

行政院國家科學委員會專題研究計畫 成果報告

利用阿拉伯芥 T-DNA Knockout 突變株，研究調控葉部形態發育之功能性基因(3/3)

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Abstract

Leaves are the most noticeable plant organs and play important roles in photosynthesis, respiration and gas exchange. However, the details of leaf development remain unclear. Therefore, our laboratory used forward genetics to identify the mutants with leaf development defect from T-DNA tagging pools in *Arabidopsis*. We use a T-DNA tagging vector, pPZP202: BAR: SK, to generate mutants and to screen for morphological mutants. In this study, we have identified a mutant, R1-2, which were selected in the T2 generation, with curled leaves and delayed flowering phenotypes. After R1-2 was crossed to the WT, none of the R1-2 phenotypes was observed in the F1 but were found in the F2 population. Therefore, it was suggested that R1-2 was a recessive mutant. Southern blotting analysis and inversed PCR identified two T-DNA linked together and inserted in the 2.5kb upstream of the *At3g22970*. Using RT-PCR and Northern blotting assay, we showed that the transcripts of *At3g22970* were down-regulated in mutant, implicating that the phenotypes of R1-2 may be resulted from down-regulation of *At3g22970*.

At3g22970 was predicted as a protein containing DUF506 domain and located in the chloroplast. There are 14 genes that contain DUF506 domain in *Arabidopsis thaliana* and all with unknown function. Using RT-PCR and Northern blotting assay, it was detected that the expression of *At3g22970* appeared rhythmic, and expressed in the whole plant, especially in flowers. However, the function of *At3g22970* is still unknown. We observe the phenotype of the transgenic plants by over-expressing or knock-outing the *At3g22970* to address its function in *Arabidopsis*. Among these over-expression lines, some lines with delayed flowering phenotype had reduced expression of *At3g22970*, but others were the same as WT. The correlation between delayed flowering time phenotype and reduced

expression of *At3g22970*, indicating that the phenotype was caused by co-suppression of the over-expressed *At3g22970*. Additionally, one of the SALK knock-out lines, SALK-085910, also exhibited a delayed flowering phenotype. Therefore, *At3g22970* might play a role to control flowering time.

Introduction

Leaf development

Leaves are the most noticeable plant organs. The early events in leaf development have been divided into three stages, i.e. organogenesis (leaf initiation), setup of the morphological domains of the leaf, and completion of the tissue differentiation. At the organogenesis stage, stage 1, cells on the flank of the shoot apical meristem are set aside as the founder cells of the initiating leaf. The region that increased cell division rates characterize will give rise to the leaf primordium. Stage 2, delimits the basic morphological domains for the growth and development of leaf parts. Cell and tissue differentiation occurs during the final stage in stage 3, which leaf development by coordinated processes of cell division, expansion, and differentiation (Sylvester et al., 1996).

The details of leaf development remain unclear. However, many mutants are isolated with alterations in the leaf morphology, and it can be accumulated information about the role of individual genes in the control of leaf development.

SAM and organogenesis: leaf initiation

The shoot apical meristem (SAM) is a population of cells located at the tip of the shoot axis and self-renewing stem cell populations that generate all of the above-ground organs of higher plants. In *Arabidopsis*, the SAM is divided into three clonally distinct layers. Cells in the outermost layer (L1) produce epidermal tissues, whereas cells of the sub-epidermal layer (L2) and the internal layers (L3) differentiate into vascular and internal tissues, respectively. Superimposed across these cell layers are distinct zones of differential

meristem activity. A central zone (CZ) at the very apex harbors the unspecialized stem cells, which maintain themselves and also replenish cells in the adjacent peripheral zone (PZ) as they are lost during the formation of lateral organ primordia on the meristem flanks.

Meanwhile, maintenance of a functional meristem between stem cell divisions and cell differentiation requires many genes to regulate. In *Arabidopsis*, two genes have been implicated in the maintenance of undifferentiated cells in the meristem: *SHOOT MERISTEMLESS* (*STM*) and *WUSCHEL* (*WUS*), both of which encode homeodomain proteins (Long et al., 1996; Mayer et al., 1998). In a strong *stm* mutant, the meristem was absent at the end of embryogenesis (Barton and Poethig, 1993), however, in the *wus* mutant, the meristem was not established during embryogenesis.

On the other hand, recent reports had shown that keeping the correct number of stem cells is achieved by a negative feedback regulation of WUS-CLV pathway in *Arabidopsis*, i.e., the *WUS* mediated signal from the organizing center (OC) that specifies stem cell identity in the outermost layers, which signal back via the CLV pathway to limit the size of the *WUS*-expressing OC (Fletcher et al., 1999; Schoof et al. 2000). Therefore, *clv* mutants that failed to repress *WUS* activity and caused an increase in the size of both shoot and floral meristems (Clark et al., 1993; Clark et al., 1995; Laufs et al., 1998).

CLV and *WUS* genes are necessary for regulating meristem size, but class-1 *KNOTTED*-like homeobox (*KNOX*) genes, such as *SHOOTMERISTEMLESS* (*STM*), *BREVIPEDICELLUS* (*BP*, formerly *KNAT1*), *KNAT2*, and *KNAT6* genes, are required for the maintenance of meristem identity and be down-regulated at the position of the next primordium (Vollbrecht et al., 1991; Long et al., 1996; Byrne et al., 2002). Mutations at the Class I *KNOX* gene *STM1* led to absence of shoot meristems in *Arabidopsis*

(Barton and Poethig, 1993).

Establishment of leaf polarity

Plant lateral organs exhibit central-peripheral axis, proximal-distal, and adaxial-abaxial polarity. The central-peripheral axis extends from the apical-basal axis at the center of the organ outward to the organ epidermis. The proximal-distal axis runs from the base of the organ to the tip. The adaxial-abaxial axis runs from the surface of the lateral organ closest to the meristem to the surface of the organ furthest from the meristem.

Genetic studies in *Arabidopsis* have identified several families of genes that play a role in promoting proper adaxial-abaxial development. The class III HD-Zip genes, in particular of the *PHABULOSA* (*PHB*), *PHAVOLUTA* (*PHV*), and *REVOLUTA* (*REV*) genes promote adaxial identity and meristem maintenance (McConnell and Barton, 1998; McConnell et al., 2001; Emery et al., 2003). The dominant gain-of-function mutants had strongly adaxialized organs and formed trumpet-shaped leaves (McConnell and Barton, 1998). In contrast, mutations in all three genes results in radial abaxial leaves, a phenotype that is complementary to the dominant mutant phenotypes (Emery et al., 2003). The other two family members, *ATHB8* and *ATHB15*, are also likely to direct vascular development, although their precise roles are yet to be uncovered (Baima et al., 2001; Ohashi-Ito and Fukuda, 2003).

The members of the *YABBY* gene family and *KANADI1-3* (*KAN1-3*) genes promote abaxial identity (Eshed et al., 2001; Sawa et al., 1999; Siegfried et al., 1999). The *YABBY* genes appear to be plant specific and at least with six family members, including *FILAMENTOUS FLOWER* (*FIL*), *CRABS CLAW* (*CRC*), and *INNER NO OUTER*, found in *Arabidopsis*. The *YABBY* genes encode a zinc finger and a helix-loop-helix motif and are predicted to be

transcriptional regulators (Siegfried et al., 1999). The *fil yab3* double mutants exhibited partial loss of abaxial characteristics (Siegfried et al., 1999; Kumaran et al., 2002). *KAN* gene encodes a protein that includes a conserved GARP domain, which binds to a specific region of DNA (Kerstetter et al., 2001). The leaves of *kan1 kan2* mutants were partially adaxialised, and *kan1 kan2 kan3* triple mutants displayed more complete adaxialisation. Genetic studies support that the roles of *KAN* genes in abaxial specification and *YABBY* genes in both abaxial fate and lamina outgrowth (Eshed et al., 2004).

The *AS1/AS2* and *YABBY* genes can repress expression of class I *KNOX* homeobox genes was reported. *AS2* (*ASYMMETRIC LEAVES2*, a *LOB*-domain family transcription factor) is predicted to be expressed in the adaxial organ domain where *AS2* forms a complex with *AS1* (*ASYMMETRIC LEAVES1*, a MYB family transcription factor), to promote repression of *KANADI* and differentiation of adaxial cell fates (Lin et al., 2003). Therefore, overexpression of *AS2* results in adaxial-abaxial polarity defects, and implicates that *AS2* also play a role in polarity establishment.

Cell division and cell expansion

Organ shape is the result of a coordination of cell division and cell expansion. For example, the *cincinnata* (*cin*) mutant in *Antirrhinum* reflected a change in the shape and the progression of a cell-cycle arrest front moving from the leaf tip toward the base. *CIN* encodes a TCP protein and is expressed downstream of the arrest front. (Nath et al., 2003). In contrast, the *struwwelpeter* (*swp*) mutant had fewer cells number but larger cell size than WT leaves and did not alter the final size of the organs in *Arabidopsis*. It has been proposed that organ size might be controlled by a compensation to achieve a more normal morphology between the amount of cells and cell size

(Autran et al., 2002).

Leaf expansion is dependent on both the division and the elongation of cells. The *angustifolia* (*an*), a mutant with narrower and thicker leaves than the WT, had altered cortical microtubule arrangements and reduced polar cell expansion in the leaf width dimension in *Arabidopsis* (Tsuge et al., 1996). *AN* encodes a CtBP/BARS-related (carboxy-terminal binding protein/brefeldin A ADP-ribosylated substrates-related) protein (Folkers et al., 2002; Kim et al., 2002) and is thought to be a key gene in regulation of the polar elongation of leaf cells in the leaf-width direction specifically (Tsuge et al., 1996). By contrast, the *rotundifolia3* (*rot3*) mutant had shorter and wider leaves, because of defecting in a novel cytochrome P450 gene (Kim et al., 1999). The *ROT3* gene appears to be the key gene that regulates the elongation of leaf cells in the leaf-length direction. In the *an rot3-1* double mutant had an additive phenotype, suggesting that the two genes act independently (Tsuge et al., 1996).

Hormone and leaf development

Although the actions of plant hormones are not specific to leaves, it is also important to regulate of leaf shape. Hormones like gibberellic acid (GA), auxin and brassinosteroid (BR) appear to be involved in leaf development.

When ectopically expressed, rice and tobacco homeobox genes cause decreased GA levels (Kusaba et al., 1998; Sakamoto et al., 2001). The decreased levels of GA correlate with the severity of leaf shape and plant stature, and the application of exogenous GA could rescue these phenotypes (Kusaba et al., 1998; Hay et al., 2002).

Mutations in the polar auxin transport (PAT) efflux carrier *PIN-FORMED1* (*PIN1*) and treatment of *Arabidopsis* or tomato with PAT inhibitors both led to a failure to initiate leaves. This phenotype implicates auxin transport in leaf

initiation and possibly as a negative regulator of *KNOX* gene expression in meristems (Abe et al., 2003).

