Current Biology *17*, 1050–1054, June 19, 2007 ©2007 Elsevier Ltd All rights reserved DOI 10.1016/j.cub.2007.05.008

Report

FT Protein Acts as a Long-Range Signal in *Arabidopsis*

Katja E. Jaeger[1] and Philip A. Wigge[1,*]
[1] John Innes Centre
Department of Cell and Developmental Biology
Colney Lane
Norwich NR4 7UH
United Kingdom

Summary

Plants are sessile organisms and must respond to changes in environmental conditions. Flowering time is a key developmental switch that is affected by both day length and temperature. Environmental cues are sensed by the leaves while the responses occur at the apex, requiring long-range communication within the plant. For many years it has been known that leaves exposed to light can trigger the floral transition of a darkened shoot, and grafting experiments demonstrated that the floral stimulus travels long distances [1]. This mobile signal was later termed "florigen," but its nature has been unclear. The gene *FLOWERING LOCUS T* (*FT*) is a major output of both the photoperiod and the vernalization pathways controlling the floral transition [2–4]. FT protein acts at the shoot apex of the plant in concert with a transcription factor, *FLOWERING LOCUS D* (*FD*) [5, 6]. A fundamental question is how FT transcription in the leaves leads to active FT protein at the apex. We have uncoupled FT protein movement from its biological function to show that FT protein is the mobile signal that travels from the leaves to the apex. To our knowledge, FT is the only known protein that serves as a long-range developmental signal in plants.

Results and Discussion

Epitope-Tagged FT Is Functional in Planta

FT mRNA is expressed in the leaves in response to long-day conditions, but it is the FT protein that is required at the shoot apical meristem to initiate the floral transition. To test the hypothesis that it is indeed the protein that is the mobile signal to stimulate flowering in plants, we created an epitope-tagged version of the protein because there are five other closely related *Arabidopsis* family members that are antigenically similar to FT [7]. We created an epitope-tagged version of FT by fusing 5× Myc tags N-terminally to FT protein. This arrangement was chosen to minimize changes to the overall size of FT, because protein size is a critical effector of protein movement [8–10]. FT itself encodes a small globular protein of less then 20 kDa with sequence similarity to the Raf kinase inhibitor protein (RKIP) family of mammals [2]. We expressed *Myc FT* under the control of the vasculature specific *SUCROSE TRANSPORTER2* (*SUC2*) promoter. *SUC2* has been shown to be specifically active in the phloem companion cells, which is the tissue, where the endogenous *FT* promoter is also active [11, 12]. To ensure protein functionality, we tested this construct in planta for its ability to stimulate flowering. *SUC2::Myc FT* in the complete loss of function line *ft-10* led to very precocious flowering, as has been reported for *SUC2::FT* previously [11]. This indicated to us that the presence of the tag has not interfered with the endogenous FT function in the plant (See Table 1 and Figure 1B). All studies were carried out in the complete loss of function background, *ft-10*, to ensure that our results cannot be perturbed by the effect of the endogenous *FT* transcript. This is important as it has been suggested that *FT* expression is subject to autoregulation through a positive-feed-forward loop between FT and FD [13].

We confirmed protein expression in these lines by western blotting. As shown in Figure 2, a band of the correct size was present and there was no detectable proteolytic cleavage of the Myc FT fusion protein in the plant. Thus, we constructed a functional tagged protein that allowed us to do detailed biochemical investigations of the protein activity domain in situ.

SUC2::Myc FT Expression Is Specific for the Vasculature

To ensure that the early-flowering lines we obtained for *SUC2::Myc FT* have companion-cell-specific expression, we used RNA in situ hybridization to analyze the expression domain for *Myc FT* mRNA. This is shown in Figures 3A and 3C where the expected vasculature-specific expression from the *SUC2* promoter is observed with no expression at the apex. This is in accordance with previously published descriptions of the *SUC2* promoter in *Arabidopsis* [11, 12] and indicates that the early-flowering phenotype observed in these lines is due a signal moving from the vasculature to the apex. However, the sensitivity of the in situ hybridization technique is limited, and we cannot exclude that very low *Myc FT* mRNA levels, which are below the detection threshold, trafficked outside the vasculature. This observation led us to conclude that mRNA movement is unlikely to cause the observed early-flowering phenotype.

