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MOLECULAR BIOLOGY AND PHYSIOLOGY

Nitrogen Metabolism in Cotton Stems and Roots During Reproductive Development

Earl Taliercio*, Prachuab Kwanyen, and Jodi Scheffler

ABSTRACT

Cotton is a perennial plant grown as an annual row crop in much of the world. Cotton stems and roots store starch prior to reproduction that is subsequently available to support reproduction. Aspects of nitrogen metabolism in cotton stems and roots were investigated to determine whether these tissues also provided nitrogen to support reproduction. Measurements of total nitrogen, soluble proteins, and individual amino acids indicated that nitrogen metabolism was altered after flowering began. Analysis of gene expression from previous microarray experiments also showed patterns consistent with a role of altered nitrogen metabolism during seed set. Changes in transcript levels of genes associated with amino acid biosynthesis, biosynthesis of nitrogenous compounds, and protein turnover also indicated that nitrogen metabolism changed in roots during seed set. We confirm that stem and roots provide nitrogen to support reproduction, and propose that the transport of nitrogenous compounds from these tissues to reproductive tissue could affect seed set by communicating the nitrogen status of the plant.

Cotton (*Gossypium hirsutum* L.) is a perennial plant grown as an annual row crop in much of the U.S. and around the world. After reproduction, wild cotton naturally sheds its leaves, usually in response to water deficiency, and regrows the following season. Wild cotton must allocate some of its carbohydrate reserves for regrowth. Domesticated cotton has been "annualized" to optimize reproductive growth in one season and although it maintains the ability to sprout from defoliated stems, domesticated cotton has been shown to direct more carbohydrates stored in stems and roots into reproduction than its wild counterparts (Wells, 2002). Redirection of stored carbohydrate reserves into reproduction is probably an important part of domestication because reserves that are not available to support reproduction are wasted in an annual row crop.

Previous analyses have shown that cotton stems and roots store starch up to the time flowering begins and then utilize these reserves to support boll development (Constable and Rawson, 1980; Taliercio et al., 2009; Wells, 2002). These experiments clearly establish cotton stems and roots as storage tissues for carbohydrates. At about the same point in development that starch is utilized from stems and roots, N is mobilized from leaves (Pettigrew et al., 2000). This N mobilization comes at the expense of protein levels and to the detriment of photosynthesis. Nitrogen levels in stems and roots increase in response to applied N early in plant development (Boquet et al., 2004; Fritschi et al., 2004). Nitrogen levels in stems are lowest near peak bloom and then increase (Fritschi et al., 2004). In general, application of N increases lint yield, but the differences decrease with increasing amounts of applied N (Clawson et al., 2006; Girma et al., 2007). Many factors affect the relationship of applied N to lint yield including soil type, initial N levels, and plant architecture (Boquet and Breitenbeck, 2000; Clawson et al., 2006; Fritschi et al., 2003). This diminishing return in yield associated with super optimal applications of N occurs even though cotton plants produce far more flowers than set fruit (Guinn and Mauney, 1984; Heitholt, 1995). The ability of cotton plants to increase N content of stems, but not increase lint yield suggests that stems may compete with reproductive tissues for N in some cases. The goal of this research is to identify changes in N metabolism in cottons stems and roots that occur during seed set that could communicate the N status of vegetative tissues to the reproductive tissues.

MATERIALS AND METHODS

Plant material. 'ST4793R' (*Gossypium hirsutum*, Bayer CropScience, RTP, NC) tissues were harvested from field-grown plants in 2004. ST4793R and 'ST gl' (glandless *G. hirsutum*, ARS germplasm release GVS5 P.0036.09) tissues were harvested

