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Isolation and Molecular Genetic Analysis of Brassinosteroid Signaling Mutants in *Arabidopsis*

조선대학교 대학원

생명공학과

Giri Raj Tripathi

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Isolation and Molecular Genetic Analysis of Brassinosteroid Signaling Mutants in *Arabidopsis*

A thesis submitted to the Graduate School of Chosun University
in partial fulfillment of the requirements for the degree of
Doctor of Philosophy

by

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Abstract

Isolation and Molecular Genetic Analysis of Brassinosteroid Signaling Mutants in *Arabidopsis*

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Plant development is one of the most important aspects of plant's life cycle that has extensively been studied at the morphological, genetic and molecular level. The generation of genetic variants, like mutants may increase genetic pool and gives the information about plant processes and their genetic control. Activation tagging is a new powerful tool to generate and identify new mutants, which emerged as an alternative for gene function analysis. This thesis reports the study on brassinosteroid hormone signaling mutants that controls the plant growth and development using mutants generated by an activation tagging-based approach in the model plant *Arabidopsis thaliana.*

Brassinosteroids (BRs) are polyhydroxylated steroid plant hormones that play essential role in growth and development. The mutant defective in BR signaling causes dwarfism, male sterility, abnormal vascular development and photomorphogenesis. Unlike animal steroid hormones, BRs are perceived at the plasma membrane by direct binding to the extracellular domain of the BRI1 receptor S/T kinase. BR pereception initiates a signaling cascade, acting through a GS3 kinase, BIN2, and the BSU1 phosphatase, which in turn modulates the phosphorylation state and stability of the nuclear transcription factors BES1 and BZR1.

An activation tagging library harboring 10,000 (F_1) independent *Arabidopsis* transformants were generated and a pool system was established in order to screen brassinosteroid hormone signaling defective mutants. At the time of activation tagging library establishment a total of 150 mutants that are defective in normal phenotype were also screened in F_1 generation. Brassinasteroid related mutants were screened using brassinazole a specific brassinosteroid biosynthesis inhibitor. In dark, brassinazole treated *Arabidopsis* develop as light grown plants and express light regulated genes, as do BR-deficient mutants. A total of 46 putative to brassinazole insensitive mutant were isolated that showed longer hypocotyls among the germinated seeds on brz at dark. All the mutants were identified T-DNA insertion by using TAIL-PCR, Adapter Ligation PCR Walking, plasmid rescue and IPCR. In this screening *atbrzi1* and *abz126* brassinszole insensitive mutants were taken for the study.

atbrzi1 has T-DNA insertion into the promoter region of gene At1g12380. In the brassinazole, hypocotyl length *atbrzi1* is nearly doubled than wild-type. ATBRZI1 gene is located in the acrocentric region of the chromosome 1 and its homology gene At1g62870 is located the opposite acrocentric region of the same chromosome. Both genes have characteristic of Zinc finger BED-type motif located at the N-terminal region. In this study we predicted that the HLH (helix-loop-helix) type structure of BED-type zinc finger motif may bind the dephosphorylated BES1 and then involve in brassinosteroid hormone signaling in the respective way. Yet, research on role of zinc finger is not reported. However, our further ongoing experimental results may show the involvement of Znf BED-type motif in brassinosteroid hormone signaling by binding dephosphorylated BZR1 in the nucleus.

abz126 is a semi-insensitive to the brassinazole and a T-DNA has been inserted into the ninth exon of the GIGANTEA (GI) gene that is involved in control of flowering time. Loss of function in GI gene makes extremely delaying in flowering in *Arabidopsis.* Results of screen on brz, RT-PCR, different hormone dose response assay presumed that GI gene is involved in brassinosteroid hormone signaling and regulate the flowering time.

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List of Abbreviations and Definitions

2,4-D.....2,4-Dichlorophenoxyacetic acid

aa .....Amino acid

BAK1 .....BRI1-Associated Receptor Kinase 1

BES1 .....Brassinosteroid Insensitive 1 EMS Supressor 1

BIN2.....Brassinosteroid Insensitive 2

BKI1.....Brassinosteroid Insensitive 1 Kinase Inhibitor 1

BRFs.....Brassinosteroid Response Factors

BL.....Brassinolide

BRI1 .....Brassinosteroid Insensitive 1

BRS1 ..... BRI1-1 SUPRESSOR1

Brz ..... Brassinosteroid biosynthesis inhibitor

BSU1 ..... Brassinosteroid Insensitive 1 Supressor Protein 1

BZR1 .....Brassinazole Resistant 1

CaMV ..... Cauliflower Mosaic Virus promoter

CDC.....Cell Division Cycle

CDK.....Cyclin Dependent Kinase

cDNA ..... Complementary DNA

df.....Degrees of freedom

DNA ..... Deoxyribonucleic Acid

ds ..... cDNA Double-stranded complementary DNA

EDTA ..... Ethylenediamine-tetraacetic acid

EGF .....Epidermal Growth Factor

et al ..... and others

GA .....gibberellins

gDNA .....Genomic DNA

GFP..... Green Fluoresecent Protein

GSK-3.....Glycogen Synthase Kinase 3
GUS .....β-glucuronidase reporter gene
IAA .....Indole-3-Acetic Acid
IPTG ..... Isopropyl-β-D-thiogalactopyranoside
JM.....Juxtamembrane Region
KPP.....a nuclear localized Kelch-Containing Protein Phosphatase
LRRs.....Lucine-rich repeats
MBP-BKI1 .....BK1-Maltose Binding Protein Fusion
MS ..... Murashige-Skoog
N .....Number of plants tested
NAA .....α-1-Naphthalene Acetic Acid
NADPH .....Dihydronicotinamide Adenine Dinucleotide Phophate reduced
NCBI .....The National Center for Biotechnology Information
ng .....Nanogram
nt .....Nucleotides
ORF .....Open reading frame
p .....protein
PCR ..... Polymerase Chain Reaction
pGEM .....Cloning vector pGEMT® Eazy from Promega
PTGS .....Post-transcriptional gene silencing
RFLP .....Restriction Fragment Length Polymorphism
RLKs ..... Receptor-like kinases
RNA .....Ribonucleic Acid
RNAi ..... RNA interference
ROS .....Reactive Oxygen Species
RT ..... Reverse Transcription
RT-PCR .....Reverse transcription PCR
SDS .....Sodium dodecyl (lauryl) sulfate

Ser ..... Serine amino acid
SERK1 ..... Somatic Embryogenesis Receptor Kinase 1
T0 ..... Plants treated with Agrobacterium culture
T1 ..... Plants produced from selfing of one Agrobacterium-treated plant
TAIL-PCR ..... Thermal asymmetric interlaced polymerase chain reaction
TAIR ..... The Arabidopsis Information Resource
T-DNA ..... A transferred DNA fragment
TGF-β ..... Transforming Growth Factor-β
Thr ..... Threonine amino acid
TRIP1 ..... TGFβ-Receptor-Interacting Protein 1
TTL ..... Transthyretin-Like Protein
Tyr ..... Tyrosine amino acid
WT ..... Arabidopsis thaliana ecotype Columbia
XET ..... Xyloglucan Endotransglycosylase
X-Gal 5-bromo-4-chloro-3-indolyl-β-D-galactoside
YFP ..... Yellow Fluorescent Protein
ZnFs ..... Zinc Fingers

Kits and Reagents

Kit/Reagent	Source	Used for/as a
Cells-to-cDNA™ II kit	Ambion-Austin, TX	cDNA synthesis
Conserve®	DowAgro-Indianapolis, IN	Insecticide
Finale™	Scotts Company- Marysville, Ohio	Herbicide
HygromycinB Tran.selection	duchefa Biochemie	Plant
1kb Plus ladder	Invitrogen	DNA standards
pGEM-T Eazy	Promega- Madison, WI	TA-cloning
PfuUltra™ High-Fidelity 3Kb) PCR	Strategene-La Jolla, CA	Inverse long (>
DNA Polymerase Freeze 'N	DNA amplification	Quantum Prep®
QIA Plant® DNA Mini-kit	Qiagen- Valencia, CA	DNA prep
QIA prep® spin	Qiagen Sciences- Valencia, CA	DNA miniprep
RETROscript™	Ambion-Austin, TX	cDNA synthesis
RNeasy® Plant Mini-kit,	Qiagen Inc, Valencia, CA	RNA miniprep
Silwet L-77	Lehle Seeds- Round Rock, TX	Wetting Agent
TOPO® TA Expression Kit Purification	Invitrogen-San Diego, CA	Protein
Wizard® plus SV	Promega- Madison, WI	DNA miniprep
X-Glu	GOLD Biotech, Inc- St Louis, MO	GUS substrate for histochem staining

On-line Molecular Tools

ABRC	http://www.biosci.ohio-state.edu/~plantbio/Facilities/abrc/abrchome.htm
AGRIS	http://arabidopsis.med.ohio-state.edu/AtTFDB/index.jsp
Ambion	http://www.ambion.com/
AthaMap	http://www.athamap.de/
Bio-Rad	www.bio-rad.com
Clontech	http://www.bdbiosciences.com/clontech/
Clustal W program	http://www.ebi.ac.uk/clustalw/
Cocalico Biologicals Inc.	http://www.cocalicobiologicals.com/
Davis Sequencing	www.davissequencing.com
DNA star	http://www.dnastar.com/
Genomatix	www.genomatix.de
GenomeNet	http://www.genome.jp/
Gramene	http://www.gramene.org/
MEROPS	http://merops.sanger.ac.uk/
NCBI	http://www.ncbi.nlm.nih.gov/
NEB	http://www.neb.com/nebecomm/default.asp
PepIdent	http://www.expasy.org/tools/peptident.html
PLACE	http://www.dna.affrc.go.jp/PLACE/
Plant energy Biology	http://www.plantenergy.uwa.edu.au/
Promega	www.promega.com
Qiagen	www.qiagen.com
RNA interference vectors	http://www.chromdb.org/plasmids/vectors2.html
Sigma	http://www.sigmaaldrich.com/
SIGNAL	http://signal.salk.edu/cgi-bin/tdnaexpress
Stratagene	www.stratagene.com
TAIR	www.arabidopsis.org
TIGR	http://www.tigr.org/
Uniport	http://beta.services.uniprot.org

Chapter I

ATBRZI1 mediates brassinosteroid regulated gene expression in *A. thaliana*

Abstract

Brassinosteroids (BRs) are plant steroid hormones that play essential role in growth and development. The mutant defective in BR signaling causes dwarfism, male sterility, abnormal vascular development and photo-morphogenesis. Plants, like animals use steroid hormones to regulate their development through changes in the expression of target genes. In this study, a brassinazole insensitive mutant *atbrzi1* was isolated from the activation tagged *Arabidopsis* and dissects ATBRZI1 by molecular genetic analysis for its possible involvement in brassinosteroid signaling. Brassinosteroids (BRs) signal through plasma membrane-localized receptor kinase to regulate plant growth and development. BRs are perceived at the plasma membrane by direct binding to the extracellular domain of the BRI1 receptor S/T kinase and initiates a signaling cascade, acting through a GS3 kinase, BIN2, and the BSU1 phosphatase, which in turn modulates the phosphorylation state and stability of the nuclear transcription factors BES1 and BZR1.

atbrzi1 mutant was identified by using TAIL-PCR and the resulting phenotype is caused by T-DNA insertion into the promoter region of the gene At1g12380. ATBRZI1 is located in the acrocentric region of the chromosome 1 and has Zinc finger BED-type motif at the N-terminal region of the gene. N-terminal region of ATBRZI1 is also characterized by proline rich region. The predicted Zinc finger motif of the ATBRZI1 gene has HLH (helix-loop-helix) type structure that can be bind the dephosphorylated BES1 and then involve in brassinosteroid hormone signaling in the respective way. The BR response element (BRRE) with the sequence CGTG(T/C)G was predicted near the Znf motif, where

BZR1could bind *CPD* promoter via this motif. Having both signaling and biosynthetic motif, it is interesting to know whether BES1 and BZR1 involve synergistically or not in BR signaling. Yet, research on role of zinc finger in brassinosteroid signaling is quite unknown. More, the presence of same two Znf motif in acrocentric region of the same chromosome made interesting to know the function of this gene motif. However, the RNAi and other further ongoing experimental evidences may prove the role of Znf BED-type in brassinosteroid signaling.

Introduction

A1.1. Activation tagging

The availability of complete genome sequence of *Arabidopsis* broadened the research activities on functional genomics in plant sciences. Out of the 26,000 genes identified in *Arabidopsis* the function of only few thousands have been defined with great confidence (Bouche & Bouchez 2001) and more than 30% of the predicted genes could not be assigned any specific function (AGI 2000). Mutant screen and analysis is the first step for elucidation of the function of gene in plant.

In the past, loss-of-function mutations have been used to identify the function of genes in *Arabidopsis.* Genetic redundancy sometimes prevents elucidation of gene function by loss-of-function approaches. Loss-of-function mutants have faced limitations, e.g., presence of functional gene redundancy caused by extensive genome duplications (AGI 2000; Bouche & Bouchez 2001). Thus, alternative approaches needed to be developed to overcome this drawback. Gain-of-function mutation is an approach that they may compensate for this type of genetic redundancy.

One of them is the generation of gain-of-function mutagenesis, in which a gene is either ectopically or constitutively overexpressed. Although in such a case a particular gene is not expressed in its normal biological context, it may provide information about biological process, in which the gene plays an important role. Activation tagging is a gain–of-function mutagenesis approach and it was first described to identify and isolate novel genes from tobacco (Walden et al. 1994), although it had been

previously reported with *in vitro* experiments to activate auxin-related plant genes (Hayashi et al. 1992). Since then, it has been extensively improved by the establishment of large collections of *Arabidopsis* lines (Kardailsky et al. 1999; Borevitz et al. 2000; van der Graaff et al. 2000; Weigel et al. 2000; Marsch-Martinez et al. 2002; Nakazawa et al. 2003). The system used for these collections are based on quadruple *CaMV35S* enhancer sequences as activator elements, either with T-DNA or transposon as insertional vehicles (Weigel et al. 2000; Marsch-Martinez et al. 2002).

Insertional mutagenesis has been widely used for cloning genes, promoters, enhancers and other regulatory sequences from *Arabidopsis.* Activation tagging is based on strong transcriptional enhancer sequences that can activate gene expression in the vicinity of the site where enhancer was inserted into the genome. The most commonly used enhancer is a quadruple combination of the cauliflower mosaic virus (*CaMV*) *35S* gene enhancer in the proximal part of the right border of T-DNA carrying vector (Weigel et al. 2000). These 4x*35S* enhancer element have been reported to strongly enhance endogenous gene expression rather than ecotypically or constitutively overexpressed gene (Neff et al. 1999; van der Graaff et al. 2000).

This is tempting technique to isolate new mutants in a random approach and analyze in more detail the overexpressed gene. The activation tagging method has also been used as a novel approach to isolate suppressor mutant of known mutant phenotype (Mora-Garcia et al. 2004). The activation of genes by T-DNA insertion causes dominant phenotype. The mutant phenotype can be screen directly in T_1 generation after the introduction of the T-DNAs where gain-of-function mutants behave dominantly. The frequency of morphological mutants varies depending on the insertion

vehicle used, with approximately 0.1% in the case of the T-DNA-based system, or about 1% when transposons are used. This low frequency in the T-DNA based activation tagging is mainly attributed to the fact that the 4x*35S* enhancer sequences are methylated (Chalfun-Junior et al. 2003). New activation tagging populations are being generated not only in *Arabidopsis*, but also in other species such as tomato (Mathews et al. 2003), rice (Jeong et al. 2002), petunia (Zubko et al. 2002) and tobacco (Ahad, Wolf & Nick 2003) demonstrating the strength of the system.

In this study, an activation tagging library harboring 10,000 independent *Arabidopsis* transformants were generated in order to screen brassinosteroid hormone signaling defective mutants. Devising specific genetic screens for the isolation of brassinosteroid hormone signaling mutants remain difficult work, although there are few *Arabidopsis* and rice mutants that are involved in brassinosteroid hormone signaling have been isolated and studied. A total of 46 putative brassinazole insensitive mutants were isolated in this screening. At the same time a total of 150 mutants that were specific to defect in morphological phenotype were also screened in F_1 generation.

A1.2. Brassinosteroid

Steroid hormones play key roles in growth and development of multicellular eukaryotes. Plant steroid hormones called Brassinosteroids (BRs) regulate cellular expansion, differentiation, and proliferation. BRs are involveed in diverse process, such as stem elongation, vascular differentiation, male fertility, timing of senescence and flowering, leaf development and resistance to biotic and abiotic stress (Li, J & Chory 1997; Clouse, Steven D. & Sasse 1998; Altmann 1999; Khripach, Zhabinskii & de Groot 2000). BRs were so named because the first compound discovered in

this group was from the pollen of *Brassica napus* L. Prior to the determination of BR structure; they were referred as *Brassins* (Mitchell et al. 1970) demonstrated that brassins could have tremendous influence on yield, photosynthetic efficiency and seed vigor in various crop plants. These observations lead to undertake the task of purifying and characterizing the active compound. In 1979, the compound was successfully purified and determined to be a steroidal lactone and was termed brassinolide (Mandava 1988). There are more than 40 free BRs and four BR conjugates have been found and fully characterized. Research findings suggested that BRs are ubiquitously distributed in the plant kingdom. Biosynthetical pathways of BRs and their counterparts in plants were already investigated and the major part of the biosynthesis of Bl has been established (Fujioka & Sakurai 1997); based on information from extensive step-wise feeding experiment and analysis of their bioconversion products.

In the beginning, the importance of BRs for the regulation of plant growth and development was not accepted like other classical plant hormones like auxins, gibberellins, cytokinins, jasmonates, ethylene and abscisic acid (Kende & Zeevaart 1997). Since identification and characterization of BR biosynthesis and signal transduction mutants, BRs are accepted as an essential endogenous hormone that has important physiological roles in growth and development (Clouse, S. D. 1996; Clouse, S. D., Langford & McMorris 1996). A new attempt to identify BR-insensitive mutants of *Arabidopsis* resulted in the isolation of the dwarf mutant named *bri1* (Clouse, S. D., Langford & McMorris 1996; Szekeres et al. 1996). BRI1 has strong homology to leucine-rich receptor kinases. *bri1* cannot be rescued by BR treatment; this is the evidence of its essential role in a signaling pathway. *bri1* mutants display a light grown morphology in

the dark, show extremely dwarfed growth in the light, and have numerous other phenotypes, all of which are also seen in strong BR biosynthetic mutants (Vert et al. 2005). The role of BRI1 was demonstrated by binding studies with radiolabeled brassinolide and mitochondrial fraction of wild-type and mutant plants, as well as from transgenic plants expressing BRI1-green fluorescent protein (GFP) fusions (Wang, ZY et al. 2001).

Like animals, plants use steroid hormones to regulate their development through changes in the expression of target genes. In contrast to animal steroid signals, BRs are perceived by plasma membrane –localized receptor kinase. This kinase is encoded by the *BRI1* gene. After the plasma membrane perception of BR, the resultant signal is then transmitted through a chain of events that leucine-rich repeat receptor kinase that spans the outer membrane of plant cells. A number of steps follow BR binding, leading to inactivation of the negative regulator of BIN2. In response to BR, BRI1 inhibits BIN2, a protein that normally attaches phosphate groups to the nuclear proteins BES1 and BZR1. These phosphate groups tag BES1 and BZR1 for rapid destruction in the proteasome, the cellular organelle that degrades unwanted proteins. Thus, inhibition of BIN2 activity promotes the accumulation of BES1 and BZR1 in the nucleus. BES1 and BZR1 then activate selected BR-responsive genes and repress the activity of others including the genes that are involved in the BR biosynthesis pathway. He, JX et al. (2005) found that BZR1 binds directly to specific sequences within the *CPD* gene and thus represses transcription and (Yin et al. 2005) showed that BES1 also binds directly to the target gene, but in this case acts as a transcriptional activator. BES1 only act associates with BIM proteins that belong to a different family of DNA binding proteins. Analysis of plant carrying *bim* mutation confirmed that BIM proteins are required for

a normal response to BR. Having 88% similarities between BES1 and BZR1 proteins, the functions of BES1 and BZR1 are partially overlaped (Yin et al. 2005).

It is important to understand the signaling pathways because brassinosteroids are the central for plant growth and development. Understanding the developmental role of the phytohormones is complex because each phytohormone controls so many responses. Furthermore, Identification of key transcription factors controlled by brassinosteroid and how they interact each other and with their target genes will crucial for understanding how BRs orchestrate in plant development.

A1.3. Zinc finger

Zinc is essential for normal healthy growth and reproduction of humans, plants and animals which is known for its ability to fight disease and to protect the immune system and helps in the prevention of stunting growth, and supports neurobehavioral development among other attributes (Hotz et al. 2003). Zinc is found in all tissues of human body with 30-40% found in the nucleus, 50% in the cytosol, with the remainder found in organelles, vesicles, and the cell membrane (Tapiero & Tew 2003). It is involved in the Krebs cycle and energy production. It is also credited with increasing male sex drive and potency because of its ability to regulate testosterone in the prostate. Unique combination of structural and thermodynamic properties of zinc make it ideally suited for a diverse array of biological roles. These properties include its ready availability, Lewis acidity, high concentration of charge (atomic radii of 0.65 A°), lack of redox reactivity and stable oxidation state (2+). Zn(II) is a d^{10} transition metal ion that forms metal

complexes of high thermodynamic stability that are often characterized by facile ligand exchange (Fraústo da Silva & Williams 2001).

In plants, Zinc is essential micro element which allows several key plant physiological pathways to function normally. These pathways have important roles in: Photosyhthesis and sugar formation, Protein synthesis, Fertility and seed production, Growth regulation, Defense against disease. Where zinc is deficient these physiological functions will be impaired and the health and productivity of the plants will be adversely affected, resulting in lower yields (or even crop failure) and in poorer quality crop products.

The folding of small globular domains within gene regulatory proteins is required for sequence-specific DNA binding, an event that underlies gene expression in all kingdoms of life (Spolar & Record 1994). The formation of zinc-ligand coordination bonds provides the major driving force for the folding of these mini-domains that lack an appreciable hydrophobic core (Berg & Godwin 1997). Over the last two decades, an explosion in our knowledge of the diverse roles that the essential trace element zinc plays in DNA metabolism, chromatin remodeling, transcriptional regulation, nucleic acid packaging and ribosome biogenesis has taken place (Katz & Jentoft 1989; Fraústo da Silva & Williams 2001; Maret 2001; Blencowe & Morby 2003; Fingerman et al. 2003).

Zinc binds nearly exclusively to three types of amino acid side chains in proteins. These are cysteines, histidines, and carboxylates (Asp and Glu) with thiolate sulfurs and imidazole nitrogens by far the most common donor atoms. Zinc sites in proteins adopt a variety of coordination structures that differ in the number of ligands bound (coordination number), the type of ligands, metal-ligand bond lengths, dihedral ligand-metal-ligand bond

angles, and in some cases, second shell interactions (Fraústo da Silva & Williams 2001). The Metalloprotein Database and Browser hosted by The Scripps Research Institute (http://metallo.scripps.edu/) reveals that among the approximately 3100 structurally characterized zinc coordination sites deposited in the Protein Data Bank (http://www.rcsb.org/pdb), the most prominent coordination number is four (1726), the most common liganding pattern tetrathiolate (C4 or S4) (484), with tetrahedral or distorted tetrahedral coordination geometries by far the most prevalent for zinc sites classified as of April 2004 (Castagnetto et al. 2002).

Zinc complexes in proteins perform structural, catalytic and regulatory functions. Structural zinc sites typically adopt tetrahedral or distorted tetrahedral coordination geometries formed by four protein-derived ligands with no bound water molecules. Cysteines and histidines are by far the preferred ligands in such sites e.g. zinc fingers (Auld 2001). Catalytic and regulatory Zn(II) sites utilize the same amino acid ligands as structural sites, but include a greater percentage of carboxylate ligands derived from aspartate and glutamate, as well as solvent molecules (H_2O), which are almost always found in catalytic sites in enzymes e.g. carbonic anhydrase (Auld 2001). Co-catalytic sites, defined as catalytic metal sites that coordinate two metal ions via a bridging ligand(s) (e.g., phosphotriesterase), are formed by ligand sets similar to those found in catalytic sites, except that cysteine residues that typify structural zinc cluster sites (e.g., metallothioneins) are never found as a bridging ligand in metalloenzymes (Auld 2001).

Structural zinc ions stabilize protein tertiary structure, protein-protein, and protein-nucleic acid interfaces. The zinc coordination bonds induce thermal and conformational rigidity to these important domains of proteins

much like disulfide bonds do in oxidizing environments. In the absence of zinc, these protein domains are unfolded and exist in a predominantly random coil conformation and are presumably subjected to rapid degradation and clearance by the cell. The most striking and seminal illustration of how “structural zinc” stabilizes tertiary structure formation in DNA binding proteins is in zinc finger (zf) proteins (Krishna, Majumdar & Grishin 2003). Classical C2H2 (Cys2His2) zinc finger domains, which mainly include DNA-binding proteins (they also mediate protein-RNA, protein-protein, and protein-lipid interactions) are small (~30 amino acid residues) domains that fold around one or more zinc ions, and are nearly exclusively found in eukaryotic organisms (Matthews & Sunde 2002; Jantz et al. 2004). Recent findings reveal that canonical C2H2 zinc finger domains in transcriptional regulatory proteins in eukaryotes are not exclusively involved in structural stability but have also been implicated in regulating the concentration of bio-available zinc in cells (Giedroc, Chen & Apuy 2001; Bird et al. 2003).

