

## Recurrent parent similarity index

Six characters (upper petal reflexing, lateral petal reflexing, pistil length, stamen length, lateral petal width and nectar volume) for which QTLs have been mapped<sup>6,7</sup> were measured on two flowers from each plant. There was a significant difference between the multivariate flower phenotypes of wild-type and 'mutant' NILs in both the *M. lewisii* (multiple analysis of variance, MANOVA,  $F = 18.18$ , Wilks'  $\lambda = 0.32$ ,  $P < 0.0001$ ) and *M. cardinalis* (MANOVA,  $F = 11.00$ , Wilks'  $\lambda = 0.56$ ,  $P < 0.0001$ ) genetic backgrounds (PROC GLM, SAS Institute). Least-squares means for each trait within each NIL genotypic class were normalized to the difference between trait means of the two parental species<sup>7</sup>, setting the recurrent parent trait value at 100% and the nonrecurrent parent at 0%. Lower recurrent parent similarity (RPS) values are evidence of linkage drag, whereas values larger than 100% represent measurement error or heterosis. In the *M. lewisii* genetic background, the wild-type plants had a mean RPS index across all traits of 91% (range 66–108%), whereas their 'mutant' sibs had a value of 80% (range 51–103%). In the *M. cardinalis* genetic background, the wild-type plants had a mean RPS index of 95% (range 49–129%), whereas their 'mutant' sibs had a value of 80% (range 46–155%). Although 'mutant' NILs show more linkage drag than the wild type, we judge the difference to be small. Nectar volume, which is known from our F<sub>2</sub> experiments to have a marked effect on hummingbird visitation<sup>8</sup>, has RPS index values that are very close to one another in the NILs: 105% and 103% in the *M. lewisii* background, and 46% and 53% in the *M. cardinalis* background. This suggests that differences in nectar production between pairs of NILs did not affect pollinator visitation patterns.

## Pollinator visitation

For each of two field experiments conducted to measure pollinator visitation, 50 pink or dark pink (*YUP/*\_\_\_) and 50 yellow-orange or red (*yup/yup*) plants were drawn at random from five BC<sub>4</sub>S<sub>1</sub> (*M. lewisii*) or BC<sub>4</sub> (*M. cardinalis*) NIL families. Assessments of pollinator visitation were performed at Mather (California, USA), the site where much of the previous work on these two species of *Mimulus* has been done<sup>5</sup>. Pollinator observations were carried out from dawn to evening, with a 1–2 h break at midday when pollinators were least active. Dates of observation were 18–30 August 1999 for *M. cardinalis* NILs, and 18–27 July 2000 for *M. lewisii* NILs. These dates correspond closely to the peak flowering times of natural populations of the two *Mimulus* species. We chose to do the experiments in different years so that pollinators were faced with a binary choice of flower phenotypes, as would be the case for a newly arisen mutation. Plants were placed at random on a 1 m × 1 m grid to produce the experimental arrays (a black bear visit reduced the total sample size in the *M. lewisii* NIL array from  $N = 100$  to  $N = 99$ ). A pollinator visit was counted if it appeared that the pollinator probed the flower and contacted the anthers or stigma. Bumblebees and hummingbirds were the only pollinators observed. We observed 1,090 bumblebee visits to the *M. lewisii* NILs, 180 bumblebee visits to the *M. cardinalis* NILs, 201 hummingbird visits to the *M. lewisii* NILs, and 3,738 hummingbird visits to the *M. cardinalis* NILs. The number of flowers on each plant was recorded daily, along with the number of hours spent observing. Visitation rates were calculated by dividing the total number of pollinator visits across all days by the aggregate number of hours in which visits could have occurred to each flower (flower-hours). For the *M. lewisii* NILs, both bumblebee and hummingbird pollinator observations were carried out simultaneously, with 47,159 flower-hours for the wild-type NILs and 138,648 flower-hours for the 'mutants'. For the *M. cardinalis* NILs, separate pollinator observation periods were required to keep track of the large number of hummingbird visits. During the bumblebee observation periods, there were 16,291 flower-hours for the 'mutant' NILs and 13,556 flower-hours for the wild-type. During the hummingbird observation periods, there were 11,505 flower-hours for the 'mutant' NILs and 9,520 flower-hours for the wild type.

