- interacting E3 ubiquitin ligase that interacts with a C-terminal region of the DELLA protein (McGinnis et al. 2003, Dill et al. 2004; Fu et al. 2004), and that targets

DELLAs of destruction by the proteasome. After all, DELLA proteins are thought to repress plant growth and gibberellins promotes growth by overcoming the repressive effects of these proteins. *Arabidopsis gai-1* mutant revealed that allele has a 51 bp deletion from the conserved DELLA domain. As mentioned above, the truncated form of GAI acts as a gain-of-function mutant that can inhibit some components of the GA signaling pathway (Peng et al. 1997). The expression of *Arabidopsis gai* in rice yielded a dwarf phenotype, suggesting that GAI is sufficiently conserved between plant families to allow it to function (Fu et al. 2001).

Haywood et al. (2005) reported that the long-distance delivery of RNA of the *Arabidopsis ΔDELLA-gai* (*Atgai*) genes. They demonstrated that specificity in the entry of these *gai* tanscripts into functional sieve elements and a highly reproducible change in leaf phenotype in tomato through the experiment in which *Atgai* mRNA transported into the shoot apex by a grafting technique (Haywood et al. 2005). Ham et al. (2009) reported that the polypyrimidine tract binding motif within the *GAI* mRNA is involved in the formation of a mobile ribonucleoprotein complex and proposed the presence of motifs that are necessary and sufficient for long-distance trafficking of the *GAI* transcript.

However, there are no details about the molecular mechanism, especially physiological function of the *GAI* mRNA long transport system. In this chapter, I focused the characterization of the *GAI* mRNA transport through phloem by using *Atgai* transgenic tobacco as the experimental materials.

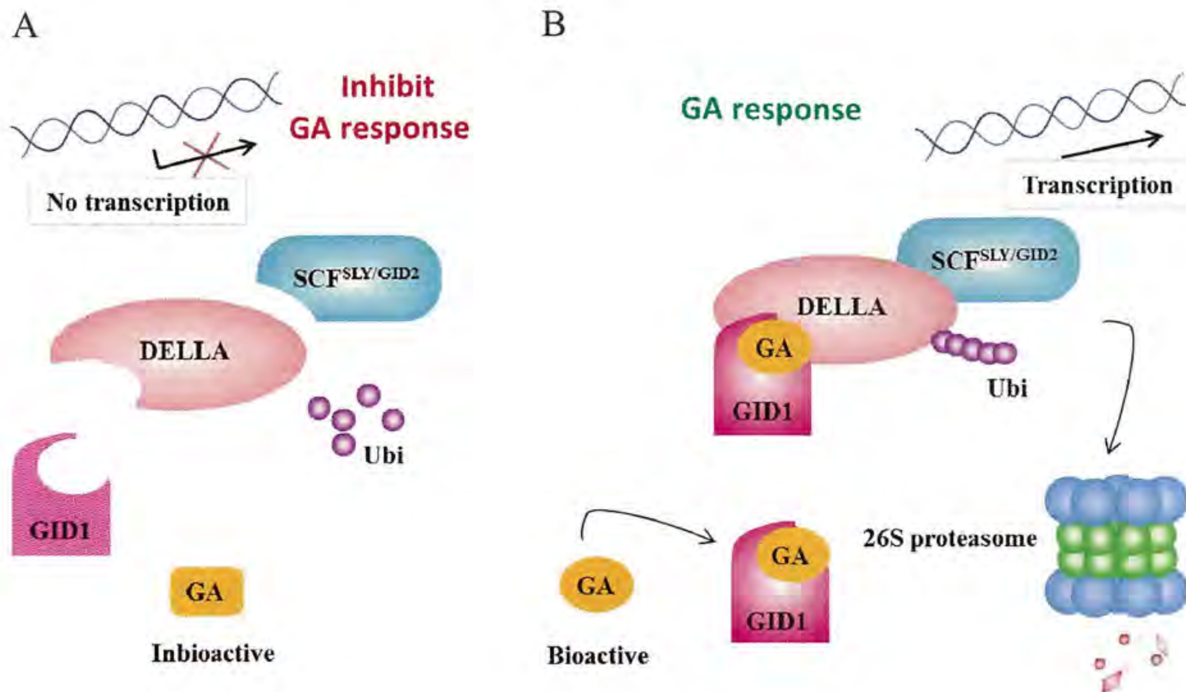

Figure I-I. Model of GA-GID-DELLA of GA response in plants.

(A) Under a low level of GA, since there is not enough bioactive GA combine with GID1 (GA receptor), GA response is inhibited by DELLA protein. (B) Increase of bioactive GA facilitates the combination between GA and GID1, which induces SCF $^{SLY/GID2}$ complex bond with DELLA. The protein complex is interacted with E3 ubiquitin ligase, and then DELLA is degradated in the ubiquitin-proteasome pathway, results in the start of the transcription of GA response genes..

I-2 Material and Methods

Plant Materials

Nicotiana benthamiana, which was kindly gifted from Professor Teruo Sano of Hirosaki University, was used in this experiment. To perform seed sterilization, seeds placed in an Eppendorf Tube, then immersed in 70% ethanol for 1 min, followed by 5 min soak in 5% hydrogen peroxide water containing 0.2% Tween 20. Seeds was then rinsed five times with sterile water, and sowed on MS (Murashige and Skoog 1962) agar (Wako Pure Chemical Industries Ltd., Osaka, Japan) plate in a Petri dish. The dish was at 24°C under a 16:8-h photoperiod under photosynthetically active radiation of about 100 μmol m^{-2} s^{-1} provided by cool-white fluorescent tubes.

Construction of binary vector

Arabidopsis seed (*gai*-1, CS63) was provided by the Arabidopsis Biological Resource Center (Ohio State University). The plantlets of the CS63 were used to extract RNA fraction for the obtaining the full cDNA of the *Atgai* gene which is a 51 bp deletion from the conserved DELLA domain (Peng et al. 1997). Restriction sites *Xba*I and *Sac*I were leaded into 5' and 3'- sites of *Atgai* fragment by PCR using primers Atgai Xba and Atgai Sac. GUS fragment was deleted with *Xba*I and *Sac*I from *pBI121* (Chen et al. 2003), and then replaced with the new *Atgai* fragment. *CaMV* (*Cauliflower mosaic virus*) 35S promoter (35S promoter) was deleted from the vector by *Sal*I and double digested by *Spe*I and *Xba*I. *Commelina yellow mottle virus* promoter (*CoYMV* pro) of pCOI (Matsuda et al. 2002; from Prof. Neil Olszewski, University of Minnesota, St. Paul, MN, USA), which is a companion cell specific expressed promoter (Medberry et al. 1992), was amplified by primers CoYMV pro FP Sal and CoYMV pro RP Spe, and then used to replace the 35S promoter sequence with *Sal*I and *Spe*I sites (Table 1). T7-epitope tag sequence (MASMTGGQQMG, Invitrogen, USA) was inserted into the 3' site of *CoYMVpro: Atgai* by PCR experiment. Forward primer was CoYMV pro Fl and the primer T7 R including T7-epitope tag sequence was used as the reward primer. After that the new vector was sequenced to ensure correct insertion. The *CoYMVp::Atgai-T7* (*CgT*) fused gene was cloned in *pBI121* (Figure I-1) carrying the nos-kanamycin resistance cassette in transgenic lines. DNA Ligation Kit (Takara Bio Inc., Otsu, Japan) was used for ligation. Transgenic plant material was obtained by *Agrobacterium* mediated transformation.

Transgenic lines were identified by PCR with primers CoYMV pro FP1 and Atgai R1 (Table I – 1).

Grafting experiment

Micrografting was performed according to the method described by Bai *et al.* (2011), briefly plantlets of 10 days after germination, were grafted under a stereomicroscope in clean bench. The plant was cut horizontally approximately 3 mm below the cotyledon. Then scion (tissue with the cotyledon) and rootstock (tissue at the bottom with root) were fastened with silicon tube (ɸ 0.5 mm× 0.1 mm, 3 mm length; TechJam, Osaka, Japan). The grafted plants were propped against agar block on MS agar medium. At 7 days after grafting, the silicon tube was cut off. Then, the grafts were cultivated in soil or using a standard nutrient solution (Otsuka House Nos. 1 and 2, Otsuka Chemical Co., Osaka, Japan).

GA_3 treatment

Grafted plants which were grown for 2 weeks in a Petri dish were transferred to pots with a nursery soil. After 1 week, they were sprayed by 0.1 mM GA_3 solution (Nakarai Tesque, Inc. Kyoto, Japan) containing 0.02% Tween 20 once every two days for three weeks. Seven days after the fifth spray, the seedling stature was measured and the shoots were sampled to extract the RNA fraction. As another method, the plantlets were grown on MS agar plate containing 0.1 mM GA_3.

RNA extraction, RT-PCR and Real Time-PCR analysis

Total RNA was extracted from leaves with TRIzol (Invitrogen, Tokyo, Japan), and genomic DNA was eliminated with a TURBO DNA-free Kit (Ambion Inc. Austin, USA). Reverse-transcribed cDNAs were prepared by SuperScript® VILO™ cDNA Synthesis Kit (Invitrogen, USA). The cDNA corresponding to 50 ng of total RNA was used in 10 μl reactions with S1000 Thermal Cycler (Bio-Rad, USA). To amplify *Atgai* mRNA in WT scion, primers (Atgai F2 and R2) and the nested primers (Atgai F3 and R3) were prepared. The amplification condition were as follows; initial denaturing at 94°C for 4 min, 25 cycles at 94°C for 30 s, 58°C for 30 s, and 72°C for 1 min; and extension at 72°C for 3 min. For the nested PCR, 1 μl of the 1st PCR product was used as the template by 30 cycles comprising. For Real Time-PCR experiment, 1 μl of the cDNA corresponding to 50 ng of total RNA was used in 20 μl reactions with iQ SYBR Green Supermix (Bio-Rad, USA). Triplicate reactions of each sample were amplified along with no-template controls on the Chromo 4 real-time

PCR detector (Bio-Rad, USA). *NbUbi* (Accession No.: AY912494) was used to normalize the expression levels of *Atgai*. Real Time-PCR Primers (Atgai QF/Atgai QR and Ubi QF/ Ubi QR) specific to the *Atgai* and *NbUbi* genes were used in this experiment. (Table I-1)

Protein extraction and immunoblotting

Protein was extracted according to the method described previously (Wang et al, 2006). About 3.0 g scion tissue (n = 5) was sampled, in which mRNA transport were positive detected, and then ground in liquid nitrogen using pre-cold mortar and pestle. The tissue powder was washed with 10% TCA/acetone once, 80% methanol, and 80% acetone, respectively. After that, dry at room temperature for 10 min. The solid matters were incubated with 1:1 phenol (pH 8.0) /SDS buffer. Keep the upper phenol phase and mixed with methanol containing 1.2 M ammonium acetate to precipitate the protein at – 20°C over night. Centrifuge at 16,000 × g for 5 min at 4 °C. Wash the precipitate with 100 % methanol and 80% acetone as the method described. Air dry briefly and dissolved by phosphate buffer. The concentration of protein was measured with the DC Protein Assay (Bio-Rad, USA). Total proteins (25 μg) were mixed with a SDS loading buffer, and heated at 95 °C for 5 minutes to denature, and fractionated by SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis). SDS-PAGE was performed in 12.5% polyacrylamide gels using Bio-Rad Mini-PROTEAN 4 equipment under 200V for 1 h. Proteins was transferred to Immun-Blot™ PVDF membrane (Bio-Rad, USA). Then, membrane was blocked with BSA (Albumin from bovine serum, Sigma, Germany) at room temperature for 1 h (Components of blocking buffer: BSA was dissolved by 1 × TBS- 0.1% Tween to a final concentration 0.02 g/ mL). For analyzing of immune blots, membrane were incubated with 0.1 μg/ mL anti T7-peptide monoclonal antibody (Novagen, USA) at 4 °C over night, and then washed 4 times in 1 hour at room temperature. After that, the membrane was incubated with a 2,000-fold dilution of anti-mouse IgG, Goat Poly., HRP (Cosmo Bio Co., Ltd., Tokyo, Japan) for 1 h, and washed 3 times in 1 hour at room temperature. The signals were detected by Amersham ECL Plus Western Blot Detected System (GE Healthcare, UK). A duplicate gel was run at the same time and then stained with Coomassie Brilliant Blue R250 as a loading control.

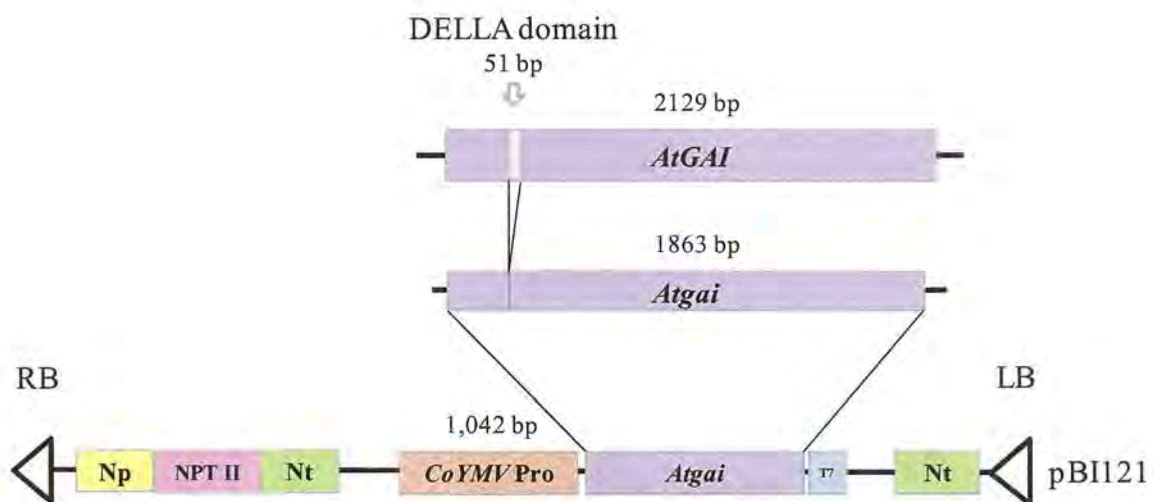

Figure I-1. Vector structure of *CgT*.

CoYMV* Pro**: *Commelina yellow mottle virus* promoter (companion cell specific expressive promoter); ***Atgai: *Arabidopsis thaliana gai* gene (a gain-of-function DELLA allele of *AtGAI*); **T7**: T7-epitope tag (an 11 amino acid peptide encoded in the leader sequence of T7 bacteriophage gene 10); **Np**: Nos promoter; **NPT II**: A gene coding for kanamycin resistance (primarily Neomycin phosphotransferase II); **Nt**: Nos terminater.

Table I-1 Primer sequences

Name of primers	Sequences
Atgai Xba	5′- GCTCTAGA ATGAAGAGAGATCATCATCATCAT -3′
Atgai Sac	5′- GCGAGCTCAGTAATTTAGGCGAGATTAAAAT -3′
CoYMV pro FP Sal	5′- GCGTCGACGGTATCGATTTCTTAGG -3′
CoYMV pro RP Spe	5′- GCACTAGTAGTAATTTAGGCGAGATTAAAAT -3′
T7 tag R	5′- AGCGGTACCCTATCCCATTTGTTGTCCTCCAGTCAT AGAAGCCATTCCTCCATTGGTGGAGAGTTT -3′
CoYMV pro F1	5′- CCTATGCCTTTATTCGCAGC -3′
Atgai R1	5′- TCAAGCCACGTGTAAAGCTCCGC -3′
Atgai F2	5′- TTGAGCTTAGACCAAGTGAGATTG -3′
Atgai R2	5′- CTGACTCAACGTTTCATGACGCTCA-3′
Atgai QF	5′- CAACTCGGCATGTTGTCCTGGTTG -3′
Atgai QR	5′- TTCGGCGAAGTAAGTAGCGAC -3′
Ubi QF1	5′- CAGGACAAGGAGGGTATC -3′
Ubi QR1	5′- CACGTCATCAACAACAGA -3′

Microarray analysis

Five WT scions in which the transported *Atgai* mRNA from *CgT* stock was detected by RT-PCR were brought together, and then total RNA was prepared from the sample. Five scions of WT/ WT with or without GA_3 treatment were also brought together and used as samples for RNA extraction. The purification, labeling of complementary RNA (cRNA), hybridization to 44K Tobacoo DNA microarray (Agilent Techologies), signal scanning, and processing were done by Hokkaido System Science Co., Ltd.(Japan) with Low Input Quick Amp Labling Kit (Agilent Technologies, USA), the Agilent Technologies Microarray Scanner (Agilent Techologies, USA). Black holes (poor pre-hybridization blocking) and probes which had weaker signals than background 13×BGSD were filter out. All processing described above was done by Hokkaido System Science Co., Ltd. (Japan). Greater than 18,588 unique genes pass the quality control. Fold changes of WT/CgT (GA+) and WT/WT (GA+) were calculated with WT/WT (GA-) as the control. Fold changes which are over 100 and below 0.01 were filter out, and below 0.5 and above 2.0 in WT/WT (GA+) vs WT/WT (GA-) were selected as up-regulated and down-regulated genes. Afterward 4, 696 corresponding genes in group WT/*CgT* (GA+) vs WT/WT (GA-) were filtering for analysis. Scatter plot Log 2 value of fold change GO (Gene Ontology, http://www.geneontology.org/) analysis was done by AgriGO analysis online (http://bioinfo.cau.edu.cn/agriGO/) following the method of Singular Enrichment Analysis (SEA). Statistical test method was Fisher. Significant level was 0.05. Heat map was generated by ArrayStar 4 of DNAStar .

I-3 Results

Tobacco transformant by *companion cell specific expressed* Atgai *transformed dwarf and less sensitive to* GA_3

Over expressed *Atgai* transgenic plants showing dwarf phenotype was already proved in *Arabidopsis*, tobacco, apple (Haywood et al. 2005; Huang and Yu 2009; Hynes et al. 2003; Zhu et al. 2007). Furthermore, since *Atgai* mRNA was proved having ability of transport through phloem, the T-DNA construct harboring companion cell specific expressing *Atgai* (*CgT*) was prepared (Fig. I-1) and it was integrated into *N. benthamiana* by Agrobacterium transformation system. Two transgenic lines (*CgT41* and *CgT81*) were confirmed by DNA gel blot (Fig. I-2). Though both *CgT41* and *CgT81*were smaller than WT, the former plant exhibited clearly semi-dwarf phenotype than the latter. Therefore, *CgT41* was used in all experiments from this point forward and *CgT41* express succinctly as *CgT*. Semi-dwarf phenotype of *CgT* plant had also smaller root volume than WT (Fig. I-3A). Accelerated growth from 7 days after the planting in WT was not obvious in the case of CgT. Additionally, the increase of *CgT* stature was still gently even after GA treatment (Fig. I-3B), resulted in the stature of *CgT* was one-fold of WT on 21 days after GA treatment. These data lead me to the conclusion that *CgT* was semi-dwarf because of less-sensitive to GA.

