Development 130, 3735-3745

doi:10.1242/dev.00577

Modes of intercellular transcription factor movement in the *Arabidopsis* apex

Xuelin Wu[1], José R. Dinneny[1,2], Katrina M. Crawford[3], Yoon Rhee[4], Vitaly Citovsky[4], Patricia C. Zambryski[3] and Detlef Weigel[1,5,*]

[1]Plant Biology Laboratory, The Salk Institute for Biological Studies, La Jolla, CA 92037, USA
[2]Department of Biology, University of California San Diego, La Jolla, CA 92093, USA
[3]Department of Plant and Microbial Biology, University of California, Berkeley, CA 94720, USA
[4]Department of Biochemistry and Cell Biology, State University of New York, Stony Brook, NY 11794-5215, USA
[5]Department of Molecular Biology, Max Planck Institute for Developmental Biology, D-72076 Tübingen, Germany
*Author for correspondence (e-mail: weigel@weigelworld.org)

Accepted 2 May 2003

SUMMARY

A recent and intriguing discovery in plant biology has been that some transcription factors can move between cells. In *Arabidopsis thaliana*, the floral identity protein LEAFY has strong non-autonomous effects when expressed in the epidermis, mediated by its movement into underlying tissue layers. By contrast, a structurally unrelated floral identity protein, APETALA1, has only limited non-autonomous effects. Using GFP fusions to monitor protein movement in the shoot apical meristem and in floral primordia of *Arabidopsis*, we found a strong correlation between cytoplasmic localization of proteins and their ability to move to adjacent cells. The graded distribution of several GFP fusions with their highest levels in the cells where they are produced is compatible with the notion that this movement is driven by diffusion. We also present evidence that protein movement is more restricted laterally within layers than it is from L1 into underlying layers of the *Arabidopsis* apex. Based on these observations, we propose that intercellular movement of transcription factors can occur in a non-targeted fashion as a result of simple diffusion. This hypothesis raises the possibility that diffusion is the default state for many macromolecules in the *Arabidopsis* apex, unless they are specifically retained.

Key words: *Arabidopsis*, Protein trafficking, Movement protein, LEAFY, APETALA1

INTRODUCTION

Intercellular communication is essential in determining and enforcing developmental fates in all multicellular organisms. In animals, this process relies mainly on secreted signaling peptides, which interact with the extracellular matrix, and specific cell surface receptors. The interaction first triggers a signal transduction response in the cytoplasm of the target cells, and eventually leads to changes in gene expression. Complex protein-protein interactions and multiple protein phosphorylation steps are involved in many of these pathways, such as the EGF, TGFβ, Notch, Hedgehog and WNT family signaling pathways. An alternative form of communication is achieved by the diffusion of transcription factors from their sources to the target nuclei through a continuous cytoplasm, such as the syncytium of *Drosophila* blastoderm embryos, one of the best understood model systems for transcriptional regulation (Rivera-Pomar and Jäckle, 1996). However, syncytial organization is restricted to only a few tissues in metazoans.

Although plants lack homologs of the well-known metazoan peptide ligands, such as EGF, TGFβ or Hedgehog, plant cells can also communicate via secreted molecules (Fletcher et al., 1999; Matsubayashi et al., 2001; McCarty and Chory, 2000). However, in contrast to animals, cytoplasmic continuity between plant cells is the rule, not the exception. Most plant cells are connected by plasmodesmata, plasma membrane-lined channels that provide cytoplasmic continuity between adjacent cells. Plasmodesmata, which are used in the transport of nutrients and signaling molecules including RNAs and proteins, can be divided into two major groups (Crawford and Zambryski, 1999; Haywood et al., 2002; Lucas, 1995). The primary plasmodesmata form during cytokinesis, whereas the secondary plasmodesmata develop between cells that are not necessarily clonally related. The size exclusion limit (SEL) of the different types of plasmodesmata can be measured using fluorescent tracer molecules. In most cases, plasmodesmata in younger tissues have larger SEL and are morphologically simpler than those in older tissue (Crawford and Zambryski, 2001).

Two modes of movement through plasmodesmata have been proposed. Targeted movement involves specific interactions between the transported macromolecules and plasmodesmata components. This leads to an increase in the SEL and is therefore not limited by the endogenous SEL of a given cell. By contrast, non-targeted movement resembles passive diffusion and is governed by the endogenous SEL of the plasmodesmata involved (Crawford and Zambryski, 2000; Imlau et al., 1999; Oparka et al., 1999). The best understood case of targeted movement is probably the trafficking of plant

viral movement proteins (MPs), which can move over long distances in plants and are key to the spreading of plant viral infections. A good example of non-targeted movement is provided by green fluorescent protein (GFP). Using transient transfection by bombardment, it has been shown that native GFP can move several cells away from its source. In addition to the SEL, multimerization and the addition of nuclear or ER localization signals can hinder, or even prevent, GFP from leaving the source cell (Crawford and Zambryski, 2000; Crawford and Zambryski, 2001).

Apart from viral proteins, studies of macromolecule movement in plants have focused traditionally on long-distance transport of photosynthates and larger molecules or complexes through the phloem (Lucas, 1995; Zambryski and Crawford, 2000). A good example is the sucrose transporter SUT1, whose mRNA is transported into the phloem before it is translated (Kühn et al., 1997). Moreover, grafting experiments have demonstrated the existence of long-distance mRNA movement in plants (Kim et al., 2001; Ruiz-Medrano et al., 1999). More recently, studies conducted in several plant species have demonstrated that non-cell-autonomous effects of transcription factors involved in plant development can be mediated by protein movement (reviewed by Haywood et al., 2002; Wu et al., 2002).

The first example of transcription factor movement was discovered through studies of the homeodomain protein KNOTTED1 (KN1) in maize. Most plant organs originate post-embryonically from meristems, which include stem cells set aside during embryogenesis. In the aerial part of the plant, new organs emerge from the shoot apical meristem (SAM), which consists of three tissue layers, L1-L3. KN1 protein is found throughout the maize SAM but *kn1* mRNA is absent from the L1 layer (Jackson et al., 1994; Smith et al., 1992). In leaf injection experiments, not only was KN1 transported to the surrounding tissue through plasmodesmata, but KN1 also increased the SEL of plasmodesmata, enabling the transport of *kn1* sense RNA and protein complexes (Kragler et al., 2000; Lucas et al., 1995). KN1 can also move away from its source of expression when expressed from heterologous promoters in *Arabidopsis* (Kim et al., 2002). There is similar evidence that the *Antirrhinum* MADS-box transcription factor DEFICIENS (DEF) moves from inner to outer tissue layers in developing flowers, although the extent of movement is stage- and organ-dependent (Perbal et al., 1996).

In *Arabidopsis*, two endogenous transcription factors move into neighboring cells: SHORTROOT (SHR) (Nakajima et al., 2001) and LEAFY (LFY) (Sessions et al., 2000). RNA of the GRAS-family transcription factor SHR is expressed in the stele of the root (Helariutta et al., 2000), but SHR protein is found in both the stele and the surrounding endodermis, which is missing in *shr* mutants. Further studies using transgenic mis-expression confirmed that movement of SHR from the root stele to endodermis is required for endodermis development (Nakajima et al., 2001). RNA of the plant-specific transcription factor LFY is expressed in all three layers of young floral primordia, which are mis-specified as shoots in strong *lfy* mutants (Weigel et al., 1992). Surprisingly, *LFY* RNA expression in the L1 of developing flowers is sufficient to fully rescue the *lfy*-mutant phenotype. In such transgenic plants, LFY protein, but not *LFY* RNA, is detected in all layers of the rescued flowers, indicating that LFY protein moves from the L1 into inner layers (Sessions et al., 2000). By contrast, the transcription factor APETALA1 (AP1), which has similar in vivo functions as LFY but is structurally unrelated, behaves largely cell-autonomously (Sessions et al., 2000).

Table 1. Transgenes used in this study

Promoter	Coding sequence	Construct
ML1	*2×GFP*	XW17
	NLS:2×GFP	XW33
	TVGV:GFP	XW14
	GFP:AP1	JD29
	AP1:GFP	JD52
	GFP:LFY	XW11(N)
	LFY:GFP	XW10
	GLFY	GLFY
	GFP:LFYΔ1	XW65
	GFP:LFYΔ2	XW66
	GFP:LFYΔ3	XW67
	glfy-2	JD4
	glfy-3	JD5
	glfy-9	JD7
	glfy-20	JD8
AP1	*GFP:AP1*	JD33
	AP1:GFP	JD51
CaMV 35S	*GFP:LFY*	XW19(N)
	LFY:GFP	JD2
LFY	*LFY*	XW44
	GLFY	XW45
*AG intron**	*2×GFP*	XW39
	NLS:2×GFP	XW13
	GLFY	XW32
	LFY	XW40

Although movement of transcription factors in *Arabidopsis* and other plants is by now well-established, there are still major gaps in understanding the underlying mechanisms. Here, we characterize the mode of LFY movement in *Arabidopsis* SAMs and floral primordia. Using functional LFY-GFP fusion proteins, we show that LFY moves more readily from the L1 into deeper cell layers than laterally into adjacent, clonally related cells. By contrast, a functional AP1-GFP fusion is unable to move from its source cells. Comparison of the dynamics of LFY-GFP fusion proteins with other GFP fusions suggests that this movement is driven by diffusion. Deletion experiments failed to identify a specific movement signal in LFY, which is compatible with the conclusion that LFY movement is non-targeted. The hypothesis of non-targeted movement is also supported by the finding of a correlation between cytoplasmic localization and the ability of these proteins to move to adjacent cells.

