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Transgenic study of chloroplast translocon gene regulation in *Arabidopsis thaliana*

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ABSTRACT. The majority of chloroplast proteins is encoded by the nuclear genome and must be accurately imported to plastids. The translocons on the outer envelope and inner envelope membrane of chloroplasts (the Toc and Tic proteins) play key roles in the machinery of protein import into the chloroplast. Among the Toc and Tic components identified in *Arabidopsis thaliana*, atToc33 and atToc34 are paralogous to pea psToc34 and play important roles in pre-protein recognition. The expression of *atToc33* and *atToc34* genes must be properly regulated, or their gene products will not be correctly integrated to their destination to allow their function. To reveal the regulatory mechanism of *atToc33* and *atToc34* gene expression, transgenes containing various lengths of the upstream regulatory sequences of *atToc33* and *atToc34* genes and GUS coding sequence were transferred into wild-type *Arabidopsis*. We found that the *atToc33* and *atToc34* genes are preferentially expressed in leaves and roots, respectively. Furthermore, *atToc34* is also regulated in an age-dependent manner. Finally, the leader intron in the 5' UTR of both genes up-regulates the gene expression in mature plants. These data suggest that atToc33 and atToc34 play distinct roles in chloroplast function and development.

Keywords: *Arabidopsis thaliana*; Leader intron; Promoter activity; Reporter gene; Stable transformation; Toc gene.

Abbreviations: ***GUS,*** β-glucuronidase; **MU,** 4-methylumbelliferone; **PCR,** polymerase chain reaction; **Tic,** translocon at the inner envelope membrane of the chloroplast; **Toc,** translocon at the outer envelope membrane of the chloroplast; **UTR,** untranslated region.

INTRODUCTION

The chloroplast is an important organelle in plant cells because it is where photosynthesis occurs. According to the endosymbiont hypothesis, chloroplasts are derived from an incorporated cyanobacterial ancestor. However, more than 90% of the plastid genes have since been transferred to the host nucleus during evolution (Martin et al., 2002). As a consequence nowadays, during the processes of chloroplast biogenesis, the products of these chloroplast protein-encoding genes have to be properly imported to the chloroplasts, otherwise the lipid synthesis, pigment synthesis, chloroplast gene expression, membrane assembly, and light perception processes required for photosynthesis will be severely inhibited. Except for a few outer membrane proteins, most of the nuclear-encoded chloroplastic proteins are imported into chloroplasts via a set of translocon components located on the outer and inner membranes (Inaba and Schnell, 2008; Jarvis, 2008). These translocon components are called Toc/Tic proteins (translocon at the outer/inner envelope membrane of chloroplasts).

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In *Arabidopsis*, translocon proteins atToc33 (At1g02880) and atToc34 (At5g05000) are paralogous to pea psToc34, a plastid protein receptor on the outer membrane of the plastid (Inaba and Schnell, 2008; Jarvis, 2008). However, atToc33 and atToc34 seem to have distinct functions in *Arabidopsis* chloroplasts: atToc33 recognizes photosynthesis-related proteins, whereas atToc34 is a receptor of non-photosynthetic proteins (Jarvis and Robinson, 2004). Based on these observations, *atToc33* and *atToc34* genes should be independently regulated during the process of vegetative growth. Indeed, Jarvis et al. (1998) showed that the mRNA level of *atToc33* was maintained at three times the level of *atToc34* in 2- to 32-day-old plants. Furthermore, Gutensohn et al. (2000) suggested that *atToc34* had higher expression than *atToc33* in the roots of 10-day-old plants. Finally, the Gene Chronologer tool in Genevestigator, which provides gene expression values in different developmental stages of *Arabidopsis* using available Affymetrix GeneChip data, also revealed that *atToc33* had higher expression than *atToc34* during vegetative development, though their expression differences fluctuated considerably (Zimmermann et al., 2004). Nevertheless, none of the above data demonstrated if the differential expression of these two genes is contributed by differential promoter

activity or different mRNA stabilities of these two genes.

