Increased Nitrogen Fixation and Remobilization May Contribute to Higher Seed Protein Content Without a Yield Penalty in a Soybean Introgression Line

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ABBREVIATIONS: DAT, days after transplanting; DAP, days after planting; LAI, leaf area index

ABSTRACT

The development of soybean varieties with higher seed protein content has been hindered by a negative correlation between protein content and yield. Benning hi-pro, a genotype that breaks this tradeoff, was recently developed by introgression of a high protein allele on chromosome 20 into a high yielding parent. Because seed protein is thought to be limited by N, field and growth chamber experiments were performed to identify the N flux(es) that enable Benning hi-pro's increased seed protein without a yield penalty. When the N source was completely controlled in growth chambers, Benning hi-pro was occasionally able to fix more N than its recurrent parent, but this response depended on the strain of the rhizobia symbiont and was not observed at all developmental stages. In the field, Benning hi-pro remobilized N from its leaves at a higher rate relative to C during seed fill, but this response was only observed in one of the two years studied. The high protein allele and surrounding region of chromosome 20 present in Benning hi-pro increases its potential for N fixation and N remobilization from vegetative tissue, but those phenotypes are not consistently expressed and may depend on environmental and sink control.

INTRODUCTION

A substantial portion of the value in soybean [*Glycine max* (L.) Merr.] seed is derived from its meal protein, which is an important source of nutrition for poultry and livestock worldwide. Soybean seed protein content ranges from 35% to over 50% among genotypes (Hwang et al. 2014, Zhang et al 2018). However, the negative correlation between seed protein and yield is a major hurdle in exploiting this phenotypic diversity to breed agronomically elite, high protein soybean varieties (Brzostowski et al., 2017). While yield has steadily increased, seed protein concentration has decreased with year of variety release (Morrison et al., 2000; Mahmoud et al., 2006). It has been hypothesized that the persistence of greenness, which prolongs CO_2 assimilation during seed fill—increasing yield—has reduced the N available for remobilization from vegetative tissue (Kumudini et al., 2002).

N is primarily transported into the seed coat in the form of ureides and from seed coat to developing seed as glutamine and asparagine (Rainbird et al., 1984b), and energy must be expended in the seed for the synthesis of storage proteins. *In vitro* studies of soybean seed development have found that seed protein accumulation is determined by the supply of N into the seed (Saravitz and Raper, 1995; Pipolo et al., 2004). Similarly, in *Brassica napus* and *Brassica carinata*, final seed protein concentration correlated positively with total amino acid content in leaf phloem sap (Lohaus and Moellers, 2000). When the soybean supply/sink ratio was altered by removing pods, seeds accumulated significantly more protein (as % of seed weight) when the assimilate supply per seed was increased (Rotundo et al., 2011).

Three N fluxes could potentially contribute to developing seeds: uptake from the soil, biological N fixation, and remobilization from vegetative tissue. The relative contribution of these components to seed N is highly variable among genotypes and environmental conditions. It

51 has been hypothesized that the large N demand in developing soybean seeds requires substantial
52 N remobilization from vegetative tissue, triggering leaf senescence and limiting the seed fill
53 period (Sinclair and de Wit, 1975, 1976). Other studies have found the proportion of seed N from
54 remobilization to be anywhere from 30% to 100%, depending on genotype (Zeiher et al., 1982).
55 Additionally, it has been shown that up to 90% of seed N may be supplied by N fixation, and that
56 N fixation continues through most of the seed fill period (Leffel et al., 1992; Mastrodomenico et
57 al., 2013). A study with three high protein and three average protein soybean genotypes found no
58 correlation between redistributed N from vegetative tissue and total N in mature seeds (Egli and
59 Bruening, 2007). Discrepancies in the estimated contribution of remobilization to seed N may
60 be partly linked to genetic improvement of soybean during the 20th century: when varieties
61 released in the 1930's were compared with varieties released in the 80's and 90's, the genotypes
62 remobilized the same amount of N from vegetative tissue, but the newer genotypes were able to
63 accumulate significantly more N during the seed fill period than the older genotypes (Kumudini
64 et al., 2002).

65 A genome-wide association study identified 17 loci on 10 chromosomes that are
66 significantly associated with seed protein content (Hwang et al., 2014). At one of the loci
67 identified in that study, on chromosome 20, an single nucleotide replacement resulted in mean
68 protein increase from 41.46% to 44.32%. In a genome-wide analysis including 934 accessions
69 from MG IV-VI, this high protein locus was refined to a 1 Mbp region (Vaughn et al., 2014), but
70 the specific gene(s) that determine this trait have yet to be resolved. A high protein locus in this
71 region of chromosome 20 (hereafter called Gm20) has also been identified in genotypes of *G.*
72 *max* and *Glycine soja* (Diers et al., 1992; Warrington et al., 2015). One of these analyses was
73 performed in a RIL population derived from a cross between Benning, a high-yielding maturity

74 group (MG) VII cultivar with moderate seed protein (Boerma et al., 1997), and Danbaekkong
75 (Kim et al., 1996), a lower-yielding MG V variety with very high seed protein. This locus
76 explained 55% of the variation in seed protein within the bi-parental population (Warrington et
77 al., 2015). From this population, the Danbaekkong Gm20 locus was introgressed into Benning to
78 create a Benning hi-pro genotype with ca. 3% increase in seed protein over Benning, similar
79 agronomic traits, and no yield penalty (Prenger, 2018). While the Gm20 locus is linked to seed
80 protein, complex epistasis limits its ability to break through the protein-yield tradeoff in most
81 genetic backgrounds (Brzostowski et al., 2017).
82 This study aimed to identify differences in N metabolism that contribute to higher seed
83 protein without a yield loss in Benning hi-pro, which possesses the Danbaekkong Gm20 locus,
84 compared to its recurrent parent Benning. N uptake and fixation were measured in growth
85 chamber experiments, while N remobilization from aboveground vegetative tissue was evaluated
86 in field experiments.
87
88

MATERIALS AND METHODS

Field experiment

Seeds were sown on 26 May 2016 and 30 June 2017 at Central Crops Research Station in Clayton, NC. Soybeans at this research station were planted in a three-year rotation with corn and cotton. In 2016 the experiment was planted in a Norfolk loamy sand, and in 2017 the experiment was planted in a field with variable Appling sandy loam and eroded Cecil sandy clay loam. Plots were arranged in a randomized complete block design with four replicates of each genotype and 38" row spacing. In 2016, plots were 3 rows wide and 12' long; in 2017, plot size was increased to 6 rows wide and 18' long to facilitate destructive aboveground biomass and leaf area measurements.

