Graft-transmissible induction of potato tuberization by the microRNA miR172

Antoine Martin*, Hélène Adam†, Mercedes Díaz-Mendoza‡, Marek Żurczak, Nahuel D. González-Schain and Paula Suárez-López§

The photoreceptor phytochrome B (PHYB) and the homeodomain protein BEL5 are involved in the response of potato tuber induction to the photoperiod. However, whether they act in the same tuberization pathway is unknown. Here we show the effect of a microRNA, miR172, on this developmental event. miR172 levels are higher under tuber-inducing short days than under non-inductive long days and are upregulated in stolons at the onset of tuberization. Overexpression of this microRNA in potato promotes flowering, accelerates tuberization under moderately inductive photoperiods and triggers tuber formation under long days. In plants with a reduced abundance of PHYB, which tuberize under long days, both BEL5 mRNA and miR172 levels are reduced in leaves and increased in stolons. This, together with the presence of miR172 in vascular bundles and the graft transmissibility of its effect on tuberization, indicates that either miR172 might be mobile or it regulates long-distance signals to induce tuberization. Consistent with this, plants overexpressing miR172 show increased levels of BEL5 mRNA, which has been reported to be transmissible through grafts. Furthermore, we identify an APETALA2-like mRNA containing a miR172 binding site, which is downregulated in plants overexpressing miR172 and plants in which PHYB is silenced. Altogether, our results suggest that miR172 probably acts downstream of the tuberization repressor PHYB and upstream of the tuberization promoter BEL5 and allow us to propose a model for the control of tuberization by PHYB, miR172 and BEL5.

KEY WORDS: Developmental timing, Flowering, miR172, Photoperiod, Phytochrome B, Potato, Tuberization

INTRODUCTION

Like flowering in many species, potato (Solanum tuberosum L.) tuberization is regulated by day length. In the S. tuberosum subspecies andigena, short days (SDs) strongly induce tuberization, short days supplemented with a night break (SD+N) are moderately inductive and long days (LDs) are repressive (Rodríguez-Falcón et al., 2006; González-Schain and Suárez-López, 2008). Very few genes involved in the photoperiodic control of tuber induction have been identified and their possible functions in the timing of tuberization have hardly been explored.

The photoreceptor phytochrome B (PHYB) plays an important role in the response of tuberization to day length. PHYB is required to inhibit tuberization under LD conditions, as PHYB antisense (anti-PHYB) plants tuberize under LDs (Jackson et al., 1996). This effect is transmitted through grafts, which has been interpreted as evidence that PHYB induces the generation of a mobile inhibitor of tuberization (Jackson et al., 1998) that must be produced in the leaves in response to LDs. However, the mechanism by which PHYB controls photoperiodic tuberization is unknown. Although it has been shown that PHYB affects the mRNA levels of a gibberellin-20-oxidase (GA20ox1), a key enzyme in gibberellin biosynthesis that affects tuberization, the alteration of GA20ox1 transcript and GA1 levels in anti-PHYB plants is not consistent with their tuberization phenotype (Carrera et al., 2000; Jackson et al., 2000; Martínez-García et al., 2002). Therefore, how PHYB negatively regulates tuberization has not been elucidated.

A homeodomain protein, BEL5, and a sucrose transporter, SUT4, are also involved in the control of tuberization by the photoperiod (Chen et al., 2003; Banerjee et al., 2006b; Chincinska et al., 2008). BEL5 overexpression promotes tuberization and overrules the inhibition caused by LDs (Chen et al., 2003; Banerjee et al., 2006b). A recent report suggests that BEL5 mRNA is graft transmissible and its movement to stolons correlates with tuber induction (Banerjee et al., 2006b). By contrast, SUT4 is a tuberization inhibitor, as SUT4 RNAi plants tuberize under non-inductive LDs (Chincinska et al., 2008). In addition, SUT4 RNAi plants flower earlier than the wild type (Chincinska et al., 2008), revealing another link in the regulation of flowering and tuber induction.

Despite the involvement of PHYB, BEL5 and SUT4 in photoperiodic tuberization, it is not known whether there are genetic or molecular interactions between them. Recently, several studies have demonstrated that microRNA 172 (miR172) plays a role in the timing of flowering and vegetative phase change (Aukerman and Sakai, 2003; Chen, 2004; Lauter et al., 2005) and is involved in a photoperiodic pathway controlling flowering in Arabidopsis thaliana (Jung et al., 2007). All the genes targeted by miR172 encode members of a subset of the APETALA2 (AP2)-like transcription factor family (Aukerman and Sakai, 2003; Schmid et al., 2003; Chen, 2004; Lauter et al., 2005; Chuck et al., 2007). Like most plant microRNAs (miRNAs), miR172 can both repress translation and induce degradation of its target mRNAs (Aukerman and Sakai, 2003; Kasschau et al., 2003; Chen, 2004; Lauter et al., 2005; Schwab et al., 2005; Chuck et al., 2007; Brodersen et al., 2008; Voinnet, 2009).