In this study, the developmental regulation of *atToc33* and *atToc34* expression during the procedure of vegetative growth was further analyzed. First, we created stable transgenic plants expressing the *GUS* reporter gene driven by *atToc33* and *atToc34* promoter sequences and determined the promoter activity of *atToc33* and *atToc34* genes in 1- to 4-week-old plants. Our results suggest that *atToc33* had constant expression, while *atToc34* had highest expression in young seedlings and expression declined afterwards. Furthermore, *atToc33* and *atToc34* might be preferentially expressed in leaves and roots of the same age, respectively. Second, the intron effect on the transcription activity was also examined by analyzing GUS activity in the stable transgenic plants. Our results show that the leader intron sequences (the intron nearest to the transcription start site) of *atToc33* and *atToc34* increased the transcription levels, especially in mature leaves.

MATERIALS AND METHODS

Plant material and growth conditions

Arabidopsis seeds from wild type (ecotype Columbia; WT) and transgenic plants were surface sterilized with 25% (v/v) commercial bleach and grown on 1x Murashige and Skoog (1962) agar medium with Gamborg's vitamins and 2% (w/v) sucrose or on soil. Plants were grown at 22°C under a 16-h light/8-h dark cycle in white light (85 μmol m^{-2} sec^{-1}) for various numbers of days.

Plasmid construction and plant transformation

The upstream sequences of *atToc33* and *atToc34* genes were amplified by PCR and ligated into β-glucuronidase (GUS)-containing binary vector pCAMBIA1391Z (GenBank accession number AF234312). The resulting plasmids were transformed into WT plants using the floral dipping method (Clough and Bent, 1998), mediated by *Agrobacterium* strain GV3101. The transformants were selected on agar plates containing 30 μg/mL hygromycin, and verified by PCR using construct-specific primers. These transformants were named 33A/33B and 34A/34B, representing different lengths of *atToc33* and *atToc34* upstream sequences, respectively. The transgenic plants were selected for more than two generations and T_3 homozygous transgenic plants were used for this study. The primer sequences specific for *atToc33* and *atToc34* upstream sequences are summarized in Table 1.

GUS activity assays

The fluorometric quantification and histochemical localization of GUS enzyme activity were performed as described by Jefferson (1987). For fluorometric quantification, cell lysate was assayed for GUS activity with fluorometer (SpectraMax Gemini XPS, Molecular Devices). For histochemical staining, the 2-week-old seedlings and mature leaves were incubated in GUS buffer for 16 h at 37°C, and stored in fix solution (0.1 M sodium phosphate, pH 7.2, 0.1% formaldehyde, 0.1% triton X-100, 0.1% β-mercaptoethanol).

RESULTS

Developmental regulation of *atToc33* and *atToc34* expression

To determine whether the expression of *atToc33* and *atToc34* was influenced by specific developmental signals, promoter-GUS fusions were constructed for both genes and analyzed in transgenic *Arabidopsis* plants. The *atToc33* and *atToc34* genes have similar gene structure, including eight exon and seven intron sequences (Figure 1A and 1B). The leader intron of both genes is located in the 5' UTR region. The *atToc33* and *atToc34* upstream sequences were individually placed into a binary vector pCAMBIA1391Z containing a GUS coding sequence as reporter. These constructs are shown in Figure 1. The p33A plasmid contained a 1.02-kb promoter and 55-bp 5' UTR sequences of *atToc33* gene. The p34A plasmid included a 532-bp promoter and 96-bp 5' UTR sequences of *atToc34* gene. The *atToc34* promoter sequence was relatively short due to adjacent to the coding sequence of clathrin adaptor-related gene (AT5g05010). Both plasmids were further transformed to WT *Arabidopsis* by the floral dipping method. Thirteen and twelve independent transgenic lines containing p33A and p34A, respectively, were obtained. These transgenic plants were named 33A-1~13 and 34A-1~12. Four homozygous 33A (33A-2-6, 33A-4-5, 33A-6-2, and 33A-7-3) and five homozygous 34A (34A-5-1, 34A-6-2, 34A-8-5, 34A-10-2, and 34A-12-5) T_3 transformants were used for further GUS analyses.