Sub-optimal or toxic (above normal physiological levels) concentrations of intracellular zinc have adverse affects on many aspects of cellular metabolism. Zinc is now known to function at the level of transcription by binding directly to regulatory proteins, i.e., metal sensor proteins that repress, de-repress, or activate the transcription of genes that encode metal transporters (importers/exporters) and/or metal chelators. This helps cells maintain homeostasis, or an optimal steady-state concentration of zinc within the cytosol and other intracellular organelles (Blencowe & Morby 2003; Eide 2003).

Protein-protein and protein-nucleic acid interactions are essential functions of many proteins. Proteins have developed different ways to bind

other molecules. Zinc-binding repeats, known as zinc fingers (ZnF), are one such molecular scaffold. Zinc finger domains are common, relatively small protein motifs that fold around one or more zinc ions. In addition to their role as a DNA-binding module, ZnFs have recently been shown to mediate protein: protein and protein: lipid interactions (Matthews & Sunde 2002). In the past few years detailed structural and functional data have been reported for several protein interactions (Table 1). Several different ZnF motifs have been characterised, and vary with regard to structure, as well as binding modes and affinities. ZnF motifs can coordinate one or more zinc atoms. They display considerable versatility in binding modes, even between members of the same class (e.g. some bind DNA, others protein), suggesting that ZnF motifs are stable scaffolds that have evolved specialised functions. Zinc-binding motifs are stable structures, and they rarely undergo conformational changes upon binding their target. Most ZnF proteins contain multiple finger-like protrusions that make tandem contacts with their target molecule, often recognising extended substrates. A few of the most common structurally defined ZnF motifs are described below.

Classical ZnF (C2H2): These are the most common DNA-binding motifs found in eukaryotic transcription factors. Transcription factors usually contain several zinc fingers capable of making multiple contacts along the DNA. Classical ZnFs comprise a short beta hairpin and an alpha helix (beta/beta/alpha structure), with a zinc atom coordinated in either Cys(2)His(2) (C2H2), Cys(2)HisCys (C2HC), or Cys (3)His (CCCH) residues (Figure 1-1A).

GATA-type ZnFs: These motifs constitute type IV ZnFs with the general sequence C-X(2)-C-X(17-20)-C-X(2)-C, followed by a highly basic region. They can be subdivided into subgroups depending upon the length of the

internal loop: type IVa has a 17-residue loop (CX2CX17CX2C), while type IVb has 18-residue loop (CX2CX18CX2C). ZnF motifs with 19 or 20-residue loops are rare and found mainly in fungi. GATA factors play essential roles in development, differentiation and control of cell growth in eukaryotes. GATA proteins often contain more than one ZnF domain, where one domain binds DNA and the other modulates DNA binding, often by binding other factors.

RanBP-type ZnFs: These motifs consist of two short beta hairpins that sandwich a single zinc atom and are related to the zinc-ribbon fold (Figure 1-1a). RanBP ZnFs domains are known to interact with ubiquitin (Meyer, Wang & Warren 2002). Recently, a structure was reported for the complex formed between ubiquitin and the RanBP domain of Np14 protein with roles that include endoplasmic-reticulum associated degradation and nuclear envelolope reassembly (Tsai, Ye & Rapoport 2002).

A20-type ZnF motifs: These motifs bind a single zinc atom (Figure 1-1A) and were first identified in protein A20 (Opipari, Boguski & Dixit 1990). These motifs are known to bind to ubiquitin, but contact a different region of ubiquitin from RanBP ZnF motifs (Penengo et al. 2006).

LIM-type ZnF motifs: LIM domains coordinate one or more zinc atoms, and are named after the three proteins (LIN-11, Isl1 and MEC-3) in which they were first found. They comprise two sequential zinc-binding motifs that resemble GATA-like ZnFs (Figure 1-1B), however the residues holding the zinc atom(s) are variable: Cysteine, Histidine, Aspartate and Glutamate residues are all known to be involved in zinc coordination and the loops between zinc ligands differ in length (Kadrmas & Beckerle 2004). LIM domains are involved in proteins with differing functions, including

gene expression, and cytoskeleton organisation and development. Protein containing LIM ZnF domains include the adaptor protein PINCH (Xu et al. 2005).

MYND-type ZnF motifs: MYND domains coordinate two zinc atoms in a sequential manner (Figure 1-1b), but they are smaller than LIM domains (~40 residues versus ~55 residues for LIM domains) and are named after the three proteins (Myeloid translocation protein 8, Nervy, and DEAF-1) in which they were first found. They consist of two zinc-binding motifs, the first containing a short beta-hairpin, while the second consists of two short alpha-helices. Like LIM motif, MYND motifs function primarily as protein binding domains and are present mainly in transcriptional regulators (Ansieau & Leutz 2002). No structural information is available on MYND-mediated interactions, although a few MYND motifs recognize a Pro-X-Leu-X-Pro (PXLXP) motif in their partner protein. Proteins containing MYND ZnF domains include the transcriptional co-repressor protein BS69 (Ansieau & Leutz 2002).

Ring-type ZnF motifs: RING [really interesting new gene](Lovering et al. 1993) domains coordinate two zinc atoms. Protein containing RING ZnF domains include KAP-1, PML, and several E3 ubiquitin ligases (catalyse final step of protein ubiquitination pathway).

PHD-type ZnF motifs: PHD domains coordinate two zinc atoms, and are named after the class of proteins (plant homeodomain) in which it was first recognized (Schindler, Beckmann & Cashmore 1993) as PHD ZnF domains differ from RING-type domains in containing a highly conserved Trp residue involved in the hydrophobic core; this residue is exposed to solvent in RING-type ZnF domains. Protein containing PHD ZnF domains include

Ing2 (inhibitor of growth protein 2), BPTF, Pygopus (Wnt signalling pathway), WSTF transcription factor, and Datf1 (Death-associated transcription factor 1).

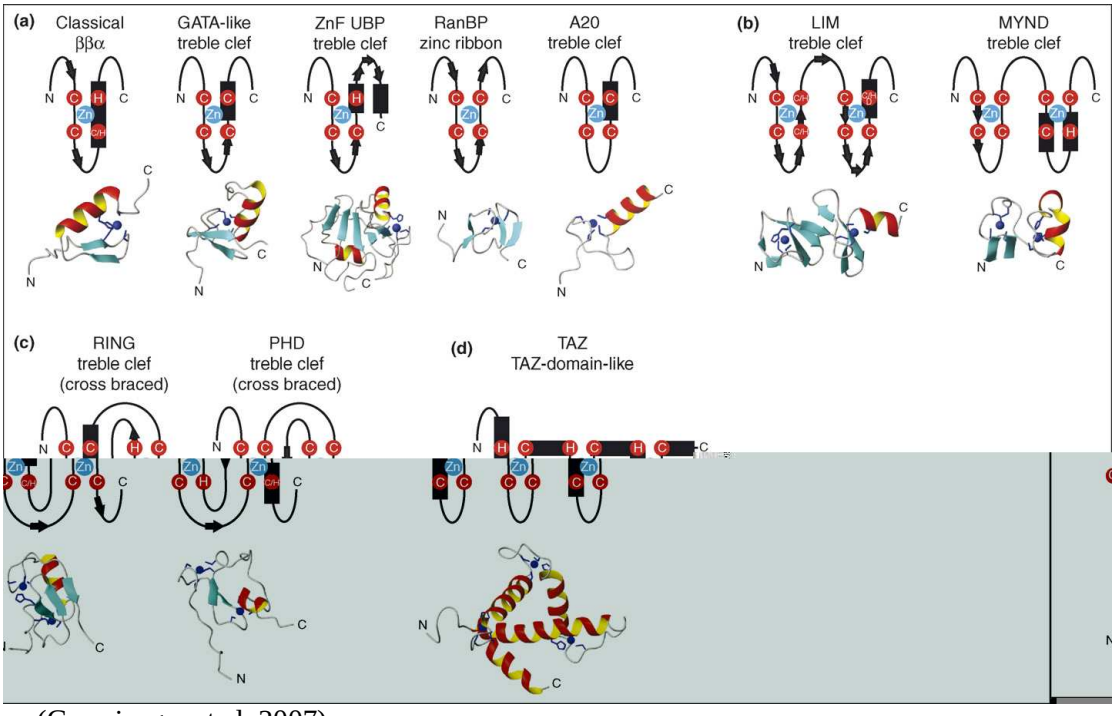
TAZ-type ZnF motifs: TAZ (transcriptional adaptor zinc-binding) domains seems to exist only in the transcriptional coactivator CBp, where two ZnF motifs form a distinct fold that is unrelated to other ZnFs (Ponting et al. 1996): namely, three zinc atom is present in a triangular structure, where each zinc atom is present in a loop region between two antiparallel alpha helices (Figure 1-1D)**.** Protein containing TAZ ZnF domains include CBP acetyltranscferase.

BED-type ZnF: The BED finger which was named after the Drosophila proteins BEAF and DREF, is found in one or more copies in cellular regulatory factors and transposases from plants, animals and fungi. The BED finger is an about 50 to 60 amino acid residues domain that contains a characteristic motif with two highly conserved aromatic positions, as well as a shared pattern of cysteines and histidines that is predicted to form a zinc finger. As diverse BED fingers are able to bind DNA, it has been suggested that DNA-binding is the general function of this domain (Aravind 2000). Some proteins known to contain a BED domain include animal, plant and fungi AC1 and Hobo-like transposases; *Caenorhabditis elegans* Dpy-20 protein, a predicted cuticular gene transcriptional regulator; Drosophila BEAF (boundary element-associated factor), thought to be involved in chromatin insulation; Drosophila DREF, a transcriptional regulator for S-phase genes; and tobacco 3AF1 and tomato E4/E8-BP1, light- and ethylene-regulated DNA binding proteins that contain two BED fingers.

Table 1. Structure of ZnF-protein complexes with cordinates in the protein Data Bank

ZnF	Protein	Partner protein	Function	Refs	PDB code ID	Kd (M)
GATA	GATA-1	FOG	Transcription regulation	Liew et al (2005)	1Y0J	10-5
ZnF UBP	IsoT	Ubiquitin	Deubiquitination	Reyes-turcu et al (2006)	2G43	10-6
RanBP	Npl4	Ubiquitin	Ubiquitination (proteolysis)	Alam et al (2004)	1Q5W	10-4
A20	Rabex5	Ubiquitin	Ubiquitination (proteolysis)	Lee et al (2006)	2FID	10-5
LIM	LMO2 (N terminus)	Ldb1	Transcription regulation	Deane et al (2003)	1J2O	NA
LIM	LMO4 (N terminus)	Ldb1	Transcription regulation	Deane et al (2003)	1M3V	NA
LIM	LMO4	Ldb1	Transcription regulation	Deane (2004)	1RUT	10-9
LIM	PINCH	Nck2 (SH3 domain)	Cell adhesion and migration	Vaynberg (2005)	1U5S	10-2
RING	c-Cb1	UbcH7	Ubiquitination (proteolysis)	Zheng (2000)	1FBV	NA
RING	Rbx1	Cul1	Ubiquitination (proteolysis)	Zheng (2002)	1LDJ	NA
RING	Cnot4	Ubch5B	Ubiquitination (proteolysis)	Dominguez et al (2004)	1UR6	NA
RING	Bmi1	Ring1B	Ubiquitination (proteolysis)	Buchwald (2006),	2CKL, 2H0D	NA
PHD	ING2	H3K4me3	Chromatin regulation	Pena et al (2006)	2G6Q	10-6
PHD	BPTF	H3K4me3	Chromatin regulation	Li et al (2006)	2FUU	10-6
TAZ1	CBP/p300	HIF-1a	Cellular hypoxic response	Dames et al (2002)	1L8C	10-7
TAZ1	CBP/p300	CITED2	Cellular hypoxic response	Freedman et al (2003)	1P4Q, 1R8U	10-8

(Gamsjaeger et al. 2007)



(Gamsjaeger et al. 2007).

Figure 1-1. Topology and structure of ZnF domains

The grouping of the ZnF domains reflects the order in which they are described in the text. (a) Classical (PDB code 1FU9), GATA (1GAT), ZnF UBP (2G43), RanBP (1Q5w), A20 (2FID). (b) LIM (1A71) and MYND (1FV6). (c) RING (1CHC) and PHD (1XWH). (d) TAZ (1R8U).

Materials and Methods

B1.1. Plant Material and Growth Condition

Plant materials used in this study were Col ecotype of *Arabidopsis* (*Arabidopsis thaliana*). F_0 seeds of Col-0 plants transformed with *Agrobacterium* GV3101 carrying pSKI015 (Weigel et al. 2000) was a generous gift from Dr. SooYoung Kim Chonnam National University, Gwangju, Korea. Seeds of the *Arabidopsis* were surface sterilized by gentle shaking in sterilization solution [70% (v/v) ethanol , 5% Clorox, containing 0.05% (v/v) Triton X-100] for 20 min, followed by rinsed three times with 95% (v/v) ethanol and finally four times with sterile water. For basta screening, between 1800 and 2000 sterilized seed for each 150Φ x 20mm sized plates were placed on half strength of MS media [2.2 g/liter Murashige and Skoog salts (Duchefa Biochemie, Netherlands), 8 g/liter Phytagar (Sigma)] containing fungicides Benomyl (5 mg/liter; Sigma) and 20 mg/L glufosinate-ammonium (Basta, SIGMA) in sterile condition. Then Petri-dishes wraped with two layers of aluminium foil and placed at 4°C for 3-4 days to synchronize germination. The plates were then transferred into growth room maintaining at 21±1°C under long day conditions (16h light/ 8h dark). Twelve and fourteen days after successive basta resistant seedlings were hand transferred into Sunshine mix 5 soil (Sun Grow Horticulture, Canada) and covered with transparent plastic cover for two days to maintain high humidity and placed into a growth room under the above controlled conditions and irrigated with mineral nutrient solution (0.1% Hyponex). After two weeks of seedling transferred to soil, plants

were again sprayed once a week with commercially available Finale, which contains 5.78% (W/V) basta, was diluted 1:1,000, for 3 weeks.

B1.2. Mutant Screening

For the screening, mutants were placed into broad classes on the basis of plant phenotype categories of number of rosette leaves, color and size of the leaf, leaf margin, length of the petiole, plant height, flower and silique shape and size, flowering time, and branching of shoot, were recorded in F_1 generation. Screening strategy and pool architecture was designed with modification of (Krysan, Young & Sussman 1999) Figure 1-2A. Brassinazole insensitive mutants were isolated as following the pool strategy of Figure 1-2B. F_2 Seeds of Columbia plants transformed with *Agrobacterium* GV3101 carrying pSKI015 (Weigel et al. 2000) screened on 20 mg/L glufosinate-ammonium (Basta, SIGMA) were germinated on one-half-strength MS media supplemented with 3μM brassinazole220 (brz220). Seven days after germination at dark, observed hypocotyls in tall phenotype were selected and transformed to half strength MS media contained 20mg/L basta plate for the confirmation of successive transformants with the help of sterilized forceps. Then selected hypocotyls were photographed and scanned for the measurement and further analysis. After one week of transformation on basta plate the seedlings were hand transferred to Sunshine mix 5 soil (Sun Grow Horticulture, Canada) and placed at growth room. The plant phenotypes were scored at the seedling and adult stages. Next, the selected mutants were back crossed with the parental line Col 0, and segregation of the selection marker and phenotypes in brassinazole and soil were followed in the progeny.

B1.3. Extraction of plant genomic DNA

The rapid plant DNA extraction, PCR grade was carried out with some modification of (Edwards, Johnstone & Thompson 1991). The leaf tissue (~100mg) was ground with liquid nitrogen in frozen Axygen tube and a small plastic pestle. Then 500 µl of extraction buffer [100mM Tris-HCl (pH 8.0), 0.5M NaCl, 50mM EDTA] was added to the ground frozen leaf tissue powder and vortex until no tissue clumps was visible and mixture was incubated for 10 min at 65°C, during the incubation mix was inverted 2 to 3 times. Following the incubation 300 µl of 5M potassium acetate was supplied to the lysate and incubated on ice for 20 min. Lysate was centrifuge for 5 min at 13,000 rpm in table centrifuge. Then cleared lysate (typically 450 µl) was pipette to clean Effendrof tube and added 500 µl isopropanol and incubate 4°C for an hour. After incubation genomic DNA was recovered by centrifugation at 13,000 rpm for 10min at 4°C. DNA pellet was washed twice with 70% ethanol, dried and resuspended in 20 µl ddH_2O.

B1.4. Determination of T-DNA insertion Site

Sequence flanking the T-DNA insertion was identified by Thermal Asymmetric Interlaced Polymerase Chain Reaction (TAIL-PCR), Plasmid Rescue and Inverse Polymerase Chain Reaction (IPCR) by using genomic DNA of *atbrzi1* mutant. The detailed procedures are as follows

B1.4.1 Thermal Asymetric Interlaced (TAIL)-PCR

TAIL PCR*:* was carried out according to (Liu et al. 1995) with three rounds of amplification using following degenerate primers AD2 primer 5'-NGTCGASWGANAWGAA-3' AD5 primer 5'-SSTGGSTANATWATW

CT-3’; DEG1 5’-WGCNAGTNAGWANAAG-3’; DEG2 5’-AWGCANG NCWGANATA-3’and T-DNA specific left border nested primers; L150 (5’-CACGTCGAAATAAAGATTTCCGA-3’), L100 (5’-CCTATAAAAC AGATCGT-3”), L50 (5’-ATAATAACGCTGCGGACATCT-3). The first amplification step was performed with 100~200 ng genomic DNA template with a primary T-DNA primer (LB150) and each of degenerate primers AD2, AD5, DEG1 and DEG2 separately. The PCR program for the first round were performed as (1) 94°C for 3 min and 95°C for 1 min (2) five cycles of 94°C for 45sec, 62°C for 1min, and 72°C for 2.5 min; (3) 94°C for 45sec, 25°C for 3min, ramp to 72°C in 3 min (0.3°C/sec) and 72°C for 3 min; (4) two cycles of 94°C for 20sec, 68°C for 1min, and 72°C for 2.5 min; (5) fifteen cycles of 94°C for 20sec, 68°C for 1min, and 72°C for 2.5 min, 94°C for 20sec, 44°C for 1min, and 72°C for 2.5 min; and (6) 72°C for 5 min. After the first amplification step, the PCR reactions were diluted with ddH_2O to 50X and an aliquot of 1 µl from each sample was added to a second round of PCR reaction of 20 µl reaction volume containing a T-DNA specific secondary (LB100) primer with each set of degenerate primer as primary TAIL PCR reactions. The second round PCR was performed using the program: (1) 94°C for 3 min (2) two cycles of 94°C for 20sec, 64°C for 1min, and 72°C for 2.5 min; (3) twenty cycles of 94°C for 20sec, 64°C for 1min, ramp to 72°C in 2.5, 94°C for 20sec, 44°C for 1min, and 72°C for 3min; and (6) 72°C for 5. The second amplification of PCR reactions were again diluted with ddH_2O to 50X and an aliquot of 1 µl from each sample were added to a third PCR reaction of 20 µl volume that containing a T-DNA specific tertiary primer (LB50) with each of degenerate primer as primary and secondary TAIL PCR reactions. In third TAIL-PCR, the reactions were subjected to a low annealing temperature

PCR program: (1) 94°C for 5 min; (2) 94°C for 38 sec, 44°C for 1 min; 72°C for 2.5 min. and a final extension at 72°C for 5 min was followed by a hold at 4°C. Takara ExTaq (Takara, Japan) was used for all amplifications. The MJ research thermocycler was utilized for all TAIL-PCR. The completed TAIL-PCR reactions were size separated by electrophoresis on a 1% agarose gel and isolated by Gel Extraction (QIAGEN). The isolated DNA fragments were sequenced with the T-DNA nested primer LB50 and obtained sequence result was blast on *Arabidopsis* genome at the TAIR blast blast (http://www.arabidopsis.org/Blast)

B1.4.2. Plasmid rescue and inverse PCR (IPCR)

T-DNA flanked insert was also isolated from genomic DNA of *atbrzi1* plant using plasmid rescue and IPCR method (Weigel et al. 2000; Li, J et al. 2001a). Genomic DNA was isolated from 2-weeks old *atbrzi1 Arabidopsis* plants with DNeasy Plant Mini Kit (QIAGEN, USA). The plasmid sequence in pSKI015 is flanked by several restriction enzyme sites that can be used for rescue of T-DNA. The restriction enzymes *KpnI*, *XhoI*, *EcoRI*, *PstI*, and *HindIII* can be used for the rescue of sequences adjacent to the right T-DNA border; and *BamHI*, *SpeI*, and *NotI* can be used for left border rescue **(**Figure 1-3C). For plasmid rescue and IPCR, 10 μg of *atbrzi1* genomic DNA was digested with *XhoI* (New England Biolabs Inc) in a 100 μl volume. After digestion, the enzyme was heat-inactivated at 70°C for 20 min and the reaction was passed through a Qiaquick Nucleotide Removal Kit (QIAGEN, USA). A ligation reaction was set up with 0.2 units of T4 DNA ligase (NEB, England) in a total volume of 100 μl at 4°C for overnight. For plasmid rescue, 20 μl of ligated aliquot was transformed by heat shock method (Sambrook, Fritsch & Maniatis 1989) into DH5α competent cells and spread on LB agar medium containing ampicillin. After incubation at 37°C overnight, 10 colonies per line were chosen and

subcultured in 5 ml of LB medium containing ampicillin. Plasmid DNAs were prepared from these liquid cultures and digested with *BamHI* to distinguish plasmids that did not contain genomic fragments (Figure 1-3 B-C).

IPCR was performed with 5μl of the ligated aliquot and 2.5 units of LA Taq DNA polymerase (TAKARA, Japan) in a total volume of 50 μl under the following conditions: (1) 94°C for 5 min; (2) thirty five cycles of 94°C for 30 sec, 60°C for 30 sec, and 72°C for 5 min (38 cycles); and final extension at 72°C for 5 min was followed by a hold at 4°C.The ligated DNA was amplified with two inverse PCR primers (T3 primer 5'-AATTAACCCTCACTAAAGG GAACAAAAG-3'; AtRB primer: 5'-GATATCTAGATCCGAAACTATCAG TG -3'). The resulting PCR products were separated by gel electrophoresis and purified (QIAquick Gel Extraction Kit, QIAFEN, USA) before it was subjected to sequence analysis

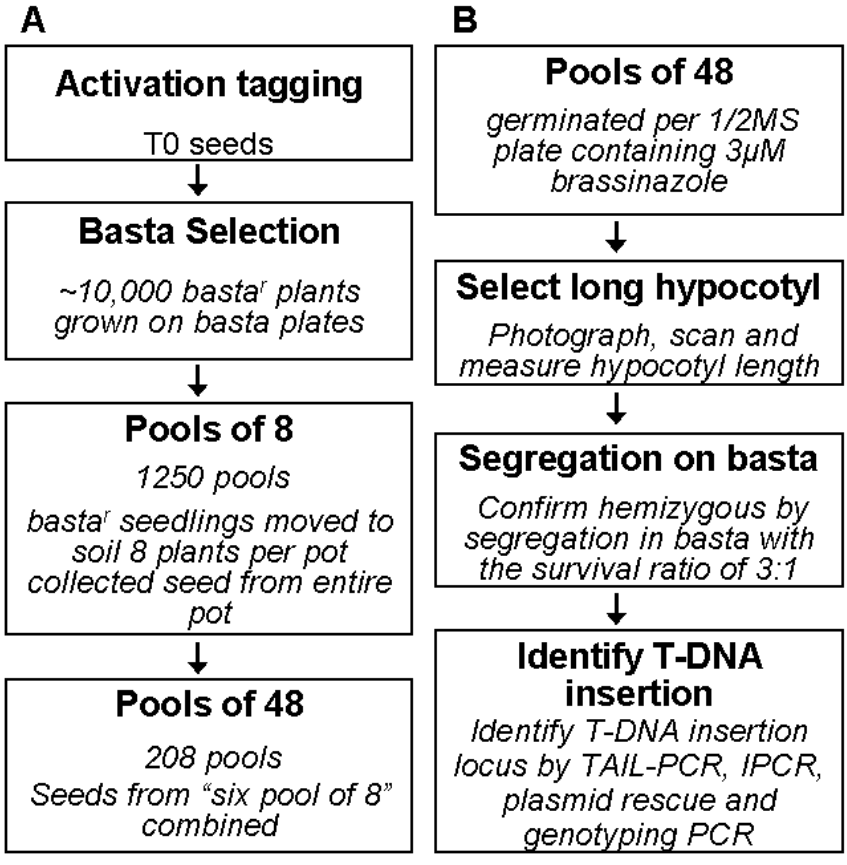


Figure 1-2. Establishment of pool system for mutant screening

(A) Strategy of pool making at the time of screening in F_1 generation.