The miR172 sequence had been previously found in a potato EST (Aukerman and Sakai, 2003), suggesting that this miRNA is produced in potato. Given the similarities in the control of flowering and tuberization (Suárez-López, 2005; Rodriguez-Falcón et al.,...
2006), we tested the hypothesis that miR172 can regulate flowering and tuber induction in this plant species. Our results show that miR172 affects the induction of tuberization, probably upstream of BEL5 and, like in Arabidopsis, is involved in the temporal control of flowering. The study of miR172 abundance and location strongly suggests that miR172 acts downstream of PHYB to regulate tuber induction and that this miRNA is a component or a regulator of long-distance signals. Furthermore, the results suggest that PHYB acts upstream of BEL5. The cloning and expression analysis of a potato AP2-like gene harboring a miR172 target site indicates that this miRNA negatively regulates its target transcripts in potato. Finally, we propose a model for a pathway regulating potato tuberization.

**MATERIALS AND METHODS**

**Plant material and growth conditions**
Solanum tuberosum L. subspecies andigena line 7540 was used as wild-type potato. Anti-PHYB plants were kindly provided by Salomé Prat (Jackson et al., 1996). Plants were propagated from single-node stem cuttings on Murashige and Skoog (MS) medium containing 20 g l–1 sucrose and 2 g l–1 Gelrite (Duchefa Biochemie, Haarlem, The Netherlands) at 23°C under LD conditions (16 hours light and 8 hours darkness). Two-week-old plants were planted in soil composed of substrate type 3 (Plantaflo) and sand (3:1) and grown in the greenhouse at 23°C under LD conditions. During autumn and winter, the light period was extended to 16 hours with high-pressure sodium vapor lamps (SON-T Agro 400 W, Philips Ibérica, Madrid, Spain). Whenever natural light intensity fell below 65 μmol m–2 second–1 during the light period, light intensity was supplemented with the same lamps. Plants were watered daily with modified Hoagland’s solution (Johnson et al., 1957) diluted 1/60.

**Prediction of miR172 precursor secondary structure**
The first 500 nucleotides of the reverse complement of a putative potato miR172 precursor (accession number BQ114970) were used to predict its secondary structure using MFold (http://www.bioinfo.rpi.edu/applications/mfold) (Zuker, 2003). Nine different structures were predicted, with ΔG values ranging from –110.77 to –99.93 kcal mol–1. The structure with the lowest AG value was chosen as representative and is shown in Fig. S1 in the supplementary material.

**Analysis of miR172 levels**
Total RNA was extracted using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s instructions with the following modification: the isopropanol precipitation was done with a high-salt solution (1.2 M NaCl and 0.8 M sodium citrate) to remove polysaccharide contaminations. Alternatively, total RNA was isolated using the Real A.R.N. spin Kit (+PVP) (Durviz, Valencia, Spain).

For RNA blots, 30 μg of total RNA were fractionated on denaturing 17.5% polyacrylamide gels containing 7 M urea, which were then electrophoresed to a charged nylon membrane (Zeta probe, Bio-Rad, Hercules, CA, USA). Blots were probed with [32P]-labeled DNA oligonucleotides, complementary to either miR172 (5′-ATGCAGGCTACATCGAAGTTTC-3′) or U6 small nuclear RNA (5′-GTCGTATCCAGTGAGTCGACGAG-3′). Probes (10 pmol) were end-labeled with [γ-32P]ATP using T4 polynucleotide kinase (Fermentas, Vilnius, Lithuania). Membranes were prehybridized and hybridized in Perfect-Hyb Plus buffer (Sigma-Aldrich, St Louis, MO, USA) at 37°C for miR172 and 50°C for U6 snRNA, and then washed twice at 50°C with 2× SSC and 0.2% SDS for 15 minutes.