MATERIALS AND METHODS

Transgenes

See Table 1 for a list of transgenes used in this study.

LFY-GFP fusions

*Pst*I sites were added to both ends of the *mGFP*5 coding sequence (Haseloff et al., 1997) using oligonucleotide primers. The *GFP Pst*I fragment was inserted into the internal *Pst*I site of a *LFY* cDNA in pAS116 (Sessions et al., 2000), which contains a *LFY* cDNA with 300 bp of *LFY* 3′UTR and a *nos* terminator, creating pBS-GLFY. The *GLFY/nos* fragment was then ligated into the binary vector pMX202,

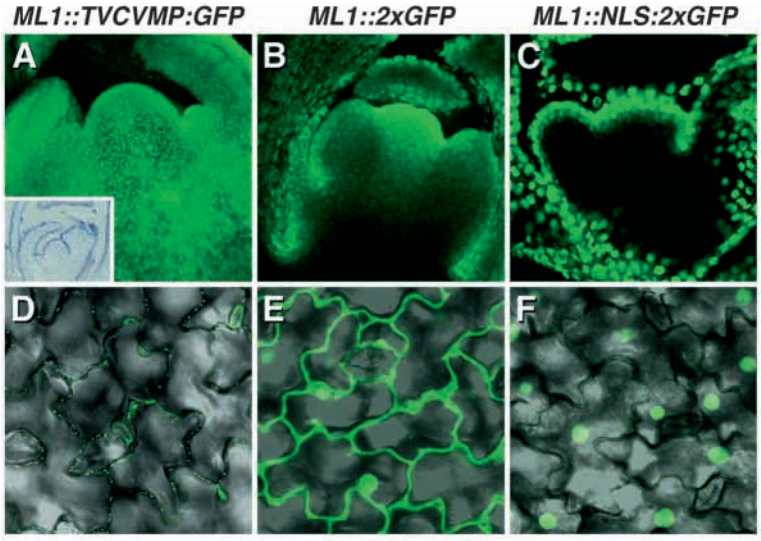

Fig. 1. Movement of TVCVMP:GFP, 2×GFP and NLS:2×GFP. Confocal images of GFP fluorescence in inflorescence apices (A-C) and leaf epidermis (D-F) of 2-week-old *ML1::TVCVMP:GFP* (A,D), *ML1::2×GFP* (B,E) and *ML1::NLS:2×GFP* (C,F) transgenic plants. Inset in A shows that *TVCVMP:GFP* RNA is restricted to the L1, as detected by in situ hybridization. In D-F, the GFP fluorescence channel is overlaid with the transmissible light channel. TVCVMP:GFP is found in all cells in the inflorescence apex (A), and it is associated with the cell wall in a punctate pattern (D). 2×GFP forms a gradient of six to ten cells in the apex, with the highest concentration in L1 (B). It is located in both the nucleus and the cytoplasm (E). NLS:2×GFP can move efficiently only one cell layer from the L1 in the apex (C), and it appears mostly nuclear (F).

which includes the RBCS terminator sequence (M. A. Busch, personal communication), along with the *ML1* promoter fragment from pAS98 (Sessions et al., 1999).

ML1::GFP:LFY was created by ligating the *ML1* promoter from pAS99 (Sessions et al., 1999) to the 5′ portion of *GFP:LFY* 5′ (up to the *Xba*I site in *LFY*) from pRTL2-sGFP:LFY, and the 3′ portion of *LFY* from pAS116 into binary vector pMX202. In this fusion, there is an additional serine inserted between *GFP* and *LFY*. *35S::GFP:LFY* was created by ligating the same *GFP:LFY* 5′ and *LFY* 3′ fragments into binary vector pCHF3, which contains a *CaMV 35S* promoter (Fankhauser et al., 1999).

ML1::LFY:GFP was created by ligating the 5′ portion of *ML1::LFY* 5′ up to the *Hin*dIII site from pAS104 (Sessions et al., 2000) and the 3′ portion of *LFY:GFP* from pBS-ML1::LFY-link-sGFP into the binary vector pJIHOON212 (J. H. Ahn, personal communication). *35S::LFY:GFP* was created by ligating the 5′ portion of *LFY* cDNA from pAS107 (up to the *Hin*dIII site) with the 3′ portion of *LFY:GFP* into pCHF3.

lfy mutant alleles

Four *lfy* alleles were included in this study: *lfy*-2, *lfy*-3, *lfy*-9 and *lfy*-20 (Weigel et al., 1992). *GFP* fusions of these mutant versions were generated in the same way as the *ML1*::*GLFY* fusion.

LFY truncations

Truncations of the LFY coding sequence were created in the context of pBS-LFY, which includes both the full-length cDNA and 300 bp of the *LFY* 3′UTR. *LFYΔ1* was generated by opening, filling-in and religating the *Bam*HI site overlapping the start codon and the *Xba*I site at position 379, which results in an in-frame deletion of amino acids 4 to 127. *LFY*Δ2 was created by opening, filling-in and religating the *Xba*I site at position 379 and the *Sty*I site at position 860, which results in an in-frame deletion of amino acids 128 to 287. *LFY*Δ3 was created by opening, filling-in and religating the *Sty*I site at position 860 and the *Hin*dIII site at position 974, which leads to a frame shift such that amino acids 289 to 424 are replaced with the sequence SFKCSQKSV. Fusions of the *GFP:LFY* truncations to the *ML1* promoter were created by combining the promoter fragment from pAS99, the 5′ fragment of *GFP:LFY* from pRTL2-sGFP:LFY and the respective *LFY* truncations in pMX202.

AP1-GFP fusions

For *AP1:GFP*, restriction sites were added to the *AP1* cDNA sequence by PCR amplification, using pAM571 as a template (M. Yanofsky, personal communication), which resulted in an *Eco*RI and a *Bam*HI site in front of the 5′ UTR, and a *Pst*I site at the 3′ end, replacing the stop codon. *GFP* coding sequence was amplified from pCAMBIA1302, replacing the start codon with a *Pst*I site and adding an *Xba*I site to the 3′UTR. For *GFP:AP1*, the start codon of *AP1* was replaced with a *Pst*I site, and an *Xba*I site was added to the 3′UTR. An *Eco*RI site was added to the 5′ end of the *GFP* coding region and a *Pst*I site replaced the stop codon. In both fusions, the *Pst*I site also created an alanine linker of 2-3 amino acids. *ML1::AP1:GFP* and *ML1::GFP:AP1* were created by ligating the *ML1* promoter from pAS99 to the *AP1* and *GFP* fragments in the background of pMX202. *AP1::AP1:GFP* and *AP1::GFP:AP1* were created in the same way as the *ML1* versions, but using the *AP1* promoter from pAM571.

Other *ML1* constructs

ML1::2×GFP was created by ligating the *ML1* promoter to *TEV5′:2×sGFP* from pRTL2-2×sGFP (Crawford and Zambryski, 2000) and inserting into pMX202. The *NLS-2×sGFP* fragment from pRTL2-NLS:2×sGFP (Crawford and Zambryski, 2000) was used to generate *ML1::NLS:2×GFP*. *ML1::TVCVMP:GFP* was generated by ligating the *ML1* promoter to coding sequences for a Turnip Vein Clearing Virus Movement Protein (TVCVMP):GFP fusion into pMX202.

Ectopic expression in the center of shoot and flower meristems

A 833 bp *Bam*HI/*Hin*dIII fragment from the 3′ end of the second *AG* intron (Busch et al., 1999) was used to drive expression in the *AG* domain. This enhancer fragment, from pMX141, carries a point mutation (from CCTTATTTGG to AATTATTTGG) that results in ectopic activity in the inflorescence meristem in a *lfy*-independent manner (Hong et al., 2003). The enhancer was placed upstream of a –46 bp cauliflower mosaic virus *35S* minimal promoter in pMX202. *AG intron*::2×GFP*, *AG intron*::NLS:2×GFP*, *AG intron*::LFY* and *AG intron*::GLFY* were created by inserting the respective coding sequence fragments into this cassette.

LFY rescue constructs

The *LFY* and *GLFY* rescue constructs were generated by expressing the *LFY* cDNA and *GLFY* under the control of the 2.3 kb *LFY* promoter (Blázquez et al., 1997).