Chamber experiment 1

Seeds of soybean genotypes Benning, Benning hi-pro, Danbaekkong, and NC-Raleigh (Burton et al., 2006) were surface sterilized with 95% ethanol and 1% NaOCl (Somasegaran and Hoben, 1985) and incubated in rolled, moistened paper towels at 29°C for two days to germinate. Seedlings were transplanted into 0.5 L pots filled with vermiculite. Three seedlings were transplanted per pot and thinned to one plant per pot a week after transplanting. Plants were grown at 26°C day/22°C night with a 9 h photoperiod and a 3 h incandescent night interruption for the first 35 days to suppress flowering. Cotyledons were removed at 8 days after transplanting (DAT) to increase reliance on the N treatments.

Plants were assigned to one of four groups: (1) NH_4NO_3 in nutrient solution, (2) inoculation with *Bradyrhizobium diazoefficiens* strain USDA 110 [formerly classified as *Bradyrhizobium japonicum* (Delamuta et al., 2013)], (3) inoculation with *Bradyrhizobium elkanii*

112 strain USDA 31, or (4) no N fertilizer or inoculation. These N-free plants were included to
113 monitor for potential *Bradyrhizobium* contamination in the growth chamber or substrate, and
114 they were harvested to measure N derived from cotyledons prior to their removal at 8 DAT. All
115 plants were fed a modified 50% Long-Ashton nutrient solution (Hewitt, 1966). Inoculated plants
116 and N-free plants received nutrient solution without a N source, and the remaining treatment
117 received the nutrient solution containing 7 mM NH_4NO_3. Inoculations were conducted
118 immediately after transplanting, by adding 1 ml of a culture containing around 10^8 CFU ml^{-1} of
119 *B. diazoefficiens* USDA 110 or *B. elkanii* USDA 31. For the first 4 DAT, each pot received 20 –
120 30 ml of deionized water. Beginning at 5 DAT, each pot received 50 ml of water daily, and this
121 was followed by 50 ml of nutrient solution every other day. At 26 DAT, water and nutrient
122 solution were both increased to 80 ml daily.

123 Plants were harvested 45 DAT. Organs were separated into shoots, roots and nodules and
124 dried at 60°C for 3 days for biomass determination, followed by C and N analysis.

125

126 **Chamber experiment 2**

127 Benning and Benning hi-pro seeds were germinated as described above. Seedlings were
128 transplanted into 9 L pots filled with vermiculite and approximately 80 g of crushed oyster shells
129 to control acidification of the rhizosphere in later developmental stages (Israel and Jackson,
130 1982). Three seedlings were transplanted per pot and thinned to one plant per pot a week after
131 transplanting. The vermiculite was saturated with water before planting and flushed with N-free
132 50% Long-Ashton nutrient solution.

133 Seedlings were inoculated with *B. diazoefficiens* USDA 110 and grown in a controlled
134 environment growth chamber as described above. For the first 4 DAT, 30 ml of deionized water

135 were added daily; then, vermiculite was flushed daily with 600ml of deionized water followed by
136 the addition of 400 ml of N-free nutrient solution. After V5, water and nutrient solution were
137 increased to 1 L and 400 mL, respectively, twice a day. Whole plants were harvested at 33, 65,
138 82, and 132 DAT, when the plants were in developmental stages V5, R5, R6, and R8 (maturity).
139 A non-inoculated set of plants was also harvested at the first sampling data (33 DAT).
140 At harvest dates, whole plants were separated into organs and dried at 60°C for a
141 minimum of 3 days. Biomass, C, and N content were measured for dry tissues. At V5, leaves and
142 stems were collected and measured together as shoots. At other stages, shoots were separated
143 into stems, pods and leaves. At R5, developing seeds were too small for individual CN analysis,
144 so whole pods were measured. At R7 and R8, pods were separated into seeds and shells. Roots
145 and nodules were separated for all plants.
146
147 **Carbon/nitrogen measurements**
148 In growth chambers, whole plants were harvested by organ at each sampling date. On field
149 sampling dates, four 2-cm diameter disks were cut from uppermost, fully expanded leaves, and
150 three pods were removed from main stem nodes in the middle (2016) or upper (2017) third of the
151 canopy. All samples were dried at 60°C for a minimum of 3 days. Nodules were ground in 2 ml
152 tubes containing 2 3-mm stainless steel grinding balls, shaken at 1400 strokes min^{-1} for 45 sec in
153 a homogenizer (Geno/Grinder 2000, Spex CertiPrep, NJ). Leaf, stem, pods and shells were
154 ground using a Wiley mill (Model 4, Thomas Scientific, NJ). Seeds were ground with a
155 centrifugal mill (ZM100, Retsch, Germany). Tissue C and N content was measured with an
156 elemental analyzer (FlashEA 1112, Thermo Scientific, Walham, MA), and the percentage of C
157 and N per sample was calculated with the instrument's software (Eager Smart, Thermo

Scientific, Walham, MA). Biomass and percent N values were multiplied to estimate total N in plant tissues.