Mature miR172 was quantified by reverse transcription quantitative real-time PCR (RT-qPCR) according to a previously described protocol (Chen et al., 2005). Reverse transcription reactions contained 1-2 μg RNA, 50 nM stem-loop RT primer (5′-GTCGTATCCAGTGAGTCGACGAG-3′), 1× RT buffer (Invitrogen), 0.25 mM dNTPs, 200 U Superscript III reverse transcriptase (Invitrogen) and 2 U μl–1 RNaseOUT (Invitrogen). The 20 μl reactions were incubated for 30 minutes at 16°C, 30 minutes at 50°C and 5 minutes at 85°C. qPCR was performed on a LightCycler 480 Real-Time PCR System (Roche Diagnostics, Mannheim, Germany). The 20 μl PCR included 0.2 μl cDNA, 1× LightCycler 480 SYBR Green I Master Mix (Roche), 0.3 μM forward primer (5′-GCGCGTGAATACCGTATGAG-3′) and 0.3 μM reverse primer (5′-GGCCAGGGTTCCAGGCT-3′). The reactions were incubated at 95°C for 10 minutes, followed by 40 cycles of 95°C for 10 seconds, 60°C for 30 seconds and 72°C for 30 seconds. For normalization, 5S rRNA was reverse transcribed (primer 5′-GTCGTATCCAGTGAGTCGACGAG-3′) and amplified (forward primer 5′-GAGTGCACTACCATACCAGCAG-3′ and the same reverse primer as for miR172) in the same conditions as for miR172. All reactions were run in triplicate. The specificity of PCR was checked with dissociation curve analysis. Data from RT-qPCR were analyzed using the 2-ΔΔCT method (Livak and Schmittgen, 2001).

**In situ hybridization**
Plant material was fixed for 8 hours at 4°C in 4% paraformaldehyde and 0.1 M phosphate buffer pH 7, dehydrated through a graded series of ethanol and butanol dilutions, embedded in Paraplast Plus (Paraplast X-Tra, Oxford Labware, St Louis, MO, USA) and sectioned to 7 μm with a microtome. Tissue sections were deparaffinized with Histoclear, rehydrated through an ethanol series, and then pre-treated with proteinase K (0.1 U μl–1) in Tris-HCl pH 7.5 at 37°C for 15 minutes. Digestion was stopped by washing with 0.2× PBS containing 0.04 M glycine and then twice with PBS for 2 minutes. After dehydrating in ethanol baths, hybridization was performed at 37°C overnight with 0.2 μM digoxigenin-labeled miR CURLY LNA probe complementary to miR172 (Exiqon, Vedbaek, Denmark) in hybridization solution (50% formamide, 2× SSC, 4× Denhardt’s solution, 20% dextran, 2 mg ml–1 tRNA). An LNA oligonucleotide complementary to mouse miR124 (5′-TAAAGCCAGGTTGAATGC-3′), with no predicted target sequences in plants, was used as negative control. After hybridization, slides were washed in 2× SSC at 37°C for 45 minutes and in 1× SSC for 15 minutes. For signal detection, samples were incubated in 10% blocking reagent (Roche, Mannheim, Germany) in PBS for 1 hour and afterwards for 30 minutes in blocking reagent containing anti-DIG alkaline phosphatase-conjugated Fab fragment antibody (Roche) diluted to 1:500. After three washes for 10 minutes in PBS, tissues were equilibrated in detection buffer (100 mM Tris-HCl pH 9.5, 100 mM NaCl, 50 mM MgCl2) for 15 minutes prior to incubating in the same buffer supplemented with 0.2 mM NBT and 0.2 mM BCIP substrates until a signal was visible.

**Generation of plants overexpressing miR172**
For miR172 overexpression, a 133 bp fragment of a putative miR172 precursor from potato was PCR-amplified using EST600546 (GenBank accession number BQ114970) as a template and primers miR172 forward 5′-GTCGTATCCAGTGAGTCGACGAG-3′ and miR172 reverse 5′-GGCCAGGGTTCCAGGCT-3′ in the same conditions as for miR172. The PCR product was digested with XbaI and SalI and cloned in sense orientation in the XbaI and SalI sites of pBIB35S-Hyg to generate pBIB-3SSS::miR172. The pBIB-Hyg vector was created by replacing the EcoRI/HindIII cassette of pBIB-HYG (Becker, 1990) with the EcoRI/HindIII cassette of pBINAR (Högen and Willmitzer, 1990). pBIB-3SSS::miR172 was electroporated into Agrobacterium tumefaciens strain C58 GV2260, which was used to transform S. tuberosum subspecies andigena essentially as described (Banerjee et al., 2006a). Hygromycin-B-resistant shoots were regenerated and then propagated from single-node stem cuttings on MS medium containing 1 mg l–1 hygromycin B, 250 mg l–1 carbenicillin and 2 g l–1 Gelrite (Duchefa Biochemie). Plants were propagated for at least two rounds on selective medium before being propagated in the absence of the antibiotic. Twenty-three putative transgenic lines were planted in soil and the level of miR172 was assessed by RNA blot. Seven 3SSS::miR172 lines and one line carrying an empty vector were selected for further analysis.