(B) Strategy of brassinazole insensitive mutant screening and identification of the T-DNA insertion site in F_2 generation

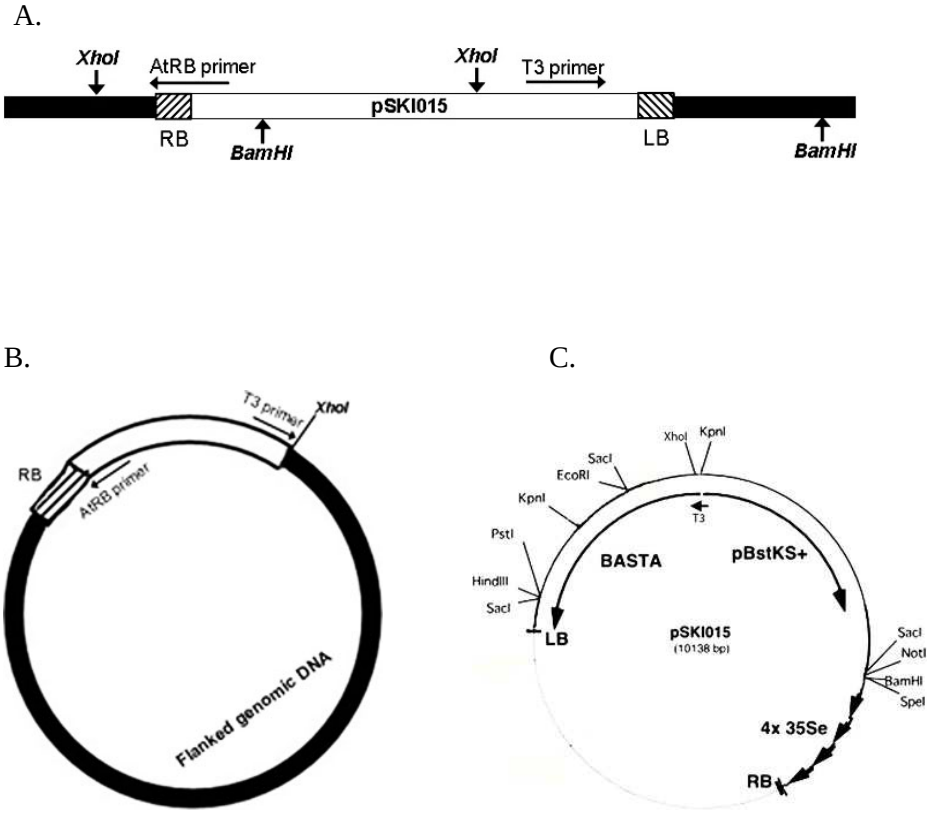


Figure 1-3. Schematic diagrams for plasmid rescue and IPCR

(A). Appropriate restriction sites of left and right border of the T-DNA, where one enzyme chopped into two sites, one is at T-DNA and another is at the possible site of genomic part. (B). Plasmid rescue and IPCR is used to amplify DNA of unknown sequence (dark line) that is adjacent to the T-DNA sequence (White blank). The genomic DNA digested with XhoI was circularized by ligation and PCR was performed by using T-DNA specific primers T3 and AtRB. (C). activation-tagging vector pSKI015 showing restriction enzyme sites and lines in bold indicate limit of T-DNA internal sequence after plasmid rescue.

B1.4.3. Genotyping PCR

The *atbrzi1* mutation was detected by using a primer pair of a T-DNA specific skLB2 (5'-CATTTGGACGTGAATGTAGACACGTCGAAAT-3') and a gene specific primer Atbrzi1-F (5'-TAGGACTGCTCTAATTATG TGGTAAGA-3'). The presence or absence of the wild-type allele was confirmed by a primer pair Atbrzi1-F and Atbrzi1-R (5'-GTGTCTGAT GAACT TGTAAATCTC-3'). The amplified PCR reactions were size separated by electrophoresis on a 1% agarose gel. Band obtained for wild type is 285 bp and band for *atbrzi1* mutant is 444 bp.

B1.5. Seed germination Experiment

Seeds of wild-type and mutant plants were placed on Murashige-Skoog medium containing 50 mg/l basta or without. Petri dishes were covered with parafilm and placed into the dark place, temperature at 4°C for 3-4 days. After that they were transferred to the growth room with conditions 16h light and 8h dark, temperature 21±2°C. Differences in seed germination between wild type and transgenic plants were obvious on 2 days. The developmental differences from the stage of seedlings between activation tagged plants and wild type were observed on the 7^{th} day.

B1.6. RNA isolation and cDNA Synthesis

Total RNA samples were isolated form 4-weeks old above ground tissue with Trizol (Life Technologies) followed by manufacturer's protocol. The concentration of the total RNA was quantified in spectrophotometer (Smart Spec 3000, *BIO-RAD,* USA) at an absorbance of 260 nm. All the extracted RNA was diluted to 1 μg/μL. cDNA was synthesized using Superscript II reverse transcriptase (Life Technologies). 2 μg of total RNA

and 1 µL of the oligo dT (500 µg/mL) were mixed in a reaction tube, heated at 70°C for 10 min to inactivate the sample, and then chilled on ice quickly. 5x first strand buffer and 0.1M DTT were added, and the mixed contents of the tubes were gently incubated at 42°C for 2 min. 1 µl (200units) of Superscript II was added to the tube, incubated at 42°C for 50 min, and the reaction stopped by inactivation heating at 70°C for 15 min. the synthesized cDNA was stored at 20°C for further use.

B1.7. RT-PCR Analysis

Semi-quantitative RT-PCR was employed in order to determine the expression of a gene adjacent to T-DNA insertion sites. RT-PCR amplifications were performed in 20 µl reaction volumes, with 200 ng of cDNA, added to 1x Taq buffer, 0.25mM of dNTPs, 0.5µM of each forward and reverse primers under the following conditions: 94°C for 5 min; 94°C for 30 sec, 55°C for 30 sec, and 72°C for 1 min 30 sec (38 cycles); and 72°C for 5 min final extension. The reverse and forward primers used for RTPCR amplifications are as follows: Atbrzi1-1F (5'-AGGCTCTCTAAA CCTCTATCTAAA-3') and Atbrzi1-1R (5'-CTAAACAATAGTTATTTG GATCAACA-3'); Atbrzi1-2F (5'-GTGAGACTAGCTAAGTTCATCAAT ACAC-3') and Atbrzi1-2R (5'-ATCATGTACTACCAACTGAATTGCCT-3') Atbrzi-3F (5'-GAGTTCTATGCGTATTCTACCTCCTAAC-3') and Atbrzi-3R (5'-TAGTGAGAAAGAGGTTAAGGACAATGTA-3'). The *Arabidopsis* actin gene was amplified as a template control using a primer pair: ACT-F (5’-GGCGATGAAGCTCAATCCAAACG-3’) and ACT-R (5’-GGTCACGACCAGCAAGATCAAGACG-3’). Actin gene was used as a positive control. PCR products were electrophoresed in a 1% agarose gel

containing 0.5 mg L^{-1} ethidium bromide (EtBr) and observed under ultraviolet light.

B1.8. *E. coli* Preparations

B1.8.1. Plasmid Preparation

Isolation of plasmid DNA from *E. coli* and *Agrobacterium tumefaciens* was done using Qiaprep Spin Miniprep Kit (QIAGEN). The procedures were followed as described by QIAGEN.

B1.8.2. Preparation of *E. coli* Competent Cells

5 ml of LB medium was inoculated with a single colony of DH5α cells and incubated overnight at 37ºC. 1ml of the overnight culture was used for the inoculation of 100ml LB medium. The culture was incubated at 37ºC by shaking until the optical density at 600 nm reached to 0.4. Cells were centrifuged for 10 min at 4ºC (4500 rpm), the pellet was resuspended in 20 ml ice cold solution 0.1M $CaCl_2$ and let on ice for 15 min. Cells were centrifuged for 15 min at 4 ºC (4500 rpm) and the pellet was resuspended in 2ml ice cold solution 0.1M $CaCl_2$ and 15% glycerol. 200 µl aliquots of heat shock competent cells were frozen in liquid nitrogen and stored at -80 ºC.

B1.9. Map-based Construct

All the cloning procedures were performed according to standard techniques described by (Sambrook, Fritsch & Maniatis 1989). All the purposed cloning is described separately as follows:

B1.9.1. cDNA Construct

For the confirmation of role of *ATBRZI1* gene overexpression, full-length cDNA of the predicted gene (At1g12380) was cloned, using the binary vector pRR 2222 (a generous gift from Dr. Hong Ma's lab, University of Park, PA, 16802 USA). Approximately 0.2 µg of total RNA from the *Arabidopsis* seedlings was used for the cDNA synthesis using TRIZOL reagent as template for the RT-PCR reaction, following the supplier's instructions (Life technologies). The cDNA fragment was obtained by *pfu* proofreading polymerase Turbo (Stratagene), using gene specific primers forward Atbrzi1_cDNA-F: 5'-TCACTAGTCTCGAGGA TGGCGACGATACGCTC -3' and reverse Atbrzi1_cDNA-R: 5'- TCGG ATCCGGCGCCCTTCACACTGAGGACGTATC- 3'. Sub-sequently, the cDNA fragment was cloned into the *XhoI* and *BamHI* sites of vector pRR2222 and the sequence was confirmed by digestion and sequencing.

B1.9.2. gDNA Construct

For the genomic construction *ATBRZI1* gene including 2029bp of gene specific promoter, 140 bp upstream of the T-DNA insertion and 961bp downstream from the stop codon as predicted terminator was amplified from the genomic DNA of ecotype Columbia. PCR was carried out with *pfu* proofreading polymerase Turbo (Stratagene), using the following primers Atbrzi1_geno-F: 5'-GTGTCTTGAACTTGAACTTGTAAATCTC-3' and reverse Atbrzi1_geno-R: 5'-CGGGTACCCATTTATGAAACTTGCCACA 3'. The obtained fragment was digested with *EcoRI* and *BamHI* presence in the specific restriction site of the genomic part and cloned into the same sites of binary vector pCAMBIA 1302.

B1.9.3. *ATBRZI1::GUS* Construct

For *ATBRZI1::GUS* construct, the putative 2.3 kb gene specific promoter including upstream of the *ATBRZI1* was amplified from the genomic DNA of Columbia-0. PCR was carried out with *pfu* proofreading polymerase Turbo (Stratagene), using the following primers Atbrzi1_GUS-F 5'-ATCTGCAGTCTTGAACTTGAACTTGT-3' and reverse Atbrzi1_GUS-R: 5'-CATGCCATGGGAGTTGAAATTAGGACA. Subsequently, the obtained fragment was cloned into the *PstI* and *NcoI* sites of vector pCAMBIA1304 and the sequence was confirmed by digestion and sequencing.

B1.9.4. RNAi Construct

ATBRZI1 RNAi construct was made on pHannibal (Wesley et al. 2001). The inverted repeat, flanked by the *35S* promoter and the OCS terminator was inserted in the *SacI* and *PstI* site of the binary vector pCAMBIA 1302 for plant transformation. To knockdown *ATBRZI1*, we choose the linker region connecting the region similar to Zinc finger, BED-type because it is very specific to the At1g12380 and At1g62870 gene. A 158 bp fragment was amplified from the Columbia cDNA with primers Atbrzi1_Hani-F: 5'-CTGGATCCTCGAGAAAGGCGCGTGGTATTG Atbrzi1_ Hani-R:5'-CCGAATTCA TCGATAGAGTTGAAATTAGGACA-3'.

B1.9.5. GFP fusion Construct

For the plant transformation, 2379 bp fragment was amplified from the Col-0 cDNA using *pfu* Turbo DNA polymerase (Stratagene) and primers Atbrzi1_GFP F: 5'-CCGAATTCGATGGCGACGACTAACGCTC 3' and Atbrzi1_GFP R: 5'-TCGGATCCACTGAGGACGTATCGACAAG-3'. The

fragment was digested with *EcoRI* and *BamHI* and fused to the enhanced green fluorescent protein (EGFP) gene in pEGAD binary vector.

B1.9.6. Protein expression Construct

A 2379 bp fragment of *ATBRZI1* gene except stop codon was amplified from the Columbia cDNA using *pfu* Turbo DNA polymerase (Stratagene) and primers Atbrzi1_GFP F: 5'-CCGAATTCGATGGCGACGACTACGC C-3' and Atbrzi1_PET R: 5'-TGCCGCGGCCGCACTGAGGACGTACG CAAG -3'. The fragment was digested with *EcoRI* and *NotI* and cloned at the multi cloning site of the C-terminal site of the His-tag of the pET-28a (+) vector (Novagen) .

B1.9.7. Yeast-two hybrid Construct

The full-length cDNA sequence was amplified from Columbia cDNA using *pfu* Turbo DNA polymerase (Stratagene) and primers Atbrzi1-pc62-F: 5'-AACTGCAGCTTG ATGGCGACGACTAACGCTCACA-3'-, and Atbrzi1-pc62-R: 5'-TCACTAGTTGTCACACTGAGGACGTATCGACA 3'. The fragment was cloned into the *PstI* and *SpeI* site of pPC62.

B1.9.8. Transformation of *E. coli* and *Agrobacterium*

Methods for preparation and heat-shock transformation of competent DH5α cells were adopted as described in (Sambrook, Fritsch & Maniatis 1989). Approximately 100-200 µl of competent cells were transferred to microcentrifuge tube and 5-10 µl aliquots of a ligation mixture were added. The samples were incubated on ice for 30 min, followed by incubation in a 42°C water bath for 90 seconds. Samples were then quickly chilled on ice and incubated for 5 minutes and 800 µl of SOC was added. Next, the

samples were incubated in 37°C water bath for 15 min and transferred to gently shaking (150 rpm) incubator in 37°C for 45 min to allow bacteria cells to recover and to express the antibiotic marker encoded by the plasmid. Two hundred µl of the putative transformed cells were transferred to LB plate containing selective antibiotics were incubated 37°C overnight.

Preparation of *Agrobacterium* competent cells was done according to TAIR protocols. The overnight culture of *Agrobacterium tumefaciens* in YEB medium was diluted with fresh medium 1:100 (final volume 200 ml) and grown at 28°C till OD 600 reached approximately 0.5-0.8. Cells were collected by centrifugation for 20 min at 3000 rpm at 4°C and resuspend gently in 10 ml of sterile cold TE. The cells were spin down for 5 min at 300 rpm and resuspend in 10 ml of sterile Millipore water. After centrifugation for 5 min at 300 rpm, cells were resuspended in 20 ml of sterile cold YEB. 500 µl aliquots of the cell suspension were frozen in liquid nitrogen for further storage at –70°C.

Freeze-thaw transformation of *Agrobacterium* with plasmid DNA was done according to Chen, Nelson & Sherwood (1994). 1-2 mg of plasmid DNA was mixed with an aliquot of competent *A.tumefaciens* cells and freeze in liquid nitrogen for 5 min. Then cells were transformed to the 37°C water bath and incubated for 5 min. 1 ml of LB medium were added to the cells. The cells were mixed and incubated for 2-3 h at 28°C with gently shaking. Then 200 µl of the cell suspension were spread onto YEB plates contained selective antibiotic and incubated at 28°C for two days and successive colonies were subculture for further transgenic confirmation.

B1.10. *Arabidopsis* Transformation

A floral dip plant transformation method was applied as described by Clough & Bent (1998). 8-10 number of *Arabidopsis plants* were grown over long days in 8x12 cm pots containing soil Sunshine mix 5 (Sun Grow Horticulture, Canada) until the first siliques were visible. Plant pots were placed under continuous light with intensity ranging from 16-20 μmol sec^{-1} m^{-1}. Seedlings fertilized weekly by irrigating with the 0.1% nutrient solution of Hyponex. Horticulture pesticides were used when necessary to control insects and spider mites according to the suppliers' recommendations. Healthy grown plants were clipped first blot to encourage proliferation of many secondary blots. Plants were ready after 4-6 days after clipping. The above ground inflorescence of the plant was dipping by inverting in *Agrobacterium* GV3101 carrying gene of interest on a binary vector solution for 20 minutes. *Agrobacterium* infiltration media was prepared on half strength MS salt (Duchefa Biochemie, Netherlands) contained 0.02% of Silwet L-77, 0.0015% BAP (1mg/ml) and 0.1% 1X Gamburgs vitamins. Dipped plants were then placed under a plastic dome cover for 16 to 24 hours for maintaining high humidity. Seeds were harvested after 2 to 3 weeks pot wise and germinated the transformants on respective selection marker.

B1.11. Computer assisted analysis and Presentation

The web-based interface software Primer3, Genomatix and AthaMap were used for primer design and gene analysis. Online resources used for functional genomics were the National Center for Biotechnology Information (NCBI), The *Arabidopsis* Information Resource (TAIR), *Arabidopsis* Gene regulatory Information Server (AGRIS), The Institute for

Genomic Research (TIGR), Salk Institute genomic Analysis laboratory (SIGnAL). Vector NTI, Gene Runner, Gentle, Plasma DNA were also used for sequence management and analysis.

Plants were photographed with a Nikon COOLPIX5000 digital camera (Nikon, Japan) and hypocotyls and all the plant parts were taken picture under the image microscope Nikon SMZ1500 (Nikon, Japan). All the figures were scanned with HP Scanjet XPA (Hewlett Packard, USA). Images were captured and analyzed with Scion Image (Scion Corporation, Frederick, MD) software. All the digital conversions of images and adjustment were done through Adobe Photoshop CS3 version.

Results

C1.1. Screening of activation tagging *Arabidopsis*

T-DNA introduced *Arabidopsis* seed (F_0) was generous gift from Dr. SooYoung Kim Chonnam National University, Gwangju, Korea. Between the 1800 and 2000 seed per plate was placed for the screening on basta as described in materials and methods. After synchronizing at 4°C, the plates were moved to light in the growth room for germination. Primary transformants harboring T-DNA with basta resistant gene (Figure 1-3C) were survived in the basta plates (Figure 1-4A). These basta resistant seedlings were carefully transferred into soil and sprayed two times with crude basta in interval of a week. After the seed harvest, the population of T-DNA transformed lines were systemized by organizing in a pool system**.** Approximately 10,000 activation tagged primary lines of *Arabidopsis* conferring resistant to basta were generated and established in a pool (Figure 1-2). In T_1 screening, in average 1.35% of Arabidopsis plants were survived in basta in (Table 2A). Among the 10,000 lines, 150 defective mutants in normal growth and development were also screened based on the phenotype characters in categories of rosette color, number of rosette leaves, size of the leaf, length of the petiole, plant height, shape and branching of shoot were recorded in T_1 generation (Appendix I) and identified and established mutant lines for future (Appendix II).

In brassinazole screening, the populations of 10,000 T-DNA insertion lines were organized in a pool system (Figure 1-2). Considering the manpower and growth room space, a total of 208 unique pools of 48 were created by scooping the equal amount of seed from all lines as estimated in

Table 2B. Seeds from the each 48 pools were then germinated in half strength MS media containing 3 μM brassinazole for 7 days in dark. Dark grown plates were carefully opened in just visible light (low light) at clean bench and selected tall hypocotyls with closed cotyledon phenotypes as in Figure 1-4B**.** Out of 10,000 insertion line, 46 were isolated as putative insensitive to brassinazole (Figure 1-5)**.** All the isolated mutants were carefully transferred onto the half strength MS plate containing basta and took photograph (Nikon COOLPIX5000 digital camera) and scanned (HP Scanjet XPA, Hewlett Packard, USA) for the measurement and analysis purposes. Among 46 putative brazzinazole insensitive lines, few lines were died on basta at T_2 generation. This brassinalole screening results reveals that some mutants are extremely and many mutants are partially resistant in the brassinazole (Figure 1-5)**.** However, we used 3 times more concentration of brasinazole than usual (Asami et al. 2000).

Table 2. Screen on basta

(A) Survival ratio in basta in F_1 Screening

(B) Estimation of Seed number in weight for screening. Each number was estimated

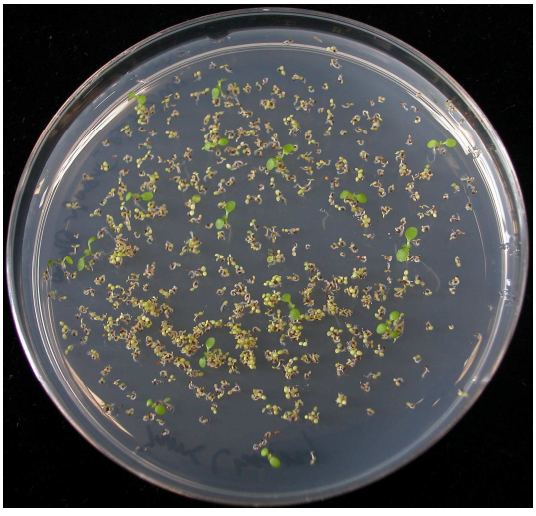
A

Plate no	alive	dead	alive%
1	32	2968	1.06
2	38	2962	1.25
3	34	2966	1.13
4	40	2960	1.33
5	60	2940	2
6	47	2953	1.56
7	54	2946	1.8
8	34	2966	1.13
9	44	2956	1.46
10	41	2959	1.36
11	37	2963	1.23
12	41	2959	1.36
13	42	2958	1.39
14	43	2957	1.43
15	39	2961	1.29
16	38	2962	1.26
17	32	2968	1.06
Average survival % in basta 1.35			

B

SN	no of seed	average wt
1	100	1.79mg
2	200	3.6mg
3	300	5.38mg
4	500	8.87mg
5	1000	17.9mg
6	2000	36mg
7	5000	90.06mg

A.



B.

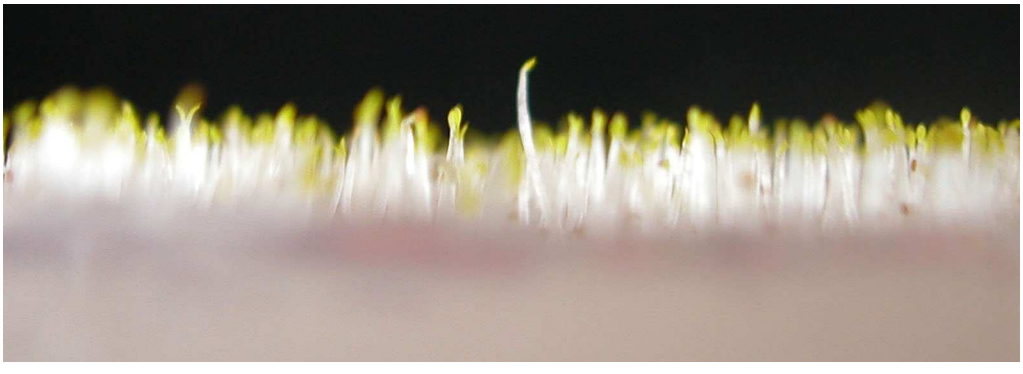


Figure 1-4 Arabidopsis screening

(A) Isolation of T_1, T-DNA insertion activation tagging mutant lines from seedlings that were grown on half strength MS medium containing 20 mg/1 basta.

(B) *Brassinazole insensitive (brzi)* mutant screened on half strength MS salt containing 3 μM brassinazole germinated for 7 days in dark: Brassinazole insensitive mutants showed long hypocotyl and closed cotyledon characters that are grown *Arabidopsis* in dark without brassinazole.