In chamber experiment 2 and in the field experiment, biomass and N content were measured at successive developmental stages. These values were used to calculate N lost from vegetative tissue or gained in reproductive tissue between developmental stages. Because total N per organ was destructively measured for separate groups of plants at each developmental stage, replicated values for N lost or N gained between stages could not be obtained and tested statistically. However, standard errors (SE) of the means were propagated using the formula:

$$SE_{difference} = \sqrt{{SE_a}^2 + {SE_b}^2} \qquad [1]$$

Leaf chlorophyll measurements

In the field, two 2-cm diameter disks were cut from uppermost, fully expanded leaves and flash frozen in liquid N. These disks were sampled from the same leaves on the same days as the disks used for leaf C and N measurements. Leaf disks were stored at -80°C until chlorophyll extraction. Chlorophyll was measured according to Porra et al. (1989). Briefly, frozen leaf disks were ground in chilled methanol. Methanol was decanted and centrifuged at 2500 rpm for 10 min. The supernatant was transferred to a cuvette, and absorbance was measured at 652.0 nm and 665.2 nm. Chlorophyll concentrations in the supernatant (µg/mL) were calculated using the equations:

$$Chlorophyll\ a = 16.29(A^{665.2}) - 8.54(A^{652.0}) \qquad [2]$$

$$Chlorophyll\ b = 30.66(A^{652.0}) - 13.58(A^{665.2}) \qquad [3]$$

$$Chlorophyll\ a + b = 22.12(A^{652.0}) + 2.71(A^{665.2}) \qquad [4]$$

Leaf gas exchange

181 In chamber experiment 2, light- and dark-adapted leaf gas exchange and chlorophyll
182 fluorescence were measured with a LI-6800 (LI-COR, Lincoln, NE). Light-adapted
183 measurements were measured at stages V5, R5 and R6, and dark-adapted measurements were
184 taken at stage R6. Light-adapted measurements were made in the middle of the light period, and
185 dark-adapted measurements were made 4 h into the dark period. Leaf chamber conditions were
186 set to ambient growth chamber conditions.
187
188 **Field biomass and yield measurements**
189 When Benning and Benning hi-pro were in early seed fill, all plants in 1 m of a center row in
190 each plot were cut down at soil level. These rows had not been used for tissue sampling. Six
191 plots were harvested at 84 days after planting (DAP), seven at 87 DAP, and the remaining three
192 plots were harvested at 88 DAP. Leaflets and pods were separated from stems and petioles, and
193 total leaf area per plant was measured with a leaf area meter (LI-3100, LI-COR, Lincoln, NE).
194 All tissues were dried at 60°C for a minimum of 7 days, and leaf, seed, pod, and stem + petiole
195 dry weight was measured for each plant.
196 At maturity, single rows were harvested with a plot combine (Almaco, Nevada, IA).
197 After seeds were weighed, seed composition was measured using near-infrared spectroscopy
198 (DA-7250, Perten Instruments North America, Springfield, IL). Seed composition parameters
199 are reported on a 13% moisture basis.
200
201 **Statistical analysis**
202 Chamber experiments 1 and 2 were completely randomized designs, with genotype and
203 either N treatment or developmental stage treated as fixed effects. Analysis of variance as

204 performed using a linear model in PROC MIXED (SAS Inc., Cary, NC). Test of effect slices
205 were calculated within N treatments and developmental stages. When these effects were
206 significant for N treatments, pairwise differences among genotypes were calculated with *t*-tests,
207 and *p*-values were adjusted to control the false discovery rate (Benjamini and Hochberg, 1995).
208 Each year of the field experiment was a randomized complete block design in which plots
209 were reps were blocked spatially across the field. When the block term was not significant, it was
210 dropped from the model. Variables measured repeatedly from each plot over the growing season
211 (C/N, seed weight, chlorophyll) were analyzed using repeated measures ANOVA. To calculate
212 the rate of N depletion from vegetative tissue during seed fill, the slopes of C/N over time were
213 analyzed leaf data from only the seed fill period (R5 and R6) in PROC MIXED.

214 **RESULTS**

215 **Growth chamber experiments**

216 In chamber experiment 1, all plants were harvested 45 DAT, at which time the plants inoculated
217 with USDA 110 were approximately at developmental stage V6, plants inoculated with USDA
218 31 were around V4, and plants fed NH_4NO_3 were around V7. Non-inoculated plants did not form
219 nodules, and the vermiculite substrate was assumed to contain no plant-available N, so the N in
220 the no-N plants was entirely derived from stored N prior to cotyledon excision. No-N plants were
221 extremely stunted and chlorotic, as would be expected for severe N deficiency. The average
222 cotyledon N value for each genotype was subtracted from total plant N in the NH_4NO_3 fertilizer
223 and rhizobia inoculation treatments to calculate total N assimilated.

224 N treatment had a significant effect on total N assimilation ($p < 0.0001$), while genotype
225 ($p = 0.0572$) and genotype × treatment ($p = 0.0820$) were not statistically significant. Plants that
226 received NH_4NO_3 assimilated the most N (Fig. 1), reflecting the 2-3 weeks after transplanting
227 and inoculation required to develop functional nodules. Plants inoculated with *B. diazoefficiens*
228 USDA 110 fixed more N than plants inoculated with *B. elkanii* USDA 31. Given the low
229 practical risk of accepting a slightly higher type I error rate in the interaction effect to consider
230 within-group tests, we further tested differences among genotypes within N treatments. Total N
231 assimilated was not significantly different among genotypes that were fed fertilizer containing
232 NH_4NO_3, nor was is it significantly different among genotypes inoculated with *B. elkanii* USDA
233 31. Genotype only had a significant effect on N assimilation among plants inoculated with *B.*
234 *diazoefficiens* USDA 110 ($p = 0.0265$), with Benning hi-pro fixing significantly more N than
235 Benning and Danbaekkong (Fig. 1).

