BARD

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**Molecular Genetic Analysis of Flowering in
Arabidopsis and Tomato**

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Project Title: Molecular Genetic Analysis of Flowering in Arabidopsis and Tomato

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Keywords *not* appearing in the title and in order of importance. Avoid abbreviations.

EARLY FLOWERING 3; SELFPRUNING; FLOWERING; CIRCADIAN CLOCK, LIGHT SIGNALLING, GATING

Abbreviations commonly used in the report, in alphabetical order:

ELF3: EARLY FLOWERING 3; PHYB: PHYTOCHROME B;

LD: LONG DAY; SD: SHORT DAY; SP: SELFPRUNING; CO: CONSTANS

Budget: IS: \$

US: \$

Total: \$

Signature
Principal Investigator

Signature
Authorizing Official, Principal Institution

BARD Final Scientific Report
Cover Page

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Refereed (published, in press, accepted)		4	1	5
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Abstracts		4		4
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Postdoctoral Training: List the names and social security/identity numbers of all postdocs who received more than of their funding by the grant.

Cooperation Summary (numbers)

	From US to Israel	From Israel to US	Together, elsewhere	Total
Short Visits & Meetings	1			1
Longer Visits (Sabbaticals)				

Description of Cooperation:

The US investigator, Dr. Wagner, visited the Israeli lab in June of 2000 to review previous work and to establish the collaboration between the existing investigators and the new Co-PI, Dr. Steve Kay.

Patent Summary (numbers)

	Israeli inventor (s) only	US inventor (s) only	Joint IS/US inventors	Total
Submitted		1		1
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ABSTRACT

The primary objectives for the US lab included: the characterization of *ELF3* transcription and translation; the creation and characterization of various transgenic lines that misexpress *ELF3*; defining genetic pathways related to *ELF3* function regulating floral initiation in *Arabidopsis*; the identification of genes that either interact with or are regulated by *ELF3*.

Light quality, photoperiod, and temperature often act as important and, for some species, essential environmental cues for the initiation of flowering. However, there is relatively little information on the molecular mechanisms that directly regulate the developmental pathway from the reception of the inductive light signals to the onset of flowering and the initiation of floral meristems. The *ELF3* gene was identified as possibly having a role in light-mediated floral regulation since *elf3* mutants not only flower early, but exhibit light-dependent circadian defects.

We began investigating *ELF3*'s role in light signaling and flowering by cloning the *ELF3* gene. *ELF3* is a novel gene only present in plant species; however, there is an *ELF3* homolog within *Arabidopsis*. The *Arabidopsis elf3* mutation causes arrhythmic circadian output in continuous light; however, we show conclusively normal circadian function with no alteration in period length in *elf3* mutants in dark conditions and that the light-dependent arrhythmia observed in *elf3* mutants is pleiotropic on multiple outputs regardless of phase. Plants overexpressing *ELF3* have an increased period length in constant light and flower late in long-days; furthermore, etiolated *ELF3*-overexpressing seedlings exhibit a decreased acute *CAB2* response after a red light pulse, whereas the null mutant is hypersensitive to acute induction. This finding suggests that *ELF3* negatively regulates light input to both the clock and its outputs. To determine whether *ELF3*'s action is phase dependent, we examined clock resetting by light pulses and constructed phase response curves. Absence of *ELF3* activity causes a significant alteration of the phase response curve during the subjective night, and overexpression of *ELF3* results in decreased sensitivity to the resetting stimulus, suggesting that *ELF3* antagonizes light input to the clock during the night. Indeed, the *ELF3* protein interacts with the photoreceptor *PHYB* in the yeast two-hybrid assay and *in vitro*. The phase of *ELF3* function correlates with its peak expression levels of transcript and protein in the subjective night. *ELF3* action, therefore, represents a mechanism by which the oscillator modulates light resetting. Furthermore, flowering time is dependent upon proper expression of *ELF3*.

Scientifically, we've made a big leap in the understanding of the circadian system and how it is coupled so tightly with light reception in terms of period length and clock resetting. Agriculturally, understanding more about the way in which the clock perceives and relays temporal information to pathways such as those involved in the floral transition can lead to increased crop yields by enabling plants to be grown in suboptimal conditions.