Myc FT Protein Is a Mobile Signal

An important question is whether the early-flowering phenotype is due to protein movement or RNA trafficking, because *Myc FT* mRNA could be the mobile signal. To investigate this possibility, we analyzed plants undergoing the floral transition by immunocytochemistry to determine whether there was evidence for Myc FT protein movement. As shown in Figure 4, it is possible to detect the presence of Myc FT protein with Myc antibody in a gradient from the vasculature toward the organ primordia that later differentiate into flowers. This signal follows the strands of provasculature leading from the vasculature bundle that terminates beneath the shoot apex to the primordia. Interestingly, it has

*Correspondence: philip.wigge@bbsrc.ac.uk

Table 1. Mobile Versions of FT Protein Stimulate Early Flowering when Expressed in the Vasculature, in Contrast to Nuclear Localized FT

Genotype	Rosette Leaves	Cauline Leaves	Total Leaf Number	SD	Range	n
Columbia-0	10.6	2.7	13.3	1.3	11–15	21
35S::Myc NLS FT ft-10 T3	3.0	1.6	4.6	0.68	4–6	21
ft-10	27.3	7.8	35.15	2.4	31–41	20
SUC2::myc FT 1-3 ft-10	4.9	1.3	6.2	1.2	5–8	13
SUC2::myc NLS FT-7 ft-10	27.6	8.4	36	1.6	33–39	20
SUC2::myc NLS FT-8 ft-10	25.6	7.6	34	1.1	31–35	20

Flowering times are measured under long days (16 hr light) for lines used in this study. Transgenic data are from individual T2 lines unless stated otherwise. Transgenic lines are all in the *ft-10* background.

recently been observed that cells of tissues destined to become vascular acquire a larger size-exclusion limit [14]. This would be consistent with the movement of Myc FT protein we observe. Moreover, this pattern of protein movement suggests that the provasculature plays a role in determining the developmental fate of the lateral organs by allowing FT protein to reach the FD domain. The provasculature strand patterning is coordinated by auxin transport [15]. These auxin maxima at the sites of future floral primordia precede *LEAFY* (*LFY*) expression. Because *LFY* is an important floral-fate marker [16], this suggests a basis for the coordination of floral fate with auxin patterning of the apex [17].

These observations led us to conclude that FT protein movement plays an important role in directing the developmental fate of emerging primordia at the plant apex.

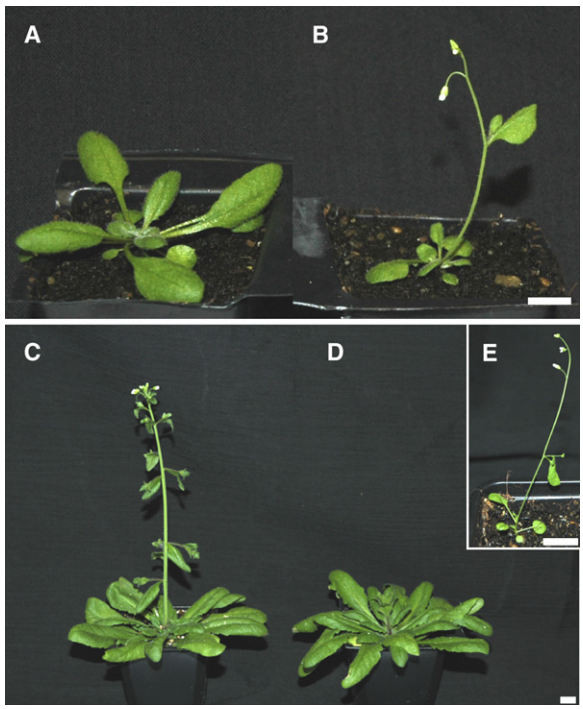

Figure 1. Expression of Mobile FT Protein Leads to an Early-Flowering Phenotype

Plants were all grown under long days (16 hr light, 23°C). (A) shows that plants carrying a complete knockout at the *FT* locus (*ft-10*) flower late and have characteristic dark green leaves. (B) shows that plants expressing *SUC2::Myc FT* in the *ft-10* background flower very early and undergo a very rapid floral transition (C) shows a plant expressing *SUC2::Myc NLS FT* in the *ft-10* background with comparable expression levels as plant in (B), but it flowers nearly as late as the *ft-10* control in (D). Inset (E) shows that a plant expressing *35S::Myc NLS FT* flowers after forming approximately three very small true leaves. The shoot apical meristem terminates after forming only a few flowers. Transgenic lines used in this study are all in the *ft-10* background. Scale bars represent 1 cm.