236 In chamber experiment 2, Benning and Benning hi-pro were inoculated with *B.*
237 *diazoefficiens* USDA 110 and harvested at developmental stages V5, R5, R6, and R8 (maturity).
238 As expected, seed protein at maturity was significantly higher in Benning hi-pro than in its
239 recurrent parent, and seed C/N was also significantly different between the two genotypes (Table
240 1). Although the average seed yield per plant was higher in Benning hi-pro, the difference was
241 not significant. Total N fixed per plant was measured at each developmental stage, but in
242 contrast to chamber experiment 1, Benning hi-pro only fixed significantly more N at maturity,
243 132 DAT (Supplemental Table 1). There were limited differences in organ-specific N between
244 the genotypes (Fig. 2, Supplemental Table 1). Benning hi-pro had significantly more N in pods
245 (seed + shell) at maturity, which drove the difference in total N fixed at this stage. At R5,
246 Benning hi-pro had significantly more N in nodules ($p = 0.0285$). Insufficient nodule tissue
247 remained for C and N measurement at R8.
248 For inoculated plants in both growth chamber experiments, total N assimilated was
249 divided by nodule weight to calculate N fixed per nodule weight. In chamber experiment 1,
250 Benning hi-pro fixed significantly more N per nodule weight than the other three genotypes
251 when inoculated with *B. diazoefficiens* USDA 110 but not when inoculated with *B. elkanii*
252 USDA 31 (Supplemental Table 2). The difference in symbiosis with *B. diazoefficiens* USDA 110
253 was due to higher total N per plant in Benning hi-pro but similar nodule mass as the other
254 genotypes. In chamber experiment 2, there was not a significant difference in N fixed per nodule
255 weight at V5, R5, or R6. N fixed per nodule weight could not be calculated at maturity because
256 virtually all nodule tissue had degenerated, although this was the only harvest point in chamber
257 experiment 2 at which total N fixed was significantly greater in Benning hi-pro.

258 In chamber experiment 2, the amount of N remobilized from vegetative tissue between
259 developmental stages, as well as the amount of N fixed between developmental stages, was
260 estimated from differences in mean N content per organ over time. Vegetative N lost was
261 calculated as the difference in mean vegetative N (leaf, stem, root, and nodule) between
262 developmental stages. Because the N remaining in abscised leaves was not measured, this value
263 represents the maximum potential N remobilization from vegetative into reproductive tissue
264 during seed development. Reproductive N gained was calculated as the difference in mean
265 reproductive N (seed and pod) between developmental stages. In this experiment, the amount of
266 reproductive N gained was always greater than vegetative N lost. This difference came from N
267 fixation, the sole N source for inoculated plants. The two genotypes lost similar amounts of N
268 from vegetative tissue and gained similar amounts of N in reproductive tissue from R5 to R6
269 (Table 2). The estimate for N fixed from R5 to R6, calculated from these values, was also similar
270 between the two genotypes. From R6 to R8, the two genotypes again lost similar amounts of N
271 from vegetative tissue. During the same period, however, Benning hi-pro gained substantially
272 more N in its reproductive tissue. Thus, from R6 to R8, Benning hi-pro may have fixed twice as
273 much N as Benning, and the propagated standard errors for these estimates do not overlap (Table
274 2).

275 Higher N fixation per nodule weight may carry a higher energy requirement per nodule
276 weight. In chamber experiment 2, light-adapted leaf gas exchange was measured at V5 and R5,
277 and light- and dark-adapted leaf gas exchange and chlorophyll fluorescence were measured at
278 R6. There were no differences between the two genotypes in CO_2 assimilation rate, dark-adapted
279 respiration rate, photosystem II operating efficiency, quantum efficiency of CO_2 assimilation, or
280 the maximum quantum efficiency of PSII photochemistry (Supplemental Table 3).

281

282 **Field experiments**

283 Seed protein was significantly higher in Benning hi-pro than in Benning for both years, while
284 yield was not significantly different between the genotypes (Table 3). Yield for both genotypes
285 was similar to check variety NC-Raleigh in both years. The increase in seed protein content in
286 Benning hi-pro was greater in 2017 than in 2016: + 4.2% compared to + 3.2%. Although the
287 seed protein rankings of Benning, Benning hi-pro, and NC-Raleigh were the same in both years,
288 average seed protein content for all three genotypes was substantially lower in 2017.
289 Danbaekkong, the high protein locus donor parent of Benning hi-pro, was included in 2017 and
290 had significantly higher seed protein than all other genotypes but lower yield. Seed C/N
291 rankings among genotypes were the reverse of those for seed protein in both years, as expected,
292 although seed C/N at harvest was not significantly different between Benning and Benning hi-
293 pro (Table 3).

294 Leaf area index (LAI) and aboveground biomass were measured in 2017 for 1 m rows
295 harvested at DAP 84-88. Benning, Benning hi-pro, and NC-Raleigh were in R5 at this time, and
296 Danbaekkong was in late R6. LAI and aboveground biomass were not significantly different
297 among genotypes (Table 4). Percent N and C were measured for aboveground organs to calculate
298 total aboveground N as well as vegetative N available for remobilization. There were no
299 differences among the genotypes in total aboveground N when Benning and Benning hi-pro were
300 at R5, despite Danbaekkong being closer to maturity. Aboveground N available for
301 remobilization, calculated as leaf N + stem N, was also not significantly different among the
302 genotypes. Genotype had a significant effect on reproductive N (seed + shell N) at this stage, but
303 only NC-Raleigh and Danbaekkong were significantly different in pairwise comparisons (Table

