Final Technical Report: Genetic and Molecular Analysis of a new control pathway in assimilate partitioning.

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Assimilate partitioning refers to the systemic distribution of photoassimilate from sites of primary assimilation (source tissue) to import-dependent tissues and organs (sinks). One of the defining questions in this area is how plants balance source productivity with sink demand. We discovered a sucrose-sensing signal transduction pathway that controls the activity of BvSUT1, a proton-sucrose symporter in sugar beet leaf tissue. Sucrose symporters are responsible for sucrose accumulation in the phloem of many plants and, therefore, they mediate the pivotal step in the long-distance transport of photoassimilate to non-photosynthetic tissues, such as roots and seed. We previously showed that sucrose transport activity is directly proportional to the transcription rate of BvSUT1 and that symporter mRNA and protein have high rates of turnover with half-lives on the order of 2 h. We further demonstrated that symporter transcription is regulated by sucrose levels in the leaf and that sucrose-dependent regulation of BvSUT1 transcription is mediated, at least in part, by a protein phosphorylation relay pathway. The goal of the experiments during this current grant were to use genetic and molecular approaches to identify essential components of this vital regulatory system. The initial objectives were to: 1) to characterize Arabidopsis mutants we've isolated that are resistant to growth inhibition by sucrose analogues that are recognized by the sucrose-sensor, 2) to screen for loss of function mutants in BvSUT1-promoter::luciferase transgenic plants that no longer respond to sucrose accumulation in the leaf using non-destructive visualization of luciferase activity, 3) to use gel mobility-shift assays and nuclease protection experiments to identify cis elements in the symporter promoter and DNA-binding proteins that are involved in sucrose regulation of symporter expression.

Objective 1

Our aim here was to screen Arabidopsis mutants for growth resistance to sucrose analogues that are not hydrolyzed within the plant. We hypothesized that several metabolic and developmental pathways are regulated specifically by sucrose abundance and that the impact of sucrose on early seedling growth can be used in a genetic screen. In addition to our work examining the phloem specific sucrose symporter, this idea is supported by the observation that the expression of a sucrose symporter that is involved in sucrose uptake by developing Faba cotyledons is regulated by sucrose, as well as the observation that plastocyanin expression in early seedling growth is also regulated by

sucrose. A problem revealed in earlier screens on high sucrose by other investigators was that sucrose is hydrolyzed by invertase in plant tissues and, therefore, resistant mutants were ultimately resistant to high glucose, not sucrose. Thus, our aim was to use sucrose analogues that are not hydrolyzed. We initially screened using phenyl-alpha-glucopyranoside, but found the isolated mutants were not stable. We then worked with an organic chemist that was going to synthesis 1'-deoxy-1'-fluorosucrose. This molecule has been previously used as a sucrose analogue that is not hydrolyzed in plant tissue. Unfortunately, after more than 24 months, the lab we contracted with was not able to generate the necessary gram quantities needed for our screen (this must be synthesized, there are no commercial sources).

Given the problems we encountered with sucrose analogues, we shifted to another strategy. Several groups reported that short exposure of 4 to 6 day old Arabidopsis seedlings to high sucrose induces anthocyanin pigment accumulation. This was reported to be sucrose specific, with little response to glucose (Plant Physiology 2005 140:637-646; Plant Physiology 2005 139:1840-1852). We repeated those experiments and confirmed their overall observations. Based on these results, we developed two strategies to identify sucrose-response mutants. In the first, we have screened over 15,000 EMS Arabidopsis 4-day old seedlings for the absence of anthocyanin accumulation after 12 hr treatments with high sucrose. Given the complexity of the anthocyanin biosynthetic pathway, this screen could easily identify mutants in the synthesis pathway that have nothing to do with sucrose-induction. Since the biosynthesis of anthocyanin is well known, there are many mutants available in that pathway so we have been able to use allelism tests and PCR to differentiate sucrose-response mutants from synthesis mutants. To date, the bulk have been mutations n the synthetic pathway, but there are a couple that we are examining that may be sucrose-sensitive.

In addition to the anthocyanin accumulation screen, we are working with PAP1, Production of Anthocyanin Pigment-1. PAP1 is a member of the MYB family of transcription factors and a key regulator of anthocyanin accumulation in Arabidopsis. Earlier studies suggested that PAP1 expression is sucrose responsive in this experimental system and PCR results showed it is strongly induced by sucrose, although there is also a limited induction by glucose. The latter observation is in contrast to earlier reports. In order to investigate sucrose induction of PAP1 expression in more detail we generated transgenic A. thaliana expressing PAP1 fused to either β-glucuronidase (GUS) or luciferase (LUC) reporter genes. Analysis of chimeric PAP1 constructs in which the PAP1 gene was fused to either GUS or LUC revealed sucrose-dependent expression of the reporter genes. We are currently trying to determine what areas of the promoter are responsive to sucrose and glucose so we can focus exclusively on the sucrose response. Once that work is completed, we'll use a PAP1::reporter construct to screen for non-response mutants in young seedlings that are easy to visualize non-destructively.