Uncoupling Intercellular Trafficking of FT Protein from Its Biological Activity

Our initial results are consistent with FT protein movement being a major factor in FT long-range signaling in the plant. These approaches are limited, however, by the detection threshold of the techniques used. Thus formally, it is possible that a small amount of *FT* mRNA might move from the vasculature to the apex and is specifically translated at the apex. Although this is not a seemingly likely scenario, there is precedent from other systems where mRNA translation occurs specifically at the location where the protein acts [18]. To clarify this, we uncoupled the biological activity of FT protein from its ability to move intercellularly. FT functions through interacting with the nuclear-localized transcription factor FD at the apex, as part of a transcriptional complex [5, 6]. Whereas FD is constitutively nuclear, FT does not possess a nuclear localization signal (NLS) and is generally cytoplasmic as well as nuclear when visualized as a GFP fusion [5]. We thus sought to target FT specifically to the nucleus with a constitutive SV40 NLS. The rationale for this experiment was that this would prevent intercellular trafficking of FT protein without interfering with its biological function.

To test this, we expressed *Myc NLS FT* under the constitutive cauliflower mosaic virus *35S* promoter (CaMV 35S) in the *ft-10* background. This construct was indeed able to cause very early flowering (Figure 1E and Table 1), as has been shown for constitutively expressed untagged *FT* [2, 3]. Overexpression of *FT* in vegetative organs such as rosette or cauline leaves causes ectopic expression of floral meristem-identity genes [19]. The extremely small leaves of the *35S::NLS Myc FT* plants suggest that this is the case here as well (Figure 1E). Interestingly, expressing *Myc NLS FT* under the *SUC2* promoter in the *ft-10* background did not lead to the dramatically early-flowering phenotype we have already described. Indeed, the plants are almost as late flowering as *ft-10* itself (Figure 1C and Table 1). A possible explanation for this lack of rescue is that these transgenic lines have a low level of protein expression. We therefore analyzed expression levels in both *SUC2::Myc FT* as well as *SUC2::Myc NLS FT* transgenic plants by western blotting to ensure that they were comparable (Figure 2 and data not shown). Additionally, analysis of these lines

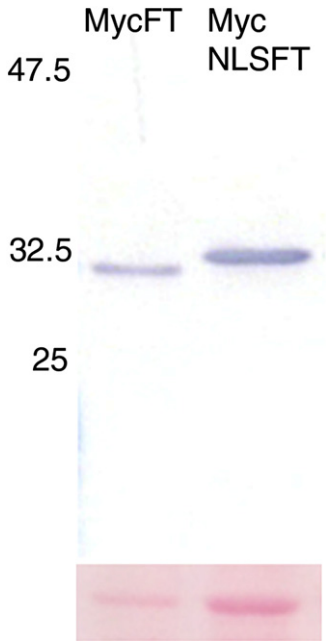

Figure 2. Myc NLS FT and Myc FT Are Stable at the Protein Level In Planta

Western blot probed with anti-Myc polyclonal (rabbit) and detected with Western Blue substrate. Ponceau-stained loading control is shown below.

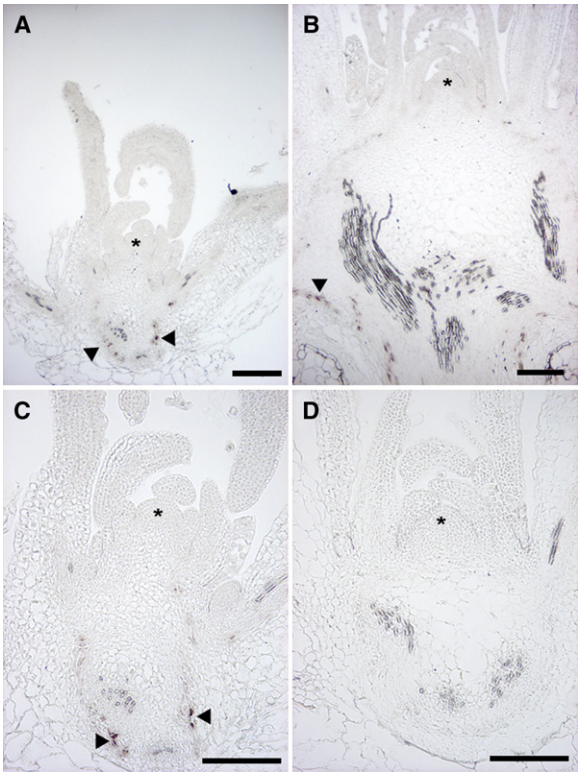

Figure 3. *FT* mRNA Expression in Plants that Are about to Flower Determined by In Situ Hybridization

