

## Mini-Review

## 14-3-3 proteins, red light and photoperiodic flowering

## A point of connection?

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**Key words:** isoform specificity, protein interaction, phosphorylation, signaling

The 14-3-3 family of proteins is well known for participating in signal transduction by binding specifically phosphorylated proteins, thereby completing their kinase-induced transition in activity or localization. This interaction-based modulation of signal flux through metabolic pathways is a critical feature of many important eukaryotic signal transduction cascades. Only recently, however, have studies in *Arabidopsis thaliana* described that some of the most fundamental plant signal transduction pathways, including the photoperiodic flowering pathway, are functionally affected by 14-3-3s. There are pivotal points in the photoperiod pathway that are characterized by the accumulation, localization and stability of critical protein factors, all of which are strongly affected by light quality and photoperiod duration. These mechanisms (localization, phosphorylation, regulated proteolysis) are the same as those regulated by 14-3-3 proteins in other systems. Yet it is only recently that well characterized 14-3-3 genetic tools have become available in sufficient diversity to make it possible to truly tie 14-3-3 interactions to light signaling and flowering. This review presents an overview of photoperiodic flowering signaling and direct 14-3-3 participation in the process, coupled with a discussion of the overlapping and specific roles of 14-3-3s which present confounding issues in the functional dissection of this family of signaling proteins.

## Light Signals and Transition to Flowering

The transition from vegetative growth to flowering is arguably one of the most fundamental and encompassing developmental switches that is exclusive to plants. Plants monitor numerous environmental cues in processing this decision, with light being among the more important causative agents. Light quality and duration directly affect the progression from vegetative to reproductive growth. Plants can be categorized by whether they will transition to flowering after exposure to long days (LD), short days (SD) or if they are insensitive to changes in photoperiod.<sup>1-3</sup> Studies in *Arabidopsis* have shown that

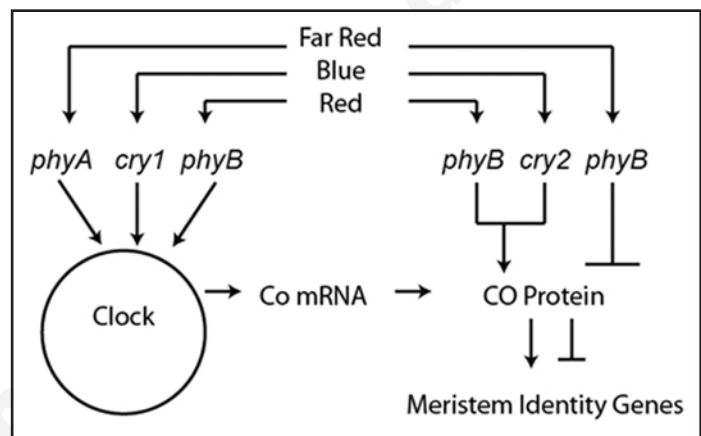


Figure 1. Photoperiodic flowering. Flowering occurs when red, far-red or blue light, acting through the phytochromes and cryptochromes, affect the clock—the circadian oscillator—to induce transcription of *Co* and accumulation of CO protein. In the LD plant the induction of CO proteins induces specific meristem identity genes, leading to flowering. In the SD plant these meristem identity genes are repressed by CO, arresting the floral transition. There is also a post transcriptional pathway of control in which blue and far-red light stabilize CO through phyA and cry2, while red light acting through phyB destabilizes the protein. 14-3-3s may influence flowering by acting at any of several testable nodes in this scheme.

the apparently simple input response is carefully regulated by at least four separate yet related pathways that guide, and sometimes gate, the forward flowering process while simultaneously attenuating inappropriate commitment to flowering.<sup>4</sup> Three pathways (“long-day”, “vernalization” and “light quality”) sense the external environment to activate flowering in response to photoperiod, temperature and red/far/red ratio.<sup>5</sup> Two other pathways use endogenous cues through the “gibberellin” and “autonomous” pathways. The next flux through these signaling schemes is integrated by proteins that regulate meristem identity, such as FLOWERING LOCUS T (FT),<sup>6</sup> SUPPRESSOR OF CONSTANS OVEREXPRESSION1/ AGL20<sup>7</sup> and LEAFY<sup>8</sup> among others. Figure 1 depicts the functional backbone of the “long-day” or “photoperiod” pathway. The complex web of effectors, pathways and their associated feedback systems offers an explanation as to why so many mutants present with phenotypes that affect flowering time.