CgT *rootstock has effect WT scion growth*

To know whether the *CgT* plant affects the grafted-WT growth by the *Atgai* mRNA transport, grafts consisting of WT/*CgT* and *CgT*/WT were treated with GA_3. Self-grafted plants of WT and *CgT* (WT/WT and *CgT*/*CgT*) were also prepared. Grafted plants grown for 7 days on MS agar medium after grafting were transferred to soil in a pot and sprayed with or without GA_3 solution. Respective growth patterns were observed among four grafting patterns (Fig. I-4). GA treated WT/WT and WT/*CgT* represented a typical GA response phenotype: fast elongation, slender shape and yellowish leaf compared with GA- group, but the response of *CgT*/*CgT* was not so obviously. Moreover, the shoot stature of WT/*CgT* was approximately half-size of WT/WT, indicating that WT scion grafted on *CgT* was less sensitive to GA_3.

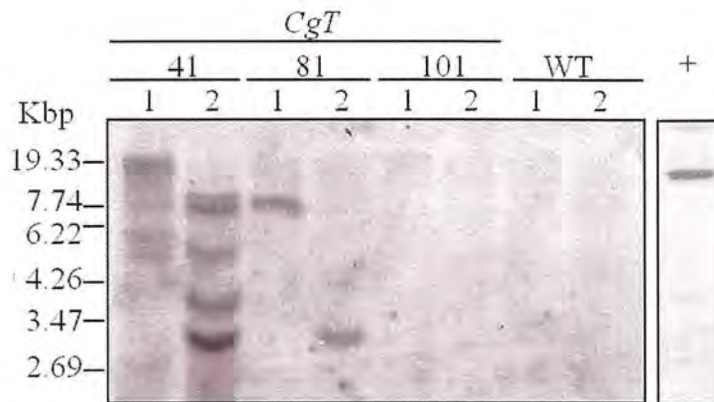

Figure I-2. DNA gel blot analyses of transgenic *CgT* lines using a specific probe for *Atgai.* Number 41, 81 and 101 represent the regenerated lines. '+' was *CgT* plasmid (positive control). WT was negative control. 15 mg of genomic DNA for each sample was digested with *Hin*dIII (lane 1) and *Eco*RV (lane 2).

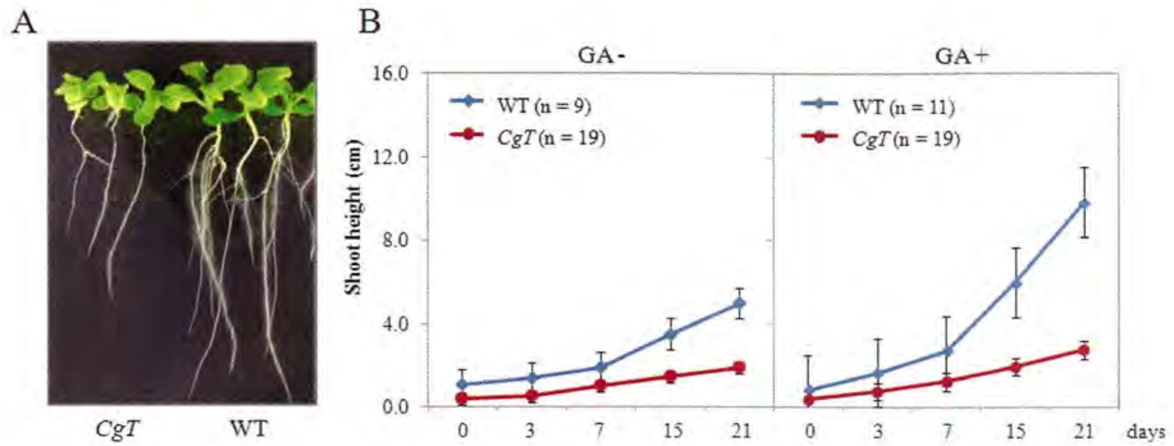

Figure I-3. The phenotypes and shoot growth rates of of *CgT* and WT.

(A) *CgT* and WT grown in MS medium. (B) Heights of *CgT* and WT shoots with and without GA treatment. *CgT* was less sensitive to GA compared to WT. In (A), germinated plants were cultured in MS for one week. Plants used in (B) were cultured in MS medium under normal condition.

Growth rates of shoot and root of the grafts were measured carefully (Fig. I-5). *CgT* root stock reduced the stature of WT, and *CgT* scion also reduced the length of WT root. (Fig. I-5A). Then, to see the effect of *CgT* to the grafted partner's volume, the fresh weights of shoot and root in four grafting combinations were measured (Fig. I-5B). The combinations with a higher weight for the both shoot and root volumes were: WT/WT, and continues WT/*CgT, CgT/CgT,* and *CgT/CgT*. As a result, *CgT/CgT* was almost one-fifth of the WT/WT (Fig. I-5 B). By using these individual grafts data, histograms and Table were constructed, respectively (Fig I-5 C, Table I-2). They showed that there were variability among the growth rates of individual grafts, but *CgT* obviously reduced the grafted WT growth rate.

Detection of* Atgai *mRNA transport through graft union

The effect of *CgT* on the grafted WT was considered to be caused by the long-distance transport of *Atgai* mRNA through the graft union. To confirm this point, detection experiments of the mRNA were done by RT-PCR. The results showed that after grafting for 21 days the RT-PCR product which was identical to the predicted size was obtained in three of six WT scion samples (Fig. I-7A). Quantitative RT- PCR to investigate the amounts indicated that a significant amount was obtained from WT scions which were undetectable the product band in agarose electrophoresis, meaning the presence of very small amount of *Atgai* mRNA in some WT scion samples. The sequencing of these amplified fragments identified the derivation from the *Atgai*. Furthermore, to understand the rate of *Atgai* transport, 63 of WT/*CgT* grafted samples were used for RT-PCR. Thirteen WT scions on *CgT* stocks produced clear amplified product (Figure I-7, TableI-2), indicating 21% of samples were positive results. To quantitative the transported *Atgai* mRNAs, we randomly chose WT/*CgT* scions (n = 6) and detected with Real-Time PCR (Fig. I-8). It was displayed in the figure that the expression level detected in WT scions were different, which suggested that transportability of the mRNA through graft unions may vary among individuals.

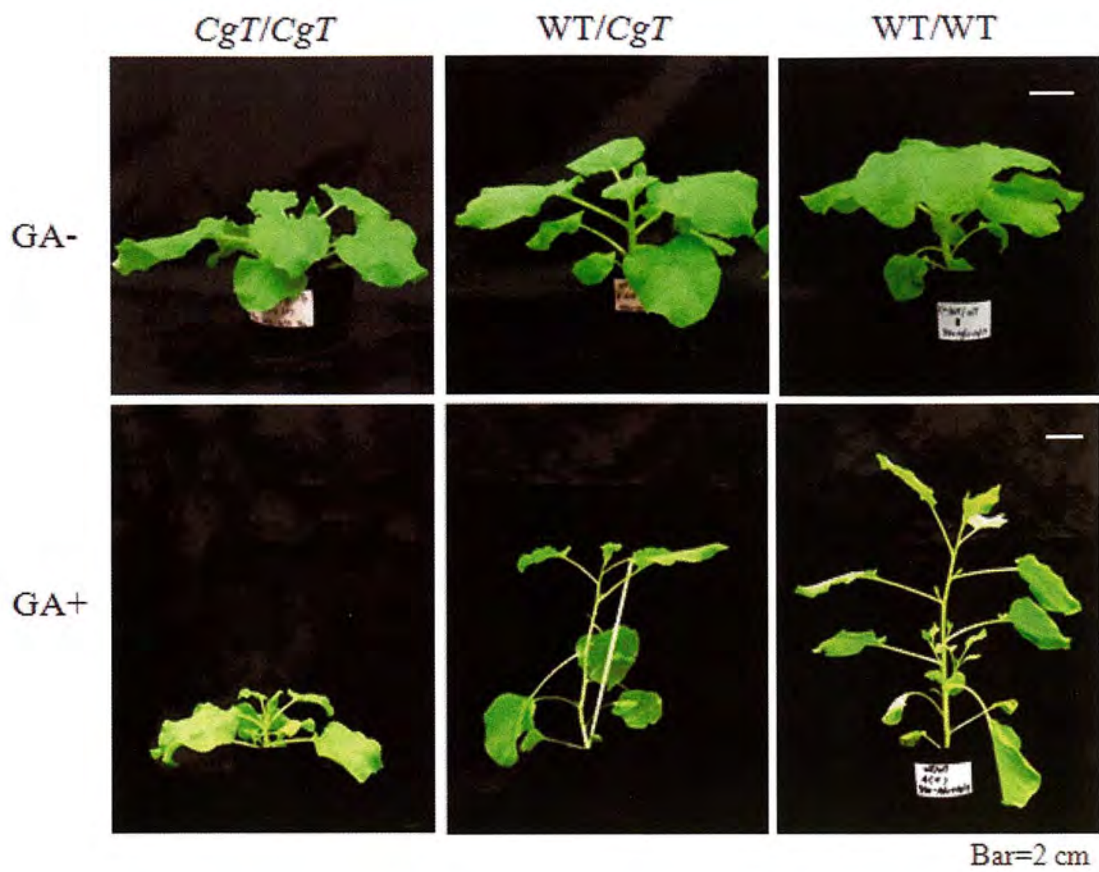

Figure I-4. Shoot phenotypes in different graft combinations.

Upper three panels show the plants grown for 21 days in respective grafting combinations. Lower three show the plants grown under the same condition on the upper unless the treatment of GA_3 spraying.

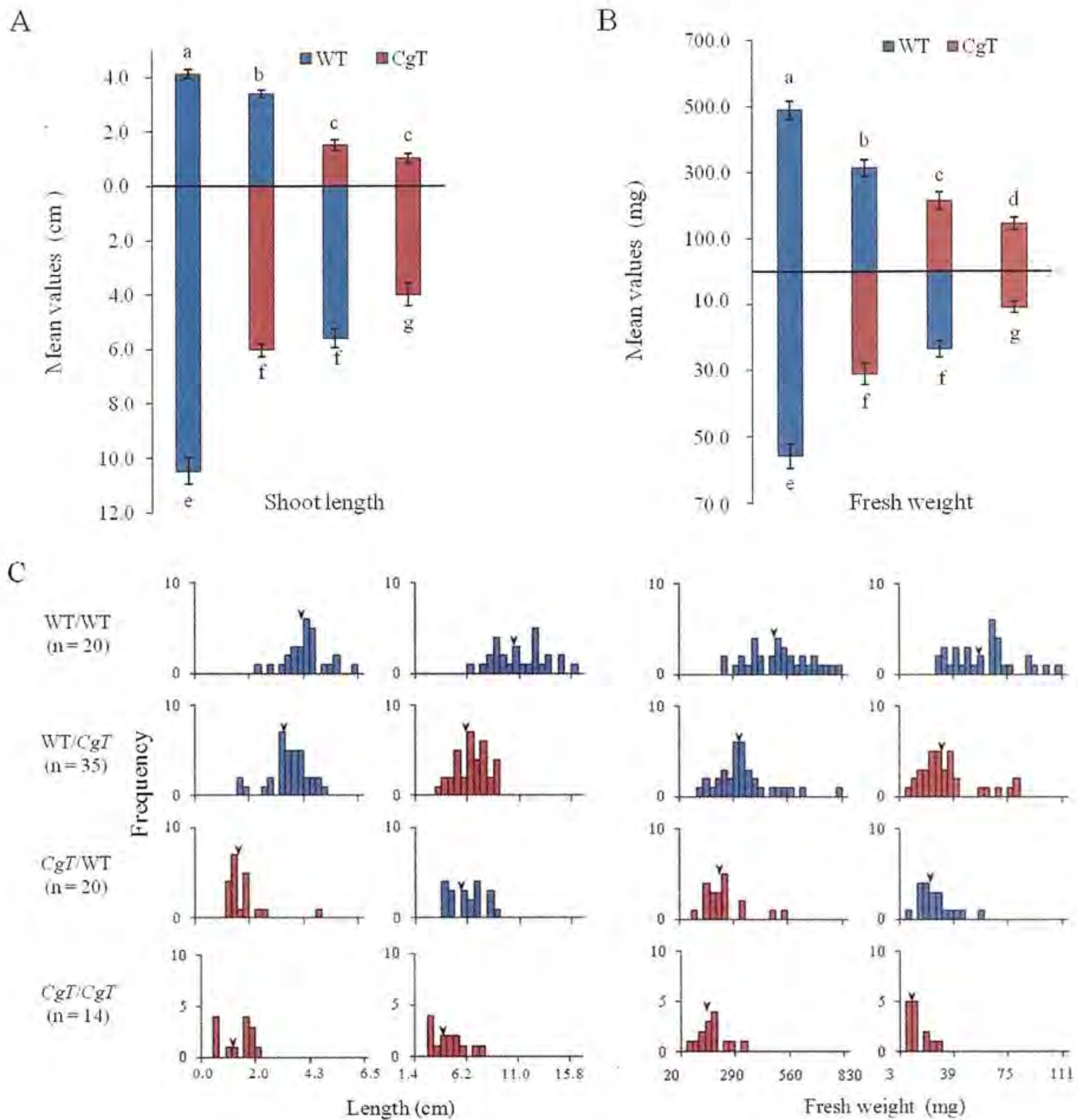

Figure I-5. Growth rates of shoot and root of the grafts after GA_3 treatment.

(A) Average shoot and root lengths. (B) Average fresh shoot and root weights. (C) Histograms of shoot lengths and root weights of different graft combination. Bars show one standard error of mean. Difference significant was determined by Student's t test with equal or unequal variances as appropriate and the same alphabet in each graph indicate no significant difference. The arrowhead in (C) indicates the average of the population.

Table I-2. Shoot and root growth rates in respective grafts after GA_3 treatment.

Grafting pattern (scion / stock)	Length (cm)		Flesh weight (mg)	
	Shoot	Root	Shoot	Root
WT / WT	4.13 ± 0.16	10.47 ± 0.48	490.0 ± 27.1	55.8 ± 3.7
WT / *CoYMVp:Atgai-T7*	3.41 ± 0.14*	6.01 ± 0.24**	314.5 ± 23.9**	30.9 ± 3.3**
CoYMVp:Atgai-T7 / WT	1.52 ± 0.19**	5.60 ± 0.34**	214.3 ± 25.0**	23.4 ± 2.4**
CoYMVp:Atgai-T7/ CoYMVp:Atgai-T7	1.03 ± 0.19**	3.98 ± 0.42**	145.1 ± 18.8**	10.9 ± 1.8**

Values are expressed as mean ± SE. Significant differences from WT/WT were determined by Student's *t* test with equal or unequal variances as appropriate (*$P < 0.01$ or **$P < 0.001$).

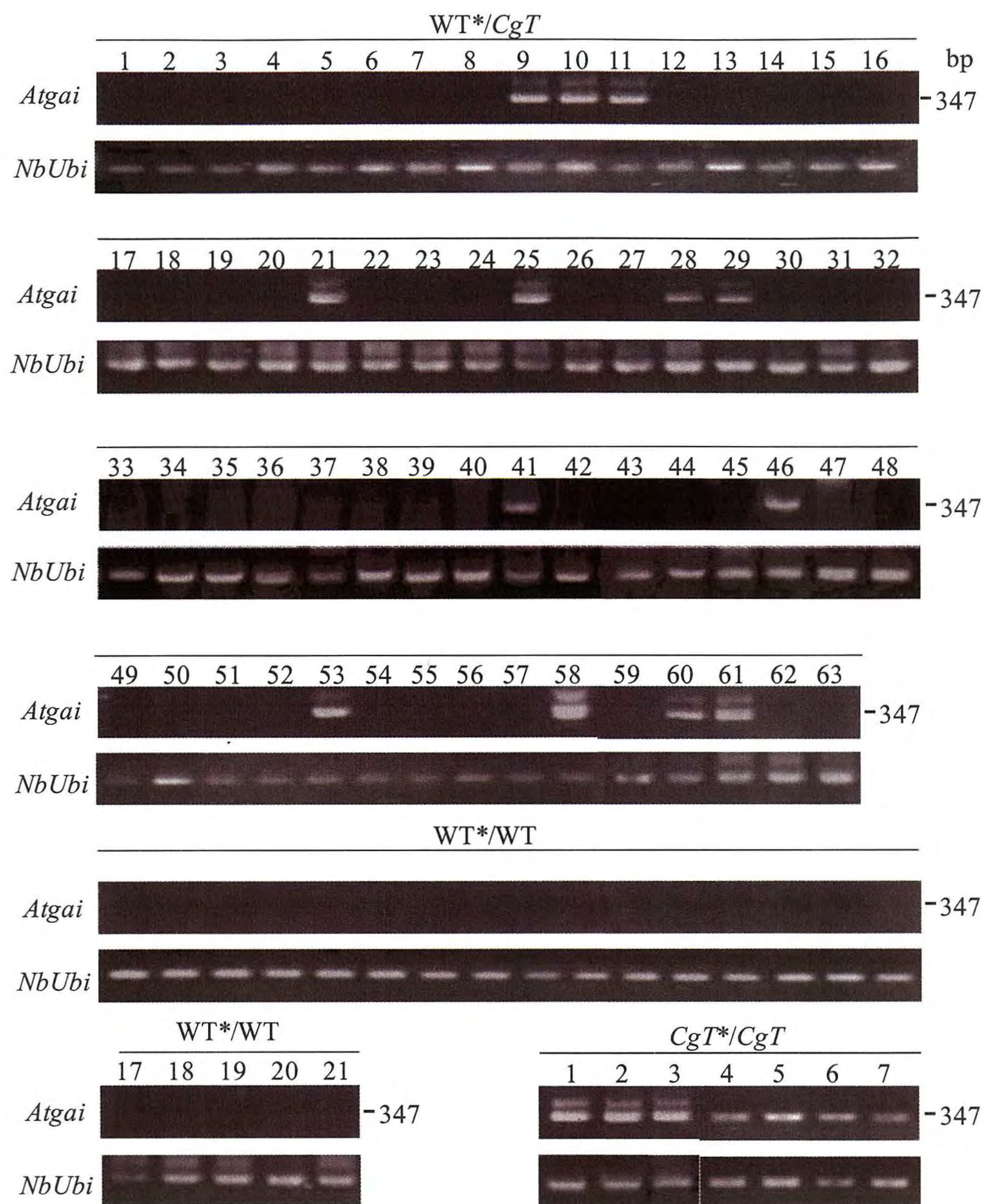

Figure I-6. RT-PCR detections of *Atgai* mRNA in scions grafted on different stocks. Asterisk shows the scion used for detection. The 347 bp fragment means the predicted amplified signal.