The phenotype of the mutants that were related to the actions of brassinosteroid (BR) had smaller leaves than the WT in *Arabidopsis*. It is believed that the main role of BR is to stimulate the elongation of cells (Altmann et al., 1998).

miRNA and leaf development

MicroRNAs (miRNAs) are important controllers of development in plants. miRNAs are ~22-nucleotide regulatory RNAs that derive from stem-loop regions of endogenous precursor transcripts (Ambros, 2004; Bartel, 2004). Many miRNAs are known to pair to the messages of protein-coding genes to target these mRNAs for posttranscriptional repression.

For example, two miRNAs from *Arabidopsis*, miR165 and miR166, match *PHB*, *PHV* and *REV* mRNA, and direct their cleavage (Emery et al, 2003). Dominant mutations in *PHB/PHV/REV* genes all disrupted the miRNA-binding site and transcripts of the dominant mutants could not be cleaved in vitro (McConnell and Barton, 1998; McConnell et al., 2001). Mutations in *ARGONAUTE1* (*AGO1*), a key component in RNA interference (RNAi)-mediated posttranscriptional gene regulation, result in adaxialisation of the leaf. The adaxial gene *PHB* and its miRNA regulator were misregulated in *ago1* mutants, demonstrating regulation of the miR165, and consequently the corresponding target, by *AGO1* (Kidner and Martienssen, 2004).

A class of TCP genes is also shown to be regulated by *MiR-jaw*. *jaw-D* dominant mutants caused overexpression of *MiR-jaw*, which targets miRNA-binding sites in the TCP transcripts for degradation, resulting in an uneven leaf surface. Therefore, miRNA-dependent regulation of TCP gene

expression may modulate the spatiotemporal characteristics of cell division arrest during leaf development. This regulation is likely to play an important role in determining final leaf shape in *Arabidopsis* (Palatnik et al., 2003).

Other factors that affect Leaf development

There are many factors that affect leaf development, such as light, water and temperature. Heterophylly is attributable to changes in leaf index because of the environmental adaptation. For example, plants that thrive at the waterside often exhibit heterophylly. Round-shaped and thick leaves with stomata are observed in dry upland conditions, while thin and elongated leaves with no or few stomata are formed under submerged conditions. Such dimorphic changes of leaves are interpreted as a kind of adaptive strategy of these aquatic and semi-aquatic plants to environmental changes, since they have to withstand two different environments of terrestrial conditions and submergence (Kuwabara, 2003).

The existence of two or more leaf types on the same plant is called heteroblastic (Sculthorpe, 1967). For example, *Arabidopsis* exhibits typical heteroblasty and leaf shape changes from the juvenile, through the early adult, to the late adult phase (Tsuge et al., 1996), with concomitant changes in the patterns of distribution of trichomes on the leaf surfaces (Chien et al., 1996; Telfer et al., 1997). The cauline leaves lack petioles (Tsukaya, 1995; Tsuge et al., 1996). The contours of the first and second true leaf resemble those of cotyledons but the leaves have trichomes and a complex vascular system. Thus, the cotyledons might be considered to be as a particular type of foliage leaf and it might be appropriate to examine the cotyledon in the context of heteroblasty.

Transition from vegetative to reproductive phase

Floral initiation is a major developmental transition from vegetative to reproductive phase in plants and it is also regulated by various environmental and physiological cues. However, recent studies have revealed that misexpression of the *PI*, *AP3*, *AG*, and *SEP3* genes, caused the curled leaf phenotype was shown in the *tfl2* mutants (Kotake et al., 2003). The *curly leaf* (*clf*) mutant also shows a curled leaf phenotype when ectopic expression of the *AG* and *AP3* was observed (Goodrich et al. 1997). Therefore, ectopic expression of these genes in the leaves would disrupt the leaf development.

Models of genetic control of flowering time in *Arabidopsis* reveal four independent floral promotion pathways: photoperiod, autonomous, gibberellin dependent, and vernalization (Koornneef et al., 1998; Simpson et al., 1999).

(1) Photoperiod

Flowering of *Arabidopsis* is regulated by daylength. Flowering occurs much earlier under long days of 16 hours light than under short days of 8 hours. *CRY2* and *PHYA*, candidates for photoreceptors that perceive the photoperiod under long days, entrain the circadian clock to oscillate within a period of 24 h (Mockler et al., 2003). Mutations affecting the photoperiod pathway delay flowering under long-day conditions, but are identical to WT under short-day conditions. For example, *CONSTANS* (*CO*) is a key player in the regulation of flowering by photoperiod. WT *Arabidopsis* plants flower earlier in long days than in short days, whereas *co* mutants flower late in both short and long days in *Arabidopsis* (Putterill et al., 1995).

(2) Autonomous

Mutations that affect the autonomous pathway delay flowering under all photoperiods. The autonomous pathway is defined by one group of late flowering mutants, such as *fca*, *fpa*, *fve*, *fld*, *ld*, and *fy*, which flower late under

both long-day and short-day conditions. However, this lateness can be suppressed by either a vernalization treatment or by growth in low red/far red light conditions (Koornneef et al., 1994). The characteristic feature of these mutants is that they all contain much higher levels of *FLC* transcript than the wild-type plant or late-flowering mutants associated with the photoperiod flowering pathway or the GA pathway (Sheldon et al., 2000).

(3) Gibberellin (GA) dependent

Mutations that strongly reduce GA biosynthesis delay flowering under long-days and almost abolish flowering under short-days. The GA pathway promotes flowering in SDs via a GAMYB-dependent (primarily MYB33) activation of *LFY* (Blázquez et al., 1998, 2001; Gocal et al., 2001). GA promotes flowering and is absolutely required for flowering in non-inductive short days. Mutations in genes involved in GA biosynthesis and signaling result in delayed flowering. A growing number of genes associated with this pathway have been identified, including *SPY*, *PHOR1*, *RGLs*, *RGA*, *GAI*, *GA4*, *GA5*, and *FPF1* (Mouradov et al., 2002; Olszewski et al., 2002).

(4) Vernalization

An exposure to cold that stimulates flowering is called vernalization. The vernalization pathway promotes flowering in response to low temperature. Many naturally occurring mutants flower very late but flower early if exposed to low temperatures. The vernalization response is mediated by dominant alleles of two genes, *FRI* and *FLC*, through reducing the expression of *FLC* (Michaels et al., 2001). Other genes involved in vernalization response include *VRN1* and *VRN2* (Gendall et al., 2001), *HOS1* (Lee et al., 2001) and *VIP1-7* (Zhang et al., 2002).

Functional study of T-DNA insertional mutants in *Arabidopsis*

Arabidopsis thaliana that belongs to the Brassicaceae family is widely used as a model organism in plant biology. Although not of major agronomic significance, *Arabidopsis* offers important advantages for basic research in genetics and molecular biology: a small genome size, a rapid life cycle, and easy cultivation in restricted space, prolific seed production, and a large number of mutant stocks that are available for researchers from a variety of stock centers.

In fact, the identification of genes that act as developmental controls in leaf ontogeny is problematic for two reasons. On one hand, the inexistence of analogous phenomena in animals forces us to discard strategies based on gene cloning by homology. On the other hand, two important processes that take place in the leaf, photosynthesis and the exchange of gases with the environment both require the participation of a large number of gene products, primarily expressing in leaves. Therefore, the better experimental approach is forward genetics to screen the mutants that affect leaf morphogenesis. Forward genetics is slow and laborious, but it is also effective and guaranteed to provide the result that is “gene and its function”.

The T-DNA can be considered a type of transposon because it moves from the Ti plasmid to the plant DNA. T-DNA tagging is a method to generate mutations in plants by random insertion of a T-DNA, which can cause knock-out, down-regulation, and over-expression of flanking plant genes. In this study, we use a T-DNA tagging vector, pZP202: BAR: SK, to generate mutants and screened for morphological mutants. We screened a R1-2 mutant with conspicuously altered phenotypes at leaves and delayed flowering from the T2 T-DNA tagging pool. Gene rescue results showed that the T-DNA inserted in the upstream region of *At3g22970*, which was annotated to encode an expressed protein, containing DUF506 domain. Besides, the transcripts of

At3g22970 were down-regulation in R1-2. Therefore, we want to find the relation between the phenotype of the mutant and *At3g22970*. Using Northern blotting, it was shown that *At3g22970* transcript levels are controlled by the circadian clock. In the overexpression lines, with delayed flowering phenotype, the transcripts were reduced compared with the WT. It was indicated that *At3g22970* might play a role to affect the flowering time in *Arabidopsis*.

Materials and Methods

Materials:

Arabidopsis thaliana ecotype Columbia was used for all experiments, and was grown in an environmental growth chamber under a photoperiod regime of 16h light/8h dark or continuous light. *Escherichia coli* DH5 α and *Agrobacterium tumefaciens* strain GV3101 were used in this research.

Methods:

T-DNA tagging screening

The T-DNA tagging lines were generated in a Columbia background with the construct of pPZP202:SK:BAR via floral dipping *Agrobacterium*-mediated transformation procedure (Clough and Bent, 1998). For mutant screening, a T1 and T2 seeds were incubated at 4°C for 4 days before being sown on the water-soaked soil then transferred to the growth chamber. Herbicide BASTA (Glufosinate ammonium 0.25 g/L) was used to screen the T-DNA tagging lines. We screened for mutants displaying abnormal leaf phenotypes.

Southern blotting analysis

Genomic DNA was isolated from rosette leaves according to Dellaporta (1993). DNA was digested with restriction enzymes, separated on a 0.8% (w/v) agarose gel, transferred to a nylon membrane (Immobilon-Ny⁺, Millipore) and hybridized at 65°C with ³²P -radiolabeled BAR or SK fragment as probe from the pPZP202:SK:BAR T-DNA tagging construct. The membrane was washed and analyzed with the phosphorimager.

Total RNA isolation and Northern blotting analysis

Total RNA was isolated from the fully expanded leaves using REzol reagent (PROtech Technologies) as described by the manufacturer. 5-10 µg of RNA was electrophoresed on a 1.0% formaldehyde agarose gel and then transferred to a nylon membrane (Immobilon-Ny+) hybridized at 65°C with gene specific ³²P-radiolabeled cDNA fragment as probe. The membrane was washed and analyzed with the phosphoimager.