The GUS activity of 34A transformants had highest expression in 7-day-old plants, at an expression level that was higher than in 33A plants. However, the expression was reduced to one-third yield of that for 33A plants in 14-day-old and older plants (Figure 2). These results suggest that *atToc33* is expressed at a constant high level through the developmental stages of vegetative growth. In

Table 1. Sequences of *atToc33*- and *atToc34*-specific primers used in this study.

	Toc33 specific primers (5′ to 3′)[a]	Toc34 specific primers (5′ to 3′)[a]
F1	GCCAAGCTTGGACCTCAATAGAGTTCCATC	GCCAAGCTTGATCCAGCGGATCTACGAGC
R1	CGGGGATCCGTTGTGTGAGGAGCTTTGA	CGGGGATCCTGAGCTAGAGTTACTAGTGG
R2	CGGGGATCCACTCCTAAACCCTTCAGCT	CGGGGATCCGGACACAAATCCTTACCTACAC

[a]The underlined sequences are additional restriction sites. *Bam*HI, GGATCC; *Hind*III, AAGCTT.

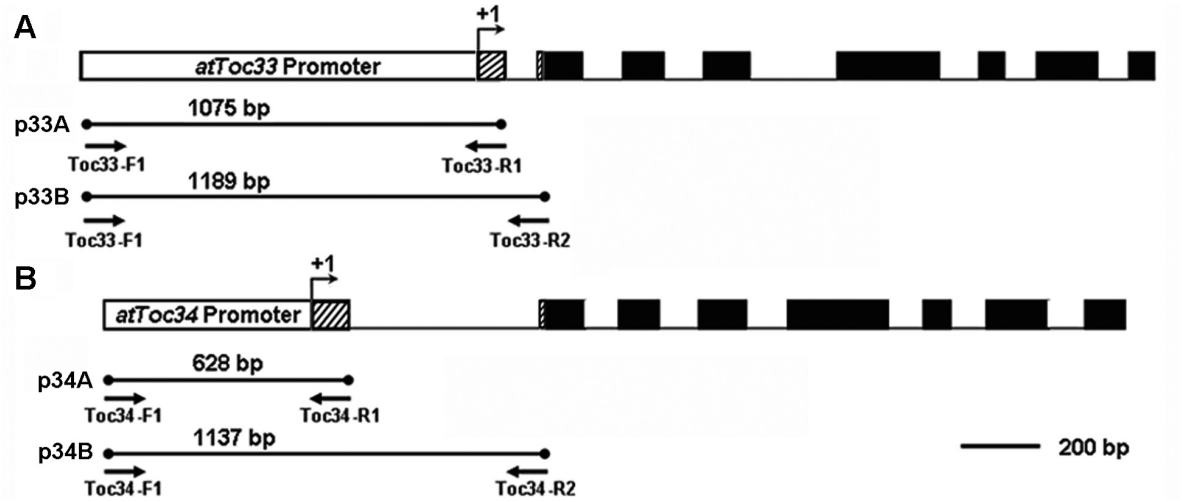

Figure 1. Gene structure and upstream construct of *atToc33* and *atToc34*. The promoter (-1020 to -1), 5' UTR (+1 to +55 and +158 to +169), and leader intron (+56 to +157) sequences of *atToc33* gene were shown in panel A. The promoter (-532 to -1), 5' UTR (+1 to +96 and +590 to +605), and leader intron (+97 to +589) sequences of *atToc34* gene were shown in panel B. The p33A and p34A constructs consist of the promoter and 5' UTR sequences prior the lead intron of *atToc33* and *atToc34*, respectively. The p33B and p34B constructs contain additional leader intron sequences of *atToc33* and *atToc34*, respectively. Arrows indicated the locations and names of primers. Open box, promoter sequence. Hatched box, 5' UTR sequence. Black box, coding sequence. Bold underline, intron sequence. +1, transcription start site.