More than a decade ago the *constans* (*co*) mutant was identified in *Arabidopsis*, identified by its conspicuous late-flowering phenotype

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Submitted: 02/05/08; Accepted: 02/12/08

Previously published online as a *Plant Signaling & Behavior* E-publication: <http://www.landesbioscience.com/journals/psb/article/5717>

under LD. The *CO* gene encodes a likely transcriptional regulator with B-box zinc fingers for protein interaction and a C-terminal domain that is required for nuclear localization.<sup>9</sup> In Arabidopsis, some *CO* overexpression lines flower very early,<sup>10</sup> consistent with *CO* itself being a positive regulator of the progression to flowering. The conspicuous phenotypes associated with *CO* overexpression and mutation demonstrate the central role of *CO* in the regulation of flowering via the photoperiod pathway.

The central function of *CO* is further underscored by the multiple levels of regulation that ultimately impact protein accumulation in the nucleus. *CO* gene expression is directed by the circadian oscillator, as transcripts peak at dawn and dusk under LD and in subjective night on SD.<sup>11</sup> Regulation by entrainment and maintenance of the oscillator is in turn dependent upon the phytochromes (phy) the cryptochromes (cry; predominantly cry<sup>2</sup>) and ZEITLUPE (ztl).<sup>12</sup> The phyA, phyB and cry1 receptors synchronize the oscillator with daily rhythms of light and darkness, whereas the LOV-domain protein ZTL adjusts the ~24 h period of the cycle.<sup>13</sup> An additional LOV-domain protein, FKF1, is required for the diurnal oscillation of *CO* transcripts.<sup>14</sup> Together with GIGANTEA, FKF1 associated directly with the *CO* promoter<sup>15</sup> opposing the influence of CDF1, a repressor of *CO* transcription.<sup>16</sup> In LD plants, when the diurnal peaks of *CO* transcript coincide with the appropriate day length, plants commit to flowering, consistent with the External Coincidence Model.<sup>17</sup>

Especially relevant to potential regulation involving 14-3-3 proteins, there is a clear post-transcriptional influence on *CO*. The mechanisms of regulation require *CO* localization to the nucleus, and modulation of *CO* stability, driven by blue, red and far-red light (transduced through cry2, phyB and phyA<sup>2,3,18</sup>). *CO* then induces expression of meristem identity genes such as FT in LD plants but acts as a repressor of these genes in SD plants, yet components of the flowering response are common among distantly-related species.<sup>19,20</sup> These findings suggest that the core mechanisms of LD and SD plants are very similar and the antithetical outcomes arise from slight modifications of the *CO* involvement in the pathway, very potentially at the posttranslational modification levels.<sup>20</sup> In summary, *CO* represents a pivotal point in the photoperiod pathway, and its downstream regulatory effects on FT and other meristem identity and signal integration genes results in regulating the decision to flower. *CO* accumulation, localization and stability are strongly affected by light quality and photoperiod duration and it is precisely these mechanistic modalities which suggest that 14-3-3s could be involved in modulating *CO*-mediated responses in the transition from vegetative growth to flowering.

### 14-3-3 Proteins

14-3-3 proteins have been shown to be absolutely required for signal-induced target transformation in many pathways, such as reductive metabolism, protein synthesis, protein folding, protein import, proteolysis, apoptosis, cellular trafficking, carbon and nitrogen metabolism, transcription, chromatin remodeling, ubiquitin metabolism, organellar/nucleocytoplasmic shuttling, blue light responses and several structure-related roles. In all of these processes 14-3-3s affect the activity or localization of the client proteins, indicating that 14-3-3s are direct and potent regulators of signal throughput. In plants, 14-3-3s have demonstrated and important roles in several metabolic systems, but functional participation in

plant developmental signaling response pathways per se are only beginning to be revealed.