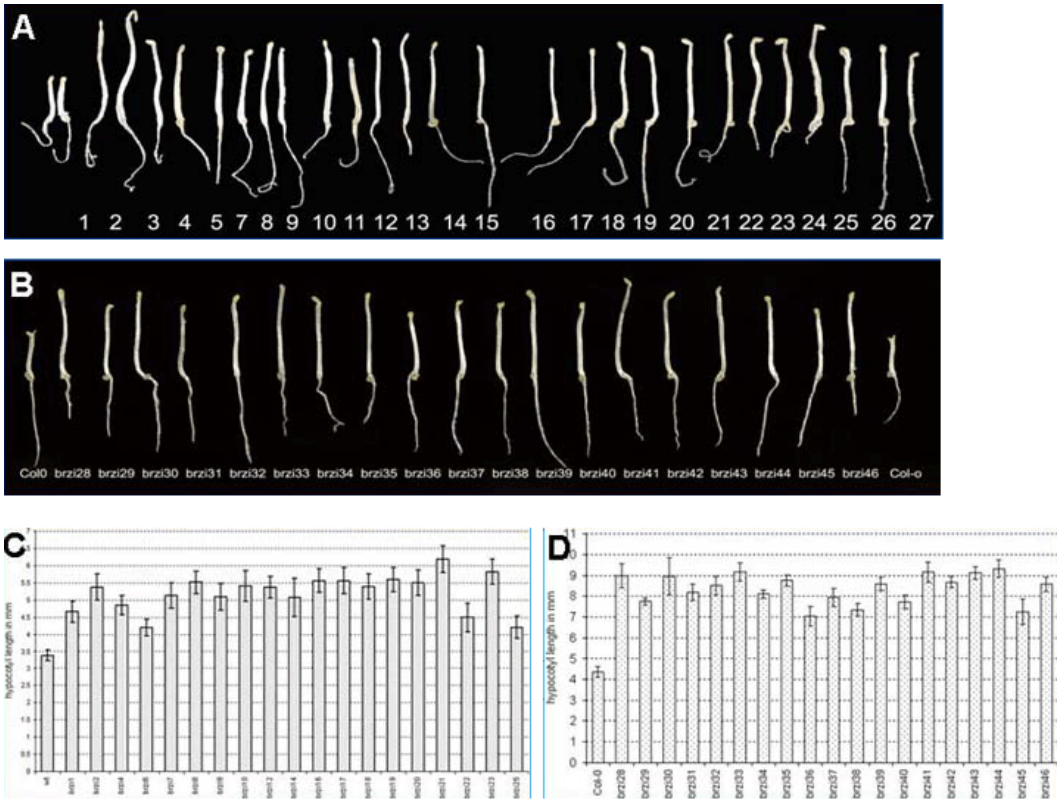


Figure 1-5. Isolation of brz insensitive mutants

Out of 10,000 basta resistant primary transformants 46 putative brassinazole insensitive lines were screened. Among the 46 brassinazole insensitive mutants, some were extremely resistant to the brassinazole**.**

C1.2. Isolation of *atbrzi1* Mutant

The seed of 46 activation tagged putative to brassinazole insensitive mutants were again organized in a pool system and screened visually that were grown on half strength MS plates containing 3 μM brassinazole for 7 days in dark. Most tallest hypocotyl with close cotyledons in brassinazole was selected and gave name *activation tagging brassinazole insensitive 1* (*atbrzi1*). The selected mutant self and T4 population was raised and checked the inheritance.

To identify the genes responsible for the mutants' phenotype, the sites of the T-DNA insertions was characterized. The junction between the T-DNA and *Arabidopsis* genomic DNA was determined by TAIL-PCR. Genomic DNA was isolated from the mutant using QIAGEN kit and performed TAIL-PCR as byLiu et al. (1995). The specific bands amplified by AD and T-DNA specific LB50 primer at TAIL III (Figure 1-6) were excised and purified using Gel Extraction Kit (QIAGEN). Purified tertiary PCR products were sent for sequencing at MACROGEN Korea. Obtained flanking DNA sequences were compared to the *Arabidopsis* data base using Basic Local Alignment Search Tool Nucleotide (BLASTN). Compared typical TAIL-PCR sequence with Genbank identified similarities of distinct regions of the TAIL sequencing read to separate and unique regions of the *Arabidopsis* genome (Figure 1-7A). Sequence analysis of the PCR product showed that T-DNA was inserted in the promoter region of gene At1g12380 which is located at the position of 48102bp of BAC F5011 of chromosome I. The T-DNA is inserted near the centre of promoter between two genes, 1576bp downstream of gene At1g12390 and 1902bp upstream of gene At12380 (Figure 1-8B).

C1.3. Genotyping PCR and T-DNA Verification

Genomic DNA from the wild-type and eight plants of *atbrzi1* was extracted. To identify the location of T-DNA insertion, primers flanking the putative T-DNA location, the left border specific primer skLB2, and gene specific primer Atbrzi1-F were used. The PCR program used 1-min extension time is sufficient to amplify up to 1000 bp fragment. The PCR program was used with a denaturation of 5 min at 94°C, 35 cycles at 94°C for 30 sec, annealing at 55°C for 30 sec and extension at 72°C for 1 min with a final extension of 5 min at 72°C. The PCR products were electrophoresed in a 1% agarose gel containing 0.5 mgL^{-1} ethidium bromide (EtBr) and observed under UV light. Figure 1-8A shows that the presence of T-DNA insertion was confirmed by the amplification of approximately 421 bp fragments. Primers flanking the T-DNA insertion were able to amplify a wild-type fragment (Figure 1-8C, Plant 3) suggesting that T-DNA insertion is hemizygous (T-DNA/WT).

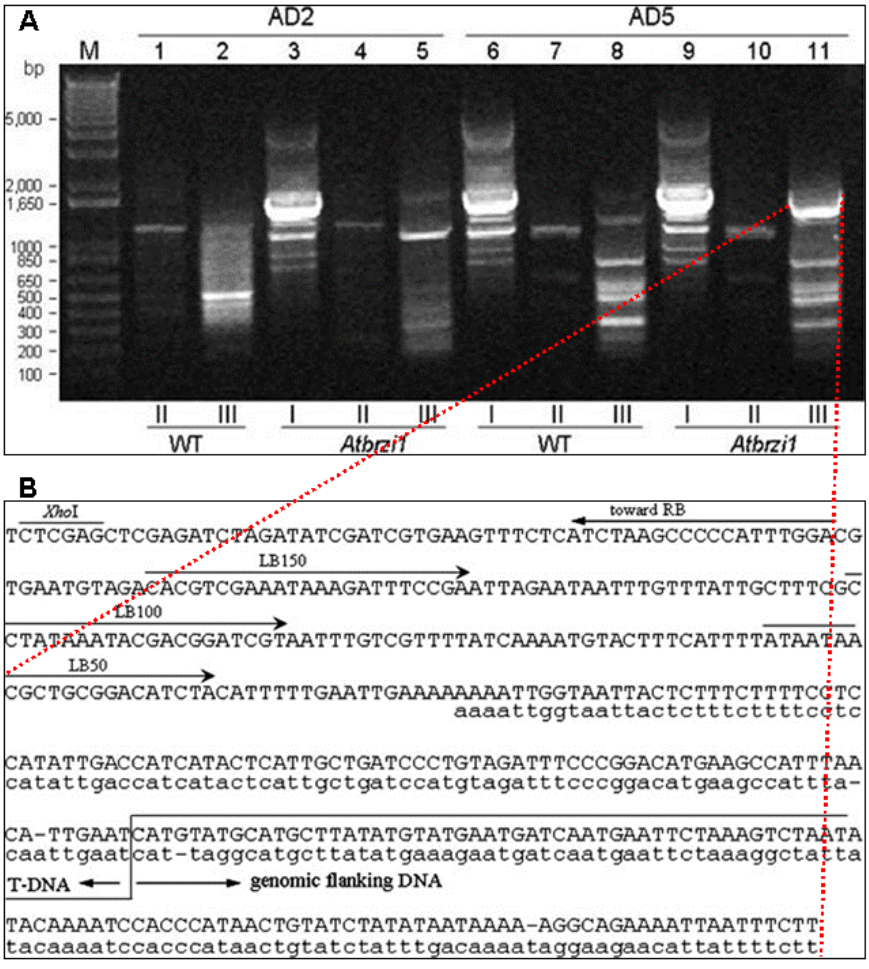


Figure 1-6. Agarose gel analysis of TAIL-PCR products

Each set of three lanes contains products from primary (I) secondary (II) and tertiary (III) reactions for a wild type and *atbrzi1* mutant. The product specificity was confirmed by the size shift between the primary, secondary and tertiary reactions. Multiple nonspecific bands observed in some lanes were nested fragments derived from annealing of the degenerate primers at more than one site along the target sequence molecules. Lane M: is the 1kb DNA ladder.

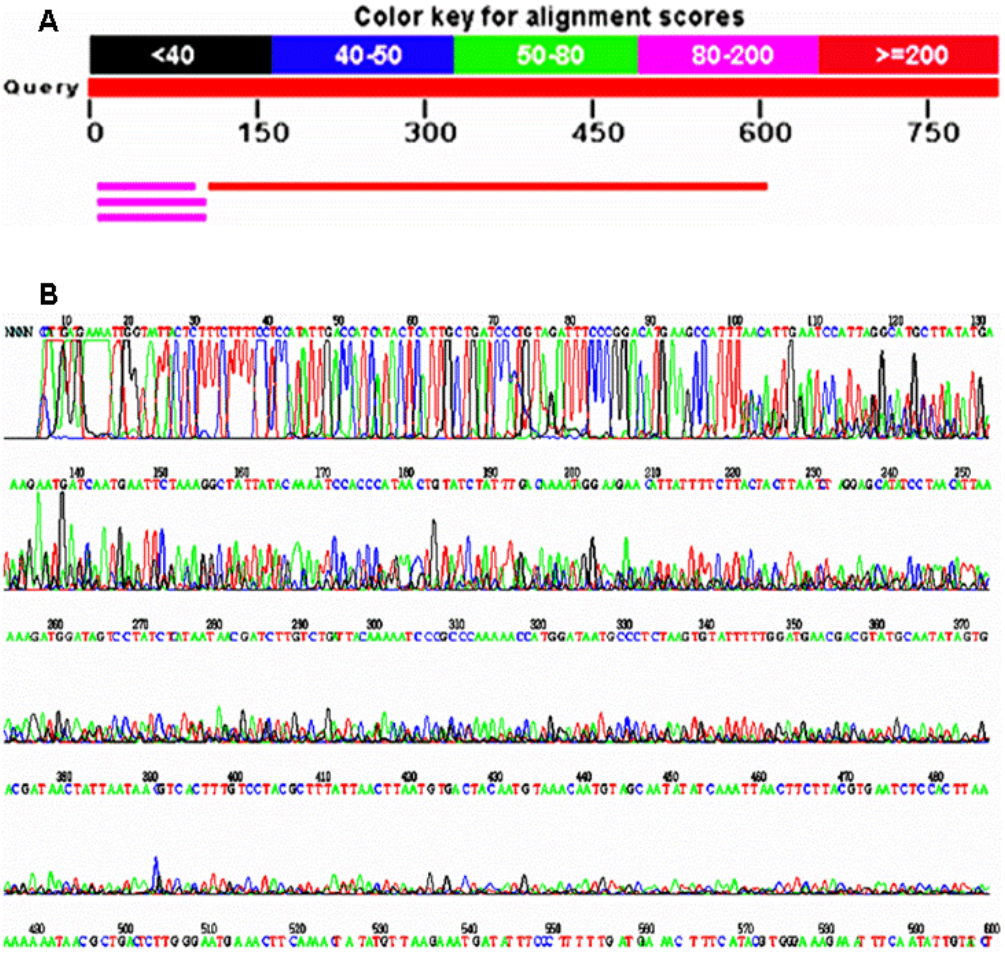


Figure 1-7. Sequence analysis of TAIL-PCR product of *atbrzi1*

(A) Scheme of BLAST hits generated for sequence from the mutant *atbrzi1*, using NCBI BLAST server (http://www.ncbi.nlm.nih.gov/BLAST). The two top hits are color coded for alignment scores, as shown at top. Positions 10 to 107 align with left border T-DNA sequences (pink), positions 109 to 608 align with an Arabidopsis thaliana BAC F5011 from chromosome I. (B) Regions of the electropherogram of the sequence: 5' sequence has higher signal intensity than 3' sequence.

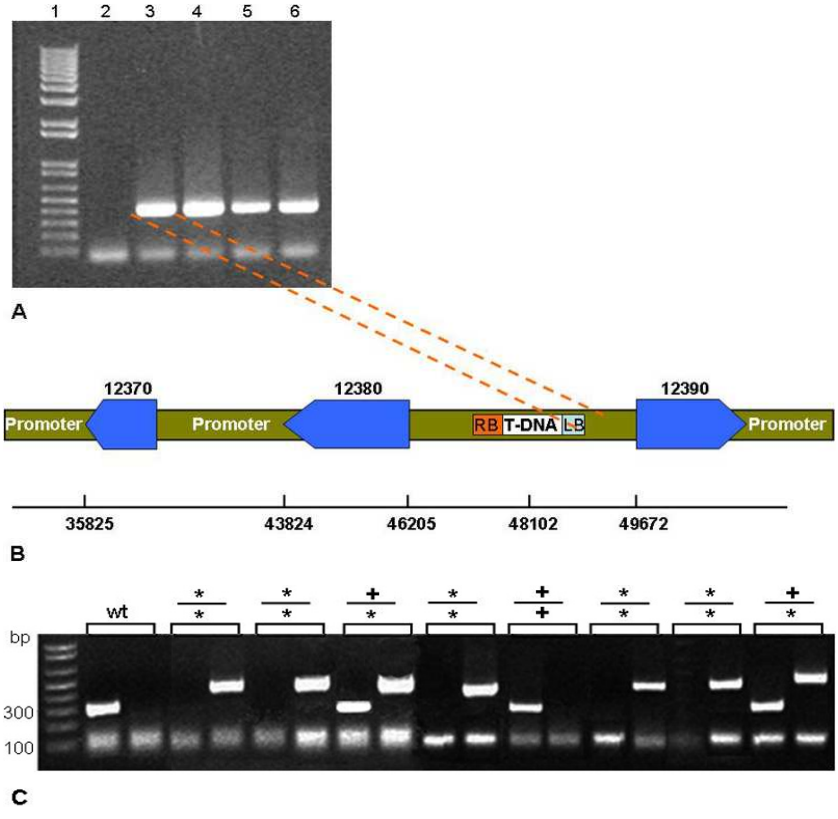


Figure 1-8. Genotyping PCR of *atbrzi1* Plants

Genotyping PCR was amplified with gene-specific primers were Atbrzi1-F & Atbrzi1-R and a T-DNA left border skLB2 (A)**.** Gel electrophoresis of PCR products. Lanes 1, 1-kb ladder; 2, wild type; 3-6 Activation tagged *atbrzi1* mutants. (B)**.** Schematic of gDNA/T-DNA junction in *atbrzi1*. T-DNA was inserted into the promoter region of At1g12380 gene at the position of 48102 of BAC F5011 from chromosome I. (C). PCR analysis of the predicted insertion in wild-type (wt) Columbia (negative control) and 8 progeny of the *atbrzi1* mutant. Products of PCR using the primer sets Atbrzi1-F/Atbrzi1-R and Atbrzi1F/skLB2 were run in adjacent lanes for each sample. Plants 3, 5, and 8 are hemizygous for the insertion, whereas 1, 2, 4, 6, and 7 are homozygous for the insertion.

C1.4 Structure of *ATBRZI1* Gene

ATBRZI1 is located on the acrocentric region of the chromosome I of *Arabidopsis thaliana* nuclear genome (AT1g12380; 4214497-4216872) and encodes a 793 amino-acid similar to the unknown protein, Zinc finger BED-type was predicted. An approximately 158-bp at the N-terminal region of *ATBRZI1* gene contains predicted zinc finger BED-type. This region terminates after the 346 bp of endogenous ATG. Therefore the *ATBRZI1* is a typical eukaryotic gene with BED-type zinc finger motif. There is also another predicted PRO_RICH motif (PPPPPPPPPAPAPP) at N- terminal site of the *ATBRZI1* protein immediately after the ATG start codon (Figure 1-9).

C1.4.1. Computer based analysis of Znf BED-type motif

Since both the 2382-bp At1g12380 and 2289-bp At1g62870 drive BED-type zinc finger motif in a acrocentric manner in *Arabidopsis*, and have 100% identical each other (Figure 1-9 B and C). A comparative analysis of both sequences using GenomeNet, SSpro version 4.5, and vector NTI advance 10 programs were conducted to identify putative zinc finger motif and its structure. The BLAST search result of ATBRZI1 protein has maximum identity with *Vitis vinifera* and *Oryza sativa* and all are uncharacterized and unknown about their function (Figure 1-10). All these proteins have Zinc finger BED type motif and located at the N-terminal region.

Figure 1-11 illustrates the analysis of the BED-type zinc finger with its predicted secondary structure and coordination of Cys-Cys-His-cys manner that is similar to the classical ZnFs comprise a short β hairpin and an α

helix coordinated with a zinc atom (Gamsjaeger et al. 2007). Each protein is labeled using its name followed by its species abbreviation. The extent of the domain in each protein is indicated by amino acid numbers on right side of the alignment. The predicted secondary structure using SSpro v 4.5 is shown that BED-type zinc finger has a short extended strand E and then α helix H (Figure 1-11).

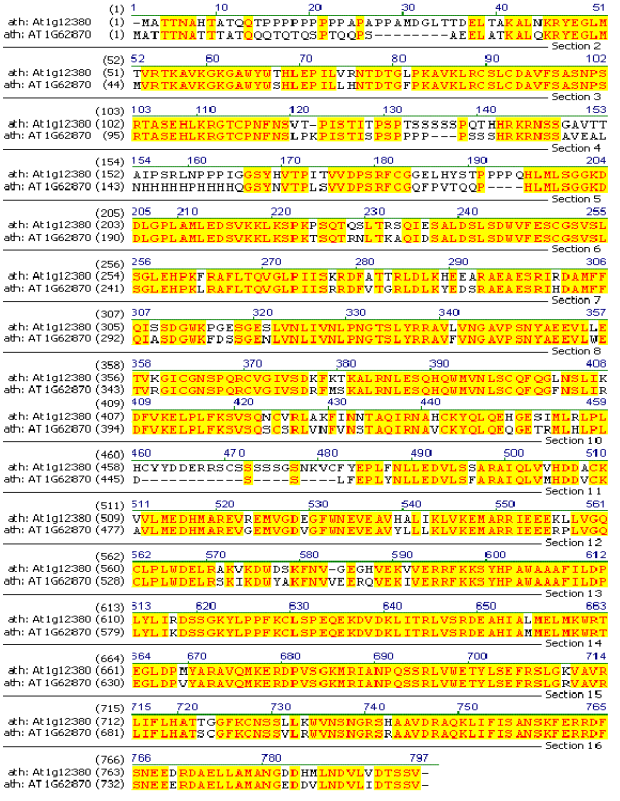
A

```
ath:AT1G12380     1 MATTNAHTATQQTPPPPPPPPPAPAPPAMDGLTTDELTAKALNKRYEGLMTVRTKAVKGK   60
ps:PRO_RICH                  <------------>

ath:AT1G12380    61 GAWYWTHLEPILVRNTDTGLPKAVKLRCSLCDAVFSASNPSRTASEHLKRGTCPNFNSVT  120
pf:zf-BED          <--------------------------------------------------->

ath:AT1G12380   121 PISTITPSPTSSSSSPQTHHRKRNSSGAVTTAIPSRLNPPPIGGSYHVTPITVVDPSRFC  180
```

B.



C.

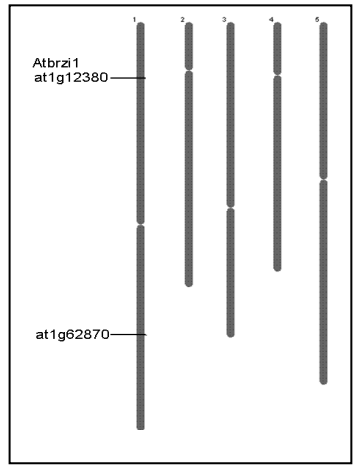


Figure 1-9. Prediction of Znf motif in *ATBRZI1* Gene

ATBRZI1 protein map with the prediction of **A**, Zinc finger BED-type motif comprises with 53aa sequence; **B**, Alignment of *ATBRZI1* with NCBI, other identical gene (At1g62870) carrying BED type zinc finger motif has positive identities with 86%; **C**, Position of two similar gene at1g12380 and at1g62870 in the acrocentric region of the chromosome I of *Arabidopsis thaliana*

Accession	Entry Name	Status	Local Alignment	Protein Name	Organism	Length	Identity (max)	Score (max)	E-Value (min)
Q9LNA8	Q9LNA8_ARATH			**F5O11.10.**	Arabidopsis thaliana (Mouse-ear cress)	856	100	1726	0.0
Q9LQ19	Q9LQ19_ARATH			**F16P17.2 protein** (Putative uncharacterized protein At1g62870).	Arabidopsis thaliana (Mouse-ear cress)	762	77	1198	0.0
A7QYT0	A7QYT0_VITVI			**Chromosome undetermined scaffold_254, whole genome shotgun sequence.**	Vitis vinifera (Grape)	774	65	1029	0.0
A5BUB2	A5BUB2_VITVI			**Putative uncharacterized protein.**	Vitis vinifera (Grape)	885	65	1017	0.0
A7QGS9	A7QGS9_VITVI			**Chromosome chr16 scaffold_94, whole genome shotgun sequence.**	Vitis vinifera (Grape)	762	60	944	0.0
A5C721	A5C721_VITVI			**Putative uncharacterized protein.**	Vitis vinifera (Grape)	762	60	942	0.0
A2X4D6	A2X4D6_ORYSI			**Putative uncharacterized protein.**	Oryza sativa subsp. indica (Rice)	810	52	810	0.0
Q6EQZ3	Q6EQZ3_ORYSJ			**Putative uncharacterized protein OSJNBa0055I13.10** (Putative uncharacterized protein) (Os02g0450000 protein).	Oryza sativa subsp. japonica (Rice)	810	52	809	0.0
A9TGN7	A9TGN7_PHYPA			**Predicted protein.**	Physcomitrella patens subsp. patens	734	54	777	0.0
A9SS37	A9SS37_PHYPA			**Predicted protein.**	Physcomitrella patens subsp. patens	733	53	775	0.0
A9RQ46	A9RQ46_PHYPA			**Predicted protein.**	Physcomitrella patens subsp. patens	747	51	758	0.0
A9SQU5	A9SQU5_PHYPA			**Predicted protein.**	Physcomitrella patens subsp. patens	742	53	755	0.0
Q01MG9	Q01MG9_ORYSA			**OSIGBa0107A02.7 protein.**	Oryza sativa (Rice)	770	52	754	0.0
A2XR40	A2XR40_ORYSI			**Putative uncharacterized protein.**	Oryza sativa subsp. indica (Rice)	770	52	754	0.0
Q7F9J2	Q7F9J2_ORYSJ			**OSJNBa0013A04.12 protein** (Putative uncharacterized protein)	Oryza sativa subsp. japonica (Rice)	770	52	753	0.0

Figure 1-10. Blast search for ATBRZI1 protein.

The ATBRZI1 protein was blast search in web (http://beta.uniprot.org) more than 40 percent identity hit was recorded for the analysis. The putative gene of *ATBRZI1 Arabidopsis* is mainly found two important economic plant *Oryza sativa* and *Vitis vinifera*. In all blast protein ZnF-BED motif is located at the N terminal of the gene.