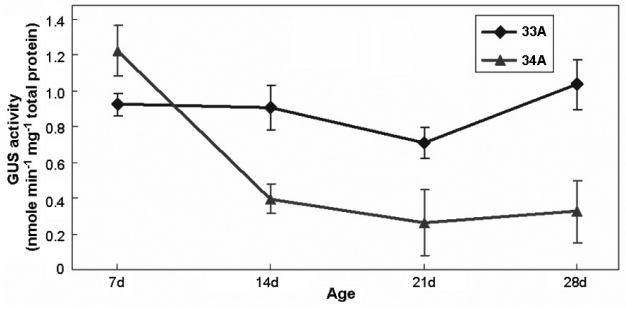

Figure 2. Expression of *atToc33* and *atToc34* genes in different developmental stages. The GUS activity of 1- to 4-week-old transgenic plants 33A and 33B was determined. Data are means ± SD of three repeat experiments from at least four individual transgenic lines (33A-2-6, 33A-4-5, 33A-6-2, 33A-7-3, 34A-5-1, 34A-6-2, 34A-8-5, 34A-10-2, and 34A-12-5). P values < 0.001 (Tukey test) were considered to represent significant differences.

contrast, *atToc34* expression was related to plant age, with highest promoter activity in the young seedling (1-week-old) and a compromised yield in the mixed tissues of mature plants (2- to 4-week-old). This indicates that *atToc34* is modulated in an age-dependent manner.

Expression of *atToc33* and *atToc34* genes in vegetative tissues

Next, we asked if *atToc33* and *atToc34* are differentially expressed in vegetative tissues. The GUS activity in leaves and roots of four 33A lines and five 34A lines was compared. The 33A-2-6, 33A-4-5, 33A-6-2, and 33A-7-3 plants revealed similar expression pattern by fluorometric quantification and histochemical staining of GUS activity, and so did 34A-5-1, 34A-6-2, 34A-8-5, 34A-10-2, and 34A-12-5 plants (data not shown). Therefore, 33A-6-2 and 34A-5-1 plants were used as representatives in Figure 3. The *atToc33* expression in leaves was approximately three times higher than in roots in both 1-week-old and 2-week-old 33A-6-2 plant. However, the overall *atToc33* expression in whole plants (the open boxes of Figure 3) of both ages was similar. This revealed that the constant expression level of *atToc33* observed in Figure 2 was a compromised result obtained from mixed plant tissues. In fact, *atToc33* was preferentially expressed in leaves and did not change significantly in roots. By contrast, *atToc34* expression in roots was about six times higher than in leaves in both 1-week-old and 2-week-old 34A-5-1 plants. Agreeing with the results in Figure 2, the overall *atToc34* expression yield in 1-week-old 34A-5-1 plants was approximately 3 to 4 times higher than 2-week-old plants. These results indicate that *atToc34* is preferentially expressed in roots and young plants. In other words, *atToc34* is regulated in both age- and tissue-specific manners.