Table I-3. RT-PCR detection of *Atgai* mRNA in scion at 21 days after grafting

Scion	Stock	No. of grafts tested	No. of grafts detected	Positive detection (%)
WT	WT	21	0	0
WT	*CgT*	63	13	21
CgT	*CgT*	7	7	100

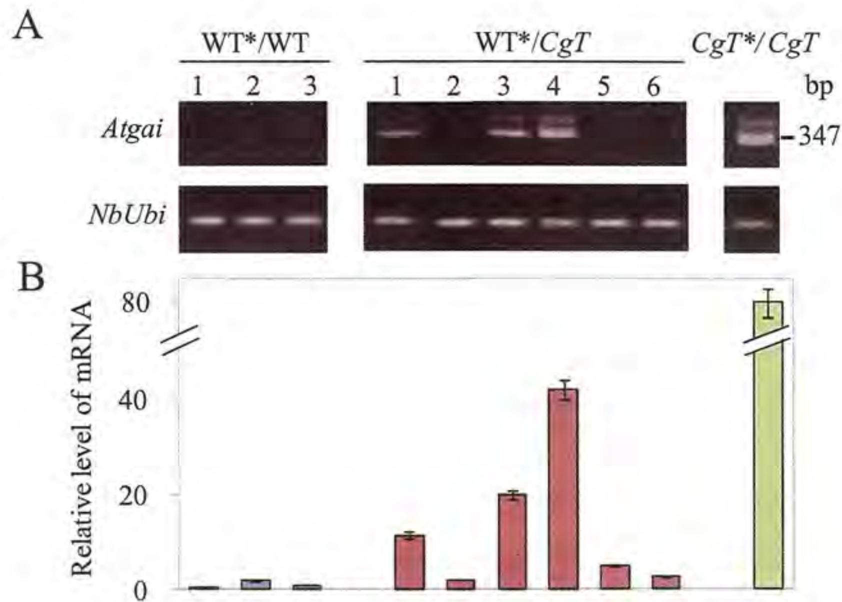

Figure I-7. Detection of *Atgai* mRNA transport at 21 days after grafting.

(A) RT-PCR detection of *Atgai* mRNA in scions randomly chosen from WT/*CgT* grafts. (B) Real Time-PCR of the respective scion samples in (A). Asterisk shows the scion for detection.

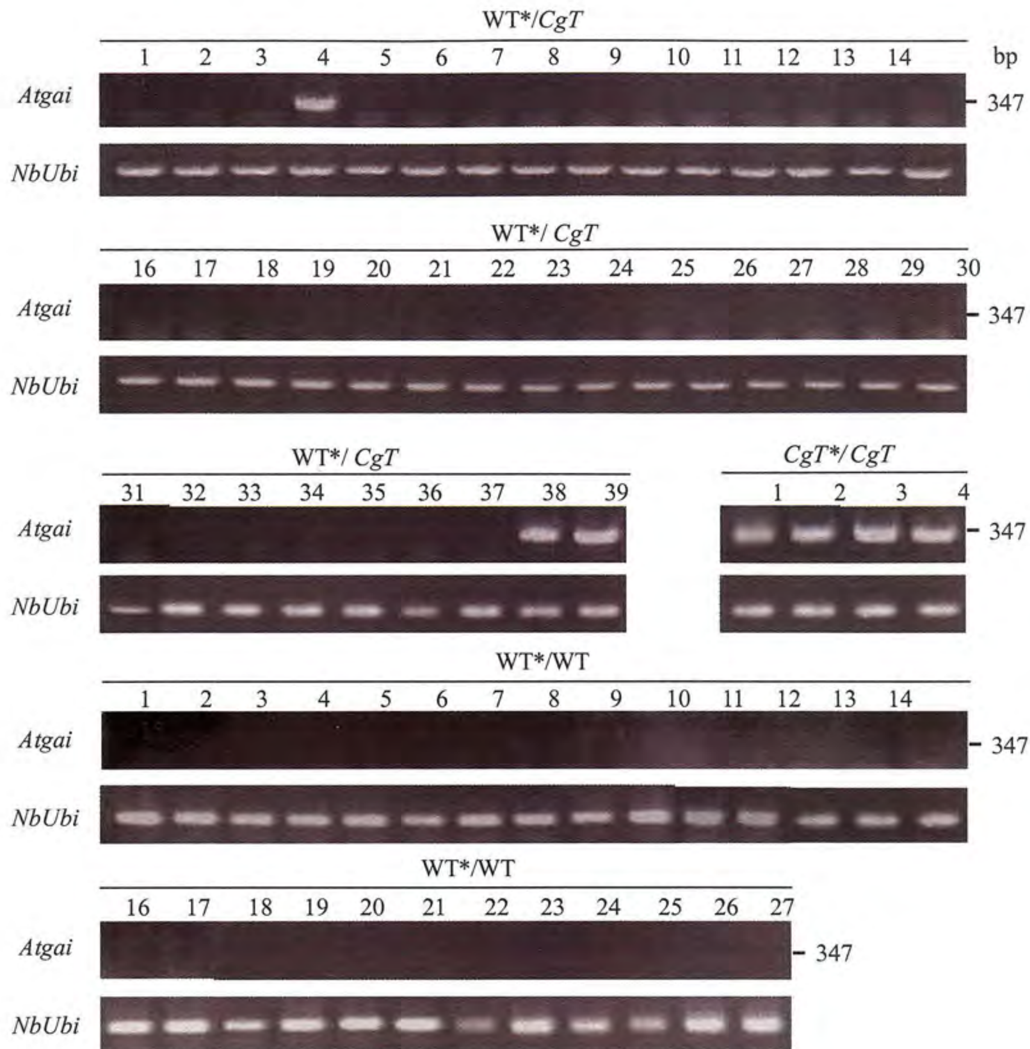

Figure I-8. Detection of *Atgai* mRNA transport at 28 days after grafting.

Asterisk shows the scion used for the detection. The 347 bp fragment means the positive amplified band.

Table I-4. RT-PCR detection of *Atgai* mRNA in scion at 28 days after grafting

Scion	Stock	No. of grafts tested	No. of grafts detected	Positive detection (%)
WT	WT	27	0	0
WT	*CgT*	39	3	8
CgT	*CgT*	4	4	100

Atgai-T7 protein is detected in scion of WT/**CgT**

Since the transport of *Atgai* mRNA was proved, I want to find out whether the long-distance transported mRNA can be translated to protein and then function at the transported sites. WT*/WT and *CgT*/CgT* (* indicated the sample extracted protein) homogeneous grafts were negative and positive controls (n > 5), respectively. Five of WT*/*CgT* were analyzed in which *Atgai* mRNA was positively detected (Fig. I-10). Using a T7-tag antibody, a clear band was detected in the WT scion on the *CgT* stock. The predicted molecular weight of Atgai-T7 was about 57 kDa, and the signal was located at little above 50 kDa standard protein. Therefore it was proved that *Atgai* mRNA can be transported from *CgT* stock to WT scion, and then translated into protein in scion tissue

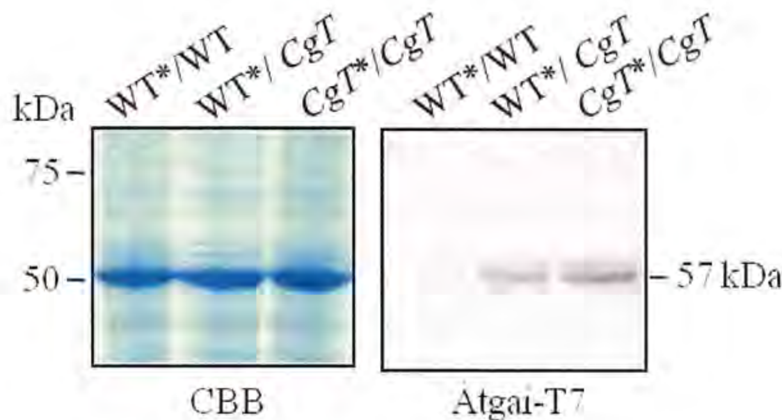

Figure I-9. Detection of Atgai-T7 protein in WT scion grafted on *CgT* stock by western blotting.

By using a T7-tag antibody Atgai-T7 protein was identified in bulk WT scion sample consisted of five scions in which *Atgai* mRNA was positively detected. Bulk samples (n = 5) of WT and *CgT* homogeneous grafts were used as negative and positive control, respectively. 25 μg protein was loaded in each lane.

Microarray data show the attenuation of GA response of WT scion on CgT *stock*

Three mRNA samples of the scions of GA treated WT*/*CgT* (n=5) in which were detected *Atgai* mRNA, GA treated WT*/WT (n = 5), and WT*/WT were prepared for microarray analysis. The marker * indicates the sample for RNA preparation. The microarray data concerning fold-changes of WT*/WT (GA+) vs WT*/WT (GA-) and WT*/CgT (GA+) vs WT/WT (GA-) were analyzed. The relationship between fold changed values of 18,588 unique genes, which changed more than 2.0 or less than 0.5, were shown in the scatter plot (Fig. I-10). The variation trend of fold change as a whole was approached to X-axis, which suggested the fold change of genes in WT*/WT (GA+) was stronger than that of WT*/*CgT* (GA+). In WT*/WT (GA+) vs WT*/*CgT* (GA+), the 415 significant different expressed genes were found (fold changes were over 2.0 and below 0.5). After GO analysis online (http://bioinfo.cau.edu.cn/agriGO/) and filter out the no-significant expressed genes, there were 153 annotated genes had different expression level between WT*/WT (GA+) and WT*/*CgT* (GA+). Heat map (Fig. I-11) of them shows the different fold change of GA response genes in scion WT/WT (GA+) and WT/*CgT* (GA+). Table I-4 shows that 88 % (134 of 153 genes) were higher in WT/WT (GA+) than that of WT*/*CgT* (GA+). One hundred twenty genes from WT*/WT (GA+) vs WT*/WT (GA-) were annotated to the GO resource IDs of GA response genes characterized in *Arabidopsis* (Cao et al. 2006). From intersect of 120 reported GA response genes (Cao et al. 2006) and significant different expressed 154 genes between WT*/WT (GA+) and WT*/*CgT* (GA+), 11 genes were found in the case, and 9 of which changed stronger in WT*/WT (GA+) than WT*/*CgT* (GA+) scions (table I-5). It was proved that the GA response in WT*/*CgT* (GA+) was weaker than WT*/WT (GA+) to a certain extent.

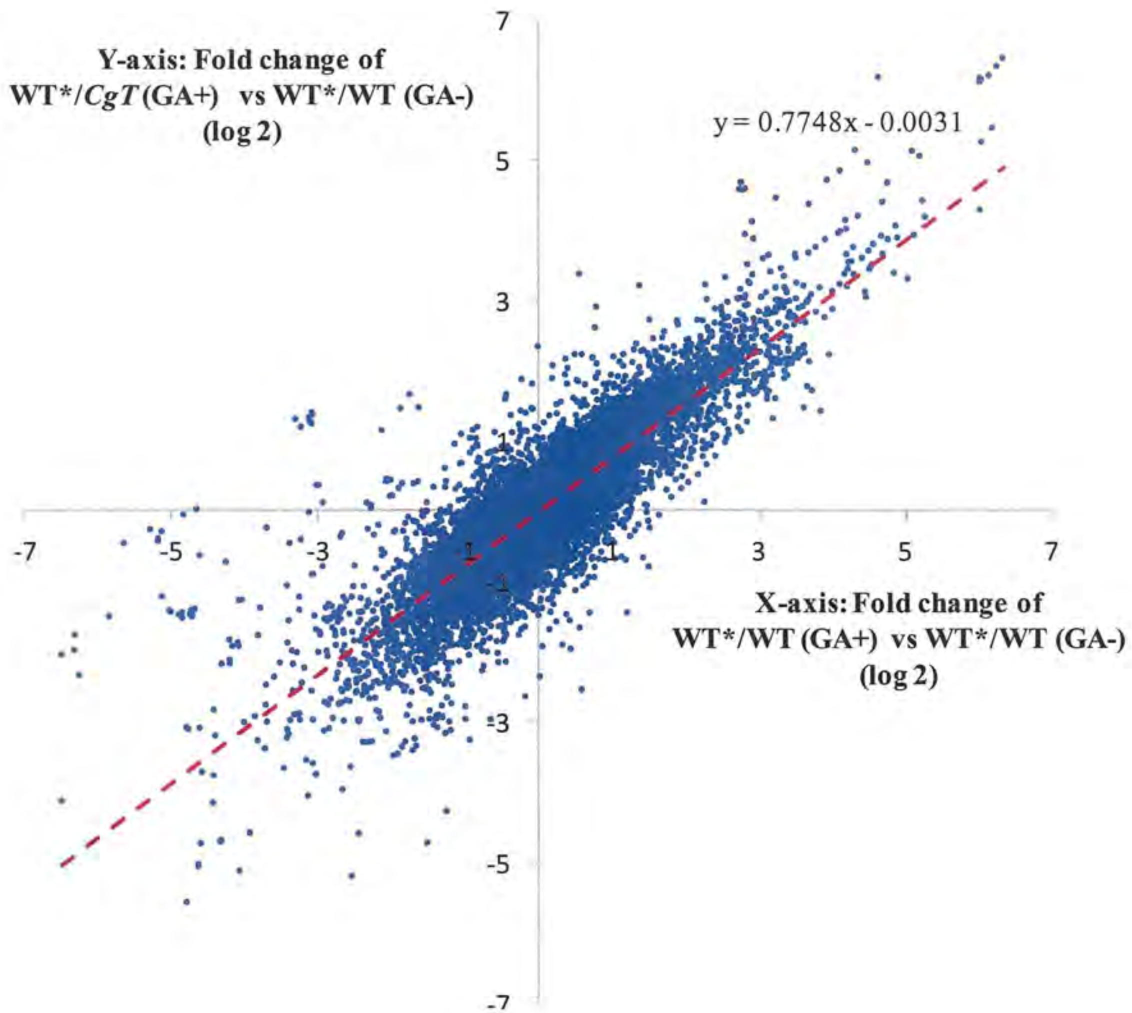

Figure I-10. Relationship of fold changes of 18,588 genes expressions in WT*/WT (GA+) vs WT*/WT (GA-) and of WT*/*CgT* (GA+) vs WT*/WT (GA-)

The numeral values were log 2 transformed and fold changes levels are plotted on the respective axis. Calculated regression line is shown by red broken line.

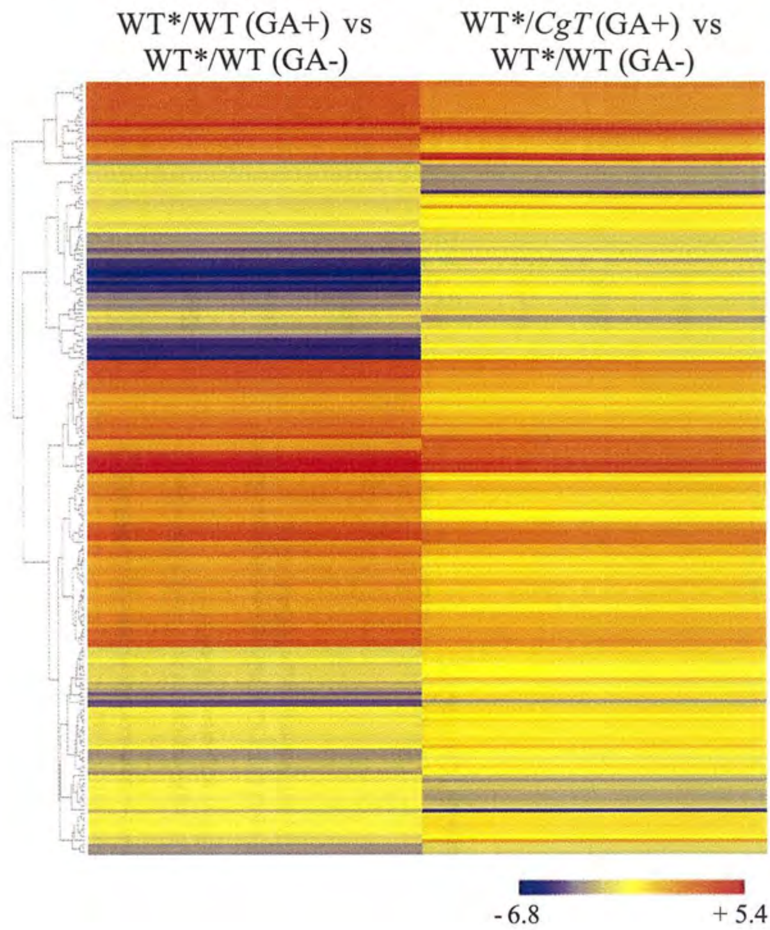

Figure I-11. Heat map of 153 significant different expressed GA response genes between WT*/WT (GA+) vs WT*/WT (GA-) and WT*/*CgT* (GA+) vs WT*/WT (GA-).

Table I-4. GA response genes which expressed at were significant different levels between WT*/WT (GA+) and WT*/*CgT* (GA+).

