IGI1 is involved in axillary branching in Arabidopsis thaliana

애기장대에서 액아발생을 조절하는 *IGI1*의
분자유전학적 연구

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황 인 덕

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ABBREVIATION

2,4-D	2,4-Dichlorophenoxyacetic Acid
AD	Arbitrary Degenerate
AP1	Apetala 1
BLAST	Basic Local Alignment Search Tool
BRC1	Branched 1
CRY	Chyptochromes
DAD	Decreased Apical Dominance
EST	Expressed Sequence Tag
FMN	Flavin Mononucleotide
IAA	Indolyl-3-Acetic Acid
IGI1	Inflorescence Growth Inhibitor 1
LOV	Light, Oxygen, Voltage
LSH1	Light-dependent Short Hypocotyls 1
MAX4	More Axillary Growth 4
MS	Murashige and Skoog
MPSS	Massively Parallel Signature Sequencing
PCR	Polymerase Chain Reaction
PERK	Proline-rich Extensin like Receptor Kinase
PHY	Phytochrome
PHOT	Phothotropins
RNAi	RNA Interference
SUB1	Short Under Blue light 1
SMS	Shoot Multiplication Signal
SPS	Supershoot
TAIL	Thermal Asymmetric Interlaced
TB1	Teosinte Branched 1
TFL1	Terminal flower 1

ABSTRACT

IGI1 is involved in axillary branching in Arabidopsis thaliana

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Genetic approach in mutant screening is an important tool for studying gene function in plant. We selected phenotypically distinct plants in the activation tagged lines and chose a mutant, *igi1* (Inflorescence Growth Inhibitor 1) for further analysis. Segregation ratio of the F2 generation, TAIL-PCR walking and genotyping PCR showed that a single T-DNA was inserted at the 200bp upstream of the *At1g23540* coding region. Real time PCR indicated that the expression level of *IGI1* gene was increased approximately by 1,000 to 3,000 fold in *igi/igi* homozygous mutant. The homozygous mutant displayed abnormal phenotypes during reproductive stgage and was sterile. The heterozygous mutant plants produced primary inflorescence with short internode, and secondary inflorescences begin to emerge straight and axillary branching was dramatically increased. We attempted RNA interference (RNAi) to revert the phenotype by reducing the mRNA levels. *IGI1* expression level was decreased in RNAi lines of *igi1* heterozygous mutants, and the phenotypes of the RNAi lines were similar to those of wild type plants. The result showed *igi1* mutants phenotypes were caused by overexpresssion of the *IGI1* gene. Auxin and cytokinin are involved in shoot branching and apical dominance. In the cytokinin and auxin response, *igi1* mutants showed opposite phenotypes in the axillary meristem development. The transcript level of auxin biosynthesis component,

CYP79B2 was increased in the *igi1* mutants. In the cell division and callus greening test for cytokinin response, *igi1* mutants showed similar pattern with wild type. Histochemical analysis by GUS staining showed tissue-specific gene expression in the anther. The expression levels of the *IGI1* gene in apical part and flower were higher than in other parts. The IGI1 possesses proline rich domain in N-terminal region and kinase domain in C-terminal region. The proline rich domain in N-terminal region interacted with SUB1, which is negative regulator of the photomorphogenesis and regulates positive regulator HY5 in the photomophogenesis. Taken together with the fact that HY5 binds the promoter region of *IGI1* gene, my results suggest that there might be a noble pathway for the axillary meristem development that involves IGI1 and is modulated by light regulated pathway.

Ⅰ. *Introduction*

A. Plant architecture

Plant architecture is influenced by genetic control and environmental conditions such as light, temperature, humidity, nutrient conditions and etc. During vegetative phase shoot apical meristem produces leaf primordia, and inflorescence meristem is initiated from shoot apical meristems and then inflorescence meristem develop into flowers or branches during reproductive phase (Smith et al., 2004; McSteen et al., 2007).

Flowering plants have determinate and indeterminate type for inflorescence architecture. The inflorescences are indeterminate shoot, and the flowers are determinate shoots. Determinate inflorescenses arise when the terminal meristem is converted to a terminal flower. The flowers in an indeterminate meristem are of lateral origin (Singer et al., 1990). Wild type *Arabidopsis* have an indeterminate growth patten. The plant generally produces 5 to 15 normal flowers in primary inflorescence and then terminates with a flower. Schultz and Haughn (1993) divided the development of the *Arabidopsis* shoot into four stages based on node morphology at maturity: juvenile rosette, mature rosette, early inflorescence, and late inflorescence stage. In the early inflorescence stage, the apical meristem initiates several lateral branches. After the primary inflorescence came out from apical meristem, lateral inflorescence (rosette and cauline leave inflorescence) is initiated in the rosette and cauline leave axils. In the late inflorescence phase, nodes lack leaves and lateral meristems develop into flowers (a number of lateral and terminal flowers) instead of lateral inflorescence (Fig. 1). The disorder of a gene involved in inflorescence architecture causes the change of inflorescence structures between indeterminate and determinate. Arabidopsis TFL1 (terminal flower) plays an important role in

maintaining proper function of the apical meristem of the inflorescence (Schultz and Haughn, 1993). *tfl1* mutant develops a terminal flower at the apex of the inflorescence (Ohshima et al. 1997). *tfl2* initiates flowering early and terminates the inflorescence with floral structures and the phenotypes were similar to that of the *tfl1* mutant. *tfl1/tfl2* double mutant terminates the inflorescccence without development of lateral flowers. *TFL2* gene product regulates the meristem response to light signals affecting the development of the plant. TFL2 function influences developmental processes controlled by APETALA1 (AP1), which is flower meristem identity gene and promotes the transition from inflorescence to floral meristem (Weigel et al., 1992; Larsson et al., 1998).

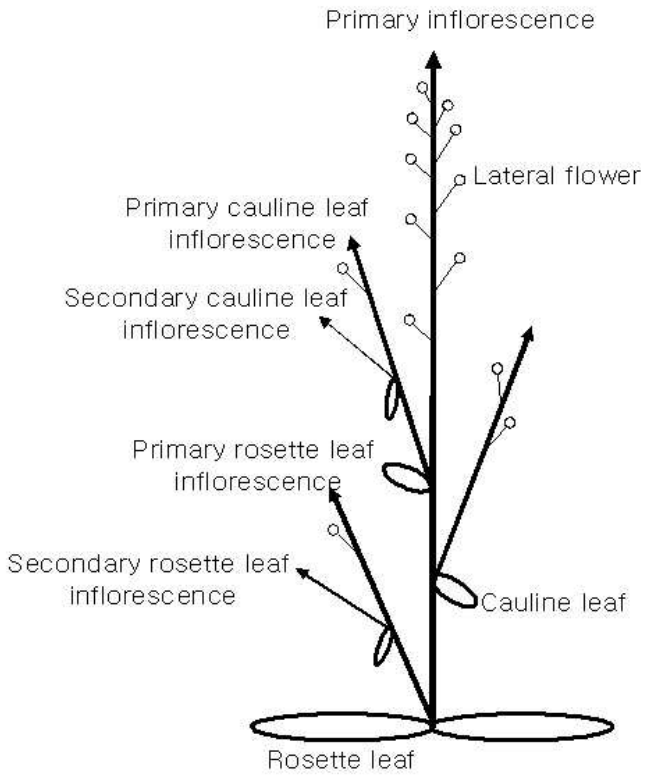


Figure 1. General development of Arabidopsis thaliana Columbia.

B. Photoreceptor for light perception

The light is one of the crucial environmental source not only for energy during photosynthesis also for plant development and growth such as seed germination, de-etiolation, phototropism, and flowering. Plant have at least three major photosensory receptors: red/far-red (600-750 nm) absorbing phytochrome (PHY), blue/UV-A (320-500 nm) absorbing chyptochromes (CRY) and phothotropins (PHOT), and UV-B (282-320 nm) absorbing unknown factors. In *arabidopsis*, there are five phytochrome members, at least three cryptochrome members, and two phototropin members (Neff et al, 2000; Nagy and Schafer, 2002; Lin and Shalitin, 2003; Huq and Quail, 2005).

1. Phytochromes

The phytochromes are dimeric chromoproteins consisting of polypeptide subunits that carry a tetrapyrrole chromophore in the amino-terminal domain. The carboxy-terminal domain functions in dimerisation and contains a region with sequence similarity to prokaryotic two-component histidine kinases. The photosensory activity of the phytochrome molecule resides in its capacity to undergo reversible, light-induced interconversion between two conformers: the biologically inactive, R-absorbing Pr form and the biologically active R-absorbing Pfr form. The phytochromes are cytosolically localised in their Pr form, but are triggered to translocate into the nucleus upon photoconversion to their Pfr form (Quail, 2002).

2. Cryptochromes

CRY were characterized in *Arabidopsis* with *hy4* mutant that was isolated from T-DNA tagged lines. When grown under blue or UV-A light, *hy4* mutant showed a longer hypocotyl than wild type (Ahmad and Cashmore, 1993). CRY1 showed sequence similarity to photolyases and the gene encodes a protein family that mediate repair of UV-damaged DNA (Sancar, 2003). CRY1 protein affects anthocyanin production, chalcone synthase gene expression, flowering time and hypocotyl elongation. CRY2 also affected hypocotyl elongation. (Ahmad et al., 1995). Analyses of *cry1cry2* double mutant and over-expression phenotype of CRY1 and CRY2 showed that these two CRY play redundant roles in photomorphogenic response to blue light response (Ahmad et al., 1998; Lin et al, 1998). In seedling stage, GUS fusion protein GUS-CRY1 was localized in the nucleus under dark condition. When the seedlings were grown under white light, the GUS-CRY1 was depleted from the nucleus and primarily founded in the cytoplasm (Yang et al., 2000). In contrast, CRY2 (second member of the Arabidopsis cryptochrome family) was localized in the nucleus in both light- and dark-grown seedlings (Guo et al., 1999).

3. Phototropins

The PHOT was most recently characterized blue/UV-A light absorbing photoreceptors in plant. The PHOT proteins have two distinct domains, a C-terminal serine/threonine kinase domain and and N-terminal region which encode two LOV (Light, Oxygen, Voltage) sub-domain. The LOV domain of POHT, which bind the chromophore flavin mononucleotide (FMN), is involved in a response for blue light absorption (Kagawa, 2003). PHOTs have been

known to regulate phototropism and chloroplast relocation movement in reponse to blue light (Sakai et al., 2001). Analyses of combinatorial multiple mutants of blue light receptors (*cry1*, *cry2*, *phot1*, *phot2* mutant) and microarray analysis suggested that CRYs play major roles whereas PHOTs play minor roles in the transcriptional regulation of blue-light-responsive genes (Ohgishi et al., 2004).

C. Apical dominance and branching

1. Apical Dominance

The shoot apex affect various developmental processes including axillary bud growth, the orientation of laterals, the growth of rhizomes and stolons, leaf abscission, and others (Imre A. Tamas). In *Arabidopsis thaliana*, apical bud generally initiate at shoot apex in shoot apical meristem. The apical bud controls (inhibits) lateral bud outgrowth. The phenomenon is the apical dominance. MG Cline divided apical dominance and its release into four developmental stages: (I) lateral bud formation, (II) imposition of inhibition on lateral bud growth, (III) release of apical dominance following decapitation, and (IV) branch shoot development. The initial bud outgrowth occurs in Stage III during the first few hours of release, which is inhibited by auxin. The lateral bud outgrowth continues in Stage IV during days or weeks after decapitation, which may be promoted by auxin and gibberellin (Fig. 2) (Cline, 1997).

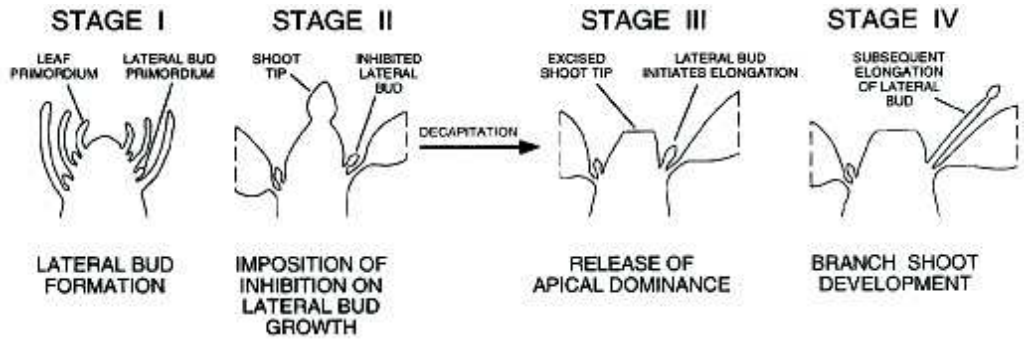


Figure 2. Developmental stages of apical dominance before and after release by decapitation of the shoot apex (Cline, 1997).

2. *DAD, MAX, SPS genes involved in branching*

The function of Petunia DAD (decreased apical dominance) regulating branching in plant architecture have been identified by plant grafting, morphology studies, double-mutant characterization, and gene expression analysis. The mutation in *DAD* gene caused an increase in basal axillary branches and a decrease in plant height (Napoli, 1996; Napoli and Rueeau, 1996, Napoli et al, 1999). Recent studies showed that *DAD* is an ortholog of the *MAX4* (More Axillary Growth 4) in *Arabidopsis* and *RMS1* (Ramosus 1) gene in pea (Sorefan et al., 2003; Snowden et al., 2005). *Arabidopsis* MAX4 has homology to carotenoid cleavage dioxygenases required to produce a mobile branch-inhibiting signal, acting downstream of auxin (Sorefan et al., 2003). *MAX3* encodes a plastidic dioxygenase that can cleave multiple carotenoids, and is required for the synthesis of a novel carotenoid-derived long-range signal that regulates shoot branching (Booker et al., 2004). MAX2 is an F-box, leucine-rich repeat-containing member of the SCF family of ubiquitin ligases (Stirnberg et al., 2002). MAX1 controls vegetative axillary bud outgrowth by the regulation of the flavonoid pathway, acts downstream of MAX3/4 to produce a carotenoid-derived branch-inhibiting hormone, and encodes a member of the CYP450 family, CYP711A1. Analysis of the *max1-max4* mutants demonstrates that branching is regulated by at least one carotenoid-derived hormone and four *MAX* genes acting in a single pathway, with MAX1, MAX3, and MAX4 acting in hormone synthesis, and MAX2 acting in perception (Booker et al., 2005).

Another branching signal component BRC1 (branched 1) involved in MAX pathway encodes TCP transcription factor in *Arabidopsis* closely related to *teosinte branched 1* (*tb1*) of maize. *BRC1* expression was localized in developing buds and down-regulated in branch outgrowth. RNAi (RNA

interference) and double mutant experiment indicated that *BRC* gene prevents rosette branch outgrowth and control branching in downstream of MAX pathway. Also, it is required of auxin induced apical dominance (Aguilar-Martínez et al., 2007).

The *sps* (supershoot) mutant has massive-overproliferation of shoots. *SPS* gene encodes a cytochrome P450 and *sps* mutant showed increased levels in Z-type cytokinins. The result indicates that SPS might modulate shoot branching by regulating cytokinin levels at sites of bud initiation (Tantikanjana et al., 2001).

3. Hormone effects on branching

It has been well-established that auxin among the plant hormones is involved in shoot branching and apical dominance (Fig. 3). The outgrowth of lateral buds was suppressed in decapitated plants with an auxin treatment (Thimann et al., 1933). It has been known that other factors such as cytokinins also regulate shoot branching and apical dominance as second messengers (Li et al., 1995, Tantikanjana et al., 2001). Cytokinin application to axillary buds induced bud outgrowth of *Pisum sativum* (King and Van Staden, 1988). When endogenous cytokinin levels was increased by overexpression of cytokinin biosynthesis genes, isopentenyl transferase, axillary bud is released in tobacco (Medford et al., 1989).

Dun et al. (2006) explained the branching control into three hypothesis, classical hypothesis, auxin transport hypothesis and bud transition hypothesis, involved a role for the plant hormone auxin (Fig. 4). The classical hypothesis suggests auxin functions to regulate shoot branching with secondary messengers such as cytokinin (Sachs and Thimann, 1967; Bangerth, 1994; Li et al., 1995). The auxin transport hypothesis suggests that the shoot branching

control is affected by auxin movement in the auxin transport stream opposed to the actual auxin level. The auxin in the shoot tip of the main inflorescence generally is loaded into the polar auxin transport stream and moved in a basipetal of plant. The axillary bud outgrowth is inhibited entirely by the auxin transported in the main inflorescence (Morris, 1977; Li and Bangerth, 1999). The bud transition hypothesis suggests that the bud enters different developmental stages that have varying degrees of sensitivity or responses to long-distance signals including auxin (Stafstrom and Sussex, 1992; Shimizu-Sato and Morris, 2001; Morris et al., 2005).