Intron effect in transgenic plants expressing *atToc33* promoter- and *atToc34* promoter-GUS fusion genes

Leader intron (also called proximal intron) has been shown to have some influence in determining the level and pattern of gene expression in *Arabidopsis* (Norris et al., 1993; Jeong et al., 2006). The mechanism of intron-mediated enhancement (IME) is largely unknown, however, it was hypothesized that IME might increase transcription efficiency or mRNA stability (Rose et al., 2008). As *atToc33* and *atToc34* have similar gene

structures but different lengths of intron sequences, we wanted to verify whether the leader intron sequences play a role in regulating the *atToc33* and *atToc34* expression in stable transgenic plants. Using similar strategies, the pCAMBIA1391Z-based transgenes containing upstream sequence and endogenous intron sequence were transformed to WT *Arabidopsis* to create 33B and 34B transgenic plants. These constructs are also shown in Figure 1. The p33B plasmid contained promoter, full-length 5' UTR, and 102-bp leader intron regions of *atToc33* gene. The p34B had promoter, full-length 5' UTR, and 493-bp leader intron regions of *atToc34* gene. Seventeen and ten independent transgenic lines containing p33B and p34B, respectively, were identified, named 33B-1~17 and 34B-1~10. Seven homozygous 33B (33B-2-5, 33B-5-1, 33B-11-3, 33B-13-1, 33B-14-5, 33B-16-1, and 33B-17-4) and five homozygous 34B (34B-1-1, 34B-5-2, 34B-7-1, 34B-9-2, and 34B-10-3) T_3 transformants were used for further GUS analyses.

The location of GUS activity from two-week-old seedlings and four-week-old mature leaves was examined histochemically (Figure 4). Transgenic plant 33B had significantly higher GUS activity in young seedlings and mature leaves (Figure 4B and 4F). Compared to 33B plants, 33A plants also showed higher GUS activity in young seedling (Figure 4A) but relatively weaker GUS activity in mature leaves (Figure 4E). GUS activity in the two-week-old seedlings and four-week-old plants were further quantified by fluorometric methods (Figure 5). In young seedlings, levels of GUS activity in 33A and 33B plants were similar, whereas those in mature 33B were 1.6 times higher than 33A 4-week-old plants. These data suggest that the intron sequence up-regulates *atToc33* expression more effectively in mature plants. Similarly,

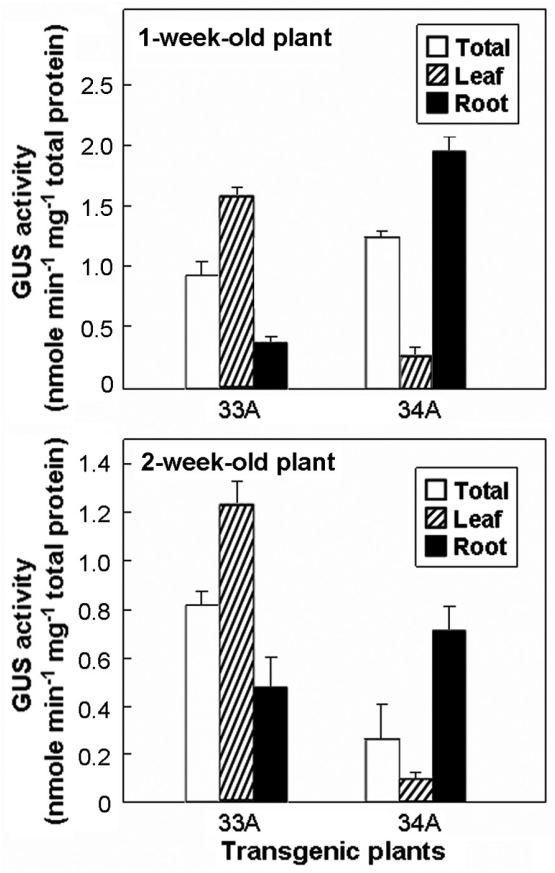

Figure 3. Expression of *atToc33* and *atToc34* genes in leaves and roots. The GUS activity of leaves and roots in 1- and 2-week-old transgenic plants 33A and 33B was determined. Data are means ± SD of three repeat experiments from 33A-6-2 and 34A-5-1 transgenic lines. *P* values < 0.001 (Tukey test) were considered to represent significant differences.

