Developmentally early and late onset of Rps10 silencing in Arabidopsis thaliana: genetic and environmental regulation

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Abstract

Transgene dosage, silencing competence of the transgene loci, and photoperiod conditions were found to regulate the onset and efficiency of Rps10 silencing in two independent transgenic lines of Arabidopsis thaliana. The Rps10 gene encodes the S10 protein which is part of the small subunit of mitochondrial ribosomes. Homozygous plants presented developmentally early onset of silencing, a very efficient decrease in the level of Rps10 transcripts, as well as a severe and uniform phenotype called P1. P1 plants either died during the vegetative growth phase or were rescued by reversion resulting from inactivation of silencing. A wide variety of morphological and developmental abnormalities observed within the hemizygous transformants allowed their classification into three categories P2, P3, and P4. The most severe and early was the P2 phenotype found in only one transgenic line and most probably resulting from high competence of the transgene loci. Developmentally late onset of silencing occurred only in the short day photoperiod and was characteristic for the P3 and P4 plants. This phenomenon was attributed to conditions favourable to silencing achieved in the short day photoperiod, e.g. a greatly prolonged vegetative phase accompanied by a gradual increase of the level of Rps10 transcripts. To the best of our knowledge, this is the first report indicating that the onset of silencing depends on the photoperiod conditions in A. thaliana.

Key words: Environmental regulation, gene silencing, photoperiod, PTGS, RNAi, Rps10, silencing reversion.

Introduction

RNA silencing is a mechanism that operates in diverse eukaryotes. In plants, two RNA silencing pathways are observed: transcriptional gene silencing (TGS) and post-transcriptional gene silencing (PTGS). TGS is associated with changes in the transcription rate, while PTGS mostly occurs via degradation of mRNA or translational repression mediated by short interfering RNAs (siRNAs) (for a review, see Baulcombe, 2004). PTGS silencing in plants has been intensively investigated in recent years, resulting in significant improvement in our knowledge of this phenomenon. Most of this research concerned transgene-mediated gene silencing. Nevertheless, some aspects of PTGS remain relatively poorly understood, including factors influencing the onset and severity of PTGS, as well as the mechanism of reversion and resetting.

There are three essential steps of PTGS: initiation, propagation, and maintenance. It is thought that initiation of silencing is spontaneous, stochastic, and localized (Vacheret et al., 2001). Silencing may be propagated either via long-distance systemic spread (Palauqui and Vacheret, 1998; Voinnet et al., 1998) or via spontaneous short-range cell to cell spread (Himber et al., 2003; Dunoyer et al., 2005). The maintenance of silencing, on the other hand, is dependent on the ability to produce a signal that induces the degradation of mRNA. Thus, the PTGS onset is a complex process requiring proper functioning of the cascade of events to achieve successful gene silencing.

Several factors that may influence silencing onset and severity of the resulting phenotypes are considered, including transgene structure, copy number, and expression level. Efficient silencing onset was achieved either with highly transcribed single transgene copies or with the DNA constructs containing two inversely repeated transgene
copies which do not require a high level of expression (Vaucheret et al., 2001). Furthermore, the frequency of silencing is often reported as being dependent on the transgene copy number (Wang et al., 2005). It was found to increase gradually as the transgene copy number rose (Mitsuhara et al., 2002). It is assumed that the expression level of the transgene, which is crucial for successful silencing (Que et al., 1997), depends significantly on the site of chromosomal integration (Vaucheret et al., 1995; Day et al., 2000; van Leeuwen et al., 2001). Silencing was usually initiated in both homo- and hemizygous transformants, but the frequency was commonly higher in homozygous plants (Dehio and Schell, 1994; Kalantidis et al., 2006). Some transgenes were able to induce silencing only when present in a homozygous state (de Carvalho et al., 1992; de Carvalho Niebel et al., 1995; Vaucheret et al., 1995; Kunz et al., 1996). Besides the factors linked to the nature of the transgene, the target gene expression may also play a role in effective onset of PTGS. Expression of the endogenous chitinase gene in tobacco shows a dramatic, transient increase just prior to the onset of silencing in co-suppressed transgenic plants containing the chitinase transgene (Kunz et al., 1996). Moreover, Kalantidis and co-workers showed that only plants with a high steady-state concentration of the target gene mRNA could enter the systemic pathway of silencing, while the remaining plants developed only short-range cell to cell silencing (Kalantidis et al., 2006). It should be emphasized that the real impact of most of these factors on gene silencing is still a matter of debate. While some reports claim their significant role in this phenomenon, according to other data they do not influence PTGS in any substantial way.

Interestingly, some environmental conditions (light intensity and temperature) were described as factors that modulate silencing onset. Low temperature may inhibit PTGS (Szütyi et al., 2003). Temperature stress (high or low) was reported to affect not only the frequency but also the severity of silencing (Meza et al., 2001). Silencing of the *Nicotiana plumbaginifolia* gnl transgene occurred earlier when plants were grown under a short day photoperiod and strong light intensity (de Carvalho Niebel et al., 1995). Under these conditions, plants develop faster, indicating that the onset of gene silencing may correspond to a certain stage of development.

Silenced genes can be reactivated in the process called resetting (Meins and Kunz, 1994). Reactivation occurs in calli induced from silenced leaves and in proliferating tissues such as meristems and developing flowers before meiosis and maturation of germ cells (Mitsuhara et al., 2002). When the proliferation is complete, the systemic silencing signal is transferred within the flower from older tissues to younger ones. However, seeds are isolated and remain free of silencing.

