Modes of intercellular transcription factor movement in the Arabidopsis apex

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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 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 knl 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 knl 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).

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**

PstI sites were added to both ends of the mGFP5 coding sequence (Haseloff et al., 1997) using oligonucleotide primers. The **GFP** PstI fragment was inserted into the internal PstI 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,

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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 XbaI 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 HindIII site from pAS104 (Sessions et al., 2000) and the 3' portion of LFY::GFP from pBS-ML1::LFY-link-sGFP into the binary vector pIHHON212 (J. H. Ahn, personal communication). 35S::LFY::GFP was created by ligating the 5' portion of LFY cDNA from pAS107 (up to the HindIII site) with the 3' portion of LFY::GFP into pCHF3.

Ify mutant alleles

Four ify alleles were included in this study: ify-2, ify-3, ify-9 and ify-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. LFYAs1 was generated by opening, filling-in and religating the BamHI site overlapping the start codon and the XbaI site at position 379, which results in an in-frame deletion of amino acids 4 to 127. LFYAs2 was created by opening, filling-in and religating the XbaI site at position 379 and the StyI site at position 860, which results in an in-frame deletion of amino acids 128 to 287. LFYAs3 was created by opening, filling-in and religating the StyI site at position 860 and the HindIII site at position 974, which leads to a frame shift such that amino acids 289 to 424 are replaced with the sequence SFKCSKSV. 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 EcoRI and a BamHI site in front of the 5' UTR, and a PstI site at the 3' end, replacing the stop codon. GFP coding sequence was amplified from pCAMBIA1302, replacing the start codon with a PstI site and adding an XbaI site to the 3' UTR. For AP1::GFP, the start codon of AP1 was replaced with a PstI site, and an XbaI site was added to the 3' UTR. An EcoRI site was added to the 5' end of the GFP coding region and a PstI site replaced the stop codon. In both fusions, the PstI 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::2xGFP was created by ligating the ML1 promoter to TEV5::2xGFP from pRTL2-2xGFP (Crawford and Zambryski, 2000) and inserting into pMX202. The NLS::2xGFP fragment from pRTL2-NLS::2xGFP (Crawford and Zambryski, 2000) was used to generate ML1::NLS::2xGFP. ML1::TCVMP:GFP was generated by ligating the ML1 promoter to coding sequences for a Turnip Vein Clearing Virus Movement Protein (TCVMP):GFP fusion into pMX202.

Ectopic expression in the center of shoot and flower meristems

A 833 bp BamHI/HindIII 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 CCTTA TTTGG to AAAGTTTGG) that results in ectopic activity in the inflorescence meristem in a Ify-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*:2xGFP, AG intron*:NLS::2xGFP, 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 s⁻¹ light provided by a 3:1 mixture of cool-white and GroLux (Osram Sylvania) fluorescent bulbs, at 21°C. Ify-12 and ap1-15 are strong alleles in the Columbia background (Huila and
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 XhoI, 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 40x or 63x 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 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 63x 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
Zambrayski, 2001; Heinlein et al., 1998). Deeper optical sections also revealed that a substantial fraction of TCVVMP: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 (2xGFP) 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 2xGFP can move at least occasionally into neighboring cells, whereas adding a nuclear localization signal (NLS) nearly fully blocks this movement (Crawford and Zambrayski, 2000). Here, 2xGFP and NLS:2xGFP, when expressed stably in the L1, behaved similarly. In vegetative and inflorescence apices, 2xGFP 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:2xGFP 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:2xGFP, a major band at about 57 kDa was observed (predicted size 58 kDa), indicating that it was stable (Fig. 4A). Similarly, 2xGFP migrated at about 50 kDa (predicted size 54 kDa), and only a small amount of degradation product was observed for 2xGFP.

Absence of movement of a functional AP1-GFP fusion
To exclude the possibility that all GFP fusion proteins can 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 promoter.
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:2xGFP (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. 4. Western blots of whole-cell extracts from 2-week-old transgenic seedlings of ML1::2xGFP and ML1::NLS:2xGFP (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 2xGFP 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.

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:2xGFP (A,D), AG intron*::2xGFP (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 ify-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 ify-12 mutants (B) are shown. Both AG intron*::LFY and AG intron*::GLFY can rescue the stamens and carpels of ify-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 \textit{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 \textit{CaMV 35S} minimal promoter fused to the mutated AG enhancer (\textit{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 \textit{AG} enhancer is active in ify mutants. When we expressed LFY and GLFY under the control of this enhancer in ify-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 \textit{AG} enhancer is active (Fig. 6), but only LFY was able to rescue petal development. All four of the ify-12: \textit{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 ify-12: \textit{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 \textit{LFY} promoter and comparing their ability to rescue ify-12 mutants. Of ten \textit{LFY::LFY} transgenic lines in the ify-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 \textit{LFY::GLFY} lines in the ify-12 background showed a similar degree of rescue; the remaining eight lines resembled weak or intermediate ify 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 ify 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 \textit{35S} and \textit{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-
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.

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 alleles mutating specific residues in GLFY. All four fusions also generated GFP fusions of four weak and intermediate lfy 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...
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.
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 MLI 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 (NtCAPP1) 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 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 Zambr设ski, 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.

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