The Israeli lab's objectives primarily dealt with the genetic and molecular mechanisms that control the vegetative/ reproductive cycles in tomato- a day- neutral plant with a sympodial shoot system. The experimental system was based on the successful cloning and developmental characterization of the *SELFPRUNING* (SP) gene which regulates the indeterminate/determinate states of the tomato shoot. In particular we proposed to study the molecular & biochemical function of SP and the relations between the SP system and the tomato orthologues of genes that

regulate the flowering/photoperiod response in Arabidopsis. In the following we will report on our studies of the *CONSTANS (CO)* gene and on CO interacting proteins in tomato. CO is a major and most studied factor that regulates the photoperiod response (long day) in Arabidopsis. To study the genetics of flowering in day-neutral plants we have isolated three CO-like genes in tomato (T-CO), studied their molecular organization and their expression patterns and identified three genes encoding T-CO interacting proteins. We continue to examine the biological role of CO and its interacting proteins in both tomato and Arabidopsis. We also describe our extensive study of the molecular function of the SP system along with the analysis of the SP interacting proteins. In this study we have presented for the first time original data showing that SP and its members of this regulatory family involved in a variety of signaling system using kinases, transcription factors and 14-3-3 modulators to calibrate the vegetative/reproductive switches in plant meristems. We have identified a kinase of the NIMA family, known to be involved in regulation of mitosis in mammals and *Aspergillus*, never studied before in plants and showed that it may regulate differential rates of cell division at least in fruits. A concise report on these efforts and a summary of additional results related to the SP interacting genes are also presented in this report.

Achievements

SIGNIFICANCE:

Both plants and animals use seasonal cues to synchronize their reproductive development with the external environment. The primary seasonal cue used by organisms is daylength, or photoperiod. Higher plants use photoperiodic cues to regulate many aspects of development, including the transition from vegetative to floral development. Because many plant species flower preferentially when grown in a certain photoperiod, it is common for the abundance and propagation of a species of plant to be limited by latitudinal and seasonal restraints. For example, a short-day plant such as rice will likely only be able to thrive at lower latitudes because at high latitudes the daylength is short enough for the transition to reproductive development only during the winter months, which may be too cold for efficient crop yield. Furthermore, since there is variation in daylength near the equator, daylengths at low latitudes may not decrease enough to elicit a robust signal for reproductive development.

By cloning and characterizing *ELF3* and *SP*'s roles in the plant circadian system and the flowering pathways, it will now be possible to address the problems related to flowering time's effect on crop yield. For example, by misexpressing *ELF3*, it is possible to alter the flowering time in *Arabidopsis*. Therefore it should be possible to alter *ELF3* ortholog expression in various crop species to effect changes in flowering time that will allow greater crop yields at sub optimal latitudes and during seasons which may not normally be associated with crop production.

POTENTIAL:

Practical applications of the work that has been conducted with this grant include altering *ELF3* levels within crop species to adapt the crops to different latitudinal and seasonal requirements, thereby extending the range over which the crop can give high yields.

DETAILS OF COOPERATION:

Cooperation between the groups in the US and Israel were based on the sharing of molecular tools and unpublished data, including clones, DNA libraries, DNA sequence information, plant genetic material, and flowering time results necessary for the research program. Once the *ELF3* gene was successfully cloned, in the US lab, sequence information was used to identify Tomato *ELF3* orthologs for use in the Israeli lab, along with promoter luciferase constructs to assay the circadian system in tomato, since *ELF3* is involved in at least circadian input in *Arabidopsis*. Since *elf3* mutants and plants overexpressing *ELF3* have distinct flowering time phenotypes, 35S::*ELF3* constructs were used to test the effect of *ELF3* overexpression on flowering time in tomato. This cooperation is enabling a better understanding of flowering and circadian clock in *Arabidopsis* and tomato and will help to describe the differential role of *ELF3* in the different plant species. The exchange has been critical for the advance of this research, and has been easily facilitated by mail, phone, and electronic mail.

PUBLICATIONS:

Covington, M.F., Panda, S., Liu, X.L., Strayer, C.A., Wagner, D.R., and Kay, S.A. (2001). ELF3 modulates resetting of the circadian clock in Arabidopsis. *Plant Cell* **13**, 1305–1315.

Hicks, K.A., Albertson, T.M., and Wagner, D.R. (2001). EARLY FLOWERING3 encodes a novel protein that regulates circadian clock function and flowering in Arabidopsis. *Plant Cell* **13**, 1281–1292.

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Pneuli, L., Gutfinger, T., Hareven, D., Ben-Naim, O., Ron, N., Adir, N., and Lifschitz E. (2001). Tomato SP-Interacting Proteins Define a Conserved Signaling System That Regulates Shoot Architecture and Flowering. *Plant Cell* **13**, 2687-2702.

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APPENDIX

Flowering in day-neutral plants- the tomato *CONSTANS* system

Following the initial cloning of three homologues of CO from tomato (related with CO, and COL2 Ledger et al., 1996, 2001, Putterill et al., 1997., Robson et al., 2001) we have isolated a cDNA clone representing the CO orthologue in tomato, hereafter TCO.