Plant material

Plants were grown in long days (16 hour light / 8 hour darkness) under ~120 μE m^{-2} $seconds^{-1}$ light provided by a 3:1 mixture of cool-white and GroLux (Osram Sylvania) fluorescent bulbs, at 21°C. *lfy-12* and *ap1-15* are strong alleles in the Columbia background (Huala and

Sussex, 1992; Ng and Yanofsky, 2001). Plant transformations were carried out using the floral dip method (Weigel and Glazebrook, 2002). For each transgene, 40-70 T1 lines were initially analyzed, and at least three plants each from three independent lines were used in further characterization and imaging. Transgenic seedlings were selected on MS agar plates containing 50 μg/ml kanamycin, then transplanted to soil. For all transgenes presented in this work, multiple samples from both agar- and soil-grown plants of at least three generations were examined, always with very similar results. In all cases where a mutant allele was involved, transgenic lines with both wild-type and mutant backgrounds have been examined. We never observed an effect of the endogenous allele on the GFP signal.

In situ hybridization

In situ hybridization was performed as described (Sessions et al., 2000; Weigel and Glazebrook, 2002). Digoxigenin-labeled antisense RNA probe for TVCVMP:GFP was generated by digesting pBS-TVCVMP:GFP with *Xho*I, then transcribing it using T3 polymerase,

Immunoblot analysis

Crude protein extract was obtained from 2-week-old seedlings and separated on 4-12% gradient gels (NuPAGE, Invitrogen) with Benchmark Protein Marker (Invitrogen). Samples were transferred to a PVDF membrane by electroblotting, and incubated with rabbit anti-GFP primary antibody (1:1000 dilution, Molecular Probes). An HRP-conjugated goat anti-rabbit secondary antibody (1:5000 dilution, BioRad) was used for signal detection with SuperSignal Chemiluminescent Substrate (Pierce).

Microscopy

GFP fluorescence images

For *ML1* transgenic plants, emerging leaves and apices were dissected from 10- to 12-day-old seedlings grown on MS agar plates. For *AG intron** transgenic plants, primary inflorescence apices were dissected from 4-week-old plants for image analysis. Confocal images were collected using a 40× or 63× oil-immersion lens on a Leica SPII spectral confocal laser scanning microscope. GFP fluorescence was excited with a 488 nm Argon laser, and images were collected in the 500-550 nm range. In some cases, images from the transmissible light channel were collected simultaneously. For *ML1* transgenic plants, both vegetative and inflorescence apices were examined. In each figure, all panels were collected during the same microscopy session from plants grown under exactly the same conditions.

Light microscopic images

Pictures of flowers were taken with a Polaroid DMC digital camera mounted on an Olympus SZH10 stereomicroscope. Images of in situ hybridization were taken with a SPOT digital camera mounted on a Nikon compound microscope.

Quantification of the subcellular distribution of GFP-LFY fusions

Confocal image series of epidermal cells in emerging leaves were collected from transgenic plants carrying ML1::GLFY, ML1::GFP:LFY, and ML1::LFY:GFP using a 63× objective, with 1 μm steps. Separate masks were generated for the nuclei and the cytoplasm using the image processing software Khoros (www.khoral.com). Total signal intensity in each part was calculated for every section and summed within the same image series. Data from approximately thirty cells were averaged for each transgene.

RESULTS

Movement of viral MP and GFP from the L1 layer

Plant viral movement proteins (MPs) and the green fluorescence protein (GFP) have been widely used in studies of intercellular protein movement in plants, and represent examples of targeted and non-targeted movement, respectively. To provide data relevant to transcription factor movement in *Arabidopsis* meristems, we first set out to examine the movement of these two types of proteins from the L1 layer when expressed in intact plants. When we expressed the Turnip Vein Clearing Virus Movement Protein fused to GFP (TVCVMP:GFP) in the L1, using the L1-specific *ML1* promoter (Sessions et al., 1999), GFP fluorescence was detected in all cells of vegetative and inflorescence apices, although the signal was slightly higher in the L1 layer (Fig. 1A). In situ hybridization (Fig. 1A, inset) demonstrated that *TVCVMP:GFP* RNA was restricted to the L1 in these plants, not only confirming the specificity of the *ML1* promoter, but also confirming that *TVCVMP* does not transport its own RNA. In the maturing leaf epidermis, which is part of the L1 layer and where cellular boundaries are clearly visible, TVCVMP:GFP was found specifically associated with the cell wall in a punctate pattern (Fig. 1D), which may coincide with the location of plasmodesmata pit fields (Crawford and

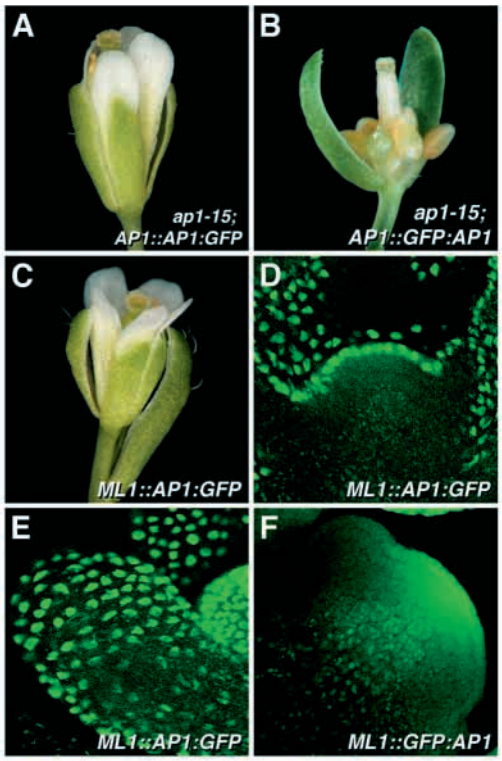

Fig. 2. Absence of movement of a functional AP1:GFP fusion. The C-terminal AP1:GFP fusion can rescue *ap1-15* mutant flowers to near wild type when expressed from the *AP1* promoter (A), and leads to the development of bracts subtending flowers when expressed in the L1 of wild type (C). The N-terminal fusion GFP:AP1 does not rescue *ap1-15* mutant flowers when expressed from the *AP1* promoter (B). Confocal images of GFP fluorescence in inflorescence apices of 2-week-old *ML1::AP1:GFP* (D,E) and *ML1::GFP:AP1* (F) plants are shown. AP1:GFP does not move from the L1 (D; optical section through the shoot apex), and is tightly associated with nuclei (E; tangential section through the L1 of a floral primordium). By contrast, GFP:AP1 is largely cytoplasmic and moves into deeper tissues layers from the L1 (F).

Zambryski, 2001; Heinlein et al., 1998). Deeper optical sections also revealed that a substantial fraction of TVCVMP:GFP can enter the mesophyll cells from the leaf epidermis (data not shown). These data confirm that plant viral movement proteins are able to move over many cell diameters (Citovsky, 1999; Haywood et al., 2002).

We then examined the behavior of GFP, which does not display targeted movement. We chose a dimerized version of GFP (2×GFP) because it has a molecular weight of approximately 54 kDa, which is similar to LFY, which is 47 kDa in size. In previous leaf bombardment studies, it has been demonstrated that 2×GFP can move at least occasionally into neighboring cells, whereas adding a nuclear localization signal (NLS) nearly fully blocks this movement (Crawford and Zambryski, 2000). Here, 2×GFP and NLS:2×GFP, when expressed stably in the L1, behaved similarly. In vegetative and inflorescence apices, 2×GFP could move at least six cells into the L2 and L3 layers, forming a gradient with the highest levels in the L1 (Fig. 1B). It appeared both cytoplasmic and nuclear in the leaf epidermis (Fig. 1E). In agreement with bombardment studies, only a small amount of NLS:2×GFP was able to enter the L2 layer in apices, with no GFP signal detected in the L3 layer (Fig. 1C). Its subcellular localization was assayed in the leaf epidermis, where most of the GFP signal came from the nuclei (Fig. 1F).

To confirm that the GFP signal was indeed from dimerized GFP instead of a breakdown product resulting in monomeric GFP, whole-cell protein extracts were prepared from transgenic seedlings after imaging and analyzed by western blot with an anti-GFP antibody. For NLS:2×GFP, a major band at about 57 kDa was observed (predicted size 58 kDa), indicating that it was stable (Fig. 4A). Similarly, 2×GFP migrated at about 50 kDa (predicted size 54 kDa), and only a small amount of degradation product was observed for 2×GFP.