E.Taliercio and P. Kwanyen, USDA/ARS, 3127 Ligon Street, Raleigh NC 27695; J. Scheffler, USDA/ARS, 141 Experiment Station Rd, Stoneville MS
*Corresponding author:Earl.Taliercio@ars.usda.gov

from field-grown plants in 2008. Irrigated plots in Stoneville, MS were overseeded and hand thinned to 1 plant 15 cm^{-1}. Nitrogen was applied in one application pre-plant, 110 units of N-sol 32% per acre. This is the normal rate and method of application for the location. Potash (K) was applied in the fall after harvest at 200 lbs per acre. No phosphorus (P) was applied. The two lines, ST4793R and ST gl, were planted in single row adjacent blocks consisting of 15 6-m plots per block. At least 3 plants were harvested from random plots at 2-wk intervals. Prior to flowering, the age of the plants was estimated by the date the first flower opened in a plot. The date the first flower opened was designated anthesis or 0 wk. After the onset of flowering, the age of the plants was determined by labeling specific plants at anthesis. Stems and tap roots were harvested at the indicated times after anthesis (- sign indicates harvest before anthesis) between 07:00 and 10:00 h and put on ice in the field. The samples were returned to the laboratory and cut into liquid N. Handling time was usually less than 30 min. Stems or roots from plots were pooled, ground in a 3-hp Waring blender in liquid N, and stored at -80 °C. Part of each sample was subsequently freeze-dried for 3 d and stored at -20 °C. This subsample was used for N analysis and amino acid determinations.

Nitrogen Analysis. Total N content of ground tissue was determined by the Dumas combustion method using an Elementar Rapid N III (Nitrogen-Analyzer Elementar Americas, Inc., Mt. Laurel, NJ). Samples were oven dried overnight at 80 °C. Samples (0.2 g) were prepared in tin foil packets for combustion analysis. A replicate was defined as a sample harvested from one plot and measured individually. Analysis was done using the SAS regression Bivariate Model option with the linear model Nitrogen = (m*Weeks after Anthesis) + b. The variation was partitioned out by using a Linear Fit Trim Model where a grouping column was selected so that further fits were done on each group (SAS, Cary, NC).

Soluble Amino Acid Analysis. Three replications of 100 mg of dried ground tissues were extracted twice with 0.5 ml of 10 mM Na_2HPO_4 buffer, pH 7.4. A replicate indicates a measurement from tissue harvested from one plot. A different plot was harvested for each replication. Samples were collected at -2 wk, 0 wk, and 4 wk after the onset of flowering. The samples were centrifuged at 18,000 x G and the supernatants pooled. The pooled supernatants were freeze dried at -20 °C until the samples were completely dry, usually 2 d depending on the total number of samples being dried. Derivatization was performed by adding 20 μL of mixture of 7:1:1:1 ethanol:water:TEA:PITC [triethylamine (TEA), phenylisothiocyanate (PITC), (v/v)] to each sample and standard, then mixing well with a vortex. The reaction between PITC and the hydrolysate to produce phenylthiocarbamyl (PTC) amino acids was incubated at room temperature for 20 min to completion. Samples were then completely dried under vacuum and stored in a freezer. PTC amino acids in each sample and standard were dissolved from the dried matrix by vortex mixing with 500 μL of 5 mM Na_2HPO_4 buffer, pH 7.4 containing 5% acetonitrile and filtered through 0.2 mm membrane. Samples were reconstituted one at a time due to the PTC amino acid sensitivity to light and ambient temperature. Ten μL of sample were injected and analyzed with a high performance liquid chromatography system (Agilent; Palo Alto, CA) equipped with column heater, autosampler, variable wavelength detector, and a data acquisition software controller. The reverse-phase column used was a Pico-Tag (Waters; Milford, MA) with an inline column filter. The column temperature was maintained at 38 °C. The PTC amino acids were separated and eluted by a gradient resulting from mixing buffers A and B. Buffer A consisted of 150 mM $CH_3COONa.3H_2O$, 0.05% TEA, and 6% acetonitrile, pH 6.4. Buffer B consisted of 6:4 acetonitrile:Milli-Q water (v/v). Both buffers were purged with ultra-pure helium gas for 10 min before use. The flow rate was 1 mL/min throughout, and the gradient consisted of the following profiles: 100% A at start, 80% A and 20% B at 5.5 min, 54% A and 46% B at 10 min, 100% B at 10.5 to 12.5 min, 100% A at 13 min. The PTC amino acids eluted from the column were detected at 254 nm and recorded. The column was regenerated and equilibrated with buffer A for 10 min. A new and freshly reconstituted sample was injected and analyzed every 23 min. Means and Student's t-tests for the entire set of amino acids were determined using EXCEL Data Analysis. Trend analysis was performed on a subset of amino acids using the SAS regression Bivariate Model option with the linear model Amino Acid = (m*Weeks after Anthesis) + b (SAS, Cary, NC).

