

## Article Addendum

# 14-3-3 isoforms participate in red light signaling and photoperiodic flowering

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Members of the 14-3-3 family of proteins participate in signal transduction by modulating flux through various pathways. Potential subfunctionalization within this family has produced a suite of related proteins with diverse client interactions and discrete localization. The associated study assesses the biological roles of two specific 14-3-3 isoforms, using genetic, biochemical and physiological assays to ascertain potential nodes of interaction. Arabidopsis T-DNA insertion mutants representing the  $\nu$  and  $\mu$  isoforms exhibited a short, yet clear delay in flowering time on long days. Tests of hypocotyl growth inhibition under narrow bandwidth light indicated a hyposensitivity to red light, while responses to blue and far-red light were normal. These physiological tests suggest a mechanistic link between 14-3-3 proteins, red light sensing, and the pathways that control photoperiodic flowering. The precise entry point into the pathway was assessed using yeast two hybrid assays targeted against specific proteins active in the circadian oscillator, light transduction and photoperiodic flowering. Yeast two hybrid interaction was observed with CONSTANS (CO), and then confirmed with coimmunoprecipitation. Functional interaction with phyB leading to defects in flowering time and direct interaction with CONSTANS circumstantially places these specific 14-3-3 isoforms into the pathway that regulates the transition between vegetative and floral development.

## A Challenge to Identify Function

Reverse-genetic studies of candidate genes, especially multigene families, benefit from identification of null alleles and sensitive detection of phenotypes in mutant lines. While these facets alone are often challenging with any gene, the problem is exacerbated when addressing a multigene family with evidence of both redundancy<sup>1,2</sup> and specificity<sup>3</sup> among members. In such cases, identification of

isoform-specific mutants and phenotypes is complicated by genetic redundancy which may preclude conspicuous changes in biochemistry, physiology and development.

For these reasons elucidation of discrete roles for 14-3-3 isoforms has been elusive in plants. 14-3-3s do exhibit functional diversity despite their core similarity and evolutionary conservation.<sup>4-6</sup> The existence of consensus 14-3-3 interaction motifs within proteomes suggests that 14-3-3s innervate a substantial suite of signaling pathways.<sup>5</sup> Current understanding has placed 14-3-3s at the terminal ends of plant signaling schemes.<sup>7-14</sup> But these examples likely represent a miniscule subset of the 14-3-3 associated regulatory networks and new tools, such as insertion mutants, can provide insight into the role of 14-3-3s in broader regulatory processes.

## Isoform Specific Tools

Hundreds of regulatory processes have been mutationally described in Arabidopsis, presenting a mature baseline to observe and compare the performance of confirmed reverse-genetic mutants to wild-type plant responses. The availability of mutant lines greatly enhances the power of other tools, such as cloned 14-3-3 genes and potential regulatory targets, as well as validated protocols for biochemical interaction and physiological effects. This combination of approaches allows for a facile dissection of 14-3-3 function and integration into established plant pathways.

The associated work by Mayfield et al. began with the identification of isoform-specific 14-3-3 T-DNA insertion mutants from public collections and the subsequent generation of homozygous, nearly-isogenic lines. Mutant identification was assisted by isoform-specific antibodies<sup>15</sup> that assessed 14-3-3 protein levels in T-DNA insertion lines, thereby demonstrating that the effect of the mutation on the accumulation of protein as opposed to the more indirect measurement of mRNA levels. Some T-DNA insertions only disrupt regulatory regions, causing a decrease in protein abundance. These “knock down” tools also are important where complete null disruptions are not available (possibly because complete loss of function is lethal). Immunological detection also makes it possible to confirm that loss of one member of the family does not result in a compensatory change in other family members that could potentially account for indirect effects of the mutation.

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## Effects of 14-3-3s on Flowering Habits

In this study, the 14-3-3  $\mu$  and  $\nu$  mutant lines growing adjacent to wild-type plants consistently flowered later by a number of days, with an associated increase in leaf number. The deviations from wild-type habits were described only under long-day conditions, and were either observed in multiple independent allelic lines or in mutant phenotypes that were then complemented with wild-type native 14-3-3 gene constructs to verify the role of these specific 14-3-3 proteins. The delay in flowering prompted direct study of the contribution of these isoforms to the flowering process.