IPCR (inverse PCR) identification of the T-DNA flanking sequence

For 1 µg genomic DNA was digested with *Hind*III or *Xba*I and purified. 100 ng DNA was used for ligation in a 100 µL reaction at 16°C overnight. 1 µL of the ligation mixture was used for IPCR. The IPCR consisted of 1X PCR buffer, 0.2 mM each dNTP, 0.5 µM each primer, 2.5 units Taq Polymerase. Thermal cycling conditions consisted of an initial denaturation at 95°C for 5 min followed by 30 to 40 cycles of 94°C for 15 sec, annealing for 30 sec, and 72°C for 2.5 min. The PCR products were subjected to nested PCR for increasing the specificity. Primer sets used for IPCR are shown as following:

IPCR	Primer sequence	Annealing temperature
1 st	35s-pro-out-0 TGCCCTTTGGTCTTCTGAGACTGTATCT	60°C
	Bar-fw-1 GGGAAGTGGCATGACGTGGGTTTCT	
2 nd	35s-pro-out-1 GTGTCGTGCTCCACCATGTTGACCTGCA	60°C
	35s-ter-out-1 CCCAAGAATATCCCAAAGCGAGTACACAAC	
3 rd	LB-0 CCGCACCGATCGCCCTTCCCAACAG	60°C
	35s-ter-out-1 CCCAAGAATATCCCAAAGCGAGTACACAAC	
4 th	LB-1 ATTCAGTACATTAAAAACGTCCGCAAT	60°C
	35s-ter-out-1 CCCAAGAATATCCCAAAGCGAGTACACAAC	

RT-PCR (reverse transcription-PCR)

The cDNA synthesis was performed with 1 µg of total RNA using Ready-to-go RT-PCR kit (Amersham international, Buckinghamshire, England, UK) combined with the oligo dT primer (18 mer). For the 50 µL RT reaction, 1 µL of the reaction was used for further PCR. PCR consisted of 1X PCR buffer, 0.2 mM each dNTP, 0.5µM each primer, 2.5 unites Taq Polymerase. Thermal cycling conditions consisted of an initial denaturation at 94°C for 3 min followed by 24 to 35 cycles of 94°C for 15 sec, annealing for 20 sec, and 72°C for 40 sec. Primer sets used for the RT-PCR are shown as following:

Genes	Primer sequence	Annealing temperature	Product size
<i>UBQ10</i>	Fw GATCTTTGCCGGAACAATT GGAGGA TGGT	60°C	489bp
	Rv CGACTTGTCATTAGAAAGAAA GAGATAACAGG		
<i>At3g22970</i>	Fw ATGCCATTTACGATGAAGATCC AA	60 °C	506bp
	Rv TTCATATCGTCTTTTCGTTTCA CC		

For RNA loading control, the exponential range of amplification was determined according to the 24 PCR cycles used for *UBQ10*, and 28 cycles used for *At3g22970*. 5 out of 25 µL PCR products were analyzed via electrophoresis.

Plasmid constructs

The *35SDE::At3g22970* construction:

The *At3g22970* genomic DNA was amplified from the start (ATG) to the

stop codon (TAA) by primers:

Primer sequence	Annealing temperature	Product size
Fw ATGCCATTTACGATGAAGATCCAA	60 °C	1296bp
Rv GGTACCTTAGGGCTTTTCCTTAAAGAGTGAA		

The PCR amplified fragment was cloned into yT&A vector for sequencing then digested with *KpnI* then subcloned into the binary vector pBIB-HYG (Becker, 1990). The expression of *At3g22970* was driven by dual enhancers of the *CaMV 35S* promoter.

35SDE:: *At3g22970*-GFP construct

The *At3g22970* cDNA was cloned by RT-PCR using primers are shown as following:

Primer sequence	Annealing temperature	Product size
Fw TCTAGAATGCCATTTACGATGAAGATCCAA	60°C	1111 bp
Rv GGATCCAGGGCTTTTCCTTAAAGAGTGAAG		

The RT-PCR cDNA was cloned into yT&A vector for sequencing and digested with *XbaI* and *BamHI* to subclone into the 326-GFP vector (acquired from Prof. In-Whan Hwang at Pohang University of Science and Technology).

Plant transformation

Plasmids for plant transformation were transformed into *Agrobacterium tumefaciens* GV3101 by electroporation. *Agrobacterium* cells containing each plasmid were transformed into the *Arabidopsis thaliana* ecotype Columbia by the floral dipping method (Clough and Bent, 1998). Transgenic plants were selected by spraying seedlings at 7-, 9-, and 11-d after germination with a

solution of 0.4% BASTA herbicide (McDowell et al., 1998) or were selected by half-strength MS medium (Murashige and Skoog, 1962) containing 25 µg mL⁻¹ hygromycin.

Transient expression into tobacco protoplasts

Transformation of protoplasts with GFP constructs was performed using optimized electroporation parameters. The DNA for transfection was prepared with the PurelinkTM HiPure Plasmid DNA Purification kit (Invitrogen). Protoplasts were transiently transfected with plasmid DNA. Protoplasts were prepared from leaves of tobacco.

The subcellular localization was analyzed using a Leica confocal laser scanning microscope (Leica TCS SP2, Heidelberg, Germany) equipped with the Ar lasers for GFP excitation and the primary beam splitting mirror 488/543/633 was used. The emitted light was detected as following: GFP, 505-520 nm; chlorophyll 650-700nm. Images were processed using Leica confocal software.

Results

1. Characterization of the R1-2 mutant

In a large-scale screening for *Arabidopsis* mutants displaying abnormally shaped leaves, a mutant named “R1-2”, which with curled leaves and delayed flowering phenotypes was obtained (Fig.1B-E). Compared with the wild type (WT), the floral organs of R1-2 were normal in shape but some with shorter stamens (Fig. 2A-C), with fewer seeds in siliques (Fig. 2E), and with reduced germination rate (64%) (Fig. 2F).

Under a long-day (LD, 16h light/8h dark) condition, the vegetative growth of R1-2 mutant was severely retarded, it took about 35 days to flowering compared with a 25 days needed for the WT. Consequently, plant size of the mutant was smaller at the early growth stage, but became comparable to that of the WT reaching/during to the flowering time (Fig.1C, D).

R1-2 showed a more obvious curled leaf phenotype under a continuous light than in a LD growth condition. During the continuous light, the rosette and cauline leaves displayed a more enhanced leaf margin serration (Fig.1D and Fig.2D).

To analyze the inheritance of the R1-2 mutant phenotype, R1-2 was crossed to the WT. None of the R1-2 phenotypes was observed in the F1 but were found in the F2 population (Fig.1C- E). Among thirty-six of the F2 seedlings, there were thirteen seedlings selected by herbicide BASTA. Twenty-three seedlings could survive after BASTA treatment, but only four seedlings showed R1-2 mutant phenotypes (homozygote). Therefore, it was suggested R1-2 was a recessive mutant.

2. Characterization and Isolation of the flanking sequence of T-DNA insertion by using Southern blotting and IPCR

Different restriction enzymes (ex., *EcoRI*, *HindIII*, *Clal*, *XbaI*, *XhoI*, and *BamHI*) were used for R1-2 genomic DNA digestion and Southern blotting analysis using the BAR or SK fragments of T-DNA as a probe (Fig. 3A). The Southern blotting results was shown that when probe with the BAR indicated there were two T-DNA insertions (Fig. 3B). However, using SK+ fragment (3 kb) as a probe, only one 6-kb hybridization signal was detected by *EcoRI*, *HindIII* and *XbaI* digestion (Fig. 3C). Therefore, the result revealed the two T-DNA was resulted in a right border (RB) to RB-linkage T-DNA. According to the results, we proposed that the model of two T-DNA inserted at same location in the R1-2 mutant (Fig. 3D).

Southern results show that the *HindIII* and *XbaI* digestion with a smaller hybridization signals and these T-DNA flanking sequence could be rescued by inverse PCR (IPCR) (Fig. 4). When to sequence the 4th-nested IPCR/PCR product and analyzed by BLAST program of TAIR (<http://www.arabidopsis.org/Blast/index.jsp>), showed that the T-DNA was tagged in a 2.5-Kb upstream of the *At3g22970* gene (Fig. 5A).

3. Molecular characterization of the *At3g22970* gene and DUF506 gene family

The T-DNA insertion site apart from the *At3g22960* and *At3g22980* gene were 7.5-kb and 11.2-Kb, respectively (Fig. 5A). Due to the insertion distance were greater than 5 kb, we therefore suggested that the interfered *At3g22970* gene may cause to the R1-2 phenotype. The RT-PCR was used to analyze the expression of the *At3g22970* in the WT and R1-2. In Figure 6, the result revealed that the *At3g22970* was down-regulated in R1-2. Therefore, we

suggested that phenotypes of the R1-2 might be resulted in a down-regulation of the *At3g22970* expression.

In TAIR, the genomic sequences of *At3g22970* was proposed to be 2065 bp, consists three exons and two introns (Fig. 5B), encoding a protein of 370 amino acids. It is a protein containing a DUF506 domain and to be translocated into the chloroplast (Fig. 5C and Fig. 7). The putative chloroplast transit peptide was predicted by the TargetP program (<http://www.cbs.dtu.dk/services/TargetP/>) based on its N-terminal amino acid sequence of the *At3g22970* protein.

Searching on the database also revealed that the *At3g22970* belongs to a gene family which containing a DUF506 domain, with 14 members in *Arabidopsis*, and all shown with unknown function (Fig. 7). The DUF506 domain was defined by a domain found toward the C-terminus in a number of the uncharacterized plant proteins. The domain is strongly conserved (more than 30% sequence identity between most pairs of members), but flanked by a highly divergent regions including stretches of low-complexity sequence. The *At3g22970* shared with the highest homology to the *At4g14620* (52.5% amino acid identity).

4. Subcellular localization of the *At3g22970* protein

To confirm that the *At3g22970* protein is translocated into the chloroplast, one GFP-coding sequence was fused to the 3'-end of *At3g22970* coding sequence by using the p326-GFP vector. This p326-*At3g22970*-GFP vector was transiently expressed in the tobacco protoplasts. The recombinant GFP and the chlorophyll auto-florescent signals were colocalized in chloroplasts, as shown in the Fig. 8. which confirmed that the *At3g22970* protein was translocated into the chloroplast.

5. Expression of the *At3g22970* displays a rhythm with the light/dark cycles

As shown in Fig. 9, one cis-acting regulatory element (CAAAtatatc) involved in the circadian control at 1829 bp upstream of the promoter region in the *At3g22970* was predicted by the PlantCARE (<http://oberon.fvms.ugent.be:8080/PlantCARE/index.html>) and was also shown to have a circadian rhythm controlled in the microarray data-base in TAIR (<http://www.arabidopsis.org/servlets/TairObject?id=37402&type=locus>). For these reasons, whether the *At3g22970* transcript was fluctuated during the day-night cycle was examined by RT-PCR and Northern blotting. The young seedling was collected every 4 to 6h over a 24h or 48h period in a LD condition and used for RT-PCR and Northern blotting analysis. Sampling time was shown in hours as the Zeitgeber Time (ZT, Zerr et al., 1990), which is the number of hours after dawn at 6:00 Am (i.e., ZT-0). RT-PCR assay indicated that the *At3g22970* transcript could be changed with time for 24h (Fig.10). Thus, Northern blotting was used to confirm that the change was rhythmic. The result revealed that the *At3g22970* transcript, indeed, has a cycled expression pattern, with the highest level at ZT -6h and ZT 18h, and the lowest level was at ZT 12 and ZT 36 (Fig.11).