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## Light-induced hormone conversion of T<sub>4</sub> to T<sub>3</sub> regulates photoperiodic response of gonads in birds

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Reproduction of many temperate zone birds is under photoperiodic control. The Japanese quail is an excellent model for studying the mechanism of photoperiodic time measurement because of its distinct and marked response to changing photoperiods. Studies on this animal have suggested that the mediobasal hypothalamus (MBH) is an important centre controlling photoperiodic time measurement<sup>1–8</sup>. Here we report that expression in the MBH of the gene encoding type 2 iodothyronine deiodinase (Dio2), which catalyses the intracellular deiodination of thyroxine (T<sub>4</sub>) prohormone to the active 3,5,3'-triiodothyronine (T<sub>3</sub>), is induced by light in Japanese quail. Intracerebroventricular administration of T<sub>3</sub> mimics the photoperiodic response, whereas the Dio2 inhibitor iopanoic acid prevents gonadal growth. These findings demonstrate that light-induced Dio2 expression in the MBH may be involved in the photoperiodic response of gonads in Japanese quail.

The molecular mechanism of photoperiodic or seasonal time measurement is not well understood in any organism studied so far. In birds, the MBH—which includes the nucleus hypothalamicus posterior medialis (NHPM), the infundibular nucleus and the median eminence—is an important centre controlling photoperiodic time measurement (Supplementary Figs 1 and 2). For example, introduction of a lesion to the nucleus hypothalamicus posterior medialis and/or the infundibular nucleus resulted in loss of photoperiodic response of the gonads<sup>1–3</sup> even though the gonadotrophin-releasing hormone (GnRH) system of the lesioned animal had been left intact<sup>4</sup>. Electrical stimulation of this area increases luteinizing hormone secretion<sup>5</sup> and induces testicular growth<sup>6</sup>. Furthermore, c-Fos expression has been reported in these structures as a result of photostimulation for one long day (20/4 h light/dark cycle)<sup>7,8</sup> and deep-brain photoreceptors are thought to be localized in the infundibular nucleus<sup>9</sup>. Recently, we have also observed the expression of circadian clock genes in the MBH, and proposed that the clock in the MBH may function as the 'photoperiodic clock'<sup>10</sup>. These observations indicate that all of the essential machinery for photoperiodic time measurement is localized in the MBH.

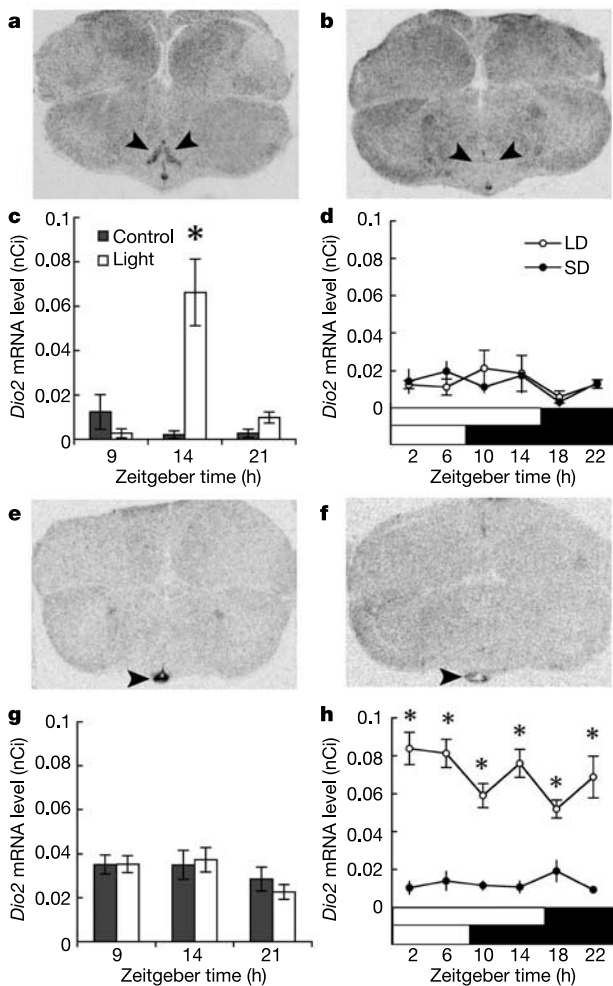
Single light pulses within the photo-inducible phase increase