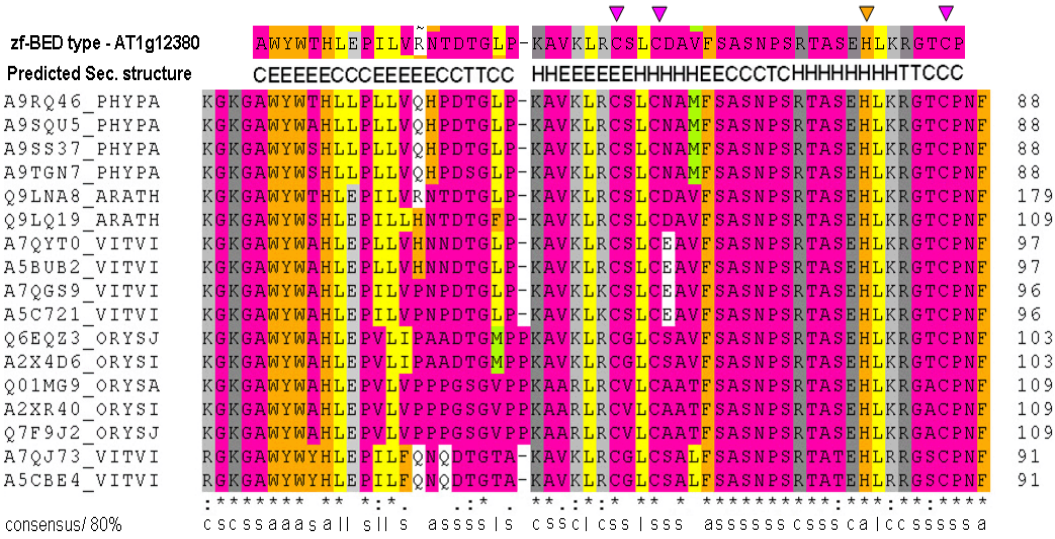


Figure 1-11. Multiple alignment of the BED-type zinc finger domain

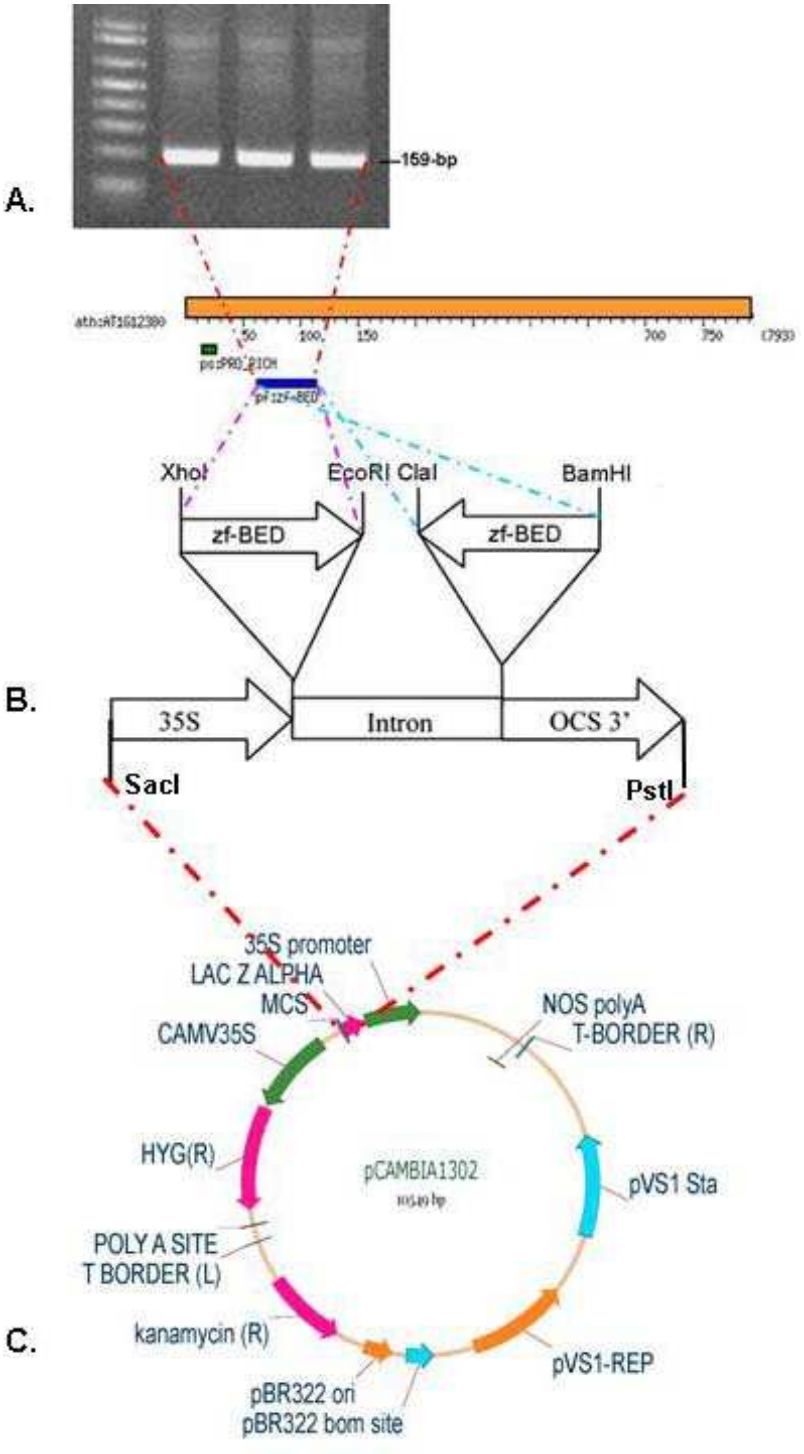
The alignment was constructed by parsing the highest-scoring pairs generated in ClustalW followed by the gene searches (http://beta.uniprot.org). Each protein is labeled using its name followed by its species abbreviation. The extent of the domain in each protein is indicated by amino acid numbers on right side of the alignment. The secondary structure predicted using SSpro v 4.5 program (http://www.ics.uci.edu/~baldig/scratch) is shown above the alignment with H for α helix, E for extended strand, T for turn and C for the rest. The shading is according to the 80% consensus using the following convention: c, conserved regions (gray and dark gray); s, small residues (violet red); a, aromatic residues (golden yellow). The zf-BED protein is arranced in Cys-Cys-His-Cys maner which is similar to the classical type of ZnFs (Gamsjaeger et al. 2007). Abbreviations: PHYPA (*Physcomitrella patens* subsp. Patens); ARATH, (*Arabidopsis thaliana*); VITVI, (*Vitis vinifera*,); ORYSJ, (*Oryza sativa* subsp japonica); ORYSI (*Oryza sativa* subsp indica); ORISA (*Oryza sativa*).A multiple sequence was analyzed with the web of (http://beta.services.uniprot.org/clustalw).

C1.5. Constructs

A 100% homology fragment of the Zinc finger motif of the *ATBRZI1* gene was amplified using the primers Atbrzi1_Hani-F: 5'- CTGGATCCTC GAGAAAGGCGCGTGGTATT G Atbrzi1_ Hani-R:5'-CCGAATTCATC GATAGAGTTGAAATTAGGACA-3' and assembled into the RNAi cloning vector pHannibal (Wesley et al. 2001) in sense and antisense direction separated by a 900 bp intron (Figure 1-12 B)**.** The assembled sense and antisense both fragment of interest gene including the promoter and OCS terminator region of the pHANNIBLA vctor was digested with *SacI* and *PstI* and cloned into the multicloning site of the binary vector pCAMBIA1302 (Figure 1-12 C) All the constructs were confirmed by digestion with respective restriction enzymes and sequencing (Figure 1-12 D-F). *ATBRZI1* silencing construct was transferred to *Arabidopsis* Col-0 using *Agrobacterium* mediated transformation floral dipping method.

cDNA overexpression construct was generated on the binary vector pRR 2222. A 2382bp of *ATBRZI1* gene fragment was amplified from Col-0 cDNA, using primers forward Atbrzi1_cDNA-F: 5'-TCACTAGTCTCGAG GATGGCGACGACTAACGCTC-3' and reverse Atbrzi1 cDNA-R: 5'-TCGGATCCGGCGCGCCTGTCACACTGAGGACGTATC- 3'. RT-PCR amplified product was purified using Gel extraction kit (QIAGEN) and subsequently, digested with restriction enzymes *XhoI* and *BamHI* and ligated into the bianary vector pRR2222 using T4 DNA ligase (NEB) (Figure 1-13 A). The successive construct was confirmed by PCR screen and digestion (Figure 1-13 B-C). cDNA overexpression construct was transferred to *Arabidopsis* Col-0 using *Agrobacterium* mediated transformation floral dipping method.

A 2379 bp of *ATBRZI1* fragment except stop codon primers Atbrzi1_GFP Fand Atbrzi1_GFP R:. The fragment was digested with *EcoRI* and *BamHI* and fused to the enhanced green fluorescent protein (EGFP) gene in pEGAD binary vector (Figure 1-14 A). The successive clone was screened using methods PCR and digestion with respective restriction enzymes (Figure 1-14 B). Following the GFP fusion construction, it was transferred to *Arabidopsis* Col 0 using *Agrobacterium* mediated transformation floral dipping method.


A.
169-bp
XhoI
EcoRI ClaI
BamHI
zf-BED
zf-BED
B.
35S
Intron
OCS 3'
SacI
PstI
C.
35S promoter
LAC Z ALPHA
MCS
CAMV35S
NOS polyA
T-BORDER (R)
HYG(R)
pCAMBIA1302
pVS1 Sta
POLY A SITE
T BORDER (L)
kanamycin (R)
pVS1-REP
pBR322 ori
pBR322 bom site

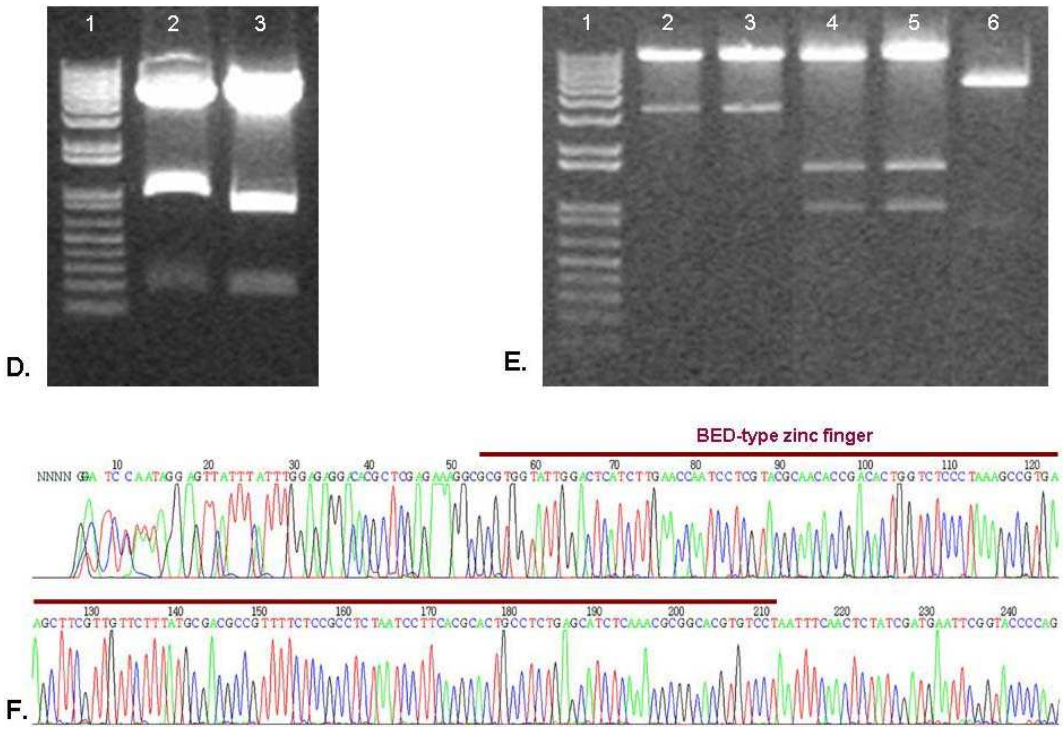


Figure 1-12. ATBRZI1 RNAi Construct

(A). A 177 bp PCR fragment of Znf BED-type motif amplified form the cDNA of Col-0. (B).The amplified fragnment was digested with *XhoI* and *EcoRI* for sense and *BamHI* and *ClaI* for anti sense cloning and cloned after the *35S* promoter for sense and before OCS terminator in antisense manner into the vector pHANNIBAL cloning vector. Sense and antisense clone is separated by 900 bp intron. (C)**.** The RNAi construct including *35S* promoter and OCS terminator region of the pHANNIBLA vctor was digested with *SacI* and *PstI* and cloned into the MCS of the binary vector pCAMBIA1302 of the same sites. (D)**.** RNAi clone in pHANNIBAL is confirmed by digestion. (E)**.** RNAi cloning on pCAMBIA1302 confirmed by digestion. (F)**.** An electropherogram diagram of RNAi cloning confirmed by sequencing

.

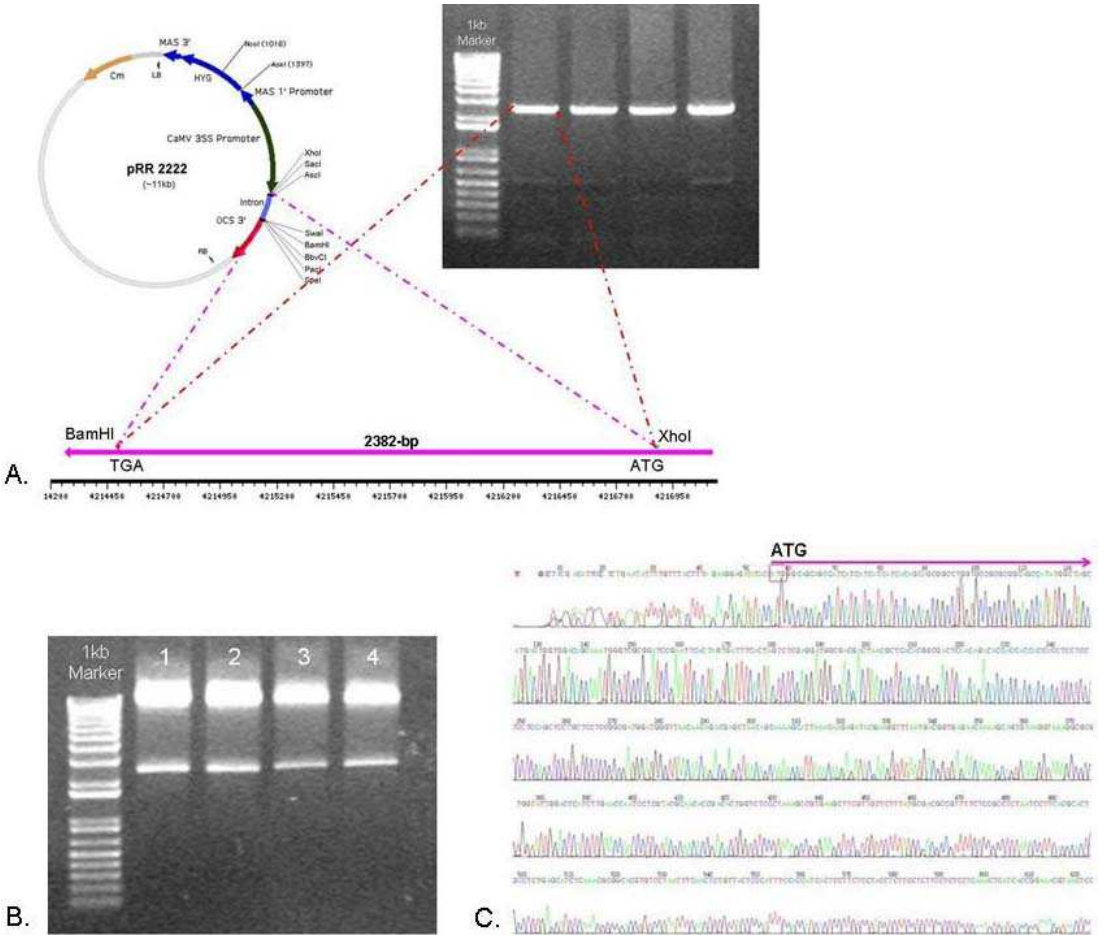


Figure 1-13. Construction for cDNA Expression

A 2380bp of PCR fragment from the Col o cDNA was cloned on binary vectory pRR222 in a sense way with the restriction enzyme site *XhoI* and *BamHI*. **A**, Map of pRR 2222 vector (a gift from Hong Ma's Lab, USA) **B**. Confirmation of cloning by digestion with restriction enzymes *XhoI* and *BamHI*, **C**. Cloning was also confirmed by sequencing, a region of the electropherogram of the sequencing.

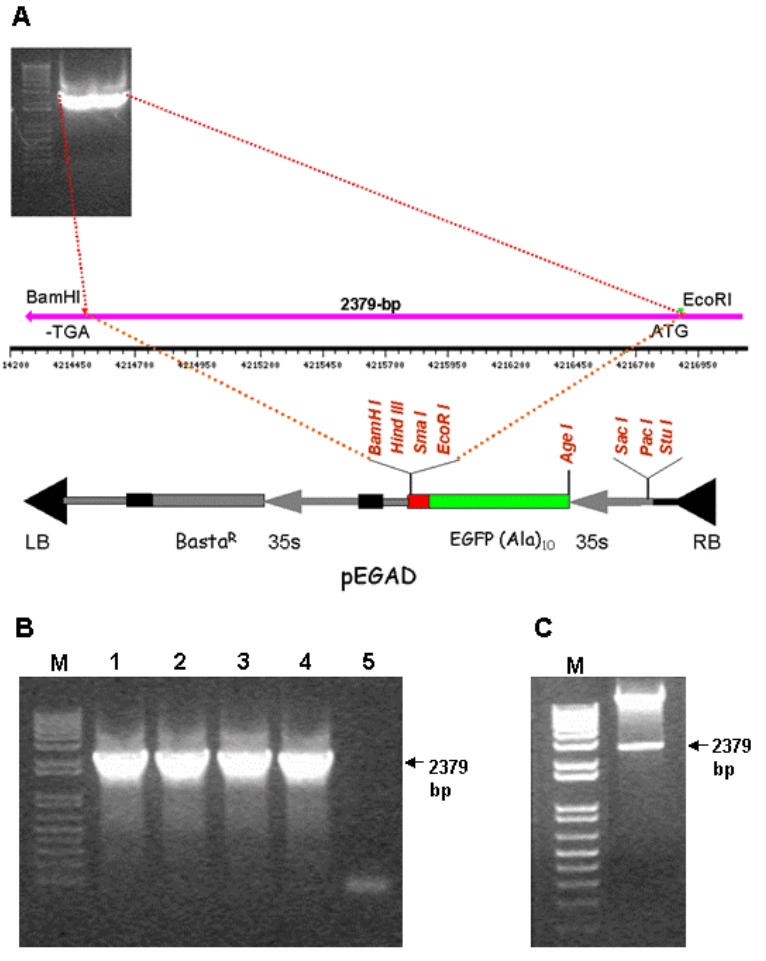


Figure 1-14. ***ATBRZI1 GFP*** **fusion Construct**

A. a 2379 bp fragment (-TGA, except stop codon) was amplified from the Columbia cDNA using primers Atbrzi1_GFP F: 5'-CCGAATTCGATG GCGACGACTAACGCTC-3' and Atbrzi1_GFP R: 5'-TCGGATCCACTG AGGACGTATCGACAAG-3'. The fragment was digested with *EcoRI* and *BamHI* and fused to the enhanced green fluorescent protein (EGFP) gene in pEGAD binary vector. **B.** Successive construct screen by PCR **C** PCR screened GFP fusion construct was again confirmed by digestion with restriction enzymes *EcoRI* and *BamHI*.

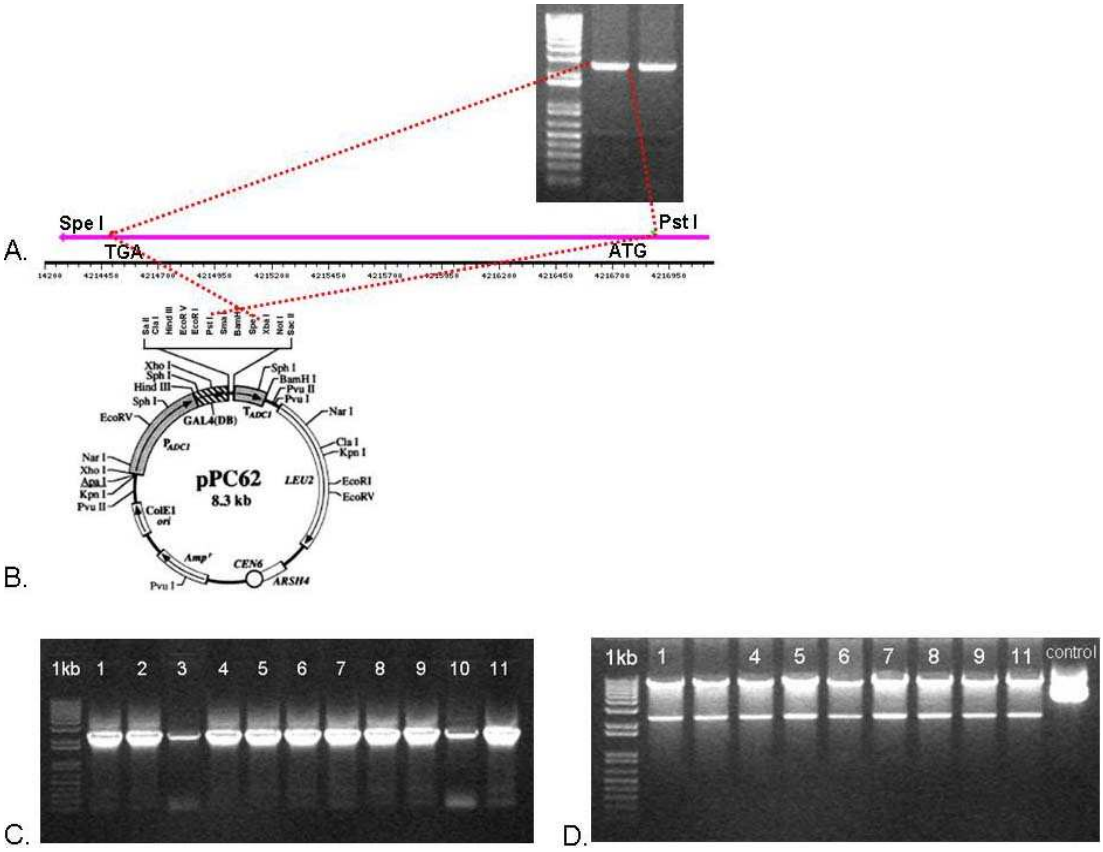


Figure 1-15. Construction for Yeast-two Hybrid

A. a 2382 bp of whole fragment of *ATBRZIN* gene was amplified from the Col-0 cDNA and colned into the *PstI* and *SacI* site of the pPC62 bector. **B.**. Successive construct screen by PCR, **C.** PCR screened constructs were again confirmed by digestion with restriction enzymes *PstI* and *SacI*.

C1.6. Phenotypes of *atbrzi1* Mutant

The T-DNA insertion into the promoter region of the At1g12380 gene made changes in phenotypes of the *Arabidospis*. In brassinazole, the *atbrzi1* mutant was found insensitive and the length of hypocotyls was neary double than the wild-type (Figure 1-16 C-D). In the absence of brassinazole, the hypocotyls length of dark grown mutant was found similar to wild-type (Figure 1-16, A-B).

Root growth assay was performed by germinating seeds of *atbrzi1* and wild type on one half strength MS media and placed plates vertically in growth room. Root length was measured 7 days after germination at light. Root length of the *atbrzi1* mutant was found longer than the wild-type (Figure 1-17 A-C). This result confirms that the gene *ATBRZI1* promotes the root elongation.

Next, we also compare the morphology of the mutant and wild-type plant. The plant height of the *atbrzi1* plant has taller than the wild-type. (Figure 1-18, A-B). The leaf phenotype of the mutant is also differing from the wild-type. The mutant leaf has long petiole and sward shaped leaf lamina and waxy leaf surcace. No of trichomes are fewer in the *atbrzi1* as compared to wild-type (Figure 1-18 C-D). Interestingly, the terminal flower of the mutant was not found at the maturation, while in wild-type it is found in acropetal manner (Figure 1-18 E-F).. The numbers of siliques were also found 1.5 times more than that of wild type (data not shown).

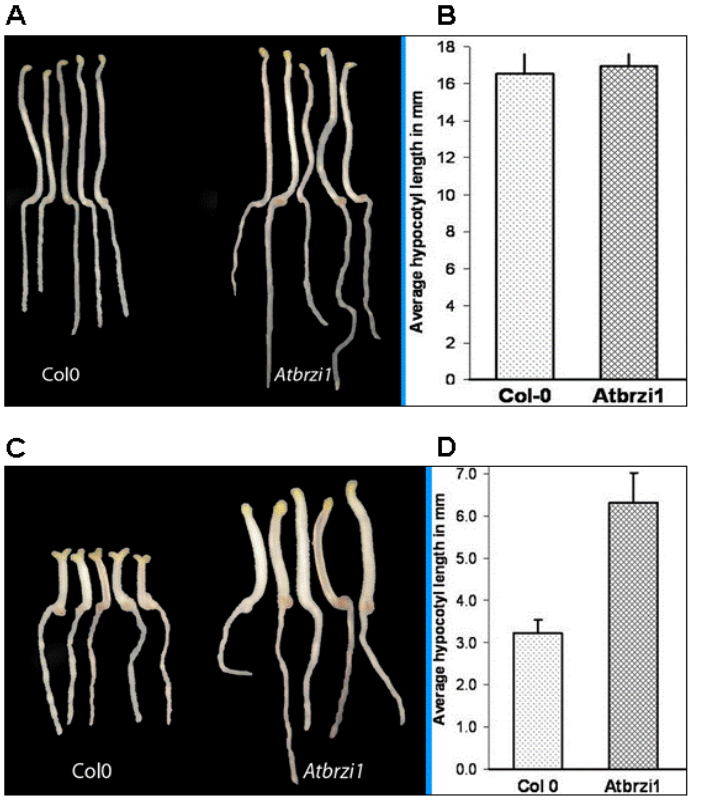


Figure 1-16. Effects of brassinazole on *atbrzi1* hypocotyl elongation

Arabidopsis seeds were germinated and grown on half strength MS as control and half strength MS containing 3 μM Brz a brassinosteroid biosynthesis inhibitor for 7 days in dark. Then hypocotyls carefully transferred into another MS plate and photograph was taken and scanned for measuring. **A-B:** Arabidopsis seeds were germinated in half strength MS media for seven days in dark. **C-D**: Arabidopsis seeds were germinated in half strength MS media containing 3 μM Brz for 7 days at dark. *atbrzi1* mutant is 100% insensitive to the brassinazole as compared to WT. Each data point represents the average hypocotyls elongation of 40-50 seedlings of three duplicate experiments. The error bar represents the SE. All the hypocotyls were measured as described in "Materials and Methods'.