Apart from resetting found in proliferating tissues, the recovery from silencing may occasionally result in reversion events (Kanazawa et al., 2007). Petunia transgenic white-flowering plants with co-suppressed chalcone synthase sporadically give rise to revertant branches with wild-type pigmentation. The revertant tissues showed repressed transcription of the transgene which normally induces co-suppression of the chalcone synthase gene. It was shown that the observed repression of the transgene resulted from the somatic conversion from PTGS to TGS associated with cytosine methylation in the promoter region of the transgene.

One of the most intriguing features of gene silencing is the commonly observed variety of silencing-associated phenotypes occurring between independent transformants or even within progeny of a single plant line (Meza et al., 2001; Wesley et al., 2001; Wang et al., 2005; Kalantidis et al., 2006; Li et al., 2007). According to some reports, the severity of silencing-induced phenotypes is correlated with depression of the target gene transcript level (Wang et al., 2005). This finding may explain the molecular background of phenotypic variety but does not answer the question of why genetically identical transgenic plants develop such a diversity of silencing efficiency. To address this question, independent transgenic *Arabidopsis thaliana* lines with a silenced *Rps10* gene encoding the S10 protein of the small subunit of mitochondrial ribosomes were analysed. These lines developed high variability of morphological anomalies. A part of this variability can be attributed to the homozygous versus hemizygous state of the silencing-inducing transgene, while the remaining diversity resulted from different timing of the onset of gene silencing. The correlation between the onset and accumulation of the target gene transcripts as well as developmental and environmental regulation of the onset of silencing are discussed.

**Materials and methods**

**Plasmid constructs**

The 355 bp of the 5′ region of the *A. thaliana* *Rps10* coding sequence was amplified from *A. thaliana* Col-0 cDNA using forward (5′-ATCTAGACTCGAGTGCGCCGATCTTCA-CTC-3′) and reverse (5′-TGGATCCGAATTCAGAGCATCAGCTCCCTCTAC-3′) primers. Primers were enriched with internal (*XhoI*, *EcoRI*) and external (*XbaI*, *BamHI*) restriction sites designed to perform a two-step cloning into the pHannibal vector (Wesley et al., 2001). The obtained plasmid contained two inverted copies of the *Rps10* cDNA fragment spaced by the pHannibal intron and inserted between the cauliflower mosaic virus (CaMV) 35S promoter and the OCS terminator. The entire DNA construction was excised with *NotI* and ligated into the binary vector pART27 (Gleave, 1992), yielding the pART27-*Rps10* plasmid.

**Agrobacterium-mediated transformation of Arabidopsis thaliana**

The pART27-*Rps10* plasmid was introduced into *Agrobacterium tumefaciens* strain LBA4404 by electroporation. The obtained bacterial strain was used for the floral dip
vacuum infiltration of the *A. thaliana* Col-0 plants as described by Desfeux et al. (2000).

**Selection of transgenic plants and plant growth conditions**

Seeds were germinated on kanamycin-containing (50 mg l⁻¹) half-strength MS medium (Murashige and Skoog, 1962) for 3 d at 4 °C in a dark place and then for 11 d in either a 16 h light/8 h dark (long day; LD) or a 8 h light/16 h dark (short day; SD) photoperiod at 22 °C. The kanamycin-resistant plants were transferred to soil. Photoperiod and temperature conditions during the growth in soil were the same. The presence of the transgene was further confirmed by PCR analysis using primers specific for the neomycin phosphotransferase gene located in the pART27 plasmid: forward (5'-TGGCAATTACCTTATCCG-3'), reverse (5'-AGAACTCGTCAAGAGGC-3').

**Genetic analysis of transgenic lines**

From the four primary T₁ transformants two plants presented phenotype alterations and they gave rise to two independent transgenic lines Rps10.1 and Rps10.2. In order to determine the number of T-DNA loci, the segregation ratio of the dominant kanamycin resistance trait was estimated in the T₂ generation seeds (collected from the primary T₁ transformants). The calculated ratios of resistant versus sensitive seedlings were compared with the segregation ratios 3:1, 15:1, 63:1 expected for one, two, and three loci insertions, respectively. In order to confirm the reliability of the selection data, statistical analysis using the χ² test was performed and the P-value was estimated.

**Nucleic acids isolation and cDNA synthesis**

Nucleic acids were extracted either from fresh plant tissue or from samples frozen in liquid nitrogen and stored at – 80 °C. The genomic DNA was isolated using a GeneMATRIX Plant & Fungi DNA Purification Kit (EURx, Poland).

The total RNA was isolated using a GeneMATRIX Universal RNA Purification Kit (EURx, Poland) according to the manufacturer’s instructions. The RNA obtained was treated with DNase (1 U of DNase, 37 °C, 30 min). DNase was inactivated by adding EDTA to a final concentration of 2.5 mM and incubation at 65 °C for 10 min. The reverse transcription reaction was performed using 5 μl of RNA, random hexamers, and a reverse transcription kit (Invitrogen) according to the manufacturer’s instructions. Resulting cDNAs were Rnase treated (1 U of Rnase, 37 °C, 30 min) and used as a template for the quantitative real-time PCR.