6. Tissue-specific expression of the *At3g22970*

To correlate the function of *At3g22970* genes with the abnormal leaf phenotype and flowering time, RT-PCR was conducted to analysis the tissue-specific expression patterns. Because the *At3g22970* was shown to be expressed at the highest level at ZT 18 in a LD condition (Fig. 10 and Fig. 11), so the WT samples were harvested at ZT 18h. In Fig. 12, the *At3g22970* transcripts could be detected in all of the tested tissues, ex. in the roots,

mature leaves, stems, flowers, and siliques, however, the highest expressed tissue was observed in the flowers.

7. Phenotypes of the *At3g22970* T-DNA knock-out lines

The loss-of-function mutants were obtained from The Salk Institute Genome Analysis Laboratory (SIGnAL) in a T-DNA insertion loss/reduction-of-function pool (Sessions et al., 2002; Alonso et al., 2003), and three T-DNA insertion alleles of the *At3g22970* (SALK-022456, SALK-085849 and SALK-085910) were analyzed (Fig.13A). Only one of the lines, SALK-085910, exhibited a delay-flowering phenotype, however, the leaves shown the same as the WT (Fig.13B). SALK-085910 is a heterozygote line and Northern blotting assay showed that expression of the *At3g22970* in the T-DNA insertion line (SALK-085910) was less than WT but more than that in R1-2 (Fig.13C).

8. Over-expression of the *At3g22970* in WT and R1-2

To further understand the functions of the *At3g22970*, the genomic clone was over-driven with the dual enhancers of CaMV 35S promoter. The 35SDE::*At3g22970* construct was transformed into WT. For all of the 16 T1 independent lines, the leaves showed no without difference as these in WT. However, 5 out of the 16 lines showed the delayed flowering phenotype (Fig.14A). The *At3g22970* expression was analyzed in these lines at ZT 18h by Northern blotting analysis and revealed that the *At3g22970* was down-regulated as that of the R1-2 (Fig.14B), suggesting that the down-regulation of the *At3g22970* expression in the transgene lines might result from co-suppression of both *At3g22970* endogenous genes and transgene. The leaf phenotype of these transgene lines were not different from WT at 30 days, but these had weak margin serration at 40 days. Therefore, We

suggest that the phenotype of leaves may be caused by dosage-dependent effect.

On the other hand, when introduced the 35SDE::*At3g22970* construct into the R1-2, only 3 independent T1 lines were obtained. Two of these T2 lines, grew very slowly than that of in the WT and R1-2 (Fig.15).

Discussion

1. Down-regulation of the *At3g22970* in R1-2

In R1-2, T-DNA was inserted in 2.5 Kb upstream of the *At3g22970* gene. Promoter was predicted by using Promoter Scan software (<http://bimas.dcrtnih.gov/molbio/proscan>), that promoter region was covered from -1712 bp to -1962 bp and that TATA box was predicted by the PlantCARE (<http://oberon.fvms.ugent.be:8080/PlantCARE/index.html>) at 32 bp upstream of the transcription start site. Therefore, one possible reason is presumed that the T-DNA might affect the enhancer of *At3g22970*, a cis-element which highly increased the expression of *At3g22970*. It is reported that the enhancer might locate upstream or downstream of a gene with hundreds to thousands of base pairs away for effecting.

Second, from previous results, when introduced the 35SDE::*At3g22970* into WT plant, five of sixteen transgenic plants showed delayed flowering phenotype and decreased the expression of *At3g22970*, indicated that the phenotype was caused by co-suppression of *At3g22970* expression (Denli and Hannon, 2003; Szweykowska-Kulinska et al., 2003). Therefore, the phenotype may be also caused by co-suppression of *At3g22970* expression in R1-2. Because two linked T-DNA had four 35S enhancers, the neighbor genes, *At3g22970*, may be over-expressed and result in the down-regulation of *At3g22970* in R1-2.

Third, recent studies have revealed that DNA (de)methylation and histone methylation are involved in the control of gene expression during development and differentiation (Bird, 1992 ; Martienssen et al., 1995). For example, the floral repressor gene *FLOWERING LOCUS C (FLC)* in *Arabidopsis* acquires methylation at H3K27 and H3K9 during an extended period of cold treatment

for floral induction (Bastow et al. 2004; Sung et al., 2004). In microarray data from the TAIR, the young seedlings were treated with 5-AC, a demethylating agent, and the *At3g22970* could be induced after treatment. Thus, T-DNA may disrupt demethylation in R1-2 and then affect the *At3g22970* expression.

Forth, T-DNA may affect the transcription factor binding to the promoter of *At3g22970*, such as the circadian regulatory element, to disrupt the rhythmic cycling of the *At3g22970* transcription.

2. *At3g22970* may play a role in seed development

The segregation ratio of F2 generation was not 3:1 (normal: abnormal), because the homozygote lines were less than expectation. It may be the reason that the R1-2 only produced 88% of seeds in one silique compared with that of WT (Fig. 2F). Among these seeds, many seeds were aborted and only 64% germinated (Fig. 2D, E). Thus, this might be the reason that only four homozygote seedlings were found among 36 of the F2 seedlings, suggested that the *At3g22970* may play a role in seed development.

3. *At3g22970* expression in wild-type plants and photoperiodic flowering

CCA1 (*CIRCADIAN CLOCK ASSOCIATED 1*) is an unusual DNA binding protein in that it recognizes an asymmetric DNA sequence (the CCA1-binding site AAAAATCT or the evening element AAATATCT) and has only one Myb-like domain for DNA binding. Meanwhile, in the *At3g22970* there are three CCA1-binding sites (AAAAATCT) in upstream of 1859 bp, 1798bp, and 498 bp in its promoter region. Analysis of the *At3g22970* promoter by PlantCARE, it also revealed at -1829 bp also with a cis-acting regulatory element, CAAAtatc, involved in the circadian control. Therefore, *At3g22970* might be regulated by CCA1 and play a role in the circadian rhythm.

Previous physiological experiments implicated the circadian clock in photoperiodic time measurement, and genetic experiments in *Arabidopsis* have also identified mutants that disrupt both circadian clock-controlled responses and photoperiodic responses (Koornneef et al., 1998). The *At3g22970* gene may be involved in the flowering photoperiodic response, because the flowering time in R1-2 mutants is delayed under long days (LD) condition. However, whether the *At3g22970* expression patterns were different between plant growth under the LD and short days (SD, 12 light/12 dark) remain unclear. Therefore, to understand the relationship between circadian clock and the *At3g22970* expression should be proceed by Northern analysis or RT-PCR in the WT and R1-2 under LD, SD, continuous light shift to dark period, and continuous dark shift to light period.

4. The leaves phenotype of R1-2, knock-out line (SALK-085910) and over-expression lines

It remain unclear which role *AT3g22970* does play in leaf development. However, recent studies have revealed that many mutants with altered flowering time also disrupt the leaf development. For example, *PIE1*, an ISWI family gene, is required for *FLC* activation and floral repression in *Arabidopsis*. In the Ws genetic background, *pie1-1* displays, in addition to early flowering, leaves that are slightly narrower and more serrated at the base (Noh et al., 2003).

We also find that the leaf phenotype of R1-2 is significant under constant light and high light intensity condition. Therefore, the leaf phenotype can be observed in the WT, R1-2, SALK_085910 and over-expression lines under LD, SD and LL and to find the relationship among the leaf phenotype, *At3g22970* and light.

Photoperiod and temperature can alter the levels of active GAs by affecting specific steps in the biosynthetic pathway. Current evidence suggests that reduced activity of the GAs growth regulator pathway promotes meristematic activity, both in the natural context of *KNOX* function in the SAM and upon ectopic *KNOX* expression in *Arabidopsis* leaves (Hay et al., 2002). On the other hand, mutations that strongly reduce GA biosynthesis delay flowering under long-days and almost abolish flowering under short-days. Therefore, *At3g22970* may affect the GAs biosynthetic pathway and result in curled leaves and delayed flowering phenotypes. We can use RT-PCR assay to study with GA synthesis gene in WT and R1-2 .

5. *At3g22970* may play a role to control flowering time

When introduced the 35SDE::*At3g22970* into WT plant, five of sixteen transgenic plants showed delayed flowering phenotype. Using Northern blotting analysis, the *At3g22970* was down-regulated as seen in the R1-2. The correlation between delay flowering phenotype and reduced expression of *At3g22970* indicated that the phenotype was caused by co-suppression of *At3g22970* expression (Denli and Hannon, 2003; Szweykowska-Kulinska et al., 2003).

By RT-PCR assay, we also detected the expression of gene controlling flowering time, such as *GI*, *CO*, *FT*, *LD*, *SOC1* and *AP1*. The results showed that *CO* and *LD* were down-regulated in R1-2 (data not show). The *CO* (*CONSTANS*) gene is found to encode a protein with similarity to GATA-1-type transcription factors and mediates between the circadian clock and the control of flowering in *Arabidopsis* (Putterill et al., 1995; Paula et al., 2001). The *LD* (*luminidependens*) gene encodes a glutamine-rich nuclear protein containing a possible homeodomain and promotes flowering by repressing *FLC* expression

(Lee et al., 1994; Sheldon et al., 2002). Recent studies have revealed that the *co* and *ld* mutants are both late flowering under LD (Koornneef et al., 1991). To understand the relationship between these gene and *At3g22970*, the expression of gene controlling flowering time should be detected by RT-PCR assay in WT, R1-2, SALK_085910 and over-expression lines with delayed flowering time phenotype under LD, SD or continuous light condition.

From previous results, *At3g22970* may play a role to control flowering time.



Figure 1. The phenotype of R1-2

- (A) WT and (B) R1-2 plants at 35 days in growth of LD (long day). Scale bar: 1 cm.
- (C) In a T2 population, plants revealed the curled leaves and delayed the flowering time phenotype. A homozygote (left) and heterozygote (right) plants at 20-day-old under continuous light condition. Scale bar: 1 cm.
- (D) R1-2 plants at 30-day-old in growth under continuous light. Scale bar: 1 cm.
- (E) The cauline leaves of R1-2 and WT at 30-day-old. The mutant with enhanced leaf margin serration compared with Wild-type under continuous light. Scale bar: 1 cm.
- (F) A homozygote (left, arrow), heterozygote (mid), and WT (right) at 20-day-old under continuous light condition. Scale bar: 5 cm.