RNA Isolation and Analysis. Total polyribosomal RNA was isolated from roots -4 wk, -2 wk, 0 wk, 2 wk, and 4 wk after flowering (Larkins and Davis, 1975). The RNA isolation included an on-column

DNase1 digestion to remove contaminating genomic DNA (Qiagen; Valencia, CA). The RNA quality was confirmed by a bioanalyzer (Agilent). The primers in Table 1 were used for quantitative polymerase chain reaction (qPCR) analysis of gene expression. The efficiencies of all primers reported in Table 1 were determined on a mixture of relevant cDNA using 4-fold or 10-fold dilutions (Pfaffl, 2001). All qPCR were performed in triplicate and confirmed on at least two different cDNA preparations isolated from different replications. Melting temperature analyses were consistent with a single amplicon produced in these PCR reactions. Relative quantification compared to expansin was calculated by the method of Pfaffl. Relative expression compared to rRNA was also confirmed for many of the genes (data not shown).

RESULTS AND DISCUSSION

Total N. Previous studies showed that cotton stems and roots stored starch just prior to flowering and degraded this starch during reproduction (Taliercio et al., 2009). The stems and roots harvested in 2004 used in the Taliercio (2009) study that demonstrated the dynamic accumulation and utilization of starch during reproductive development were used in this study to evaluate N levels. Figure 1 shows that in the weeks prior to flowering total N levels are consistently about 1% of the dry weight of stems and roots. At about the time of first anthesis and during the weeks following the onset of flowering, the total N level dropped 20 to 40%. A similar pattern of N loss was observed for the same genotype harvested in 2008 and another genotype (ST gl) harvested only in 2008 during the same period of development. These data are consistent with observations that N levels were lowest in stems when plants are at peak flowering (Fritschi et al., 2004).

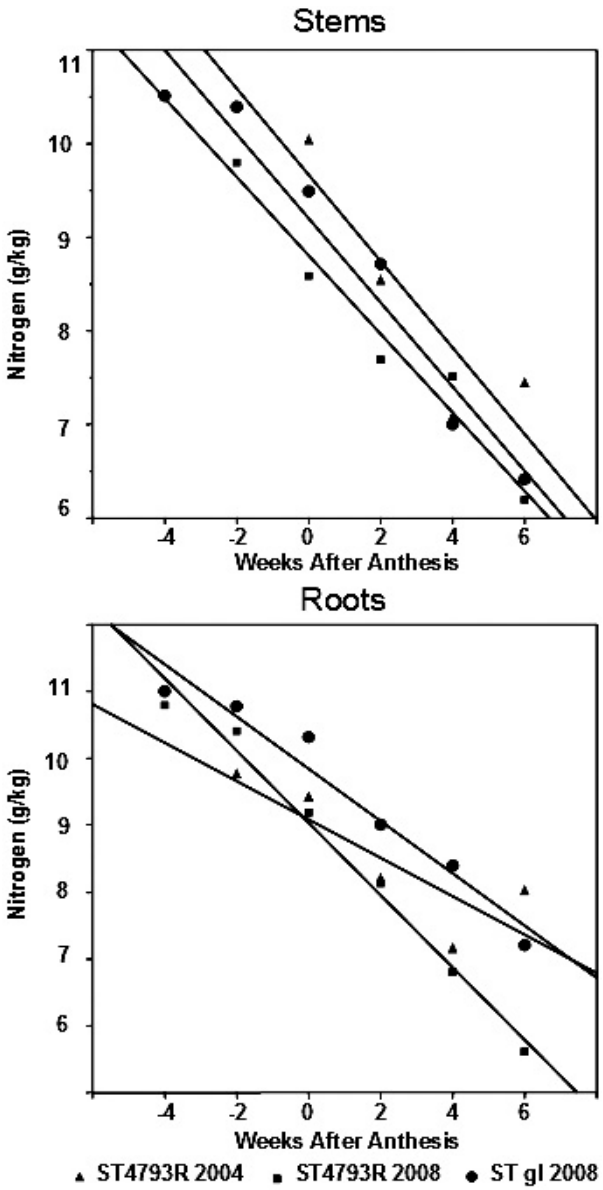

Figure 1. Total N levels determined for developing stems and roots of two cotton cultivars. There is a statistically significant difference in N levels in roots or stems between all 0-wk and 4-wk samples of the same genotype ($p \leq 0.05$).