The shoot branching was increased and plant height was decreased in *axr1* mutants. The mutant seedlings are resistant to 2,4-Dichlorophenoxyacetic Acid (2,4-D) and Indolyl-3-acetic acid (IAA) than wild type (Lincoln et al., 1990). The outgorwth of lateral inflorescences from excised cauline nodes of wild type plants is inhibited by apical auxin, but *axr1-12* nodes are resistant to the inhibition. The means that the auxin may inhibit bud outgrowth (Stirnberg et al., 1999).

An activation-tagging vector, pSKI015 contains four multimerized transcriptional enhancers derived from the cauliflower mosaic virus (Fig 5). The four multimerized transcriptional enhancers can induce the overexpression of genes near to the randomly inserted T-DNA, causing new phenotypes. (Weigel et al., 2000). A phenotypically distinct plant, *igi1* (Inflorescence Growth Inhibitor 1), was selected in the activation tagged lines. The mutant was heterozygous line, and the homozygous mutant lines were sterile, with no inflorescence and no seeds. The mutant also displayed abnormal flower development. In *igi1* heterozygous mutant, the internode elongation was reduced in the primary inflorescence. Additionally, secondary inflorescences begin to emerge straight from the axils of both rosette and cauline leaves, and, as a result, the mutant interestingly showed increased axillary branching pattern. These phenotypes were induced by overexpression of a gene encoding proline-rich extensin-like

receptor kinase (PERK) named IGI1. *API* gene expression levels was decreased and *CYP79B1* gene was overexpressed in the mutants. SUB1 (short under blue light) and LSH1 (Light-dependent Short Hypocotyls 1) was identified by yeast-two-hybrid screening, and *igi1* mutants showed similar pattern with wild type in hypocotyl growth under dark condition, suggesting that IGI1 modulate axillary branching through not only the plant hormone auxin but also light signal.

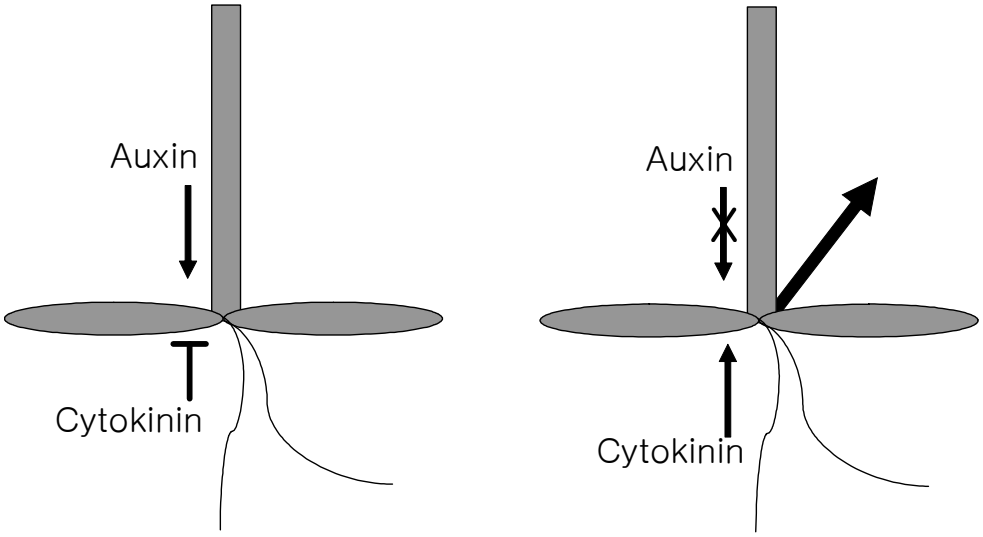


Figure 3. The scheme of auxin and cytokinin function in the control of bud outgrowth. The axillary buds were suppressed by auxin signal and cytokinin induces bud outgrowth.

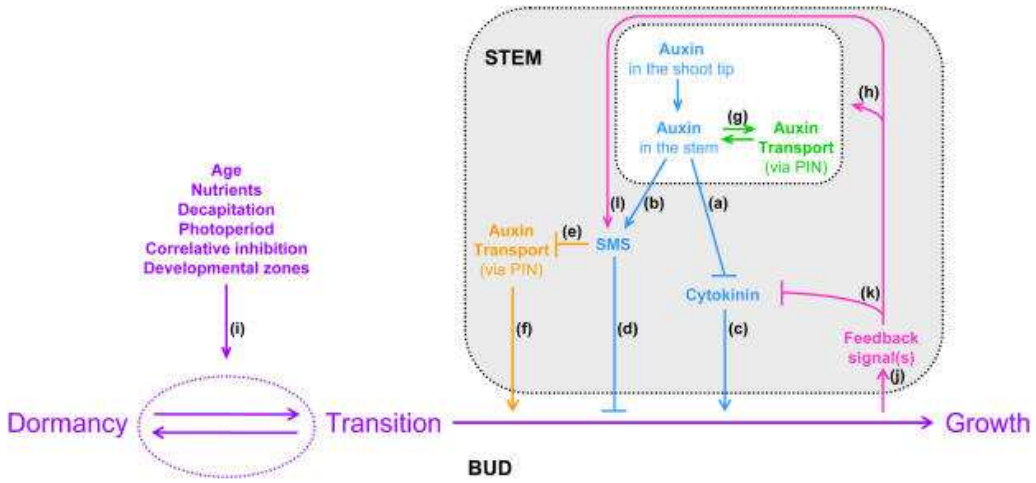


Figure 4. Model for regulation of stages of bud outgrowth. The classical hypothesis is illustrated in a, b, c, and d; the auxin transport hypothesis is illustrated in e and f; an alternative interpretation of the auxin transport hypothesis is illustrated in g; hypothesized feedback interactions are illustrated in h, j, k, and l; and the bud transition hypothesis is illustrated in a, b, c, d, g, h, i, j, k, and l. Arrowhead lines indicate promotion and flat-ended lines indicate inhibition. Location of cytokinin is not specified. Long-distance feedback regulation of cytokinin resulting from the perception of SMS (shoot multiplication signal) by RMS4 (not depicted explicitly) refers to xylem-sap cytokinin, whereas auxin regulation of cytokinin occurs in shoots and roots. Feedback regulation of auxin, which may be from an independent feedback process, is not shown because it may affect auxin levels or transport (Dun et al., 2006).

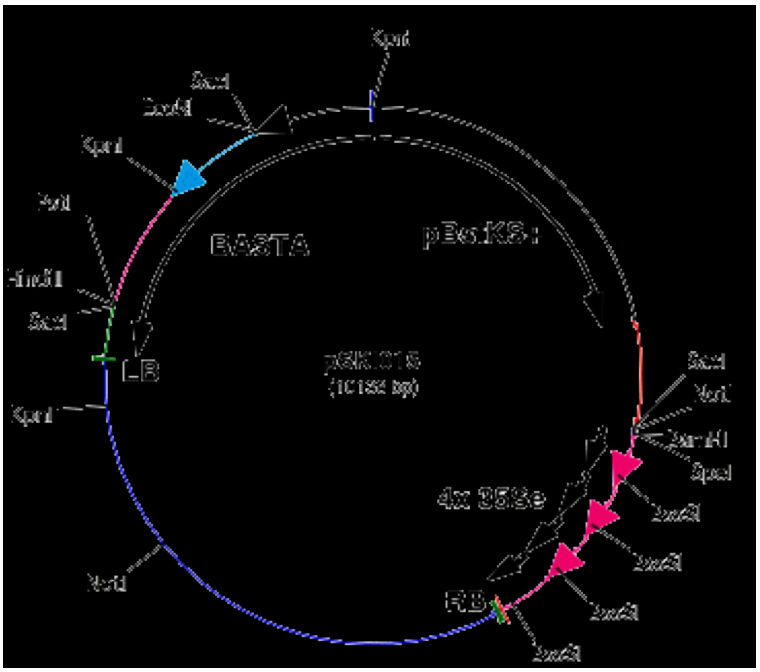


Figure 5. The map of pSKI015 vector. Activation-tagging vector, pSKI015 constructed by Igor Kardailsky in the Weigel lab (Weigel et al., 2000). This vector is composed with the CaMV 35S enhancers, basta resistance gene for selection in plants, and ampicillin resistance gene for plasmid selection in *E. coli* and in *A. tumefaciens*.

Ⅱ. *Materials and Methods*

A. Plant materials and growth conditions

Arabidopsis thaliana Columbia-0 (Col-0) was used as the wild type. Seeds were surface sterilized with 70% Ethanol containing 0.05% tritonX-100 and 95% Ethanol. After plating on the medium, the plate was warped with aluminium foil and cold treated at 4℃ for 3 days. All seeds were germinated on half strength Murashige and Skoog (MS) medium supplemented with 1% sucrose (1/2MS · 1S) and thean transferred to soil. Transferred plants were grown in the growth room at 22℃ under 16 hrs light and 8 hrs dark cycle.

B. Mutants screening and selection

1. The screening for morphological mutant

Activation-tagged columbia seeds were provided by Dr. Soo Young Kim (Chonnam National University), and the plants were screened by examining morphological phenotypes on soil. Selected mutants were back-crossed with Col-0 for searching single copy lines. The mutants were then back-crossed again with Col-0 to confirm single copy integration and compared with the original mutant phenotypes.

C. TAIL-PCR

1. Plant genomic DNA preparation

The DNeasy plant mini kit (QIAGEN) was used for the plant genomic DNA preparation. Plant leaf (0.1 g) was ground in liquid nitrogen to a fine powder using the mortar and pestle. Ground plants were transferred into the 1.7㎖ microtube, and 400㎕ of buffer AP1 and 4㎕ of RNase A stock solution were added. The mixture was incubated for 10min at 65℃. 130ul of buffer AP2 was added to a tube containing the lysate, and the tube was incubated for 5min on the ice. The lysate was transferred to the spin column and centrifuged for 2min at the maximum speed. The flow-through fraction was transferred to a new tube and added 1.5 volumes of buffer AP3/Ethanol to the cleared lysate. Again the flow-through was transferred to a new column and centrifuged at the maximum speed. To wash, buffer AW was added to the column and centrifuged. 100㎕ of preheated (65℃) buffer AE was directly added onto the membrane, and it was incubated for 5min at the room temperature and centrifuged for 1 min for elution.

2. TAIL-PCR condition

TAIL(Thermal Asymmetric Interlaced)-PCR (polymerase chain reaction) (Liu et al, 1995) was performed with long specific primers on the T-DNA and short arbitrary primers on the genomic DNA (Table 2). Three PCR reactions are carried out by using the LB150, LB100, and LB50 (Table 1). Figure 6 shows that T-DNA specific primers-LB150, LB100, LB50, and AD primer pairs make the specific product of the genomic sequence flanking a T-DNA insertion. The last amplification band is eluted and carried out

sequencing. T-DNA insertion sites of the transgenic lines were identified by basic local alignment search tool (BLAST) search.

D. Genotyping

T-DNA insertion sites and inserted T-DNA direction were determined by PCR procedure using T-DNA primers and genomic DNA primers. Also, the TAIL-PCR results give us the information whether the lines are homozygous or heterozygous. Figure 7 shows the principle of Genotypting. If the T-DNA present, the PCR product isn't present when the PCR reaction was carried out with a genomic F primer and R primers, and when the PCR reaciton was carried out with the T-DNA primer (RB and LB primer) and genomic primer (R and F primer) the PCR product is present. If it was a heterozygous-line, the PCR product is also present when the PCR reaction was carried out with the genomic F primer and genomic R primer.

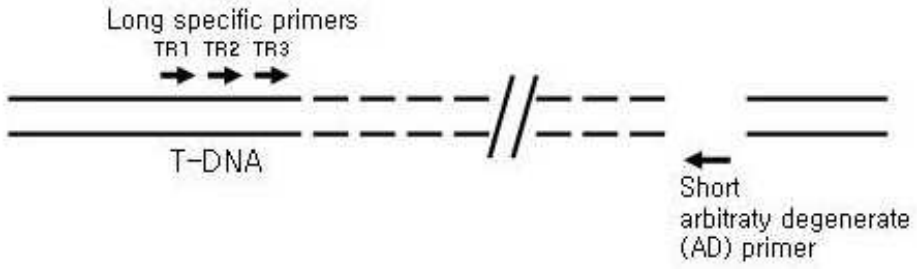


Figure 6. TAIL-PCR procedure for the specific amplification of genomic sequence flanking a T-DNA insertion. T-DNA specific primers and genomic random primer were used for TAIL-PCR. TAIL-PCR tertiary reaction products are sequenced and T-DNA insertion sites of the transgenic line can be analyzed by BLAST search.

Table 1. Cycling conditions used for the TAIL-PCR (Liu *et al.*, 1995).

Reaction	File no.	Cycle no.	Thermal condion
Primary	1	1	93°C(1min)→95°C(1min)
	2	4	94°C(45sec)→62°C(1min)→72°C(2.5min)
	3	1	94°C(45sec)→25°C(3min)→ramp to 72°C in 3min (0.3°C/sec)→72°C(2.5min)
	4	14[a]	94°C(20sec)→68°C(1min)→72°C(2.5min)→94°C(20sec)→68°C(1min)→72°C(2.5min)→94°C(20sec)→44°C(1min)→72°C(2.5min)
	5	1	72°C(5min)
Secondary	6	11[a]	94°C(20sec)→64°C(1min)→72°C(2.5min)→94°C(20sec)→64°C(1min)→72°C(2.5min)→94°C(20sec)→44°C(1min)→72°C(2.5min)
	5	1	72°C(2.5min)
Tertiary	7	20	94°C(38sec)→44°C(1min)→72°C(2.5min)
	5	1	72°C(5min)

Note. The program files in each reaction were linked automatically. [a]These are nine-segment super cylces.

Table 2. Arbitrary degenerate(AD) primers on the genomic DNA and long specific primers(LB150, LB100 and LB50) on the T-DNA (Liu et al., 1995).

arbitrary degenerate primers on the genomic DNA	AD2	5′-NGTCGASWGANAWGAA
	AD5	5′-SSTGGSTANATWATWCT
long specific primers on the T-DNA	LB150	5′-CACGTCGAAATAAAGATTTCCG
	LB100	5′-CCTATAAATACGACGGATGCT
	LB50	5′-ATAATAACGCTGCGGACATCA
N=A, T, C or G S=C or G W=A or T		

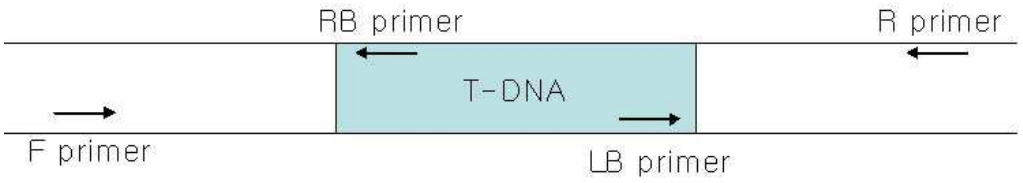


Figure 7. A general principle of Genotyping. In case of homozygous line, the PCR product is not present for the T-DNA insertion when the PCR reaction was carried out with a genomic F primer and R primer. But, when the PCR reaction was carried out with the T-DNA primer(RB and LB primer) and genomic primer(R and F primer), the PCR product is present. If it was a heterozygous line, the PCR product present when the PCR reaction was carried out with a genomic F primer and R primer. Also when the PCR reaction was carried out with the T-DNA primer (RB and LB primer) and genomic primer(R and F primer), the PCR product is present.

E. Constructs

1. Recapitulation

pMN20 vector including the four enhancers were used for recapitulation of *igi1* phenotypes (Fig. 8) (Weigel et al., 2000). The *IGI1* gene were produced by the PCR amplification. PCR reactions are carried out by using the 50KC1F (5′-AAc tgc agA GGT ATC TGT TAC TTT CAC CTA-3′) and 50KC1R (5′-TTc tgc agG CCC ATC TAG ATT TCA CAT CAT-3′). PstI restriction sites was used for cloning the *IGI1* gene. The clones were confirmed by PCR reaction with genomic part primer and T-DNA part primer, digestion with several restriction enzymes and sequencing.