serum luteinizing hormone concentration and cause photoperiodic response of the gonads<sup>11</sup>. Therefore, it is expected that some molecular events take place in the MBH when animals are exposed to light within the photo-inducible phase. To identify genes that are responsible for the regulation of photoperiodic time measurement in birds, we performed a differential subtractive hybridization analysis. A total of 40 8-week-old male Japanese quail were raised under short day conditions (8/16 h light/dark cycle). From this group, 20 animals were exposed to a 1-h light pulse at zeitgeber time 14 (ZT14), whereas the other 20 animals were kept in darkness. One hour after the end of the light pulse (ZT16), both groups of animals were killed and the MBH was removed (Supplementary Fig. 1). Differential subtractive hybridization analysis was carried out and 150 clones were sequenced. Expression of all of these genes was verified using *in situ* hybridization. Among them, expression of only

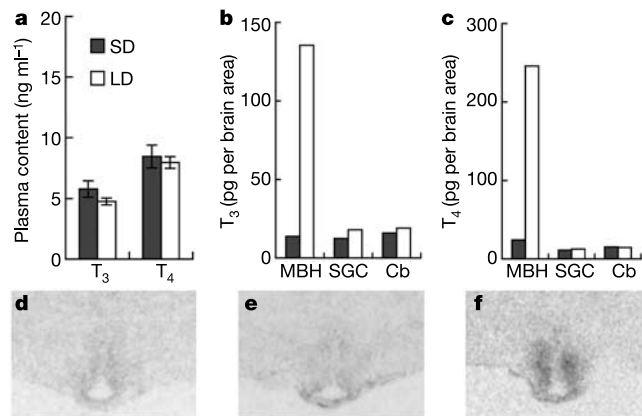
one gene, *Dio2*, was significantly induced by the light pulse within the photo-inducible phase (ZT14) in the dorsal hypothalamus around the paraventricular organ (PVO) as well as the lateral hypothalamus<sup>12</sup> (Mann–Whitney *U*-test,  $P < 0.01$ ; Fig. 1a–c). (More details of localization and the schematics of quail brain can be seen in Supplementary Fig. 2.) We then examined the effect of light exposure at times outside the photo-inducible phase (ZT9 and ZT21). However, light-induced expression of *Dio2* was not observed at ZT9 or ZT21 (Mann–Whitney *U*-test;  $P > 0.05$ ), which indicates that the light-induced expression of *Dio2* is specific to the photo-inducible phase (Fig. 1c).

We next examined the expression profiles of *Dio2* in the dorsal hypothalamus under short and long day conditions. Unexpectedly, expression of *Dio2* in the dorsal hypothalamus was almost undetectable at any time of day, whether examined under long or short day conditions (Fig. 1d). Notably, expression of *Dio2* was observed in the infundibular nucleus and the median eminence (both of which are collectively known as the basal tuberal hypothalamus; BTH) (Fig. 1e, f; see also Supplementary Fig. 2). Consequently, we examined the effect of light on the expression of *Dio2* in these regions. Although acute induction of *Dio2* expression by light pulse was not detected at any of the phases examined (Mann–Whitney *U*-test,  $P > 0.05$ ; Fig. 1g), significant differences in the level of expression between short day (weak expression) and long day (strong expression) conditions were observed in these regions (two-way analysis of variance (ANOVA),  $F_{1,41} = 219.917$ ,  $P < 0.0001$ , \* $P < 0.05$ , Mann–Whitney *U*-test; Fig. 1e, f, h). No clear day/night difference in expression was observed in either long day or short day conditions (one-way ANOVA: long day,  $F_{5,24} = 2.273$ ,  $P = 0.0795$ ; short day,  $F_{5,17} = 0.861$ ,  $P = 0.5269$ ). It has been shown that introduction of a small lesion to the nucleus hypothalamicus posterior medialis (including the PVO) or the infundibular nucleus, or a combination of both lesions, completely blocks testicular development<sup>1</sup>. The expression sites of *Dio2* revealed in our study are consistent with these results. Although it remains to be determined how these two structures are related to each other, these results suggest that expression of *Dio2* in both regions is of critical importance for the photoperiodic response of the gonads.