The interactions among 14-3-3s and their client proteins are increasingly recognized as a layer of network organization and regulation, a layer that complements, for example, network organization at the transcription level. There are very well understood interaction networks in animal systems where 14-3-3s have a large influence. One major example is the network of regulation surrounding apoptosis and the mitogen-activated protein kinase (MAPK) pathway, 14-3-3s exert control through multiple interaction points in the pathways, regulating subcellular localization and phosphorylation of many proteins within the network.<sup>21</sup> As 14-3-3 interactions are revealed within plant pathways such as photoperiodic flowering, it is likely that such protein interaction networks will provide a similar overlay of pathway regulation.

The biochemical functions of 14-3-3s are well characterized, particularly in the regulation of enzymes, where the binding of 14-3-3s activates or inhibits enzyme activity.<sup>22</sup> In these cases phosphorylation marks the enzyme for change and the activation or inactivation process is completed by the binding of a 14-3-3 to the phosphorylated enzyme.<sup>23-25</sup> Another well characterized cellular function of 14-3-3s is governing client protein localization in response to phosphorylation, such as in nucleocytoplasmic shuttling.<sup>26-30</sup> The kinase Chk1, for example, contains both a nuclear export signal (NES) and a nuclear localization signal (NLS) and the function of 14-3-3 is to regulate which signal is exposed through molecular interference.<sup>26</sup> In a variation of this mechanism Cdc25 becomes excluded from the nucleus when a 14-3-3 acts as a transient nuclear export signal.<sup>27</sup> The presence of 14-3-3s in nuclei,<sup>31</sup> chloroplasts,<sup>32</sup> mitochondria<sup>33</sup> and in all membrane bound organellar fractions,<sup>34</sup> is consistent with a role in multi-organellar translocation. 14-3-3s also modulate target activity by influencing protein stability, and partnering target proteins together through the use of the two binding domains in a single 14-3-3 dimer. 14-3-3 binding can alter the target conformation such that a second target can be bound either in conjunction with the 14-3-3 or by the original target.<sup>35,36</sup>

The crystal structure has been solved for several mammalian and plant 14-3-3s,<sup>37,38</sup> and the extreme conservation of the central core region of 14-3-3s makes it very likely that this structure is a common feature of all 14-3-3s in all eukaryotes (Fig. 2).<sup>39,40</sup> However, all of the known crystal structures fail to resolve the N and C termini, which (along with several small regions within the molecule) are highly divergent among isoforms. Thus it is possible to consider the model as generally applicable to all plant 14-3-3s while recognizing that divergent areas might well contribute to specific structures and regulatory functions. The main feature of the 14-3-3 structure is a double-barreled clamp formed from the essentially parallel helices of the dimer pair. Each monomer barrel is sized to accept a phosphorylated helix from the target protein, and the main phosphoserine interaction zone is in the base of the barrels.<sup>41</sup>

In plants, 14-3-3s are encoded by a moderately large multigene family. Arabidopsis, for example, encodes thirteen expressed 14-3-3 proteins. The complexity of the Arabidopsis family suggests that the diversity among members reflects a divergence along functional lines, thereby producing a set of highly related 14-3-3 proteins with potential for diverse client interactions and discrete tissue and cell expression and subcellular localization. This situation makes