**Analysis of flowering and tuberization**
To analyze flowering time, plants were either grown in the LD greenhouse or in controlled environment chambers with the following photoperiods: SD (8 hours light and 16 hours darkness); and SD supplemented with a 30 minutes white-light night break given 8 hours after the start of the dark period (SD+NB). Lighting was provided by high-pressure sodium vapor lamps (SON-T Agro 400 W). The shoot apex was carefully checked for...
visible signs of flowering every 2 or 3 days. Flowering time was measured as the number of days from planting in soil to the appearance of the floral bud. Leaf number was also recorded at the time the floral bud was visible.

For tuberization experiments, potato plants grown in the greenhouse were transferred to SD or SD+NB environment chambers at the 10-12 leaf stage, approximately 4 weeks after potting. LD experiments were performed in the greenhouse. Tuberization was analyzed once per week under SD and SD+NB conditions and after 4 months of growth under LDs. Tuberization time was measured as the number of days from transfer to SD or SD+NB conditions to the appearance of tubers. Leaf number was also recorded when tubers were first visible.

**Grafting experiments**

Five-week-old LD-grown 35S::miR172 (line 8) and wild-type plants were used. V-shape grafts were made, bound with paper surgical tape, and immediately covered up with a transparent plastic bag to preserve humidity. Grafts were maintained in the LD greenhouse. Humidity was slowly released after 48 hours. Ten days after grafting, when graft unions had healed, the stock leaves were removed and plants were transferred to SD+NB conditions. Twenty grafts of each of the following types were analyzed: 35S::miR172 scions onto wild-type stocks, wild-type scions onto 35S::miR172 stocks, wild-type scions onto wild-type stocks and 35S::miR172 scions onto 35S::miR172 stocks. Ungrafted plants were also used as controls. Tuberization time was determined as described above.

**Identification of RAP1, a putative miR172 target gene**

In order to isolate potential target genes of miR172, total RNA was extracted from potato tissues with low miR172 levels. RNA was reverse transcribed using the RNase-ligase-mediated method from the Gene Racer Kit (Invitrogen). First, a forward primer corresponding to the miR172 sequence (5'-AGAATCTTGATGATGCTGCAT-3') was used in a 3' RACE reaction to amplify the 3' part of cDNAs representing potential targets of miR172. A PCR product was gel-extracted, cloned and sequenced. Then, a reverse primer (5'-GTCAAGAGTTGTCGAAGCAATGTA-3') was used for 5' RACE to obtain the full-length sequence of the cDNA. This sequence was named RAP1 and has been submitted to the EMBL Nucleotide Sequence Database under the accession number FM246879.

**Reverse transcription quantitative real-time PCR analyses**

RAP1 and BEL5 mRNA accumulation was analyzed by RT-qPCR in wild-type, anti-PHYB and 35S::miR172 plants grown under different photoperiods. Total RNA was purified from frozen tissues by Trizol extraction, as described above. cDNA synthesis was performed in a 20 μl volume, containing 2 μg RNA, 25 μg ml-1 oligo(dt)2-18 (Invitrogen), 0.5 mM dNTP Mix (Invitrogen), 10 mM DTT (Invitrogen), 40 U RNase Inhibitor (RNA Guard, Pharmacia) and 200 U M-MLV Reverse Transcriptase (Invitrogen). qPCR was performed on an ABI Prism 7000 Sequence Detection System (Applied Biosystems, Foster City, CA, USA) with gene-specific primer pairs. The reactions, performed in triplicate, contained 0.2 μl of cDNA, 1× SYBR Green PCR Master Mix (Applied Biosystems), 0.3 μM forward primer and 0.3 μM reverse primer, and were incubated at 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. The specificity of PCR was checked with dissociation curves and quantification was standardized to ACTIN (ACT) mRNA levels. Data from RT-qPCR were analyzed using the 2-ΔΔCt method (Livak and Schmittgen, 2001). Primers used for PCR were as follows: ACT forward, 5'- CCTTGATGCTAGTGTCG-3'; ACT reverse, 5'-GTCAAGAGTTGTCGAAGCAATGTA-3'; BEL5 forward, 5'-TTTATGGCAGCATA-3'; BEL5 reverse, 5'-TTAAATGGCTGTCATGTGTTTC-3'; RAP1 forward, 5'-AGGGAACAGCATTAGGGAAGGGT-3'; RAP1 reverse, 5'-AGTCAAGAGTTGTCGAAGCAATGTA-3'.

**RESULTS**

**A miR172 precursor is present in potato**

A putative miR172 precursor, which contains a sequence identical to miR172a from Arabidopsis, had been previously identified in potato (Aukerman and Sakai, 2003). A prediction of the secondary structure of this putative precursor using Mfold showed that the miR172 sequence is present, paired to the miR172* sequence, in an arm of the stem of a stem-loop structure (see Fig. S1 in the supplementary material), as expected for a miRNA precursor. This suggests that miR172 can be produced in potato.