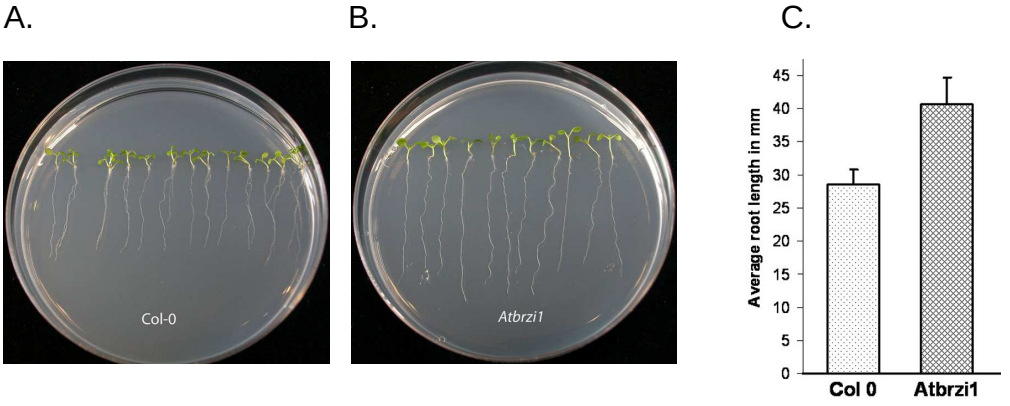


Figure 1-17. Root growth experiment

Invitro root growth experiment with *Arabidopsis atbrzi1* homozygous line compared to its wild type ecotype Col-0.**A.** Wild type ecotype Col 0 **B.** *atbrzi1* mutant. **C**. Average root length of Col0 and *atbrzi1*. *Arabidopsis* seedlings were grown on half strength MS media by placing vertically in growth room. Root length was measured 7 days after germination at light. Each data represents the average root length of 40 to 50 seedlings of three duplicate experiments. Error bar represents the SE. Seed sterilization, growth condition and root measurement that was done as described in "materials and methods"

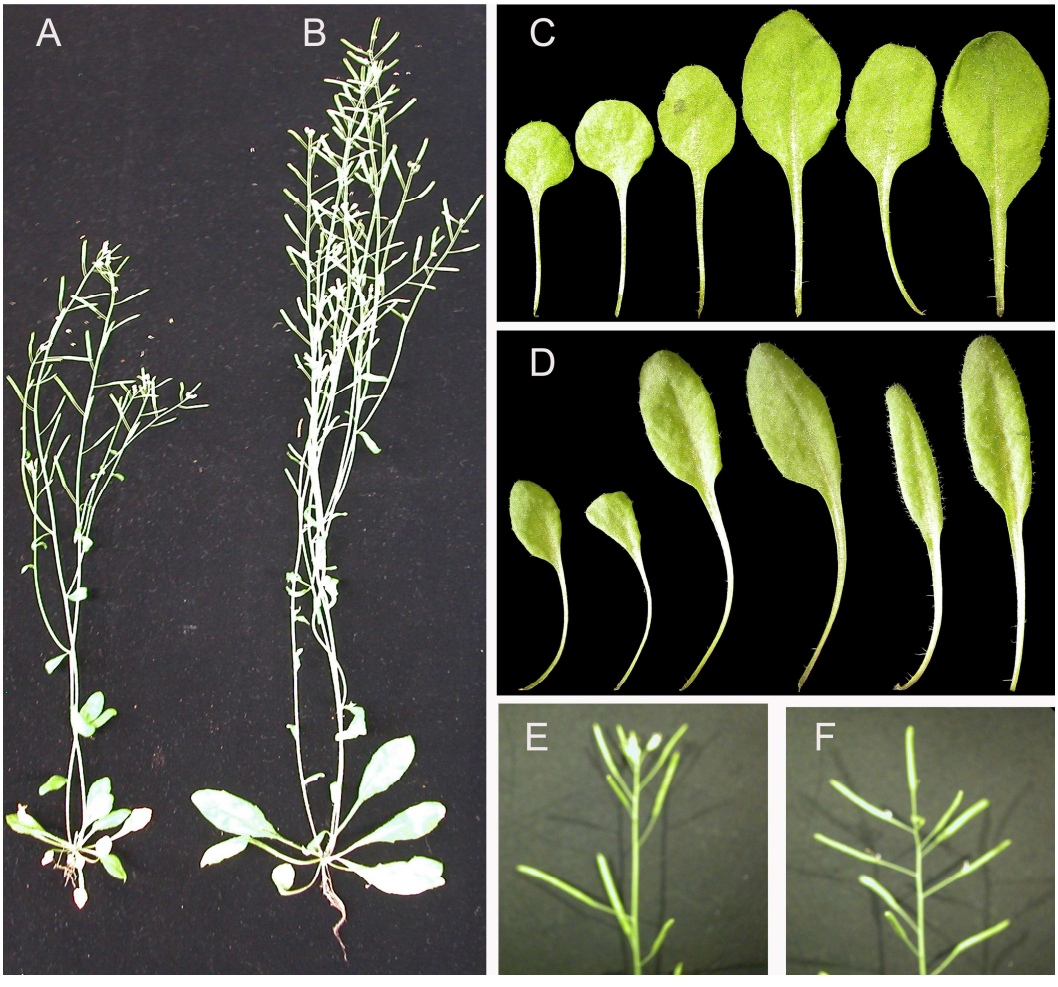


Figure 1-18. ***atbrzi1*** **plant phenotype**

atbrzi1 mutant produced by activation tagging in *Arabidopsis* ecotype Col 0. (A) Wild-type plant (B) *atbrzi1* plant;. **C-D**:, Rossette leaves of 4 week old wild type (C), *atbrzi1* (D); **E-F**: are inflorescence top view of wild type (E), and *atbrzi1* (F). All compared images were taken under the same magnification.

Discussion

D1.1. Mutant Screening

After the completion of *Arabidopsis* genome sequence, (AGI 2000) the challenge for plant biologist is to understand the function of all the genes in the *Arabidopsis* genome, and their mechanism underlying the development of plant (Chory et al. 2000). Recently TAIR8 released 27,235 protein coding genes, 4759 pseudogenes or transposable elements and 1288 ncRNAs (33,282 genes in all 38,963 gene models (http://www. arabidopsis.org). A total of 1291 new genes and 2009 new gene models were added. Updates were also made to 3811 gene structures of which 625 gene models had CDS updates, a total of 4007 exons were modified and 683 new exons incorporated. There were 33 gene splits and 41 gene merges. Overall 23% of all existing TAIR7 genes (7380 genes) were updated (includes split, merged and deleted genes as well as locus type changes, structural updates and sequence updates) for TAIR8.

The manner of systematization screening and establishment of useful resource for future, a modified pooling architecture was designed as suggested by (Feldmann & Marks 1987; Krysan, Young & Sussman 1999). Activation tagging is a common approach to generate mutants for specific trait of interest. In this study, populations of 10,000 T-DNA transformed lines were established and organized by simple strategy outlined in (Figure 1-2A). The collection lines were created by using *Arabidopsis* ecotype Columbia transformed with a derivative of the pSKI015 T-DNA vector that carries tetramerized CaMV 35S enhancer sequences at the T-DNA right border (Figure 1-3 C).

Between the 1800 and 2000 seeds for each 150Φ x 20mm sized plates were placed onto half strength MS media containing 20mg/L glufosinate-ammonium (Basta, SIGMA). Plates were synchronizing at 4°C for 4 days and placed at growth room at 21±2°C under lights (80–100 μE/sec/m^2) with a 16-hr light and 8-hr dark photoperiod for the germination. Twelve and fourteen days after the germination, successive basta resistant seedlings were hand transferred to soil with eight seedlings per 8x12xm sized pot. 150 mutants that are defective in normal phenotypes were screened in T_1 generation. These mutants were screened on the basis of broad classes of plant phenotype categories of: number of rosette leaves, color and size of the leaf, leaf margin, length of the petiole, plant height, flower and silique shape and size, flowering time, and branching of shoot (Appendix I and II).

In case of brassinosteroid hormone signaling related mutant screening, a conditional screening was performed using brassinazole by establishing a pool organization system as designed in Figure 1-2. Considering the limitation of manpower and space, Seeds of T_2 generation were collected in bulk from each pot containing eight plants and gave name “pools of 8” because each pool represents seeds collected from eight independently transformed parent lines. For considering the cost of screening in brassinazole, a pool of 48 was created. This pool of 48 is the batch of seed that was derived from 48 independently transformed parent plants. These pools of 48 were created by scooping equal portion of seeds (~5mg Table 2B**)** from 6 separate pools of eight into the single container. In this manner entire population of 10,000 T-DNA insertion lines were reduced to an ordered collection of 208 pools of 48. Seeds from the each 208 unique pools of 48 were then germinated onto half strength MS media containing 3 μM brassinazole for 7 days in dark.

Brassinazole is a triazole derivatives that inhibits brassinosteroid biosynthesis and has been shown to be useful for investigating brassinsoteroid function in plants (Asami & Yoshida 1999). In both light and dark brz induced morphological changes and were nullified by the addition of brassinosteroid hormone brassinolide (Sekimata et al. 2001). In dark brassinazole treated *Arabidopsis* plant develop as light grown response and express light-regulated genes (Asami et al. 2000). In this screening, instead of 1 μM as used by (Sekimata et al. 2001) we used 3 μM brz with the consideration of reducing the no of semi-insensitive lines, manpower and growth room space. In this order, from the population of 10,000 T-DNA insertion lines we exhaustively screened 46 putative to brassinazole insensitive mutants in T_2 generation (Figure 1-5). Again, a pool was made from 46 putative brassinazole insensitive mutants and screened on 3 μM brz and isolated a tallest brassinazole insensitive line and named the mutant *atbrzi1*.

To identify the genes responsible for the mutant phenotypes, the sites of the T-DNA insertions were characterized. The junction between the T-DNA and *Arabidopsis* genomic DNA was determined by thermal asymmetrical interlaced (TAIL)-PCR (detail in Materials and Methods). T-DNA insertion of the right border (contains the 35S enhancer regions) was positioned at the 5’ end of the predicted genes, as illustrated in Figure 1-8. TAIL-PCR sequencing results reveals that the junction of gDNA and T-DNA insertion in *atbrzi1* was predicted at the 48102 bp poison of the BAC F5011 of the chromosome. This promoter region is shared by two genes Atg12380 and At1g12390 (Figure 1-8B**).** The direction of T-DNA insertion was identified using genotyping PCR with genomic flanking primer and T-DNA left border primer (skLB2). Genotyping PCR results shows that T-DNA right

flank is inserted towards the at1g12380 gene. Genotyping PCR was performed on gDNA extracted from eight lines of the mutant *atbrzi1*. Out of eight lines 3 were found hemizygous and 5 were homozygous insertion (Figure 1-8C). Next, we attempted Reverse transcription based PCR (RT-PCR) to identify the expression level of neighbor genes. RT-PCR result did not show the different expression level as compared to wild-type (data not shown). After then *ATBRZI1* gene was analyzed on blast search on TAIR and identified that *ATBRZI1* has homology with another gene At1g62870 located at the opposite distal end of the same chromosome (Figure 1-9 B and C). Next, bioinformatics analysis was applied to predict the structure of At1g12380 gene for showing brassinazole resistant phenotype. *atbrzi1* gene is located at the N- terminal acrocentric region of the chromosome 1, encodes 793 amino-acid named unknown protein, zinc finger-BED type predicted (www.arabidopsis.org). A 158 bp of zinc finger BED-type motif is located at the N-terminal region after the PRO_RICH motif (PPPPPPPPPAPAPP) of the *ATBRZI1* gene (Figure 1-9).

Since the occurrence of duplicate gene having similarity with 86% and same motif in an acrocentric manner at the two distal end of the chromosome I (Figure 1-9 C) it is hard to predict the function of *ATBRZI1* gene by simply mutant analysis. To know the role of ZnF-BED-type we cloned this motif in RNAi approach (Figure 1-12) and transferred to the wild-type. BLAST result using SSpro version 4.5 found that ZnF BED-type motif is occurred in many plant species in a duplicate or more patterns at the N-terminal region of the particular gene (Figure 1-10). Secondary structure of the BED-type zinc finger was also predicted using SSpro version 4.5 reveals that it is has short extended strand E and then α helix H (Figure 1-11). The predicted secondary structure is in coordation of Cys-

Cys-His-cys manner that is similar to the classical ZnFs comprise a short β hairpin and an α helix coordinated with a zinc atom (Gamsjaeger et al. 2007) Figure 1-1**.**

Zinc finger was first reported by (Miller, McLachlan & Klug 1985) a repeated zinc-binding motif, containing conserved cysteine and histidine ligands, in *Xenopus* transcription factor IIIA (TFIIIA). Since then many other zinc finger motif, widely in structure and function ranges form DNA or RNA to protein–protein interactions and membrane associations have been identified. In addition to their roles as a DNA binding module, ZnFs are now known to have additional activities such as RNA and other protein interactions. Zinc fingers are reporting extremely abundant in higher eukaryotes as progress on genome sequencing of different species. N-terminal DNA-binding domains of proteins like DREF, BEAF32A showed shared pattern of cystine and histidine which could form metal-chelating structure highly conserved with aromatic positions is named as BED finger, after the BEAF and DREF (Aravind 2000). Classical Znf is coordinated in Cys-Cys-His manner. BED-type zinc finger of ATBRZI1 has also coordinated in same manner (Figure 1-17 B)**.** This kind of ZnFs has been shown to interact with RNA and DNA/RNA heteroduplexes with high affinity, although no biological significance of heteroduplex interactions has yet been established (Shi & Berg 1995).

D1.2 Possible involvement of ATBRZI1 in brassinosteroid Signaling

Polyhydroxylated steroid hormones are widely distributed in nature and have been identified in fungi, plants and animals (Vert et al. 2005). Steroid hormones play key roles in growth and development of multicellular

eukaryotes. Plant steroid hormones called Brassinosteroids (BRs) are small growth promoting molecules found at low concentrations throughout the plant kingdom that are involved in diverse process, such as stem elongation, vascular differentiation, male fertility, timing of senescence and flowering, leaf development and resistance to biotic and abiotic stress (Li, J & Chory 1997; Clouse, Steven D. & Sasse 1998; Altmann 1999; Khripach, Zhabinskii & de Groot 2000). Plants lacking a type of steroid-brassinosteroid are likely to be dwarfed with curled leaves and exhibit an ineffective growth pattern in the dark.

Unlike animal steroid hormones, which are mainly perceived by nuclear receptors, plant steroid hormones are recognized at the cell surface by BRASSINOSTEROID INSENSITIVE 1 (BRI1) a leucine-rich repeat receptor kinase (LRR-RK) (Li, J & Chory 1997; He, Z et al. 2000; Wang, ZY et al. 2001). BRI1 is an extracellular domain annotated with predicted putative N-terminal lucine-zipper followed by 24 LRRs, a single transmembrane domain, and an intracellular serine-threonine kinase domain followed by a short C-terminal extension. However, overall structure of BRI1 is representing a receptor kinase (Li, J & Chory 1997).BRI1 is the major BR-binding activity of *Arabidopsis*. The minimal BR-binding domain is a 94–amino acid subdomain that includes the 70 amino acid “island” just proximal to LRR20 and the atypical LRR, LRR21 (Kinoshita et al. 2005).

Direct binding of the brassinolide (BL), a most active brassinosteroid, to the extracellular domain of BRI1 activates and performed homo-oligomer. Auto or trans-phosphorylation of the C-terminus of BRI1 then enhances kinase activity and affinity of BRI1 for BAK1, its proposed co-receptors (Li, J et al. 2002; Nam & Li 2002; Wang, X et al. 2005a; Wang, X et al. 2005b).

In the absence of steroids (BRs) BK1, a plasma membrane-associated protein, interacts directly with the kinase domain of BRI1 to negatively regulate the signaling pathway (Wang, X & Chory 2006).

Binding of BRs to preformed BRI1 homo-oligomers triggers the rapid dissociation of BKI1 from the plasma membrane (Wang, X et al. 2005b; Wang, X & Chory 2006). Subsequently, BRI1 autophosphorylates and associates with second LRR-RK called BAK1 (BRI1-associated receptor kinase1) (Nam & Li 2002; Russinova et al. 2004). BAK1 has a short extracellular domain comprising five LRRs and lacks the critical 70-amino acid 'island" domain responsible for BR binding (Vert et al. 2005). It has been proposed that the physical interaction between BRI1 and BAK1 leads to the formation of a signaling component hetero-oligomer (Li, J et al. 2002; Nam & Li 2002; Russinova et al. 2004). Although SERK1, a BAK1 homolog, also found interacts with BRI1, and genetic experiments have implicated this protein in the signaling pathway (Karlova & de Vries 2006). Thus, the function of the SERK family in BR signaling may be to facilitate the entry of BRI1 into these intracellular compartments. In addition to the plasma membrane, a signaling-competent BRI1-BAK1 heterooligomer was detected in bonafide endocytic compartments of plant protoplasts (Karlova et al. 2006). However, physiological role of an endosome-localized BRI1-BAK1 hetero-oligomer is not known.

The signals transmitted from the plasma membrane-localozed BRI1-BAK1 hetero oligomer negatively regulate the activity of Glycogen Synthase Kinase 3 (GSK-3; also known as Shaggy like Kinases), called BIN2 (Li, J et al. 2001b; Li, J & Nam 2002). Although the mechanism is as yet uncharacterized, single loss-of-function alleles revealed no effect on BR signaling (Belkhadir, Wang & Chory 2006; Vert & Chory 2006), reduced

expression of the entire subfamily results in plants with enhanced BR responses, supporting a role for these three kinases as negative regulators of BR signaling (Vert & Chory 2006). BIN2 localizes to multiple subcellular compartments but appears to exert its largest effects on BR signaling when it is retained in the nucleus. Inactivation of BIN2 leads to the desphosphorylation of BES1 and BZR1, members of a new family of plant specific transcription factor (Wang, ZY et al. 2002; Yin et al. 2002; He, JX et al. 2005; Vert & Chory 2006). BES1, and likely other family members, are further dephosphrylated through the activity of a nuclear localized klech-containing protein phosphatase BSU1 (BRI1 suppressor 1) (Mora-Garcia et al. 2004). BSU1 was identified as a dominant suppressor of a weak *bri1* mutant (Vert et al. 2005; Belkhadir, Wang & Chory 2006). BSU1 overexpression substantially suppresses the dwarf phenotypes associated with either the bri1 or bin2 mutations, which suggests that BSU1 is a positive regulator of BR signaling that acts on the same process or downstream of BIN2. BSU1 encodes a nuclear-localized serine-threonine phosphatase with an N-terminal domain comprising Kelch repeats. Loss of BSU1 function has no effect on *Arabidopsis*, but when the expression of BSU1 and three related genes is reduced by RNA interference, the resulting plant is dwarfed (Wang, ZY et al. 2002; Li, L & Deng 2005). The substrates of BIN2 and BSU1 are likely to be a family of plant-specific transcription factors.

Therefore recent models propose that the balanced activities of BIN2 and BSU1 directly control the phosphorylation state of BES1 and BZR1. BIN2 induced phosphorylation of BES1 inhibits its transcriptional activity through impaired multi merization and DNA binding activity at BR-responsive target promoters. In contrast, in the presence of BRs, BES1 and

BZR1 are dephosphorylated by the combined inactivation of BIN2 and the phosphatase activity of BSU1, which allows them to homo-or heterodimerize and bind more efficiently to the BR-responsive elements to either positively or negatively regulate BR-responsive target genes (Li, L & Deng 2005; Vert & Chory 2006).

Next, all the above data suggest that dephosphorylated BES1 is then able to form homo or hetero-dimers with the basic helix-loop-helix (bHLH) transcription factor BIM1, to bind E-box elements in the promoter of BR-regulated genes (Yin et al. 2005). Then dephosphorylated BZR1 binds to a novel element in the promoter of BR biosynthetic genes to repress their expression (He, JX et al. 2005). Because BES1 is 89% identical to BZR1, and then it is expected that BES1 and BZR1 will have both activating and repressing activities (Li, L & Deng 2005).In summary, Brassinosteroids bind to receptors at the plant cell surface and initiate a signaling cascade that involves nuclear factors including BZR1 and BZR2. (He, JX et al. 2005; Sablowski & Harberd 2005) have now characterized aspects of the signaling pathway for brassinosteroids in detail and find that BZR1 is a DNA binding protein that functions as a transcriptional repressor

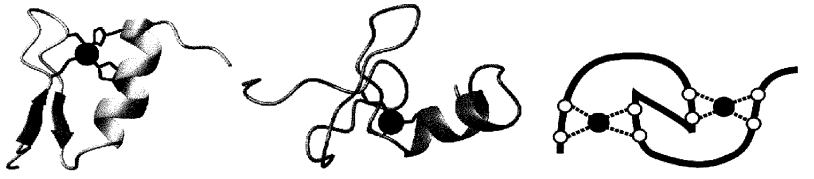
Only the BRI1 locus was defined by loss-of function mutations that caused BR-resistant dwarfism; however, several additional components of the pathway were identified by analysis of gain-of function phenotypes or plants with increased sensitivity to BRs. In this study we also isolated a mutant named *atbrzi1* from activation tagging *Arabidopsis* a gain of function approach. *atbrzi1* mutant shows insensitive to the brassinazole a brassinosteroid biosynthetic inhibitor (Figure 1-16 C-D)**.** Results of TAIL-PCR, genotyping PCR and bioinformatics analysis identified that *atbrzi1* has features of Zinc finger BED-type binding motif at the N-terminal region

of the ATBRZI1 gene. The role of zinc finger motif for brassinosteroid hormone signaling is not characterized yet. However, it is known that zinc ion has crucial role to stabilize the structure of small domain, with out the zinc ions, the domains unfold. The folding of small globular domains within gene regulatory proteins is required for sequence-specific DNA binding, an event that underlies gene expression in all kingdoms of life (Spolar & Record 1994). The formation of zinc-ligand coordination bonds provides the major driving force for the folding of these mini-domains that lack an appreciable hydrophobic core (Berg & Godwin 1997).

After reviewing the brassinosteroid hormone signaling pathways, suggest that Steroid receptors are the first members identified of a group of transcription factors in which the protein is activated by binding a small hydrophobic hormone. The activated factor becomes localized in the nucleus, and binds to its specific response element, where it activates transcription. ATBRZI1 has a BED-type zinc finger DNA-binding motif that is located in the N-terminal region of the gene (Figure 1-9A)**.** It has HLH (helix-loop-helix) type os structure (Figure 1-11) which is similar to the structure of classical type of Zinc finger (Figure 1-19A). The dephosphorylated BES1 is then may able to bind with HLH (helix-loop-helix) form as like in BIM1 of the zinc finger BED type motif and this protein has amphipathic helices that are responsible for dimerization adjacent to basic regions that bind to DNA. This dephosphorylated BZR1 binds to Zin finger motif could be a novel element in brassinosteroid hormone signaling (Figure 1-20). Further, it is yet to be confirmed whether ATBRZI1 is responsible to bind from the dephosphorylated BZR1 or to the other site. Results form RNAi and GFP will make further clear about the involvement of Znf BED type motif in BR signaling.

A

Family	Zinc-ligation	Structure	Function/Binding targets
Classical/C2H2	C2-H[C/H]	Classical ββα	DNA, Protein, RNA
GATA	C2-C2	Treble clef	GATA sites, protein
Nuclear hormone receptor	$C2_A$-$C2_A$-$C2_B$-$C2_B$	Treble clef	DNA
FYVE	$C2_A$-$C2_B$-$C2_A$-$C2_B$	Treble clef (x)	Lipid
Protein kinase C	H_A-$C2_B$-$C2_A$-[H/D]C_B-C_A	Treble clef (x)	Phorbol esters, diacylglycerol
RING	$C2_A$-CH_B-[C/H]C_A-$C2_B$	Treble clef (x)	Ubiquitin ligation, protein
LIM	$C2_A$-[H/C]2_A-$C2_B$-C[C/H/D]$_B$	Treble clef	Protein
PHD[1]	$C2_A$-$C2_B$-HC_A-C[H/C]$_B$	Treble clef (x)	Protein, ubiquitination.
TAZ[1]	HC_A-CC_A-HC_B-CC_B-HC_C-CC_C	TAZ	Protein



ββα[2] Treble clef[3] Treble clef (x)

B

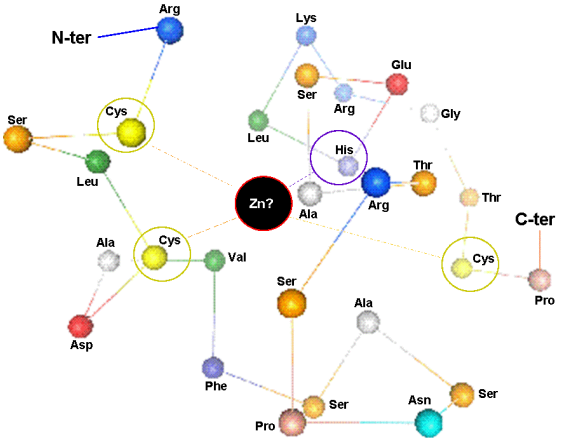


Figure 1-19. Features of zinc Fingers.