2. Constructs for gene silencing

The pHANNIVAL vector was used for gene cloning (Fig. 9). Intron containing hairpin RNA construct shows silencing of targeting gene. KpnI restrction enzyme is used for sense orientation insertion and BamHI restrction enzyme is for anti-sense orientation. PCR amplifications were carried out on cDNA. The sequence of the PCR primers were: for the sense strand amplification, a forward primer 5′-CGg gta ccA GAA TGT CTA ACA CAT GCA GC-3′ named RIKpnI2F and a reverse primer 5′-CGg gta ccT AGC GCC GGA ATA TGC ATC A-3′ named RIKpnI2R, and for the anti-sense strand, a forward primer 5′-CGg gat ccA GAA TGT CTA ACA CAT GCA GC-3′ named RIBamHI2F and a reverse primer 5′-CGg gat ccT AGC GCC GGA ATA TGC ATC A-3′ named RIBamHI2R. These amplification products were digested with KpnI and BamHI restrction enzymes and directly cloned into the pHANNIVAL. The construct were digested with SacI and PstI

restrciton enzymes. The fragment subcloned into the pUC18 multi cloning site of a binary vector pCAMBIA1302 (Fig. 10 and Fig. 11). All constructs were confirmed by PCR reaction, enzyme digestion and sequencing.

3. Constructs for histochemical assay of GUS activity

The predicted promoter region of the *IGII* gene was amplified from wild type genomic DNA by the PCR amplification with the primers 5′-TCg gat ccG GCG ACT CGC CTA AGT CTG ACA T-3′ named KCPCF6 and 5′-AGg gat ccT ATA CTA AGA TCA CGT TAC TTG CC-3′ named KCPCR5. The PCR product was digested with BamHI restriction emzyme and then diretly cloned into pBI101.2 binary vector (Fig. 12).

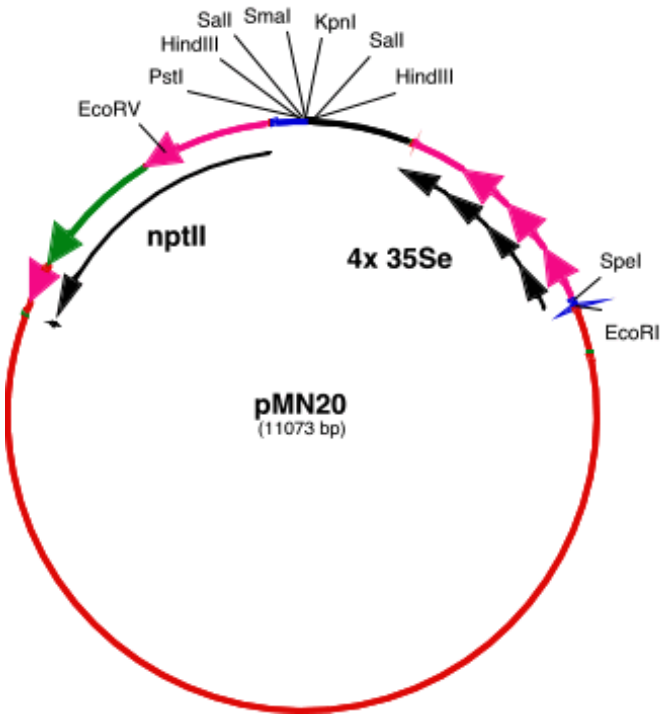


Figure 8. The map of pMN20 vector. The vector includes 4 copies of CaMV 35S enhancer and are used to confirm activation-tagged genes. Also the vector contains nptII gene for plant selection on MS media and spectinomycin gene for plasmid selection in *E. coli* and *Agrobacterium tumefaciens*.

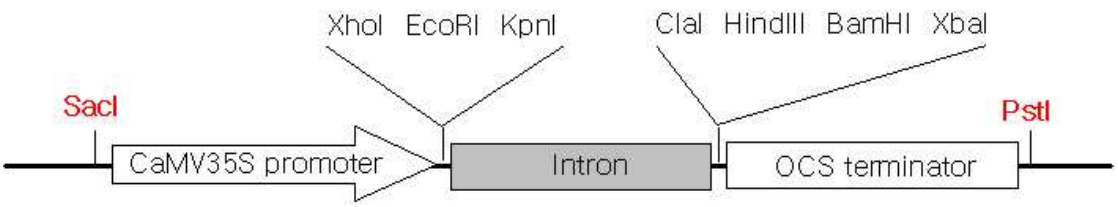


Figure 9. The map of pHANNIBAL vector functional region. The PCR fragment could be inserted in the sense orientation into the XhoI.EcoRI.KpnI polylinker and in the anti-sense orientation into ClaI.HindIII.BamHI.XbaI polylinker. The construct will produce an hairpin RNA for silencing with targeting gene.

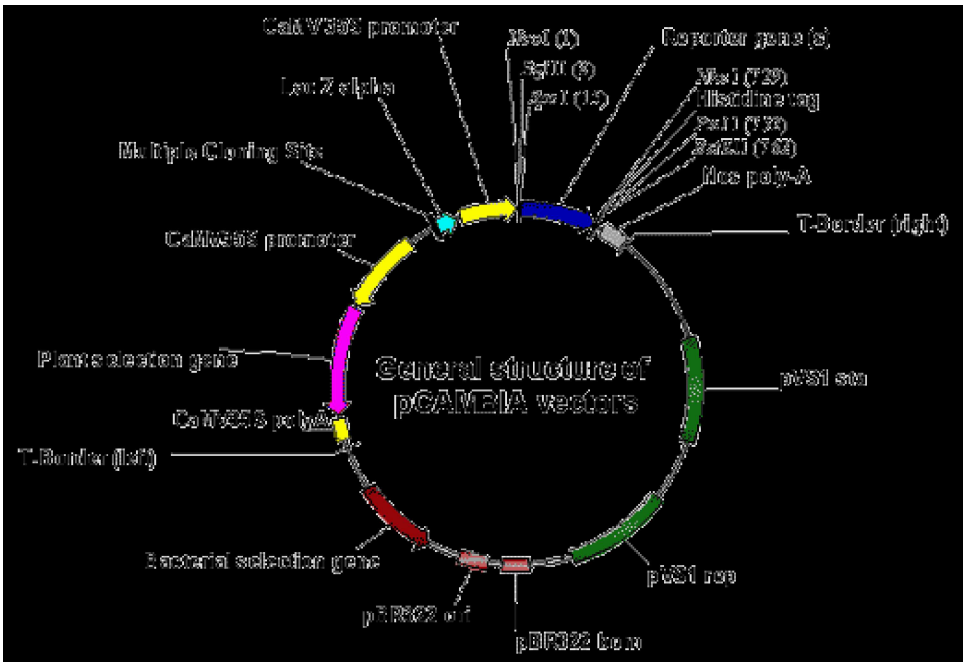


Figure 10. General structure of pCAMBIA vector. The pCAMBIA vector backbone is derived from the pPZP vectors. pCAMBIA vectors offer high copy number in E.coli for high DNA yields, pVS1 replicon for high stability in *Agrobacterium*, bacterial selection with chloramphenicol or kanamycin, and plant selection with hygromycin B.

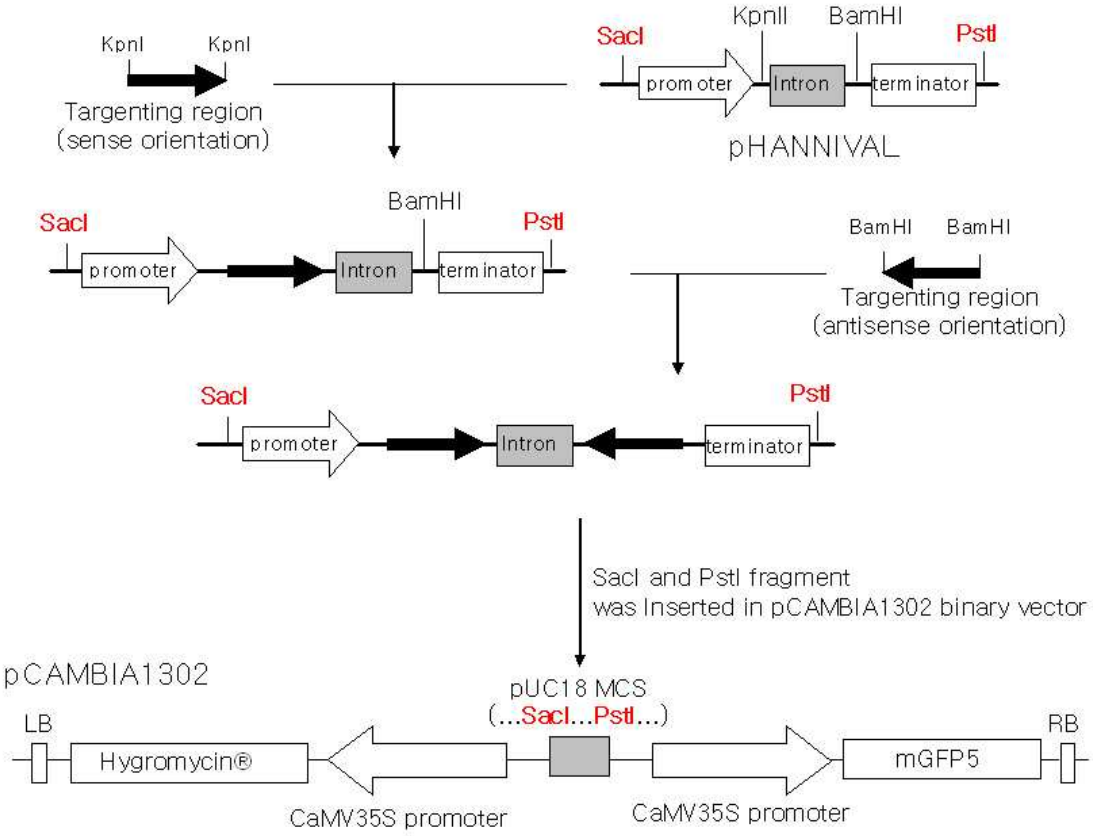


Figure 11. Schematic diagram for RNAi constructs. The KpnI and BamHI fragment for gene silencing is cloned into the pHANNIVAL and subcloned into the binary vector pCAMBIA1302.

F. Plant transformation

All cloned vectors were introduced into the A*grobacterium tumefaciens (A. tumefaciens),* GV3101 stain by freeze and thaw method. Competent cell of *A. tumefaciens* was mixed with about 1 ㎍ DNA in micro-tube. The mixture was frozen with liquid nitrogen (LN_2) for 2 minutes (min) and moved in 37℃ water. The mixture tube was incubated for 5 min in water bath to melt. The step, freeze and melt, was repeated one more time. The tube was incubated in ice for 30 min. After incubation in ice, The cell mixture was spreaded with a bent glass rod on YEP (Bacto-peptone 10 g/ℓ, Bacto-yeast extract 10 g/ℓ, and NaCl 5 g/ℓ) plates containing antibiotics. Colonies were confirmed by digestion and PCR reaction. *Agrobacterium*-mediated transformation was carried out by a dipping transformation protocol by Kim Hanson 5/95 for Vacuum Infiltration, and adapted from protocol by Andrew Bent (*Arabidopsis* Network 1/11/94 based on work of Nicole Bechtold, Jeff Ellis and Georges Pelletier) with suggestions from Peter Doerner and Takashi Araki. Agrobacterium was grown to mid-log phase in YEP medium, pelleted and resuspended with infiltration medium (Half strength MS salt, Gamborg's vitamin 112 ㎎/ℓ (Duchefa, G0415), and 0.44 uM benzylamino purine). Plants was inverted in infiltration media for 15 minute. Immediately, the plants were loosely covered with plastic wrap to maintain humidity. The plastic wrap was removed after a day. The soil was sufficiently dried about a week and than watered. T1 seeds were selected on the 1/2MS medium with antibiotics to get transformed plant, T2 to select single copy lines, and T3 to distinguish homozygous and heterozygous lines.

G. RNA preparation and cDNA synthesis

Total RNA was isolated from plant tissues using Trizol reagent (Life Technologies). 100 ㎎ of the plant tissue was homogenized by freezing with the liquid nitrogen and ground to the very fine powder. 1 ㎖ of TRIzol reagent was added to homogenized tissue samples, mixed, incubated for 10min at the room temperature. 0.2 ㎖ chloroform was added to those samples, mixed and incubated for 3min at the room temperature. The samples were centrifuged at 13,000 rpm for 15 min at 4℃ and a colorless upper aqueous phase was transferred to the new tube. 0.5 ㎖ of isopropyl alcohol was added to the samples and the samples were centrifuged at 13,000 rpm for 10 min at 4℃. The supernatant was removed, the pellet was washed with 70% ethanol and dried. cDNA was synthesized by using the superscript Ⅱ reverse-transcriptase (Invitrogen). 4 ㎕ (about 2 ㎍) of total RNA and 1ul of the oligo dT (500 ㎍/㎖) were mixed in the reaction tube and it was heated at 65 ℃ for 10 min. The enzyme was added into the tube and incubated at 42℃ for 50 min. The reaction tube was incubated at 70 ℃ for 15 min for inactivation.

H. Real-time (Quantitation) PCR

Real-time PCR amplifications were carried out using cDNA from mutants and wild type. For the *IGII* gene amplification, the forward primer 5′-AGG CGA TTG AAG ATC TTG AGA CGG AGG A-3′ named EPKF and a reverse primer 5′-ATG TCA GAC TTA GGC GAG TCG CCG AGT TCT-3′ named EPKR. All the primers were selected by computer analysis with the PRIME program. We used QuantiTect SYBR Green PCR Kit (QIAGEN) containing 2x SYBR Green PCR Master Mix with ROX as a passive reference dye,

HotStarTaq DNA Polymerase and dNTP Mix. 5 pmol of both primers (Forward and Reverse), 1 ㎕ cDNA and RNase-free water were added. For *IGI* gene, PCR amplified with a denaturation of 15 min at 95℃ 40 cycles at 95℃ 20 s; 5 5℃ 20 s 72℃ 40 s; and a final extension of 5 min at 72℃. It was measured on a real-time DNA detection system (Corbett co, RG-3000, Australia). For the multiple branching control genes, cytokinin response genes, and auxin biosynthesis genes primer pairs were described in Table 3.

I. Histochemical analysis

For GUS activity assay, MUG assay was performed. Sample was ground with 150㎕ extraction buffer (50 mM sodium phosphate buffer (pH 7.0), 10 mM EDTA, 0.1% triton X-100, 0.1% sarcosyl, 10 mM β-mercaptoetahnol). The samples were centrifuged at 13,000 rpm for 10 min at 4℃ and upper aqueous phase was transferred to the new tube. Protein concentration is measured with Bradford reagent using 25 ㎍ sample. 8 ㎕ MUG stock solution was added to the samples and the mixture was incubated for 1 hour at 37 ℃. After 1 hour, 40 ㎕ sample mixture and 160 ㎕ 0.2 M Na_2CO_3 were mixed to stop reaction, and the activity was measured at 420 nm wavelength.

Histochemical analysis using GUS staining was performed by incubation tissue in GUS staining buffer containing 2 mM cyclohexylammonium salt (Duchefa), 100 mM sodium phosphate buffer (pH 7.0), 10 mM EDTA, 0.5 mM potassium ferrocyanide, 0.5 mM potassium ferricyanide, and 0.1% triton X-100 (volume in volume, v/v). Samples were incubated for 16 hours in the reaction buffer and destained with 70 % (v/v) ethanol.

J. Decapitation experiment (Apical dominance test)

Arabidopsis wild type plants, Col-0, were grown under long day condition. After primary bolts come out (<10 cm), the primary shoot was removed. RNA was extracted from decapitated plants in 0 hour (hr), 1 hr, 2 hr, 4 hr, 8 hr, 12 hr, 24 hr, 36 hr, and 48 hr after decapitation. The experiment was repeated two times.

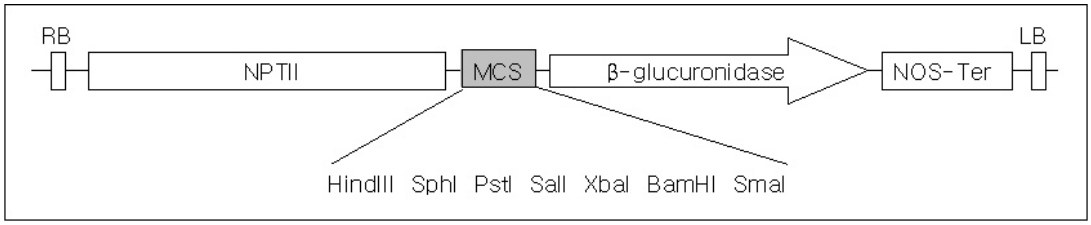


Figure 12. pBI101.2 T-DNA region. The vector contains promoterless, 1.87kb GUS cassette, and bacterial and plant selection with kanamycin.

Table 3. Primers for detection several gene expression level.