Mollecular Function	Probe ID	GO source	Description (Direct description or transfer from target)	P-value	Fold change (log 2)	
					WT*/WT(GA+) vs WT*/WT(GA-)	WT*/*CgT*(GA+) vs WT/*WT(GA-)
Catalytic activity	A_95_P114712	AT3G28180	Unknown	1E-59	2.9	1.3
	A_95_P007466	AT4G11280	gb|Nicotiana tabacum mRNA for 1-aminocyclopropane-1-carboxylate synthase [AJ005002]	0	2.6	1.1
	A_95_P230964	AT3G28180	gb|TT-41_A15 K326 early senescent leaf library Nicotiana tabacum cDNA, mRNA sequence [FG643198]	4E-83	2.7	1.2
	A_95_P226799	AT2G23910	gb|KF8C.106N10F.051215T7 KF8 Nicotiana tabacum cDNA clone KF8C.106N10, mRNA sequence [EB426691]	7E-52	3.1	1.6
	A_95_P146262	AT4G14130	Unknown	6E-95	4.5	3.1
	A_95_P252979	AT3G44190	gb|CHO_SL010xh07f1.ab1 CHO_SL Nicotiana tabacum cDNA, mRNA sequence [EH617697]	3E-89	3.3	2.1
	A_95_P160667	AT2G02800	Unknown	3E-45	3.5	2.2
	A_95_P233549	AT4G19230	Unknown	2E-95	4.9	3.4
	A_95_P121982	AT1G80160	Unknown	3E-45	5.0	3.3
	A_95_P161872	AT5G27930	Unknown	3E-72	2.5	1.3
	A_95_P247422	AT4G25810	Unknown	6E-35	5.1	3.9
	A_95_P146182	AT4G25810	Unknown	1E-87	3.6	2.3

(Table continues on following page.)

Table I-4. b *(Continued from previous page.)*

Mollecular Function	Probe ID	GO source	Description (Direct description or transfer from target)	P-value	Fold change (log 2)	
					WT*/WT(GA+) vs WT*/WT(GA-)	WT*/*CgT*(GA+) vs WT*/WT(GA-)
Catalytic activity	A_95_P010671	AT4G30270	tc|Rep: Xyloglucan endotransglucosylase-hydrolase XTH3 - Solanum lycopersicum (Tomato) (Lycopersicon esculentum), partial (82%) [TC79020]	1E-114	4.5	3.1
	A_95_P303148	AT1G60190	gb|TT-48_C07 K326 late senescent leaf library Nicotiana tabacum cDNA, mRNA sequence [FG645303]	1E-46	3.3	2.2
	A_95_P101828	AT4G11280	Unknown	2E-67	3.1	1.7
	A_95_P257129	AT3G46940	gb|CHO_SL027xp09f1.ab1 CHO_SL Nicotiana tabacum cDNA, mRNA sequence [EH622025]	3E-71	2.2	0.5
	A_95_P203527	AT1G11910	gb|KR3B.110O23F.051111T7 KR3B Nicotiana tabacum cDNA clone KR3B.110O23, mRNA sequence [DW005228]	8E-60	1.5	-0.2
	A_95_P228979	AT2G32990	gb|KP1B.104P14F.050725T7 KP1B Nicotiana tabacum cDNA clone KP1B.104P14, mRNA sequence [DV161055]	1E-105	1.5	0.1
	A_95_P115427	AT4G25980	Unknown	5E-75	1.4	-0.2
	A_95_P080310	AT5G65280	tc|Rep: Chromosome undetermined scaffold_431, whole genome shotgun sequence - Vitis vinifera (Grape), partial (67%) [TC118116]	4E-25	1.3	0.0
	A_95_P271601	AT3G11420	tc|Rep: Chromosome chr8 scaffold_23, whole genome shotgun sequence - Vitis vinifera (Grape), partial (46%) [TC101209]	1E-100	1.2	-0.1
	A_95_P085270	AT5G07920	Unknown	1E-27	1.2	0.0
	A_95_P149062	AT5G65280	tc|Rep: Chromosome undetermined scaffold_431, whole genome shotgun sequence - Vitis vinifera (Grape), partial (91%) [TC82088]	1E-116	1.2	0.2
	A_95_P148382	AT3G48540	gb|KG9B.102G18F.051126T7 KG9B Nicotiana tabacum cDNA clone KG9B.102G18, mRNA sequence [EB677673]	1E-86	1.1	-0.2

(Table continues on following page.)

Table I-4. c *(Continued from previous page.)*

Mollecular Function	Probe ID	GO source	Description (Direct description or transfer from target)	P-value	Fold change (log 2)	
					WT*/WT(GA+) vs WT*/WT(GA-)	WT*/*CgT*(GA+) vs WT*/WT(GA-)
Catalytic activity	A_95_P238509	AT2G36190	gb|Nicotiana tabacum NtINV mRNA for invertase, partial cds [AB055500]	9E-89	-1.0	0.2
	A_95_P255149	AT5G24530	gb|CHO_SL015xh04f2.ab1 CHO_SL Nicotiana tabacum cDNA, mRNA sequence [EH618915]	1E-111	-1.0	0.7
	A_95_P122017	AT5G11880	gb|N.tabacum mRNA for ornithine decarboxylase [Y10472]	6E-18	-1.2	0.0
	A_95_P154187	AT1G27320	tc|Rep: Histidine kinase 1 - Lupinus albus (White lupin), partial (27%) [TC113970]	1E-111	-1.3	0.0
	A_95_P001761	AT4G34131	gb|Nicotiana tabacum immediate-early salicylate-induced glucosyltransferase (IS5a) mRNA, complete cds [U32644]	1E-151	-1.5	-0.3
	A_95_P193832	AT5G07990	gb|Nicotiana tabacum clone D224-BD11 CYP92B2v1 mRNA, complete cds [DQ350327]	1E-103	-1.8	-0.1
	A_95_P006386	AT5G54160	gb|KR2B.112A09F.051230T7 KR2B Nicotiana tabacum cDNA clone KR2B.112A09, mRNA sequence [EB446561]	5E-73	-1.8	-0.4
	A_95_P042806	AT3G48990	Unknown	7E-68	-1.8	-0.5
	A_95_P078565	AT1G14220	Unknown	1E-15	-1.8	-0.8
	A_95_P012116	AT4G35090	gb|Nicotiana tabacum Petit Havana SR1 catalase (CAT-1) mRNA, complete cds [U07627]	0	-2.3	0.1
	A_95_P159477	AT2G39030	Unknown	8E-20	-2.3	0.1
	A_95_P153502	AT3G28580	gb|KR3B.113G16F.060119T7 KR3B Nicotiana tabacum cDNA clone KR3B.113G16, mRNA sequence [EB683753]	8E-90	-2.7	-0.3
	A_95_P002681	AT3G12500	Unknown	4E-26	-2.9	0.1

(Table continues on following page.)

Table I-4. d *(Continued from previous page.)*

Mollecular Function	Probe ID	GO source	Description (Direct description or transfer from target)	P-value	Fold change (log 2)	
					WT*/WT(GA+) vs WT*/WT(GA-)	WT*/*CgT*(GA+) vs WT*/WT(GA-)
Catalytic activity	A_95_P004526	AT1G15520	gb|CHO_SL024xg11f1.ab1 CHO_SL Nicotiana tabacum cDNA, mRNA sequence [EH622485]	2E-29	-2.9	-0.8
	A_95_P157492	AT1G05010	tc|Rep: 1-aminocyclopropane-1-carboxylate oxidase 3 - Petunia hybrida (Petunia), partial (98%) [TC92717]	3E-18	-3.2	-0.4
	A_95_P028711	AT3G12500	tc|Rep: Endochitinase 3 precursor - Nicotiana tabacum (Common tobacco), partial (70%) [TC85045]	2E-37	-4.6	-0.6
	A_95_P164712	AT4G16260	tc|Rep: Glucan endo-1,3-beta-glucosidase, basic vacuolar isoform GLB precursor ((1->3)-beta-glucan endohydrolase) ((1->3)-beta- glucanase) - Nicotiana tabacum (Common tobacco), partial (85%) [TC116956]	5E-60	-4.8	0.0
	A_95_P001721	AT4G16260	gb|N.tabacum beta-1,3-glucanase mRNA, clones pGL[28,30,31] [M20618]	4E-82	-5.1	-0.3
	A_95_P305858	AT3G12500	gb|basic chitinase [Nicotiana tabacum=tobacco, cv Samsun nn, floral bud day 7 explant, mRNA, 1156 nt] [S44869]	1E-113	-5.2	-0.4
	A_95_P105517	AT3G12500	Unknown	2E-73	-5.2	-0.4
	A_95_P179847	AT3G12500	gb|Tobacco (N.tabacum) endochitinase mRNA, partial cds [M15173]	1E-143	-5.6	-0.4
	A_95_P016576	AT1G60420	gb|KP1B.103C08F.050725T7 KP1B Nicotiana tabacum cDNA clone KP1B.103C08, mRNA sequence [DV160405]	2E-95	-2.0	-1.0
	A_95_P227954	AT1G05010	gb|Nicotiana tabacum mRNA for ACC oxidase, complete cds [AB012857]	1E-144	-2.6	-1.3
	A_95_P220957	AT2G37130	gb|KT7C.104O02F.051219T7 KT7 Nicotiana tabacum cDNA clone KT7C.104O02, mRNA sequence [EB449799]	2E-84	-2.7	-1.2
	A_95_P000344	AT2G39030	gb|Nicotiana tabacum mRNA for tyramine hydroxycinnamoyltransferase, partial [AJ131767]	6E-37	-3.0	-1.5

(Table continues on following page.)

Table I-4. e *(Continued from previous page.)*

Mollecular Function	Probe ID	GO source	Description (Direct description or transfer from target)	P-value	Fold change (log 2)	
					WT*/WT(GA+) vs WT*/WT(GA-)	WT*/*CgT*(GA+) vs WT*/WT(GA-)
Catalytic activity	A_95_P177857	AT1G78340	gb|CHO_SL008xp22f1.ab1 CHO_SL Nicotiana tabacum cDNA, mRNA sequence [EH621190]	4E-42	-3.2	-1.4
	A_95_P018851	AT1G17180	tc|Rep: Probable glutathione S-transferase parA - Nicotiana tabacum (Common tobacco), complete [TC92678]	4E-53	-3.4	-1.5
	A_95_P007891	AT1G23820	gb|Nicotiana tabacum mRNA for putrescine N-Methyltransferase, complete cds [D28506]	1E-120	-3.8	-1.8
	A_95_P158327	AT4G23160	Unknown	8E-29	-4.2	-1.9
	A_95_P180922	AT3G09270	gb|KL4B.101D17F.051103T7 KL4B Nicotiana tabacum cDNA clone KL4B.101D17, mRNA sequence [DV999163]	1E-47	-4.4	-3.2
	A_95_P246738	AT2G47880	gb|KL4B.105P10F.051104T7 KL4B Nicotiana tabacum cDNA clone KL4B.105P10, mRNA sequence [DW000487]	1E-33	-4.8	-3.1
	A_95_P158977	AT5G58390	Unknown	3E-37	-4.8	-1.5
	A_95_P004536	AT5G58390	gb|AGN_RNC012xl06f1.ab1 AGN_RNC Nicotiana tabacum cDNA 5', mRNA sequence [FG164960]	1E-47	-5.1	-1.2
	A_95_P164182	AT4G02340	tc|Rep: Epoxide hydrolase - Nicotiana tabacum (Common tobacco), partial (34%) [TC106386]	2E-32	-5.8	-1.5
Binding	A_95_P163062	AT1G76650	Unknown	6E-17	4.0	2.2
	A_95_P287973	AT4G37260	gb|AGN_RNC030xp08f1.ab1 AGN_RNC Nicotiana tabacum cDNA 5', mRNA sequence [FG155793]	1E-45	3.9	1.4
	A_95_P000091	AT1G07790	gb|TT-06_B09 Burley21 trichome library Nicotiana tabacum cDNA, mRNA sequence [FG638714]	4E-38	3.6	2.3
	A_95_P107002	AT5G65360	gb|TT-31_K08 K326 trichome library Nicotiana tabacum cDNA, mRNA sequence [FG641470]	5E-71	3.6	2.3

(Table continues on following page.)

Table I-4. f *(Continued from previous page.)*

Mollecular Function	Probe ID	GO source	Description (Direct description or transfer from target)	P-value	Fold change (log 2)	
					WT*/WT(GA+) vs WT*/WT(GA-)	WT*/*CgT*(GA+) vs WT*/WT(GA-)
Binding	A_95_P110157	AT2G28740	Unknown	9E-38	3.6	2.2
	A_95_P195022	AT5G65360	tc|Rep: Histone H3.2 - Encephalartos altensteinii (Altenstein's bread tree) (Cycad), complete [TC89811]	5E-71	3.4	2.2
	A_95_P107987	AT3G45980	Unknown	1E-10	3.4	2.3
	A_95_P016011	AT5G27670	tc|Rep: H2A histone - Nicotiana tabacum (Common tobacco), complete [TC86759]	2E-61	3.4	2.0
	A_95_P103522	AT3G46030	Unknown	1E-65	3.4	2.0
	A_95_P002516	AT5G27670	gb|AGN_RNC001xi15f1.ab1 AGN_RNC Nicotiana tabacum cDNA 5', mRNA sequence [FG170570]	7E-62	3.4	1.9
	A_95_P117777	AT1G62510	tc|Rep: HyPRP2 - Gossypium hirsutum (Upland cotton) (Gossypium mexicanum), partial (92%) [TC82793]	1E-41	3.2	1.3
	A_95_P185322	AT5G59910	gb|TT-10_I08 Burley21 trichome library Nicotiana tabacum cDNA, mRNA sequence [FG638902]	1E-69	3.1	1.7
	A_95_P017396	AT5G65360	gb|ntb0510 Tobacco cultivar SR1 basal cell cDNA library Nicotiana tabacum cDNA, mRNA sequence [HS083569]	5E-71	3.1	1.7
	A_95_P033754	AT1G07790	tc|Rep: Histone H2B - Capsicum annuum (Bell pepper), partial (97%) [TC91050]	2E-43	3.1	1.9
	A_95_P000741	AT2G28740	tc|Rep: Histone H4 [Contains: Osteogenic growth peptide (OGP)]. - Rattus norvegicus, complete [TC108297]	1E-53	3.1	1.9
	A_95_P110737	AT2G28740	gb|ntb1876 Tobacco cultivar SR1 basal cell cDNA library Nicotiana tabacum cDNA, mRNA sequence [HS084935]	6E-19	2.9	1.7

(Table continues on following page.)

Table I-4. g *(Continued from previous page.)*

Mollecular Function	Probe ID	GO source	Description (Direct description or transfer from target)	P-value	Fold change (log 2)	
					WT*/WT(GA+) vs WT*/WT(GA-)	WT*/*CgT*(GA+) vs WT*/WT(GA-)
Binding	A_95_P245527	AT1G14900	gb|KF8C.104A14F.051214T7 KF8 Nicotiana tabacum cDNA clone KF8C.104A14, mRNA sequence [EB425696]	1E-47	2.9	1.7
	A_95_P301618	AT2G38540	gb|TT-06_L15 Burley21 trichome library Nicotiana tabacum cDNA, mRNA sequence [FG638745]	2E-25	2.9	1.8
	A_95_P110442	AT1G08880	tc|Rep: Histone H2AX - Picea abies (Norway spruce) (Picea excelsa), partial (95%) [TC92091]	3E-50	2.7	1.4
	A_95_P127082	AT2G45190	gb|KF8C.106E17F.051215T7 KF8 Nicotiana tabacum cDNA clone KF8C.106E17, mRNA sequence [EB426500]	2E-41	2.7	1.4
	A_95_P241195	AT1G51060	gb|AGN_RPC008xm20f1.ab1 AGN_RPC Nicotiana tabacum cDNA 5', mRNA sequence [FG146706]	2E-64	2.4	1.0
	A_95_P178467	AT5G02560	gb|KG9B.106H02F.051129T7 KG9B Nicotiana tabacum cDNA clone KG9B.106H02, mRNA sequence [EB679141]	4E-62	2.1	1.4
	A_95_P131117	AT4G30080	gb|TT-06_J07 K326 early senescent leaf library Nicotiana tabacum cDNA, mRNA sequence [FG641981]	1E-33	2.4	1.0
	A_95_P201792	AT2G28740	tc|Rep: Histone H4 - Tetraodon nigroviridis (Green puffer), partial (45%) [TC101472]	1E-53	2.3	0.7
	A_95_P140702	AT5G23420	tc|Rep: Chromosome undetermined scaffold_133, whole genome shotgun sequence - Vitis vinifera (Grape), partial (50%) [TC88622]	3E-37	2.2	0.8
	A_95_P116842	AT5G23420	tc|Rep: Chromosome undetermined scaffold_133, whole genome shotgun sequence - Vitis vinifera (Grape), partial (50%) [TC88622]	4E-48	2.2	0.9
	A_95_P264166	AT1G01370	gb|Nicotiana tabacum NtCENH3-1 mRNA for centromere specific histone H3 variant, complete cds [AB366152]	3E-40	2.0	0.9
	A_95_P094463	AT5G45550	tc|Rep: Mob1-like protein - Medicago falcata (Sickle medic), partial (84%) [TC101485]	6E-74	2.0	0.5

(Table continues on following page.)

Table I-4. h *(Continued from previous page.)*

Mollecular Function	Probe ID	GO source	Description (Direct description or transfer from target)	P-value	Fold change (log 2)	
					WT*/WT(GA+) vs WT*/WT(GA-)	WT*/*CgT*(GA+) vs WT*/WT(GA-)
Binding	A_95_P105207	AT2G28740	Unknown	7E-48	2.0	0.8
	A_95_P007641	AT2G29570	gb|Nicotiana tabacum mRNA for proliferating cell nuclear antigen [AJ012662]	1E-135	2.0	0.3
	A_95_P132937	AT3G20670	tc|Rep: Histone H2A - Vitis vinifera (Grape), partial (96%) [TC94217]	3E-27	1.9	0.5
	A_95_P139492	AT2G45660	Unknown	9E-70	1.7	0.6
	A_95_P152122	AT2G28740	tc|Rep: Histone H4 [Contains: Osteogenic growth peptide (OGP)]. - Rattus norvegicus, complete [TC104938]	1E-46	1.7	0.5
	A_95_P114782	AT3G12630	Unknown	1E-33	1.7	0.5
	A_95_P277018	AT5G61000	gb|AM826144 COL, cold overnight library Nicotiana tabacum cDNA clone nt006170050, mRNA sequence [AM826144]	1E-84	1.6	0.3
	A_95_P032471	AT3G54810	gb|Nicotiana tabacum AGP1 mRNA for AG-motif binding protein-1, complete cds [AB107689]	3E-61	1.5	0.5
	A_95_P148457	AT3G14450	Unknown	7E-50	1.5	-0.8
	A_95_P109467	AT1G76100	Unknown	7E-44	1.5	0.4
	A_95_P272296	AT1G01200	gb|KT7C.101J16F.051216T7 KT7 Nicotiana tabacum cDNA clone KT7C.101J16, mRNA sequence [EB449005]	1E-33	1.5	-0.1
	A_95_P293653	AT1G19180	gb|AGN_RNC015xl11f1.ab1 AGN_RNC Nicotiana tabacum cDNA 5', mRNA sequence [FG163164]	6E-16	1.5	0.0

(Table continues on following page.)