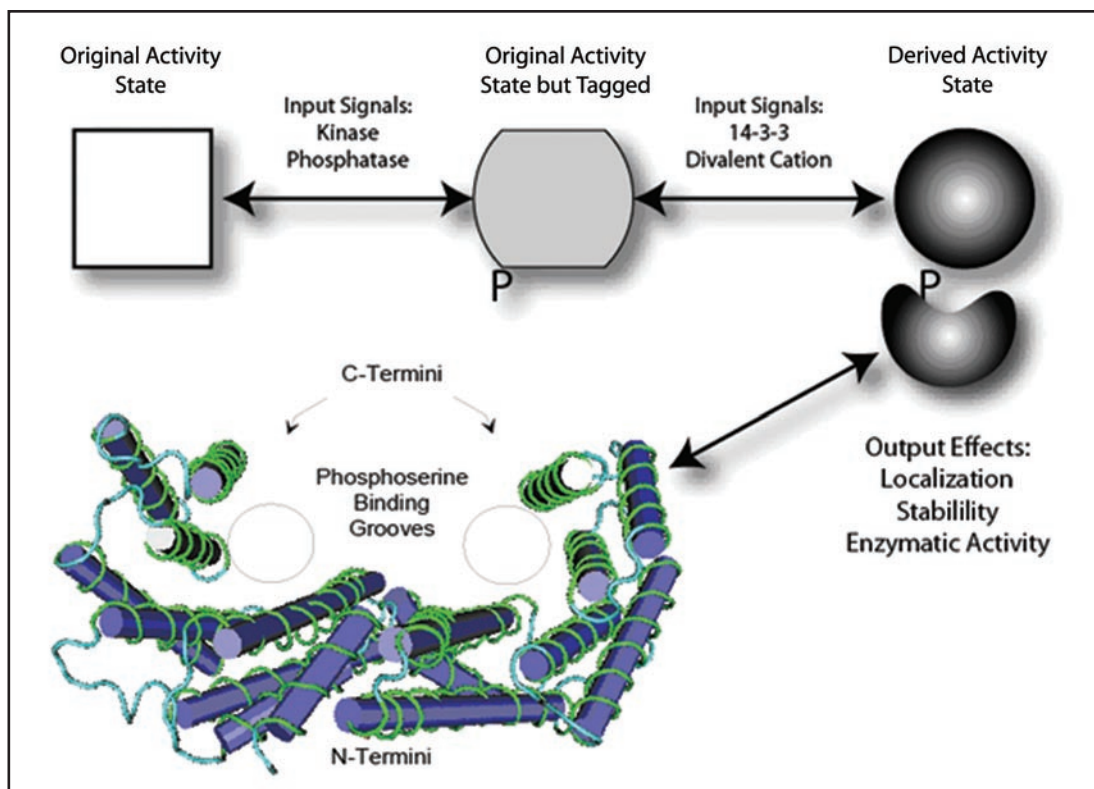


Figure 2. Model for 14-3-3 structure and function. The fundamental structure of 14-3-3s is a conserved core (51% identity among Arabidopsis isoforms) that is flanked by highly divergent N and C terminal regions. The structure of the conserved central region is known to be a “w”-shaped clamp formed of two monomers, each capable of binding phosphorylated proteins or peptides. The complete structure and positions of the N and C termini (with reduced overall identity but with blocks of sequences that are conserved among evolutionary branches) are unresolved in the crystals, but the C termini likely form a movable flap that can seal the top of the clamp, perhaps depending upon divalent cation binding. The 14-3-3 shown is human Eta PDB:2C74 viewed with Cn3D v4.1 National Center for Biotechnology Information <http://www.ncbi.nih.gov>.

functional analysis of 14-3-3 mutations in plants a challenging exercise in the dissection of redundancy, specificity and function. Elucidation of discrete functional roles for 14-3-3 isoforms has, therefore, been elusive in plants even though the broad distribution of consensus 14-3-3 binding motifs within proteomes suggests that 14-3-3s interact with a great number of signaling pathways.<sup>42</sup>

### 14-3-3 Proteins Intersect Flowering Pathways

Biochemical evidence for 14-3-3 participation in flowering first appeared in elegant studies of tomato *SELF PRUNING* (*SP*), a gene that encodes a homolog to Arabidopsis FT, part of the downstream effectors regulated by CO. Specific tomato 14-3-3s were found to be interactors within in a network of associations involving SP, SP Associated Kinase, a bZip transcription factor, and a novel 10 kDa protein.<sup>43</sup> In the same study, yeast two hybrid studies demonstrated interactions between 14-3-3s and Arabidopsis FT. These biochemical interactions suggest a role for 14-3-3s in the downstream flowering regulatory events involving FT, FT homologs<sup>44</sup> and potentially other members of the CETS family of proteins.<sup>43</sup> Phenotypically, the *sp* mutation changes the timing of shoot meristem development, resulting in earlier flowering and a determinant growth habit.<sup>45</sup> While direct complementation or epistasis tests with 14-3-3 mutations is not yet possible in tomato, overexpression of two different 14-3-3s partially compensate for the *sp* mutation, essentially delaying flowering and suppressing the transition from vegetative to

reproductive growth.<sup>43</sup> These data indicate that at least one point of entry of 14-3-3s into the flower signaling pathways is likely to be at the level of determining meristem identity, well downstream of CO.