**Real-time PCR analysis of the transgene copy number, transgene transcript level, and Rps10 transcript level**

The real-time PCR analyses were performed using the DNA or cDNA samples obtained from the T₄ generation of the segregating Rps10.1 line and homozygotes of the Rps10.1.1 line. Relative quantification analysis using the second derivative maximum method of the LightCycler v 4.0 software was used. The wild-type *A. thaliana* plants or the control plants containing the empty vector served as the calibrator, and the actin gene *Act2* (At3g18780) was used as the reference. The values of amplification efficiency of the analysed amplimers were calculated by the LightCycler v 4.0 software based on the standard curve. The standard curves were generated using six serial 2-fold dilutions of the cDNA or the DNA sample obtained from the tissue of the wild-type or control plant. The LightCycler 2.0 instrument and the Real-Time 2×PCR Master Mix SYBR version B (A&A Biotechnology, Poland) were used. Reactions were carried out in a total volume of 10 μl with a final concentration of 0.5 μM primers. The following oligonucleotides were used as primers: Rps10 specific (forward 5'-ATTITCTCCAGAAATGTCA-3', reverse 5'-CTCATCTCAATCGTCTCA-3'), transgene specific (forward 5'-TGTAGAAATCCATGTC-3', reverse 5'-AAATGTAAGAATGTAAC-3'), actin specific (forward 5'-ATCGAGAGATGATGATTAC-3', reverse 5'-AAAGTCCTGTATTCTT-3'). Primers specific for the transgene-targeted pHannibal intron sequence allowed a distinction to be made between Rps10 and the transgene transcripts. The protocol consisted of three programs: denaturation, 95 °C for 1 min; amplification, 40 cycles at 95 °C for 8 s, 51°C (actin and Rps10) or 57 °C (transgene) for 8 s, 72 °C for 12 s with single data acquisition; cooling, 40 °C for 30 s. The specificity of the amplification products was verified by analysis of the melting curve.

**Statistical analysis:** Fisher’s exact test was performed using online tools available at http://www.langsrud.com/fisher.htm. A P-value <0.05 was considered statistically significant.

**Results**

**Selection of transgenic *A. thaliana* lines**

In *A. thaliana* the mitochondrial S10 ribosomal protein is encoded by one nuclear gene *Rps10* (At3g22300). The aim of this study was to silence the *Rps10* gene using the binary vector pART27. From the single transformation experiment, four primary T₁ transformants were obtained, but only two of them exhibited morphological abnormalities. These two plants gave rise to two transgenic lines named Rps10.1 and Rps10.2. The presence of the transgene was confirmed by PCR analysis. The segregation analysis of the T₂ generation of both Rps10.1 and Rps10.2 lines based on kanamycin resistance revealed a ratio of resistant:sensitive plants close to 3:1 (99:26, P=0.3 for Rps10.1; and 109:35, P=0.85 for Rps10.2). This segregation ratio indicates a single locus insertion for each line. In the T₃ and following generations of both transgenic lines, the seeds were collected from individual plants. Consequently, plants used in each of the described experiments were siblings of a single parental plant. For four generations,
plants of both lines produced kanamycin-resistant and sensitive offspring, indicating that Rps10.1 and Rps10.2 are segregating lines. Within the T4 generation of the Rps10.1 line, one homozygous plant able to produce all kanamycin-resistant seeds was found. It gave rise to the homozygous line named Rps10.1.1.

Zygosity of five morphological phenotypes found in transgenic lines

It was expected that silencing of the Rps10 gene would be related to phenotypic abnormalities including plant morphology since the gene product could be essential for mitochondrial biogenesis. To characterize plant abnormalities putatively related to silencing of the Rps10 gene, transformants of the Rps10.1, Rps10.1.1, and Rps10.2 lines were grown in the LD photoperiod optimal for A. thaliana and in the SD photoperiod which favours vegetative growth. Plant phenotypes were analysed under both photoperiods since in the segregating lines a significant increase in the proportion of plants with altered morphology was observed under SD compared with LD conditions. The morphology of transgenic plants was evaluated versus wild-type plants or plants transformed with an empty vector. The phenotypes observed in the SD photoperiod were classified into five categories (P1, P1r, P2, P3, and P4) depending on the time of their onset and severity of morphological abnormalities (Fig. 1). Details of these phenotypes will be described later with reference to Fig. 1. Since the homozygous Rps10.1.1 line consisted only of P1 and P1r plants, the P1 plants of the segregating lines were also expected to represent transgenic homozygotes. The P2,

Fig. 1. Phenotype categories P1–P4 observed under short day conditions in the Rps10.1 line. (A–F) Comparison of the phenotypic categories observed during the vegetative growth phase between the third and 13th week. (G) Magnification of 13-week-old P2, P3, and P4 plants. (H) Leaves from 13-week-old plants of different phenotype categories. (I) Variability of severe (P2), mild (P3), and weak (P4) phenotypes observed in the generative growth phase.
P3, and P4 plants (found only in segregating lines) were predicted to be hemizygous. To verify this possibility, real-time PCR analysis was applied using the transgene-specific primers and genomic DNA of the P1 and P1r plants of the Rps10.1.1 line and P1–P4 plants of the Rps10.1 line. The Rps10.1 plants used in this experiment were siblings from the same hemizygous parent. This experiment proved the homozygosity of all the tested P1 and P1r plants (including P1 plants from both Rps10.1 and Rps10.1.1 lines), while the P2, P3, and P4 plants from the Rps10.1 line appeared to be hemizygous (Table 1).

**Homozygous plants present severe morphological abnormalities and developmentally early onset of silencing**

Most (83%) of the homozygous Rps10.1.1 plants displayed the P1 phenotype characterized by the developmentally early onset of severe morphological abnormalities. By the third week the P1 plants were distinguished by small size and the production of small undulated leaves which became yellow with time (Fig. 1A). They were unable to reach the generative phase and even the vegetative growth phase was prematurely inhibited. Seventeen percent of the Rps10.1.1 plants presented a phenotype very similar to P1 for the first 4–5 weeks; however, after that time, they started to produce normal leaves, flowers, and siliques. This plant phenotype was named P1r (Fig. 1B) suggesting that reversion from the silenced state has occurred during the vegetative growth.