Absence of movement of a functional AP1-GFP fusion

To exclude the possibility that all GFP fusion proteins can move, we examined N- and C-terminal fusions of GFP to the transcription factor AP1. We have previously reported that AP1 has largely cell-autonomous effects when expressed in the L1 layer, indicating that AP1 does not move into internal layers, although protein localization was not directly examined (Sessions et al., 2000). In strong *ap1* mutants, sepals and petals fail to develop, and secondary flowers develop in the axil of sepals that have been converted into bracts. A 1.7 kb promoter fragment drives expression of a reporter gene in a pattern similar to that of endogenous *AP1* (Hempel et al., 1997), and when fused to an *AP1* cDNA can largely rescue the phenotype of the strong *ap1-15* allele (M. Yanofsky, personal communication). Expression of the C-terminal AP1:GFP fusion from the *AP1* promoter rescued most aspects of the *ap1-15* mutant phenotype, although rescue was variable in different lines (Fig. 2A). In contrast, the N-terminal GFP:AP1 fusion was unable to rescue the *ap1-15* phenotype (Fig. 2B).

We subsequently expressed both fusions under the control of the *ML1* promoter to test for their movement. When AP1:GFP (55 kDa) was expressed under the *ML1* promoter, we observed some gain-of-function phenotypes associated with the overexpression of AP1 in the L1 (Sessions et al., 2000), such as the development of bracts subtending the flowers (Fig. 2C). The GFP signal in apices from these plants was restricted to the L1 layer (Fig. 2D), and was very tightly associated with the cell nuclei (Fig. 2E). This observation supports the previous conclusion that AP1 does not move from the L1 layer into the inner tissue, and that only some transcription factors can move between tissue layers in the *Arabidopsis* apex. GFP:AP1, which appeared non-functional when expressed from the *AP1* promoter, moved into all tissue layers from the L1 in *ML1::GFP:AP1* apices (Fig. 2F). The subcellular localization of GFP:AP1 was also abnormal; instead of being exclusively nuclear like the functional AP1:GFP fusion, it produced a mostly cytoplasmic signal. The cytoplasmic localization may well be causally related to the ability of this fusion to move and to the inability to rescue the mutant phenotype; however, it is unlikely that movement itself interferes with AP1 function. Western blots probed with an anti-GFP antibody showed that both fusions migrated at approximately 52 kDa (expected size 55 kDa; arrow, Fig. 4B). There was only a small amount of free GFP (27 kDa; arrowhead, Fig. 4B), confirming that the observed fluorescence signal reflected the behavior of the fusion protein.

Movement of LFY-GFP fusions from the L1 layer

To avoid artifacts caused by the addition of GFP to a specific domain of the LFY protein, we generated three different LFY-GFP fusions: GFP:LFY, an N-terminal fusion; GLFY, with an insertion of GFP at amino acid 31; and LFY:GFP, a C-terminal fusion. We used these fusions, which increase the size of LFY by about half, from 47 to 74 kDa, to further characterize the movement of LFY, which has been previously detected using anti-LFY antibodies (Sessions et al., 2000). The fusions were introduced into *lfy-12*/+ plants under the control of the *ML1*

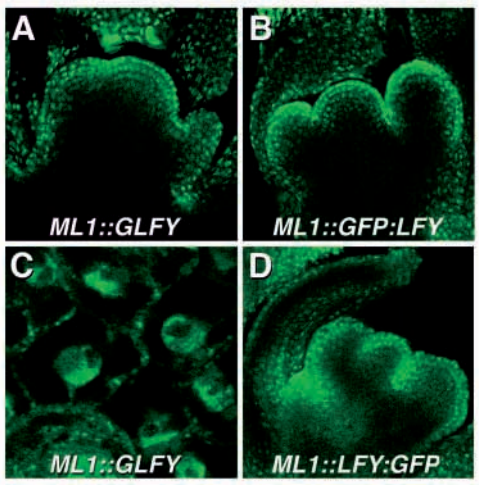

Fig. 3. Movement of LFY-GFP fusions. Confocal images of GFP fluorescence in inflorescence apices (A,B,D) and leaf epidermis (C) of 2-week-old *ML1::GLFY* (A,C), *ML1::GFP:LFY* (B) and *ML1::LFY:GFP* (D) transgenic plants. GLFY moves several cell layers into the underlying tissue from the L1 in the apex (A). GFP:LFY shows a similar gradient from the L1 as GLFY (B). The signal from LFY:GFP, which has the longest moving range, appears 'fuzzy' because of its higher cytoplasmic localization (D). All three fusions are localized to both the nucleus and cytoplasm in leaf epidermal cells, and bright spots are sometimes found along the cell wall with GLFY (shown in C).

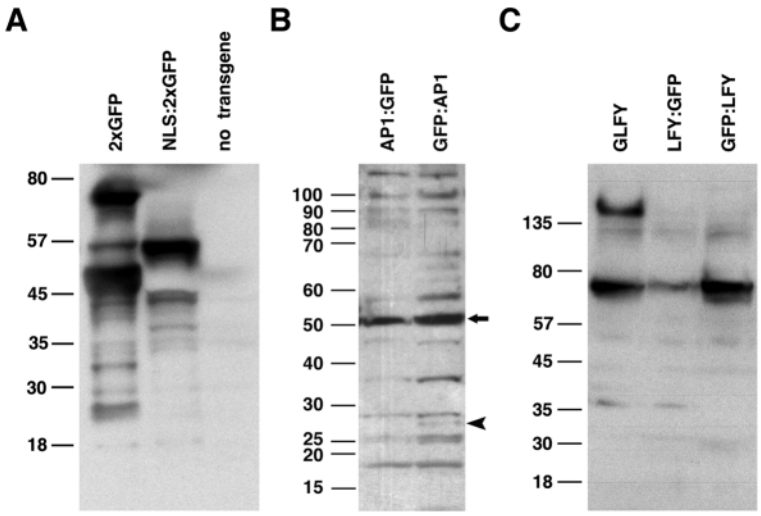

Fig. 4. Western blots of whole-cell extracts from 2-week-old transgenic seedlings of *ML1::2×GFP* and *ML1::NLS:2×GFP* (A), *ML1::AP1:GFP* and *ML1::GFP:AP1* (B), and *ML1::GLFY, ML1:LFY:GFP* and *ML1::GFP:LFY* (C) probed with anti-GFP antibody. Extract of wild-type Col-0 seedlings was used as a negative control in A. Blots were deliberately overexposed to reveal the presence of any minor bands. No major degradation products in the form of single GFP were found. Bands detected at higher molecular weight in the 2×GFP and GLFY lanes probably represent dimers formed during the extraction procedure. In B, GFP:AP1 bands are marked with an arrow and the minor single GFP band in GFP:AP1 is marked with an arrowhead.

promoter. To test independently for functionality, the N- and C-terminally tagged versions were also expressed under the control of the constitutive *CaMV 35S* promoter. All five transgenes were able to rescue the *lfy-12* mutant phenotype, and to cause the typical gain-of-function phenotypes associated with overexpression of LFY (Weigel and Nilsson, 1995), which indicates that the three GFP fusions were fully functional.

Using the *ML1* promoter lines, we examined the subcellular localization of the LFY-GFP fusions, as well as their movement from the L1 layer (Fig. 3). All three fusions were detected in both the nucleus and cytoplasm, which was best seen in leaf epidermal cells (GLFY shown in Fig. 3C), and all produced more cytoplasmic signal than NLS:2×GFP (Fig. 1F). In leaf epidermal cells, a punctate signal that appeared along the cell wall was observed with all three fusions (GLFY shown in Fig. 3C), which suggests a possible association with plasmodesmata pit fields. GLFY and GFP:LFY moved three to four cell layers into the L2 and L3 in both the vegetative and inflorescence apices, forming a gradient with the highest concentration in the L1 (Fig. 3A,B). Both GLFY and GFP:LFY were restricted to the epidermal layer in maturing leaves (data not shown). LFY:GFP moved further, approximately 10 cell layers in apices (Fig. 3D). In leaves, its distribution in the epidermis was similar to that of GLFY and GFP:LFY, but it could also be detected in the underlying mesophyll cells (data not shown). Overall, LFY:GFP appeared more cytoplasmic than GLFY and GFP:LFY. This observation was confirmed by quantifying the total signal intensity in the nucleus and cytoplasm of the epidermal cells of emerging leaves from all three fusions (see Materials and Methods). For GLFY and GFP:LFY, the nuclear to cytoplasmic signal ratios were very similar, 1:2.3 and 1:2.5, respectively. By contrast, the nuclear to cytoplasmic ratio of LFY:GFP was 1:5.4, a twofold increase compared with GLFY and GFP:LFY. Western blots probed with an anti-GFP antibody demonstrated that there was little degradation of the fusion proteins, indicating that the in vivo fluorescence signal came from the intact fusion proteins (Fig.