**Analysis of miR172 levels in potato plants**

The relative abundance of miR172 was analyzed by RT-qPCR and RNA blot, using a miR172 antisense probe, in potato plants grown under LD, SD+NB and SD conditions. As shown in Fig. 1 and Fig. S2 in the supplementary material, miR172 is present in all organs studied (leaves, stems and stolons), with a higher accumulation in stems under all photoperiods. miR172 levels were higher in all organs under tuber-inducing conditions (SDs) than under non-inducing LDs (Fig. 1A), whereas under moderately inductive SD+NB conditions, levels were lower than under LDs (Fig. 1B). Both under SD and SD+NB conditions, an increase in miR172 levels correlated with tuber initiation, as its abundance was higher in swollen than unswollen stolons (Fig. 1; see Fig. S2 in the supplementary material).

To examine the spatial distribution of miR172, we performed in situ hybridization on leaves, stems, stolons and swollen stolons. In leaves, miR172 was mainly detected in the epidermis and vascular cells (Fig. 2A). By contrast, in the stem, a signal corresponding to miR172 was detected only in the internal and external phloem (Fig. 2B). In stolons collected under LDs, miR172 was present mainly in vascular cells and in the apical meristem (Fig. 2C). Under SDs, however, miR172 was detected in all cell types of unswollen stolons (Fig. 2D) and its localization was restricted to the vasculature and apex when stolons started to swell (Fig. 2E).
Altogether, these experiments show that: (1) miR172 is present in the vascular bundles of all the organs studied; (2) miR172 levels are highest under tuber-inducing SD conditions; and (3) miR172 distribution changes and its levels increase in stolons at the onset of tuberization.

**Effect of PHYB on miR172 levels**
The photoreceptor PHYB is involved in tuberization control in potato. To study whether PHYB regulates miR172 levels, we compared the accumulation of miR172 in different organs of wild-type and anti-PHYB plants under LD conditions by RNA blot and RT-qPCR. These experiments showed that the level of miR172 is reduced in the leaves of anti-PHYB plants (Fig. 3A). Moreover, miR172 abundance was dramatically higher in swollen than unswollen stolons of anti-PHYB plants (Fig. 3A). This difference was confirmed by RT-qPCR by comparing miR172 levels in stolons at 35 and 39 days in wild-type and anti-PHYB plants (Fig. 3B). The difference of miR172 abundance between swollen and unswollen stolons is more dramatic in anti-PHYB plants under LDs (Fig. 3) than in the wild type under SD or SD+NB conditions (Fig. 1). These experiments show that in plants in which PHYB is silenced, miR172 levels are reduced in leaves and increased in swollen stolons.

**miR172 affects flowering time in potato**
To understand the function of miR172 in potato, we generated plants that constitutively express miR172 from the cauliflower mosaic virus 35S promoter. Seven 35S::miR172 lines and a control line transformed with an empty vector were selected for analysis. The level of miR172 in the transgenic lines was examined by RNA blot and RT-qPCR. No change in the abundance of miR172 was observed in the control line compared to the wild type. By contrast, an increase in the amount of this miRNA was clearly visible in all the 35S::miR172 lines (see Fig. S3 in the supplementary material).

Since miR172 is a regulator of flowering time (Aukerman and Sakai, 2003; Chen, 2004; Lauter et al., 2005), we investigated the flowering time of potato 35S::miR172 plants. All the 35S::miR172 lines flowered earlier than control plants under LDs when flowering time was estimated by counting the number of leaves at flowering (Fig. 4A), and most lines were also statistically different from the controls in the number of days to flowering (Fig. 4B). Similar results were obtained under SD+NB and SD conditions (see Fig. S4 in the supplementary material). These results indicate that miR172 promotes flowering in potato, as previously shown in other plant species.

**Overexpression of miR172 promotes tuberization in a photoperiod-dependent manner**
Given that the regulation of flowering and tuberization shows several similarities (Suárez-López, 2005; Rodríguez-Falcón et al., 2006), we investigated whether the increase in miR172 levels could have an impact on tuber induction in potato. Under strongly inductive SD conditions, no significant differences in tuberization time were observed between 35S::miR172 lines and the controls (Fig. 5A,B). In moderately inductive SD+NB conditions, several 35S::miR172 lines (6, 8 and 22) showed an early tuberization.
maximum levels set to 1. All samples were collected 4 hours after dawn. Samples and s.e.m. are indicated. Relative samples were not swollen. Mean values calculated from triplicate samples and s.e.m. are indicated. Relative miR172 levels are given, with maximum levels set to 1. All samples were collected 4 hours after dawn.