The enzyme *Dio2* converts  $T_4$  to  $T_3$ , which is primarily responsible for thyroid hormone action. *Dio2* has an essential role in the local control of levels of  $T_3$  in the brain through mechanisms that operate under a variety of conditions to maintain  $T_3$  concentrations within a narrow range<sup>13</sup>. To determine the levels of  $T_3$  and  $T_4$  in the



**Figure 1** Identification of a gene that controls photoperiodic time measurement. **a, b**, Representative autoradiograms for light-induced expression of *Dio2* (**a**) and a control animal without light (**b**) in the dorsal hypothalamus (arrowhead). A single 1-h light pulse was given at ZT14, and brain was collected 1 h after the end of the light pulse. **c**, Light-induced *Dio2* expression in the dorsal hypothalamus was specific to the photo-inducible phase. Values are mean  $\pm$  s.e.m. ( $n = 5$ ). Asterisk,  $P < 0.01$ . **d**, Temporal expression profiles of the *Dio2* gene under short day (SD) and long day (LD) conditions in the dorsal hypothalamus. Values are mean  $\pm$  s.e.m. ( $n = 3-5$ ). **e, f**, Representative autoradiograms of the BTH (arrowhead) of animals housed under long day (**e**) and short day (**f**) conditions. **g**, Effect of the light pulse on *Dio2* expression in the BTH. Values are mean  $\pm$  s.e.m. ( $n = 5$ ). **h**, Temporal expression profiles of the *Dio2* gene in the BTH in animals kept under short day and long day conditions. Values are mean  $\pm$  s.e.m. ( $n = 3-5$ ). Asterisk,  $P < 0.05$ .



**Figure 2** Locally generated  $T_3$  acts on the BTH. **a**, Plasma content of  $T_3$  and  $T_4$  under long day and short day conditions. **b, c**,  $T_3$  (**b**) and  $T_4$  (**c**) contents are increased in the MBH under long day conditions, whereas they are not changed in the stratum griseum centrale (SGC) and the cerebellum (Cb). The MBH collected from ten animals were pooled. **d-f**, Expression of thyroid hormone receptor genes *Thra* (**d**), *Thrβ* (**e**) and *Rxra* (**f**) in the BTH.

MBH, we measured the contents of pooled MBH collected from ten animals in each group under short day and long day conditions. Although the plasma concentration of  $T_3$  and  $T_4$  was not significantly different between short day and long day conditions (Mann–Whitney  $U$ -test,  $P > 0.05$ ; Fig. 2a), the contents of both in the MBH were about tenfold higher in long day than in short day conditions (Fig. 2b, c). This difference was not observed in other parts of the brain, such as the stratum griseum centrale and the cerebellum (Fig. 2b, c). Recently it has been reported that expression of the gene encoding transthyretin ( $Ttr$ ), which regulates thyroid hormone uptake, increases in Siberian hamster hypothalamus under long day conditions<sup>14</sup>. Although we could not detect expression of  $Ttr$  in the hypothalamus, strong expression was observed in the choroid plexus in Japanese quail; however, there was no difference in the expression of  $Ttr$  in the choroid plexus between long day and short day conditions (Supplementary Fig. 3). Therefore, the content of other type(s) of thyroid hormone transporters may increase in the MBH of Japanese quail under long day conditions that subsequently aid the generation of  $T_3$  through increased  $T_4$  uptake.