Table 1. Primer sequences, amplification efficiencies, best cotton EST representative, best BLASTx cognate, and E value of cotton gene putatively encoding expansin, ASP synthase, GLU synthase, nitrate transporter, ammonia transporter, ubiquitin conjugating enzyme and a proteasomal subunit expressed in cotton roots

Gene	forward (5'-3')	reverse (5-'3')	efficiency	cotton est	BLASTX	E value
Exp	TTGCTTTGTATTGTTCTGTGGTGTG	ACTTGGGCTGCGGGCTAC	1.89	DW505162.1	gb|AAR09168.1|	2.E-139
AS	TACAGGAAGGAAAGCAGAACACATG	CATAAACATCGCTTATAGTACACATC	2.07	DW496959.1	gb|AAL91002.1|	3E-39
GS	CTTAAGAGGGGGTGGAAATTGAA	CCACTGGCCCAACAAAGAAAG	1.90	DW243709.1	emb|CAA71316.1|	0
NT	ACTGCTGAGAATAAGTGCTAAACTAC	GAAGAAAAAGAGAGAGAAATGGAAC	1.89	DW501886.1	emb|CAD33927.1|	2E-16
AT	GGCTGCTCGGTGGTTGAAC	CTTGACTTTCTCGGCTAGTTTGTTG	1.94	DW240017.1	gb|ABI52423.2|	4E-71
UCE	CTGTTCTGTCACTTCAATTTCTAAGG	TCCATACAACAATAGCAAAAGCAGC	1.98	DW500590.1	gb|ABW24024.1|	2E-41
PS	TTGTTTTGAGACTTTCCAATTCGG	TCGATTACAGTTCTAGCATCATGC	1.89	DW239775.1	gb|EEF48777.1|	2.E-125

Soluble Protein Measurements. Part of the N transported from cotton leaves to support reproduction comes from turnover of soluble proteins in leaves (Pettigrew et al., 2000). Soluble protein levels in leaves drop as total N levels in leaves drop. Soluble protein levels were measured from cotton stems and roots harvested at 0 wk and 4 wk after anthesis from ST4793R harvested in 2004 and 2008 and ST gl harvested in 2008 (Fig. 2). The levels of soluble protein were always reduced in cotton stems at 4 wk after the first flower. A similar reduction in soluble protein levels was observed in roots from both genotypes harvested in 2008, but no such difference was observed from roots harvested in 2004. Levels of insoluble proteins did not vary across developmental times in 2004 and therefore were not measured in the 2008 samples (data not shown). More detailed analyses and better quantitative methods will be needed to determine if there is a specific soluble vegetative storage protein degraded to provide N for cotton reproduction. However, analyses of soluble protein by 1-dimentional PAGE failed to identify a candidate for a vegetative storage protein (data not shown).

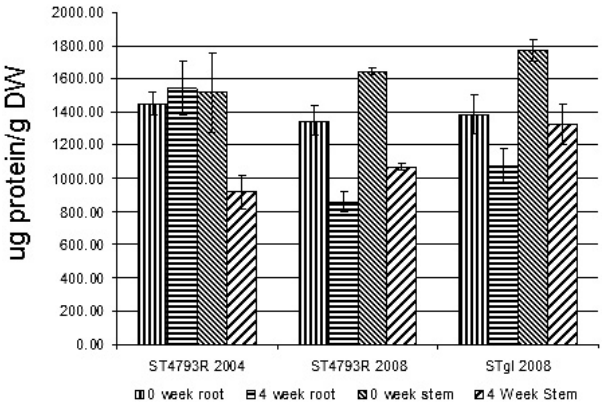

Figure 2. The amount of soluble proteins (µg/g dry weight) was measured in stems and roots of two cotton cultivars over 2 seasons. The differences between the 0-wk and 4-wk tissues are significantly different ($p \leq 0.05$) except for STgl root ($p \leq 0.06$) and root harvested in 2004 where there is not a statistical difference.