FT mRNA is specifically localized to the *SUC2* promoter expression domain. (A) and (C) show that plants expressing *SUC2::Myc FT* undergoing the floral transition show localization of *FT* only in the vasculature and not in the apex. (B) shows that plants expressing *SUC2::Myc NLS FT* flower much later than plants expressing *SUC2::myc FT* but have similar RNA expression levels as apices in (A). (D) shows the nontransgenic Col-0 wild-type control. All slides are probed with *FT* anti-sense probe. Arrowheads indicate presumed companion cells. Note the absence of transcript signal near the SAM (*). Scale bars represent 100 μm.

by in situ hybridization with an *FT* antisense probe revealed comparable expression levels to our previously described early flowering *SUC2::Myc FT* lines (Figure 3B). Remarkably, protein-localization analysis of lines expressing *Myc NLS FT* under the *SUC2* promoter with Myc antibody revealed a pattern comparable to that seen for the mRNA, with strong vasculature expression, but no staining of the provasculature leading to the primordia as it is the case for *SUC2::Myc FT* (Figures 4B, 4D, and 4F). Close ups of the companion cells reveal the nuclear localization of the signal (Figure 4H) in individual companion cells. FT protein that is targeted to the nucleus via the NLS is visible as a signal tightly restricted to individual companion cells, unlike the FT protein that is generated from the *SUC2::Myc FT* construct and that exists in a continuous gradient from the vascular bundle up to the floral anlagen. This suggests to us that the Myc FT protein is freely mobile within the vasculature, whereas with the *Myc NLS FT* construct, we have created a version of FT that is completely functional when expressed in its target cells but that has no ability to traffic intercellularly.

Taken together, we could show that a version of FT that is targeted to the nucleus is still able to act in a transcriptional complex with FD and subsequently switch on downstream genes but is not longer able to act as a non-cell-autonomous signal.

Conclusions

In previous studies, it could be demonstrated that *FT* mRNA overexpressed specifically in the companion cells of the vasculature leads to early flowering, but these studies could not distinguish between mRNA or protein movement. In our study, we could show that it is indeed the FT protein that travels from the vasculature to its site of action at the flanks of the shoot apical meristem, where it acts with FD in a transcriptional complex.

Our experiments with an immobile FT protein suggest to us that it is the FT protein whose movement is crucial for triggering the floral transition. If *FT* mRNA movement was the key factor, then one would expect movement of the *Myc NLS FT* mRNA from the vasculature to the apex to be sufficient to trigger the floral transition because the presence of the short NLS coding sequence in the mRNA would not be expected to hinder any potential RNA trafficking. Accordingly, we note that the report of *FT* mRNA being a mobile signal in *Arabidopsis* has recently been retracted [20]. Our results are also consistent with complementary studies involving grafting experiments and a FT-related protein in tomato [21] as well as similar recent studies in rice and *Arabidopsis* ([22, 23]; see also [24], this issue of *Current Biology*). Additionally, in a study in *Brassica napus*, a close relative to *Arabidopsis thaliana*, FT protein could be identified among other potential signaling proteins by analysis of the soluble fraction of the sieve-tube exudates of