The transition from a vegetative to floral program is regulated by a complicated orchestration of at least four independent pathways that together influence reproductive timing.<sup>16-18</sup> The photoperiod pathway is one mechanism that connects the ambient environment to the circuits that influence the transition. Progression from vegetative growth to reproductive growth is dependent upon flux through photosensors, a circadian oscillator and a series of proteins that eventually alter the fate of meristematic cells destined to arise as an inflorescence.<sup>19</sup> The complex web of effectors explains why so many light-signaling-associated mutants present flowering defect phenotypes. The many post-translational processes associated with light signaling and photoperiodic flowering control define numerous potential regulatory points for 14-3-3 participation and it is not surprising that at least a subset of 14-3-3 isoforms would immix with these carefully regulated processes.

## A Tie to Phytochrome B

Dystrophy in flowering behavior alone does not allow placement of 14-3-3s within a specific floral transition mechanism. Flowering is influenced by many factors that affect plant stasis, including plant health, nutrition, growth conditions, and a handful of factors that induce floral progression in a manner independent of photoperiodic cues. However, a series of simple and proven tests can support or refute such an assignment. Analysis of early stem elongation under various light quantities and qualities provides information about how a certain regulator interacts with plant sensory systems.<sup>20</sup> In *Arabidopsis*, analysis of hypocotyl length after days of growth in red, blue or far-red light can be quite useful in defining functional interaction with discrete photosensory pathways. In the case of this study, 14-3-3  $\nu$  and  $\mu$  T-DNA insertion mutants exhibited decreased hypocotyl growth inhibition in comparison to wild-type seedlings when grown under low-fluence rate red light for four days. 14-3-3 mutant seedlings grown in darkness, blue or far-red light maintained comparable lengths to wild-type plants. These findings point to a hyposensitivity in red light input, a process initiated primarily by the phytochrome B (phyB) photosensor and transduced by a well populated pathway. 14-3-3  $\nu$  T-DNA insertion mutants also exhibit a more vertical directional growth habit under red light, presenting an additional, independent phenotype pointing to the phyB pathway.

## Direct Ingress to the Photoperiodic Pathway

Many regulatory proteins represent candidate nodes of 14-3-3 interaction, starting with phyB and progressing through the photoperiod pathway to the proteins that remodel meristem identity. Direct interaction with phyB (or other photosensors), phyB pathway components (e.g., PIF3) the circadian oscillator, clock-associated

proteins (such as ZTL or FKF), or photoperiodic flowering regulators (CO, SOC1) could account for the observed lateness in flowering. These possibilities were directly tested using protein-protein interaction studies in yeast. The results demonstrated direct interaction with the photoperiodic regulatory protein CONSTANS and no other experimental targets in the test. Direct interaction with PHYB was not observed in these yeast two hybrid studies, providing no support for a hypothesis that the 14-3-3 proteins are a scaffold for PHYB and CO interaction.

## Areas of Further Exploration

Hypocotyl elongation assays indicate a negative-regulatory effect on red-light sensing via the phyB signaling pathway. Based on this finding we might predict that 14-3-3 mutants would flower early, as phytochrome B negatively regulates CO accumulation and nuclear localization.<sup>21</sup> However, the mutants flower later. Results of these genetic tests do not conveniently agree with physiological observations, and can only be reconciled by suggesting different roles in different tissues, or under different developmental contexts. For instance, 14-3-3  $\nu$  and  $\mu$  may negatively regulate phyB activity in early development or in elongating stems, but may antagonize phyB activity in rosette leaves. The single isoforms used in this study may articulate with sensory pathway components, but the biological manifestation of the interaction may be determined or influenced by other isoforms specific to that tissue or developmental context. The fact that the 14-3-3 mutants in this study are not immediately and completely subsumed into a simple, extant model is an exciting indicator of the need for further inquiry, namely the careful evaluation of a larger suite of 14-3-3 mutations, their interactions with each other and well characterized biological processes.

## Conclusions

In the referenced study, functional, genetic and biochemical data align to place two 14-3-3 isoforms in the signal transduction pathways associated with light sensing and the regulation of photoperiodic flowering. Logical extensions of this study seek to identify other reverse-genetic 14-3-3 mutants and test their effects on these processes. In particular, it will be of interest to perform crosses to pyramid multiple 14-3-3 isoform mutations and evaluate what will likely be increasingly severe phenotypes in these specific pathways. In this sense, the work detailed in the associated report stands as an inroad into elucidating isoform specific 14-3-3 contributions to critical biological processes; a starting point for discovery leveraged by a comprehensive set of tools and techniques, and the well defined physiology of an established model plant system.

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