304 4). At the time of biomass harvest, DAP 84-88, Benning and Benning hi-pro seeds had gained
305 one-third or less of their final weight (Fig. 3).
306 The seed N gained from R5 (Benning, Benning hi-pro, and NC-Raleigh) or R6
307 (Danbaekkong) to R8 was calculated for each genotype, as it was for growth chamber
308 experiment 2 (Supplemental Table 4). For Benning and Benning hi-pro in the field, the amount
309 of seed N gained from R5 to R8 was smaller than the amount of aboveground N available for
310 remobilization from vegetative tissue at R5 (Table 4). The standard error estimates for seed N
311 gained overlap greatly. Thus, it was not possible to estimate from these data how much N
312 fixation or soil N uptake occurred during this time period.
313 Seed growth in Benning and Benning hi-pro was examined in both years. Seed fill began
314 fewer DAP in 2017, because the planting date was much later that year. In 2016, the overall
315 effect of genotype on seed dry weight was significant (Fig. 3A). Seed weight tended to be higher
316 in Benning late in seed fill, but pairwise comparisons were not significant on any specific
317 sampling date in 2016, and genotype did not have a significant effect on seed weight in 2017
318 (Fig. 3B). Genotype did not have a significant effect on N per seed in either year, although the
319 pairwise difference was significant at 104 DAP in 2017 (Fig. 3C, 3D). However, genotype had a
320 significant effect on seed C/N during seed fill in both years, and the pairwise difference between
321 genotypes was significantly different on almost every measurement day during the seed fill
322 period in 2016 (Fig. 3E), and on every measurement day in 2017 (Fig. 3F). In 2017, seeds were
323 too small on the first sampling date to analyze C and N content.
324 Leaf C/N was not significantly different between genotypes during vegetative
325 development or during flowering, but leaf C/N was significantly higher in Benning hi-pro late in
326 seed fill in 2016, suggesting a steeper slope for N depletion relative to C (Supplemental Fig. 1A).

327 Leaf C/N data from the seed fill period were then analyzed as a regression to determine the
328 relative rates of C and N remobilization from vegetative tissue. In 2016, genotype and
329 genotype*DAP significantly affected leaf C/N during seed fill (Fig. 4A). The slope of leaf C/N
330 over DAP was higher for Benning hi-pro than for Benning, showing that N was depleted at a
331 higher rate relative to C in Benning hi-pro leaves during seed fill. However, genotype and
332 genotype*DAP did not significantly affect the relative C and N remobilization rates during seed
333 fill in 2017 (Fig. 4B).

334 Leaf chlorophyll was measured alongside leaf CN to determine if differences in N
335 remobilization could also be detected through leaf chlorophyll content. Despite the differences in
336 leaf CN and in the rate of N depletion, the decline of leaf chlorophyll was not significantly
337 different between Benning and Benning hi-pro (Supplemental Fig. 2A). Additionally, no
338 significant correlation was observed between CN and chlorophyll over the growing season
339 (Supplemental Fig. 2B).

340 **DISCUSSION**

341 The primary goal of this study was to identify the N flux(es) that contribute to higher seed
342 protein without a yield penalty in Benning hi-pro compared to its recurrent parent, Benning. In
343 these experiments, Benning hi-pro supplied more N to developing seed through greater N
344 fixation capacity as well as a higher rate of N depletion from vegetative tissue, but these traits
345 were not observed concurrently or consistently. Strikingly, Benning hi-pro plants inoculated with
346 *B. diazoefficiens* USDA 110 in growth chambers fixed over twice as much N on average between
347 early R6 and maturity. Differences in seed protein between two genotypes of similar yield
348 potential have previously been linked to higher N fixation during seed fill period (Leffel et al.,
349 1992). In a greenhouse study, N fixation across reproductive development, measured by
350 integrating acetylene reduction measurements over time, was found to be significantly and
351 positively correlated with seed protein content, while integrated N fixation was not significantly
352 correlated with yield (Fabre and Planchon, 2000).

353 *B. diazoefficiens* USDA 110 is more efficient at fixing N than *B. elkanii* USDA 31, and
354 this has been linked to the presence of a hydrogenase in the nodule which recycles H_2 evolved
355 from the nitrogenase-catalyzed reaction (Caldwell and Vest, 1970; Schubert et al., 1978). As
356 expected, plants inoculated with *B. diazoefficiens* USDA 110 in this study fixed more N and had
357 greater dry weight across genotypes than those inoculated with *B. elkanii* USDA 31. The lower
358 N fixation efficiency of *B. elkanii* USDA 31 seemed to restrict the higher N fixation potential of
359 Benning hi-pro that was observed with *B. diazoefficiens* USDA 110. The native rhizobia
360 population was not characterized for the field experiment, but a prior analysis of a different soil
361 type at the same research station found the majority of nodules to be infected with rhizobia
362 belonging to serotypes 31/94, 46/76, and 76 (Ramirez et al., 1997). In that study, about 15% of

363 the samples isolated from the research station had high N fixation capacity, as does *B.*
364 *diazoefficiens* USDA 110, while ca. 20% had low N fixation capacity like *B. elkanii* USDA 31,
365 and the rest had intermediate N fixation capacity. Thus, it is possible that in our field experiment,
366 Benning hi-pro's potential to supply more seed N from N fixation was restricted by the
367 efficiency of rhizobia strain(s) that were present in soil.

368 The rate of N remobilization from Benning hi-pro leaves was only higher in one of the
369 two years of the field experiment, despite Benning hi-pro maintaining higher seed protein and
370 similar yield to Benning in both years. This variability in N remobilization between years,
371 together with the growth chamber results, do not entirely support the self-destructive hypothesis,
372 which suggests that high seed N requirements in soybean trigger leaf senescence and thus limit
373 the seed fill period (Sinclair and de Wit, 1975). Under the artificial, but optimal, environmental
374 conditions of the growth chambers, the high N requirement in developing seeds was met by
375 sustained N fixation though seed fill in both Benning and Benning hi-pro, as remobilized N
376 accounted for less than half of the N in each genotype at maturity. In the field, Benning hi-pro
377 produced the same seed yield as Benning despite remobilizing N from leaves at a higher rate in
378 2016. This suggests that under favorable environmental conditions—moderate temperatures and
379 plenty of light, water, and nutrients, excepting soil N—leaves can continue to assimilate carbon
380 and thus fuel N fixation well into the seed fill period, and that environmental cues may play a
381 larger role than seed N demand in triggering leaf senescence. Because the carbon demand for N
382 fixation is high (Rainbird et al., 1984a), this implies that leaf senescence may actually have been
383 delayed relative to seed development if photosynthesis continued at rates necessary to sustain
384 nodule function.