To verify that the P1 phenotype was attributed to the decrease of the Rps10 transcripts and that the P1r phenotype resulted from reversion of silencing, real-time PCR was applied. As expected, at the age of 3 weeks, all plants of the homozygous line Rps10.1.1 presented strong silencing (Fig. 2A). This result indicated that the silencing, detected as a decrease of the Rps10 transcript level, was correlated with the onset of the morphological abnormalities. However, 10 weeks later, a lack of silencing was detected in young leaves of most of the analysed P1r plants (Fig. 2B). Only in two cases was weak silencing observed when compared with the control plants. Apparently, such a weak decrease in Rps10 transcripts was above the threshold needed to cause the phenotype disturbances. A more diverse pattern of silencing was observed in old leaves displaying the morphological alterations. Three out of eight P1r plants showed strong silencing, while the remaining plants displayed weak or no silencing. Taken together, reversion of young leaves to the wild-type phenotype observed in the late vegetative growth of P1r plants was associated with the inactivation of silencing. This inactivation may also happen in old leaves; however, old leaves could not regain the normal phenotype even if the Rps10 transcript level was increased to its normal value.

Three phenotype categories (P2, P3, and P4) were identified among the hemizygous plants of the segregating lines (Fig. 1). As described above, the homozygous plants of these lines presented the P1 phenotype. The P2, P3, and P4 phenotypes were characterized for the Rps10.1 plants grown in the SD photoperiod since this line under these conditions produced the greatest variety of morphological alterations.

The P2 category, similarly to P1, comprised plants of the developmentally early phenotype recognized between the third and the fifth week of growth (Fig. 1C). Initially, these two plant categories had been indistinguishable, but with further development the difference became clear. The P2 plants (Fig. 1C) grew almost as poorly as P1 and also produced undulated leaves (Fig. 1H), but these leaves were green. In older plants, the induction of numerous axillary buds located in the leaf axils was observed. This resulted in an abnormal phenotype characterized by many small rosettes with undulating leaves (Fig. 1G). Significantly shorter inflorescences producing a reduced number of small siliques were noticed during the generative phase (Fig. 1I).

To ascertain whether the early onset of the P2 phenotype corresponds to the decrease of the Rps10 transcript level, real-time RT-PCR was performed. Three-week-old seedlings were used to find the proportion of plants exhibiting

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**Table 1. Zygosity of plants presenting P1–P4 phenotypes from the Rps10.1.1 and Rps10.1 lines**

Plants exhibiting the most severe phenotype disturbances (P1 and P1r) irrespective of the zygosity of parental plants were confirmed to be homozygous due to double transgene dosage when compared with P2–P4 plants. At least two plants of each phenotype category were tested.

<table>
<thead>
<tr>
<th>Line</th>
<th>Plant</th>
<th>Estimated copy number</th>
<th>Homozygosity</th>
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<td>Rps10.1.1 (homozygous)</td>
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<td>1.00</td>
<td>+</td>
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<td>+</td>
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<td></td>
<td>P1 (3)</td>
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</tr>
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<td></td>
<td>P1 (4)</td>
<td>1.00</td>
<td>+</td>
</tr>
<tr>
<td></td>
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<td>+</td>
</tr>
<tr>
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<td>+</td>
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<tr>
<td></td>
<td>P2 (1)</td>
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<td>−</td>
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<td></td>
<td>P2 (3)</td>
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<td>−</td>
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<td>P2 (4)</td>
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<td>−</td>
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<tr>
<td></td>
<td>P3 (1)</td>
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<td></td>
<td>P3 (2)</td>
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</table>

**Similarly to homozygotes, the hemizygous P2 transformants present developmentally early onset of silencing but the morphological abnormalities are less severe**

Three out of eight P1r plants showed strong silencing, while the remaining plants displayed weak or no silencing. Taken together, reversion of young leaves to the wild-type phenotype observed in the late vegetative growth of P1r plants was associated with the inactivation of silencing. This inactivation may also happen in old leaves; however, old leaves could not regain the normal phenotype even if the Rps10 transcript level was increased to its normal value.

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**Gene silencing in Arabidopsis thaliana**

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silencing in the early stage of development (P1 or P2). A decreased transcript level (Rps10 transcript level <75% of the wild type) was detected in ~50% of the analysed plants in both photoperiods (Fig. 3A, B). This percentage is similar to the proportion of P1 and P2 phenotypes found among all plants of the Rps10.1 line in both photoperiods. This correlation allows the assumption that the 3-week-old plants exhibiting down-regulation of the Rps10 transcript level represent the P1 and P2 categories. Moreover, this result suggests that the onset of silencing responsible for the appearance of the P1 and P2 phenotypes occurs at a very early stage of vegetative growth.

**Fig. 2.** Silencing onset and reversion observed within homozygous plants of the Rps10.1.1 line. (A) All tested 3-week-old plants presented strong Rps10 silencing when compared with the control plants. (B) The Rps10 silencing estimated in 13-week-old revertant P1r plants was weak or was not detected in young leaves, while in the old leaves silencing was either strong or was not active. (C) The accumulation of the transgene transcripts in young and old leaves of the P1r plants.
Morphological abnormalities appearing developmentally late in hemizygous P3 and P4 transformants are linked to the late onset of silencing

Hemizygous plants growing under the SD conditions developed the late onset phenotypes classified as P3 or P4. The morphology of P3 plants was normal for at least 7 weeks (~17 leaves visible). After that time, a gradually increasing pool of plants with characteristic rosettes composed of two parts diverging in architecture was observed (Fig. 1D, G). The lower and older part was similar to the wild-type rosette. The architecture of the upper and younger part resulted from the induction of the axillary buds, which gave rise to numerous small rosettes of curly leaves resembling those of the P2 plants (Fig. 1H). Numerous rosettes of the P3 plants produced many shortened inflorescences. The increased number of inflorescences resulted in the characteristic bushy phenotype (Fig. 1I). Siliques were more abundant compared with the wild-type plants, but they were also the smallest of all those observed. The P4 category was characterized by plants developing phenotypic alterations restricted to generative organs only. The P4 plants had normal rosettes and usually one, albeit shortened, inflorescence (Fig. 1E, H, I); the siliques were more numerous and slightly smaller.