Table I-4. i *(Continued from previous page.)*

Mollecular Function	Probe ID	GO source	Description (Direct description or transfer from target)	P-value	Fold change (log 2)	
					WT*/WT(GA+) vs WT*/WT(GA-)	WT*/*CgT*(GA+) vs WT*/WT(GA-)
Binding	A_95_P112757	AT1G12090	Unknown	1E-32	1.2	0.2
	A_95_P003546	AT5G67300	gb|Nicotiana tabacum B19 mRNA for Myb-like protein, complete cds [AB032540]	1E-73	1.2	0.0
	A_95_P133842	AT3G20670	Unknown	9E-36	1.2	-0.1
	A_95_P276573	AT3G02150	tc|Rep: Chromosome chr14 scaffold_9, whole genome shotgun sequence - Vitis vinifera (Grape), partial (25%) [TC101833]	1E-39	-1.2	-0.1
	A_95_P150647	AT3G12560	gb|KL4B.111F11F.060128T7 KL4B Nicotiana tabacum cDNA clone KL4B.111F11, mRNA sequence [EB680776]	6E-15	-1.3	0.0
	A_95_P159487	AT5G14040	tc|Rep: Mitochondrial phosphate transporter - Lotus japonicus, partial (88%) [TC81227]	1E-105	-1.3	-0.1
	A_95_P145322	AT1G56210	gb|gzyksnj3-B123 Normalized cDNA Library Tobacco Leaves and Flowers Nicotiana tabacum cDNA clone B123 similar to similar to Nicotiana tabacum cDNA clone, mRNA sequence [HO663983]	2E-11	-1.4	-0.2
	A_95_P153262	AT3G17100	gb|KR3B.112N15F.060119T7 KR3B Nicotiana tabacum cDNA clone KR3B.112N15, mRNA sequence [EB683540]	1E-46	-1.4	-0.2
	A_95_P039071	AT2G01690	Unknown	5E-20	-1.4	-0.2
	A_95_P310908	AT1G27840	gb|AGN_RNC127xb09f1.ab1 AGN_RNC Nicotiana tabacum cDNA 5', mRNA sequence [FG172547]	2E-91	-1.5	-0.2
	A_95_P125307	AT2G40140	tc|Rep: Cys-3-His zinc finger protein - Capsicum annuum (Bell pepper), partial (22%) [TC87722]	6E-21	-1.5	-0.5
	A_95_P269096	AT2G40140	gb|KR3B.107K05F.051110T7 KR3B Nicotiana tabacum cDNA clone KR3B.107K05, mRNA sequence [DW004053]	7E-19	-1.7	-0.3
	A_95_P112757	AT1G12090	Unknown	1E-32	1.2	0.2

(Table continues on following page.)

Table I-4. j *(Continued from previous page.)*

Mollecular Function	Probe ID	GO source	Description (Direct description or transfer from target)	P-value	Fold change (log 2)	
					WT*/WT(GA+) vs WT*/WT(GA-)	WT*/*CgT*(GA+) vs WT*/WT(GA-)
Binding	A_95_P159652	AT3G43810	gb|CHO_SL011xa07f1.ab1 CHO_SL Nicotiana tabacum cDNA, mRNA sequence [EH618070]	1E-51	-2.0	0.2
	A_95_P202642	AT1G48160	gb|BP131040 MAT001 Nicotiana tabacum cDNA clone BY3062, mRNA sequence [BP131040]	2E-47	-2.8	-1.7
	A_95_P120617	AT1G68520	gb|KL4B.108E04F.051105T7 KL4B Nicotiana tabacum cDNA clone KL4B.108E04, mRNA sequence [DW000578]	2E-42	-3.3	-2.0
	A_95_P249267	AT3G04720	gb|Nicotiana tabacum pathogen- and wound-inducible antifungal protein CBP20 (CBP20) mRNA, complete cds [S72452]	4E-75	-3.8	-1.4
	A_95_P154892	AT5G65310	Unknown	4E-43	-4.2	-2.1
	A_95_P161357	AT1G62790	gb|CHO_SL010xf02f1.ab1 CHO_SL Nicotiana tabacum cDNA, mRNA sequence [EH617763]	2E-24	-6.5	-2.0
	A_95_P103227	AT5G07090	Unknown	6E-29	1.5	0.4
Structural molecule activity	A_95_P100938	AT1G21310	gb|N.tabacum mRNA for extensin [X71602]	1E-101	-4.7	-1.4
	A_95_P006201	AT1G21310	tc|Rep: PAP8 product - Nicotiana tabacum (Common tobacco), complete [TC95737]	4E-30	-4.7	-1.5
	A_95_P100928	AT1G21310	gb|Nicotiana tabacum clone PR42 mRNA sequence [AF154654]	1E-22	-4.7	-1.4
	A_95_P225452	AT1G21310	gb|Nicotiana tabacum clone PR38 mRNA sequence [AF154651]	1E-44	-5.0	-1.4
Transporter activity	A_95_P147092	AT4G10310	tc|Rep: Chromosome chr11 scaffold_118, whole genome shotgun sequence - Vitis vinifera (Grape), partial (52%) [TC92492]	2E-52	1.0	-0.4

(Table continues on following page.)

Table I-4. k *(Continued from previous page.)*

Mollecular Function	Probe ID	GO source	Description (Direct description or transfer from target)	P-value	Fold change (log 2)	
					WT*/WT(GA+) vs WT*/WT(GA-)	WT*/*CgT*(GA+) vs WT*/WT(GA-)
Transporter activity	A_95_P163192	AT2G38940	Unknown	4E-91	-1.2	0.0
	A_95_P259986	AT3G21690	gb|Nicotiana tabacum NtMATE1 mRNA for multi antimicrobial extrusion family protein, complete cds [AB286961]	0	-1.3	0.0
	A_95_P164107	AT2G38940	Unknown	1E-107	-1.6	-0.3
	A_95_P007331	AT2G38940	gb|Nicotiana tabacum NtPT2 mRNA for phosphate transporter, complete cds [AB042950]	0	-1.8	-0.8
	A_95_P019116	AT5G40780	gb|KR2B.103D07F.051227T7 KR2B Nicotiana tabacum cDNA clone KR2B.103D07, mRNA sequence [EB444274]	1E-103	-1.9	0.3
	A_95_P219747	AT1G71680	gb|CHO_SL015xh07f2.ab1 CHO_SL Nicotiana tabacum cDNA, mRNA sequence [EH618831]	5E-13	-2.0	0.5
	A_95_P054931	AT5G65380	tc|Rep: Chromosome chr18 scaffold_1, whole genome shotgun sequence - Vitis vinifera (Grape), partial (40%) [TC106687]	3E-54	-2.7	-1.5
	A_95_P195642	AT5G65980	gb|CHO_SL025xe13f1.ab1 CHO_SL Nicotiana tabacum cDNA, mRNA sequence [EH621524]	8E-65	-3.4	-1.0
	A_95_P268451	AT1G30690	gb|KR2B.105P21F.051227T7 KR2B Nicotiana tabacum cDNA clone KR2B.105P21, mRNA sequence [EB445078]	4E-10	-3.5	-2.1
Enzyme regulator activity	A_95_P111642	AT2G02100	Unknown	1E-20	1.0	-0.4
	A_95_P185272	AT1G17860	gb|KF8C.104N02F.051214T7 KF8 Nicotiana tabacum cDNA clone KF8C.104N02, mRNA sequence [EB425965]	6E-20	-3.0	0.4
Molecular transducer activity	A_95_P151872	AT4G02510	gb|AGN_RNC030xi19f1.ab1 AGN_RNC Nicotiana tabacum cDNA 5', mRNA sequence [FG155337]	4E-68	-2.5	-1.1

Table I-5. Fold changes of GA response genes in WT*/WT (GA+) scion which significant different from that of WT*/*CgT* (GA+) compared with Microarray data of *Arabidopsis* (Cao et al. 2006)

Mollecular Function	Probe ID	GO source	GO Term	Molecular function description	P-value	Fold change (log 2)	
						WT/WT(GA+) vs WT/WT(GA-)	WT/*CgT*(GA+) vs WT/WT(GA-)
Catalytic activity	A_95_P178107	AT5G13870	GO:0016798	Hydrolase activity, acting on glycosyl bonds	1E-127	2.0	0.9
	A_95_P193832	AT5G07990	GO:0009411	Response to UV	1E-103	-1.8	-0.1
			GO:0019825	Oxygen binding			
			GO:0016711	Flavonoid 3'-monooxygenase activity			
			GO:0009813	Flavonoid biosynthetic process			
Binding	A_95_P112757	AT1G12090	GO:0008289	Lipid binding	1E-32	1.2	0.2
			GO:0006869	Lipid transport			
	A_95_P277018	AT5G61000	GO:0005634	Cell nucleus	1E-84	1.6	0.3
			GO:0006260	DNA replication			
			GO:0003677	DNA binding			
	A_95_P301618	AT2G38540	GO:0006869	Lipid transport	2.00E-25	2.9	1.8
			GO:0009505	Plant-type cell wall			
			GO:0005516	Calmodulin binding			
Transporter activity	A_95_P019116	AT5G40780	GO:0016020	Membrane	1E-103	-1.9	0.3
			GO:0043090	Amino acid import			
			GO:0015171	Amino acid transmembrane transporter activity			
	A_95_P054931	AT5G65380	GO:0015238	Drug transmembrane transporter activity	3.00E-54	-2.7	-1.5
			GO:0006855	Drug transmembrane transport			
			GO:0016020	Membrane			
			GO:0015297	Antiporter activity			
			GO:0009835	Ripening			
	A_95_P054931	AT5G65380	GO:0005342	Organic acid transmembrane transporter activity	3.00E-88	-1.1	1.0
			GO:0046943	Carboxylic acid transmembrane transporter activity			
Enzyme regulator activity	A_95_P185272	AT1G17860	GO:0005618	Cell wall	6E-20	-3.0	0.4
			GO:0048046	Apoplast			
			GO:0008150	Biological_process			
			GO:0004866	Endopeptidase inhibitor activity			

I-4 Discussion

The lack of the DELLA domain of *GAI* causes a constitutively active mutant growth inhibitor whose genetically dominant action can no longer be opposed by GA. Since *gai* is a semidominant and gain-of-function mutation (Peng and Harberd 1993), the integration of *gai* into WT leads to semidwarf phenotypte which have contributed for the Green Revolution (Peng et al. 1999). Therefore, to reduce the stature, several crops were transformed by ΔDELLA GAI such as *Arabidopsis gai-1* allele, rice (Fu et al. 2001), *Chrysanthemum* (Petty et al. 2003), tobacco (Busou et al. 2006), and apple (Zhu et al. 2008). In all these reports, *ΔDELLA GAI* was expressed under *CaMV 35S* (*Cauliflower mosaic virus* promoter 35S). Although DELLA protein genes are expressed ubiquitously in all tissues (Tyler et al. 2004), detailed expression site on a cell level is little known.

On the other hand, *GAI* mRNA can move long-distantly through phloem and is considered to function at the transported site(s) (Haywood et al. 2005; Ham et al. 2009; Huang and Yu 2009). As plasmodesmata inter connect the functional enucleate sieve elements of the phloem to their neighboring companion cells (Turgeon 1996), this pathway is considered to allow the selective entry of information macromolecules into the phloem translocation stream. In this study, the *Atgai* was drived by the promoter of a plant virus CoYMV, which expresses strongly in only companion cells (Giffins et al. 2003). Intriguingly, the *CgT* transgenic tobacco plants also exhibited semidwarf phenotypes as well as the cases using of 35S promoter, indicating that only companion cell expression of the *gai* can function to lead the dwarf phenotype. Therefore, this result suggested that *gai* transcript in companion cell acts non-cell-autonomously to the growth in plants. The less-sensitive response to GA_3 treatment of *CgT* plants also supports the non-cell-autonomous effect of *gai* transcript, because gibberellic acid affects to the whole cells in plant. Furthermore, the experiments with GA_3 treatment to the grafts clearly proves that the effect of *gai* gene exhibits transmissible action through graft union. Haywood et al. (2005) proposed that the harmonious integration of growth modalities between plant organs might be balanced through the transport of *GAI*.
The low efficiencies of graft transmission have been reported for RNA movement (Sjölund et al. 1997; Brosnan et al. 2007; Pant et al. 2007; Lin et al. 2008). In this study, not only the integrated *Atgai* but also the indigenous *N. benthamiana GAI* are considered to transport by the same molecular mechanism. Through the intimate analysis of both genes transcripts transported via graft union, the physiological role of GAI long-distantly movement would be

elucidated.

Approximately one-fourth of grafts could not show the RT-PCR product derived from the transported *gai* mRNA from the stock. Long-distance transport of RNA in sieve tubes appears to be mediated by RNA-binding proteins (Carlsbecker et al. 2010; Martin et al. 2009). Ham et al. (2010) identified RNA-binding proteins involved in mRNA transport, and proposed a model in which a ribonucleoprotein complex moves in the phloem. It is clear that RNA also binds to chaperone proteins for stability and delivery to target tissues. As a matter of course, this large complex must pass the graft union, where vascular bundles are developed in the callus at the union. The *de novo* sieve tube passage is prone to be unorthodox, showing features such as a winding path, disrupting the passage of the large ribonucleoprotein complex. In an extreme case, the complex would become clogged. Since the conductance of a vessel is proportional to the fourth power of the vessel radius (Hagen-Poiseulle law), a slightly reduced diameter would pose an obstacle to passage. On the other hand, grafting of many horticultural crops is a well-developed technology; these plants may experience less of a problem.

There was no report concerning the identification of the translated protein derived from the transported *gai* mRNA using graft experiment. In the WT scion on the *CgT* stock, the fused-*gai* protein with the T7 tag peptide was clearly detected. Its amount was considered to be approximately one-third of that in CgT stock, indicating an effective translation of the gai mRNA in the scion. This resultant is the first report on the detection of translated product of long-distant transported mRNA in plants. It is interesting to investigate how far the translated product can be traveled from the graft union. The microarray experiment was also the first report concerning the long-distant transport mRNA. The whole picture of the results clearly suggested that in the WT scion on the *CgT* stock the transcriptions of many GA-responsive genes were affected to reduce the responses. Trough this study, the characterization of the long-distant *gai* mRNA in plants made substantial progress. This achievement could help researchers better understand the molecular mechanism of not only GAI but also other phloem transportable mRNA.

I-5 Summary

The transcript of *GIBBERELLIC ACID INSENSITIVE* (*GAI*), a negative regulator of gibberellic acid (GA) responses, was identified the transport ability through phloem. Therefore, the *gai* gene formed by deletion of *GAI* DELLA domain induces dwarf or semi-dwarf phenotypes, and the transport ability could explain why scions grafted onto *gai*-overexpressing transformants show dwarfism. To make practical use of this system, I wanted to characterize the *gai* mRNA transport through graft union. *Arabidopsis gai* gene (*Atgai*) was fused with T7 epitope tag and expressed under control of a companion cell specific expression promoter, *Commelina yellow mottle virus* promoter (*CoYMV*p). The resulting construct *CoYMVp: Atgai-T7* (*CgT*) was transformed into *Nicotiana benthamiana*. These transforms showed a dwarf phenotype and less insensitivity to GA_3. About three weeks after grafting between wild type (WT) scion and *CgT* rootstock, *Atgai* mRNA was detected in the WT scion by RT-PCR and qRT-PCR which revealed that the transported amount of the mRNA was varied among individuals. Atgai-T7 protein was also identified in the WT scion grafted on the transgenic rootstock which revealed that *gai* transcripts can be translated after transport through graft junction. Microarray analysis to find out the effect of *CgT* stock on the gene expression pattern in the scion clearly revealed that *CgT* stock reduced the GA-responsiveness of the WT scion. The new knowledge concerning the mRNA long-transport would be useful for the application of this system in horticulture crops using grafting.

Chapter II

Long-distance transport of *GAI* mRNA in *Malus* species

II-1 Introduction

Grafting is a common means of asexual propagation of fruit trees (Westwood 1993). It involves joining two genetically distinct plants—the rootstock and the scion—so that they continue growth as a single plant. Over the centuries, orchardists have selected rootstocks that confer advantageous characters, such as stress tolerance on scions (Tubbs 1973). Rootstocks that cause dwarfing of the scion are popular in apple production (Olien and Lasko 1984). However, the molecular mechanisms of how rootstocks bring about these effects are still largely unknown. Many studies have been undertaken to understand and improve the effects of rootstocks on scion growth and most of the studies have argued that the graft union controls the circulation of hormones, the movement of assimilates, and the uptake of water and nutrients (Webster 1994).

Advanced molecular biological techniques have revealed the presence of mRNAs in the phloem sap of castor bean (Doering-Saad et al. 2006), melon (Gomez et al. 2005; Omid et al. 2007), barley (Gaupels et al. 2008), cucumber (Ruiz-Medrano et al. 1999), and *Arabidopsis* (Deeken et al. 2008). Grafting experiments have proven the long-distance transport of mRNAs via the sieve element, which would appear to affect the graft partners (Kim et al. 2001; Haywood et al. 2005; Banerjee el al. 2006; Banerjee et al. 2009). Therefore, elucidation of the molecular basis of long-distance mRNA transport in plants and its importance in crop species propagated through grafting has attracted a lot of attention.

GIBBERELLIC ACID INSENSITIVE (*GAI*) negatively regulates responses to gibberellic acid (GA) (Harberd et al. 2009). A gain-of-function mutant (*gai*) of *GAI* induces dwarfing in some plant species. The GAI mRNA transport via the phloem could explain why scions grafted onto *gai*-overexpressing transformants show dwarfism (Haywood et al. 2005; Huang and Yu 2009). However, the molecular and cellular mechanisms of this transport and action are poorly understood. To make practical use of this system, we have studied the transport of *GAI* in apple (*Malus* species). Here we report that *GAI* transcripts travel in both upward and downward directions through the graft union in apple.