Amino Acid Measurements. The degradation of soluble protein should release amino acids that could be transported or further metabolized to release N for transport. Levels of 20 amino acids were measured in cotton stems and roots at the time of first flower (0 wk) and 4 wk later (Fig. 3). Levels of most amino acids were low and did not vary much during this period of development in either genotype. Trends of three of the amino acids—aspartic acid/aspargine (ASP/ASN), glutamic acid/glutamine (GLU/GLN), and threonine (THR)—were evaluated at -2 wk, 0 wk and 4 wk after anthesis (Fig. 4). For both genotypes and tissues, GLU/GLN showed a small but significant linear increase across the three developmental stages (R^2 values ranged from 0.64–0.99 with $p < 0.01$). ASP/ASN levels did not follow a linear change over developmental stages. In stems, ASP/ASN decreased from -2 to 0 wk and then increased to its highest level at 4 wk. Root levels were consistently low at -2 and 0 wk and then increased sharply between 0 and 4 wk. Evaluating differences across weeks after anthesis, levels of significance ranged from $p = 0.04$ (ST gl stems) to $p = 0.12$ (ST4793R roots). ASP/ASN increased as much as 70% in stems and 55% in roots. Levels of THR were variable but fell below acceptable levels of significance (data not shown).

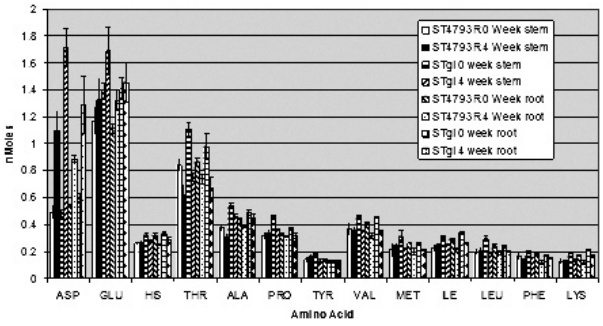

Figure 3. Levels of amino acids at 0 and 4 wk after anthesis in cotton stems and roots. Serine, glycine, arginine, and cystein levels were not detectable.

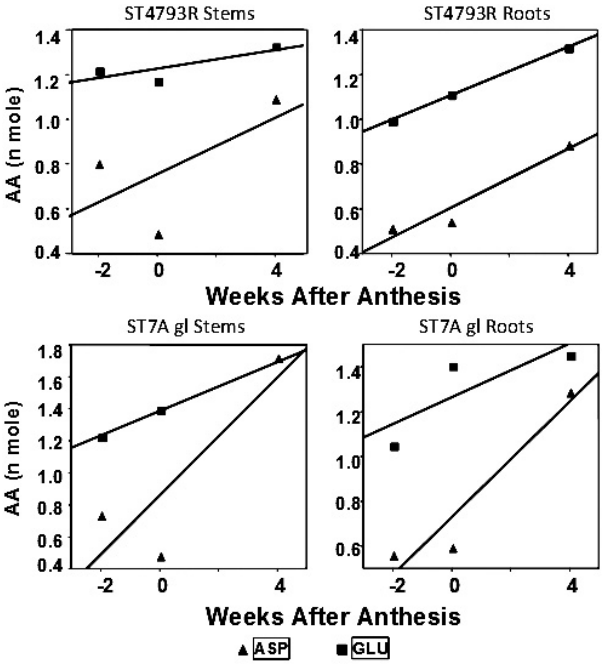

Figure 4. Changes in ASP and GLU levels (n moles) measured at -2, 0, and 4 wk after anthesis. Two cotton genotypes (ST4793R, ST gl) were analyzed.