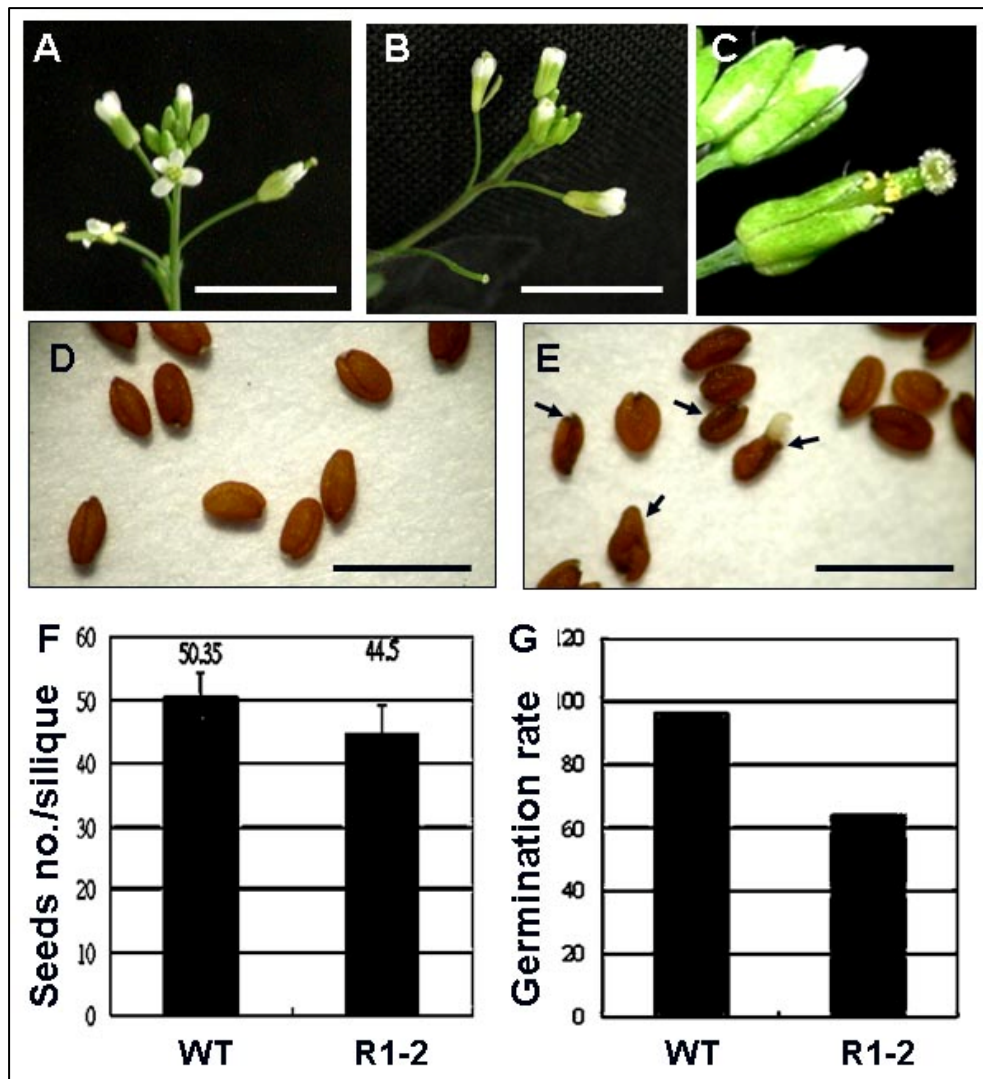


Figure 2. The phenotype of R1-2

The flowers in (A) WT and (B) R1-2. Scale bar: 1 cm.

(C) R1-2 flowers with short stamens

The seeds in (D) WT and (E) R1-2. There are more abortive seeds (arrows) in R1-2 than WT. Scale bar: 1 mm.

(F) Seeds number in siliques of R1-2 and Wild-type. It was shown that the silique had fewer seeds in R1-2 than WT.

(G) A graph displaying percentage of seeds that could germinate. It was shown that only 64% of R1-2 seeds could germinate.

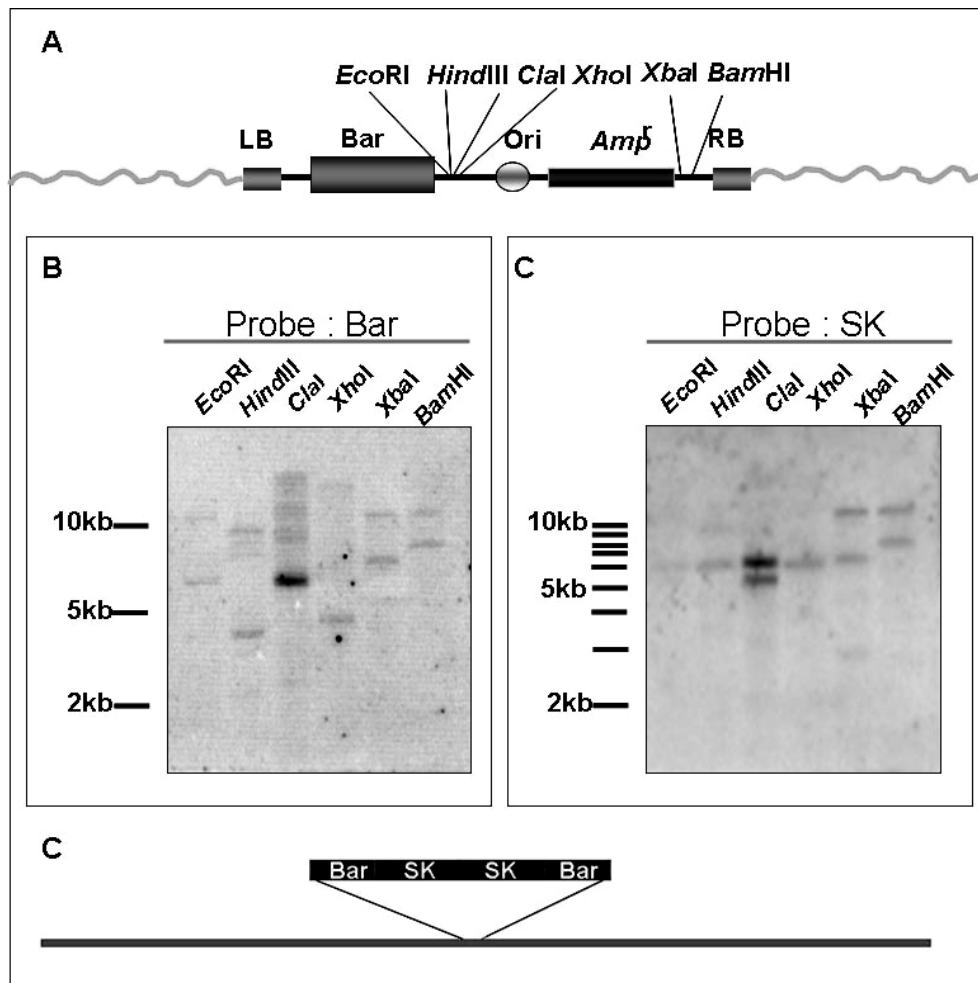


Figure 3. Southern blotting to analyze the T-DNA insertion numbers

(A) The restriction enzyme site in the T-DNA, including *EcoRI*, *HindIII*, *ClaI*, *XhoI*, *XbaI*, and *BamHI*.

Southern blotting analysis by BAR (B) or SK (C) fragments as a probe, respectively; shows that there were two T-DNA were linked together inserted in the R1-2.

(D) The model of two T-DNA linked together with SK fragments inserted in the DNA sequence in R1-2 mutant

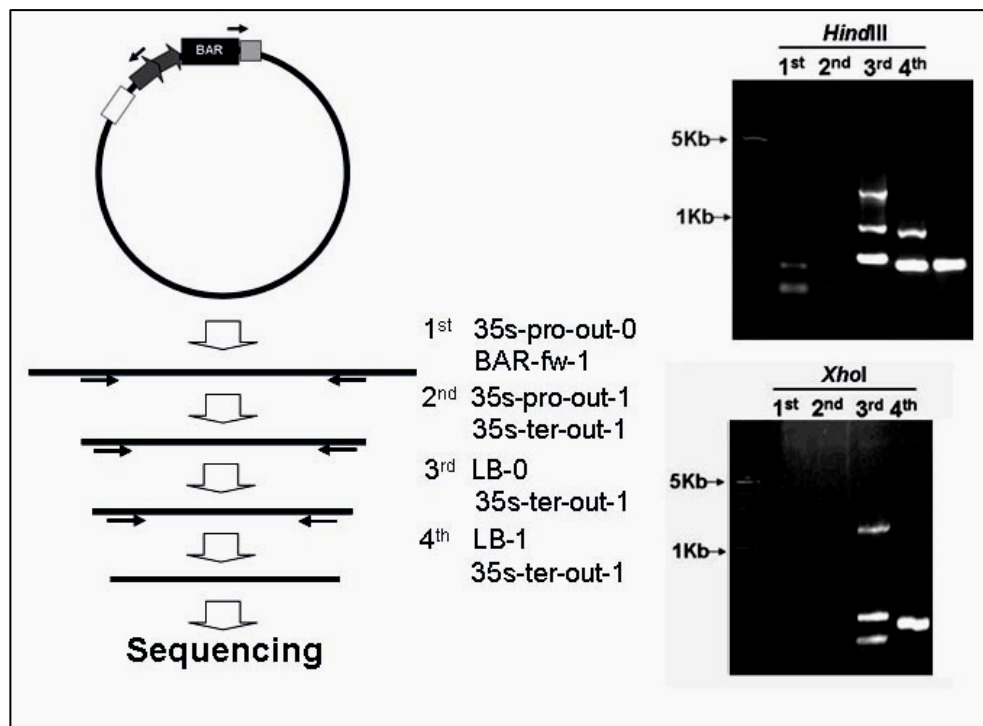


Figure 4. IPCR to clone the T-DNA flanking sequence

Using different nested-PCR primers, the final T-DNA flanking sequence were amplified and sequenced.

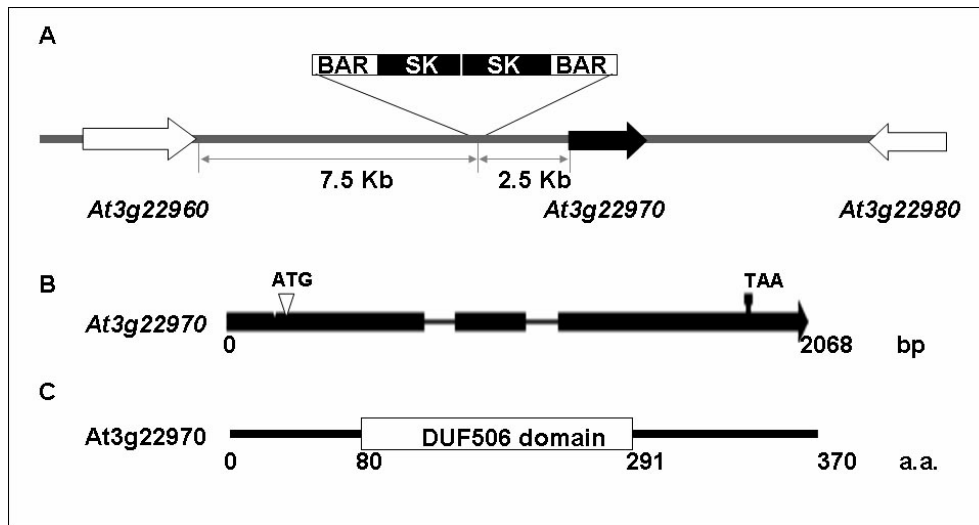


Figure 5. Molecular cloning of the *At3g22970*

- (A) In R1-2, the T-DNA is inserted between genes *At3g22960* and *At3g22970*
- (B) Schematic of *At3g22970* gene. The genomic length is 2068 bp. There is three exons (boxed) and two introns (lined) in *At3g22970*
- (C) Schematic of *At3g22970* protein. *At3g22970* gene encodes a protein of 370 amino acids and contains the DUF506 domain from 80 a.a. to 291 a. a.