Concomitant with the generation of transgenic tomato plants expressing the gene in various orientations we have also used the TCO gene to isolate CO interacting proteins. Such proteins reveal components that are differentially important for the function of CO in day-neutral plants. These components are not critical in Arabidopsis. Since a complete CO protein is a strong activator of transcription in yeast we used a chimeric gene from which a major activation element was deleted (Fig 1). In panel #1 of Fig 1 we have identified several interacting proteins and choose to concentrate our analysis of four Tomato CO Interacting Proteins (TCIP) candidates. Three are transcription factors, whereby TCIP2a and TCIP2b belong to the same family and are quite close to one another. Antibodies generated against TCIP2a proved that the protein is localized to the nuclei. TCIP3 is a third transcription factor, carries, just like CO a B-box like zinc-finger domain (Robson et al., 2001) but there is no homology with TCO outside this domain while TCIP3 encodes a small protein with a presumptive modulating role.

The identity of all genes along with their complete sequence and the base sequence of all T-CO genes is available upon request.

The complete set of interactions between the TCIPs and T-CO is shown in the second panel of Fig 1. Note that TCIP2a&b as well as TCIP3 interact with the complete T-CO protein. The interactions between the N and C-ter domains of T-CO and its interacting proteins are domain specific. Thus TCIP2a&b interact only with the C-ter domain while the other two interact with the N-terminal domain only. As shown in the left hand bottom panel (Fig 1) TCIPs 1, 2a and 2b interact with the Arabidopsis *CONSTANS* protein and with the same domain specificity.

Daily expression pattern of TCO is regulated by light in the opposite way to that of Arabidopsis.

To examine the possible role of TCO on the day –neutral response of tomato we followed its expression pattern under long day condition. As shown in just one example of this (Bottom right Fig 1.), expression of *TCO* is upregulated at the end of the dark period and peaks about 4 hours of light. The gene is being shut down toward the end of the light period. This pattern presents a mirror image of the pattern observed by Suarez-Lopez et al., (2001) for the CO gene in Arabidopsis. A similar daily cycle is also displayed by TCIP1, the transcription factor with the B-box domain. Other results suggest that as in Arabidopsis, the expression of TCO is under the control of the circadian clock. By contrast the expression of the other two interactors TCIP2a and TCIP3 is nearly insensitive to light.

We are also studying the daily expression pattern of members of the *SP* family, including the tomato *FT* gene (see Samach et al, 2000) as well as in plants expressing the Arabidopsis *ELF3* (studied by Dr. Meeks-Wagner).

Is *TCO* the real *CONSTANS* orthologue?

The three T-CO genes are much more related to one another than the homologue series in Arabidopsis. In cooperation with Alon Samach we are studying now the effect of all three genes

both species. We will have these results in about 6 months time. The effect of the *TCIPs* on flowering time is being studied now in *Arabidopsis* transgenic plants over expressing *CO* and

Part 2- SP interacting proteins- tools to regulate vegetative/reproductive balance

A recessive mutation in the *SELF PRUNING (SP)* gene confers accelerated termination of stem units until the shoot is eventually terminated by two consecutive inflorescences resulting in a bushy compact constitution and nearly homogeneous fruit setting. Introduction of the recessive *pruning(sp)* gene into tomato cultivars revolutionized the tomato industry because the 'determinate' growth habit facilitates homogeneous fruit setting and mechanical harvesting. The *SELF PRUNING (SP)* gene of tomato was cloned (Pnueli et al., 1998) and shown to be a member of a novel CEN family of regulatory genes (*CEN*, *TFL1*, *FT*, Bradley et al., 1996, 1997) that controls shoot architecture and are related to PEBPs, from mammals (Grandy et al., 1990; Schoentgen et al., 1995).

To obtain additional molecular tools for the manipulation of shoot architecture we identified and analyzed several proteins that interact with SP (SIPs) and with its homologues in other species; **SIP2 and SIP74** represent isoforms of the **14-3-3** family. 14-3-3s proteins involved in signaling, transcription and compartmentalization via their ability to stabilize, dimerize or bind their substrates (Pan et al., 1999, Yang, et al., 1999). **SIP3** is a serine/threonine kinase, designated **SPAK** which shares 60–65% with the NIMA- kinase (**Never In Mitosis A**) which regulates early to late progression of G2 stages in *Aspergillus* and mammalian cells (Wu et al 1998). **SIP4** is a 10 kD (99 amino acid) novel protein related to two anonymous genomic sequences from *Arabidopsis*. **SIP8**, named **SPGB**, is a G-Box (CCACGTGG) binding proteins (Menkens et al 1995). These findings formed the basis for our program.