385 The data from these experiments cannot conclusively determine if seed composition
386 differences in Benning hi-pro are source- or sink-driven. However, differences in seed C/N
387 between Benning and Benning hi-pro were established early in seed development, when seeds
388 had gained less than a third of their final weight. However, the amount of N per seed was not
389 different between genotypes this early in seed development, and leaf C/N had not yet diverged
390 between the two genotypes this early in seed development. These observations, alongside the
391 fact that differences in N fixation and N remobilization rate were only observed intermittently,
392 suggest that the difference in seed C/N may be driven in part by differences in N sink strength
393 rather than by constitutively higher N fixation or N remobilization in Benning hi-pro. In growth
394 chamber experiment 2, the higher total N assimilation in Benning hi-pro was accompanied by
395 substantially higher seed yield (ca. 20%), congruent with a previous finding that N accumulation
396 is positively correlated with yield across genotypes (Rotundo et al., 2014). If higher N fixation in
397 growth chambers was driving seed protein gain, then a greater protein increase (>4%) may have
398 been expected, considering Benning hi-pro's large increase in N fixed. Instead, the seed protein
399 gain for chamber-grown Benning hi-pro was similar to that in the field, ca. 3-4%. Perhaps the
400 capacity for greater N fixation during seed fill in Benning hi-pro contributes to its yield
401 maintenance despite higher seed protein.
402 In the field, N accumulation per seed closely mirrored seed dry weight accumulation, and
403 significant differences were rarely present between the two genotypes on any particular sampling
404 date. Benning hi-pro also tended to produce smaller seeds than Benning, although the pairwise
405 differences were not significant in either year. Based on these findings, we hypothesize that
406 Benning hi-pro's higher seed protein concentration is related to reduced C accumulation in seed
407 relative to N, but that yield is maintained because the of the plant's ability to remobilize or fix

408 sufficient N to support a similar weight of seeds. Genotypic differences in seed sink strength for
409 N were also observed in an *in vitro* study comparing cotyledon N uptake by normal and high
410 protein genotypes at a range of N concentrations (Hayati et al., 1996). In that study, cotyledon N
411 concentration in both genotypes responded positively to N availability in solution, but the high
412 protein genotype's seeds were able to take up more N at every concentration. Seed C/N was
413 significantly different between Benning and Benning hi-pro through most of seed development,
414 although the differences were not significant at harvest. The lack of significance for seed C/N
415 differences at harvest is probably the result of variability through the canopy (Huber et al., 2016).
416 Harvest samples were taken from the combine, which homogenized all the seed from a row. In
417 contrast, samples during seed development were selected from the same canopy position.
418 Directly measuring leaf N requires specialized, expensive instrumentation, and requires
419 destructive sampling. Chlorophyll content also declines as leaves senesce, and chlorophyll can be
420 chemically extracted in relatively simple assay (Porra et al., 1989) or estimated non-destructively
421 in the field (e.g., Lichtenthaler et al., 1996; Cassol et al., 2008; Steele et al., 2008). The N in
422 chlorophyll is not directly exported from senescing leaves (Hörtensteiner and Feller, 2002), but
423 N from the concurrent breakdown of chloroplast proteins is an important source of N for
424 remobilization to vegetative tissue (Fischer, 2007; Liu et al., 2008). If a relationship could be
425 established, measuring the decline in leaf chlorophyll content might be a convenient proxy for
426 estimating N remobilization in future studies. Thus, we measured leaf chlorophyll in 2016 to
427 determine if it could be used as a surrogate for N remobilization rates. However, the decline of
428 leaf chlorophyll content during seed fill did not reveal the difference in N depletion that was
429 measured in the field in 2016, and chlorophyll did not significantly predict leaf C/N in field-
430 grown Benning and Benning hi-pro over the course of the field season. These results indicate

431 that chlorophyll would not be a reliable proxy for detecting subtle but significant differences in
432 leaf N metabolism.

433 These experiments revealed that the introgressed region of chromosome 20 from
434 Danbaekkong in the Benning background can increase N fixation as well as the rate of N
435 remobilization from senescing soybean leaves, but that the phenotypic expression of these traits
436 may depend on environment and biotic interactions. Whether the changes in these N fluxes are
437 source or sink driven remains unresolved, and the molecular mechanisms behind these changes
438 have yet to be elucidated. Although the Gm20 high protein locus has been narrowed to a <1Mb
439 region (Vaughn et al., 2014), the protein gain combined with maintaining the high yield potential
440 of Benning evidently involves multiple genes, as evidenced by large region of Danbaekkong's
441 chromosome 20 that is present in Benning hi-pro (Prenger, 2018). If the gene(s) involved in this
442 epistasis can be identified, a new path to improving soybean seed composition through breeding
443 or biotechnology could be illuminated.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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453 **SUPPLEMENTAL MATERIAL**

454 **Supplemental Table S1.** ANOVA results (*p*-values) for main effects, interactions, and slices
455 within effects for total N fixed and N per organ in chamber experiment 2. Analysis corresponds
456 with data shown in Figure 2.

457 **Supplemental Table S2.** Total N fixed per nodule weight in chamber experiments 1 and 2.

458 **Supplemental Table S3.** ANOVA results (*p*-values) for differences between Benning and
459 Benning hi-pro in leaf gas exchange and chlorophyll fluorescence in chamber experiment 1.
460 Light-adapted leaf gas exchange measurements were made at V5, R5, and R6. Light-adapted
461 chlorophyll fluorescence as well as dark-adapted gas exchange and chlorophyll fluorescence
462 parameters were measured at R6. Table abbreviations: photosystem II operating efficiency
463 (ΦPSII), quantum efficiency of CO_2 assimilation (ΦCO_2), maximum quantum efficiency of PSII
464 photochemistry (F_v/F_m).