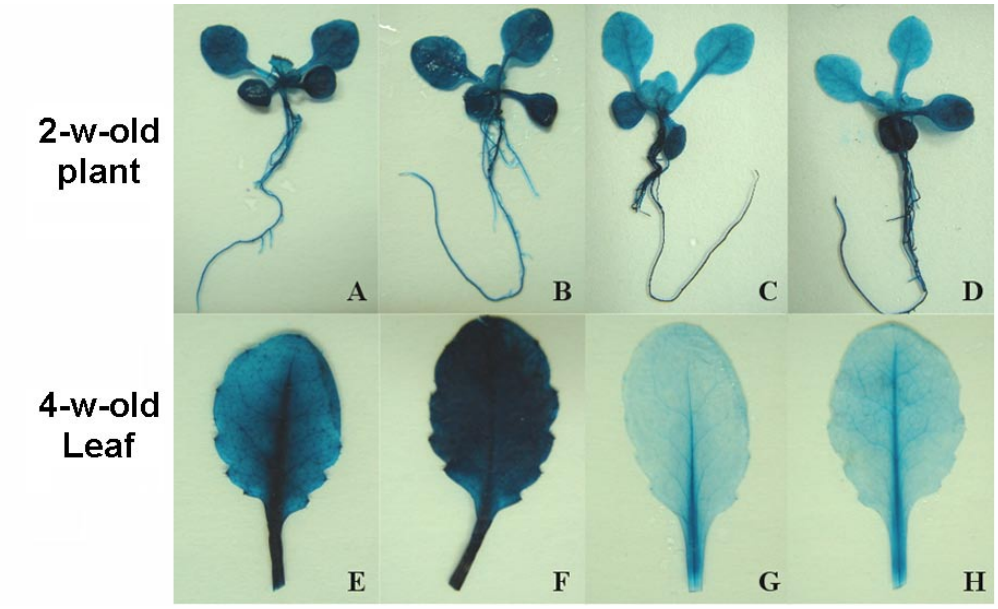

Figure 4. Intron effect of *atToc33* and *atToc34* gene expression. The GUS expression of 2-week-old seedling and 4-week-old leaves was demonstrated by histochemical staining. 33A-6-2 (A, E), 33B-16-1 (B, F), 34A-5-1 (C, G), and 34B-5-2 (D, H) were homogenous T_3 transgenic lines expressing p33A, p33B, p34A, and p34B constructs, respectively.

the GUS activity of 34A and 34B plants was alike in young seedlings (Figures 4C and 4D). However, the mature 34B leaves had slightly higher GUS activity than mature 34A leaves (Figures 4G and 4H). The fluorometric quantification was consistent with the GUS staining data (Figure 5). Again, these results suggest that *atToc34* leader intron plays a more important role in up-regulating *atToc34* expression in mature plants.

DISCUSSION

Due to their recognizing different substrate proteins during protein import into chloroplasts, the closely related *atToc33* and *atToc34* genes are independently regulated in response to developmental signals to ensure the proper import of chloroplast proteins depending on different developmental needs. Based on results from stable transgenic plants expressing GUS reporter under the control of *atToc33* and *atToc34* upstream regulatory sequences, we conclude that the promoter regions of paralogous *atToc33* and *atToc34* genes govern the differential expression of these two genes among different tissues. Furthermore, both endogenous intron sequences up-regulate gene expression in mature plants.

According to the GENEVESTIGATOR database, the expression level of *atToc33* and *atToc34* fluctuates during vegetative growth (Figure 6; Zimmermann et al., 2004). Disagreeing with the database, our GUS activity assays in transgenic plants revealed that both genes had similar GUS