The next experiment was performed to verify if the delay in the phenotype onset observed between P2 versus P3 and P4 plants results from the delayed decrease of $Rps10$ transcripts. The transcript level in old and young rosette leaves was compared in 13-week-old P2, P3, P4, and control plants grown under the SD conditions. At this time of development, the P2 phenotypic alternations were visible in old and young leaves while in P3 they were restricted only to the young leaves. The rosette leaves of the P4 phenotype resembled those of the wild type. As expected, the decreased level of $Rps10$ mRNA was found only in P2 when old leaves were examined (Fig. 4A). However, when the transcript level was evaluated in young leaves, it was decreased in all P2 and P3 transformants analysed. Surprisingly, from two P4 plants examined, one presented decreased and one showed normal levels of $Rps10$ mRNA in young leaves. Taken together, the above observations support the previous conclusion that the early onset of silencing was restricted to the P1 and P2 plants and indicated that onset of $Rps10$ silencing in P3 was delayed until the late vegetative phase. The picture was not so clear for the P4 plants. Therefore, the $Rps10$ mRNA level in young rosette leaves, cauline leaves, and flowers of P2, P4, and control 16-week-old plants was checked (Fig. 4B). Three plants of each category were analysed. As expected, the $Rps10$ transcript levels were decreased in all tested organs of the P2 plants. The P4 plants contained a normal level of $Rps10$ mRNA in the rosette leaves, but significantly reduced amounts of transcripts were found in the cauline leaves and flowers when compared with the level of transcripts in the control plants. Thus, based on the two last experiments, it can be

**Fig. 3.** Developmentally early onset of silencing occurs in 50% of all tested 3-week-old plants grown in the short (A) and long (B) day photoperiod. It was assumed that silencing is active when the $Rps10$ transcript level was <75% of the wild-type (WT) level.
Fig. 4. The phenotype–silencing correlation assay based on real-time RT-PCR. Expression of the Rps10 gene and transgene was evaluated in Rps10.1 plants of various phenotypic categories grown in the short day photoperiod. (A) The developmentally late onset of silencing. The Rps10 transcript level in young and old leaves of 13-week-old P2, P3, and P4 plants was compared with the level in the control plants. While in the P2 plants silencing was found in both young and old leaves, in the P3 and P4 plants it was observed only in young leaves. (B) The developmentally late onset of silencing in the P4 phenotype. The Rps10 transcript level was compared in rosette leaves, cauline leaves, and flowers of the 16-week-old P2 and P4 with the level in the control plants. Rps10 gene silencing was observed in all analysed organs of P2 plants and only in cauline leaves and flowers of the P4 plants. (C) The correlation between transgene transcript level and silencing. Transgene transcript accumulation was evaluated using the same cDNAs as presented in B. The decreased level of the transgene transcript correlated with the Rps10 silencing in all analysed organs of the P2 plants. In the P4 plants, correlation was observed in rosette leaves and flowers. Only in the P4 cauline leaves was the Rps10 silencing–transgene correlation not observed.
concluded that the silencing onset in the majority of the P4 transformants occurs in the late vegetative or early reproductive developmental phase.

Except for the P4 category, the transgenic Rps10.1 plants presented delayed development which became particularly marked in the generative growth phase (the appearance of the first flower bud, the time of the first flower opening, and the completion of flowering). The developmental delay was visible in P3 and was the greatest in the P2 category (Fig. 5), correlating with the phenotype onset.

**P1, P3, and P4 phenotypes are not dependent on the site of transgene integration in the Arabidopsis genome**

The abnormal phenotypes observed in the segregating Rps10.2 line grown in the SD photoperiod were also characterized. It was possible to distinguish characteristic features of three phenotype categories equivalent to those described for the Rps10.1 line: P1, P3, and P4. The P2 phenotype was not observed. Consequently, it is probable that at least P1, P3, and P4 phenotypes result from the silencing of the Rps10 gene rather than from the transgene position effect.

**The late onset of Rps10 silencing resulting in the P3 and P4 phenotypes is not observed in the long day photoperiod**

While the Rps10.1 plants were grown in the SD photoperiod they all presented altered phenotypes: P1, P2, P3, or P4. However, it should be emphasized that within the same phenotypic category some variations were observed. The P1 plants differed in the intensity of yellowing of leaves and their age at death. The P2 category comprised plants of various rosette sizes. In the P3 plants, the phenotype alterations occurred at different stages of rosette development, resulting in diverse numbers of altered leaves. Finally, the P4 plants differed in the height of the inflorescence.