465 **Supplemental Table S4.** Estimated seed N gained from R5 to R8 in the field experiment in
466 2017. Estimated nitrogen gained was calculated as the difference in mean seed N between R5
467 and R8. Values are mean ± standard error; errors were propagated as described in the Methods;
468 $n = 4$.

469 **Supplemental Figure S1.** Leaf CN across the growing season for Benning and Benning hi-pro
470 grown in the field in 2016 (A) and 2017 (B). Leaf tissue was sampled from the uppermost, fully
471 expanded leaf in the canopy. Significant pairwise differences (adjusted $p < 0.05$) between the
472 genotypes within days are indicated by asterisks. Points and error bars are means ± standard
473 error.

474 **Supplemental Figure S2.** (A) Leaf chlorophyll content during the 2016 seed fill period, R5 and
475 R6. Slopes were not significantly different between the two genotypes. (B) Relationship

476 between leaf C/N and chlorophyll content throughout the 2016 growing season, including
477 vegetative and reproductive developmental stages. Leaf chlorophyll content did not predict leaf
478 C/N.

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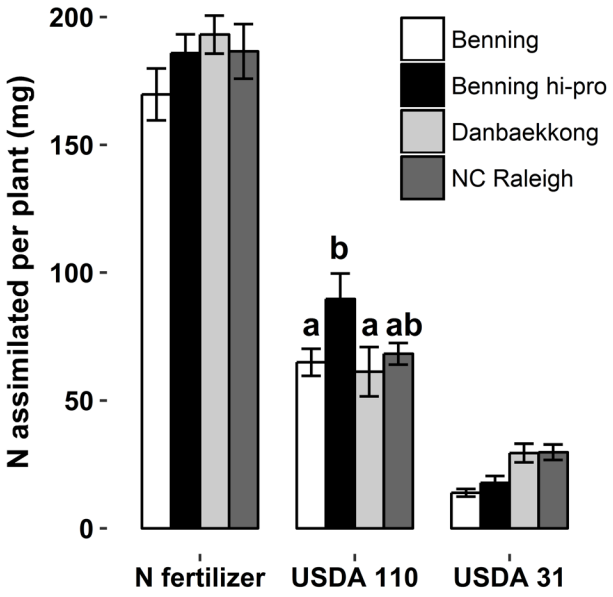
583

584 **FIGURES AND TABLES**

585 Figure 1. Total nitrogen assimilated per plant (mg) for plants supplied with NH_4NO_3 fertilizer,
586 inoculated with *B. diazoefficiens* USDA 110, or inoculated with *B. elkanii* USDA 31 in chamber
587 experiment 1. Whole plants were harvested 45 days after transplanting. Values are mean ±
588 standard error; $n = 4$. Letters indicate significant differences (adjusted $p < 0.05$) between
589 genotypes within N treatments.



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597 Table 1. Final seed yield, seed protein, and seed C/N from plants relying on nitrogen fixation

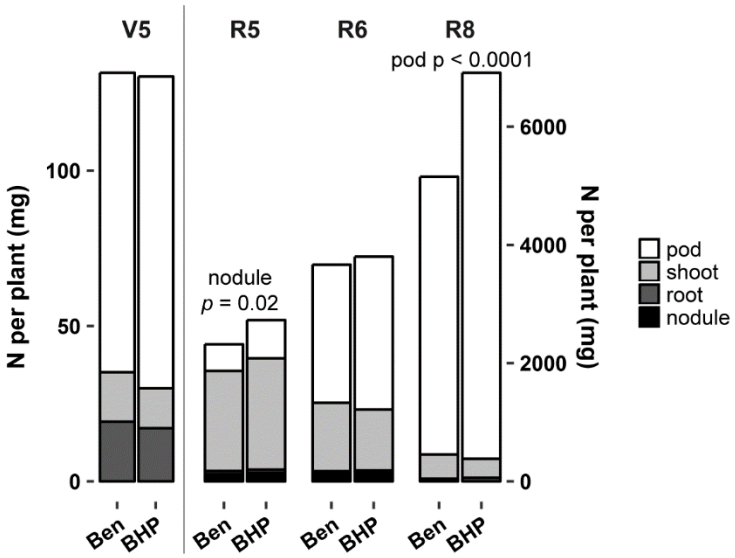
598 with *B. diazoefficiens* USDA 110 in chamber experiment 2. Letters indicate significant

599 difference ($p < 0.05$) between genotypes. Values are means for each genotype; $n = 5$.

Genotype	**Yield**	**Seed protein**	**seed C/N**
	g plant^{-1}	%	
Benning	69.0	43.1 **a**	8.2 **a**
Benning hi-pro	82.8	47.4 **b**	6.8 **b**
p-value	0.1554	**<0.0001**	**0.0022**

600

601 Figure 2. Total nitrogen (mg) in Benning and Benning hi-pro plants relying on nitrogen fixation
602 with *B. diazoefficiens* USDA 110 in chamber experiment 2. Values are means (n = 5).
603 Annotations indicate p-values for significant differences in organ N content between the two
604 genotypes. Scale on left applies to V5; scale on right applies to R5 – R8. Ben = Benning; BHP =
605 Benning hi-pro.



606

607 Table 2. Nitrogen lost per plant (mg) from vegetative tissues and nitrogen gained in reproductive
608 tissues between developmental stages in chamber experiment 2. Estimated nitrogen fixed was
609 calculated as the difference between reproductive N gained and vegetative N lost (representing
610 potential remobilization). Values are mean ± standard error; errors were propagated using
611 equation 1 as described in the Materials and Methods.