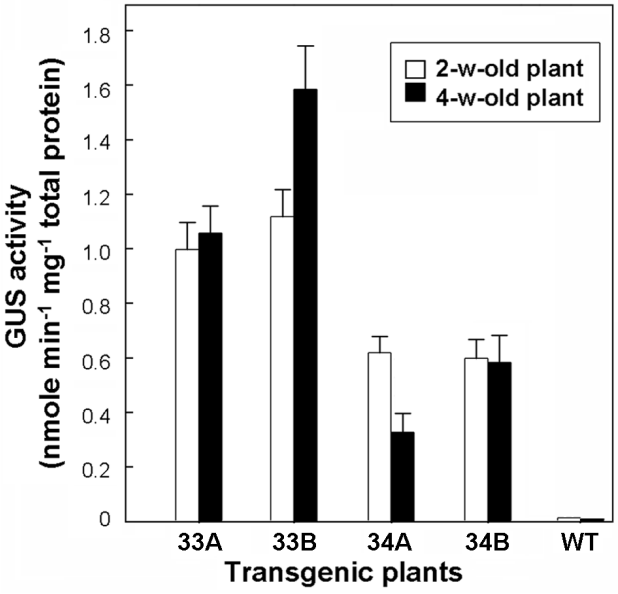

Figure 5. Induction of *atToc33* and *atToc34* gene expression by endogenous intron. The GUS activity of 2- and 4-week-old transgenic plants was analyzed. Data are means ± SD of three repeat experiments from three individual transgenic lines (33A-2-6, 33A-6-2, 33A-7-3, 33B-11-3, 33B-14-5, 33B-16-1, 34A-5-1, 34A-6-2, 34A-10-2, 34B-5-2, 34B-7-1, and 34B-9-2. *P* values < 0.001 (Tukey test) were considered to represent significant differences. WT, Columbia as control.

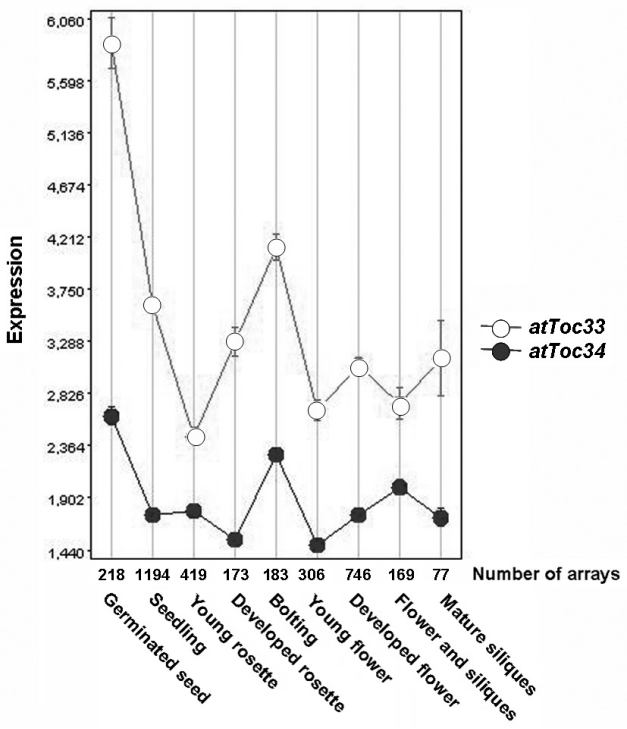

Figure 6. Expression profile of *atToc33* and *atToc34* genes during *Arabidopsis* development. This expression profile was downloaded from GENEVESTIGATOR database (Zimmermann et al., 2004).

enzyme activity in 2- to 4-week-old plants (Figure 2). One may argue that perhaps translational results from chimeric GUS fusions are different from the real endogenous steady state mRNA levels of *atToc33* and *atToc34* genes. However, correlating with our GUS data, other Northern blot analyses have indicated stable expression levels of *atToc33* and *atToc34* from 4- to 32-day-old plants (Jarvis et al., 1998). Therefore, the expression level of closed related genes concluded from the ATH1 GeneChip should be reconsidered. According to previous reports and this study, we suggest that *atToc33* has higher expression in germinating seedlings, but similar expression in young rosette, developed rosette, and even bolted rosette leaves. Similarly, *atToc34* has the same expression pattern but lower expression yield compared to *atToc33*. This provides a more accurate insighs into the developmental regulation of *atToc33* and *atToc34* genes during vegetative growth.