**The effect of miR172 on tuberization is graft transmissible**

The presence of miR172 in vascular bundles (Fig. 2), the change in distribution in stolons at the onset of tuberization (Fig. 2) and the opposite effect of PHYB on miR172 levels in leaves and stolons (Fig. 3) suggested that this miRNA might be involved in long-distance signaling to control tuberization. Therefore, we tested whether the tuberization phenotype of 35S:miR172 plants is graft transmissible. 35S:miR172 scions grafted onto wild-type stocks (35S:miR172/WT grafts) tuberized as early as 35S:miR172/35S:miR172 controls (Fig. 5D), showing that the effect of miR172 on tuberization is transmissible through grafts and indicating that miR172 overexpression in aerial organs is sufficient to promote tuberization. By contrast, WT/35S:miR172 grafts tuberized like WT/WT controls (Fig. 5D), indicating that the overexpression of miR172 in underground parts is not sufficient to affect this process.

**Identification of RAP1, a potato gene with a miR172 binding site**

All of the target genes of miR172 identified so far belong to the AP2-like family (Aukerman and Sakai, 2003; Schmid et al., 2003; Chen, 2004; Lauter et al., 2005; Chuck et al., 2007). In order to further investigate the role of miR172 in potato, we used an approach to identify potato genes possessing a miR172 binding site, which therefore represent candidate genes involved in the miR172 pathway. The miR172 sequence was used as a primer in a 3′ RACE reaction, leading to the amplification of a cDNA fragment containing a miR172 binding site. The full-length CDNA sequence, as well as validation of the miR172 binding site, were obtained by subsequent 5′ RACE. Using this approach, we identified a gene encoding a 454 amino acid protein showing strong homology with AP2 and AP2-like proteins. This protein was named RAP1 (RELATED TO APETALA2 1) and it contains two AP2 domains highly similar to those of previously characterized members of the AP2 family (Fig. 6A). The RAP1 coding sequence displays a miR172 binding site, and alignments with known targets of this miRNA indicated a partial conservation of mismatches between miR172 and its targets (Fig. 6B). This suggests that RAP1 is a likely target of miR172 in potato.

**Transcript levels of RAP1 are regulated by miR172 and PHYB**

To determine whether miR172 regulates RAP1, RT-qPCR analyses were performed. In wild-type plants grown under SD+NB or LD conditions, the mRNA abundance of RAP1 was higher in leaves than in stems and stolons (Fig. 7A). Therefore, RAP1 transcript levels show an inverse correlation with miR172 accumulation, suggesting that this miRNA can target RAP1 mRNA for degradation. The only significant difference in RAP1 mRNA abundance between SD+NB and LD conditions was observed in leaves, with higher levels under LD than SD+NB conditions (Fig. 7A).

To test whether RAP1 is a target of miR172, the levels of RAP1 transcript were analyzed in 35S:miR172 plants. RAP1 mRNA abundance was significantly lower in 35S:miR172 than in wild-type leaves (Fig. 7C), indicating that miR172 negatively regulates RAP1. By contrast, RAP1 mRNA levels were not significantly altered in the stems and stolons of 35S:miR172 plants (Fig. 7C).

Then, levels of RAP1 mRNA were examined in anti-PHYB plants, as these plants showed altered miR172 accumulation (Fig. 3). Levels of RAP1 were drastically reduced in all analyzed organs of anti-PHYB plants in contrast with the controls.
Nevertheless, anti-PHYB plants still have lower levels of RAP1 in stems and stolons than in leaves (Fig. 7D). These results indicate that PHYB regulates RAP1.

**Transcript levels of BEL5 are regulated by miR172 and PHYB**

Transcript levels of BEL5 were analyzed in different organs and photoperiodic conditions because BEL5 induces tuberization in potato (Chen et al., 2003; Banerjee et al., 2006b). RT-qPCR experiments showed that the levels of BEL5 mRNA were higher in leaves and stems than in unswollen stolons of wild-type plants under SD+NB and LD conditions (Fig. 7B), and an increase in BEL5 in stolons correlated with the onset of tuber development under SD+NB conditions (Fig. 7B; compare stolons with swollen stolons). This is consistent with a previous analysis of BEL5 transcript abundance (Chen et al., 2003). Wild-type leaves and stems showed a higher accumulation of BEL5 mRNA under LD than SD+NB conditions, whereas the opposite was found in stolons (Fig. 7B).