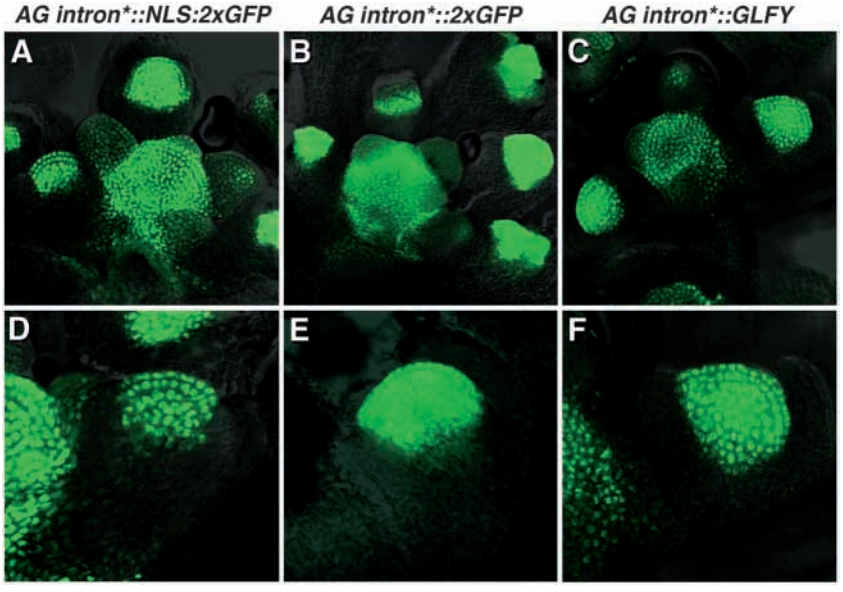

Fig. 5. Restricted lateral protein movement in the *Arabidopsis* shoot apex. Confocal images of GFP fluorescence for inflorescence meristems with surrounding young floral primordia from *AG intron*::NLS:2×GFP* (A,D), *AG intron*::2×GFP* (B,E) and *AG intron*::GLFY* (C,F) plants. The confocal images have been overlaid with images from the transmitted-light channel for orientation only. Note that the confocal images are optical sections, which is not true for the transmitted-light images. The mutated *AG* sequences in the reporters activate expression in the shoot apical meristem and the center of young flowers. Close-up views of stage 3 flowers reveal discrete lateral boundaries of the GFP signal (D-F). No lateral movement is obvious in any of the three cases.

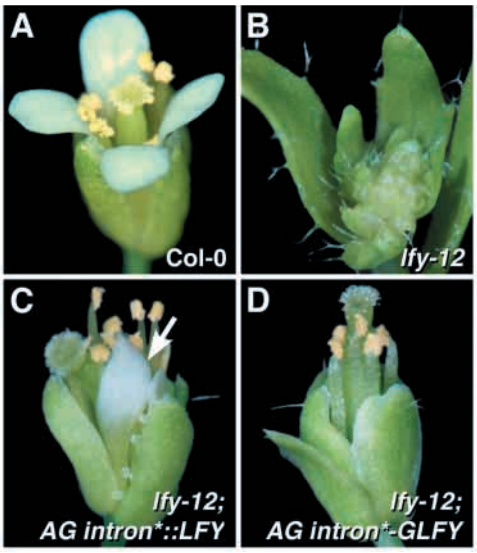

Fig. 6. Rescue of *lfy-12* mutant flowers by LFY and GLFY expression in the center of the floral primordia. A wild-type *Arabidopsis* flower (A) and a shoot-like structure that replaces a flower in *lfy-12* mutants (B) are shown. Both *AG intron*::LFY* and *AG intron*::GLFY* can rescue the stamens and carpels of *lfy-12* mutant flowers (C,D), but only LFY rescues petals completely (arrow in C).

4C). Taken together, the behavior of LFY-GFP fusions is intermediate between that of 2×GFP and NLS:2×GPF, both with respect to their cytoplasmic localization and their ability to move from the L1 layer.

Lateral movement of GLFY within tissue layers

Movement of LFY-GFP fusions from the L1 to deeper layers indicated that they can pass through the secondary plasmodesmata, which connect cells that are not clonally related. To study the lateral movement of LFY within each tissue layer, we took advantage of a mutated enhancer from the *AGAMOUS* (*AG*) gene, which drives reporter gene expression in the inflorescence meristem in addition to the normal *AG* domain in the center of floral meristems (Hong et al., 2003).

We expressed 2×GFP, NLS:2×GFP and GLFY under the control of the *CaMV 35S* minimal promoter fused to the mutated *AG* enhancer (*AG intron**). Except for the subcellular localization, the expression patterns of NLS:2×GFP (Fig. 5A) and 2×GFP (Fig. 5B) were indistinguishable, with fluorescent signal in the inflorescence meristem and central domain of young floral primordia. The signal in both the shoot and floral meristems had discrete boundaries, indicating that GFP neither moved from the inflorescence meristem into emerging floral primordia, nor moved from the center of stage 3 flowers to the periphery (Fig. 5D,E). However, a gradient of 2×GFP could be seen extending into deeper cell layers in L3 in stage 3 flowers (Fig. 5E), which is consistent with our earlier observation. Thus, compared with movement from the L1 to internal layers, lateral movement of 2×GFP and NLS:2×GFP within tissue layers is much more limited, or possibly even absent. The much reduced movement of 2×GFP and NLS:2×GFP within L1 and L2, compared with movement between layers, suggests that the plasmodesmata SEL within these two layers is lower than that between layers.

Similarly, GLFY could not move laterally within the same tissue layer (Fig. 5C,F). We have previously found that periclinal, as well as anticlinal, sectors of LFY can rescue mutant parts of flowers, indicating that LFY moves in both directions (Sessions et al., 2000). One possibility for the apparently different behavior of LFY and GLFY is that the greater size of GLFY, compared with endogenous LFY, reduces its ability to move laterally within tissue layers.

We used a functional assay to test this assumption. For this, we took advantage of the fact that the mutated *AG* enhancer is active in *lfy* mutants. When we expressed *LFY* and *GLFY* under the control of this enhancer in *lfy-12* plants, the same fraction of transgenic lines showed rescue in flowers (4 out of 12 lines for LFY and 4 out of 11 lines for GLFY; no significant difference using Fisher's exact test), suggesting that GLFY has similar activity to LFY. However, the rescued flowers differed in phenotype. Both GLFY and LFY rescued the development of the two inner whorls, which contain stamens and carpels, and in which the *AG* enhancer is active (Fig. 6), but only LFY was able to rescue petal development. All four of the *lfy-12; AG intron*::LFY* lines that showed phenotypic rescue produced at least some flowers with petals, and many flowers had the normal complement of four petals. By contrast, none of the four *lfy-12; AG intron*::GLFY* lines produced flowers with petals. As GLFY appears to be as active as LFY in the inner two whorls where it is produced, the difference in their activity in the outer two whorls is consistent with the conclusion that LFY can move more extensively than GLFY.

Requirement of LFY movement for normal flower development

Although it has been established that LFY can move within flowers (Sessions et al., 2000) (this work), it is unclear whether LFY movement is required for normal flower development, because LFY protein and RNA are expressed throughout young flowers (Parcy et al., 1998). The difference between LFY and GLFY in their apparent ability to move within layers allowed us to address this question by expressing LFY and GLFY under the control of the *LFY* promoter and comparing their ability to rescue *lfy-12* mutants. Of ten *LFY::LFY* transgenic lines in the *lfy-12* background, three showed complete rescue, and the remaining seven showed nearly complete rescue, with only a few flowers having reduced petal or stamen number. By contrast, only six out of fourteen *LFY::GLFY* lines in the *lfy-12* background showed a similar degree of rescue; the remaining eight lines resembled weak or intermediate *lfy* mutants and were sterile. This difference between their ability to rescue is statistically significant based on Fisher's exact test. Although only the opposite result – full ability of movement-compromised LFY to rescue *lfy* mutants – would have been entirely conclusive, our finding is consistent with the notion that LFY movement may be required for normal flower development. However, we cannot exclude the possibility that GLFY is somewhat less active as a transcription factor than native LFY, although no difference in activity could be observed when tested from the *35S* and *AtML1* promoters.