Recently a reverse genetic approach was applied to the study the functional biological roles of two Arabidopsis 14-3-3 isoforms, specifically with regard to 14-3-3 isoform-specific flowering effects.<sup>46</sup> Candidate gene studies in reverse-genetic mutant backgrounds, especially mutants affecting in individual members of multigene families such as the 14-3-3s, are made possible by isolation of null mutants and sensitive phenotypic detection assays in mutant lines. These requirements can be experimentally challenging with any gene, and are especially so in a multigene family like the 14-3-3s which exhibits evidence of both redundancy<sup>47,48</sup> and specificity.<sup>49</sup> In such cases phenotype detection for any given isoform can be obscured by functional redundancy, and the need for tools that are specific for individual isoforms. However, in this study, Arabidopsis T-DNA insertion mutants from public collections were confirmed for two members of the 14-3-3 family, the  $\nu$  and  $\mu$  14-3-3 isoforms, using isoform specific antibodies<sup>32</sup> that directly measured the accumulation of the specific 14-3-3 isoform, confirming its disruption or suppression. Immunological detection had the added benefit of confirming that loss or reduction of one family member did cause other family members to increase in abundance to compensate for the loss of one member a condition that could introduce confounding phenotypic effects.

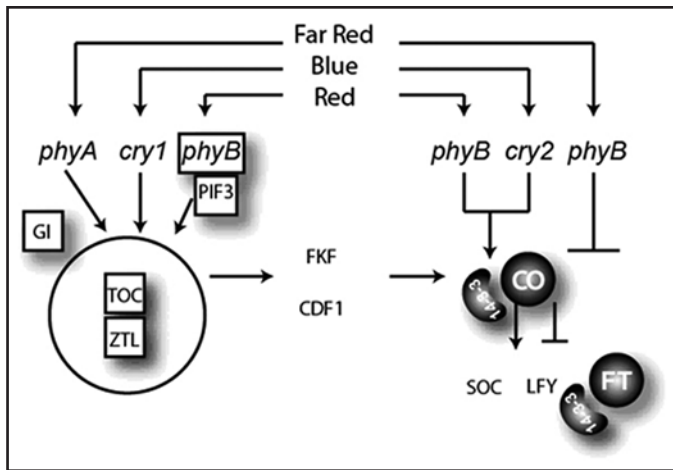


Figure 3. Proteins in the photoperiodic flowering pathway. 14-3-3s are currently known to connect to photoperiodic flowering by interaction with CO and FT proteins, as indicated by the darkened symbols in the diagram. Some of the other proteins discussed in the text as being involved in flowering are shown in their approximate position in the pathways. Those within boxes have been tested for 14-3-3 interaction, but to date no interaction with these proteins and 14-3-3s has been reported.

The 14-3-3 mutant lines flowered later than wild-type by a number of days, and also displayed an increase in leaf number at the time of flowering. The delayed flowering was not observed under SD conditions, so the problem was not in flowering per se, it was in the photoperiodic regulation of flowering. In order to confirm that the effect was due to the 14-3-3 lesion, multiple independent allelic lines were assayed or insertion lines were complemented with wild-type native 14-3-3 gene constructs. Fluence rate-response tests of hypocotyl growth inhibition, demonstrated that the mutants also have a hyposensitivity to red light, with wild-type growth in darkness, blue light or far-red light. This set of growth assays suggested a mechanistic link between 14-3-3 proteins and the phytochrome system, most likely phyB. Because phyB input affects photoperiodic flowering this finding represented a genetic link, possibly bridging the two processes, or acting between phyB and other elements of the photoperiod pathway.