Gene name	*Primer name*	*Primer sequence (5′→3′)*
MAX1 (At2g26170)	MAX1F MAX1R	ACATCAATGGTGGGAAGTTCTTGATCCA ACCATAAGAGATGGCCATATATACCA
BRC1	BRC1F BRC1R	TTCCCAGTGATTAACCACCAT TCCGTAAACTGATGCTGCTC
BRC2	BRC2F BRC2R	TCAAAGAGAAGAACAAAGACTATGGA CCGAGGTCTCAAATAATCTCATC
CYP79F1 (At1g16410)	SPS1F SPS1R	ACATCATGATGAGCTTTACCACATCAT GGAGTTTATGGTGATGGCACGGATGCCGGC
CYP79F2 (At1g16400)	CYP79F2F CYP79F2R	TGGCCGACCAGGATGGCCCATCCTCGG TGTCTCCAATGGACTCTACGATGGAAAGT
REV (At5g60690)	REVF REVR	AGTGTTGTTCGTTCAGAGTCTTCAA AGACCAACGACATGTGGATACGAGA
ARR4 (At1g10470)	ARR4F ARR4R	AGCTCGTCTATGGCCAGAGACGGTGG AGGCATACAGTAATCAGTGATGATC
ARR5 (At3g48100)	ARR5F ARR5R	CCCGAGATGTTAGATATCTCTAACGAC ATCCAGTCATCCCAGGCATAGAGTA
CYP79B2 (At4g39950)	CYP79B2F CYP79B2R	TGTGGCTATAACCTTAGTGATGCTACT GCCGTTAGAGAGGATCTTCTGAGCGTAAG
CYP79B3 (At2g22330)	CYP79B3F CYP79B3R	ACGACCAAGTCAAGTCTCGGAATGTCGT GGGATCACGTGAGTGTTTCCTAGACGCA

Ⅲ. *Result*

A. Mutant screening

Genetic approach in mutants screening is an important tool for studying gene function in plant. We got morphological mutant plants in the activation tagged lines and chose a mutant. The mutant exhibited a number of phenotypes: smaller silique, semisterility, bunchy stem and shortened inflorescence. When selfed, three phenotypic classes of progeny were observed with an approximately 1:2:1 ratio. The first class was sterile and severely defective, the second class had many branching like original mutant, and the third class was normal, similar with wild type. To conform single copy, back-crossing with wild type to original mutant was performed. In F1 generation, the progeny showed a segregation ratio of approximately 1:1 (survival plants : dead plants) in basta plate. All survival plant showed phenotypes similar to original mutant in soil. In F2 generation, the seven plants, which survived in F1 generation, the progeny showed a segregation ratio of approximately 3:1 ratio (survival plants : dead plants) in basta plate (Table 4). The viable plants also segregated severely defective phenotypes and resemble phenotypes with original mutant with approximately 1:2 ratio in soil. These results indicate that original mutant was single copy line and heterozygous, and three phenotypic classes in next generation of original mutant corresponded to plants containing a homozygous, a hetetozygous, or no mutation. Upon self pollination, plants looking wild type produced only wild type progeny, whereas all original mutant segregated out three phenotypic classes. In *igi1/IGI1* heterozygous mutant, the phenotype of young seedlings is similar to wild type, and *igi1/igi1* homozygous mutant showed curled and smaller leaf (Fig. 13A). The homozygous mutant showed sterility and had no inflorescence, no seeds and abnormal flower organ after the plants start to flower (Fig. 13B). The heterozygous mutant plants produce

primary inflorescence with reduced internode elongation, and secondary inflorescences begin to emerge straight. The number of inflorescence developing from the rosette leaves in wild type is usually 1 to 5 and fewer than 10 including inflorescence of cauline leaves. In contrast, heterozygous mutant plants continue to produce axillary inflorescence from the axils of both rosette and cauline leaves. Consequently, the heterozygous mutant have dramatically increased number of axillary branches (Fig. 14).

Table 4. Segregation ratio of the igi1 progeny. Screened mutant didn't have single T-DNA. After the mutant was back-crossed with wild type and the genotyping PCR was performed in F2 generation for searching single copy lines. After fixing the original mutant which have single T-DNA insertion, again the mutant was back-crossed with wild type to test the single copy line or not. After seedling *igi/IGI1* heterozygous mutant (expected) in 1/2MS medium containing basta 20mg/l, the plants were counted.

Plant(s)/cross	Number of plants		
	Similar with wild type	Defective phenotype	dead plants
The original mutant(*igi1/IGI1*)/self	226	119	150
igi1/IGI1 × wild type			
F1	38	-	31
F2 (7 plants)	1638	910	941

A B

Figure 13. igi1 mutants phenotype. A. 10 day old plants. B. 25 day old plant. From left to right, *Col-0, igi1/IGI1* heterozygous, and *igi1/igi1* homozygous mutant plant.

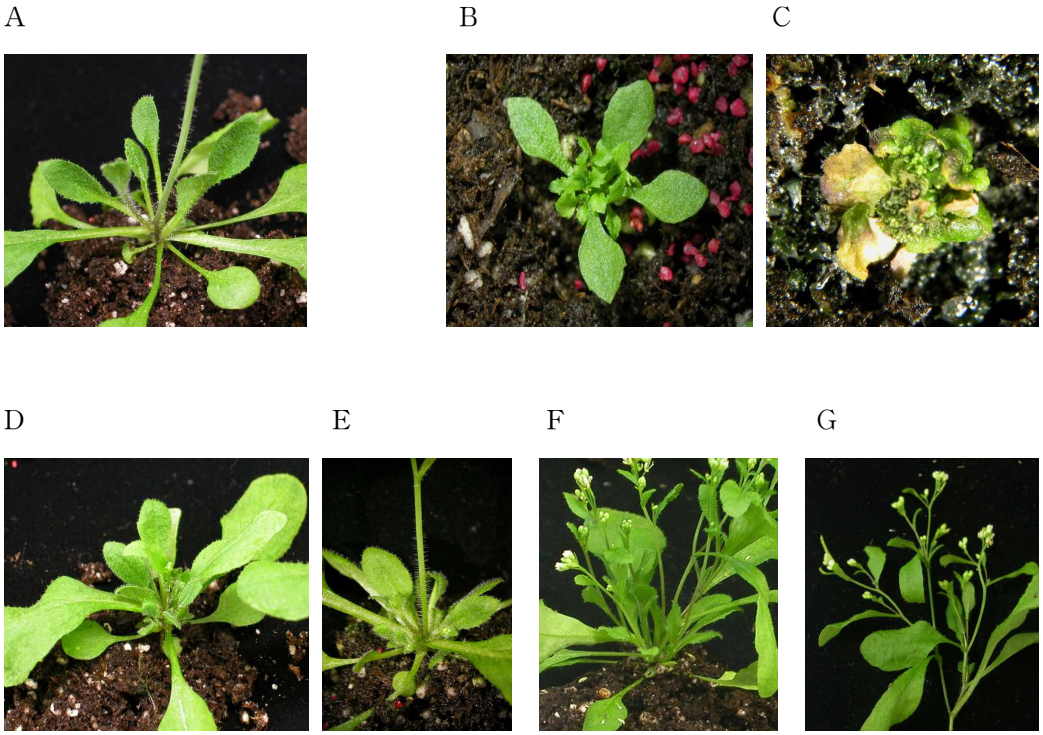


Figure 14. Axillary branching pattern in igi1 mutants. A. Wild-type axillary inflorescence from rosette leaves. 20 day-old plant (B) and 40 day-old plant (C) of *igi1/igi1* homozygous mutant are shown. The homozygous mutant have no inflorescence and abnormal flowers. D-F. *igi1/IGI1* heterozygous mutant axillary inflorescence from rosette leaves. G. Multiple axillary inflorescences emerge from each cauline leaf of *igi/IGI1* mutant.

B. Cloning of the IGI1

To determine T-DNA locus of the mutants, we extracted genomic DNA from heterozygous mutant, and performed thermal asymmetric interlaced-PCR (TAIL-PCR) using arbitrary genomic primers and T-DNA specific primers. Segregation ratio analysis, TAIL-PCR walking and genotyping PCR results indicated a single T-DNA insertion at the 200bp upstream of the *At1g23540* coding region in the BAC F5O8 (Fig. 15). We referred to *At1g23540* gene as *IGI1* (inflorescence growth inhibitor 1) and the mutation as *igi1/IGI1* for heterozygous and *igi1/igi1* for homozygous mutant. After determining T-DNA insertion site by sequencing of TAIL-PCR products containing the T-DNA/plant genomic DNA junction, Genotyping PCR was carried out to identify whether the plant assayed is wild type, homozygous or heterozygous mutant. Figure 16 is results of the genotyping PCR in genomic DNA samples of the wild type and igi1 mutants. Because wild-type plants don't have the T-DNA, there are no band in the lane 3 and lane 5. When only the genomic DNA primer pairs were used, the PCR product in *igi1/igi1* mutant did not amplify because of T-DNA insertion (lane 2), but it was amplified in *igi1/IGI1* mutant. The homozygous and heterozygous plants were selected by segregation analysis and genotyping PCR reaction, and T-DNA direction also was determined.

After the T-DNA position and direction were determined, expression levels of nearby genes flanking T-DNA were investigated by Real time PCR reaction. The expression level for *At1g23540*, *IGI1* gene was increased approximately by 1,000 to 3,000 fold in *igi/igi* homozygous mutant and 500 to 1,000 fold in *igi1/IGI1* heterozygous mutant (Fig. 17). Expression of other neighboring genes also increased in *igi1/igi1*, but not in *igi1/IGI1* mutant. These results indicate that the over-expression of the *IGI1* gene cause the *igi1* mutants phenotypes such as steriility, lack of inflorescence and seeds, and abnormal flower organ in *igi1/igi1* mutant and dramaticially increased axillary branches in *igi1/IGI1* mutant.

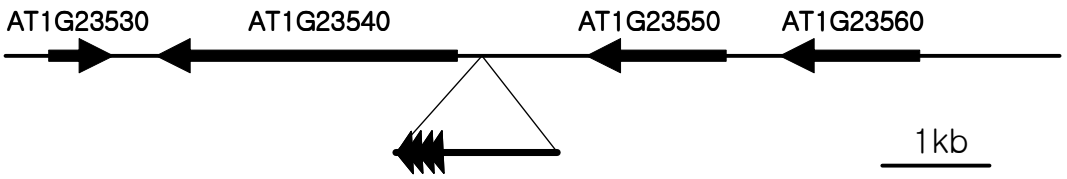


Figure 15. T-DNA position in the igi1 mutants. BAC F5O8 clone are shown. There is T-DNA insertion in the 200 bp upstream of the *At1g23540* gene coding region.

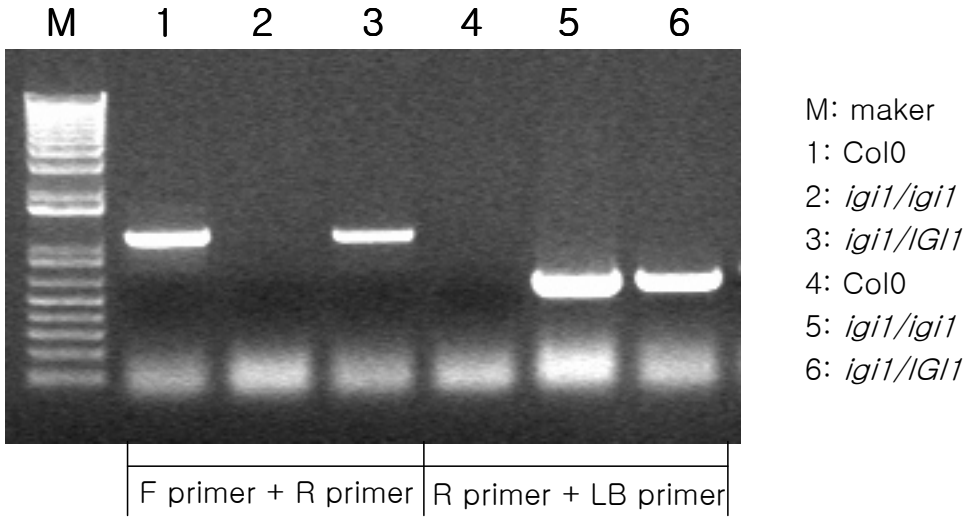


Figure 16. The genotyping result for T-DNA locus in the igi1 mutants. Genomic DNA was amplified by forward and reverse genomic primers in lain 1 to 3, and by reverse genomic primer and T-DNA specific primer in lain 4 to 6.

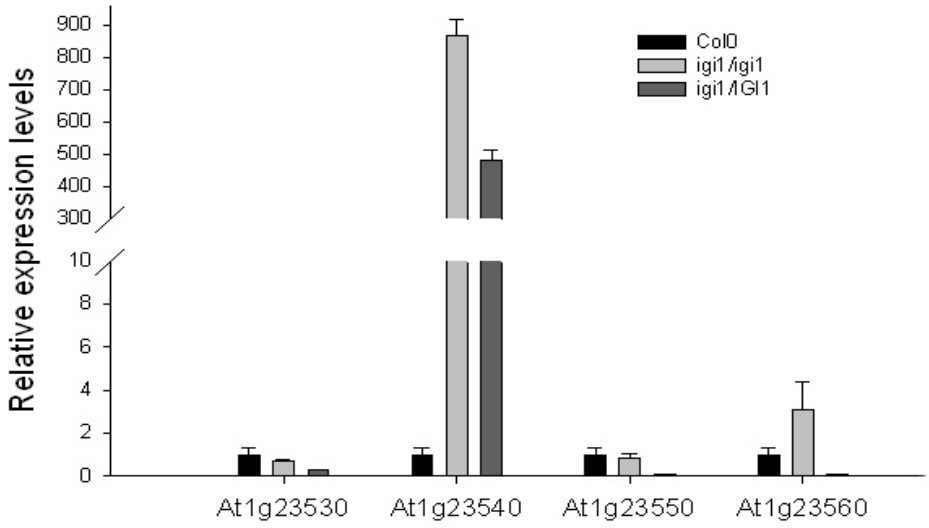


Figure 17. The expression level of neighboring genes near the T-DNA. Real time PCR result show that *At1g23540* gene transcript was dramatically increased in *igi1* mutants. Actin was used for normalization and error bars indicated standard deviation.

C. Recapitulation

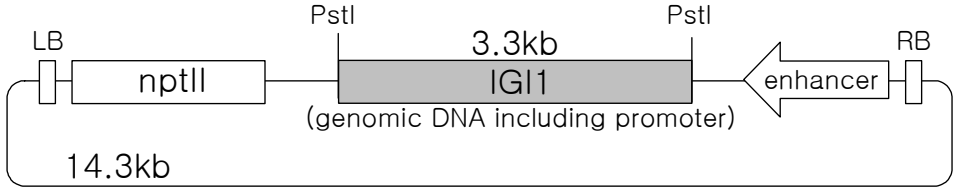
To rescue the *igi1* mutants phenotypes, recapitulation construct was generated. Recapitulation vector, pMN20, including the four enhancers was used (Weigel *et al.*, 2000) for this experiment. 3.3kb *IGI1* gene containing own promoter was amplified by PCR reaction from wild type genomic DNA and cloned into the *Pst*I restriction site of pMN20 vector (Fig. 18A). To confirm the construct, it was digested by several restriction enzymes, *Pst*I, *EcoR*I, and *Kpn*I (Fig. 18B). After cloned, the construct was transformed to the wild type plant. T3 homozygous lines were generated from T2 individuals carrying single insertion, which was identified in 3:1 segregation ratio on antibiotics medium. The mRNA level of the recapitulation mutants was analyzed by quantitative real-time PCR. *IGI1* recapitulation lines #1 (*IGI1-RC#1*) and *IGI1-RC*#5, which showed higher and middle expression levels among other recapitulation lines, were selected. The expression levels of *IGI1* gene were lower in *IGI-RC#1* and *IGI-RC#5* than that of *igi1* mutants (Fig. 21). Figure 19 and Figure 20 show the phenotypes of *IGI-RC#1* and *IGI-RC#5*. Unexpectedly, we didn't find same phenotypes with *igi* mutants in population of recapitulation mutants. The *IGI-RC#5* mutant showed same phenotype with wild type. However, *IGI-RC#1* showed phenotypes somewhat similar to those of *igi1/IGI1* mutant. Significantly, the inflorescence number was increased and plant height was reduced in *IGI-RC#1* mutant (Fig. 22). It couldn't completely rescue *igi1* mutants phenotypes possibly because of lower expression levels in recapitulation mutants.