Movement of mutant LFY proteins

To further test whether LFY movement is regulated, we investigated whether deleting parts of the LFY protein abolishes intercellular movement. Three large, non-

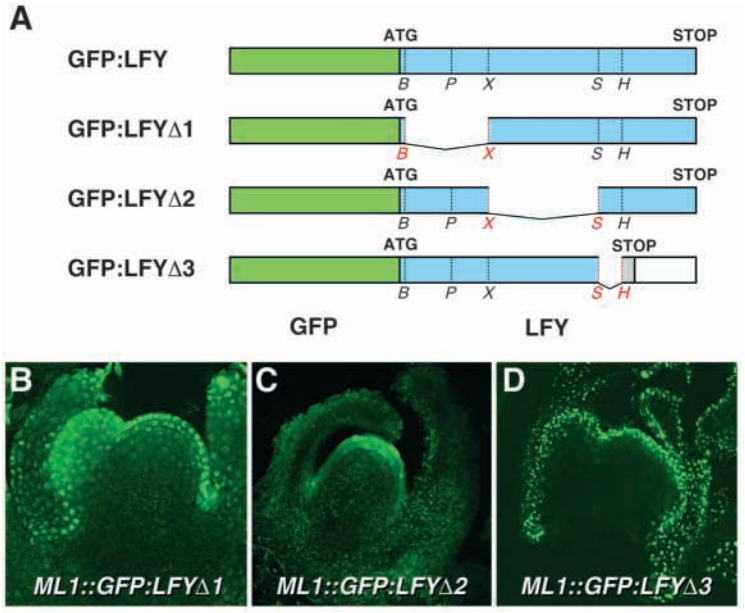

Fig. 7. Movement of truncated LFY from the L1. (A) Diagram of GFP:LFY and the three truncated forms of the GFP:LFY fusion protein. GFP-coding sequence is shown in green and LFY-coding sequence in light blue, along with restriction sites used to make the deletions: B, *Bam*HI; P, *Pst*I; X, *Xba*I; S, *Sty*I; H, *Hin*dIII. (B-D) Confocal images of GFP fluorescence in inflorescence apices of 2-week-old plants. All three forms of truncated GFP:LFY move from the L1 to the inner tissue layers. GFP:LFYΔ1 is mostly located in the nucleus (B), whereas GFP:LFYΔ2 appears to be largely cytoplasmic (C). Both can move several cell layers from the L1. GFP:LFYΔ3 is mainly found in the form of large aggregates, but can still move one to two cell layers from the L1 (D).

overlapping deletions were made in the LFY coding sequence, each removing approximately one third of the protein (Fig. 7A). All three were linked to GFP at the N terminus and expressed under the *ML1* promoter. Although they differed in the extent with which they moved from L1 to inner layers, all three deletion variants were still able to move from L1 into the inner layers in both vegetative and inflorescence apices. GFP:LFYΔ1, with an N-terminal deletion, behaved very similar to GFP:LFY. It was mostly located in the nucleus, and formed a gradient of four to five cell layers into the L2 and L3 (Fig. 7B). GFP:LFYΔ2, with a central deletion, was expressed at lower levels and was largely cytoplasmic, presumably because of the deletion of the NLS. GFP signal could be clearly detected for at least three cell layers into the L2 and L3 (Fig. 7C), but its low expression levels may have been limiting our ability to determine its actual range of movement. GFP:LFYΔ3, with a C-terminal deletion, showed the least degree of movement, moving only one to two cell layers from the L1 (Fig. 7D). However, most of the GFP signal was found in large aggregates, sometimes associated with the cell membrane when imaged in the leaf epidermis (data not shown), suggesting that GFP:LFYΔ3 is improperly folded and localizes to a specific subcellular compartment, which may affect its movement. Furthermore, all three truncated versions were able to enter mesophyll cells from the epidermis in maturing leaves (data not shown).

As large deletions may cause mis-folding of a protein, we also generated GFP fusions of four weak and intermediate *lfy* alleles mutating specific residues in GLFY. All four fusions were functional, as they rescued *lfy-12* plants to the phenotype corresponding to the *lfy* allele used for the fusion. When expressed in the L1 layer, all of them could move into the underlying tissue layers. Products of the fusions of the two weak *lfy* alleles, *glfy-2* and *glfy-20*, showed near wild-type movement (Fig. 8A,D; compare with Fig. 3A), whereas the products of the two intermediate alleles, *glfy-3* and *glfy-9*, moved somewhat less well into the L2 and L3, about two to three cell layers (Fig. 8B,C). Taken together, these data suggest either the presence of redundant movement signals, or the absence of a specific movement signal.

DISCUSSION

Since the discovery of transcription factor movement in plants almost a decade ago, questions have arisen regarding how they move and whether movement is regulated. There are at least two scenarios for how transcription factors reach neighboring cells: targeted movement guided by a specific movement or export signal, or non-targeted movement by diffusion (Crawford and Zambryski, 1999). We have performed several tests to determine whether there is evidence for targeted movement of LFY. None of our results point to specific regulation of LFY movement, which suggests that LFY movement is non-targeted. Furthermore, we have found important differences in the dynamics of apical-basal and lateral movement in the apex.

Mode of LFY movement

Several lines of evidence are compatible with the view that LFY movement is non-targeted. First, GFP-LFY fusions produced in the L1 formed limited gradients extending into deeper layers. Their movement range was between that of 2×GFP and NLS:2×GFP, which move in a non-targeted fashion (Crawford and Zambryski, 1999; Crawford and Zambryski, 2000; Kim et al., 2002). This is in contrast to the nearly uniform distribution in the shoot apex (reflecting an active mechanism of cell-to-cell transport) that is observed when a viral movement protein fusion is expressed in the L1. Second, size affects LFY movement, as native LFY was more effective in rescuing *lfy* defects in adjacent cells in the same tissue layer than the larger GLFY fusion. The effect of size is one of the prominent characteristics of non-targeted movement (Zambryski and Crawford, 2000). Third, although differing in stability and sub-cellular localization, all three GFP-LFY truncations and fusions of four mutant *lfy* alleles were able to

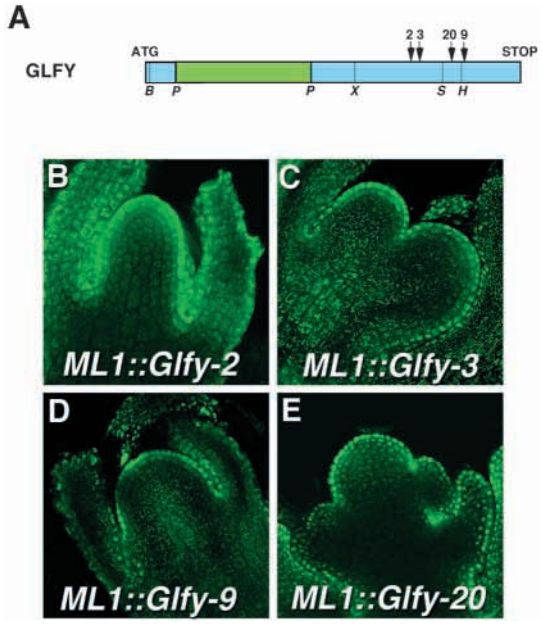

Fig. 8. Movement of mutant GLFY fusions from the L1.
(A) Diagram of fusions of GFP to weak alleles *lfy-2* (P240L) and *lfy-20* (N306D), and intermediate alleles *lfy*-3 (T244M) and *lfy*-9 (R331K) in the GLFY background. (B-E) Confocal images of GFP fluorescence in inflorescence apices of 2-week-old plants. All fusion proteins can move from the L1 into interior layers, although to different degrees.

move from the L1 into interior cell layers. Although one cannot exclude the possibility that LFY has several redundant movement signals, the simpler explanation is that the LFY protein sequence does not contain a specific movement or export signal.

It has been suggested that non-targeted movement, like targeted movement, occurs through plasmodesmata. The potential localization of foci of GFP-LFY fusion proteins along the cell wall in the leaf epidermis supports this hypothesis. This also suggests that the size exclusion limit of the secondary plasmodesmata connecting tissue layers in the *Arabidopsis* apex is greater than 74 kDa (the size of the LFY-GFP fusions), which is consistent with previous estimates for nascent leaves (Zambryski and Crawford, 2000).

Movement within and between tissue layers

An important new finding is that GFP variants, as well as GLFY, move more easily in the apical-basal direction than laterally. Using in vivo function as a criterion, we found that the GLFY fusion was less efficient than the native LFY in moving from the center of floral primordia to the periphery. This functional difference is most likely a result of their size difference, because both proteins are fully functional in the cells where they are produced. Similar to previous studies that have demonstrated that the inner central zone (L3) does not allow fluorescent tracer uploading from the vascular tissue (Gisel et al., 1999; Gisel et al., 2002), we observed much more limited lateral movement within the L3. Thus, intercellular movement needs to be considered in the context of the specific location and developmental stage within the plant.

That lateral movement is less easily achieved than apical-basal movement may also explain the fact that LFY does not move out of floral primordia into the inflorescence meristem in wild type (Parcy et al., 1998; Sessions et al., 2000), as lateral movement would be required for efficient protein exchange between the two tissues. The inflorescence meristem may even form a symplasmic domain that is insulated from emerging floral primordia (Rinne and van der Schoot, 1998), thus restricting movement of all macromolecules. Alternatively, there may be selective gating, such that movement of only certain macromolecules from floral primordia into the inflorescence meristem (and vice versa) is permitted. A similar mechanism may also be responsible for maintaining discrete whorl boundaries within the flower. In this context, it is noteworthy that floral homeotic proteins that are expressed in distinct whorls of the developing *Arabidopsis* flower, such as AP1 and AP3, do not move, whereas LFY, which is expressed throughout the flower, does (Jenik and Irish, 2001; Sessions et al., 2000).