II-2 Materials and methods

Plant materials

Cultures of apple (*Malus ×domestica* Borkh. cv. Fuji) and wild apple (*Malus xiaojinensis* Cheng et Jiang) were subcultured every five weeks on MS medium (Murashige and Skoog 1962) containing 0.5 mg L^{-1} benzylaminopurine, 0.5 mg L^{-1} indole acetic acid, and 0.8% (w/v) agar (Difco Bacto, Kansas, USA) at 25°C day, 23°C night, with a 14-h photoperiod of 50 μmol m^{-2} s^{-1} photosynthetically active radiation provided by cool-white fluorescent tubes.

Grafting of shoots

Four-week-old shoots of *M. xiaojinensis* (*M.x*) as stock and 'Fuji' as scion were used. About 1.5 cm of the stock was prepared with a horizontal transverse cut, in which a snick of about 5 mm deep was made. About 1.5 cm of the scion was cut from its culture in a wedge shape and then V-grafted onto the stock. The graft union was fastened with aluminum foil (10 mm wide × 7 mm high), and the grafted plants were grown on 1/2 MS medium with 0.1 mg L^{-1} indole butyric acid, 0.1 mg L^{-1} GA_3, and 0.8% (w/v) agar (Figure II-1 A). Tissues were sampled every day from 1 to 5 days, and every five days to 35 days after grafting (Figure II-2 A). As the control, Fuji/Fuji and *M. x/M. x* were made. Tissues sampled were shoot apexes, scion leaves, scion stem phloem, graft junction, stock stem phloem, and stock roots.

DNA and RNA extraction

Total genomic DNA was extracted from the shoots by the CTAB (cetyltrimethylammonium bromide) method of Doyle and Doyle (1990). The DNA fraction was treated with RNase A. Total RNA was isolated by the CTAB method of Chang et al. (1993) and treated with RNase-free DNase I (TaKaRa, Dalian, China).

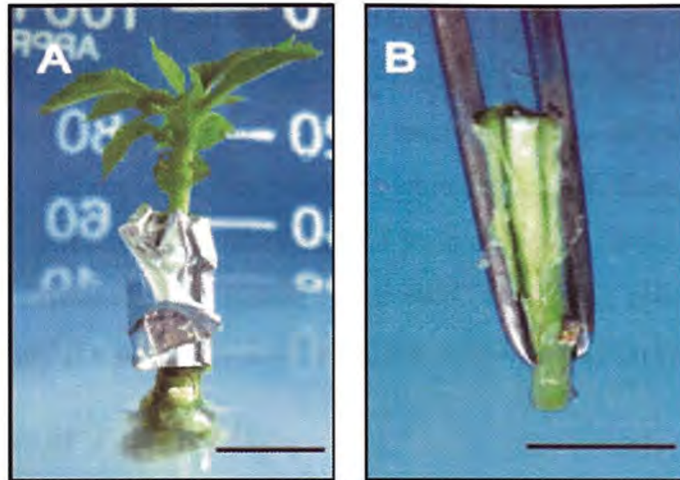

Figure II-1. Grafting materials.

(A) 'Fuji' scion on *M. x* rootstock at 35 days after grafting. (B) Separation of inner tissue (xylem) and outer tissue (phloem) of the scion stem. Bars=1 cm.

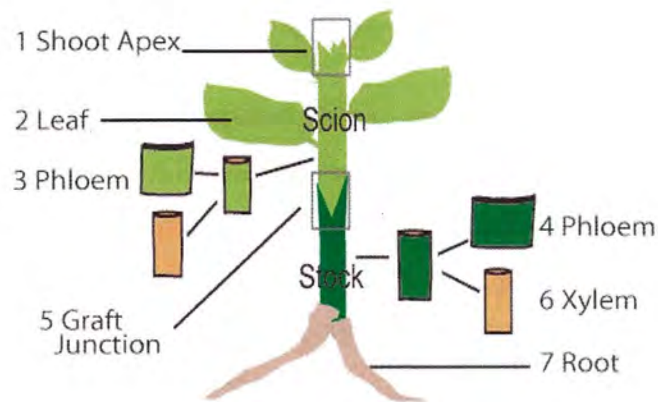

Figure II-2. Sampled materials.

'Fuji' scion was grafted onto *M. x* rootstock, and then tissues were harvested for analysis.

MdActin *PCR using gDNA and cDNA*

In order to confirm that RNA fractions prepared by us were no contamination of DNA, we used an intron sequence of MdActin (Apple EST Mdfwf, accession no. GO513076.1).The sequence was amplified from 'Fuji' gDNA fraction using primers MdActinF1 and MdActinR1 (Table 1), and the amplified products were sequenced. Then, primers MdActinF2 and R2 and the nested primers MdActinF3 and R3 were prepared (Table 1). The amplification conditions were as follows: initial denaturation at 94°C for 4 min, followed by 30 cycles at 94°C for 30 s, 58°C for 30 s, and 72°C for 2 min, and a final extension at 72°C for 3 min.

Cloning of GAI alleles of **'Fuji'** *and* **M. x**

Degenerate primers GAIdg5 and GAIdg3 (Table 1) were designed from *GAI* sequences of other plants in the NCBI database. Polymerase chain reaction (PCR) using these primers amplified 1710-bp DNA fragments from both 'Fuji' and *M. x*. The 3′ and 5′ ends of these PCR products were produced by DNA walking using TaKaRa LA PCR in vitro Cloning Kit (TaKaRa) and 5′-RACE using the SMART RACE cDNA Amplification Kit (Clontech, Palo Alto, CA, USA).

RT-PCR and CAPS (cleaved amplified polymorphic sequence) analysis

Reverse-transcribed cDNAs were prepared with oligo(dT) in an M-MLV cDNA Synthesis Kit (Promega, Madison, WI, USA). The shared primers GAIf and GAIr (Table 1) were designed to amplify the GAI coding regions in *MdGAI1*, *MdGAI2*, and *MxGAI1*. The reverse transcriptase (RT)-PCR product was 1912 bp. PCR was performed using the following reaction conditions: 5 min at 94°C followed by 35 cycles at 94°C for 40 s, 60°C for 40 s, and 72°C for 40 s, and a final extension at 72°C for 7 min. The RT-PCR products were digested with SduI (MBI Fermentas, St. Leon-Rot, Germany) and fractionated by electrophoresis on a 3% agarose gel.

Table II-1. Primer sequences used in this study.

Primers	Sequences
GAIdg5	5′-GCKGTKYTDGGKTACAAGGT-3′*
GAIdg3	5′-AGCCGAGGTGRCDATGAGCG-3′
GAIf	5′-AAATGAAAAGGGAGCACCAG-3′
GAIr	5′-CAGTGAGCGATGACCGAGTT-3′
MdActinF1	5′-TTGGGTTTAGAGGCTCATGG-3′
MdActinR1	5′-CACCCACGCTGAATACATTG-3′
MdActinF2	5′-CAAATCTTCCCAGGCAAAAA-3′
MdActinR2	5′-CAGAAGAAGTTGGCAGTCCA-3′
MdActinF3	5′-GTGTTTGGCGTTTCCATTCT-3′
MdActinR3	5′-CACCAGATCAATGCAATTCCT-3′
MdGAIprobe5	5′-CCTCTTCCAACTCGGTCATC-3′
MdGAIprobe3	5′-ACACTTTGACGTGCCCACA-3′

*K (G, T); Y (C, T); D (G, A, T); R (A, G).

In situ-*hybridization*

The segment between the apex and base of the 'Fuji' scions (~6 cm long) was used for *in situ* hybridization. Segments were fixed in ethanol: acetic acid (3: 1) for 15 h at 4°C, and then dehydrated through graded ethanol series containing 25%, 50% and 75% Lemosol (Wako Pure Chemical Industries, Ltd., Osaka, Japan). Finally, the materials were embedded in Paraplast Plus (Sherwood Medical, St. Louis, MO, USA). Ten-micrometer slices were cut by microtome and mounted on glass slides. To create the *in situ* hybridization probe for *MdGAI*, a 403-base fragment consisting of the 3′ coding region (29 bp) and 3′-UTR (374 bp) was generated by PCR amplification using the primers MdGAIprobe5 and MdGAIprobe3. PCR condition: initial denaturing at 94°C for 1 min; 35cycles at 94°C for 30 s, 58°C for 30 s, and 72°C for 2 min; denatured at 72°C for 2 min 30 s. Digoxigenin (DIG) -labeled antisense and sense RNA probes were made by following the method of Digoxygen in RNA labeling kit (Roche Applied Science, Penzberg, Germany). Hybridization and immunological detection of the hybridized probes followed the methods of Banerjee et al. (2006). Signals were observed and recorded by light microscopy (BX61 Olympus, Tokyo, Japan).

II-3 Results

MdGAI *and* MxGAI *gene structure*

Genomic PCR to obtain *MdGAI* and *MxGAI* clones successfully amplified a 1710-bp product from each of 'Fuji' and *M. x* using degenerate primers. DNA sequencing revealed that each product was composed of two *GAI* sequences, which we considered to have been derived from the alleles. These results indicate, as in other variety (Foster et al. 2007), that the *GAI* genes in *Malus* species have no introns. By genomic walking and 5′-RACE, we fully sequenced *GAI* from 'Fuji'—*MdGAI1* (FJ535245) and *MdGAI2* (GQ384423), and from *M. x*—*MxGAI1* (FJ535244). Each fragment was 1911 bp long and encoding 637 amino acids with high sequence identity (about 98%). The *MxGAI2* (GQ384426) allelic sequence corresponded to positions +166 to +1876 of the *MxGAI1* coding region. Between the coding regions of *MdGAI1* and *MdGAI2*, there were 12 single nucleotide polymorphisms (SNPs), whereas there were 117 SNPs between the *M. x* sequences, indicating that the wild *M. x* has a much higher genomic heterogeneity than 'Fuji' when respective *GAI* alleles were compared.

Discrimination between **MdGAI1** *and* **MxGAI1**

Figure II-3 A shows the *Sdu*I cleavage sites of both alleles of *MdGAI* and *MxGAI* and the resulting fragment sizes. The *Sdu*I-digested products of genomic DNA showed fragments of the predicted sizes but those less than 100 bp products could not be see (Fig. II-3 B). Although the sequences at both ends of *MxGAI2* are not determined yet, the results suggest that PCR amplified *MxGAI2* too because of the presence of a 121-bp fragment specific to *MxGAI2* in the *M. x* genome (Fig. II-3 B). The same products were obtained from RT-PCR of total mRNA extracted from both species (Fig. II-3 C). These results identified a 618-bp fragment from 'Fuji' and a 387-bp fragment from *M. x*, thus allowing discrimination between the transcripts of *MdGAI* and *MxGAI*.

MdGAI has no introns like *GAI* gene in other plant species. Therefore, to confirm that RNA fractions prepared in this study did not contain genomic DNA (gDNA) which can function as template by our RT-PCR protocol, we performed a PCR experiment of a locus of *MdActin* intron sequence (Fig. II-4 A). As shown in Fig 4B, even nested PCR showed no amplification from cDNA fractions. These results proved that RNA fractions prepared by us did not contain any gDNA which may function as PCR template.

Grafting and the analysis of **GAI** *mRNA transport*

The grafted scions remained vigorous without wilting (Fig. II-1 A). The tissue-cultured stem can be dissected into the outer and inner tissues of the interfascicular cell layer with relative ease using a scalpel and forceps (Fig. II-1 B). We sampled the outer tissue of scion 'Fuji' and rootstock, where the phloem cells lie (Fosket 1994), in addition to the shoot apex and leaf of the scion. RT-PCR-RFLP showed that all three tissues of 'Fuji' (Fig. II-5, lanes 1–3) and the phloem tissue of *M. x* (Fig. II-5B, lane 4) exhibited unique restriction band patterns until 3 days after grafting. However, the patterns at 4 days incorporated the grafting partner's bands. The new bands were initially weaker than the own bands, but became clear by 10 days and continued to 35 days. At 35 days, the roots showed a mixed pattern (Fig. II-5, lane 7), but the stock xylem showed only the *M. x* pattern (lane 6). As a control, we grafted 'Fuji' on itself (lanes 8, 9) and *M. x* on itself (lanes 10, 11). Each scion and stock showed its own RT-PCR-RFLP pattern, indicating that the grafting procedure did not affect the pattern.

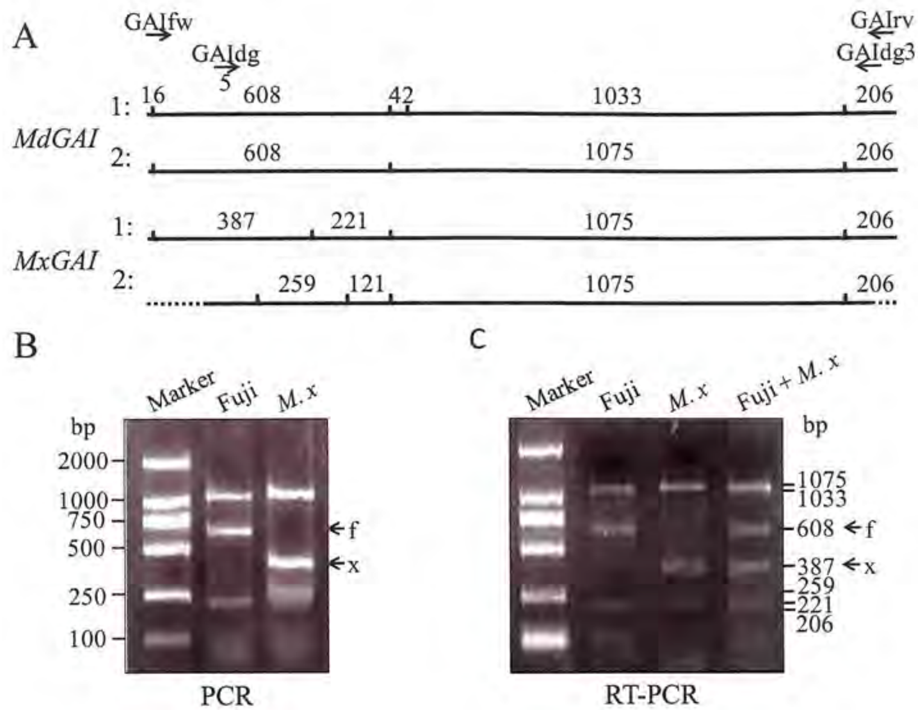

Figure II-3. CAPS analysis by *Sdu*I digestion of *MdGAI* and *MxGAI*.

(A) Fragment sizes (bp) of each *GAI* allele. The broken lines indicate unsequenced regions. (B) Electrophoresis pattern of *Sdu*I digests of genomic PCR product. (C) *Sdu*I digests of RT-PCR product. Marker: DNA marker. Fuji- and *M. x*-specific fragments are indicated with f and x, respectively.

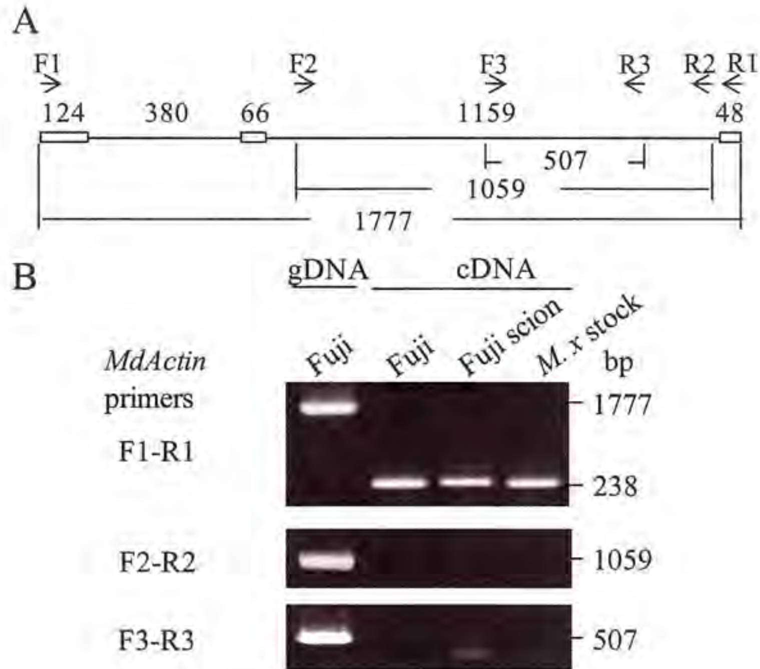

Figure II-4. Evidence of no contamination of gDNA which may function as the PCR-templates.

(A) The genomic structure of a part of *MdActin* gene. Boxes and lines indicate exons and introns, respectively. The positions of primers used are shown above the structure. 'Fuji' gDNA and cDNA were prepared from cultured shoots. (B) PCR and RT-PCR products from gDNA and cDNA templates. The gDNA fraction amplified the part of *MdActin* contains two introns, while the cDNA fractions amplified the 283 bp *MdActin* sequences without any intron. There are no PCR products derived intron when the cDNAs prepared in this study were used as the templates. The scion and stock cDNA fractions were came from plants after 25 days grafting.

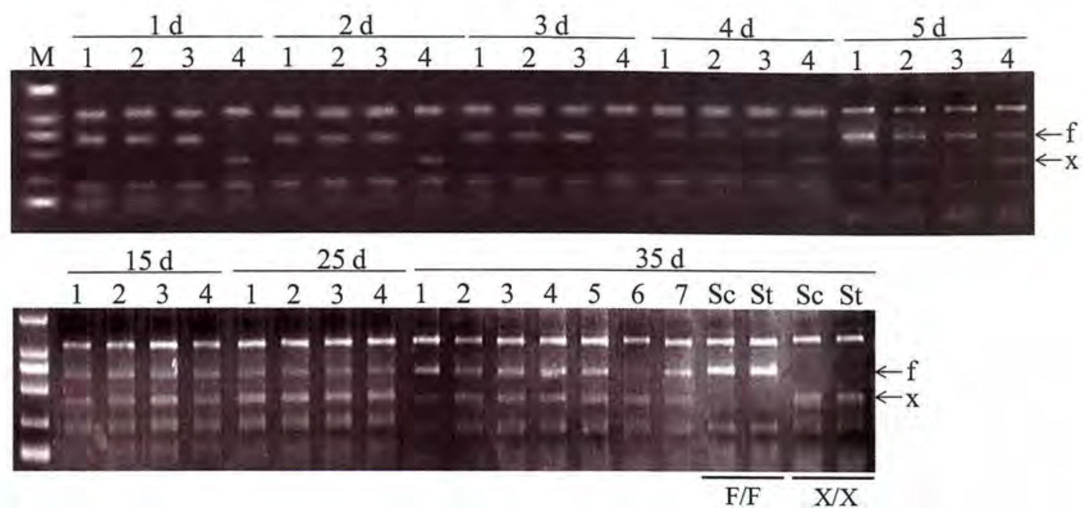

Figure II-5. Detection of *GAI* mRNAs transport by RT-PCR-CAPS.