We attempted to revert the phenotype of *igi1* plants by reducing the mRNA levels with RNA interference (RNAi). The 300 base pairs (bp) of *IGI1* gene cording region were amplified from wild type plant using primers that was added *Kpn*I site on the ends of one product (sense strand) and *Bam*HI site on the ends of one the other product (anti-sense strand). These amplification

products were digested with *Kpn*I and *Bam*HI restrction enzymes and directly cloned into the pHANNIVAL (Fig. 23). The construct was subcloned into the binary vector pCAMBIA1302 (Fig. 24) and transformed into *igi1/IGI1* mutant plants to revert the phenotype of *igi1* mutants. The cloning region of pHANNIVAL vector contains a intron which induced self complementarity between the sense and anti-sense targeting RNA strand.

To select the *igi1/igi1-RNAi* mutant, segregation test in the medium supplement with hygromycin for RNAi single locus (data not shown) and genotyping PCR for *igi1* locus in F2 generation (Fig. 25) were performed. *IGI1* expression level was efficiently decreased in RNAi transformed mutant, *igi1/igi1-RNAi* (Fig. 27), and the phenotype showed similar to wild type (Fig. 26). The phenotype was reverted by reducing the mRNA levels, suggesting that *igi1* mutants phenotypes were caused by overexpression of the *IGI1*.

A



B

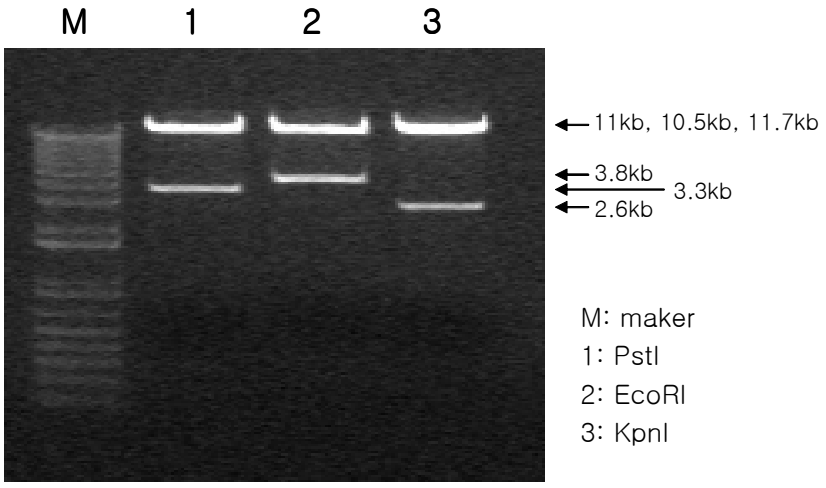


Figure 18. The map and confirmed image for IGI1-RC construct. A. The construct for recapitulation containing genomic part including the gene promoter. B. DNA fragments of *Pst*I-, *Eco*RI-, and *Kpn*I-digested *IGI1-RC* construct in 1% agarose gel. The restriction enzyme sites of *Pst*I was chosen to confirm the insert sizes. The lower fragments in lain 1 indicate the insert. M, 1kb DNA size maker; 1, *Pst*I restricted fragment; 2, *Eco*RI restricted fragment; 3, *Kpn*I restricted fragment.

Figure 19. Recapitulation plants phenotypes. The plants were grown for 15 days (upper panel) or 20 days (below panel). From left to right, Col-0, *igi1/IGI1, igi1/igi1, IGI1-RC#1, and IGI1-RC#5.*

Figure 20. Recapitulation plants phenotypes. The plants were grown for 25 days. From left to right, Col-0, *igi1/IGI1, igi1/igi1, IGI1-RC#1, and IGI1-RC#5.*

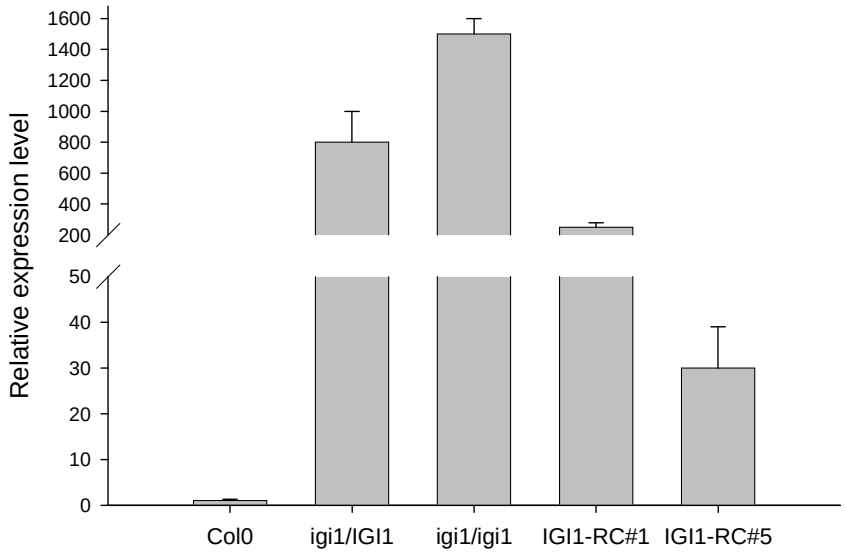


Figure 21. Relative expression level for recapitulation lines. Actin was used for normalization and error bars indicated standard deviation.

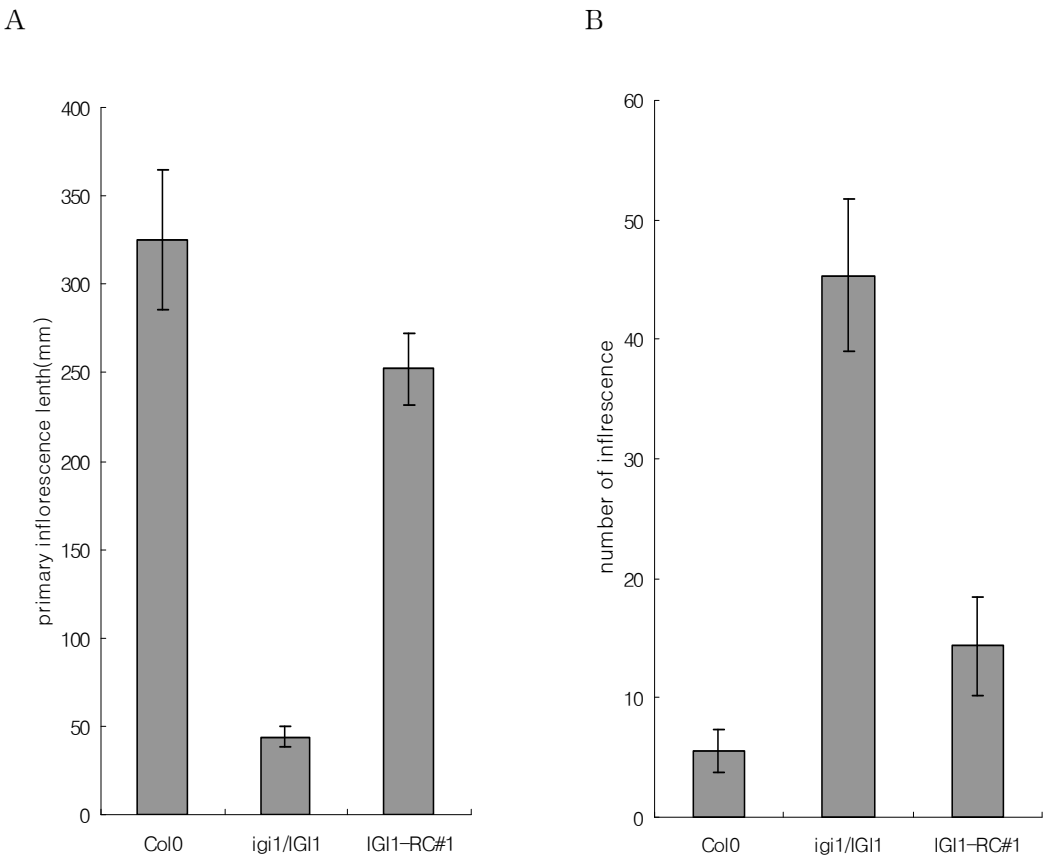
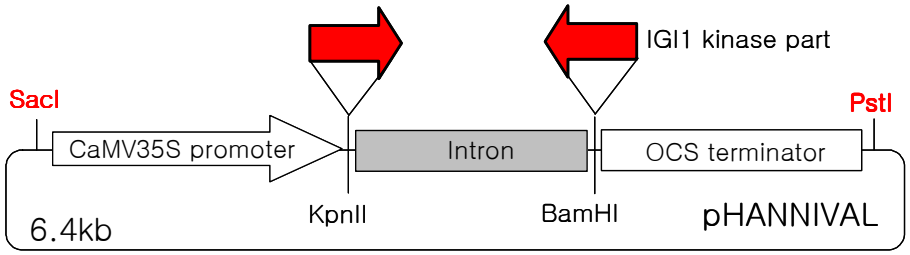


Figure 22. The plant height (A) and number of inflorescence (B) in 40-day-old *igi1/IGI1* mutant and *IGI1-RC#1*. Error bars represent the standard errors of the means.

A



B

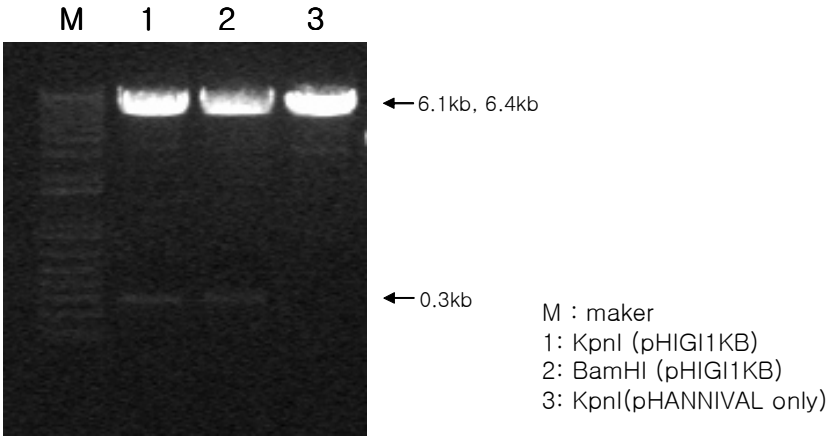
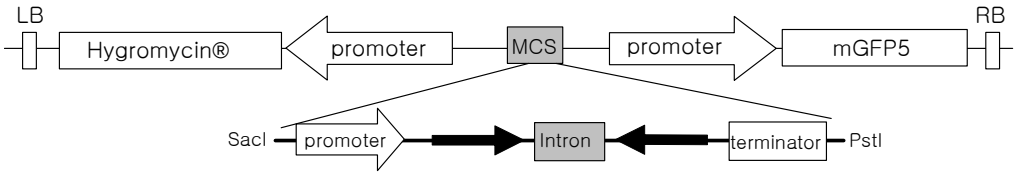


Figure 23. The map and confirmed image for IGI1-RNAi intermediate construct. A. The construct map for RNA interference including targeting site. B. DNA fragments of *Kpn*I and *Bam*HI digested *IGI1-RNAi* intermediated construct in 1% agarose gel. The restriction enzyme sites of *Kpn*I and *Bam*HI were chosen to confirm the insert sizes of sense and antisense fragment. There are no lower fragment (0.3kb) in lain 3 (vector only). M, 1kb DNA size maker; 1, *Kpn*I restricted fragment; 2, *Bam*HI restricted fragment; 3, *Kpn*I restricted fragment with vetor only.

A



B

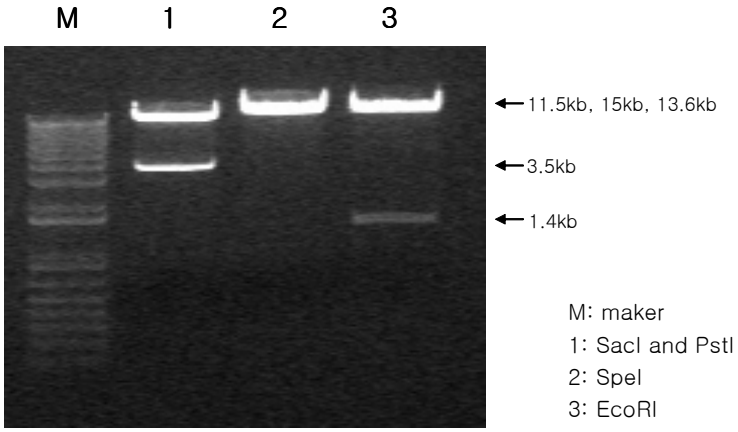


Figure 24. The map and confirmed image for IGI1-RNAi construct. A. The construct map for RNA interference in binary vector pCAMBIA1302. B. DNA fragments of *Sac*I, *Pst*I, *Spe*I, and *Eco*RI digested *IGI1-RNAi* construct in 1% agarose gel. The restriction enzyme sites of *Sac*I and *Pst*I were chosen to confirm the insert sizes. The lower fragments in lain 1 should be insert. M, 1kb DNA size maker; 1, *Sac*I and *Pst*I double restricted fragment; 2, SpeI restricted fragment; 3, *Eco*RI restricted fragment.

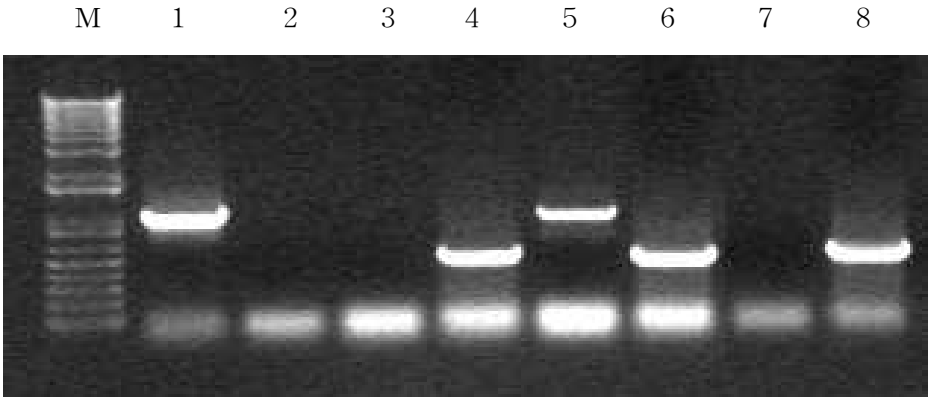


Figure 25. igi1/igi1-RNAi mutant genotyping for igi1 locus. The PCR reaction was performed with genomic primer pairs (lain 1, 3, 5, and 7), and genomic primer and T-DNA primer (lain 2, 4, 6 and 8). M, 1kb DNA size maker; lain 1 and 2, Col0; lain 3 and 4, *igi1/igi1*; lain 5 and 6, *igi1/IGI1*; lain 7 and 8, *igi1/igi1-RNAi*.

Figure 26. The phenotype of igi1/igi1-RNAi mutant. Col-0, *igi1/IGI1*, *igi1/igi1*, and *igi1/igi1-RNAi* (left to right).

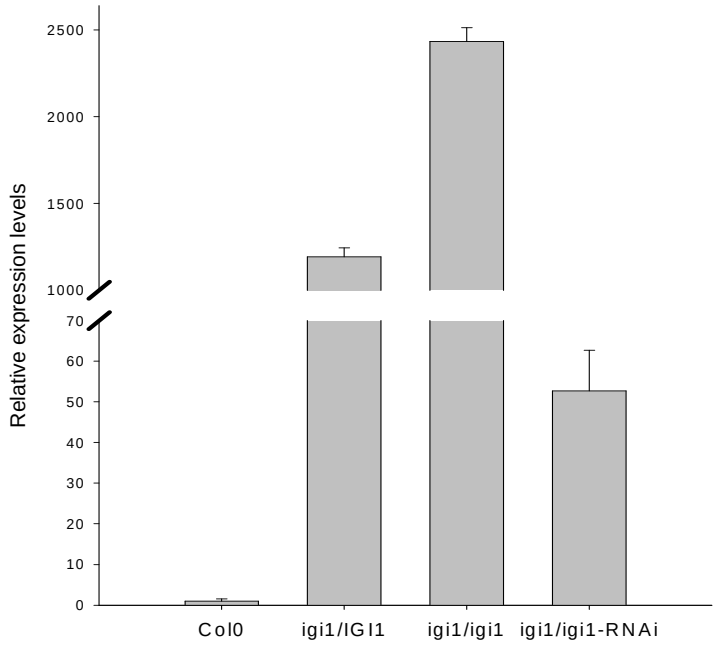


Figure 27. Real-time PCR result for igi1/igi1-RNAi mutant. Actin was used for normalization and error bars indicated standard deviation.