Gene Expression Analyses. Previously, gene expression has been analyzed in stems and roots using microarrays during the period just prior to the beginning of reproduction (Taliercio et al., 2009). During this period starch accumulated and many genes associated with starch biosynthesis increased in expression to establish cotton stems and roots storage sites for carbohydrates. Analyses of the ontologies of genes that increased in expression at least 3 fold were associated with carbohydrate metabolism as expected (GO:0005975, $p = 0.06$) (Beissbarth and Speed, 2004). Changes in N metabolism were also implicated in these analyses because ontologies associated with transaminase activities were also significantly increased in expression in these same tissues (GO:0008483, $p = 0.0345$) at even more significant levels. Expression of selected genes was confirmed by qPCR on RNA from roots to extend information about their expression to include the period after flowering. Table 1 shows the cotton EST, BLASTx cognate, and expected value of the cognate that represent the selected genes. Figure 5 shows that the levels of ASP/ASN synthase mRNA increased in the 2 wk prior to flowering then remained at a fairly constant level. However glutamine synthase transcripts remained at about the same level during this period of development. The change in the relative expression of these genes was consistent with the change in ASP/ASN observed after flowering. A ubiquitin conjugating enzyme mRNA increased in expression at the same time as ASP/ASN synthase. This gene may play a role in protein turnover by adding ubiquitin molecules to proteins targeted for degradation. Interestingly, a subunit of the proteasome that might degrade ubiquitinated proteins does not appear to be differentially regulated at the mRNA level. The decrease in the N content of cotton stems and roots during flowering may indicate that N is transported from these tissues to bolls to support reproduction. Primers were identified that amplified mRNA encoding putative ammonia and nitrate transporters. The ammonia transporter increased in expression prior to the decrease in N and was fairly constant during the period of N loss. Levels of nitrate transporter mRNA increased substantially in cotton roots during the reproductive stage of development, overlapping with the reduction in N levels in stems and roots. Based on the magnitude of increase in mRNA levels and broad overlap with decrease in N levels, nitrate transport may play a role in translocation of N during the reproductive stage of development. Another candidate for transport is amino acids. Unfortunately we could not identify primers that amplified a unique band at detectable levels the represented amino acid transporters. A role for amino acid transport particularly ASP/ASN is likely.

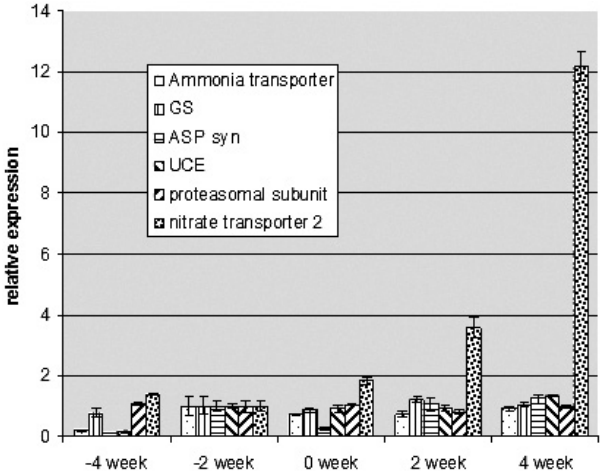

Figure 5. QPCR was performed to measure the levels of a putative ammonia transporter, glutamine synthase, aspartate synthase, ubiquitin conjugating enzyme, proteasomal subunit, and a nitrate transporter.

CONCLUSION

Taken together these data indicate that N stored in the stem and root was available to support reproduction. At least part of the reduction in N, particularly in stems, came from the degradation of soluble proteins as has been reported in cotton leaves. However, other forms of N appeared to be mobilized, particularly in roots, because changes in soluble protein levels were not always associated with total N loss. There was a reliable increase in ASP/ASN 4 wk after first flower making it a good candidate for export to reproductive tissues. Additionally transport of nitrate may also play a role in translocating N during reproduction based on a substantial increase in the expression of a gene encoding a nitrate transporter. The form of transport of N from the stem, root, and possibly leaves during flowering is important because it could act as a signal to inform the reproductive sinks of the N status of the plant thereby allowing fruit sets consistent with available N levels. This signal could impact seed set and the time of cut-out. Interestingly, cotton yield levels plateau in response to N, and N in stems can increase with application of N fertilizer (Clawson et al., 2006; Girma et al., 2007) . It should be possible to determine if excess N is diverted to storage forms of N in stems and roots during reproduction.

We note that the allocation of metabolic resources to stems in competition with reproduction may be unique to perennial plants and that most row crops are annual species. Identification of germplasms that do not divert N to stem and roots during reproduction could substantially affect yields. Additionally, identification of germplasm with higher levels of soluble protein (or other nitrogenous compounds) at first flower may provide more N to support reproduction.

DISCLAIMERS

Mention of trade names or commercial products in this article is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the USDA.

ACKNOWLEDGMENTS

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