Electrophoresis results: RT-PCR–CAPS results of tissues sampled from 1 to 35 days after grafting. Numbers of Lanes represent tissues used for RT-PCR-CAPS (showed in Fig. II-2): 1 scion shoot tip, 2 scion leaf, 3 phloem tissue from scion stem, 4 phloem tissue from stock stem, 5 graft junction, 6 xylem tissue from stock stem, 7 root. F/F and X/X indicate self-grafting materials of 'Fuji' and *M. x*, respectively. 'Fuji'- and *M. x*-specific fragments are indicated with an 'f' and 'x', respectively.

GAI* in situ-*hybridization

To identify the precise location of *MdGAI* transcripts, we performed *in situ* hybridization on transverse sections of 'Fuji'. *MdGAI* mRNA was detected in the phloem by antisense probe (Fig. II-6 B, C), but no signal was detected in the xylem or when a sense probe was used (Fig. II-6 A). Most of the signal was detected as small dots in the gap between large cells. The signal sizes suggest that the positive signals correspond to the companion cells.

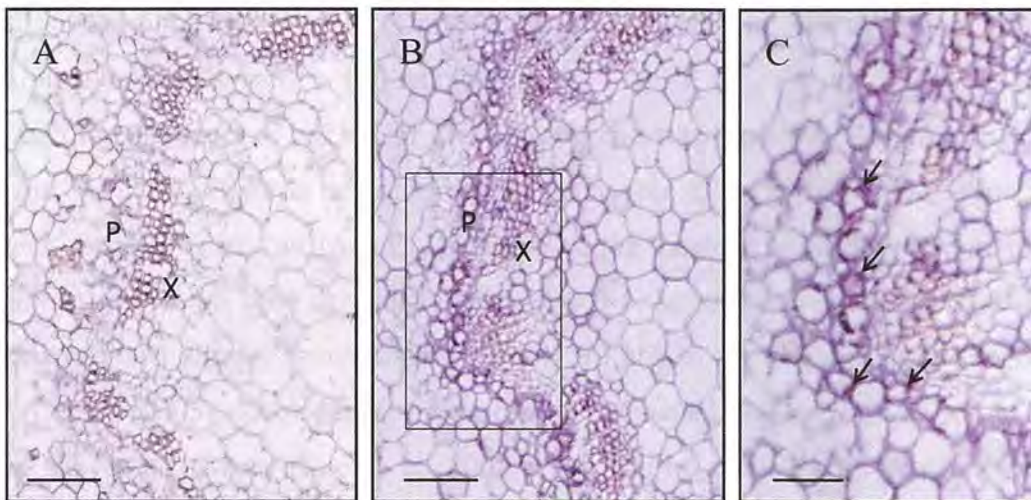

Figure II-6. *In situ* hybridization of transverse sections of 'Fuji' shoots.
Sections were hybridized with a digoxygenin-labeled 403-base RNA copy of a sequence from the 3′-untranslated region of *MdGAI1*. (A) Sense probe. (B) and (C) Antisense probe. The boxed area in (B) is enlarged in (C). The arrows in (C) indicate positive *MdGAI1* signal. P = phloem, X= xylem. Bars: (A) and (B), 50 μm; (C), 25 μm.

II-4 Discussion

The phloem long-distance translocation system of plants appears to function as both a nutrient delivery route and an information pathway (Behnke and Sjolund 1990). The latter system involves not only traditional signaling factors such as hormones, but also RNAs (Oparka and Turgeon 1999; Chen and Kim 2006; Lee and Cui 2009). Grafting experiments revealed that the stock-to-scion transport of some mutated gene mRNAs invoked phenotypic changes in the scion (Kim et al. 2001; Haywood et al. 2005; Kudo and Harada 2007), suggesting the functional importance of long-distance mRNA transport in the growth and development of plants. However, although several mRNAs have been reported to travel to distant tissue through the phloem (Omid et al. 2007; Kehr and Buhtz 2008), the precise picture of this delivery system is still unknown.

Although the transport of mRNA of a *GAI* transgene was reported (Haywood et al. 2005; Huang and Yu 2009), there is no information on the role of endogenous *GAI*. Our grafting experiment revealed firstly the two-way transport of the innate *GAI* mRNA. The involvement of the phloem tissue has been demonstrated in Fig. II-6. In addition, our in situ hybridization experiment showed similar accumulation of *GAI* mRNA in the phloem to that reported by Haywood et al. (2005). RNA gel-blot hybridization revealed *GAI* transcripts at steady-state levels in almost all tissues (Lee et al. 2002). The use of a transgenic plant expressing a reporter gene driven by the *GAI* promoter would clarify whether *GAI* is expressed exclusively in the phloem tissue or if mRNA expressed in surrounding tissues accumulates in the phloem. There are subfamily genes (Foster et al. 2007) of *GAI* in apple corresponding to *RGA* and *RGL* in *Arabidopsis* (Lee et al. 2002). Currently, no information is available on the transportability of these transcripts. Huang and Yu (2009) reported that the transcripts of five *GAI* paralogous genes in Arabidopsis did not efficiently move long distances. It would be interesting to see if the transportability of mRNA of the *GAI* subfamily genes in *Malus* shares similar features with that of *Arabidopsis*.

The characteristics conferred by various rootstocks on fruit tree scions include dwarfing, fruiting at an earlier age, improved fruit quality, and increased disease resistance (Wertheim 1998). Different rootstocks trigger distinct, reproducible scion gene expression patterns (Jensen et al. 2003, 2010), and it is possible that RNA molecules delivered from the stock could be responsible for this. Conversely, different scions could have different effects on

rootstocks (Zhou et al. 2005), suggesting that there could be some downward signal like *GAI* mRNA from scion to stock. Another intriguing question is where and how much of the transported *GAI* mRNA is translated. Moreover, why do the *GAI* transcripts move long distances in the sieve element? DELLA proteins such as *GAI* play a masterfully orchestrated role in regulating the growth response to environmental variables (Alvey and Harberd 2005; Harberd et al. 2009). The transport of *GAI* mRNA might achieve the harmonious integration of growth between organs. Further research is necessary to determine why *GAI* mRNA is involved in long-distance transport of molecules between the scion and rootstock.

II-5 Summary

Sieve tube in higher plant functions as infrastructure for long-distance transport of nutrients, photo-assimilates and growth regulators like hormones. Recently, it was revealed that some protein and RNA molecules also function as movable growth regulator in the sieve tube. In the case of the mRNA of *GIBBERELLIC ACID INSENSITIVE* (*GAI*), the transport evidence was obtained through identifying of the over-produced transgene transcript so far. In this work, we investigated the transport of apple (*Malus×domestica* cv. Fuji and *Malus xiaojinensis*) endogeneous *GAI* mRNA by grafting experiments. Each *GAI* mRNA of scion and stock plants was detected in the partners from 5 days after grafting, indicating the *GAI* mRNA moves in both upward and downward directions via graft union.

Chapter III

Breeding improvement of apple rootstock Marubakaidou (*Malus prunifolia*) by using *Atgai*

III-1 Introduction

Semi-dwarf rootstocks are highly recommended for both backyard and commercial orchard planting. Trees on dwarfing root-stocks are much easier to maintain and care for due to their smaller size. Fruit can be thinned and harvested mostly form ground level, and pest management is both easier and less expensive. Dwarf rootstocks are also useful for high-yeield fruit production because trees can be planted close together. Cultivars like M9 and M26, the most common dwarf and semi-dwarf rootstocks nowadays, have been developed for this purpose, but these dwarf cultivars are difficult to propagate from cutting because of their poor rooting ability. Some rootstocks are too vigourous to be used for commercial purposes, and several dwarf rootstocks have problems with adaptability. Since ideal rootstocks are not yet available, there is much interst in improving apple rootstocks.

Marubakaido (*Malus prunifolia* Borkh. var. ringo Asami Mo84-A) is an apple rootstock, not a dwarf rootstock, which is often used in Japan and is readily propagated from stem cuttings. Therefore, we attempted to transform Marubakaidou by the introducing of a dwarf inducing gene.

In the chapter I, I demonstrated that *Atgai* moves from the over produced-stock to the scion, and reduced the stature of the scion through the suppression by GA responsiveness. Furthermore, in the chapter II, it was proved that apple tree also can transport the *GAI* mRNA through graft union. This study investigated whether *Atgai* is effective in producing dwarf apple rootstocks and whether such rootstocks maintain high rooting ability.

The *Atgai* gene over expressed or driven by its own promoter could change the phenotypes of transformants not only to a dark green leaves, dwarf or semi-dwarf height, tobacco, and apple, but also less roots in *Arabidopsis* and *Populus* (Hynes et al. 2003; Haywood et al. 2005; Huang and Yu 2009; Busov et al. 2006; Zhu et al. 2007). But the over expressed *Atgai* cause a totally dwarf phenotype, which is not suitable for rootstock using. Therefore *CoYMV pro* promoted *Atgai* gene was transformed into *M. prunifolia*. Phosphinothricin acetyl

transferase (*Bar*) gene originally cloned from *Streptomyces hygroscopicus* was also inserted as a selectable marker gene, which have a function of herbicide resistance (Becker et al, 1992). Herbicide bialaphos widely used in nurseries and orchards is a tripeptide antibiotic employed in transformation experiments in plants (Thompson et al. 1987; Szankowski et al. 2003; Sripaoraya et al. 2010).

In this experiment, since the expression level of this *CoYMV pro:Atgai-bar* (*Cgb*) gene is not as strong as *CaMV 35S* promoter, I wanted to breed a new stock with moderately dwarf phenotype, having ability to regulate scion development by transported *Cgb* mRNAs, and also less-sensitive to GA.

III-2 Materials and methods

Plant materials

Apple rootstock variety (*Malus prunifolia* var. ringo Asami Mo 84-A) and scion cultivar 'Orin', both were gifted from Aomori Green-Bio Center as sub-cultured shoot, were used in this experiment. Shoots were sub-cultured every 4 weeks on MS (Murashige and Skoog, 1962) with 1 mg · L^{-1} 6 - benzyladenine and 0.8 % (w/v) agar (Wako Pure Chemical Industries Ltd., Osaka, Japan) at 25°C day, 23°C night, with a 14-h photoperiod of about 100 $\mu mol\ m^{-2}\ s^{-1}$ photosynthetically active radiation provided by cool-white fluorescent tubes.

Construction of binary vector

Restriction sites *Sal*I and *Spe*I were leaded into 5' - site of Nos promoter promoted bar sequence and 3'- sites of T7 terminator in pGPTV-Empty vector type II with primer Nos R Sac (5'-GCG AGC TCG ATC ATG AGC GGA ATT -3' and T7 F Spe (5'- GAA CTA GTT TAA TTC CCA TCT TGA -3'). After that, the new sequence was inserted at 5'-site of *CoYMV pro:Atgai* sequence in pBI121 which created in Chapter I, and then checked the correct insert by sequencing. New fused gene was called *CoYMV pro:Atgai-bar* (*Cgb*).

Agrobacterial infection of apple

Cgb was induced in *Malus prunifolia* following the methods of Komori et al. (2009).

DNA extraction

The method has been described in Chapter II-2.

PCR detection

PCR amplification was carried out using gene-specific primer CoYMV pro F1 and Atgai R3. PCR was performed as follows: 3 min at 94°C; 35 cycles at 94°C for 40 s, 58°C for 40 s, and 72°C for 2 min; denatured at 72°C for 5 min.

Southern blot

Fifteen µg of genome DNA was used for Southern blot for each plant sample. WT and *Cgb* plasmid were used as negative and positive controls, respectively. Probe was synthesised by PCR using primers Atgai SF and SR, following the manual of DIG DNA labeling and Detection Kit (Roche, USA). An aliquot of genomic DNA of *Cgb* and WT was digested either with 15 U *Eco*RV or *Bam*HI at 37°C overnight. After that the DNA was fractionated on a 1 % agarose gel and subsequently transferred to a nylon membrane (Pall Corporation, USA).

Plant growth and GA treatment

CgT transformants were rooted in medium of 1/2 MS with 0.5 mg · L^{-1} 3-Indolebutyric acid (Wako Pure Chemical Industries Ltd., Osaka, Japan) and 50 mg/mL MEPM (Dainippon Sumitomo Pharma Co. Ltd. Japan) and 0.8 % (w/v) agar. Rooted *CgT* cultivated in Jiffy pot (Jiffy Preforma Production, Yokohama, Japan) for four weeks were used for GA treatment. Plants were sprayed with 0.1 mM Gibberellin $Acid_3$ (Nacalai Tesque, Inc. Kyoto, Japan) solution once a week for four weeks.

Measurements of Phenotype observation

Increment of WT (n = 5) and *Cgb* (n = 5) were measured 8 weeks after the cultivation in Jiffy pot.

Grafting

Rooted *CgT* in Jiffy pot were used as stock. Four-week-old shoots of 'Orin' were used as the scions. About 3 cm of the stock of which leaves were cut off was prepared with a horizontal transverse cut, in which a snick of about 1 cm deep was made. About 2 cm of the scion was V-shape cut and wedged into the stock. The graft union was fastened with Parafilm (American National Can., USA), and grafted plants were preserve moisture with plastic bag for about one week. 'Orin'/WT grafts were made as the control. Phenotype observation was measured two months after the grafting.

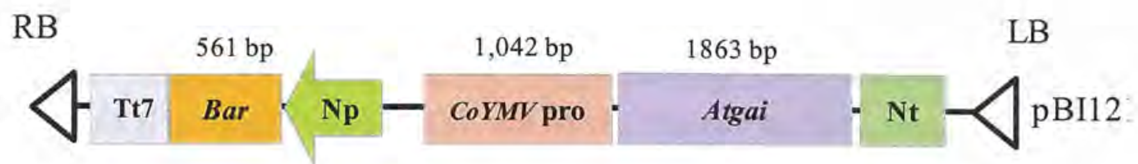

Figure III-1. Vector structure of *CoYMVp:Atgai-bar*.

CoYMV* Pro**: *Commelina yellow mottle virus* promoter (companion cell specific expressive promoter); ***Atgai: *Arabidopsis thaliana gai* gene (a gain-of-function DELLA allele of *AtGAI*); ***Bar***: *Bar* gene have function of bialaphos resistance; **Np**: Nos promoter; **NPT II**: A gene coding for kanamycin resistance (primarily Neomycin phosphotransferase II); **Nt**: Nos terminater.

III-3 Results

Cgb ***transgenic apple rootstock displays a semi-dwarf phenotype***

Through five transformation experiments, 67 regenerants were successfully proliferated on selective media. The transformation frequency was calculated as 0.1%. However, only one transgenic line, *Cgb*26 (*Cgb*), was confirmed by PCR (a 1266 bp target band was amplified, Fig. III-2). Actually, the *Cgb*26 transgenic rootstock grew vigorously on the medium containing even 3 mg bialafos. Furthermore, the *Cgb* gene integration in the genome was confirmed by a gel-blot experiment. The result showed that there were two bands detected in the *Cgb* lane (Fig. III-3) in both *Eco*RV and *Bam*HI digested products, suggesting that at least two *Cgb* genes were integrated into the Marubakaido genome.

The size of the *Cgb* was obviously smaller, and root growth rate was less than WT (Fig. III-4), which were corresponded with phenotype caused by *Atgai*. There were significant differences of shoot length and weight between *Cgb* and WT (Fig. III-5). The differences between *Cgb* and WT were also observed in internode lengths (Fig. III-6 and Table III-1). Moreover, on four weeks after GA treatment, the stature of *Cgb* was grown slower than that of control Marubakaido, which suggested less GA response of the *Cgb* line (Fig. III-7).

Dwarfing of 'Orin' scion on **Cgb** ***rootstock***

Since *MdGAI* has ability of two directions transportation through phloem, the grafting experiment was carried out to know the effects from *Cgb* semi-dwarf stock on the scion growth (Fig. III-8). The mean height of 'Orin' scion on *Cgb* stock was 3.61 cm that was about half size of 'Orin' on WT stock (Fig. III-9, Table III-2) at 4 weeks after grafting (wag). Therefore, it was demonstrated that *Cgb* semi-dwarf stock could make the growth of 'Orin' scion slower, which was just the same as *CgT* stock affected WT scion in tobacco grafting system (Chapter I).

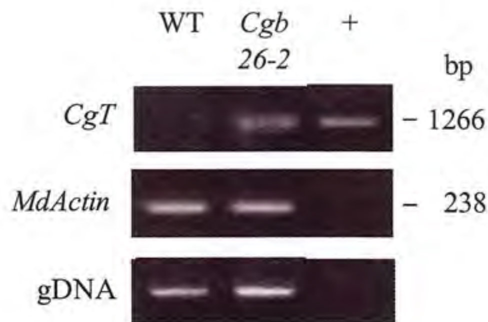

Figure III-2. Detection of *Atgai* gene in *Cgb*.

A 1266 bp target band was detected by PCR in Cgb 26-2. +: plansmid of *Cgb*.

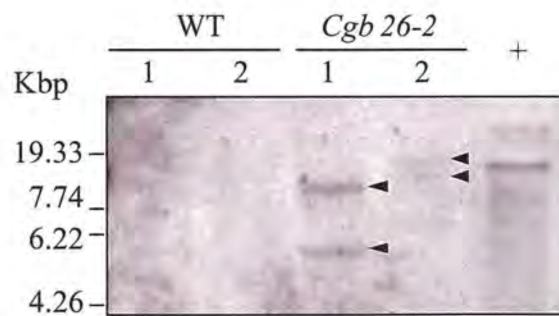

Figure III-3. Southern blot result of *Cgb* with *Atgai* probe.

15 μg of gDNAs were loaded in each lane. Lane 1: digested by *Eco*RV. Lane 2: digested by *Hin*dIII. +: plasmid of *Cgb*.