D. IGI1 gene is strongly expressed in anther

To study the expression pattern of the *IGI1* gene, we developed a construct for histochemical GUS reporter assay. In order to insert predicted *IGI1* promoter region into pBI101.2 binary vector, PCR was carried out for predicted *IGI1* promoter region. The PCR product and pBI101.2 vector were designed with *Bam*HI restricting enzyme (Fig. 28). These two fragments were ligated and named as *IGI1p::GUS*. To confirm the *IGI1p::GUS* construct, *Bam*HI and *Xba*I resteciton enzymes were used (Fig. 29). Lane 1 and lane 2 were the fragments of *Bam*HI- and *Xba*I-digested *IGI1p::GUS*. The construct was introduced into wild type plant. In mature plants, GUS expression was detected strongly in anther part and detected rarely in upper part of stem, immature siliques, and root. However the GUS expression was not detected in other part such as rossette and cauline leaves, mature siliques, and middle and lower stem.

The expression was detected at low level in only hair zone of primary root and not detected in other part of 5-day-old seedlings (Fig. 30A-D). The expression was detected more strongly than 5-day-old seedlings in hair zone of lateral root but was not detected in primary root of 10-day-old seedlings (Fig. 30E-G). *IGI1p::GUS* expression also observed only flower parts when the plants are about to start bolting (about 20 days after seedling). In the 33-day-old plants, the expression was strongly observed in anther of flower organ and weakly observed in upper stem and immature siliques (Fig. 31). *IGI1p::GUS* expression showed tissue-specific gene expression which strongly expressed in the anther part. To confirm the expression patten of *IGI1* gene, the expression level was examined in different tissues by the real time PCR. The expression level in flower part was much higher than in other parts such as the rosette and cauline leave, and stems (Fig. 32).

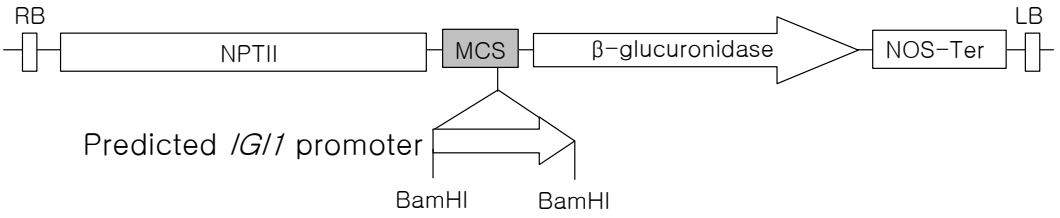


Figure 28. The construct map for GUS reporter assay in binary vector pBI101.2. The promoter region of *IGI1* gene was amplified with genomic specific primers containing the *Bam*H I restriction site and directly cloned into binary vector pBI101.2.

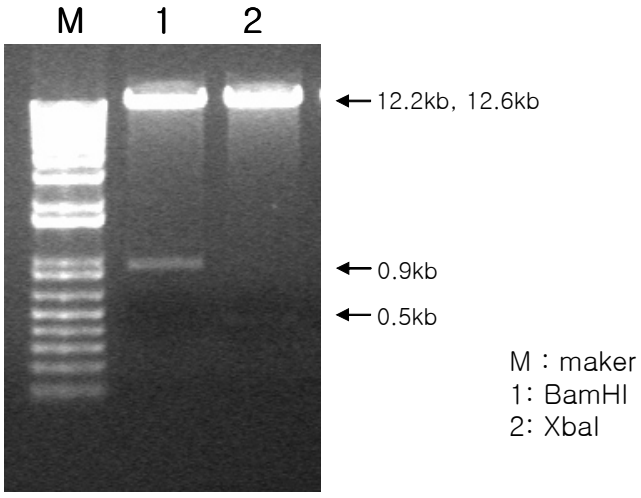


Figure 29. DNA fragments of BamH I- and Xba I-digested IGI1p::GUS construct in 1% agarose gel. The restriction enzyme sites of *Bam*H I and *Xba* I were chosen to confirm the insert sizes and orientation. The lower fragments in lain 1 should be insert. M, 1kb DNA size maker; 1, *Bam*H I restricted fragment; 2, *Xba* I restricted fragment.

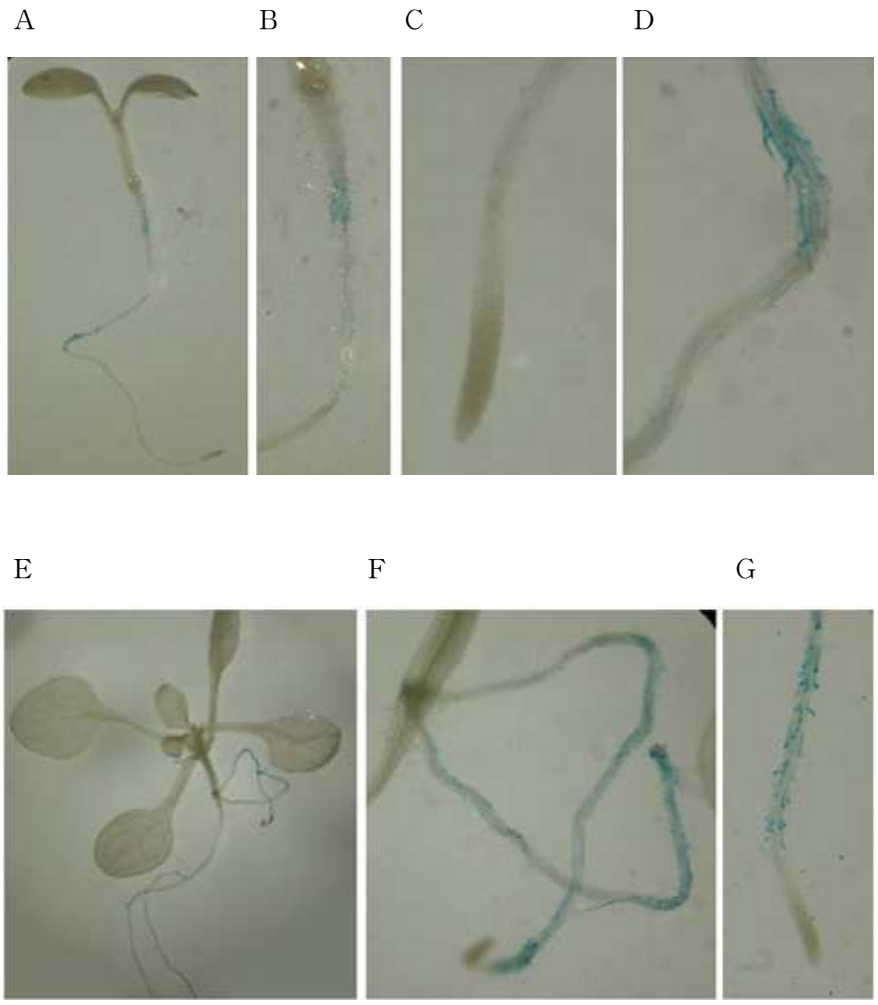


Figure 30. Expression pattern of the IGI1 gene. The expression pattern was detected with GUS reporter gene under control of *IGI1* gene promoter. A-D. 5 day old plant. E-G. 10 day old plant.

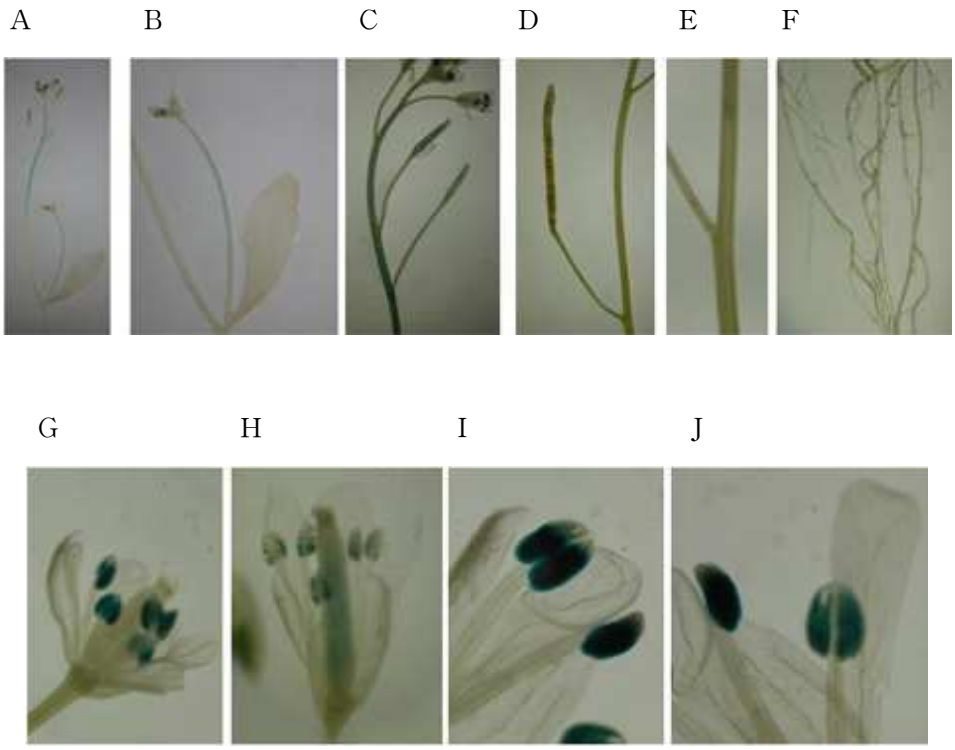


Figure 31. Expression pattern of the IGI1 gene in 33 day old plant. The expression pattern showed tissue specific gene expression. A. Primary inflorescence. B. Secondary inflorescence. C. Primary inflorescence with silique. D. Mature seed. E. Middle stem. F. Root. G-H. Flower. I-J. Anther.

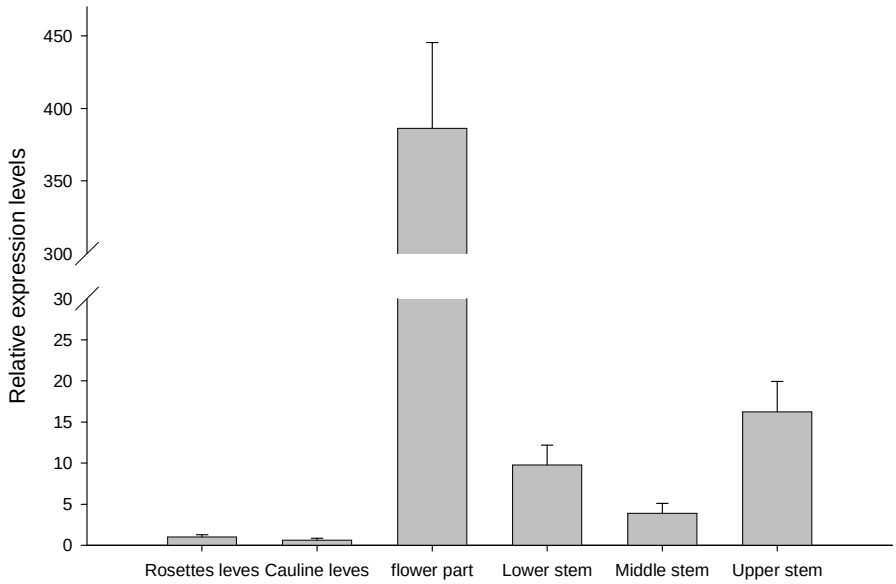


Figure 32. The relative expression levels for IGI1 gene in different tissues. The flower part has higher expression level than other parts. Actin was used for normalization and error bars indicate standard deviation.

E. IGI1 interact with LSH1 and SUB1

The *IGI1* gene consists of 8 exons with over 2,800 base pair (bp). The predicted IGI1 protein contains 720 amino acid residues possessed proline rich domain in N-terminal region and kinase signature domain in C-terminal region (Fig. 33 and Fig. 34).

To investigate protein-protein interactions, yeast two-hybrid screening was performed with IGI1 proline rich domain in the N-terminal and kinase domain in the C-terminal as baits. Table 5 summarizes the protein which identified by the yeast two hybrid screening. Interestingly, light response protein SUB1 and LSH1 were identified. LSH1 do interact with both proline rich domain and kinase domain in the yeast two-hybrid screen. The SUB1 interacts with only the proline-rich domain in the N-terminal, implying that IGI1 might be a nuclear receptor and the light source may influence the phenotype of *igi1* mutants.

F. Hormones and axillary branching

To investigate the apical dominance and bud outgrowth, decapitation was performed with wild type plant. After primary bolts come out (<10 cm), the primary shoot was removed. After Decapitation, lateral bud outgrowth is stimulated by the apical source as auxin (IAA) or various component in genetic pathway in less than several hours. The *IGI1* gene expression levels were markedly increased in 4 hours after decapitation. *BRC2* also highly increased in 4 hours after decapitation (Fig. 36).

Different gene expression profiles were analyzed in *igi1* mutants to test the expression of the gene which is related axillary bud outgrowth or branching.

There are no visible detectable increase in the expression levels of branching components, such as *BRC1*, *BRC2*, *MAX1*, *REV*, and *SPS1,* and cytokinin response genes, such as *ARR4* and *ARR5*, cytokinin signaling component. In contrast, the expression level of auxin biosynthesis component *CYP79B2* is significantly increased, and flower meristem identity component *API* is decreased in *igi1* mutants (Fig. 35, Fig. 37, Fig. 39).

Cytokinin normally stimulate cell division and greening of hypocotyl-derived calli (Higuchi et al, 2004). To test cytokinin sensitivity, callus induction assay was performed. For the callus induction assay, the plant hypocotyls grown in dim light for 15 days were excised with scissors and cultured for 20 days in 1/2MS · 1S supplemented with 50 nM 2,4-D and varying concentrations of kinetin. The *igi1/igi1*, *igi1/IGI1* mutants responded normally to cytokinin in the assay (Fig. 38).

When grown in dark, Col-0 and *igi1/igi1* mutant showed similar patterns in hypocotyl growth and in 2,4-D and kinetin dose response. Interestingly, the root growth showed inhibition pattern in *igi1/igi1* mutant under light condition (0nM hormone concentration) and the relative deference in root growth between Col0 and *igi1/igi1* mutant diminished in BAP dose response (Fig. 40). In the medium supplemented with 2,4-D, the growth showed no response (Fig. 41). The effect of the auxin and cytokinin on roots elongation was examined in 7 days grown seedlings.

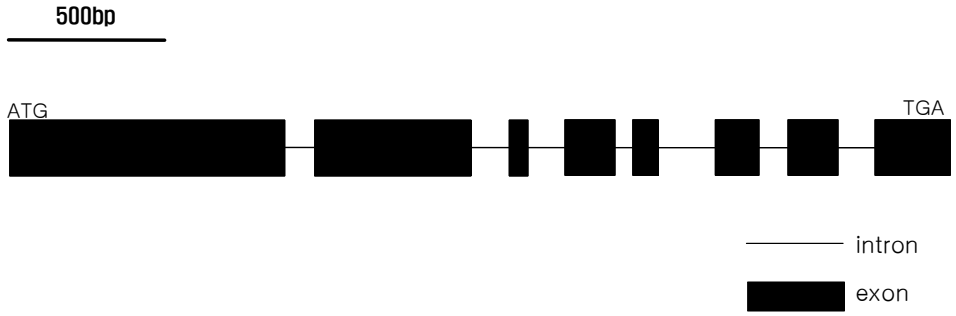


Figure 33. Diagram of IGI1 genomic DNA. The *IGI1* gene consists of 8 exons (black rectangles) and 7 introns.

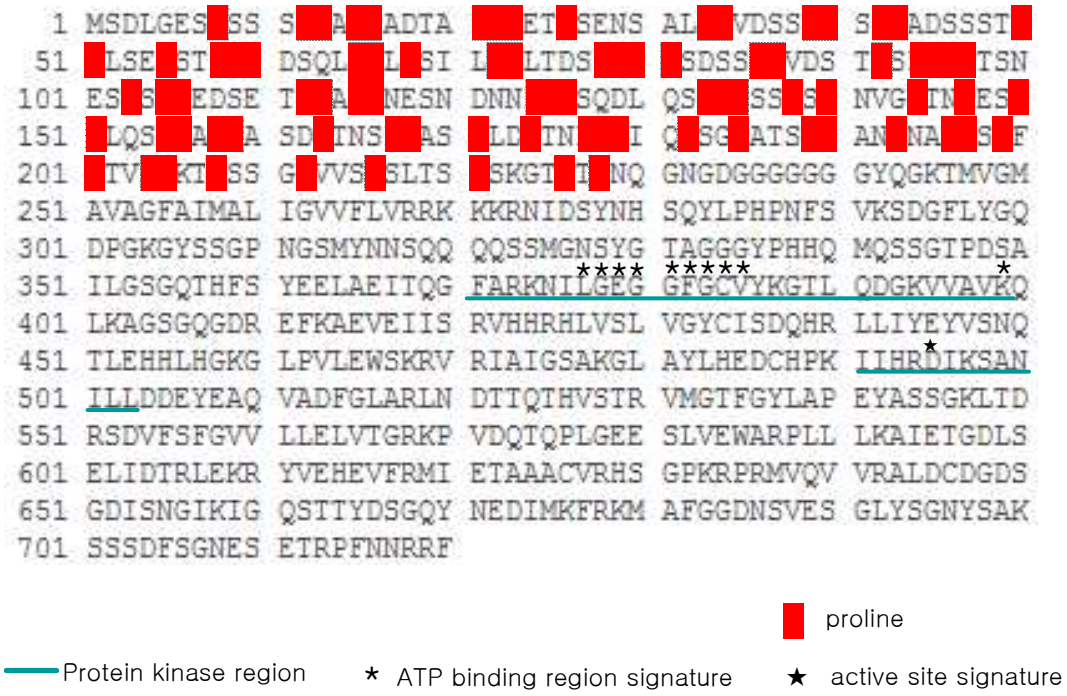


Figure 34. Amino acid sequence of the IGI1. The predicted IGI1 protein contains 720 amino acid residues composed proline rich domain in N-terminal region and kinase domain signature in the C-terminal region. The ATP binding region signature is marked (★), the protein kinase region is underlined, and acive site signature is maked (*).