Objective 2

This approach was based on a screen for loss of function mutants by looking for BvSUT1-promoter::luciferase transgenic plants that no longer respond to sucrose accumulation in the leaf using non-destructive visualization of luciferase activity. Although we generated the necessary transgenic plants with this reporter construct, we had difficulty getting a reliable report from luciferase in the mature leaves we had to

screen. We have generated new transgenic plants with a modified construct in hopes of increasing our ability to resolve the luciferase signal.

Objective 3

The aim of this objective was to use gel mobility-shift assays and nuclease protection experiments to identify cis elements in the sucrose symporter promoter that are responsible for sucrose regulation of its expression. This biochemical approach would also allow us to identify DNA-binding proteins that are involved in sucrose regulation of symporter expression. In spite of months of effort and many modifications of the basic protocols to enhance sensitivity, we could not achieve the goal of this objective.

New work from our lab provided convincing evidence that sucrose regulation of symporter expression is mediated by a protein phospho-relay involving a protein kinase and protein phosphatase (Ransom-Hodgkins et al. 2003). These would be good targets for getting a better understanding of this pathway if we could identify the kinases and phosphates involved. Regretfully, the companion cells where these signal-transduction enzymes control symporter expression are only 1-2% of all the leaf cells and, moreover, they are surrounded by mesophyll cells, thus making direct biochemical analysis virtually impossible. An indirect approach for identifying these signaling proteins would be using reverse genetic where we obtain knock-out kinase and phosphates mutants and then score them for an impact on sucrose signaling. However, since the Arabidopsis genome codes for hundreds of kinases and many phosphatases, that isn't a viable approach without some early filtering to decrease the number of plants we'd have to examine. To simplify the search, it would be essential to know which kinases and phosphatases are present in the companion cell. Therefore, we developed a new experimental objective in which we isolated companion cells from leaves and then used expression profiling to determine what sub-group of kinases and phosphatases are expressed in these unique cells.

To obtain a companion cell specific profile of gene expression, we were faced with the same problem of how to get at these cells. To over come the leaf tissue complexity, we used transgenic Arabidopsis in which a companion cell specific promoter (for galactinol synthase) is used to drive the expression of GFP (green fluorescent protein). In these plants, the companion cells in the leaf glow green when illuminated with uv light. We made protoplasts of mature leaf tissue from these plants in which all the leaf cells are in a heterogeneous mixture. We then used a cell sorter to separate the GFP-expressing companion cell protoplasts form the rest of the cells in the mixture and then used the Affymetrix AHA1 chip to determine which Arabidopsis are expressed in the companion cell. We've just completed that analysis and are writing up the results. Most importantly, there are fewer than 80 kinases and 20 phosphatases differentially expressed in the companion cell (relative to the mesophyll) that are targets for functional analysis via reverse genetics.

With a small amount of university funding, we will complete the new experiments with the aim of identifying one or more key players in this critical sucrose signaling system.

<u>Publications resulting from this funding period:</u>

Ransom-Hodgkins W, MW Vaughn, and DR Bush 2003. Protein phosphorylation mediates a key step in sucrose-regulation of the expression and transport activity of a beet proton-sucrose symporter. *Planta* 217:483-489

Harrington GN and Bush DR 2003. The bifunctional role of hexokinase in metabolism and glucose signaling. *Plant Cell* 15: 2493-2496

Bush DR 2004. Functional analysis of proton-coupled sucrose transport. In: Membrane Transport in Plants. Ed. Michael Blatt. p. 135-147 Blackwell Publishing

Bush DR and Leach J 2007. Translational Genomics for Bioenergy Production: There's room for more than one model. *Plant Cell* 19: 2971-2973

DOE Report Co-Author. Carbon Cycling and Biosequestration: Report from the March 2008 Workshop. p. 1-138. I co-chaired the session on plant productivity.

Harrington G and DR Bush 2009. AtSUC5 knockout plants exhibit multiple phenotypes related to cell expansion. *Plant Physiology* (working on revision)

Kumar A, DR Bush. Expression profile of Arabidopsis companion cells. (in preparation)