The switch from the SD to the LD photoperiod resulted in a dramatic decrease in the proportion of plants showing altered phenotypes. Based on observations carried out over three generations, it was estimated that nearly half of the plants developed phenotypic abnormalities under LD conditions (Table 2B). These plants represented either P1 or P2 phenotype categories (Fig. 6). The remaining plants, which did not develop any phenotypic changes, although they were observed until the late reproductive growth phase, were named P0 (Table 2). It was not clear if the P0 plants showed the wild-type phenotype due to the lack of silencing or due to inefficient silencing. To discriminate between these two possibilities the levels of the Rps10 transcripts in the rosette leaves (Fig. 7A) and flowers (Fig. 7B) of 7-week-old P0, P2, and control plants were compared. The results clearly demonstrated that the Rps10 gene was silenced in both the rosette leaves and flowers of the P2 plants. However, any sign of silencing was found in the P0 plants, indicating that the LD conditions prevent the late onset of silencing. Thus, the SD photoperiod not only stimulated more transformants to develop the altered phenotype, but it also induced P3 and P4 phenotypes characteristic only for this photoperiod (Table 2). The loss of the late silencing in LD compared with SD conditions was shown to be statistically significant using Fisher’s exact test ($P=1.55\times10^{-5}$ for T3; $P=4.72\times10^{-7}$ for T4; $P=8.47\times10^{-4}$ for T5). The decrease in the incidence of silencing-induced phenotype observed in LD versus SD conditions was also shown to be statistically significant ($P=2.89\times10^{-3}$ for T3; $P=9.92\times10^{-3}$ for T4; $P=5.86\times10^{-3}$ for T5).

Subsequently, the distribution of early (P1+P2), late (P3+P4), and P0 phenotype categories in plant populations of all generations grown under SD and LD conditions was analysed (Table 2). In most cases, the proportion of early...
and late phenotype categories observed under the SD conditions as well as early and P0 phenotype categories (LD conditions) was similar in the analysed generations ($P > 0.05$, Supplementary Table S1, available at JXB online). The distribution of phenotype categories was disturbed only in plants of the T5 generation grown under the SD conditions. It should be emphasized that a similar disturbance in distribution was not observed within plants of the same generation grown under the LD conditions, although all these plants were obtained from the same seed pool. Since a significantly different distribution of phenotypic categories was restricted only to a fraction of a single generation, it seems to be accidental.

Taking the statistical analyses together indicated that the number of P3 and P4 plants (late phenotype) in the SD corresponded to the number of the P0 plants in the LD. This analysis also suggested that the P0 plants maintaining a normal morphology in the LD photoperiod would develop phenotypical abnormalities of the P3 or P4 categories if grown under the SD conditions.

The correlation between severity of phenotype and efficiency of silencing

The real-time PCR experiments (Figs 4A, B, and 7) indicated that irrespective of the phenotype category, silencing in morphologically altered organs of hemizygous plants resulted in the $Rps10$ transcript level decreasing to 15–30% of the level found in the control plants. P2 plants with a $Rps10$ transcript level <15% of the control value were observed only sporadically. Thus the P2–P4 plants present a very similar silencing efficiency defined as the percentage difference between the $Rps10$ mRNA levels measured in the wild-type and transgenic plants. Consequently, the phenotype variety of the hemizygous P2, P3,
and P4 plants cannot be attributed to the silencing efficiency (which is very similar in all the plant categories) but rather to the time of silencing onset, which is significantly different.

On the other hand, the correlation between severity of phenotype and efficiency of silencing was observed when homozygous and hemizygous plants were compared. The Rps10 mRNA level in four 8-week-old dying P1 plants varied in the range of 2–12% (average 6%) of the control level (Fig. 8). The Rps10 transcript level was also measured in the hemizygous P2 plants capable of surviving the vegetative growth phase. When compared with the control plants, the P2 plants contained between 10% and 28% (average 18%) of the Rps10 transcripts. These data indicated that silencing efficiency was generally higher in homozygous than in hemizygous plants.

The correlation between transgene transcript level and silencing

Transgene transcript accumulation was tested in different organs which developed either before or following the onset of silencing. To address this issue, the transgene mRNA level in young rosette leaves, cauline leaves, and flowers of the 16-week-old P2 and P4 plants was investigated (Fig. 4C). As shown in the experiment presented in Fig. 4B, the Rps10 silencing was active in all examined organs of the P2 plants, but in the P4 plants it was observed only in cauline leaves and flowers. As expected, the level of transgene transcripts measured in the rosette leaves was much lower in the P2 plants already presenting active silencing than in the P4 plants where silencing was not observed in these organs. It was also not surprising that the transgene transcript levels were similarly low in flowers of both P2 and P4 plants since

Fig. 7. Developmentally late onset of silencing does not occur in the long day photoperiod. The Rps10 transcript level was compared in the rosette leaves (A) and flowers (B) of 7-week-old P0, P2, and control plants. The silencing was detected in both leaves and flowers of the P2 plants, while it has not been found in the P0 plants.
the \textit{Rps10} silencing was detected in flowers of both plant categories. However, the fact that the transgene transcripts were still abundant in P4 cauline leaves was rather intriguing considering that silencing was already active in these plant organs.

As shown in Fig. 2B, the phenotypic reversion detected in the P1r plants is associated with the inactivation of silencing. In the following experiment, the same plants as in the experiment presented in Fig. 2B were tested for the level of the transgene transcripts (Fig. 2C). The accumulation of the transgene mRNA in young and old leaves of the P1r plants was higher than in the same organs of the P2 plants. Thus, the reversion observed in the P1r plants did not result from the complete inactivation of transgene transcription.