Table 5. The result of yeast-two hybrid screening. The protein identified from the cDNA library in Arabidopsis.

Bait; IGI1 N-terminal		
Locus	Description	
At5g28490	***LSH1 (Light-dependent short hypocotyl 1)***	***Full-length***
At4g08810	***SUB1 (Short under blue light 1)***	***Full-length***
At5g08720		Partial
At3g25070	RIN4 (RPM1-interacting protein 4)	Full-length

Bait; IGI1 C-terminal		
Locus	Description	
At2g42610	***Unknown, homologous to LSH1***	***Full-length***
At5g28490	***LSH1 (Light-dependent short hypocotyl 1)***	***Full-length***
At5g11740	AGP15 (Arabinogalactan protein 1)	Full-length
At2g21230	bZIP protein similar to CREB protein	Partial
At3g02780	IPP2 (Isopentenyl pyrophosphate 2)	Full-length
At4g18610	Unknown	Partial
At3g11100	Transcription factor, 6b-interacting protein 1	Full-length

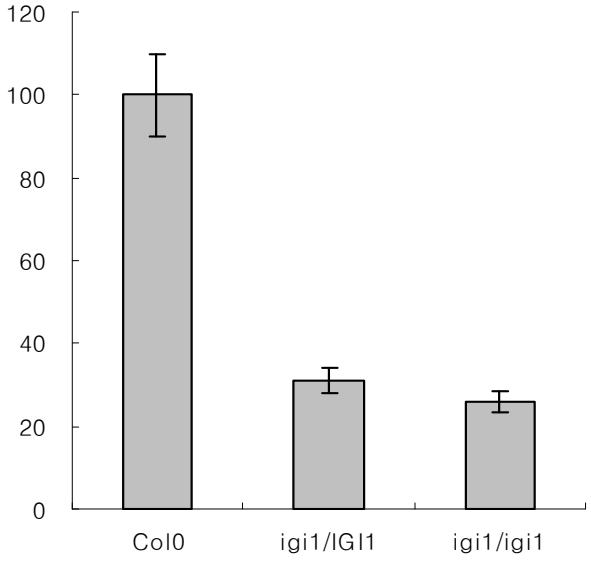


Figure 35. AP1 expression levels in igi1 mutnats. Actin was used for normalization and error bars indicate standard deviation.

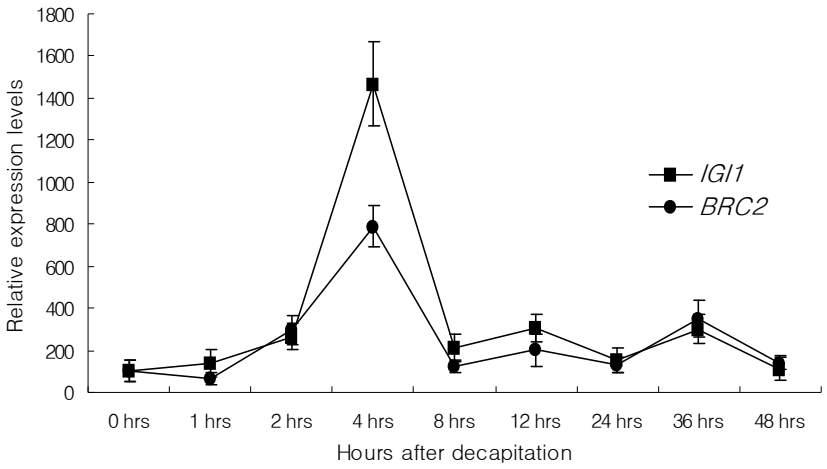


Figure 36. Relative expression level of IGI1 and BRC2 after decapitation. *BRC2* was included as positive control. Actin was used for normalization, and error bars indicate standard deviation.

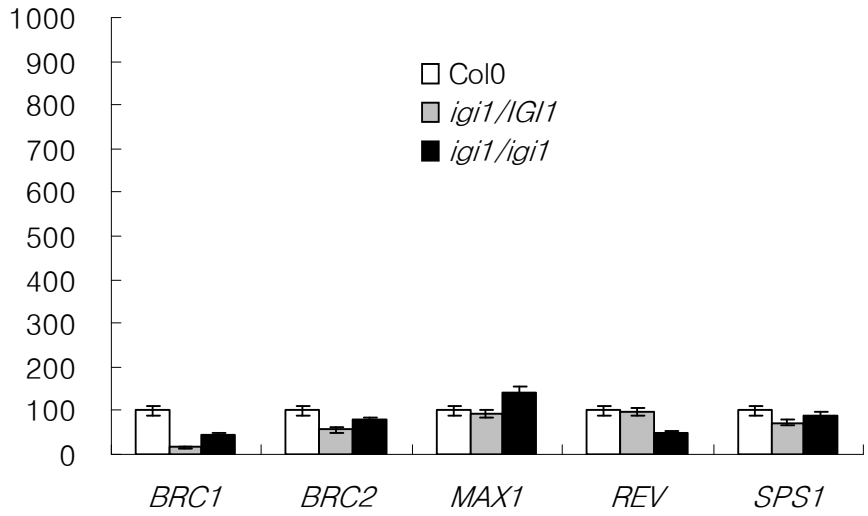


Figure 37. Real-time PCR analysis of multiple branching control genes. Graph shows relative expression levels of branching control genes. The PCR reaction was performed using cDNA from 10-day-old plant wild type and *igi1* mutants. The actin transcript levels were used for normalization. Error bars indicate standard deviation.

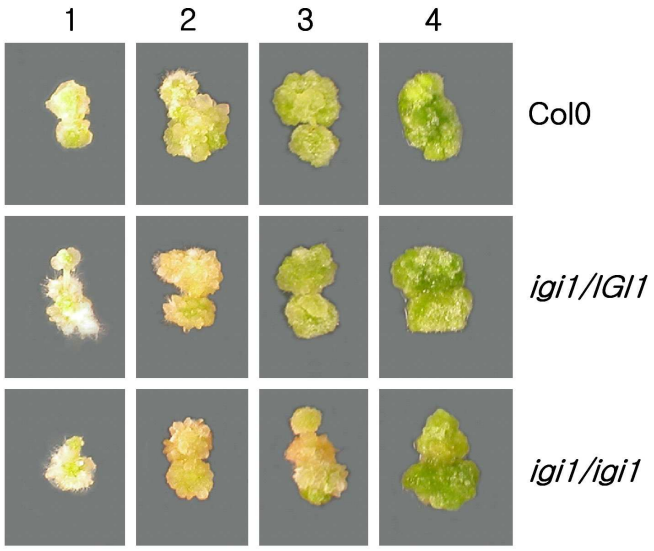


Figure 38. Cytokinin response. 1, 0 nM Kinetin + 50 nM 2,4-D; 2, 50 nM Kinetin + 50 nM 2,4-D; 3, 200 nM Kinetin + 50 nM 2,4-D; 4, 1000 nM Kinetin + 50 nM 2,4-D.

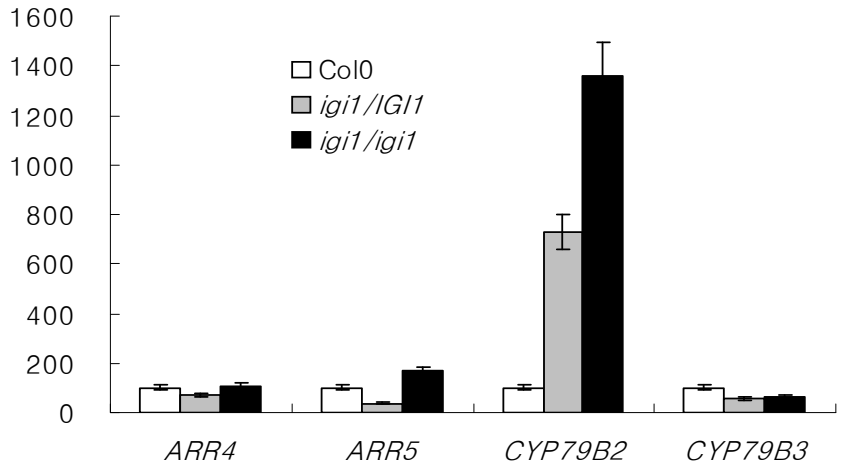


Figure 39. Real-time PCR analysis of control genes of hormones. Graph shows relative expression levels of control genes of hormones. The PCR reaction was performed using cDNA from 10-day-old plant wild type and *igi1* mutants. The actin transcript levels were used for normalization. Error bars indicate standard deviation.

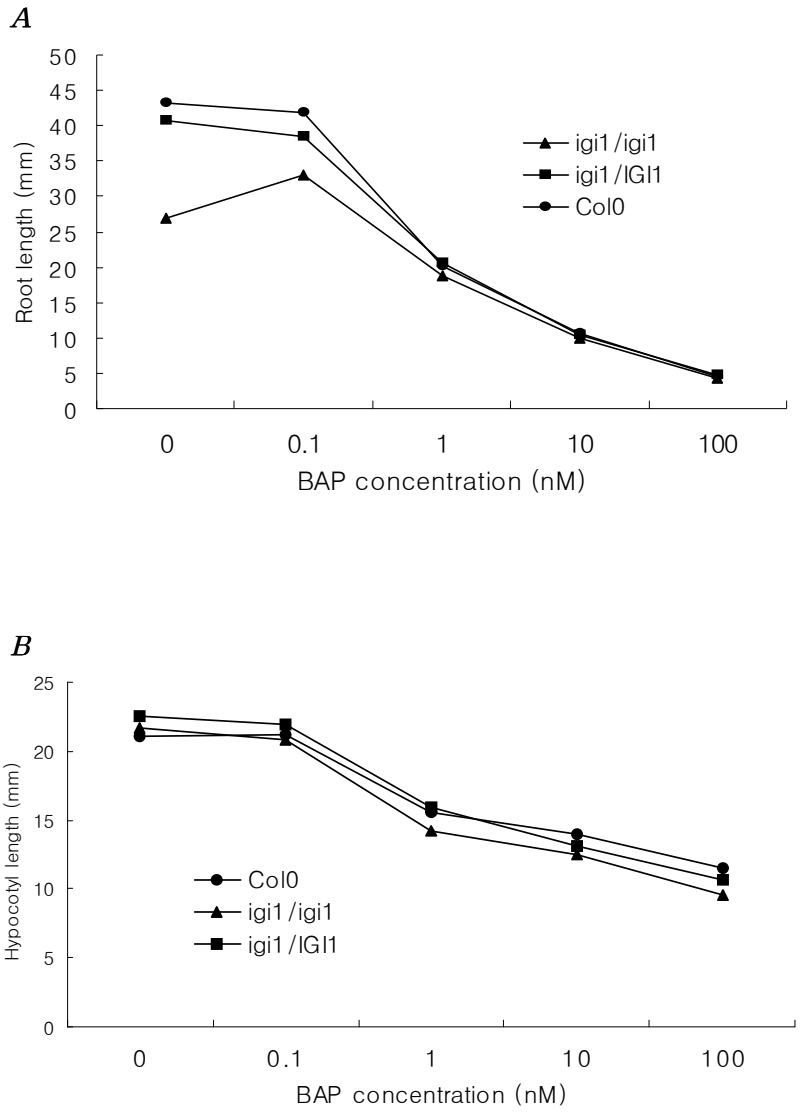


Figure 40. Cytokinin dose response under light and dark condition.

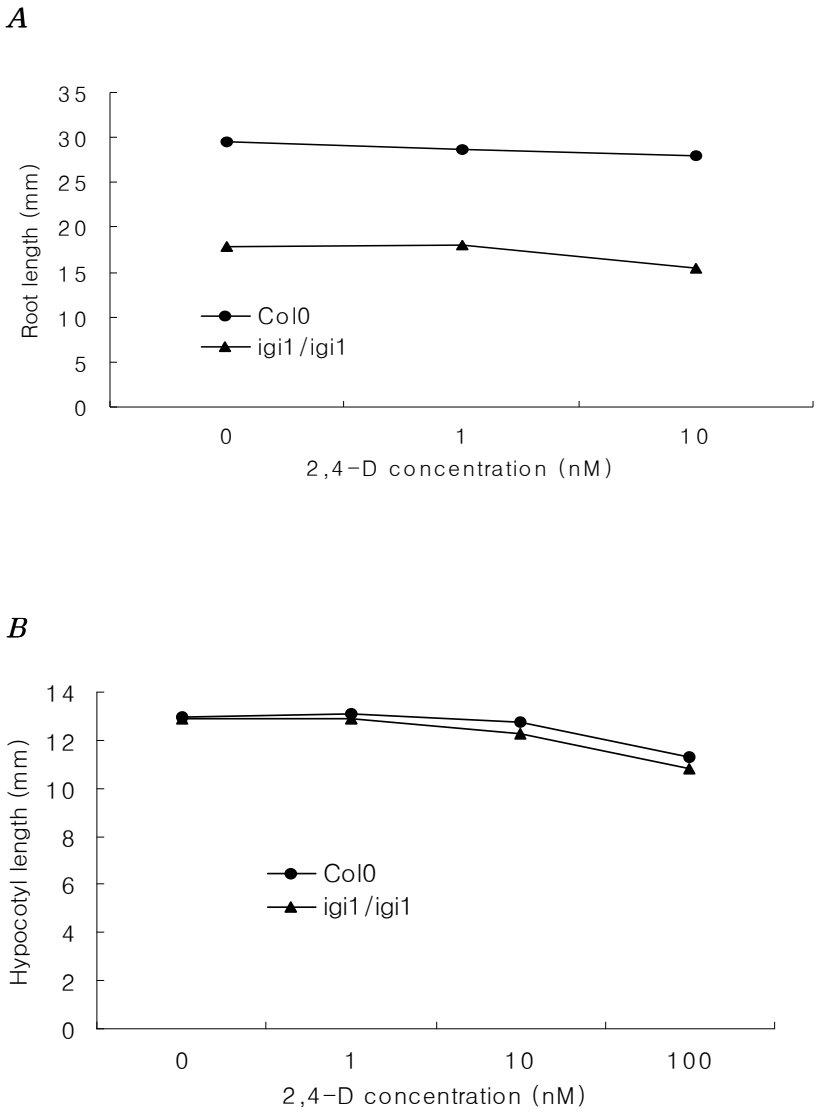


Figure 41. Auxin dose response under light and dark condition.

Ⅳ. *Discussion*

In plants, mutations can be caused by copying errors or changing mRNA expression level in the genetic material using some methods such as exposure to ultraviolet, chemical mutagens like EMS, or activation-tagging using T-DNA (agrobacterim mediated transformation). Despite the limitations such as functional redundancy and early embryonic or gametophytic lethality in organisms, the loss of function mutation technique still plays an important role in studying genetic pathway. Activation tagging, a method that enables gain of function mutation, can be used for genetic screening to overcome such limitations of loss of function mutaion. An activation-tagging vector containing four multimerized transcriptional enhancers derived from the cauliflower mosaic virus 35S gene was used to generate the gain of function mutants. Over-expression of the genes near to the randomly inserted T-DNA may result in new phenotypes, and the relevant genes can be identified. The T-DNA of the activation tagging vector, pSKI015, can enhance the expression level of neighboring genes in the insertion position (Weigel et al., 2000). Additionally, the T-DNA including enhancers can induce knock-out of the gene by T-DNA insertion (Hwang et al., 2007). *igi1* mutants have T-DNA insertion in 200bp upstream of *IGI1* gene start codon (Fig. 15), that indicate *IGI1* gene overexpression which was confirmed by Real-time PCR (Fig. 17). The phenotype was reverted by reducing the mRNA levels (i.e., RNA interference, RNAi) (Fig. 26). The recapitulation was failed in *IGI1-RC* lines. This is probably because the expression level was not high enough to recapitulate the phenotype (Fig. 21). The higher level expression may be necessary to get the *igi1* mutants phenotype.