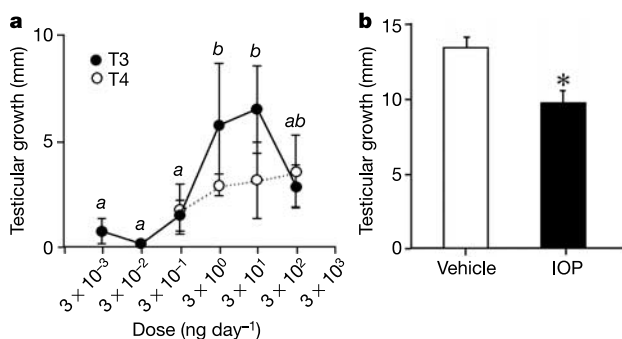
To explore the target site that locally generated  $T_3$  acts upon, expression of thyroid hormone receptor genes ( $Thra$ ,  $Thrb$ ,  $Thrb2$ ,  $Rxra$  and  $Rxrg$ , which encode thyroid hormone receptor- $\alpha$ , - $\beta$ , - $\beta 2$  and retinoid X receptor- $\alpha$  and - $\gamma$ , respectively) were examined. Among these genes, weak expression of  $Thra$  and  $Thrb$  and strong expression of  $Rxra$  was observed in the BTH, whereas expression of  $Thrb2$  and  $Rxrg$  was undetectable (Fig. 2d–f). Expression of  $Thra$ ,  $Thrb$  and  $Rxra$  was observed for the entire day under both short day and long day conditions, and did not show a difference between day and night (data not shown). These results indicate that locally generated  $T_3$  acts on the BTH. Although we have also examined expression of these genes in the preoptic area, where cell bodies of GnRH neurons are located, no signal was detectable under any conditions (data not shown). It has been suggested that photoperiodic GnRH release could be controlled at the GnRH terminals by glia<sup>8,15</sup>, and thyroid hormones are known to have a critical involvement in the development, plasticity and function of the central nervous system<sup>13</sup>. Therefore, we examined the morphological changes in the median eminence under long day and short day conditions. Immunoelectron microscopy revealed that GnRH nerve terminals are in close proximity to the basal lamina in birds subjected to long day conditions (T.Ya., K.H., S.E. and T.Yo., unpublished observations). We also found that the GnRH terminal was enclosed by the end feet of glia in animals subjected to short day conditions (T.Ya., K.H., S.E. and T.Yo., unpublished observations). These morphological changes may allow the neurons to secrete GnRH owing to the increased access of their terminals to the

perivascular area surrounding the fenestrated portal capillaries under long day conditions.

To assess whether  $T_3$  mediates the photoperiodic response of the gonads, we studied the effect of intracerebroventricular infusion of  $T_3$  on testicular growth. Vehicle and several doses of  $T_3$  and  $T_4$  were infused into the third ventricle by an osmotic mini-pump, and the testicular size was measured before and after infusion.  $T_3$  infusion induced testicular growth in a dose-dependent manner, even though the animals were kept under short day conditions (one-way ANOVA,  $F_{5,18} = 4.008$ ,  $P = 0.0128$ ; Fisher's LSD post-hoc test,  $P < 0.05$ ), whereas  $T_4$  infusion had only a minor effect (one-way ANOVA,  $F_{3,16} = 0.32$ ,  $P = 0.8111$ ;  $n = 3–6$ ; Fig. 3a). However, significant testicular growth was not observed in the largest dose of  $T_3$ . Iopanoic acid is known to inhibit the conversion of  $T_4$  to  $T_3$  (ref. 16), thus we examined the effect of iopanoic acid infusion and found that it reduced testicular growth under long day conditions ( $n = 4–5$ ; Mann–Whitney  $U$ -test,  $P < 0.05$ ; Fig. 3b).