Since miR172, PHYB and BEL5 influence tuberization, we examined whether miR172 and PHYB have an effect on BEL5. Under SD+NB conditions, the levels of BEL5 mRNA were higher in all organs of 35S::miR172 than wild-type plants (Fig. 7E). Therefore, miR172 overexpression leads to the upregulation of BEL5. In addition, in anti-PHYB lines the levels of BEL5 were increased in stems and stolons when compared with the wild type, whereas in leaves transcript levels were decreased in one of the transgenic lines, anti-PHYB-10 (Fig. 7F). In other experiments, a reduction in BEL5 mRNA was also observed in the leaves of transgenic line anti-PHYB-4. Interestingly, there was a remarkable increase in BEL5 levels in anti-PHYB stolons at the onset of tuberization (Fig. 7F, compare stolons with swollen stolons). These results indicate that PHYB controls BEL5 transcript abundance in several organs. The reduction in BEL5 mRNA levels in the leaves of anti-PHYB plants and the increase in mRNA levels in stolons when these start to swell is consistent with the reported movement of BEL5 mRNA (Banerjee et al., 2006b).

**DISCUSSION**

It has been reported that miR172 regulates flowering time, flower development, vegetative phase change, sex determination and meristem cell fate (Aukerman and Sakai, 2003; Chen, 2004; Lauter et al., 2005; Chuck et al., 2007; Zhao et al., 2007). We show here that, in potato, miR172 affects not only flowering time, but also the timing of tuberization, another developmental process related to reproduction.

We have overexpressed a member of the miR172 family that had been previously identified in potato (Aukerman and Sakai, 2003). Recently, this miR172 member has been named miR172b, and four additional potato miR172 precursors have been computationally

**Fig. 5. Tuberization of 35S::miR172 plants under different photoperiods.** (A,B) Control (WT and plants carrying an empty vector, c; black bars) and 35S::miR172 (white bars) plants were grown under LDs for 4 weeks and then transferred to SD or SD+NB conditions. Tuberization time was measured as days to tuberization (A) or as the total number of leaves produced before tuberization (B). Mean and s.e.m. for at least six plants per line and condition are shown. Asterisk indicates a statistically significant difference between the 35S::miR172 lines and the controls at P<0.05 (Bonferroni’s test). (C) Underground parts of 35S::miR172 and control (WT, c) plants grown under LDs for 12 weeks. Green arrowheads indicate tubers. Wild-type plants did not tuberize under LDs for at least 4 months. (D) Tuberization time of grafts. Five-week-old LD-grown 35S::miR172 (line 8) and wild-type plants were grafted. Ungrafted plants were also used as controls. Grafts and controls were transferred to SD+NB conditions and tuberization time was measured as days to tuberization under SD+NB conditions. Mean and s.e.m. for 17-19 grafts of each type and 10 ungrafted control plants are shown.
identified (Zhang et al., 2009). The predicted secondary structure of pre-miR172b shown in Fig. S1 in the supplementary material is identical to that reported by Zhang et al. (Zhang et al., 2009). The sequence of potato miR172b is identical to that of Arabidopsis miR172a and b and maize miR172a-d. However, overexpression of this miRNA in potato does not cause any obvious floral organ identity phenotype (not shown), suggesting that the function of this miRNA in potato does not cause any obvious floral organ type plants (Fig. 1). In addition, tuberization of anti-miR172b plants and an increase in its levels in stolons correlates with early stages of tuber development under SD and SD+NB conditions in wild-type plants (Fig. 7), both of which tuberize earlier than wild-type plants (Jackson et al., 1996) (Fig. 5). In addition, 35S::miR172 plants exhibit early flowering (Fig. 4; see Fig. S4 in the supplementary material). We hypothesize that RAP1 might be involved in the repression of tuberization and/or flowering downstream of PHYB and miR172.

Silencing of PHYB completely abolishes the repression of tuberization under LDs (Jackson et al., 1996) (see Fig. S5 in the supplementary material), indicating that PHYB is essential for the inhibition of tuberization under this photoperiod. miR172 levels are altered in anti-PHYB plants (Fig. 3), strongly suggesting that miR172 acts downstream of PHYB to control tuberization. However, changes in miR172 levels are not sufficient to explain the strong effect of PHYB on tuberization, as the tuberization phenotypes of 35S::miR172 plants are weaker than those of anti-PHYB plants and, in addition, 35S::miR172 plants are still partially sensitive to day length (Fig. 5; see Fig. S5 in the supplementary material). Similarly, Arabidopsis plants overexpressing miR172 also show a residual photoperiodic flowering response (Aukerman and Sakai, 2003). Given that BEL5 promotes tuberization (Chen et al., 2003; Banerjee et al., 2006b), we examined the possibility that miR172 and PHYB could regulate this gene. We found increased BEL5 mRNA levels in all organs of 35S::miR172 plants and in stems and stolons of anti-PHYB plants (Fig. 7E,F). Therefore, BEL5 might also contribute to the regulation of tuber induction by miR172 and PHYB. Taken together, these results suggest that the regulation of BEL5 by PHYB is at least in part mediated by miR172. Our results do not rule out that additional genes might act downstream of PHYB and miR172 to mediate their effects on tuber formation.