Auxin and cytokinin are important phytohormones for axillary branching and apical dominance (Cline, 1997). The main inflorescence grows more strongly

than other rosette and cauline inflorescence. Apical bud is stimulated to grow while the lateral buds are inhibited. Auxin treatment inhibits the lateral bud outgrowth of decapitated plant which is stimulated by cytokinin treatment and overexpression of the cytokinin biosynthesis gene (Thimann et al., 1933; Medford et al., 1989). The branching phenotypes were observed in auxin and cytokinin mutants (Booker et al., 2003; Tantikanjana et al., 2001). The phenotype of *igi1/IGI1* mutant includes increased branching and reduced plant height (Fig. 22). To test the effect of endogenous auxin and cytokinin in the mutants, the transcript levels of auxin biosynthesis genes and cytokinin response genes were examined. In *igi1* mutants, the transcripts level of the auxin biosynthesis component, *CYP79B2,* was increased and those of the cytokinin response genes *ARR4* and *ARR5* were similar with that of wild type (Fig 37, Fig 39). Although the transcript level of auxin biosynthesis component was increased, *igi1/IGI1* mutant showed opposite phenotype in the axillary branching pattern. Also, exogenous 2.4-D didn't influence the root elongation of the *igi1/igi1* mutant (Fig. 41). Cytokinin induced cell division and greening of hypocotyl-derived calli were partially inhibited in cytokinin receptor mutant such as *cre1* and *ahk2* mutants (Higuchi et al, 2004). When the cytokinin sensitivity was tested with callus induction assay, the cytokinin response of the *igi1* mutants were slightly reduced in the greening and induction of hypocotyl-derived calli (Fig. 40). These phenotypes were also opposite response to cytokinin similar to the auxin response in the axillary branching pattern. These results indicate not only the hormonal regulation by auxin and cytokinin but also other pathway influenced the axillary branching.

Decapitation, shoot tip removal, has been used to study bud outgrowth. After removing the shoot tip, lateral shoot come out from axillary bud. The outgrowth of axillary buds after decapitation may be essential to provide replacement sites for reproductive development. The bud outgrowth could be quickly led after decapitation and might also divert resources away from

reproductive structures developing elsewhere on the plant. This implies communication between axillary buds and the shoot tip (Shimazo-Sato and Mori, 2001; Christine et al. 2003). *IGI1* transcript was dramatically increased in 4 hour after dcapitation (Fig. 36). The result indicates that IGI1 is involved in the communication between axillary buds and the shoot tip for axillary bud development.

The increased branching patterns were observed in *max, brc, sps*, and *tfl* mutants. MAX-dependent carotenoid hormone moves up the plant from root and prevents bud outgrowth (Bainbridge et al., 2005; Stirnberg et al., 2002). BRC1 and BRC2 prevent axillary bud outgrowth in the downstream of the MAX pathway (Aguilar-Martinez et al., 2007). SPS1 is the cytokinin biosynthesis regulator (Reintanz et al., 2001; Tantikanjana et al., 2008). It is thought that the pathway through IGI1 is not connected with these pathway like MAX and SPS pathway.

The amino acid of *IGI1* contains the proline rich domain in N-terminal region and kinase domain signature in the C-terminal region (Fig. 34). The proline rich region is present in the receptor protein family such as PERK in *Arabidopsis*. In the study using three different approaches, EST (expressed sequence tag), MPSS (massively parallel signature sequencing), NASCArrays detabases, and RNA blot analyses, by Alina Nakhamchik et al., *Arabidopsis* proline-rich extensin like receptor kinase (*PERK*) gene family was classified with 15 predicted receptor kinases. They classified *At1g23540* into *AtPERK12*. The basic structural features of conserved sequence regions is consisted of proline-rich domain, transmembrane domain and kinase domain. Some of *AtPERK* members were identified as tissue-specific genes while others were more broadly expressed. The EST and MPSS data indicated that *AtPERK* members have variable expression patterns. Some were more highly expressed based on the number of ESTs detected and the higher MPSS values. For example, *AtPERK1* and *8* showed the highest value, *AtPERK3, 7, 11* showed

no data, and *AtPERK2, 4, 12* showed low values in MPSS. Also, in NASCArrays, strong expression was seen for *AtPERK4, 5, 6, 7, 11* and *12* in flower buds. RNA gel blot analyses demonstrated that the majority of the *AtPERK* famly are expressed in buds (Nakhamchik et al. 2004). Our results also showed similar results with PERK family expression patterns, which were highly detected in flower buds by real time PCR and GUS staining.

Interestingly, light response components such as SUB1 and LSH1 were screened in yeast two-hybrid screening (Table 5). SUB1 is a Ca^{2+} binding protein involved in cryptochrome and phytochrome coaction. The *sub1* mutant showed no sign of photomorphogenic development in the dark and had same phenotype with the wild type plant in red light. The *sub1cry1*, *sub1cry2* and *sub1phyA* double mutant experiment showed SUB1 act as signal transducer of cry1 and cry2 but as a modulator of phyA signal transduction. SUB1 is apparently enriched in the nuclear periphery region surrounding the nucleus. Guo et al. proposed a hypothesis, which SUB1 is a negative regulator of photomotphogenesis, whereas the cryptochromes suppress the activity of SUB1 to activate the light response. The analysis of *sub1hy5* double mutant indicated that *hy5* is epistatic to *sub1* (Guo et al., 2001). SUB1 interacted with only N-terminal region of IGI1 including proline rich domain, which was expected cytoplasmic region, suggesting that there is possibility that IGI1 is the receptor related with a signal transduction (Becker et al., 2003). The transctiption factor, HY5 is the bZIP protein and promotes photomorphogenesis (Quail, 2002; Chen et al., 2004; Oyama et al., 2008). HY5 binding targets tend to be enriched in the early light-responsive genes and transcription factor genes (Lee et al., 2007). In the transcription factor-promoter interactions analysis by Gao et al., the promoter region of *IGI1* gene exhibited high HY5 binding affinity (Gao et al., 2004). AP1 belong to the class A floral organ identity genes, which is required for activation of B class and C class genes (Gomez-Mena et al., 2005; Sridhar et al., 2006; Weigel and Meyerowitz, 1993). In *igi1* mutant, *AP1* is

down-regulated by the overexpression of *IGI1* gene (Fig. 35), suggesting that the function of IGI1 is associated with AP1 expression in the floral meristem transition. These results indicate that IGI1 might be the nuclear receptor and regulated by the light response and flowering. The developmental phenotypes of heterozygous mutant become noticeable after transition from vegetative to reproductive phase. The heterozygous mutant plants produce primary inflorescence with reduced internode elongation, and secondary inflorescences begin to emerge straight. The heterozygous plants continue to produce axillary inflorescence from the axils of both rosette and cauline leaves. Consequently, the heterozygous mutant have dramatically increased axillary branches. These results suggest that there may be a noble pathway of the axillary meristem development throuth IGI1, which was modulated by light regulated pathway (Fig. 43, Fig. 44).

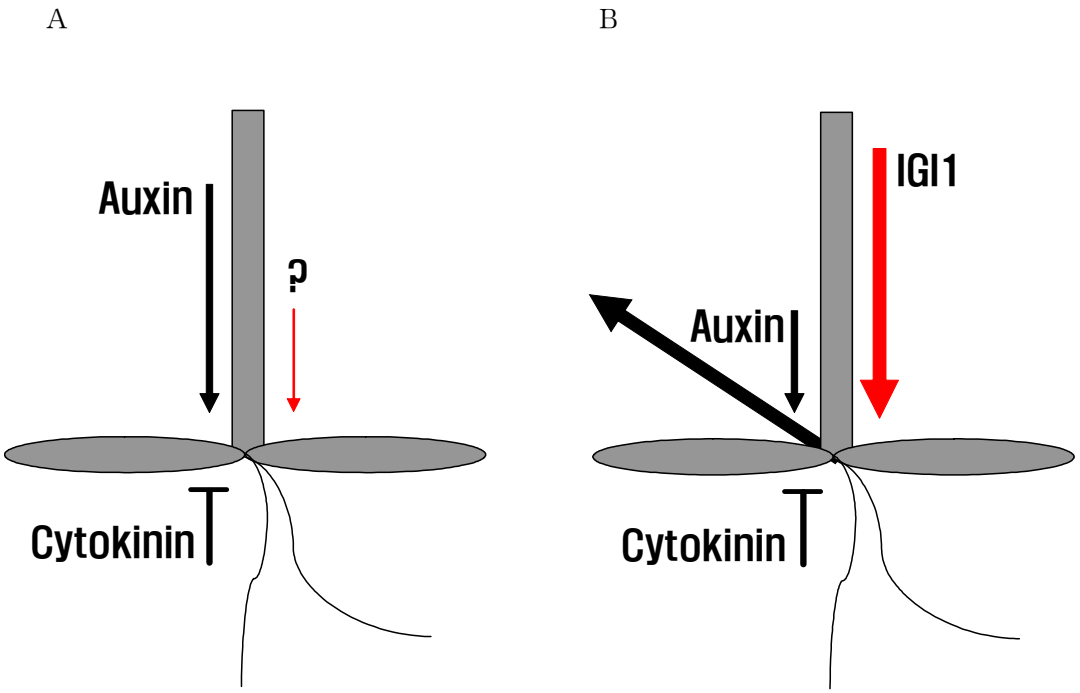


Figure 42. Diagram summarizing the pathway that control branching in Arabidopsis. Activation componant is indicated by lines with arrowheads and repression is indicated by lines with bars.

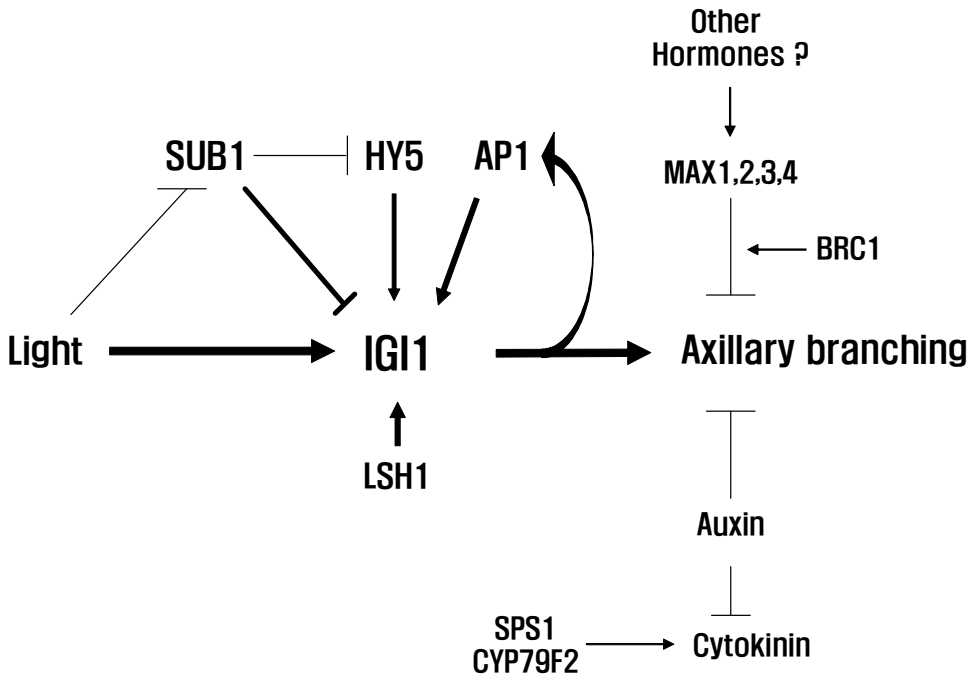


Figure 43. Diagram summarizing the pathway that control axillary branching in Arabidopsis. Activation componant is indicated by lines with arrowheads and repression is indicated by lines with bars.

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감사의 글...

먼저 논문이 있기까지 제 2의 부모님 역할을 해 주시고 지도해 주신 정현숙 지도교수님께 감사드립니다. 부족한 부분이 많았지만 10년 가까이 믿어주시고 이끌어 주셔서 감사드립니다. 많은 부분을 배울 수 있었습니다. 학부때부터 성심껏 지도해 주시고 논문 심사해 주신 생명공학과 박열교수님, 양영기교수님, 김성준교수님, 이정섭교수님, 전홍성교수님, 박윤경교수님과 친할아버지 같으신 전남대학교 황백교수님, 실험 부분에 있어 도와주시고 지도해 주신 김수영교수님, 짧게나마 논문에 대해 지도해주시고 여러 모양으로 도움을 주신 강훈승교수님, 김철수교수님, 서미정교수님께 감사드립니다.

미국으로 공부하러 간건지 여행을 간건지 잘은 모르지만 시카고에 있는 재성이형, 같은 나라에 있는 송규형선배님, 같이 했던 시간이 오래토록 기억에 남을 것입니다. 그리고 실험실 식구들, 실력은 없지만 잘 따라주었던 현진이, 그래도 선생님이라 불러주면서 배우려했던 가영이, 열심히 실험실 살림 잘 꾸려주었던 영민이, 창수에게도 고마움을 전합니다. 반년이라는 시간동안 같이 해준 재영이, 주영이..., 수없이 많이 스쳐 지나간 학부 학생들 모두 모두 감사드립니다.

실험에 많은 도움을 준 정은이와 그 무리들(?), 김승 박사님, 세은이, 봉석이 이하 그 일당들(?), 왕래는 별로 없었지만 보이지 않게 도움을 준 신경분자 생물학 실험실 식구들에게도 감사의 마음을 전합니다. 실험에 많은 도움을 주지 못했지만 영어에 있어 도움을 준 Dill Prasad Paudyal, Giri Raj Tripathi 에게도 감사드립니다. 감사드려야 할 분이 많이 있는 것 같습니다.

무엇보다 바쁘다는 핑계로 집안에 별 도움이 못됐지만, 큰아들이라고 믿어주시고 후원해 주신 존경하신 부모님 감사드립니다. 동생 황인천, 비슷한 분야는 아니지만 비슷한 재료로 실험하기 때문에 후에 둘이 합치면 뭐 하나 만들지 않을까...? 결혼했지만 든든한 후원자 누님께도 감사드립니다.

뒤에서 보이지 않게 후원해 준 백설이~, 7년이란 시간 동안 옆에서 지켜봐주고 지쳐도 지치지 않게 힘을 실어주며...앞으로도 변치 않길...

뒤돌아보면 하나님은 언제나 옆에 계셨습니다. 감사드립니다.

항상 제 자리를 지키려 노력했지만, 잘 되지 않았던 듯 싶습니다. 인내함으로 꾸준히 한 발 한 발 앞으로 나가도록 하겠습니다. 모두에게 감사드립니다.

저작물 이용 허락서

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논문제목	한글 애기장대에서 액아발생을 조절하는 IGI1의 분자유전학적 연구				
	영문 IGI1 is involved in axillary branching in *Arabidopsis thaliana*				

본인이 저작한 위의 저작물에 대하여 다음과 같은 조건 아래 조선대학교가 저작물을 이용할 수 있도록 허락하고 동의합니다.

- 다 음 -

1. 저작물의 DB구축 및 인터넷을 포함한 정보통신망에의 공개를 위한 저작물의 복제, 기억장치에의 저장, 전송 등을 허락함.
2. 위의 목적을 위하여 필요한 범위 내에서의 편집과 형식상의 변경을 허락함. 다만, 저작물의 내용변경은 금지함.
3. 배포·전송된 저작물의 영리적 목적을 위한 복제, 저장, 전송 등은 금지함.
4. 저작물에 대한 이용기간은 5년으로 하고, 기간종료 3개월 이내에 별도의 의사 표시가 없을 경우에는 저작물의 이용기간을 계속 연장함.
5. 해당 저작물의 저작권을 타인에게 양도하거나 출판을 허락을 하였을 경우에는 1개월 이내에 대학에 이를 통보함.
6. 조선대학교는 저작물 이용의 허락 이후 해당 저작물로 인하여 발생하는 타인에 의한 권리 침해에 대하여 일체의 법적 책임을 지지 않음.
7. 소속 대학의 협정기관에 저작물의 제공 및 인터넷 등 정보통신망을 이용한 저작물의 전송·출력을 허락함.

동의여부 : 동의(O) 반대( )

2008 년 8 월 25 일

저작자 : 황 인 덕 (인)

조선대학교 총장 귀하