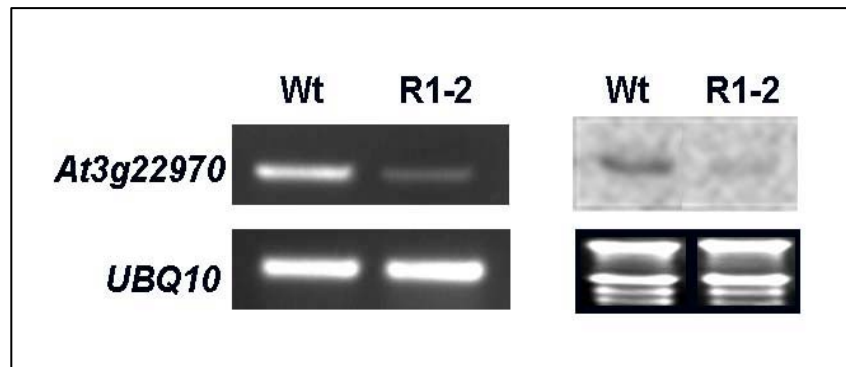


Figure 6. The expression of *At3g22970* in Wild-type and R1-2

RT-PCR and Northern blotting assay were performed on RNA from rosette leaves. The result was shown that the *At3g22970* was down-regulated in the R1-2. The samples were collected at ZT 18h. *UBQ10* was used as a control.

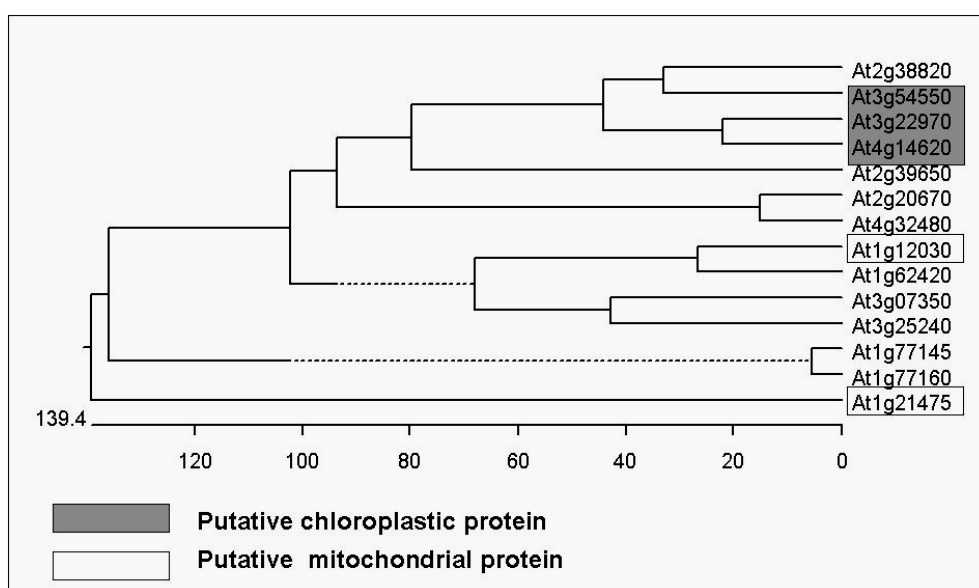


Figure 7. Phylogenetic analysis of genes with the DUF506 domain in *Arabidopsis*.

A phylogenetic tree of the gene with DUF506 domain based on amino acid sequences. The plot was obtained by the Cluster Method of the MegAlign program (DNASTar, Madison, WI).

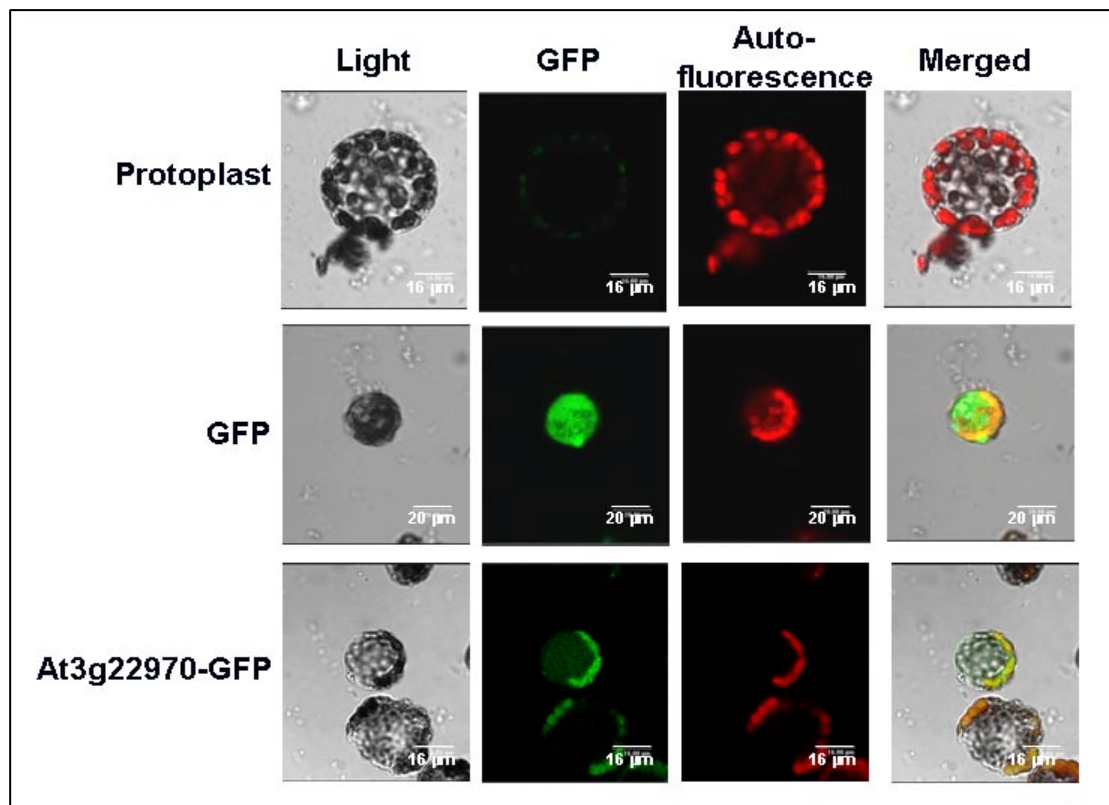


Figure 8. Confocal imaging for the colocalization of the chloroplast auto-fluorescence and recombinant At3g22970- GFP

Confocal fluorescence images of (A) protoplast, (B) transient expressing of p326-GFP, and (C) p326-At3g22970-GFP. From the left to the right, a bright-field, GFP fluorescence image, chlorophyll auto-fluorescence, and the merged confocal images, respectively. Bars represent 16 μm in (A) and (C) and 20 μm in (B).

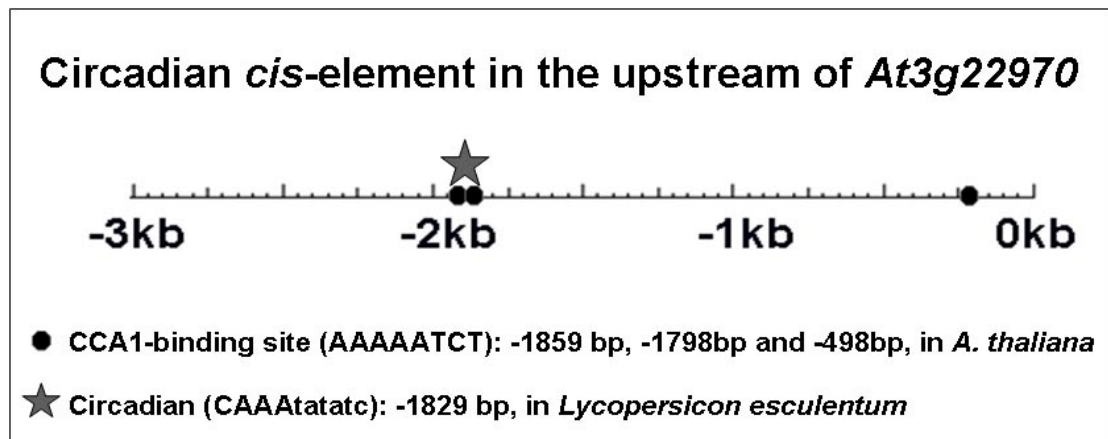


Figure 9. The *cis*-elements related to the circadian in 3 kb upstream of *At3g22970*

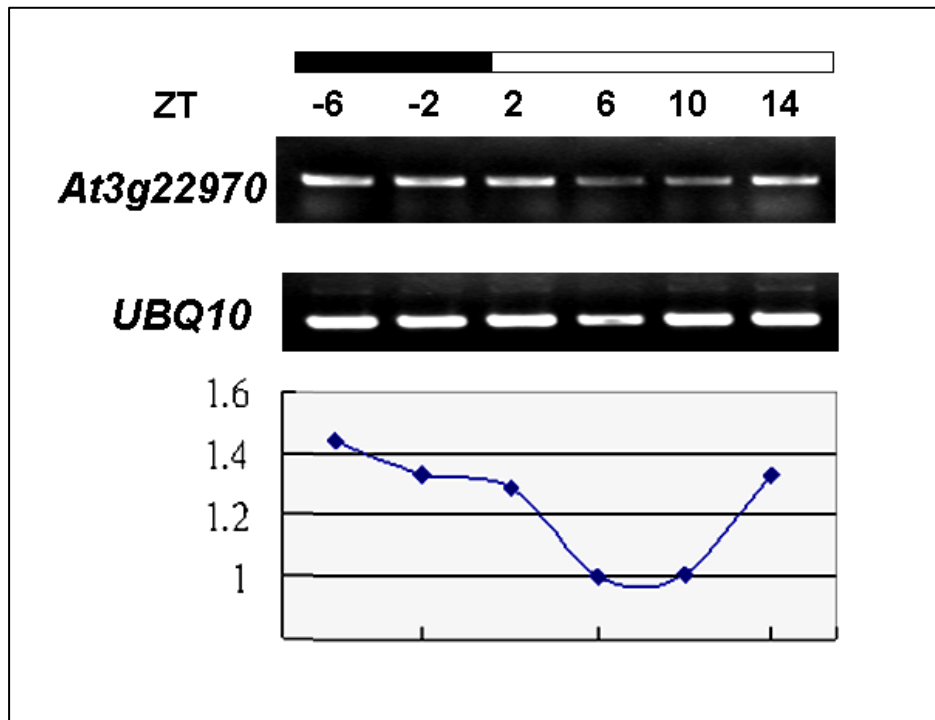


Figure 10. RT-PCR analysis of the *At3g22970* gene expression during one day interval.