The tomato SIPs bind to CETS proteins from *Arabidopsis* and *Antirrhinum* and form a network of bipartite interactions.

We have shown that *CEN* of *Antirrhinum* and the *TFL1* and its *FT* antagonist in *Arabidopsis* (Kardeilsky et al., 1999) display the same binding pattern.

In addition to interacting with CETS proteins, SIPs form associations among themselves, some of which are phosphorylation dependent. *Such differential affinities may form a basis for subtle modifications of the SP system by 14-3-3 factors.*

Expression domains of *SPAK*, *SPGB* and *SIP4* overlap with that of *SP*.

In situ hybridization experiments proved that the expression pattern of *SPAK* overlaps spatially and temporally with the patterns observed for *SP* and for the tomato *LEAFY* gene. The in situ localization of transcripts of the *SPGB* gene and the *SIP4* gene also overlap throughout with that of *SPAK* and *SP*. The same pattern was seen for 14-3-3/2.

In vitro and phosphorylation dependent interactions among SIPs.

In vitro assays showed that all the yeast interactions are also formed in vitro, that *SP* phosphorylates itself as well as *SIP4* and that in vitro translated 14-3-3/74 binds only phosphorylated *SPAK*.

SP and 14-3-3/74 may be interchangeable at, or even compete for a six amino acid binding site in the SPAK protein.

Raf1, provides a precedent as it is also associated with 14-3-3 and with a mammalian homologue of SP (Muslin et al., 1996). The results indicate that A) The C-terminal domain of SPAK, amino acids 511-609, is required for homo-dimerization. B) A six amino acid peptide with a perfect match with 14-3-3 consensus binding site, is required for the binding of both SP and 14-3-3/74.

Over expression of 14-3-3 genes compensates for the loss of function of SP and the suppression of SPAK induces ovate fruits.

In transgenic plants partial rescue of the indeterminate phenotype is evident in the shoot inflorescence of *sp an* plants over-expressing either of the two genes. It will be interesting to test if over-expression, of 14-3-3/2 and 14-3-3/74 which form strong heterodimers, will result in a complete complementation of the determinate phenotype. Antisense expression of *SPAK* has no obvious effect on growth habit but plants expressing antisense or dominant negative constructs of *SPAK* (i.e. amino acids 403-609) form ovate pear-shaped fruits instead of the normal round fruits. This is the first evidence for kinases involved in regulation of fruit size. Using the services of the University of Madison center we have successfully identified a knockout line of *Arabidopsis* with an insertion that interferes with the reading frame of the best homologue of SPAK. We will examine the plants under different light regimes and in combination with other mutants.

Mutations in conserved structural domains annul the SP/SIPs interactions and identify the protein association domain required for SP function.

To substantiate a functional need for SP to interact with SIPs we took advantage of the recently determined crystal structures of three CETS (PEBP) proteins, from mammals and plants (Banfield and Brady, 2000) and of single amino acid alterations which are known to inactivate *CETS* genes (Bradley et al., 1997). The SP model was constructed by N. Adir, using homology based modeling (Peitsch, 1996) and the binding properties of SP proteins carrying three key mutations in particularly critical and conservative domains of all CETS/PEBP proteins were determined. The nice correlation between mutant sites and binding potentials of the SP proteins is discussed in detail by Pnueli et al., 2001.

We concluded that the *CETS* genes encode a new family of modulator/adaptor proteins analogous to the 14-3-3 family. They seem likely to participate in many signaling pathways (see Young et al., 2000) but their developmental role may be revealed only in systems and organs where their function is responsible for a rate-limiting process.

Further studies:

The tomato SP family.

In cooperation with the lab of D.Zamir we have identified six members of the SP family in tomato (Carmel-Goren, et al., in prep). Here we choose to concentrate on two SP homologues located on chromosomes II and III one is the functional orthologue of FT. These are being studied at all levels as discussed for SP itself.

Inflorescence architecture in Tomato

To study the regulation of meristem proliferation and formation of flowers in the inflorescence shoot we have generated the following critical double mutant genotypes which several criteria may be important for the understanding of the regular cycling of the vegetative/reproductive switches in the tomato plant; *sp fals*, *an bl*, *an j*, *an ls* and *sp ls*, *bl sf*, *sft ro*, *ro j*. *an sp* and *sp j* double mutants were constructed a year ago. *sp* for example was shown to be a suppressor of *falsiflora*, while *jointless* is an enhancer of *anantha* converting the *anantha* inflorescence into a *falsiflora* phenotype. These studies form an advanced stage of the BARD program and will be pursued in the coming years.

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