It is notable that *atToc33* expression levels in leaves are three times higher than in roots, while *atToc34* expression levels in roots are six times higher than in leaves of 1- to 2-week-old plants (Figure 3). The *atToc33* has higher expression in leaves because its expression is regulated by CIA2 (chloroplast import apparatus 2), a leaf-specific transcription factor. Reverse transcription-PCR (RT-PCR) and chromatin immuno-precipitation analyses proved that CIA2 was able to bind to *atToc33* promoter sequence and up-regulate its gene expression in leaves (Sun et al., 2009). However, CIA2 has no effect on regulation of *atToc34* expression. Furthermore, Yu and Li (2001) used

RT-PCR to examine the expression level of these two genes in 30-day-old plants, and found the expression level of *atToc33* and *atToc34* were approximately 20% higher in leaves and roots, respectively. These observations support the previous hypothesis of atToc33 and atToc34 function, which indicated that atToc33 and atToc34 were photosynthetic and non-photosynthetic receptors, respectively (Jarvis and Robinson, 2004). It is no surprise that *atToc33* has to be up-regulated in leaves to import more photosynthesis-related proteins to allow leaves to maintain their photosynthetic functions due to sufficient supply or replacement of photosynthesis-related proteins. By contrast, *atToc34* only has a basal expression level in leaves. This is possibly because the demand for structural proteins to be imported by atToc34 into leaf chloroplasts is considerably lower.

Mascarenhas et al. (1990) investigated the enhancing effects of two maize introns and defined the function of IME. Even though the mechanism of IME remain largely unknown, the effect of IME has been broadly observed in eukaryotes, including animals and plants (Jonsson et al., 1990; Palmiter et al., 1991; Morello et al., 2002; Norris et al., 2003). Our stable transformation assay suggested the intron sequence did not increase the expression yield of both genes in young plants, while we did actually see a gradual increase in the gene expression in mature plants (Figure 5). This implied that the intron effect of *atToc33* and *atToc34* is more effective in mature plants in order to maintain chloroplast function in mature leaves. We thus hypothesize that these introns might contain age-specific enhancers. However, more experiments are required to prove this hypothesis.

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以轉殖植物研究阿拉伯芥葉綠體運輸蛋白基因表現的調節

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絕大多數的葉綠體蛋白是從細胞核內的基因體解碼合成後，再精準地送入葉綠體中。位於葉綠體外膜及內膜上的運輸蛋白（Toc 及 Tic 蛋白）則負責辨識及轉運這些葉綠體蛋白質。在阿拉伯芥中已知的運輸蛋白機組成員裡，atToc33 和 atToc34 是豌豆 psToc34 的旁系同源蛋白，在運輸蛋白質進入葉綠體的過程中，扮演辨識蛋白質的角色。因此運輸蛋白基因必須被適當的調節，否則這些運輸蛋白機組將無法準確的組裝以執行適當的功能。為了解 *atToc33* 和 *atToc34* 基因表現的調節的機制，*atToc33* 和 *atToc34* 基因的上游調節序列接上 GUS 報導基因後送入野生型阿拉伯芥植株。結果顯示這兩個基因傾向於分別在葉中及根中表現。此外，*atToc34* 基因亦受到植株年齡而影響其表現。最後，在 5’ 端非轉譯區內的第一內插子可促進其基因在成熟植株內的表現。因此二基因的表現受到不同的機制調節，我們總結 atToc33 和 atToc34 在葉綠體的功能及發育中扮演不同角色。

關鍵詞：阿拉伯芥；引導內插子；啟動子活性；報導基因；穩定轉殖；葉綠體外膜運輸蛋白基因。