Follett *et al.* have shown that peripheral injection of pharmacological doses of thyroid hormone ( $T_4$  and  $T_3$ ) can mimic photoperiodically induced gonadotrophin secretion and gonadal growth<sup>17,18</sup>. In these previous studies, however,  $T_4$  was more effective than  $T_3$  in mimicking gonadotrophin release. The findings seem to stand in contradiction to our results. In blood, however, about 33% and 45% of  $T_4$  is known to be converted to  $T_3$  and reverse  $T_3$  ( $rT_3$ ), respectively<sup>19</sup>. Furthermore,  $T_3$  is converted to 3,3'-diiodothyronine (3,3'- $T_2$ ) in the blood. Therefore, in the studies of Follett *et al.*, most of the peripherally delivered  $T_4$  might be converted to  $T_3$  and act on the central nervous system. In addition, we have shown that iopanoic acid, an inhibitor of Dio2, reduces testicular growth under long day conditions. Thus, our results are consistent with the hypothesis that Dio2 is important for regulation of the photoperiodic response of the gonads. Of note,  $T_3$  infusion did not maximize testicular size and iopanoic acid did not block testicular growth completely. There are two possible explanations for this finding. One is that  $T_3$  and iopanoic acid were subject to partial inhibition or partial loss through imprecise delivery. It is impossible to infuse at exactly the same location in all birds, and drugs may be diffused and metabolized through the cerebrospinal fluid in some. Variability in the effect of  $T_3$  seems to support this idea. The other possibility is the existence of other regulatory pathways or compensatory mechanisms. This possibility is supported by previous studies showing that after thyroidectomy, quail can still respond to photostimulation when transferred from short day to long day conditions, although increases in their follicle-stimulating hormone levels and in the sizes of testes and the cloacal gland are attenuated<sup>20,21</sup>. This is in contrast with the observation that after starlings and house sparrows are photoperiodically blind thyroidectomy<sup>22,23</sup>. The exploration of this mechanism is important for our eventual understanding of the complete molecular machinery of photoperiodism. Although thyroid hormone is of critical importance in the regulation of photoperiodically induced gonadal growth, as shown here, this hormone is also known to be involved in the regulation of photorefractoriness (insensitivity to previously stimulatory day-length)<sup>15,24</sup>. The observation that no significant testicular growth is induced at the highest dose of  $T_3$  (Fig. 3) suggests the possibility that photorefractoriness occurs as a result of downregulation of thyroid hormone activity. This hypothesis remains to be tested.

Although exogenous thyroid hormones mimic the effect of long day conditions, their precise role remains uncertain<sup>15</sup>. The present study has determined the target site and molecular events of  $T_4$  to  $T_3$  conversion that take place in the brain. Finally, it is also of interest to note that multiple studies indicate that thyroid hormones are essential for the maintenance of seasonal reproductive changes in a number of mammals<sup>25</sup>. Thus, our present study may provide the means for a collective understanding of the molecular mechanism of photoperiodic time measurement in all photoperiodic vertebrates. □



**Figure 3** Intracerebroventricular  $T_3$  infusion mimics photoperiodically induced testicular growth. **a**,  $T_3$  and  $T_4$  were infused under short day conditions. Testicular length was measured before and after infusion ( $n = 3–6$ ). Different characters (*a*, *b*) indicate significant differences ( $P < 0.05$ ). **b**, Iopanoic acid infusion reduces testicular growth under long day conditions ( $n = 4–5$ ). Asterisk,  $P < 0.05$ .

Methods

Animals

Four-week-old male Japanese quail (*Coturnix coturnix japonica*) were obtained from a dealer and kept under short day conditions (8/16 h light/dark cycle) in light-tight boxes. The boxes were placed in a room at a temperature of 24 ± 1 °C. Light was supplied by fluorescent lamps with a light intensity of 200 lx measured at the head level of the birds. Short day animals were kept under short day conditions whereas long day animals were transferred from short to long day conditions (16/8 h light/dark) for 2 weeks. Food and water were available *ad libitum* and were replenished at least twice a week. Animals were treated in accordance with the guidelines of Nagoya University.