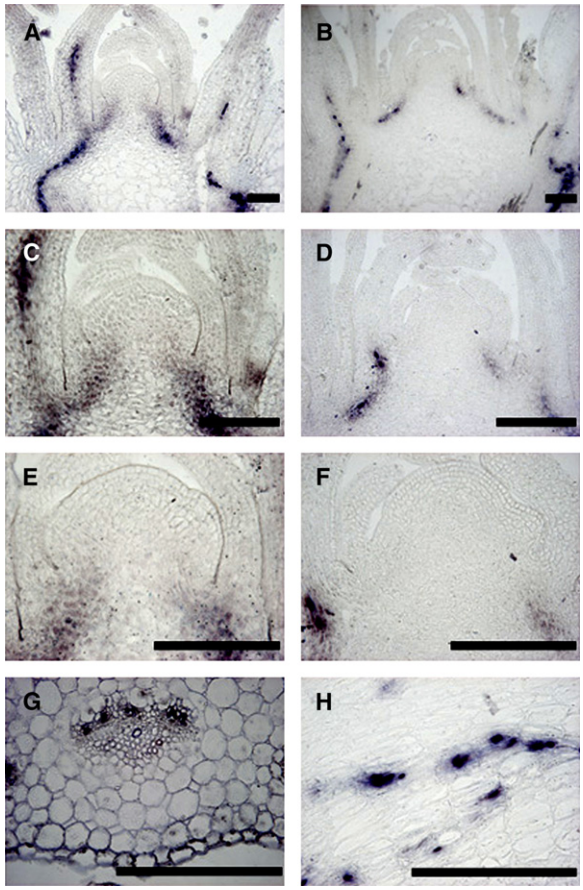

Figure 4. Myc FT Protein Moves from the Vasculature to the Apex

Transgenic lines were tested for FT protein localization and are in the same developmental age as plants in Figure 3.The protein localization at the apex differs significantly in plants expressing *Myc FT* from plants expressing *Myc NLS FT* under the *SUC2* promoter during the floral transition. (A), (C), and (E) show Myc FT expressed from the *SUC2* promoter. Note the diffuse extension of signal from the vasculature up into the primordia; such a signal indicates movement of the protein from the site of *SUC2* transcription in the vasculature. The streaky distribution implies that Myc FT is cytoplasmic as well as nuclear. (B), (D), (F), and (H) show Myc NLS FT expressed from the *SUC2* promoter. Note the sharp punctate staining pattern indicative of nuclear localization. Although the *SUC2* promoter is clearly active, protein movement away from the sites of transcription appears predominantly absent. (G) shows a cross section through a vascular bundle displaying characteristic phloem-companion-cell staining for *SUC2*. Scale bars represent 50 μm.

Brassica phloem sap [25]. Our results indicate that FT protein is a very interesting example of a key developmental signal that acts over significant distances in plants. The use of a constitutive NLS might be a useful approach for studying other FT protein family members because it provides a means to separate their tendency to traffic intercellularly from their biochemical activity, especially because a recent study indicates that another member of this family also acts as a mobile signal during plant development [26].

Experimental Procedures

Plant Material

We used Columbia-0 (Col-0) accession as the wild-type; *ft-10* and *35S::FT* have been described elsewhere [2, 27]. All plants were grown on soil at 23°C constant temperature, 16 hr light and 8 hr dark, or under short-day conditions with 16 hr dark and 8 hr light, under a mixture of Philips Cool White and Osram fluorescent lights, with a fluency rate of 120 μmol m^{-2} s^{-1}. Relative humidity was 70%. Plants were grown on Scotts levington F2 Intercept containing 1 liter of 4 mm grit in 10 liter of Levington. Transgenic lines for analysis were selected by comparison of protein expression levels in the T1 generation, and selected lines carried on in the next generation. For each construct, two lines with comparable expression levels were chosen and used for all subsequent analysis. Plants for in situ or immunocytochemistry experiments were grown under LD conditions for 5 weeks in the case of *SUC2::Myc NLS FT* or under SD conditions for 2 weeks for the *SUC2::Myc FT* lines. All seeds were germinated on MS plates and transferred to soil at cotyledon stage. All transgenic lines used for the experiments are in the *ft-10* background.

Plant Transformation and Selection

Plants were transformed with the floral-dip method [28] and selected on germination medium containing 0.5× Murashige and Skoog salt mixture, 0.7% agar, and 50 μg ml^{-1} Kanamycin (Melford, UK) (pH 5.7).