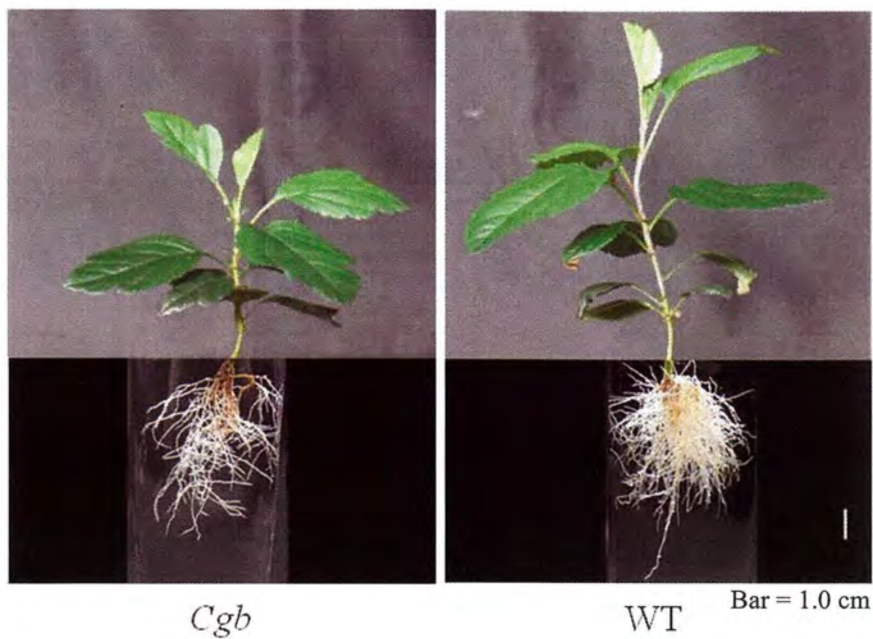

Figure III-4. Phenotype observation of *Cgb* transgenic Marubakaido.

Left is *Cgb* which displayed: semi-dwarf and less roots compared with WT in right figure. Bar = 1.0 cm.

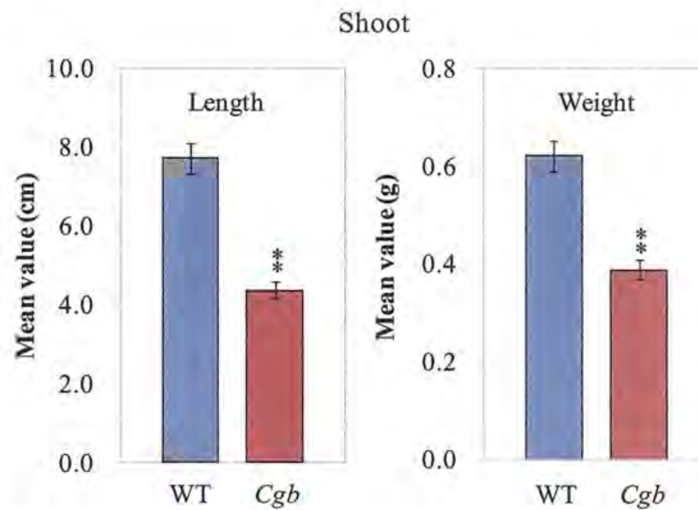

Figure III-5. Height and weight of WT and *Cgb* transgenic rootstock at 8 weeks after cultivation in Jiffy pot.

Both of mean length and weight of *Cgb* were significantly different from WT. Significant differences from WT was determined by Student's *t* test with equal or unequal variances as appropriate (**$P < 0.01$, n = 5).

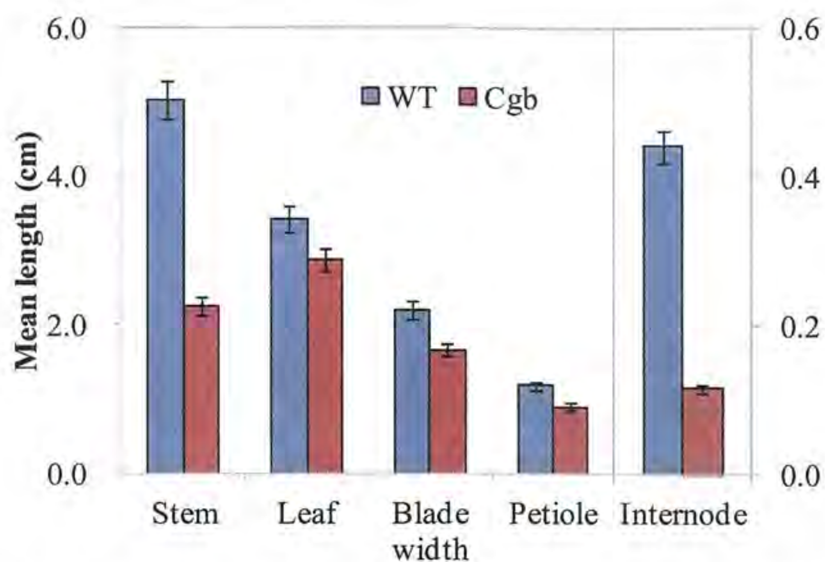

Figure III-6. Mean growth of WT and *Cgb* transgenic rootstock at 8 weeks after the cultivation in Jiffy pot.

Both of mean length and weight of *Cgb* were significantly different from WT. Significant differences from WT was determined by Student's *t* test with equal or unequal variances as appropriate (*$P < 0.05$, **$P < 0.01$, n = 5).

Table III-1. Growth of *Cgb* and WT 8 weeks after the cultivation in Jiffy pot

	Stem (cm)	Internode (cm)	Leaf (cm)	Blade width (cm)	Petiole (cm)
WT	5.01 ± 0.88	0.47 ± 0.15	3.40 ± 0.26	2.19 ± 0.17	1.16 ± 0.11
Cgb	2.24 ± 0.54 **	0.11 ± 0.05 **	2.86 ± 0.04 *	1.65 ± 0.17 *	0.90 ± 0.24

Values were expressed as mean ± SE. Significant differences from WT was determined by Student's *t* test with equal or unequal variances as appropriate (*$P < 0.05$, **$P < 0.01$, n = 5).

Figure III-7. Stature increasment of *Cgb* and WT by GA_3 treatment.

At four weeks after fourth GA_3 treatment (once every two days), the shoot statures were measured .of WT (up-panel) was visibly faster than that of *Cgb*. Bar = 5 cm.

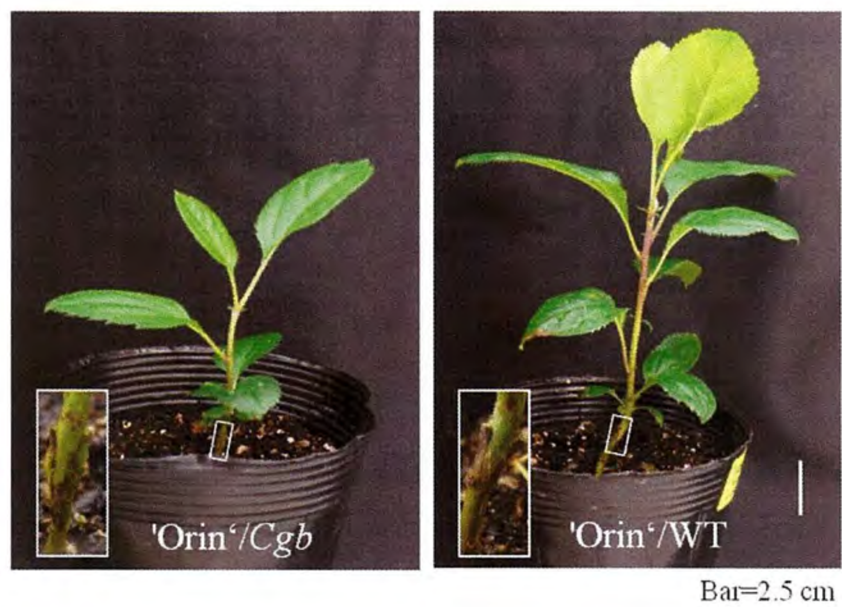

Figure III-8. Typical phenotypes of 'Orin' grafted on *Cgb* or WT.

Grafts were four weeks after grafting. The grafting junctions were magnified at the left side of pictures.

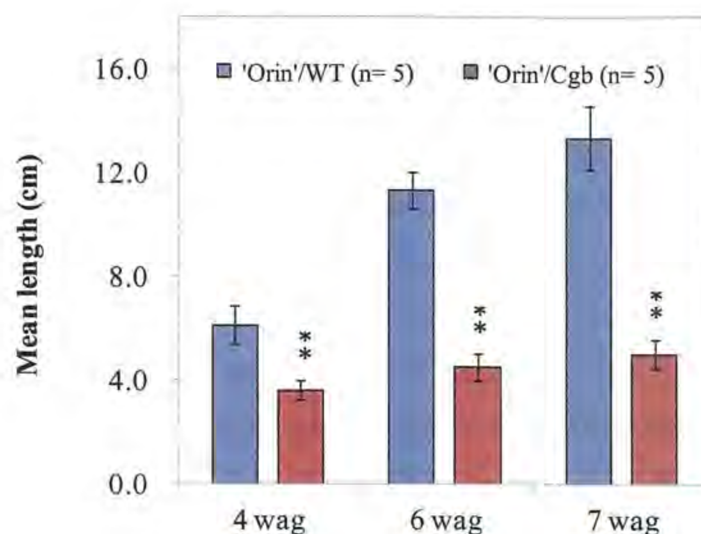

Figure III-9. Shoot length of 'Orin' grafted on *Cgb* or WT after grafting.
The shoot length of 'Orin'/*Cgb* grafts were obviously shorter than that of 'Orin'/WT at 4 wag, 6 wag and 7 wag. The data were shown in Table III-2.

Table III-2. Shoot length of 'Orin' grafted on *Cgb* or WT 4 weeks after grafting.

Scion	Stock	Mean height (cm)		
		4 wag	6 wag	7 wag
'Orin'	WT	6.12 ± 0.73	11.30 ± 0.71	13.37 ± 1.21
'Orin'	*Cgb*	3.61 ± 0.38 **	4.50 ± 0.52 **	5.03 ± 0.55 **

Values were expressed as mean ± SE. Significant differences from WT was determined by Student's *t* test with equal or unequal variances as appropriate (** $P < 0.01$, n = 5).

III-4 Discussion

In this chapter, *Cgb* transformed apple rootstock exhibited the dwarf phenotype with small and little dark green leaf, short internodes length, and less GA sensitiveness. These characteristics were also showed in the other *Atgai* transgenic plans (Hynes et al. 2003; Haywood et al. 2005; Huang and Yu 2009; Busov et al. 2006; Zhu et al. 2007). In this experiment, however, *Atgai* gene was derived by a companion specific promoter, while all others used universal expression promoter cauliflower mosaic mottle virus 35S promoter. Therefore, effective dwarfing by *Cgb* construct indicates that the *Atgai* expression at only companion cells is sufficient for the leading to dwarf phenotype. This may be related with the accumulation of *GAI* mRNA in phloem cells which was clarified by *in situ* hybridization (Chapter II).

In addition, the reduced stature of 'Orin' scion on *Cgb* Marubakaidou indicates that this transgenic plant has a possibility to become a new apple dwarf variety because of the remaining of active rooting ability. Thus, it is intriguing to investigate whether the *Cgb* gene does not affect other significant characters such as fruit loading number and sizes.

I obtained only one *Cgb* transgenic Marubakaido until now. Generally, the transgenic efficiency of *Malus* is very low, approximately 0.1% (Zhu et al. 2007). Furthermore, it is considered that dwarfing gene may further decrease the efficiency. In breeding, the most excellent clone should be selected from the candidate population.

III-5 Summary

In this study, I attempted to get a new apple dwarf rootstock, *Cgb* Marubakaido, which reduces the scion stature by long-distantly transported *Cgb* mRNAs. One *Cgb* transformant was obtained through 4 times *Agrobacterium* transformation experiments. It exhibited semi-dwarf character with shortened internodes, little smaller leaves, and less root volume. Furthermore, GA treatment experiment proved that the *Cgb* transformant was less sensitive to GA_3 than WT. In addition, the *Cgb* stock reduced the stature of 'Orin' scion. These results clearly indicate that the *Cgb* Marubakaidou would become a new apple dwarf rootstock, if it is free of defect in the grafting cultivation.

General discussion

The long-distance transport of mRNA is not occurring only in plants. In *Drosophila*, *oskar* mRNA localization at the oocyte posterior pole is essential for correct patterning of the embryo. This gene can be transported from nurse cells to oocyte before translation. After transport from nucleus to cytoplasm of nurse cells, *oskar* mRNA was loaded with two motor proteins, kinesin and dynein which responsible for transport of the RNPs within the oocyte (Trucco et al. 2009). In *Escherichia coli*, certain mRNAs are targeted to the future destination of their encoded proteins, cytoplasm, poles, or inner membrane in a translation-independent manner (Trucco et al. 2009). Therefore, the transport system of mRNA through plant sieve tube is not so surprising. Next question is how many and what sort of mRNAs are moving in the tube. Although some reviews reported the mRNAs which were identified as phloem movable molecules so far, the total numbers are around only ten (Harada 2010). I convince that the numbers will increase definitely when many researchers adopt a serious stance on the finding them.

Transport of RNA, including virus and viroid RNAs, via sieve tubes is considered to involve certain proteins, such as RNA-binding protein and/or chaperonin (Ding et al. 1992; 2003, 2005; Zhong et al. 2007; Gopinath and Kao 2007). Indeed, a pumpkin RNA-binding phloem protein (CmPP16) has been proposed as an analog of viral movement protein, mediating the movement of RNA between companion cells and sieve elements (Xoconostle-Cázares et al. 1999). Gómez and Pallás (2004) have demonstrated that a cucumber phloem protein, PP2, is able to bind a viroid RNA *in vivo* and that it may be involved in its long-distance translocation to non-host pumpkin, grafted onto infected cucumber stock (Gómez et al. 2005). Moreover, through inter-generic grafting between pumpkin and melon, they have demonstrated the involvement of translocatable melon phloem lectin (CmmLec17) as a putative component of vascular transport. Ham et al. (2009) identified RNA-binding proteins involved in mRNA transport, and proposed a model in which a RNP complex moves in the phloem. A lot more study needs to put the whole-picture of the molecular mechanism by which RNA can transport long-distantly through phloem in plants.

Grafting has provided conclusive evidence of long distance messengers since the first experiments on the photoperiodic regulation of flowering (Lang et al. 1977). Through the grafting experiments, I could also provide clearly the function of *GAI* mRNA which was

transported long-distantly. Furthermore, I created a new apple dwarf rootstock, Marubakaido, in which a *GAI* mutant gene was integrated. When this transgenic Marubakaido is used as apple rootstock, it does not have own flowers because of the exclusive existence in the basement, meaning no fear about gene flow through pollen. Moreover, terrestrial part including fruits does not harbor the integrated gene. Although the general public is not familiar with recombinant DNA crops, the usage of transgenic crop as the stock may reach acceptance easily.

From December 1st of this year, Japan started to import gene modified (GM) papaya from Hawaii. This became one of head-line news because the papaya is the first raw eating GM crop in Japan. I hope that this momentum would lead the beginning of actual usage and cultivation of GM crops in Japan. If it becomes, a new apple dwarf rootstock, Marubakaido, which was created through this study, would spread widely in Japanese apple farmers, and also in the world.

General summary

Some of RNAs posses ability to transport in the phloem and control accommodative development of organs. One of them is *GIBBERELLIC ACID INSENSITIVE* (*GAI*) mRNA. *Atgai*, a gain of function gene of *GAI* in *Arabidopsis*, induces dwarf or semi-dwarf phenotypes, and the transcript of which have the transport ability. Therefore the long-distance transport of *Atgai* mRNA could explain why scions grafted onto *gai*-overexpressing transformants showed dwarfism. However, little is known about the molecular mechanism.

To characterize the *gai* mRNA transport through graft union, a new fused gene was created, in which *Atgai* was fused with T7 epitope tag and driven by a companion cell specific expression promoter, *Commelina yellow mottle virus* promoter (*CoYMV*p). *Nicotiana benthamiana* introduced the resulting construct *CoYMVp: Atgai-T7* exhibit dwarf, less root, and less insensitivity to GA_3. *Atgai* mRNA was detected in WT scion onto *CgT* rootstock by RT-PCR and qRT-PCR at 21days after grafting. The amount of the transported mRNA was varied among individual grafts. Furthermore, by using the antibody of T7-tag, Atgai-T7 protein was identified in WT scion on *CgT* rootstock, revealing that *gai* transcripts can be translated after transport through graft junction. Microarray analysis was used to identify different GA response between WT scions on *CgT* and that of WT stocks. The results showed clearly the decrease of GA response in the WT scion resulted in smaller shoot of WT/*CgT* than WT homo-grafts. There were also significant differences of root growth between *CgT*/WT and WT/WT or *CgT*/*CgT* homo-grafts. The new knowledge concerning the mRNA long-transport would be useful for the application of this system in horticulture crops using grafting.

The transport of apple (*Malus×domestica* cv. Fuji and *Malus xiaojinensis*) endogeneous *GAI* mRNAs were investigated by grafting experiments. Using RFLP-RT-PCR, each *GAI* mRNA of scion and stock plants was detected in the partners from 5 days after grafting, and *in-situ* hybridization showed the exclusive location of the *MdGAI* mRNA in companion cells. This result indicated that the *GAI* mRNA moves in both upward and downward directions via graft union through phloem.

To breeding new dwarf rootstock with ability that control the scion height by long-distance transported *Atgai* mRNA, *CoYMVp:Atgai-bar* with bialaphos-resistant *bar* gene were transformed into rootstock cultivar Marubakaido (*Malus prunifolia* var. ringo Asami Mo

84-A). One positive regenerated line probably harboring two copies of the *Cgb* gene was obtained. The transgenic plant exhibited semi-dwarf with little shortened internodes, smaller leaves, less root volume, and less responsiveness to GA_3 than WT. Finally, stock of the *Cgb* Marubakaido reduced the stature of the scion cultivar *Malus* × *domestica* 'Orin'. As the result of grafting with scion cultivar, scion growth of 'Orin'/*Cgb* was slower than 'Orin'/WT. It was revealed that *Cgb* was a semi-dwarf rootstock which was GA less- responsiveness and reduce the grow speed of 'Orin' scion. Therefore, new dwarf apple cultivar was created by using the mRNA long-distant transport system.

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