Differential subtractive hybridization analysis

Brain slices (3 mm) of quail were generated by mouse brain matrix (ASI), and the MBH was punched out (3 mm diameter). Total RNA was prepared from 20 pooled MBH using Trizol reagent (Gibco BRL). Poly(A)<sup>+</sup> RNA was purified using oligotex-dt30 Super (Takara). Differential subtractive hybridization analysis was performed according to the manufacturer's instructions (PCR-select complementary DNA subtraction Kit, Clontech). Final PCR products were inserted into a TA cloning vector (Invitrogen) and sequenced by an ABIPrism 373 using the Big Dye Terminator kit (ABI).

In situ hybridization

Animals were killed by decapitation and the brains were immediately removed to avoid acute changes in gene expression. *In situ* hybridization was carried out as previously described<sup>26</sup>. Antisense and sense 45-nucleotide oligonucleotide probes were labelled with [<sup>33</sup>P]dATP (NEN) using terminal deoxyribonucleotidyl transferase (Gibco BRL). *Dio2*, 5'-GATGGTTCAGCCTCAATGAATATCAAGACGGAAATACATTCTGTA-3'; *Thra*, 5'-TTGATGGAATTGCCGGTGAATGGAA CAGAAGCCAGCAGCCCTGGAC-3'; *Thrb*, 5'-CGGAGTGAGAGAACAGAAAATGAAGCTCTAGTAAGGTGGCAGTGG-3'; *Thrb2*, 5'-TGAAGTGCACCCAGCTGCTGGTATGCAATTGCTACATGCAGTCCAC-3'; *Rxra*, 5'-GGCATGAGTTAAGCACCAGCGATGGACACCAAAACACTTCCTGCCA-3'; *Rxrg*, 5'-ATTCCCTGTTCATGCCAGCTCCACGCTCTGTGAGCCCATCATCCA-3'; *Ttr*, 5'-ACAGTACGTTAGCTGCAGGACTTCTCTGACTGCATCCAGCACT-3'.

Hybridization was carried out overnight at 42 °C. After glass slides were washed, they were air dried and apposed to Biomax-MR film (Eastman Kodak Co.) for 2 weeks with <sup>14</sup>C standards (American Radiolabelled Chemicals). Relative optical densities were measured by using a computed image-analysing system (MCID Imaging Research), and were converted into the relative radioactive value (nCi) by <sup>14</sup>C standards. Specific hybridization signals were obtained by subtracting background values obtained from adjacent brain areas that did not exhibit a hybridization signal.

Quantification of T<sub>3</sub> and T<sub>4</sub>

Thyroid hormones in the brain were extracted with ethanol as described<sup>27,28</sup>. Concentrations of T<sub>4</sub> and T<sub>3</sub> in the brain and plasma were determined by radioimmunoassay as described previously<sup>27,28</sup>.

Intracerebroventricular infusion

Animals were raised under short day conditions to an age of 8 weeks. T<sub>3</sub> (T-2877, Sigma) and T<sub>4</sub> (T-2376, Sigma) were dissolved in a solution containing 0.001 M NaOH and 0.9% NaCl. Iopanoic acid (I 0300, Tokyo Kasei) was dissolved in 0.05 M NaOH and 0.2 M HCl was added. Intracerebroventricular infusion was carried out using an ALZET 2002 osmotic mini-pump with a brain infusion kit according to the manufacturer's instructions (ALZET). Animals infused with iopanoic acid were transferred into long day conditions 2 days after the beginning of the 2-week infusion period. Testicular size was measured before and 3 weeks after the beginning of infusion. Placement and patency of the canula were verified by injecting Evans blue dye after the experiment, as suggested by the manufacturer.

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APL regulates vascular tissue identity in *Arabidopsis*

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Vascular plants have a long-distance transport system consisting of two tissue types with elongated cell files, phloem and xylem<sup>1</sup>. Phloem has two basic cell types, enucleate sieve elements and companion cells. Xylem has various lignified cell types, such as tracheary elements, the differentiation of which involves deposition of elaborate cell wall thickenings and programmed cell death<sup>1–4</sup>. Until now, little has been known about the genetic control of phloem–xylem patterning. Here we identify the *ALTERED PHLOEM DEVELOPMENT (APL)* gene, which encodes a MYB coiled-coil-type transcription factor that is required for