Movement and subcellular localization

LFY and LFY-GFP fusions can move into the inner tissue layers from the L1. By contrast, AP1:GFP does not move between tissue layers. This cannot be simply due to size, because an N-terminal fusion, GFP:AP1, could move well. Furthermore, GFP:AP1 (55 kDa) is smaller than either NLS:2×GFP (57 kDa) or LFY-GFP fusions (74 kDa). Therefore, if LFY is moving by diffusion, AP1 and AP1:GFP must be actively retained in the cells where they are expressed. One way to achieve the retention may be by subcellular localization, such as nuclear or ER localization. From this study, we have found that there is a good correlation between nuclear localization and movement: 2×GFP, which is highly cytoplasmic, can move a considerable distance from the L1, and the same is true for the predominantly cytoplasmic GFP:AP1 fusion. AP1:GFP, which appeared to be exclusively nuclear, did not move. Between these two extremes, NLS:2×GFP showed little cytoplasmic localization and moved only one cell layer. The GFP-LFY fusions all showed more cytoplasmic localization than NLS:2×GFP, and all moved farther than NLS:2×GFP but less than 2×GFP. Among the three GFP-LFY fusions, LFY:GFP had the most cytoplasmic localization and moved the farthest.

Another possible mechanism for retaining a protein could be through the formation of large protein complexes with more exclusive subcellular localization, or simply with sizes above the SEL of plasmodesmata. This may contribute to the retention of MADS domain proteins such as AP1, as several of them, including AP1, are known to form heteromultimers in the absence of DNA (Egea-Cortines et al., 1999; Honma and Goto, 2001). In addition, it has been shown that AP3, a MADS-domain transcription factor that does not move between tissue layers, needs to heterodimerize with another MADS-domain protein, PISTILLATA (PI), in order to localize to the nucleus (McGonigle et al., 1996). It is possible that the GFP:AP1 fusion disrupts such interactions, thereby interfering with biological activity and nuclear localization, as well as with retention in the cells where it is produced.

In this context, it is noteworthy that SHR is found in both the nucleus and cytoplasm of the stele, where it is produced. From there, SHR moves exactly one cell diameter, into the adjacent endodermis, where it is located entirely in the nucleus

(Nakajima et al., 2001). This observation is consistent with a model in which SHR gets trapped in the nuclei of the endodermis through interaction with a partner that causes translocation to the nucleus, similar to the AP3/PI interaction (McGonigle et al., 1996).

Mechanisms of movement

Our results are compatible with the view that LFY movement is driven by diffusion. However, it remains unclear whether the same conclusion can be drawn regarding the intercellular movement of other transcription factors. Another well-studied example of a trafficking transcription factor is KN1 of maize (Kim et al., 2002; Kragler et al., 2000; Lucas et al., 1995). As with LFY, KN1-GFP fusions are detected in the nucleus and cytoplasm, and in a punctate pattern associated with the cell wall (Kim et al., 2002). In contrast to LFY, for which various deletions did not prevent movement, a simple mutation in the homeodomain and the potential NLS of KN1 abolished its movement. Furthermore, experiments with tobacco have indicated the presence of a cellular component that is limiting for KN1 movement, and have suggested that the mode of KN1 trafficking may be related to the targeted movement of viral movement proteins (Kragler et al., 2000). However, other results are consistent with KN1 moving in a non-targeted fashion. In bombardment assays, KN1-GFP moved considerably less well than a fusion of GFP to the movement protein of TVCV (Kim et al., 2002). Furthermore, movement of KN1 expressed from the *SCR* promoter in the shoot apex of transgenic *Arabidopsis* plants was rather limited (Kim et al., 2002), similar to that of NLS:2×GFP or GFP:LFY expressed from the *ML1* promoter (this work). Further studies in a non-heterologous system will be required to clarify the mechanisms behind KN1 movement. A complex relationship between targeted and non-targeted movement is also indicated by a recent study, in which a dominant-negative form of the tobacco NON-CELL-AUTONOMOUS PATHWAY PROTEIN 1 (NtNCAPP1) was overexpressed. In such transgenic plants, trafficking of viral movement protein, but not of KN1, was affected (Lee et al., 2003). Interestingly, tobacco LFY protein appears more uniform in such transgenic plants, which also have phenotypes reminiscent of LFY overexpressing plants. The causal relationship between these observations needs further investigation, but it will be interesting to combine the dominant-negative *NtCAPP1* overexpressing plants with the tools presented here.

It is also worth noting that the determinants underlying transcription factor movement may be species-dependent. Like LFY, its *Antirrhinum* ortholog FLORICAULA (FLO) has non-cell-autonomous effects in mosaic studies (Carpenter and Coen, 1995). In contrast to LFY, the extent to which FLO can rescue mutant flowers varies depending on the layer in which FLO is expressed (Hantke et al., 1995; Sessions et al., 2000). As FLO protein has not been examined in these mosaics, it is unknown whether the differential rescue ability is caused by differences in FLO movement, or is only a result of downstream effects, such as the documented abnormalities in target gene expression in the mosaics (Hantke et al., 1995). Another example of interspecific differences is provided by DEF of *Antirrhinum*, which was found to move from the L2 to the L1 in a stage- and organ-dependent manner (Perbal et al., 1996). However, the DEF ortholog AP3 of *Arabidopsis* does not move between layers (Jenik and Irish, 2001), which indicates that subtle differences in sequence, or interspecific differences in the translocation machinery, affect transcription factor movement, the latter being consistent with the interspecific differences that have been reported for GFP movement (Crawford and Zambryski, 2001). These observations highlight that care must be taken when extrapolating from one transcription factor assayed in a single species or single tissue.

In conclusion, we have presented evidence that the transcription factor LFY moves in a non-targeted fashion. We are proposing the testable hypothesis that movement is a default mechanism for many proteins in the *Arabidopsis* shoot apex, unless they are either efficiently targeted to specific subcellular locations or retained through formation of protein complexes. More case studies are needed to determine whether our results can indeed be generalized to include other proteins, other tissues and other species.

We thank: Steve Barlow for help with confocal microscopy; Virginia Butel for generating GLFY; Ji Hoon Ahn and Marty Yanofsky for gifts of material; and Ray Hong, Jan Lohmann, Alexis Maizel, Javier Palatnik, Markus Schmid, Jon Werner and Philip Wigge for discussions and critical reading of the manuscript. We especially acknowledge our comrade *in plasmodesmatis*, Bill Lucas, for spirited discussions and exchange of ideas on intercellular protein movement. This work was supported by: fellowships from the Life Sciences Research Foundation/US Department of Energy (X.W.) and the Howard Hughes Medical Institute (J.R.D.); a grant from the NIH (GM45244) to P.C.Z.; grants from the NIH (GM50224), NSF (9975717 and 0210992), USDA (00-35304-9333), BSF (0100476) and BARD (334702) to V.C.; and a grant from the NIH (GM62932) and funding by the Max Planck Society to D.W., who is a Director of the Max Planck Institute.

REFERENCES

Blázquez, M. A., Soowal, L., Lee, I. and Weigel, D. (1997). *LEAFY* expression and flower initiation in *Arabidopsis*. *Development* **124**, 3835-3844.

Busch, M. A., Bomblies, K. and Weigel, D. (1999). Activation of a floral homeotic gene in *Arabidopsis*. *Science* **285**, 585-587.

Carpenter, R. and Coen, E. S. (1995). Transposon induced chimeras show that *floricaula*, a meristem identity gene, acts non-autonomously between cell layers. *Development* **121**, 19-26.

Citovsky, V. (1999). Tobacco mosaic virus: a pioneer of cell-to-cell movement. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* **354**, 637-643.

Crawford, K. M. and Zambryski, P. C. (1999). Plasmodesmata signaling: many roles, sophisticated statutes. *Curr. Opin. Plant Biol.* **2**, 382-387.

Crawford, K. M. and Zambryski, P. C. (2000). Subcellular localization determines the availability of non-targeted proteins to plasmodesmatal transport. *Curr. Biol.* **10**, 1032-1040.

Crawford, K. M. and Zambryski, P. C. (2001). Non-targeted and targeted protein movement through plasmodesmata in leaves in different developmental and physiological states. *Plant Physiol.* **125**, 1802-1812.

Egea-Cortines, M., Saedler, H. and Sommer, H. (1999). Ternary complex formation between the MADS-box proteins SQUAMOSA, DEFICIENS and GLOBOSA is involved in the control of floral architecture in *Antirrhinum majus*. *EMBO J.* **18**, 5370-5379.

Fankhauser, C., Yeh, K. C., Lagarias, J. C., Zhang, H., Elich, T. D. and Chory, J. (1999). PKS1, a substrate phosphorylated by phytochrome that modulates light signaling in *Arabidopsis*. *Science* **284**, 1539-1541.

Fletcher, J. C., Brand, U., Running, M. P., Simon, R. and Meyerowitz, E. M. (1999). Signaling of cell fate decisions by *CLAVATA3* in *Arabidopsis* shoot meristems. *Science* **283**, 1911-1914.