**Late onset coincides with the increased \textit{Rps10} transcript level**

It was reported that the onset of RNA silencing may positively correlate with the endogenous transcript level of the silenced gene (Kunz \textit{et al.}, 1996; Kalantidis \textit{et al.}, 2006). The developmental stage associated with silencing of the \textit{Rps10} gene was determined only for the late onset of silencing (17–20 leaves visible in SD) occurring exclusively in the SD photoperiod. To establish if the late onset of \textit{Rps10} silencing is correlated with an increase in the \textit{Rps10} transcript level, the accumulation of the \textit{Rps10} transcripts was followed in wild-type \textit{A. thaliana} plants grown under LD and SD conditions. The total RNA was isolated from the tissue samples collected at the time points corresponding to the appearance of the cotyledons, second, fourth, and seventh leaf, and in the SD photoperiod also the 12th, 22nd, 46th, and 70th leaf. In the reproductive growth phase, the cauline leaves, young flowers, open flowers, and siliques were analysed. At the stages of cotyledons and two-leaf rosettes RNA was isolated from the aboveground part of the plant. At the remaining growth stages, the youngest plant organ available at the moment of sampling was always tested for the \textit{Rps10} transcript level. The profiles of development-dependent transcript accumulation under the SD and LD conditions were different (Fig. 9). In the LD photoperiod the vegetative phase was completed shortly after the morphological alterations connected with the onset of early silencing became visible. In contrast, in the SD photoperiod the vegetative phase was much longer and the noticeable increase in abundance of the \textit{Rps10} transcripts was observed at the growth stage corresponding to 12–22 leaves visible in the rosette. Thus, the increase in the abundance of \textit{Rps10} transcripts is correlated with the time when the first plants of the P3 category presented the onset of late silencing.

**Discussion**

Since it is generally believed that silencing is not inherited but established \textit{de novo} in each plant, it could be assumed that neither silencing efficiency nor the resulting phenotype of the individual plant can be predicted based on the characteristics of the parental transgenic plants. However, data concerning morphological alterations and diverse levels of the \textit{Rps10} gene silencing, collected over several generations of transgenic plants, allow prediction of the frequency and efficiency of silencing at the population level. Despite the fact that the fate of the single transformant cannot be foreseen, the proportion of the silencing-associated phenotypes can be predicted. The phenotype onset and its severity depend on intrinsic factors (transgene dosage and silencing competence of the transgene loci), but are also regulated by environmental conditions (day length). The present results have shown that the silencing-related phenotype of the homozygous transplants was uniform while the hemizygous plants presented a spectrum of morphological and developmental abnormalities. This phenotypic variety was observed only in the SD photoperiod and resulted from at least two events, separated in time, of the
onset of Rps10 silencing, while in the LD photoperiod only the early onset was initiated.

The silencing construct used in these studies contained an inversely repeated fragment of the Rps10 coding sequence. Consequently, the transgene locus was expected to produce dsRNAs corresponding only to the transcribed sequences of the target gene leading to PTGS. However, it is known that PTGS may be switched to TGS if the DNA methylation, originally present only in the coding sequence, is introduced into the promoter (Fojtova et al., 2003). It has also been shown that the same transgene locus may result in both TGS and PTGS (Mourrain et al., 2007). Thus, since neither siRNA measurements nor nuclear run-on assays were performed, the possibility cannot be excluded that TGS contributes to gene silencing detected in the present experiments.

One of the factors influencing the Rps10 silencing efficiency as well as the phenotypic diversity of transgenic plants was the transgene dosage. The silencing efficiency was always higher in the homozygous plants (the Rps10 transcript level decreased to 2–12% relative to the control plants) compared with the hemizygous plants (15–30%). In contrast to the hemizygous transformants, all progeny of the homozygous plants exhibit silencing very early in both applied photoperiods (P1 phenotype) and developed nearly homogenous severe phenotypic aberrations leading to premature death. A higher frequency and greater severity of gene silencing in the homozygous transformants have already been reported (de Carvalho Niebel et al., 1995; Kunz et al., 1996; Kalantidis et al., 2006). It can be attributed to much more frequent occurrence of spontaneous short-range silencing observed in homozygous versus hemizygous transgenic plants (Kalantidis et al., 2006).

In contrast to the homozygous transformants displaying only one phenotype (P1), hemizygotes presented quite diverse morphology (P2, P3, and P4). It should be emphasized that the correlation between silencing efficiency and the resulting phenotypes was not observed within hemizygotes. The present data rather indicated that these phenotypes were associated with timing of the onset of silencing. The morphological phenotype, classified as P2, resulted from the early onset of silencing occurring most probably at the beginning of plant development, whereas the P3 and P4 phenotypes developed significantly later, with the first onsets observed in the middle vegetative growth phase. To date timing variability in silencing onset either between different lines or within individual transgenic lines has rarely been described (Correa et al., 2004; Kalantidis et al., 2006).

The variation in timing of the onset of silencing observed between independent Rps10.1 and Rps10.2 transgenic lines may result from different silencing competence of the given transgene loci determined by the positional effects (de Carvalho Niebel et al., 1995; Vaucheret et al., 2001). The hemizygous Rps10.1 plants developed either early or late onset, while all hemizygous transformants of the Rps10.2 line developed only the late onset of silencing. Thus, it is possible that the early onset responsible for the appearance of the P2 phenotype may result from higher silencing competence of the transgene loci present in the Rps10.1 line. The two remaining hemizygous phenotypes—P3 and P4—are observed in both transgenic lines but only in the SD photoperiod. It seems that the absence of the P3 and P4 phenotypes in the LD photoperiod was caused by the plants’ inability to develop gene silencing under these conditions. Thus, the late Rps10 silencing can be described as conditional on the environment. This finding is especially interesting because environmental control of gene silencing in plants is still hardly known. Low temperature is the only environmental factor known to inhibit PTGS (Li et al., 2007). A photoperiod characterized by day length decrease from 16 h to 14 h accompanied by increased light intensity was found to result in earlier initiation of gnl gene silencing in...
N. plumaginifolia (de Carvalho Niebel et al., 1995). However, since two environmental clues (day shortening and increased light intensity) were applied, it is difficult to compare these results with the present observations, especially as N. plumaginifolia plants developed more rapidly, while A. thaliana develops more slowly in the SD photoperiod. Moreover, in the case of the gnl gene, the environmental conditions resulted in a shift in the onset of silencing, while the present data indicated that the selected photoperiod was the crucial factor determining whether or not late onset of silencing occurred.