Seedlings of wild-type was collected every 4 h from ZT -6h to ZT 14h were used for RT-PCR analysis. *UBQ10* was used as loading control. Results are presented as a proportion after normalization with *UBQ10* signal.

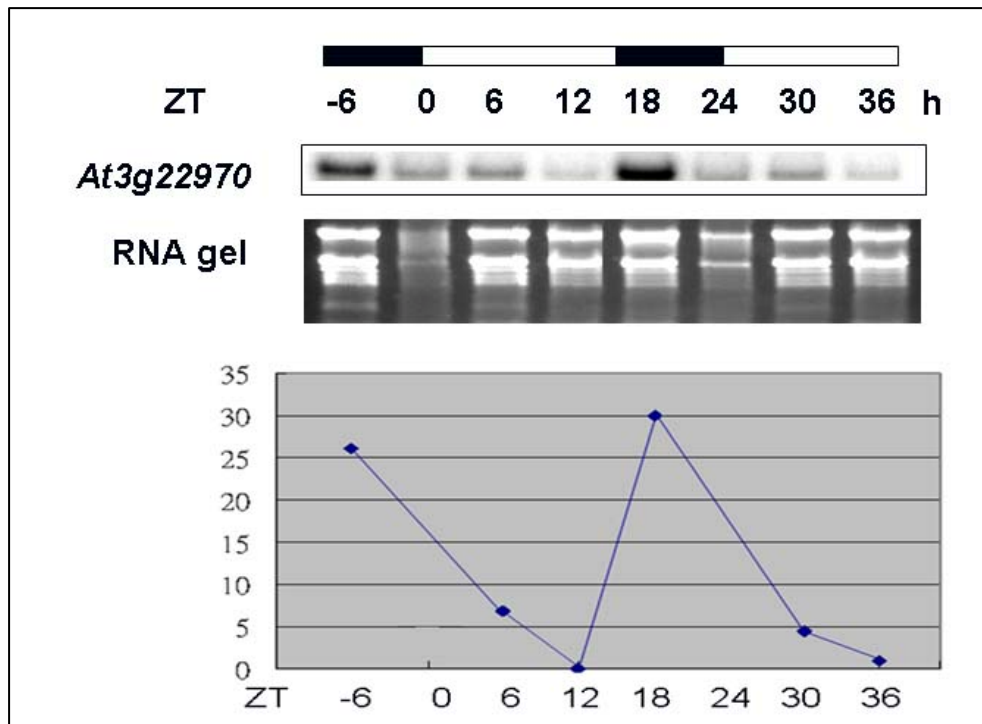


Figure 11. Northern blotting analysis of the *At3g22970* gene expression with a circadian rhythm

Seedlings of wild-type were collected every 6 h from ZT -6h to ZT 36h and were used for Northern blotting analysis. Results are presented as proportion after normalization with RNA stained gel.

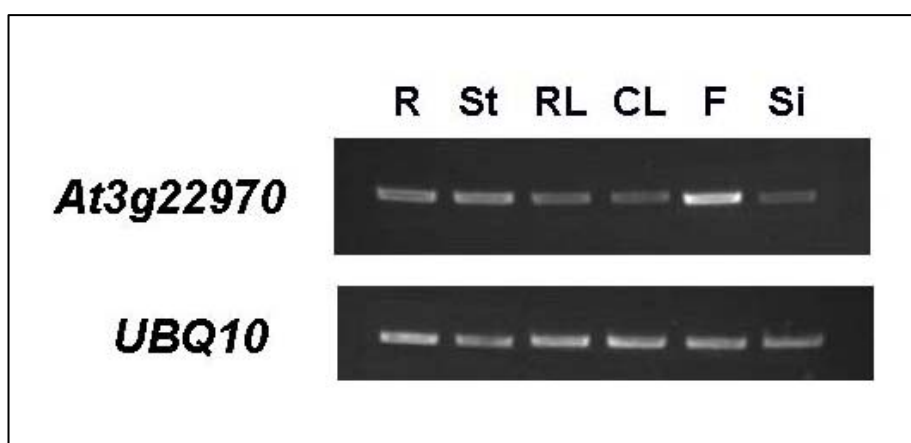


Figure 12. Tissue-specific expression of the *At3g22970*

RT-PCR was performed to analyze the *At3g22970* gene expression in the roots (R), stems (St), rosette leaves (RL), cauline leaves (CL), flowers (F) and siliques (Si). The samples were collected at ZT 18h. *UBQ10* was used as loading control.

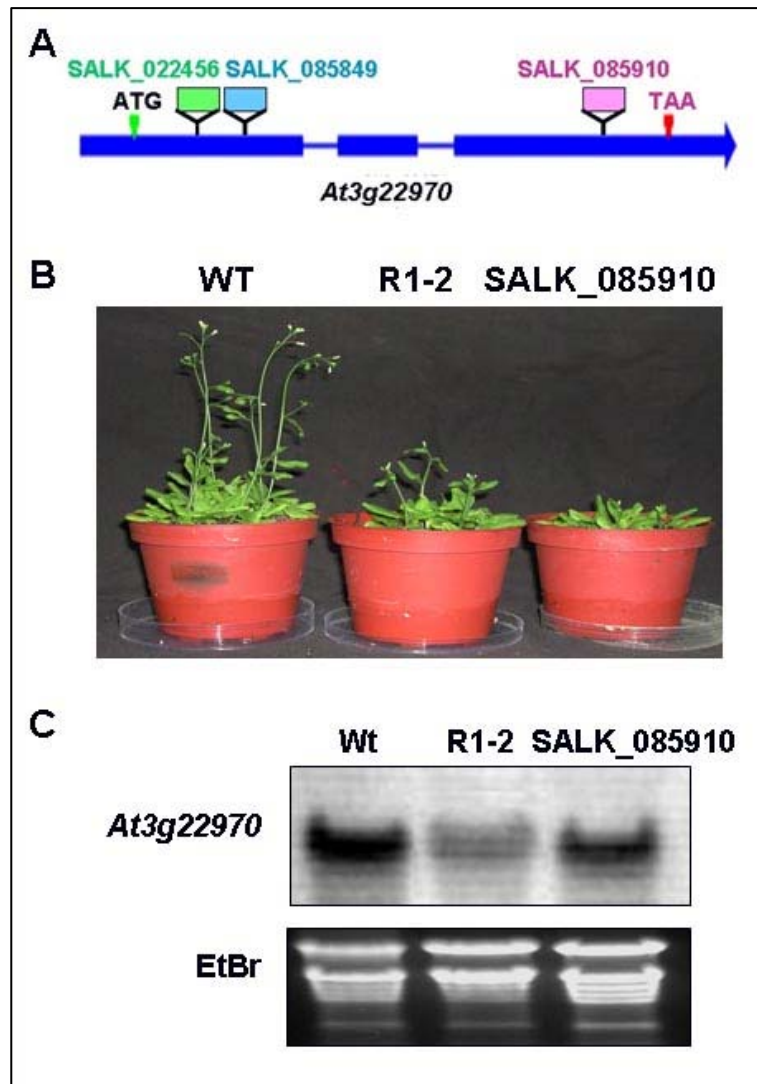


Figure 13. The phenotypes of the *At3g22970* T-DNA knock-out line

- (A) The T-DNA insertion site of SALK knock-out line
- (B) SALK-085910, a *At3g22970* T-DNA knock-out line, exhibited a delayed-flowering phenotype but with normal leaves phenotype as the WT.
- (C) Northern blotting analysis the expression of *At3g22970* in WT, R1-2 and the SALK- 085910 line. The samples were collected at ZT 18h.

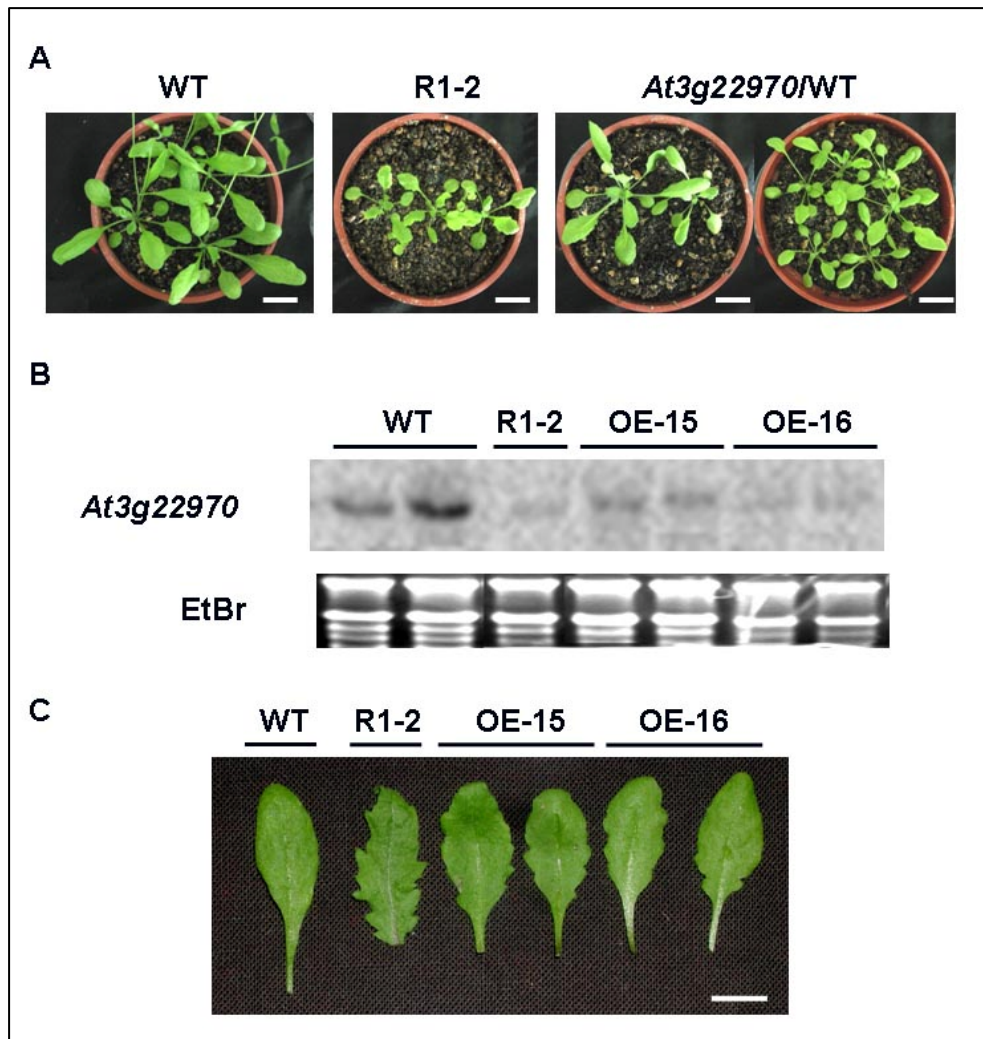


Figure 14. The phenotype of over-expression *At3g22970* in WT

- (A) WT, R1-2, and the line with *At3g22970* over-expressed in WT were growth in LD for 30 days. Scale bar: 1 cm.
- (B) Northern blotting analysis the expression of *At3g22970* in WT, R1-2, and the *At3g22970* over-expressed in WT. The samples were collected at ZT 18h.
- (C) The rosette leaf phenotype of WT, R1-2 and over-expressed lines at 40 days. Shown are the fifth rosette leaves. Scale bar: 1 cm.