In Situ Hybridization and Immunocytochemistry

For the Paraplast-embedding steps, an automated tissue processor was used (TissueTek, USA). Plant material for in situ hybridization and immunocytochemistry was fixed in FAA and embedded overnight (70% ethanol 1 hr, 90% ethanol [2× 1 hr], 99% ethanol [2× 1 hr], absolute ethanol for 1 hr, xylene [3× 1 hr], histowax [3× 1 hr]). In situ hybridization was carried out in accordance with previously published protocols with modifications [29]. Digoxigenin-labeled RNA probes were used for detecting transcript patterns. A probe against whole FT cDNA sequence was subcloned in pBS with reagents from Roche diagnostics. For immunocytochemistry, slides with 8 μM sections were dewaxed with two washes of Histoclear. Sections were rehydrated with 2 min washes of ethanol of concentration 100%, 95%, 85%, 70%, 50%, and 30% and then rehydrated with 2× deionized water. The slides were boiled for 10 min in 10 mM citrate buffer (pH 6.0) (9 ml 100 mM citric acid, 41 ml 100 mM sodium citrate, 450 ml water). Sections were allowed to cool to room temperature and then rinsed in deionized water.

Sections were blocked in 5% nonfat dry milk, PBS 0.3% Triton X-100 for 3 hr. Sections were rinsed in deionized water. Myc polyclonal antibody (AbCam, Cambridge, UK, ab9106) was used at 1:1000 and incubated for 1 hr at 4°C and 1 hr at 37°C in a humidified chamber. Slides were washed 3× 20 min in PBS 0.3% Triton X-100. Alkaline phosphatase linked secondary anti-rabbit IgG (AbCam, ab6722) was diluted 1:2000 and incubated on the slides overnight at 4°C. Further washes in PBSX (3× 20 min) and TBS (pH 7.5) were followed by 100 mM Tris, 100 mM NaCl, and 50 mM MgCl2 (pH 7.5). Signal was detected with Western Blue alkaline phosphatase. Slides were mounted in 50% glycerol and viewed with Nikon Eclipse 800 microscope under bright field. Images were captured with a Pixera Pro ES600 digital camera.

Western-Blot Analysis

Total protein from plant material were extracted by shock freezing samples in liquid nitrogen and after grinding mixed with 5× Laemmli buffer (20 mM Tris, 1% SDS, 0.05% bromophenol blue, and 10% glycerol [pH 6. 8]) and incubated at 100°C for 5 min. The proteins were separated by SDS-PAGE through a 12% acrylamide gel. The proteins were blotted onto a Hybond nitrocellulose membrane (Amersham Biosciences, UK). Total protein was assessed by staining of the membrane with Ponceau Red (0.2% Ponceau in 1% acetic acid) and blocked in 5% skim milk and TBST (10 mM Tris, 150 mM NaCl, and 0.2% Tween-20 [pH 7.5]). Primary antibody (Myc polyclonal antibody, AbCam, UK, AB9106) was diluted 1:10,000 and incubated for 1 hr at RT. After three washes with TTBS, the membrane was incubated with the AP-linked secondary antibody (Abcam, UK, AB6722) for 1 hr at RT. After washing 3× with TBST, the membrane was overlaid with Western Blue (Promega Inc., USA) and allowed to develop.

Plasmid Constructions

All plant constructs were cloned into the binary vectors pMLBART or pART27 and transformed into *Arabidopsis* plants by the floral-dip method. Constructs were cloned with standard PCR and restriction-enzyme-digestion/ligation methods as described [19]. The 5× Myc repeat was amplified by PCR. The NLS construct was created by annealing the following oligonucleotide primers together: W61: 5′-TCGACCCCCAAAGAAGAAGCGTAAGGTTGC-3′ and W62: 5′- TC GAGCAACCTTACGCTTCTTCTTTGGGGG-3′ and cloned between the Myc and the FT cDNA at an XhoI site. This created the NLS sequence RPPKKKRKVAR. The 2 kb *SUC2* promoter was amplified by PCR and cloned into BJ36 as a SalI PstI fragment.

Acknowledgments

We thank M. Schmid and D. Weigel for sharing unpublished work. We are grateful to all the members of the Wigge lab for stimulating discussions and support, A. Graf for involvement in the early stages of the project and synthesizing the *FT* antisense probe, D. Bradley for advice on immunocytochemistry, and Timothy Wells and Ruth Williams for excellent care of *Arabidopsis* plants. This work was funded by the start-up funds for P.W. and Biotechnology and Biological Sciences Research Council grants C0610C10A and BB/D010047/1.

Received: April 1, 2007
Revised: May 7, 2007
Accepted: May 8, 2007
Published online: May 31, 2007

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