To further test this hypothesis, potential entry points of 14-3-3 regulation into the photoperiod pathway upstream of and including CO were tested for direct interaction with yeast two hybrid assays. Specific interaction between 14-3-3 isoforms and specific proteins of the circadian oscillator, light sensing and transduction, and photoperiodic flowering were tested. A series of proteins in the photoperiod pathway represent potential points of 14-3-3 interaction; starting with phyB and progressing through the circadian oscillator and photoperiod pathway to the proteins such as FT and other activators that function to remodel meristem identity. Mis-regulation of the phyB pathway or associated components like PIF3, the proteins influencing the circadian oscillator, clock-associated proteins such as ZTL or FKF, or photoperiodic flowering regulators such as CO or SOC1, could all cause dystrophy in appropriate photoperiodic flowering response. In the yeast two hybrid analyses, interaction was observed only with CO. Co-immunoprecipitation of CO and 14-3-3s from extracts from leaves confirmed the interaction. While the absence of yeast two hybrid interaction does not rule out

14-3-3 regulation at other points, the positive interaction with CO is consistent with the observed phenotype. Combined with the phyB data these data suggest that these specific 14-3-3 isoforms may participate in connecting a sensor of the light environment to the central pathway that regulates the transition to flowering. This is one interpretation of the data.

Yet if hypocotyl elongation assays indicate a negative-regulatory effect on red-light sensing via the phyB, the 14-3-3 mutants should flower early. Phytochrome B negatively regulates CO accumulation and localization,<sup>3</sup> opposing flowering progression. The 14-3-3 mutants flower later obscuring these simple models and interpretations. One explanation might be that 14-3-3 isoforms have contrasting roles in various developmental contexts, negatively regulating phyB throughput in the developing seedling, while positively influencing phyB activity in mature rosette leaves. These contrasting roles may also be based on the degree of potential heterodimerization available in each of these contrasting conditions, tissues and developmental contexts. Examination of a larger set of 14-3-3 isoforms may better define the roles of 14-3-3 isoforms in this process.

In the broader picture, flowering is influenced by many factors, including plant health, nutrition, growth conditions, and other factors that induce floral progression in a manner that is not solely associated with the photoperiodic cues discussed to this point. Therefore changes in flowering behavior alone do not allow placement of 14-3-3s within any specific or unique floral transition mechanism or branch of the flowering signaling pathway(s). However, the biochemical interaction with CO allows assignment of a specific role in the photoperiod pathway per se and the interaction with SP/FT suggest a role downstream of CO in the photoperiod pathway (Fig. 3).

## Conclusions

Physiological, genetic and biochemical data are beginning to align in a way to firmly suggest that 14-3-3s interact with the signal transduction pathways associated with light sensing and the regulation of photoperiodic flowering. By its very nature, the complex pathways leading to the decision to flower present logical places for the extended regulation offered by 14-3-3 interaction. Indeed, it would seem truly exceptional if such a pathway were to escape regulation by 14-3-3s. With the development and deployment of characterized 14-3-3 mutations as well as other isoform specific tools, the dissection of 14-3-3 function is greatly enhanced, as biochemical studies can now be complemented by genetic studies that join physiology and phenotype to biochemistry and cell biology.

It is likely that as the flowering pathways are more fully elucidated, and as the tools for 14-3-3 research continue to be refined, the understanding of 14-3-3 involvement in flowering will continue to expand. Many questions remain unanswered. Some are specific: What is the nature of the CO- 14-3-3 interaction and what does the interaction impart to the pathway? What cell, tissue and environments promote or impede the interaction? Some are general: How do the various flowering signals reach the pathway? How are non-light signals integrated into the pathways? What other 14-3-3s, CO-Like genes, other FT-like genes and other gene families are involved in the flowering responses? Are there other direct 14-3-3 interactions in the pathway besides CO and FT? Extensions of the concepts and

studies presented here will seek to identify answers to these and other related questions, but it is clear that the fields of study involving 14-3-3 biochemistry, cell biology and signal transduction are now firmly intertwined with those involving the most fundamental developmental signals in plants.

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