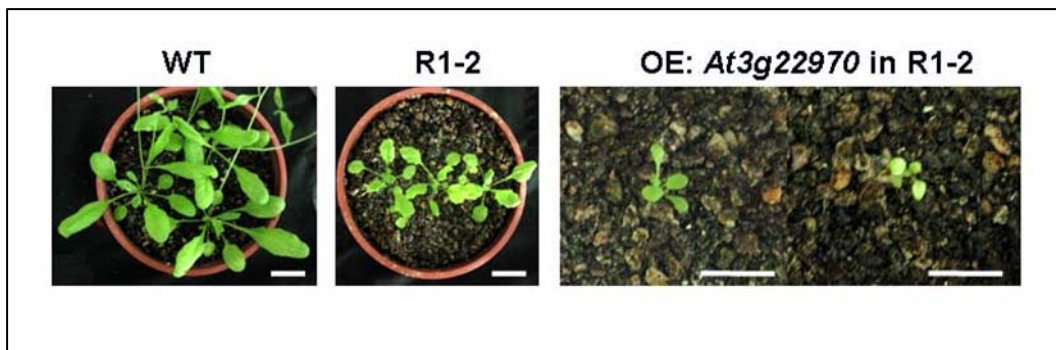


Figure 15. The phenotype of over-expression *At3g22970* in R1-2

WT, R1-2, the line with *At3g22970* over-expressed in R1-2 grew in LD for 30 days. Scale bar: 1 cm.

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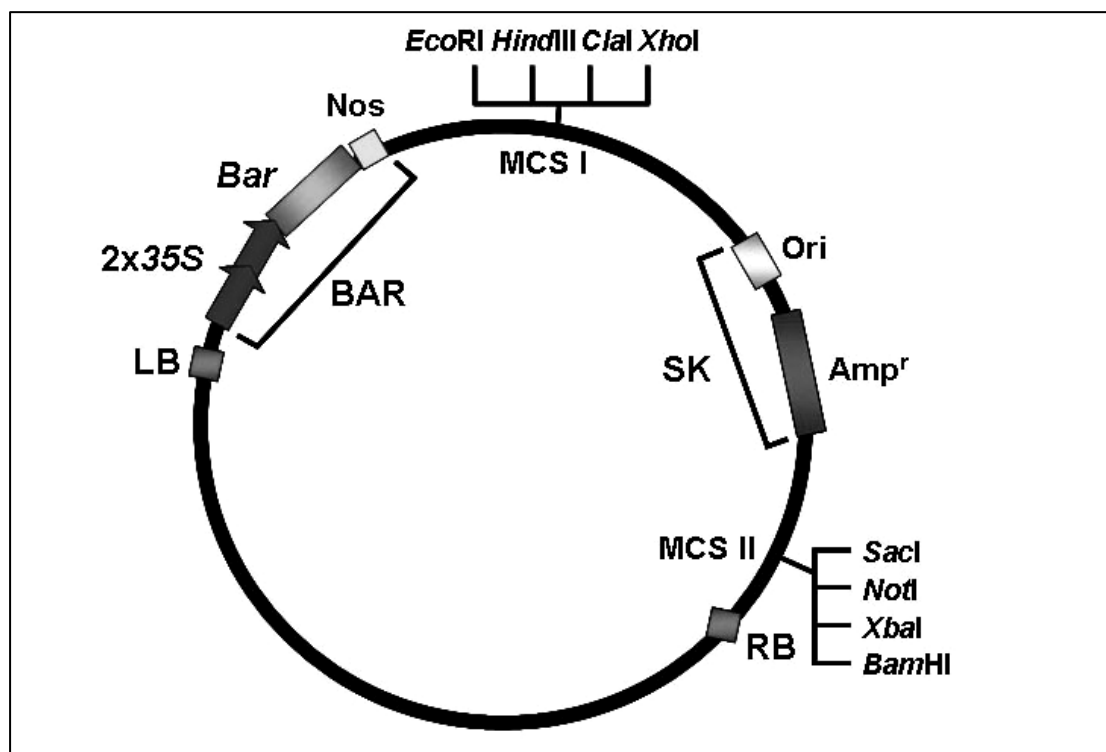
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Appendixes

pPZP:SK:BAR vector



- RB: T-DNA right border
- 35S: CaMV 35S promoter
- Bar*: Basta-resistant gene
- Nos*: Terminator
- MCS: Multiple Cloning Sites
- Ori*: Replication Origin
- Amp^r*: Ampicillin-resistant gene
- LB: T-DNA left border

Promoter cis-element prediction by the PlantCARE
(<http://oberon.fvms.ugent.be:8080/PlantCARE/index.html>)

Site Name	Function	Organism	Strand	Position
A-box	sequence conserved in alpha-amylase promoters	<i>Oryza sativa</i>	+	-1319, -1906
			-	-987, -991, -1444, -1594, -2230
AACA_motif	involved in endosperm-specific negative expression	<i>Oryza sativa</i>	-	-1522
ABRE	cis-acting element involved in the abscisic acid responsiveness	<i>Arabidopsis thaliana</i>	+	-213
			-	-111
ACA-motif	part of gapA in (gapA-CMA1) involved with light responsiveness	<i>Arabidopsis thaliana</i>	-	-675
ACE	cis-acting element involved in light responsiveness	<i>Petroselinum crispum</i>	+	-113, -215
			-	-246, -341, -972, -1102, -2446
AE-box	part of a module for light response	<i>Arabidopsis thaliana</i>	+	-2098
			-	-691, -1376
AT1-motif	part of a light responsive module	<i>Solanum tuberosum</i>	+	-1523, -2488
ATCT-motif	part of a conserved DNA module involved in light responsiveness	<i>Arabidopsis thaliana</i>	-	-1530
AuxRE	part of an auxin-responsive element	<i>Glycine max</i>	+	-2480
AuxRR-core	cis-acting regulatory element involved in auxin responsiveness	<i>Nicotiana tabacum</i>	+	-2054
			-	-743
Box-W1	fungal elicitor responsive element	<i>Petroselinum crispum</i>	-	-732, -1198
CATT-motif	part of a light responsive element	<i>Zea mays</i>	+	-2154
DRE	cis-acting element involved in dehydration, low-temp, salt stresses	<i>Arabidopsis thaliana</i>	-	-238
EIRE	elicitor-responsive element	<i>Nicotiana tabacum</i>	-	-1469
ELI-box3	elicitor-responsive element	<i>Brassica oleracea</i>	-	-2075, -1259
ERE	ethylene-responsive element	<i>Dianthus caryophyllus</i>	+	-507, -561, -1622, -2029
			-	-111,
G-Box	cis-acting regulatory element involved in light responsiveness	<i>Pisum sativum</i>	+	-213, -339
		<i>Triticum</i>	-	-114

		<i>aestivum</i>		
G-box	cis-acting regulatory element involved in light responsiveness	<i>Arabidopsis thaliana</i>	+	-341
GA-motif	part of a light responsive element	<i>Arabidopsis thaliana</i>	+	-2450
			-	-1206, -1334
GAG-motif	part of a light responsive element	<i>Arabidopsis thaliana</i>	-	-47, -49, -69
GATA-motif	part of a light responsive element	<i>Arabidopsis thaliana</i>	+	-984, -1455, -2451
			-	-2280,
GC-motif	enhancer-like element involved in anoxic specific inducibility	<i>Zea mays</i>	+	-187, -265
			-	-110
GCN4_motif	cis-regulatory element involved in endosperm expression	<i>Oryza sativa</i>	+	-24, -872
			-	-216, -1042, -1141, -2427
GT1-motif	light responsive element	<i>Arabidopsis thaliana</i>	+	-654
Gap-box	part of a light responsive element	<i>Arabidopsis thaliana</i>	-	-1340
I-box	part of a light responsive element	<i>Arabidopsis thaliana</i>	+	-32, -330, -899, -1777, -1928, -2480
			-	-421, -1189, -1840
LAMP-element	part of a light responsive element	<i>Pisum sativum</i>	+	-35, -2334
			-	-651
		<i>Spinacia oleracea</i>	-	-1668, -2067
LTR	cis-acting element involved in low-temperature responsiveness	<i>Hordeum vulgare</i>	+	-1946
			-	-237, -284, -1579, -1587, -1998, -2132
MBS	MYB Binding Site	<i>Zea mays</i>	+	-733
MRE	MYB binding site involved in light responsiveness	<i>Petroselinum crispum</i>	+	-1895, -2019
			-	-1565, -1578, -1597, -1602, -1607, -1670, -1681, -1721, -1980, -2048, -2063, -2073, -2201, -2227

MSA-like	cis-acting element involved in cell cycle regulation	<i>Catharanthus roseu</i>	+	-135, -738
O2-site	cis-acting regulatory element involved in zein metabolism regulation	<i>Zea mays</i>	-	-563
P- box	gibberellin-responsive element	<i>Oryza sativa</i>	+	-471, -488, -810
			-	-201, -1449
Pc-CMA2c	part of a light responsive element	<i>Pisum sativum</i>	-	-2161
Skn-1_motif	cis-acting regulatory element required for endosperm expression	<i>Oryza sativa</i>	+	-869
			-	-1141, -2428
TATA-box	core promoter element around -30 of transcription start	<i>Pisum sativum</i>	+	-32
TATC-box	cis-acting element involved in gibberellin-responsiveness	<i>Oryza sativa</i>	+	-116, -1824, -1835, -1906
			-	-282, -902, -1572, -1755, -2090
TCA-element	cis-acting element involved in salicylic acid responsiveness	<i>Brassica oleracea</i>	—	-485, -571, -760, -868
TCCC-motif	part of a light responsive element	<i>Spinacia oleracea</i>	+	-47, -49
			-	-1390
TCT-motif	part of a light responsive element	<i>Arabidopsis thaliana</i>	+	-43
			-	-2109, 2171, -2403
TGA-element	auxin-responsive element	<i>Brassica oleracea</i>	+	-171
chs-CMA1a	part of a light responsive element	<i>Daucus carota</i>	+	-333, -349, -414, -1349, -1806, -1869
			-	-648, -697, -2039, -2175, -2312
chs-CMA2a	part of a light responsive element	<i>Petroselinum crispum</i>	-	-1461
Circadian clock	cis-acting regulatory element involved in circadian control	<i>Lycopersicon esculentum</i>	+	-1830