A. Classical ZnFs: according to (Matthews & Sunde 2002); Upper classes of ZnFs and zinc-binding ligands; Bottom, Examples of structure

B. Structure of BED-type zinc finger located in *ATBRZI1* gene.

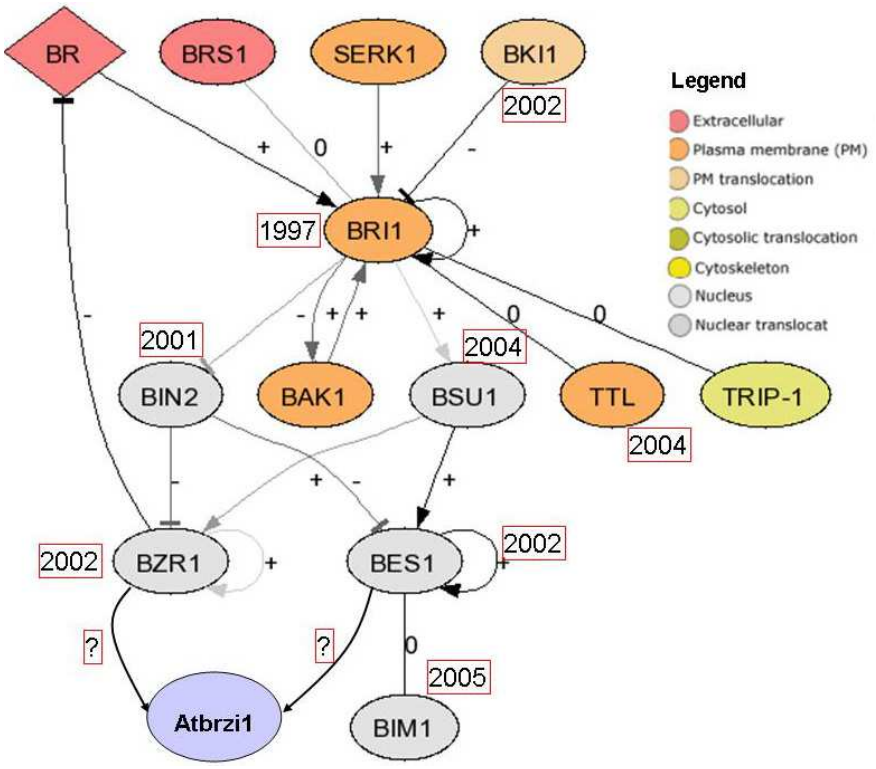


Figure 1-20. A purposed model for BR signaling in addition to ATBRZI1

The BZR1-BES1 family of transcription factors regulates BR-responsive genes in *Arabidopsis.* BR perceived by the receptor kinase BRI1 and BAK1 at the plasma membrane initates the BR signal transduction pathway that leads to dephosphorylation and accumulation of BZR1 and BES1 in the nucleus. The phosphorylation state of BZR1 and BES1 is regulated by the counter actions of the BIN2 kinase and the BSU1 phosphatase. BZR1 and BES1 represent a class of plant-specific transcription factors that bind to and activate the promoters of BR responsive genes, with the facilation of other transcription factor BIM1 in the case of BES1. BZR1 also specifically binds to and repress BR biosynthetic genes to reduce BR levels in the cell. Wheather BZR1 binding requires co-factors such as the BIM proteins have not yet been determined. This model is based on (Belkhadir, Wang & Chory 2006)

D1.3. Application and limitations of the Study

In addition to mutant screening performed in this study, the activation tagging library (which took almost two and half year) may prove useful in several contexts. First, this mutant population can be used to screen for conditional mutants (here we already isolated some mutants for defective in root growth, and heavy metal resistant mutants) that do not occurred under normal condition. Second the isolated 50 mutants that are defective in normal growth and development might be very useful for the further research in plant science. Third, the rest 45 putative brassinazole insensitive mutants that were isolated in this study might give more clues for the understanding in brassinosteroid hormone signaling and also could be strengthen our knowledge on plant growth and development.

In conclusion, isolation and analysis of brassinazole insensitive mutant *atbrzi1* and *abz126* from activation tagging *Arabidopsis* displayed the roles in brassinosteroid hormone signaling. Next, the established mutant library including 45 additional brassinazole insensitive mutants may useful in the isolation of genes that play roles in plant growth and development.

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Chapter II

GIGANTEA is involved in brassinosteroid signaling in *Arabidopsis*

Abstract

Brassinosteroids (BRs) are plant steroid hormones that play essential role in growth and development. The mutant defective in BR signaling causes dwarfism, male sterility, abnormal vascular development and photo-morphogenesis. Transition from vegetative to reproductive growth is a critical phase change in the development of a flowering plant. In a screen of activation-tagged Columbia in *Arabidopsis*, we screened a mutant that displayed tall hypocotyls on MS media contained BRs biosynthetic inhibitor brassinazole (Brz) in the dark and named *abz126.* We have cloned the mutant locus by using adapter ligation PCR walking and identified that a single T-DNA had been integrated into the ninth exon of the *GIGANTEA* (*GI*) gene as being involved in control of flowering time. This insertion made loss of function in *GI* gene caused exhibited phenotype of long petiole, tall plant height, many rosette leaves and late flowering. RT-PCR assay on *abz126* mutant showed that the T-DNA insertion on GIGANTEA made loss of mRNA expression in *GI* gene. In hormone dose response assay, *abz126* mutant showed an insensitive to pacrobutrazole (PAC), an altered response with 6-benzylaminopurine (BAP) and less sensitive with Brassinolide (BL) as compared to wild-type. Based on these results, we propose that the tall phenotype and late flowering *mutant abz-126* is caused by loss of function in *GI* gene is associated with brassinosteroid hormone signaling.

Introduction

Brassinosteroids (BRs) are plant hormones that ubiquitously distributed throughout the plant kingdom and play essential roles in growth and development (Clouse 2002; Thummel & Chory 2002). BR mutants in *Arabidopsis* show a characteristic phenotype that includes dwarfism, round dark green leaves, delayed development, reduced fertility and altered vascular structure (Clouse, Langford & McMorris 1996; Schumacher & Chory 2000). In BR deficient mutants, all of these phenotypic defects can be rescued by exogenous application of BRs (Altmann 1999), demonstrating that the plant steroid hormone has an essential role for normal plant growth and development.

Molecular genetic studies demonstrated that mutation in single locus, BRASSINOSTEROID INSENSITIVE 1 (BRI1), caused the phenotype of steroid-deficient mutants but cannot be rescued by treatment with BRs (Clouse, Langford & McMorris 1996; Li & Chory 1997). BRI1 is a Leucine Rich Repeat (LRR) Receptor Kinase(RK), with an extracellular domain containing 24 LRRs and a 70 amino acid island motif between twenty-first and twenty-second LRRs, a transmembrane domain and a cytoplasmic kinase domain (Li & Chory 1997). Brassinosteroids are perceived by a plasma membrane-localized receptor kinase Serine/Threonine Kinase is encoded by *BRI1* gene (Clouse, Langford & McMorris 1996).

BRI1-ASSOCIATED RECEPTOR KINASE1 (BAK1) is another component of the BR co-receptor complex and downstream target of BRI1 (Wang, X et al. 2005). The cell surface receptor kinase BRI1 and BAK1, perceive the BR signal and initiate the signal transduction cascade. The two

nuclear proteins, BZR1 and BES1 which activate growth response and are dephosphorylated and stabilized by BR signaling (Wang, ZY et al. 2002; Yin et al. 2002). The BIN2 kinase regulates negatively in BR responses by phosphorylating BZR1 and BES1 and targeting them for degradation by the proteasome (Li & Nam 2002; Yin et al. 2002). Another BSU1 phosphatase, was recently identified as positively regulator in the BR signaling by dephosphorylating and stabilizing the BES1 protein (Mora-Garcia et al. 2004). This suggests BES1and BZR1 is found in the nucleus and modulates the transcription of BR regulated genes, which kept in check by BIN2, and fine tune the signal strength. Dephosphorylated BZR1 binds to a novel element in the promoters of BR biosynthetic genes to repress their expression (He, JX et al. 2005). Although, the molecular target of the BR signaling pathway is unknown.

In this study, we isolated the late flowering mutant from the activation tagged Col-7, which showed the characteristics of the BR signaling mutant and named *abz126.* Further, molecular genetic analysis of *abz126* mutant shows that T-DNA was inserted in GIGANTEA and made knockout the gene. GIGANTEA is a nuclear-localized protein that is involved in several processes including the induction of flowering by long days, inhibition of hypocotyls elongation by red light (Huq, Tepperman & Quail 2000), and the circadian clock (Araki & Komeda 1993; Koornneef et al. 1998; Fowler et al. 1999; Park et al. 1999; Huq, Tepperman & Quail 2000). Here, we identified that GIGANTEA could be either transcribe or modify the BR signaling.

Materials and Methods

B2.1. Plant Material and Growth Condition

Arabidopsis thaliana (ecotype Col 7) were mutagenized by *Agrobacterium tumefaciens* (strain GV3101) mediated T-DNA transformation using the activation tagging vector pSKI015 (Weigel et al. 2000). Seeds of T_2 plants that were resistant to basta (20 mg/L) were used for subsequent experiments. T_2 seeds were surface-sterilized in a solution of 70% ethanol containing 0.05% Triton X-100 by gently shaking for 20 min and rinsed three times with 95% ethanol and then five times with sterile water. The seeds were resuspended in an appropriate volume of sterile water (until seeds were free floating) before being sown in rows onto agar plates. The agar medium contained Murashige and Skoog (1962) (MS) salts ((DUCHEFA BIOCHEMIE, The Netherlands) with 1% (w/v) sucrose and phytoagar (Sigma), pH 5.8. The plates then were wrapped into three layers by aluminum foil and placed at 4°C for 4 to ensure uniform germination before being placed in growth room for germination. The appropriate (two to three week old) seedlings were transferred to autoclaved soil (Sunshine mix 5, Sun Grow Horticulture, Canada) and grown to maturity in a growth room. Plants were watered twice a week with 0.1% Hyponex solution. The growth room temperature was maintained at 21±1°C. Light provided by cool-white florescent tubes was 50 to 70$\mu E \cdot m^{-2} \cdot s^{-1}$ (constant) for seedlings on agar plates and ~10070$\mu E \cdot m^{-2} \cdot s^{-1}$(16 h of light and 8 h of dark) for potted plants.

B2.2. Isolation of *abz126* Mutant

Approximately 6000 T_2 mutated lines isolated form basta resistant in T1 plants were screened for brassinazole resistance mutants using a hypocotyl measurement assay. Seeds were surface sterilized and sown by spreading on one-half-strength MS medium containing 2 uM brassinazole (brz220) and kept 21±1°C for germination at dark. Seven days after germination in dark, tallest a seedling was observed in brz and this mutant was given name as *abz126.*

B2.3. Hormone Assay

For hormone dose response, seeds of the wild-type and mutants were germinated and grown on half-strength MS plates containing the different concentrations of plant hormones Pacrobutrazol [Pac (0M, 0.4uM and 4uM)], benzyl aminopurine [BAP (0M, 0.1uM, 1uM and 10uM)], brassinolide [BL (0M, 1nM, 10nM, 100nM, 1uM and 5uM)] (all plant hormones except BL were purchased from Sigma, St. Louis). Hypocotyl lengths of individual seedlings were measured after 7 days grown in dark. Each plate contained at 40 to 50 seedlings of mutant and wild-type. Three replicate plates were used for each treatment. Hypocotyls were scanned and measured using SCION Image software and values were plotted in average with ± SE.

B2.4. Determination of T-DNA Insertion Site

Genomic DNA was isolated form dark grown T_2 seedlings based on magnetic beads method (BioSprint 96, QIAGEN, USA) according to the manufacturer's manual. The prepared DNA samples were digested with *ApoI* at 50°C for 4 hours and purified once with phenol/ chloroform (v/v)

and precipitated by addition of 0.1 vol. 3M Na acetate and 2.5 vol. of 100% ethanol kept on ice for 10 min and centrifuged at 13000 rpm for 10 min. The obtained pellet was washed once with cold 70% ethanol and air dried. The dried pellet was dissolved in 10µl of DW. 3µl of DNA was then ligated with mixed adaptors (5'-GTAATACGACTCACTATAGGGCACGCGT GGTCGACGGCCCGGGCTGC 3', 5'-AATTGCAGCCCG-(NH_2)-3', 5'-AGCTGCAGCCCG-(NH_2)-3') and subsequently subjected to PCR and nested PCR. The first PCR was performed with a primer set of AP_1 (5'-AGAATACGACTCACTATAGGGC-3') and SKLB1 (5' TCGATCGTGA AGTTTCTCATCTAAGCCC 3'), and the PCR cycle conditions were (94°C for 25sec, 72°C for 3 min) x 7 cycles and (94°C for 25 sec, 67°C for 3 min) x 32 cycles. The resultant PCR products were diluted approximately 30 fold in the second PCR mixture. The nested PCR was performed with primers AP_2 (5'-TCGACGGC CCGGGCTGCAATTC-3') and SKLB2 (5'-CCATTTGGACGTGAATGTAGACACGTCT-3') and the cycle conditions were (94°C for 30 sec, 67°C 30 sec with touch-down of 0.5°C per cycle, 72°C for 3 min) x 8 cycles, (94°C for 30 sec, 63°C for 10 sec, 72°C for 3 min) x 25 cycles, and (72°C for 10 min) x 1 cycle. The product of the nested PCR was examined on 1 % agarose/EtBr and excises the band and purified using QIAGEN Gel Extraction Kit. Then purified DNA was sent subjected for sequencing with the T-DNA LB primer. The sequenced data was mapped to the *Arabidopsis* genome at the TAIR blast (http://www.arabidopsis. org/Blast/).

B2.5. RNA Isolation and cDNA Synthesis

Total RNA was extracted using Trizol (Life Technologies) from 100 mg of two weeks old leaves of *Arabidopsis* grown on soil according to the

manufacturers' protocol. The concentration of the total RNA was quantified in spectrophotometer (Smart Spec 3000, *BIO-RAD,* USA) at an absorbance of 260 nm. All the extracted RNA was diluted to 1 µg/µL. cDNA was synthesized using Superscript II reverse transcriptase (Life Technologies). 2µg of total RNA and 1 µL of the oligo dT (500 µg/mL) were mixed in a reaction tube, heated at 70°C for 10 min to inactivate the sample, and then chilled on ice quickly. 5X first strand buffer and 0.1M DTT were added, and the mixed contents of the tubes were gently incubated at 42°C for 2 min. 1 µL (200units) of Superscript II was added to the tube, incubated at 42°C for 50 min, and the reaction stopped by inactivation heating at 70°C for 15 min. The synthesized cDNA was stored at -20°C for further use.

B2.6. RT-PCR Expression Analysis

Semi-quantitative RT-PCR was employed in order to determine the expression of a gene adjacent to T-DNA insertion sites. RT-PCR amplifications were performed in 20 µL reaction volume, with 200ng of cDNA, added to 1x Taq buffer, 0.25mM of dNTPs, 0.5µM of each forward 5'-GAACTCGGCTGTACTATATCCTTGTCCCA-3'and reverse 5'-ATC C ACTGAAGACTAAACACCAGACGCACA-3' primers, and 1 unit of Taq DNA polymerase were used. *GI* gene was amplified with a denaturation of 5 min at 94°C; 30 cycles at 94°C for 30 sec, annealing at 55°C for 30 sec and extension at 72°C for 1min with a final extension of 5 min at 72°C. Actin gene was used as a positive control. PCR products were electrophoresed in a 1% agarose gel containing 0.5 mg L^{-1} ethidium bromide (EtBr) and observed under ultraviolet light.

Results

C2.1. Screening of *abz-126* Mutant in T-DNA tagging Lines

The activation tagging lines were generated by random T-DNA insertion into the Arabidopsis genome (Weigel et al. 2000). Arabidopsis activation tagged seed ecotype Columbia (Col-7) was purchased from Arabidopsis Biological Research Centre (ABRC) and examined co-segregation of the mutant phenotype and the basta resistance marker. To obtain homozygous mutant the resulting F_1 progeny were analyzed on one-half strength MS-1% Sucrose medium (MS-Suc) containing basta (20 mg/litre). Analysis of the basta resistant phenotype segregated with a 3:1 ratio, indicated that there was only one T-DNA insertion at a single locus in the mutant.

Brassinazole (BRZ) is a triazole compound that specifically blocks brassinolide biosynthesis by inhibiting the cytochrome P450 steroid C-22 hydroxylase encoded by the *DWF4* gene, which causes deetiolation and dwarf phenotypes similar to those of BR-deficient mutants (Asami et al. 2000; Asami et al. 2001). Mutants that are insensitive to BRZ and fail to deetiolate on medium containing BRZ, may have reduced BRZ uptake, overproduce BRs, be hypersensitive to BRs, or have constitutive BR responses (Wang, ZY et al. 2002). We screened activation tagged Col-7 seeds and isolated a dominant mutant that had a long hypocotyl when grown on BRZ in dark and named this mutant as *abz126*. The phenotypes of *abz126* mutant plants grown on brz are shown in Figure 2-1. Compared with wild-type seedlings, dark grown *abz126* mutant seedlings had normal hypocotyl length when grown on Brz unsupplemented medium (Fig 2-1A and 1B).

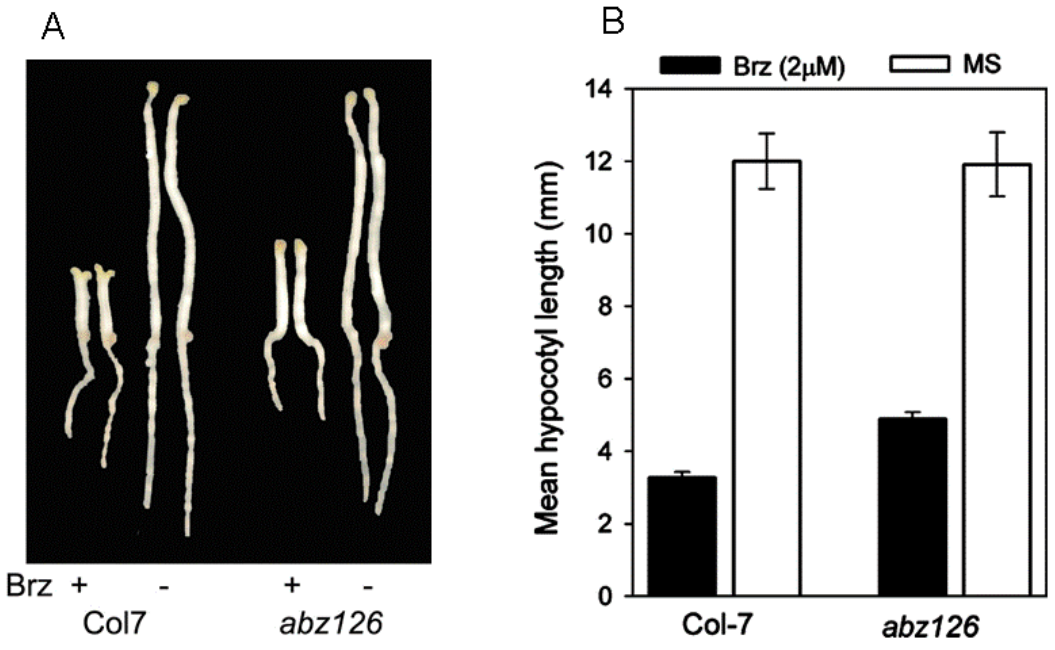

Figure 2-1. Effect of Brz on *abz126* hypocotyl Elongation

Wild-type and *abz126* mutants were germinated and grown on one-half-strength Murashige and Skoog medium containing 2 μM Brz for the 7 days in dark. (**A**) Hypocotyl elongation of *abz126* mutant seedling is partially insensitive to Brz. Data are the means ± SE obtained from 40 seedlings. All the hypocotyls were measured as described in "Materials and Methods". (**B**) Comparison of hypocotyl phenotype of the wild-type and *abz126* mutant grown on half-strength MS media supplemented with 2 μM Brz.

C2.2. Identification of T-DNA insertion Site

Next, we isolated the sequence flanking the T-DNA insertion site by using adopter ligation-based PCR mediated Walker. Approximately a 1.7kb fragment was isolated by using a T-DNA specific primer located near the left border. BLAST searches using the sequence of this fragment showed identity to a region on NCBI gene locus AF076686 that is located on chromosome 1. Sequence analysis indicated that the T-DNA was inserted in the *GIGANTEA* gene that was located at the 9^{th} exon of *GI* gene, 3045 bp far from its putative translation start codon (Figure 2-2A).

C2.3. Expression of *GI* and neighboring Genes

RT-PCR amplification was carried out to investigate the expression of *GIGANTEA* and other neighbor genes on either side of the T-DNA insertion of *abz126* mutant. Both neighbor genes on either sides of the T-DNA insertion were expressed with wild-type, at the left border, adjacent to the enhancers, At1g22760 encoding putative poly A binding protein, and at the right border, At1g22780, coding for putative 40S Ribosomal protein S18 respectively. But T-DNA insertion on At1g22770, encoded GIGANTEA protein made knockout in *abz126* mutant (Figure. 2-2B). Thus, *abz126* mutant phenotype caused by T-DNA insertion on At1g22770 locus made knockout of the GIGANTEA gene. However T-DNA insertion do not changed the expression of neighboring genes.

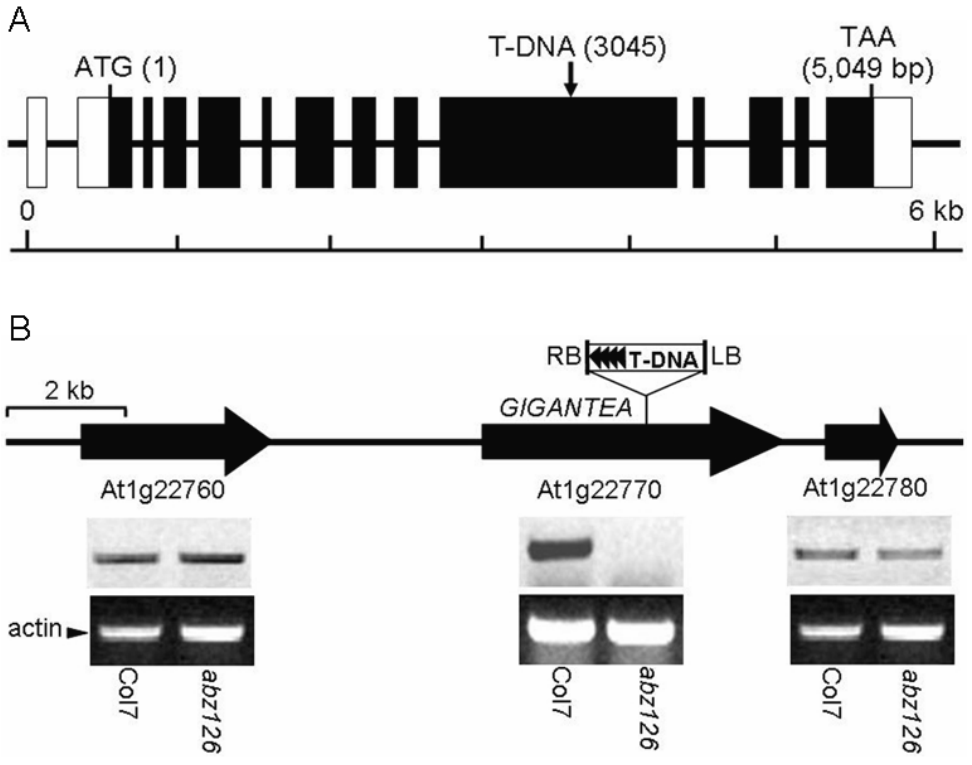

Figure 2-2. Identification of *abz126*

(A) Structure of the *GIGANTEA* gene. The *GIGANTEA* coding sequence (3,455 bp) consists of thirteen exons (black rectangles). White rectangles are untranslated regions. The exons and introns ranged in length from 54 to 1532 and 79 to 302 bp, respectively. Black arrow indicates T-DNA insertion position of *GIGANTEA.*

(B) Three genes surrounding the T-DNA insertion were knockout in the *GIGANTEA.* Arrows indicate the direction of transcription. (*Below*) RT-PCR with *GIGANTEA* and each of its neighbour gene in wild-type Col7 and *abz126*. *Actin* was used as control.

C2.4. *GI* Gene Effects on Plant Size and Flowering Time

To determine whether the expression of *GI* gene is related with plant size and flowering time, we isolated total RNA from the leaves of wild-type (Col-7), and *abz126* mutant and mRNA expression levels were analyzed by RT-PCR. A 3,468 bp size of *GI* gene was expressed in wild-type but completely absent in the *abz126* mutant (Figure 2-3).

Next, we analyzed the functional importance of the *GI* gene on *Arabidopsis* plant phenotype. In the *abz126* mutant had many rosette leaves (under the long day condition), elongated stature and petiole length and delayed flowering compared with wild-type (Figure 2-3, B-E).