The most intriguing issue is why some hemizygous Rps10.1 plants developed early onset of silencing while in the remaining hemizygotes the Rps10 gene became silenced much later. It is possible that this difference may result from the timing and precise location of silencing initiation. The chances of a successful systemic spread of silencing is higher if initiation occurs early and close to the vascular tissue (Kalantidis et al., 2006). It is proposed that early onset of gene silencing in the hemizygous plants occurs only if the transgene loci are of high silencing competence (a condition fulfilled by all hemizygous Rps10.1 plants) and if the initiation takes place in the vicinity of vascular tissue (since the location of silencing initiation is stochastic, this condition is fulfilled only by the random pool of the Rps10.1 plants).

The late onset of silencing of the Rps10 gene occurred only if plants were grown under the SD conditions. The candidate factors responsible for the late onset of silencing are the length of the vegetative phase and the transcript level of the Rps10 gene. In the LD photoperiod, the vegetative phase was very short (4 weeks) and the level of the Rps10 transcripts decreased with the transition from the vegetative to the generative phase. Under the SD conditions, the vegetative growth phase of Arabidopsis was much longer (up to 14 weeks) and the level of the Rps10 mRNA gradually increased until the end of the generative phase. Consequently, beginning from the middle vegetative growth phase, plants grown in the SD photoperiod were continuously exposed to circumstances favourable for successful silencing onset. This period was long enough to allow each plant to develop phenotypic abnormalities associated with gene silencing not later than in the early generative growth phase. Assuming that the dynamics of the increase in Rps10 transcript may vary in individual plants, the diverse timing of onset observed between the P3 and P4 plants can be explained. This model of gene silencing initiation dependent on the level of the gene transcripts is similar to the threshold model described by Smith et al. (1994). The authors propose that plant cells have a threshold below which they can accommodate a specific RNA species. Once this threshold is exceeded, the concentration of transcripts is lowered by specific targeting and mRNA degradation. It is proposed that the successful onset of silencing not only depends on the mRNA level exceeding the threshold but it also requires that this favourable condition is maintained for longer time periods. The relatively high abundance of the Rps10 transcripts observed in the SD photoperiod may be one of the elements of the response of plant mitochondria to the stress induced by the elongated dark phase. Shortened photosynthesis and an elongated dark phase enhance the role of the mitochondria in energy production. Since S10 is one of the protein constituents of mitochondrial ribosomes, a deficiency of this protein can disturb mitochondrial functions.

Another possible explanation for the differences observed in silencing onset in the LD and SD photoperiod could be the properties of the transgene promoter region. The transgene expression is driven by the CaMV 35S promoter which has been shown to be up-regulated by the SD photoperiod (Schnurr et al., 2000).

It was found that ~17% of the homozygous P1 plants were able to switch off silencing in the late vegetative growth stage, resulting in normal or close to normal levels of the Rps10 transcripts and wild-type morphology of young leaves. Silenced genes can undergo spontaneous and developmentally regulated reactivation by a process called resetting (Meins and Kunz, 1994; Kunz et al., 1996). Release from PTGS was found in meristems and developing leaves and flowers (Mitsuhara et al., 2002). When cell proliferation is completed, a systemic silencing signal is transferred from older to young tissue and induces gene silencing. The results indicate, however, that silencing inactivation is not restricted only to young newly developed organs. It occurs even in tissues where silencing onset has already occurred, resulting in severe morphological abnormalities. Thus, the reversion observed in the case of the P1r plants is apparently not associated with cell proliferation. Reversion of the PTGS phenotype was previously described by Metzlafl et al. (1997) and Kanazawa et al. (2007) in petunia. In this species, reversion was linked to the transcriptional repression of the transgene which normally induces silencing. It was demonstrated that epigenetic changes occurred in the PTGS line and it was suggested that these changes interfere with the initiation of transgene transcription, leading to a reversion of the PTGS phenotype. However, despite transcription repression, the transgene mRNA level detected in flowers of revertants was higher than in the flowers of silenced petunia. This observation is very similar to what was found in the present experiments—the transgene inducing the silencing of the Rps10 gene accumulates in the revertant plants at a level higher than in the silenced transformants. Thus it cannot be excluded that the reversion mechanism described by Kanazawa et al. (2007) also operates in the case of the P1r plants. Interestingly, under the present experimental conditions, inactivation of silencing was limited only to the homozygous plants, suggesting that it is induced by a very low Rps10 transcript level found only in homozygotes.

Based on the present results, it is concluded that the onset of Rps10 silencing depends on genetic and environmental factors. The main novelty of this work is the finding that the photoperiod conditions could influence the onset of gene silencing. It was demonstrated that the photoperiod length is crucial for the developmentally late Rps10 silencing onset resulting in the appearance of P3 and P4 plants.
Supplementary data

Supplementary data are available at JXB online.

Table S1. Statistical analysis of the distribution of the early (P1+P2) and late (P3+P4) or P0 phenotypes in Rps10-silenced plants in various generations (T2–T5) and photoperiods (SD, LD). Statistically significant P-values from Fisher’s exact test are shaded.

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