The presence of miR172 in the vascular tissue of wild-type potato plants and its detection in the phloem sap of Brassica napus (Buhtz et al., 2008) and the graft transmission of the tuberization phenotype of 35S::miR172 plants (Fig. 5D) show that this miRNA is mobile or it regulates long-distance signals to induce tuberization. Our grafting experiments, together with the analysis of RAP1 and BEL5 mRNA levels in miR172-overexpressing plants, indicate that miR172 can act at least in the leaves to promote tuberization and regulate the mRNA levels of these two genes. Although overexpression of miR172 in the stocks of WT/35S::miR172 grafts does not promote tuberization (Fig. 5D), this does not rule out the possibility that endogenous miR172 can act in these organs. The clear correlation of tuber induction with an increase in miR172 levels in stolons is consistent with this idea. It is possible that the overexpression of miR172 in stocks is not sufficient to counteract repressive signals coming from the wild-type scions.
It has been established that the FT protein is a component of the long-range signals for flowering (Turck et al., 2008). However, its counterpart for tuberization has not been identified so far. It has been reported that BEL5 mRNA is graft transmissible and its movement correlates with tuber induction (Banerjee et al., 2006b). Interestingly, anti-PHYB plants show a reduction in BEL5 transcript levels in leaves in comparison with wild-type plants and an increase in the levels in stolons when these start to swell (Fig. 7F). Similarly, we have observed reduced miR172 levels in leaves and increased levels in swollen stolons of anti-PHYB plants (Fig. 3). One interpretation of these results is that PHYB promotes miR172 and BEL5 expression in leaves and represses their expression in stolons. However, this is inconsistent with the fact that PHYB represses tuberization, whereas overexpression of miR172 and BEL5 in aerial organs promotes it. Therefore, we hypothesize that BEL5 mRNA and miR172 move from leaves to stolons under SDs to induce tuberization. Under LD and SD+NB conditions, this movement would be repressed by PHYB, thereby preventing or delaying tuberization. Previously it had been proposed that PHYB induces the production of a graft-transmissible inhibitor of tuberization (Jackson et al., 1998). Our data suggest that PHYB might inhibit the transport of promoters of tuberization. Although the idea that miRNAs can act as systemic signals is still under debate (Voinnet, 2009), at least one miRNA has been shown to be a long-distance signal (Pant et al., 2008).

Our results allow us to propose a model for the regulation of tuberization. miR172 is regulated by PHYB and these both regulate RAP1 and BEL5. This strongly suggests that PHYB acts upstream of miR172 and BEL5, and BEL5 acts at least in part downstream of miR172, to control tuberization. As suggested above, PHYB might repress the movement of BEL5 mRNA and miR172 from leaves to stolons under LDs. Under SDs, this repression would be released to allow BEL5 and miR172 to induce tuberization. This is consistent with the notion that PHYB acts in LD conditions to repress a tuber-inducing pathway that is active under SDs (Jackson et al., 1996). miR172 promotes tuberization probably via the upregulation of BEL5 in leaves and stolons. Moreover, this miRNA inhibits RAP1. We postulate that the upregulation of BEL5 by miR172 might be mediated by a miR172 target gene, perhaps RAP1, which would repress BEL5. Interestingly, the BEL5 promoter contains two CAACA sequence motifs (in reverse orientation, TGGTG) that have been described as binding sites for the AP2 domain of an AP2-like transcription factor (Kagaya et al., 1999), and RAP1 has two AP2 domains. Increased miR172 levels lead to the inhibition of its targets (e.g. RAP1) and therefore probably to a release of BEL5 repression. This release would lead to more BEL5 mRNA in stolons, therefore inducing tuberization. However, we cannot rule out an effect of miR172 target genes on tuberization through a BEL5-independent pathway. In addition, PHYB regulates RAP1 probably through miR172, but also through a miR172-independent pathway, as the effect of PHYB on RAP1 mRNA levels is much more dramatic than that of miR172 (Fig. 7). This complex network probably confers robustness to the regulation of tuberization by reinforcing signals, as PHYB acts under LDs to repress the function of two tuberization inducers, BEL5 and miR172, and to promote the expression of a putative negative regulator of BEL5 (RAP1), ensuring that tuberization does not occur under LD conditions.

This work establishes that miR172 is another regulator shared by flowering and tuberization, shows that this miRNA affects a long-distance signaling pathway and strongly suggests that it acts between PHYB and BEL5 to control tuber induction. In Arabidopsis, several miR172 target genes are involved in the
regulation of flowering time. Further research will help to determine whether different miR172 targets are specialized for the control of flowering and tuberization.

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Supplementary material

Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/136/17/2873/DC1

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