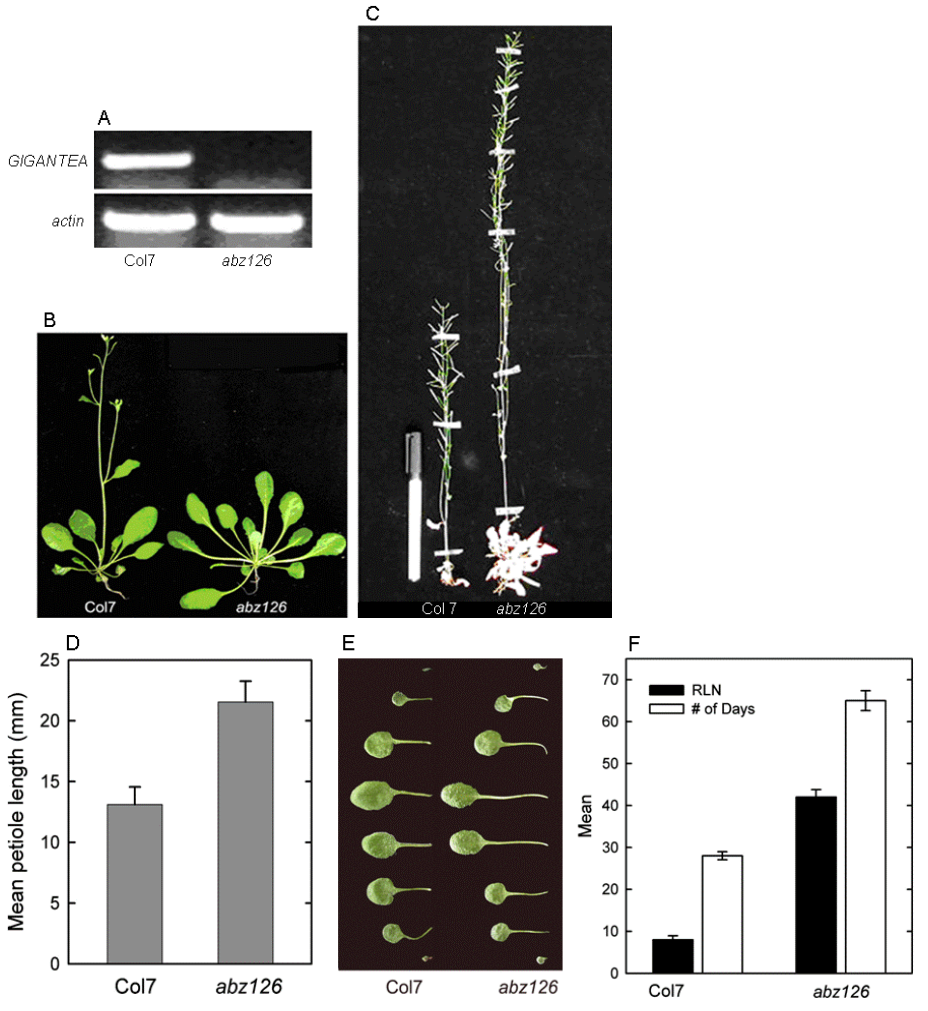

Figure 2-3. RT-PCR and Plant Morphology

All the plants depicted are at the same age. **A,** RT-PCR analysis of wild-type (Col7), *gi1*, *gi2* and *abz126* mutants against *GIGANTEA*. Reducing the expression of *GIGANTEA* gene increased the late flowering phenotype. *Actin* was used as a control; **B,** Flowering time for the wild-type (Col7), *abz126* mutant; **D and E,** mean petiole length of col 7 and *abz126;* **F,** Rosette leaf number (RLN) and days were counted when plants bloted (16h light/8h dark) at 21°C. Datas are expressed as mean ±SE calculated from three independent experiments with at least 15 plants each.

C2.5. Response of Plant Hormones on Exogenous Application

To test the involvement of *abz126* in BR signaling, response of BL in the mutant plant was determined using a hypocotyl elongation assay in dark grown seedlings. We used a BL biosynthesis mutant *det2* as a control. *det2* has shorter hypocotyls than WT in both dark and light. Hypocotyl elongation in BR-deficient mutant *det2* plant was rescued by increasing the concentration of BL in both light and dark conditions (Takahashi et al. 2005). The hypocotyl length of *det2* increased till the concentration of 100nM BL, where as the hypocotyl lengths of *abz126* mutant and wild-type were altered at 10 nM BL concentration (Figure 2-4A). BL-induced hypocotyls growth was not found sensitive in *abz126*, suggested that BL-induced cell elongation is impaired in the mutant. We also observed that the *abz126* mutant was less-sensitive than the wild-type at all the concentrations of BL (Figure 2-4A).

We examined the effect of exogenous 6-benzylaminopurine (BAP) on the elongation of *abz126*, *bzr1* and wild-type seedlings. The elongation of *abz126* hypocotyls were markedly relatively less inhibited than that of wild-type, when increased the concentrations (Figure 2-4B).

We also tested *abz126* mutant under GA deficiency (induced by treatment with the GA biosynthesis inhibitor paclobutrazol [PAC]), exhibited GA-independent phenotype that are distinct from the wild-type, including resistance to the PAC-induced effects of hypocotyls elongation (Figure 2-4C). These results suggest that brassinosteroids signal transduction negatively act for hypocotyls elongation through *GI*.

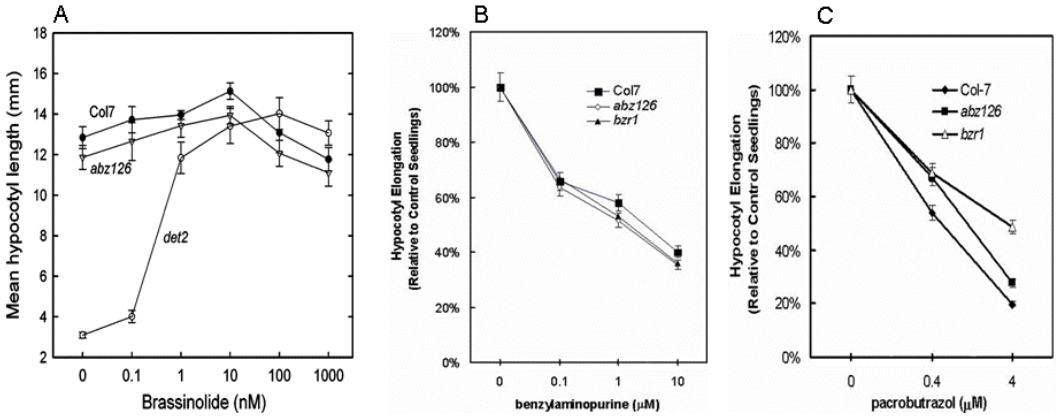

Figure 2-4. Analysis of Hormones Sensitivity on *abz126*.

Seedlings of wild-type (Col7), *det2*, *bzr1* and *abz126* mutants were germinated and grown on half-strength MS medium containing increasing concentrations of Hormones. **(A)** Hypocotyl length of wild-type (Col7), *det2*, *bzr1* and *abz126* mutants in the presence of different concentration of BL in the dark. **(B)** Effects of BAP on hypocotyls elongation in the presence of 0, 0.1, 1 and 10 μM concentration. **(C)** Hypocotyl length of wild-type (Col7), *bzr1* and *abz126* mutants in the presence of 0, 0.4, 1 and 4 μM concentration of PAC. Hypocotyl elongation was measured 7 days after germination at dark. Each data point represents the average hypocotyls elongation of 40 to 50 seedlings of three duplicate experiments. Controls of wild-type and each mutant were grown on medium containing the same volume of 80% (v/v) ethanol used to dilute from a stock solution. Inhibition of hypocotyls elongation by BAP and PAC are expressed relative to the hypocotyls elongation of the same genotype of control. Error bar represents the SE. All the hypocotyls were measured as described in "Materials and Methods".

Discussion

Increasingly, hundreds of genes involved in brassinosteroid (BR) signaling are being linked to physiological and developmental process such as stem elongation, vascular differentiation, seed size, fertility, flowering time, senescence and resistance to biotic and abiotic stresses. Despite the rapid progress in recent years in identifying several BR signaling components and BR-regulated genes, there is no information on how brassinosteroid related genes that regulates the normal flowering process. In this report we described the role of *GIGANTEA* gene that has an unknown function in BR signaling.

Previously it was shown that *GI* transcript is detected throughout development in all parts of mature plant (Fowler et al. 1999). The analysis of *GI* expression may be involved in controlling of photoperiodic responses (Fowler et al. 1999).*GI* was previously shown to be required for inhibition of hypocotyls elongation specifically under red light condition (Huq, Tepperman & Quail 2000). In addition, *GI* gene has been implicated in abiotic stress, because the *GI* gene is upregulated in response to low temperature (Fowler & Thomashow 2002; Cao, Ye & Jiang 2005). Interestingly, *gi* mutants show enhanced tolerance to oxidative stress (Kurepa et al) suggests that *GI* gene mediates in circadian clock, cold stress and oxidative stress tolerances through the different signaling pathways.

Here, we describe a knockout of *GIGANTEA* gene phenotype is involved in brassinosteroid hormone signaling. First we isolated *abz126* from the screening of activation tagged Col-7 seeds in brassinazole. Brassinazole is a BR biosynthesis inhibitor that induced dwarfism in *Arabidopsis*, mutants

that resembled BR biosynthesis mutants that can be rescused by BR (Asami et al. 2000). In the dark brassinazole treated *Arabidopsis* seedling induced retardation of hypocotyls growth. Our feeding experiment on *abz126* mutant demonstrated that hypocotyls elongation in brassinosteroid was as compared to wild-type (Figure 2-4A).

The loss of function of GIGANTEA in *abz126* significantly increase the plant architecture like leaf petiole, no of rosette leaves, plant height and flowering time (Figure 2-3). The phenotypic analysis of the *abz126* mutant demonstrated that the GI gene is could be negatively regulate in divergent set of overall developmental process in plant (Figure 2-3).

The plant steroid hormones brassinosteroids (BRs) are perceived by the cell surface receptor kinase BRI1 (Bishop & Koncz 2002). BRI1 is a leucine-rich-repeat receptor-like kinase (LRR-RLK) located on the cell surface (Li & Chory 1997). BRI1 has an extracellular domain containing 25LRRs, a transmembrane domain, and a cytoplasmic serine/threonine kinase domain (Friedrichsen et al. 2000). When, BRI1 perceives the BR signal through its extracellular domain and initiates a signal transduction cascade through its cytoplasmic kinase activity (He, Z et al. 2000; Wang, ZY et al. 2001). BIN2 encodes a cytoplasmic protein kinase and regulate negatively in BR signaling pathway (Li & Nam 2002). Two nuclear proteins, BZR1 and BES1, were identified as positive regulators of the BR signaling pathway downstream of *bin2*. BZR1 and BES1 are mostly in phosphorylated forms, and BR treatment indicates dephosphorylation and accumulation of the proteins (Wang, ZY et al. 2002; Yin et al. 2002). These studies demonstrate a BR signal transduction pathway leading from cell surface receptors to the nucleus. There could be many genes involved in BRs signal transduction in nucleus. GI is localized to the nuclear

membrane, where the region between the residues 543 and 783 contain four separate cluster of basic amino acid that function as nuclear localization signals (NLSs) (Huq, Tepperman & Quail 2000). Studies of the *abz126* mutant suggested that GIGANTEA is negatively involved in growth promotion but it has positive role for flowering time. The mutation in GIGANTEA causes insensitivity to the BR biosynthetic inhibitor brassinazole, indicating a negative role of GI in BR signaling. *abz126* mutant, when grown on light, displayed constitutive BR response phenotypes including long and bending leaf petioles and tall in plant height. In BL

As a localized in nuclear membrane, GI is likely may regulate negatively in BR signaling pathway. The role(s) of GI gene in growth and development may also involve the identification of the new additional set of BR-responsive gene. Finally, the identification and analysis of GI gene mutation in *abz126* mutant showed that *GI* gene is involved negatively in BR signaling and results in late flowering and increase in number of rosette leaves and plant height provides an excellent elucidate that the same gene could be involved differently.

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Appendix I

Phenotypic character of activation tagged mutant

Mutant line	basta resistant ratio (T_2)	hypocotyl length in brz (T_2)	plant phenotype (T_1)
M3	3:1	like WT	plant ht 2 cm, silique length 2cm, no of rosette leaves ~30, length of leaf lamina 2-3cm, shoot branches 8-10
M4			No infloresence, leaves are backwardly folded, no of rosette leaves 10
M5			plant height 12cm, short petiole, leaf length 2cm, no of rosette leave 14
M6	1:1	like WT	plant height 8cm, short petiole, leangth of leaf 3 cm, rosette leag is looks like GI mutant
M7			plant height 20cm, leaf length cm, petiole is short, leaf emerged horizontally along the soil surface, no branched, no of rosette leaf 14
M8	3:1	like WT	plant height 21cm, rosette leaves 12, no of shoots many, at least 3 infloresence branches per shoot so plant looks bushy, silique many and hanging downward, seed yield is very good, might be useful for crop/legume improvement.
M9	3:1		plant height 38cm (at the time of flower ceasing), rosette leaf number 14, average leaf length 3cm, no of shoot 7, small seized silique, hairy branched infloresence

M10			plant height 6cm, possibly this mutant will be serile
M11			plant height 8cm, leaf length 2.5cm, short petiole, dark geen leaf, no of shoots 5
M12	all died		plant height 27cm, no of rosette leaf 14, lef length 3cm, leaf is elongated along the soil surface, no of shoots 5
M13	3:1	like WT	plant height 40cm, no of rosette leaf 12, leaf length 2.5cm, leaf color is dark green, silique is curved and shorter than WT
M14	3:1	like WT	plant height 26cm, no of rosette leaf 18, leaf length 1.8cm, no of shoot 3, whole plant is albino.
M15	all died		plant height 24cm, leaf length 2.5cm, silique ripening early
M16	3:1	like WT	plant height 39cm, no of rosette leaves 10, leaf length 4cm, dark green leaves, leaf shape is tapering towards the end, basal lamina is serrate, petiole is short,
M17	all survived	like WT	plant height 21cm, no of rosette leaves 12, leaf lamina is flat
M18	3:1	like WT	plant height 32cm, dark green leaf color, leaf length 3 cm, leaf surface is rough, and one side of leaf lamina is zigzagly folded bacawardly, no of shoot 5
M19			tiny plant and sterile
M20			tiny plant and sterile
M21	3:1	like WT	plant height 25cm, leaf size 2cm, no of rosette leaf 14
M22	3:1	like WT	plant height 38cm, no of rosette leaf 14, leaf length 3cm, ovate in shape, no of shoot 5
M23	3:1	like WT	plant height 24cm, no of rosette leaf 10, leaf length 1cm, no of shoot 5
M24	3:1	like WT	plant height 38cm, no of rosette leaf 14, leaf length 3.5cm, no of shoot 5

M25			plant height 9cm, no of rosette leaf 8, leaf length 1cm, infetile
M26			plant height 23cm, no of rosette leaf 4, infertile
M27			plant height 14cm, leaf length 1cm, plant stature like bri1-5, round shape leaf and dark green in color, looks infertile
M28	all died		plant height 22cm, rosette leaves 14, plant color is slightly albino
M29	3:1	like WT	plant height 29cm, no of rosette leaves 14, leaf length 4cm, right side leaf lamina is upwardly folded, stem bending in zigzag shape, length of silique is shorter than WT, no of silique are few but have enough seed
M30			plant height 12cm, thin, unfertile
M31	all survived	like WT	plant height 28cm, leaf size 1.5cm long small, silique early ripening
M32	3:1	like WT	plant height 33cm, leaves are many and shorter than WT, shoot nos are many
M33	3:1	like WT	plant height is 28cm, no of rosette leaves 8, no of shoot 3
M34	3:1	like WT	plant height 35cm, leaf length 2cm, leaf shape is round, ovate and dark green, no of shoot 3 and each shoot is branched into 3-4 infloresences so the plant stature is looks like a bunch
M35	3:1	like WT	plant height 25cm, many cauline leaves, floral buds are originated from each axis of the cauline leaves, leaf length 3cm, long petiole
M36	3:1	like WT	plant height 28cm, rosette leaves are elongated along the soil surface leaf length 3cm, no of shoot 3, each shoot has branch
M37	3:1	like WT	plant height 29cm, leaves are narro and elliptical in shape, no of shoot 10

M38	3:1	like WT	plant height 28cm, leaves are same as WT, stem color purple, silique numbers are many than Wt
M39			phenotype is looks M40, but this is not fertile
M40	3:1	like WT	plant height 18cm, no of rosette leaves 14, leaf length 1.5 cm, siliqie length is about half of the Wt, no of shoots 8
M41	3:1	like WT	plant height 32cm, no of rosette leaves 14, leaf length 2.5cm, leaf size small, ovate and elliptical in shape, stem color purple, no of shoots 6
M42	3:1	like WT	platn height 33cm, no of rosette leaves 10, length of leaf 3cm, leaf shape ovate, stem color purple, no of shoot 5
M43	all survived	like WT	plant height 11cm, no of rosette leaves 8, stem color purple, few silique (9), single shoot
M44	3:1	like WT	plant height 31cm, short petiole, leaf seath is connect until the base of the petiole, leaf length 3cm, distance between the siliques are close
M45	3:1	like WT	plant height 34cm, leaf length 2~3cm, smaller than WT, lamina margin smooth, ovate shape, no of shoot 5
M46	3:1	like WT	plant height 18cm, no of rosette leaves 7, no of shoot 2
M47	3:1	like WT	plant height 32cm, small leaves, no of shoots 5, flowers many but infertile
M49	3:1	like WT	
M51	3:1	like WT	
M52	3:1	like WT	
M54	3:1	like WT	purple stem color and late flowering

M55	3:1	like WT	phenotype looks same as M76
M56	3:1	like WT	plant height 27cm
M59	3:1	like WT	plant height 21cm, short silique but many
M61	3:1	like WT	
M62	3:1	like WT	
M63	3:1	like WT	plant height 43cm, healthy silique and many seeded
M66	3:1	like WT	
M68	3:1	like WT	purple leaf color, short petiole
M69	all died		
M71	3:1	like WT	
M72	1:1	like WT	
M73	all survived	like WT	
M75	3:1	like WT	plant height 36cm, no of silique are many but smaller in size than WT
M76	3:1	like WT	plant height 47cm, dark green leaves, many rosette leaves, stem diameter is bigger than WT
	3:1	like WT	many cauline leaves
M79	3:1	like WT	
M80	3:1	like WT	plant height 28cm

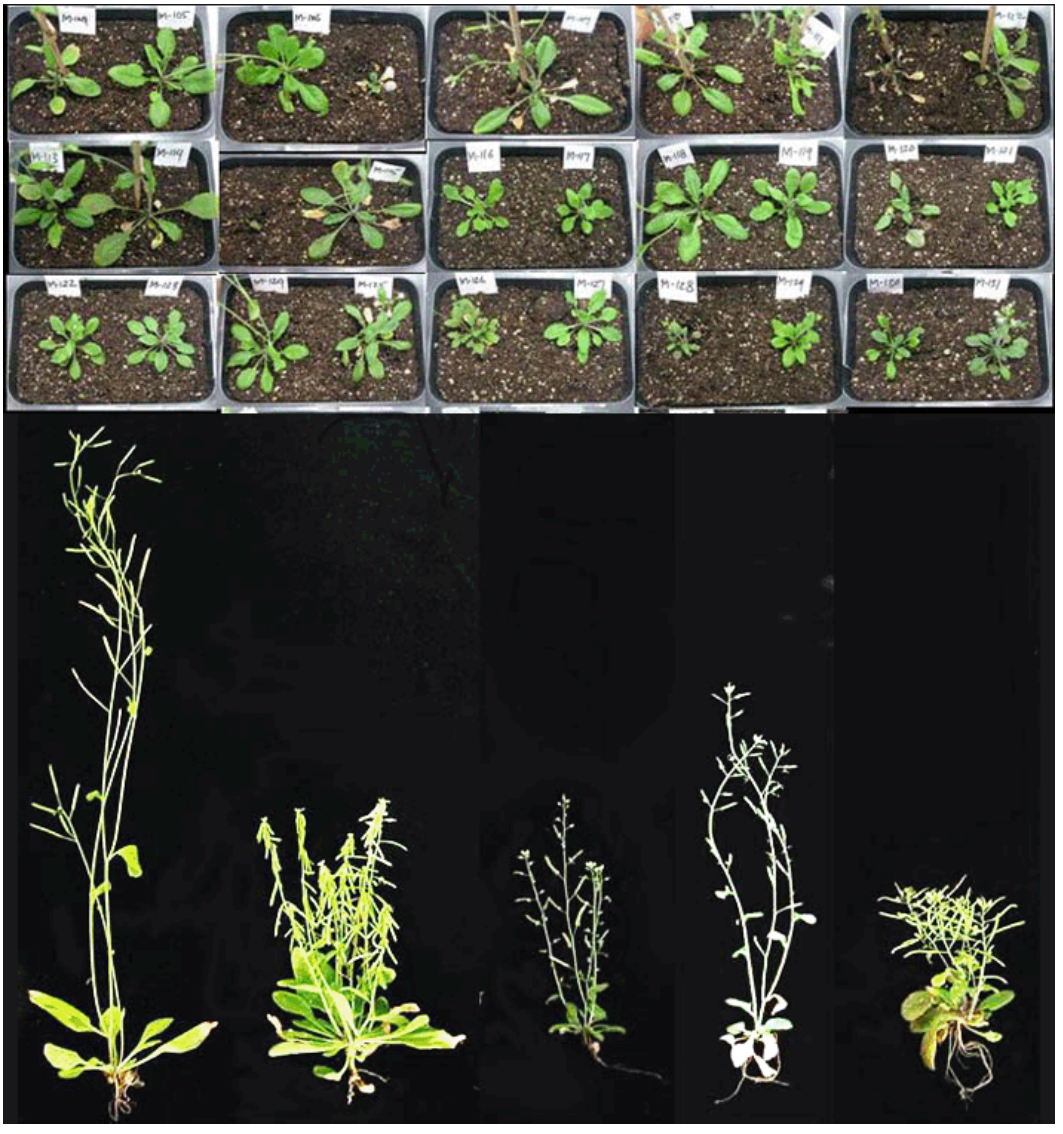
M83	3:1	like WT	plant height 16cm
M84	3:1	like WT	
M86	3:1	like WT	plant height 38cm, cauline leaves are purple in color, few silique are viable
M87	3:1	like WT	many rosette leaves and different is shape each other
M88	3:1	like WT	plant height 38cm
M90	3:1	like WT	
M91	3:1	like WT	plant height 30cm, silique no many but shorter than WT, all siliqua are viable
M92	3:1	like WT	plant height 41cm, small and short silique
M93			plant height 43cm, many rosette leaves
M94			plant height 30cm
M95			plant height 43cm,
M97	3:1	like WT	plant height 30cm
M98	3:1	like WT	
M99	3:1	like WT	
M100	3:1	like WT	
M101	3:1	like WT	
M102	3:1	like WT	plant height 43cm

M103	3:1	like WT	plant height 38cm
M105	all survived	like WT	plant height 36cm
M106	4:1	like WT	
M107	1:3	like WT	
M108	3:1	like WT	plant height 22cm
M109	3:1	like WT	
M110	3:1	like WT	
M112	3:1	like WT	
M113	3:1	like WT	
M114	3:1	like WT	
M115	all died		
M117	3:1	like WT	
M118	all survived	like WT	
M119	3:1	like WT	
M121	3:1	like WT	plant height 38cm
M122	3:1	like WT	Mutant 122 to 133 was changed phenotypes to WT in T2 generation, therefore no described in here
M133	3:1	like WT	

M134	all survived	like WT	
M135	3:1	like WT	
M136	3:1	like WT	plant height 15cm
M137	all survived		
M138	Contaminated		
M139			plant height 18 cm, infooresence color purple
M140			plant height 34cm
M143			plant height 44cm
M144			many leaves
M145			plant height 48cm
M146			plant height 42cm
M147			small and thin and tiny plant
M149			plant height 40cm, small sized silique but many numbers
M150			survived in extream drought

Appendix II

Mutants phenotype in T1 screening



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2008.08.25, Gwangju

Giri Raj Tripathi

Dedication

I would like to dedicate this work to my nearest and dearest brother Gyanendra Tripathi who is a National Hero and fighter for the Democratic-Republic movement of Nepal. He was made disappeared by the ex-Kings' regime since 2003 for timidity from the people's power. His situation is still unknown; however his movement is succeed and recently established a Democratic-Republic of Nepal.

Similarly this work is also dedicated to my parents Dol Nath Tripathi and Bhagawati Tripathi, they've supported me continuously through my entire life. I can't express how much this has meant for me. I would like to express my gratitude to my wife Malika Tripathi, son Santanu Tripathi, sister in law Sharmila Tripathi and niece Chunauti Tripathi. I did it for you.

I love all of you.

저작물 이용 허락서

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논문제목	한글 : 활성표지 애기장대에서 브라시노스테로이드 신호 돌연변이체의 분자유전학적 연구 영어 : Isolation and Molecular Genetic Analysis of Brassinosteroid Signaling Mutants in *Arabidopsis*				

본인이 저작한 위의 저작물에 대하여 다음과 같은 조건아래 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 편집·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 또는 출판을 허락을 하였을 경우에는 1개월 이내에 대학에 이를 통보함.
6. 조선대학교는 저작물의 이용허락 이후 해당 저작물로 인하여 발생하는 타인에 의한 권리 침해에 대하여 일체의 법적 책임을 지지 않음
7. 소속대학의 협정기관에 저작물의 제공 및 인터넷 등 정보통신망을 이용한 저작물의 전송·출력을 허락함.

동의여부 : 동의(O) 반대( )

2008년 8 월 25 일

저작자 : Giri Raj Tripathi 서명 또는 인) Giri Raj Tripathi

조선대학교 총장 귀하