Gisel, A., Barella, S., Hempel, F. and Zambryski, P. (1999). Temporal and spatial regulation of symplastic trafficking during development in *Arabidopsis thaliana* apices. *Development* **126**, 1879-1889.

Gisel, A., Hempel, F. D., Barella, S. and Zambryski, P. (2002). Leaf-to-shoot apex movement of symplastic tracer is restricted coincident with flowering in *Arabidopsis*. *Proc. Natl. Acad. Sci. USA* **99**, 1713-1717.

Hantke, S. S., Carpenter, R. and Coen, E. S. (1995). Expression of *floricaula* in single cell layers of periclinal chimeras activates downstream homeotic genes in all layers of floral meristems. *Development* **121**, 27-35.

Haseloff, J., Siemering, K. R., Prasher, D. C. and Hodge, S. (1997). Removal of a cryptic intron and subcellular localization of green fluorescent protein are required to mark transgenic *Arabidopsis* plants brightly. *Proc. Natl. Acad. Sci. USA* **94**, 2122-2127.

Haywood, V., Kragler, F. and Lucas, W. J. (2002). Plasmodesmata: pathways for protein and ribonucleoprotein signaling. *Plant Cell Suppl.* **14**, S303-S325.

Heinlein, M., Padgett, H. S., Gens, J. S., Pickard, B. G., Casper, S. J., Epel, B. L. and Beachy, R. N. (1998). Changing patterns of localization of the tobacco mosaic virus movement protein and replicase to the endoplasmic reticulum and microtubules during infection. *Plant Cell* **10**, 1107-1120.

Helariutta, Y., Fukaki, H., Wysocka-Diller, J., Nakajima, K., Jung, J., Sena, G., Hauser, M. T. and Benfey, P. N. (2000). The *SHORT-ROOT* gene controls radial patterning of the Arabidopsis root through radial signaling. *Cell* **101**, 555-567.

Hempel, F. D., Weigel, D., Mandel, M. A., Ditta, G., Zambryski, P., Feldman, L. J. and Yanofsky, M. F. (1997). Floral determination and expression of floral regulatory genes in *Arabidopsis*. *Development* **124**, 3845-3853.

Hong, R. L., Hamaguchi, L., Busch, M. A. and Weigel, D. (2003). Regulatory elements of the floral homeotic gene *AGAMOUS* identified by phylogenetic footprinting and shadowing. *Plant Cell* **15**, 1296-1309.

Honma, T. and Goto, K. (2001). Complexes of MADS-box proteins are sufficient to convert leaves into floral organs. *Nature* **409**, 525-529.

Huala, E. and Sussex, I. M. (1992). *LEAFY* interacts with floral homeotic genes to regulate Arabidopsis floral development. *Plant Cell* **4**, 901-913.

Imlau, A., Truernit, E. and Sauer, N. (1999). Cell-to-cell and long-distance trafficking of the green fluorescent protein in the phloem and symplastic unloading of the protein into sink tissues. *Plant Cell* **11**, 309-322.

Jackson, D., Veit, B. and Hake, S. (1994). Expression of maize *KNOTTED1* related homeobox genes in the shoot apical meristem predicts patterns of morphogenesis in the vegetative shoot. *Development* **120**, 405-413.

Jenik, P. D. and Irish, V. F. (2001). The Arabidopsis floral homeotic gene *APETALA3* differentially regulates intercellular signaling required for petal and stamen development. *Development* **128**, 13-23.

Kim, J. Y., Yuan, Z., Cilia, M., Khalfan-Jagani, Z. and Jackson, D. (2002). Intercellular trafficking of a KNOTTED1 green fluorescent protein fusion in the leaf and shoot meristem of *Arabidopsis*. *Proc. Natl. Acad. Sci. USA* **99**, 4103-4108.

Kim, M., Canio, W., Kessler, S. and Sinha, N. (2001). Developmental changes due to long-distance movement of a homeobox fusion transcript in tomato. *Science* **293**, 287-289.

Kragler, F., Monzer, J., Xoconostle-Cázares, B. and Lucas, W. J. (2000). Peptide antagonists of the plasmodesmal macromolecular trafficking pathway. *EMBO J.* **19**, 2856-2868.

Kühn, C., Franceschi, V. R., Schulz, A., Lemoine, R. and Frommer, W. B. (1997). Macromolecular trafficking indicated by localization and turnover of sucrose transporters in enucleate sieve elements. *Science* **275**, 1298-1300.

Lee, J. Y., Yoo, B. C., Rojas, M. R., Gomez-Ospina, N., Staehelin, L. A. and Lucas, W. J. (2003). Selective trafficking of non-cell-autonomous proteins mediated by NtNCAPP1. *Science* **299**, 392-396.

Lucas, W. J. (1995). Plasmodesmata: intercellular channels for macromolecular transport in plants. *Curr. Opin. Cell Biol.* **7**, 673-680.

Lucas, W. J., Bouchepillon, S., Jackson, D. P., Nguyen, L., Baker, L., Ding, B. and Hake, S. (1995). Selective trafficking of KNOTTED1 homeodomain protein and its mRNA through plasmodesmata. *Science* **270**, 1980-1983.

Matsubayashi, Y., Yang, H. and Sakagami, Y. (2001). Peptide signals and their receptors in higher plants. *Trends Plant Sci.* **6**, 573-577.

McCarty, D. R. and Chory, J. (2000). Conservation and innovation in plant signaling pathways. *Cell* **103**, 201-209.

McGonigle, B., Bouhidel, K. and Irish, V. F. (1996). Nuclear localization of the *Arabidopsis* APETALA3 and PISTILLATA homeotic gene products depends on their simultaneous expression. *Genes Dev.* **10**, 1812-1821.

Nakajima, K., Sena, G., Nawy, T. and Benfey, P. N. (2001). Intercellular movement of the putative transcription factor SHR in root patterning. *Nature* **413**, 307-311.

Ng, M. and Yanofsky, M. F. (2001). Activation of the Arabidopsis B class homeotic genes by *APETALA1*. *Plant Cell* **13**, 739-754.

Oparka, K. J., Roberts, A. G., Boevink, P., Santa Cruz, S., Roberts, I., Pradel, K. S., Imlau, A., Kotlizky, G., Sauer, N. and Epel, B. (1999). Simple, but not branched, plasmodesmata allow the nonspecific trafficking of proteins in developing tobacco leaves. *Cell* **97**, 743-754.

Parcy, F., Nilsson, O., Busch, M. A., Lee, I. and Weigel, D. (1998). A genetic framework for floral patterning. *Nature* **395**, 561-566.

Perbal, M.-C., Haughn, G., Saedler, H. and Schwarz-Sommer, Z. (1996). Non-cell-autonomous function of the *Antirrhinum* floral homeotic proteins *DEFICIENS* and *GLOBOSA* is exerted by their polar cell-to-cell trafficking. *Development* **122**, 3433-3441.

Rinne, P. L. and van der Schoot, C. (1998). Symplasmic fields in the tunica of the shoot apical meristem coordinate morphogenetic events. *Development* **125**, 1477-1485.

Rivera-Pomar, R. and Jäckle, H. (1996). From gradients to stripes in Drosophila embryogenesis: filling in the gaps. *Trends Genet.* **12**, 478-483.

Ruiz-Medrano, R., Xoconostle-Cázares, B. and Lucas, W. J. (1999). Phloem long-distance transport of CmNACP mRNA: implications for supracellular regulation in plants. *Development* **126**, 4405-4419.

Sessions, A., Weigel, D. and Yanofsky, M. F. (1999). The *Arabidopsis thaliana MERISTEM LAYER 1* promoter specifies epidermal expression in meristems and young primordia. *Plant J.* **20**, 259-263.

Sessions, A., Yanofsky, M. F. and Weigel, D. (2000). Cell-cell signaling and movement by the floral transcription factors LEAFY and APETALA1. *Science* **289**, 779-782.

Smith, L. G., Greene, B., Veit, B. and Hake, S. (1992). A dominant mutation in the maize homeobox gene, *Knotted-1*, causes its ectopic expression in leaf cells with altered fates. *Development* **116**, 21-30.

Weigel, D. and Nilsson, O. (1995). A developmental switch sufficient for flower initiation in diverse plants. *Nature* **377**, 495-500.

Weigel, D. and Glazebrook, J. (2002). *Arabidopsis: A Laboratory Manual.* Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

Weigel, D., Alvarez, J., Smyth, D. R., Yanofsky, M. F. and Meyerowitz, E. M. (1992). *LEAFY* controls floral meristem identity in Arabidopsis. *Cell* **69**, 843-859.

Wu, X., Weigel, D. and Wigge, P. A. (2002). Signaling in plants by intercellular RNA and protein movement. *Genes Dev.* **16**, 151-158.

Zambryski, P. and Crawford, K. (2000). Plasmodesmata: gatekeepers for cell-to-cell transport of developmental signals in plants. *Annu. Rev. Cell Dev. Biol.* **16**, 393-421.