Genotype	R5 – R6			R6 – R8		
	Vegetative N lost	**Reproductive N gained**	**Estimated N fixed**	**Vegetative N lost**	**Reproductive N gained**	**Estimated N fixed**
	——— mg plant^{-1} ———					
Benning	540 ± 213	1887 ± 153	1373 ± 262	875 ± 164	2363 ± 488	1488 ± 515
Benning hi-pro	867 ± 169	1940 ± 121	1073 ± 208	833 ± 46	3945 ± 359	3112 ± 362

612

613 Table 3. Mean yield, 100 seed weight, seed protein, and seed C/N at developmental maturity in
614 field experiments. 100-seed weight was measured at approximately 8% moisture, and seed
615 protein is expressed on a 13% moisture basis. Letters indicate significant pairwise differences
616 (adjusted $p < 0.05$) among genotypes within years; $n = 4$.

	2016				**2017**			
Genotype	**yield**	**100 seed weight**	**seed protein**	**seed C/N**	**yield**	**100 seed weight**	**seed protein**	**seed C/N**
	g m^{-2}	g	%		g m^{-2}	g	%	
Benning	206.5	16.9	45.0 **a**	7.8 **ab**	218.1	16.1	34.8 **a**	8.4 **a**
Benning hi-pro	214.6	14.8	48.2 **b**	7.2 **a**	194.3	15.1	39.0 **b**	7.9 **a**
NC-Raleigh	192.4	14.9	41.1 **c**	8.5 **b**	210.5	15.6	32.5 **c**	9.4 **b**
Danbaekkong	—	—	—	—	131.3	16.4	43.7 **d**	6.3 **c**
p-value	0.6307	0.0570	**<0.0001**	**0.0114**	0.0504	**0.0479**	**<0.0001**	**<0.0001**

617

618 Table 4. Leaf area index, aboveground biomass, and aboveground N measured at 84 – 88 DAP

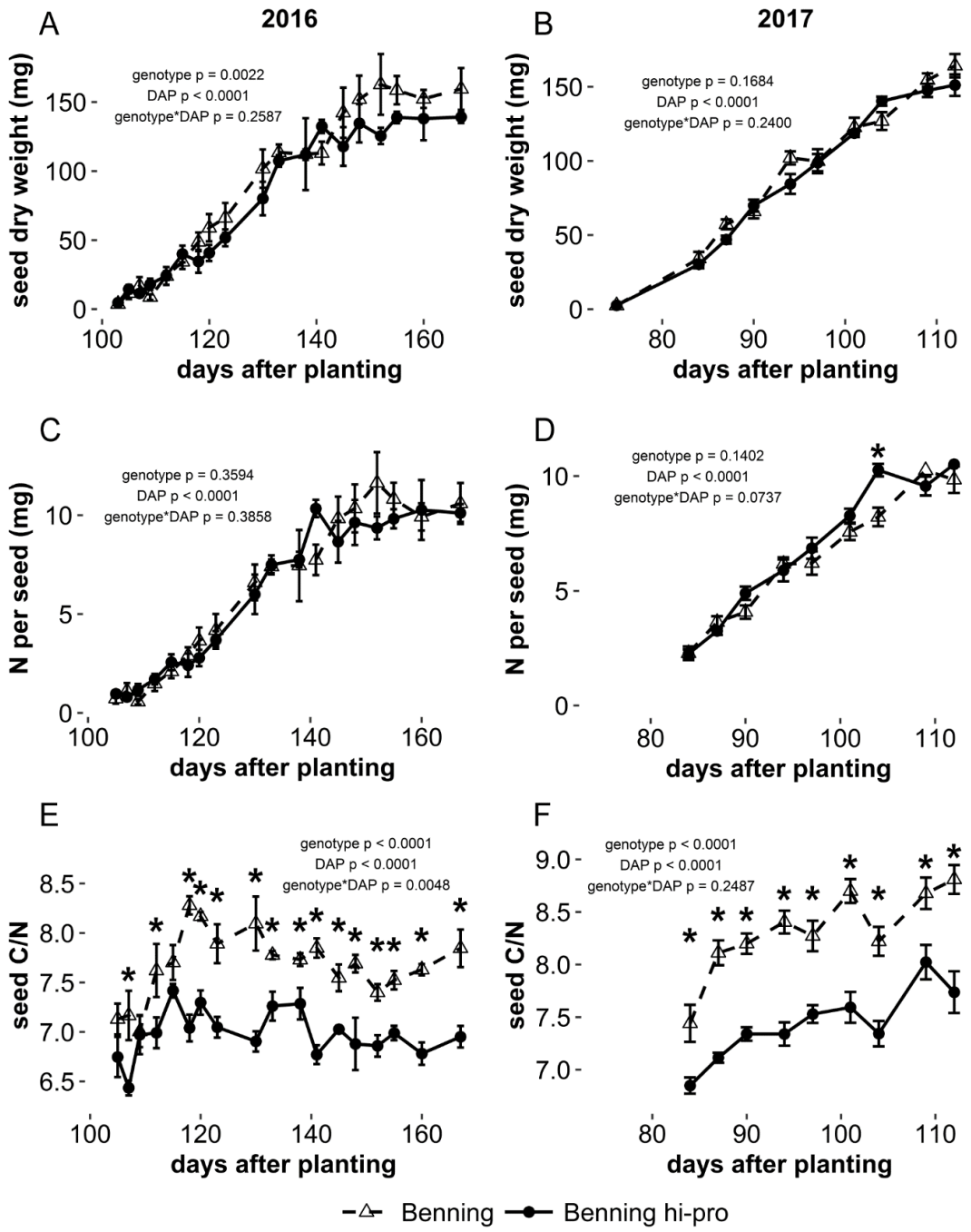
619 in 2017, when Benning and Benning hi-pro were in developmental stage R5. Letters indicate

620 significant pairwise differences (adjusted $p < 0.05$) among genotypes; $n = 4$.

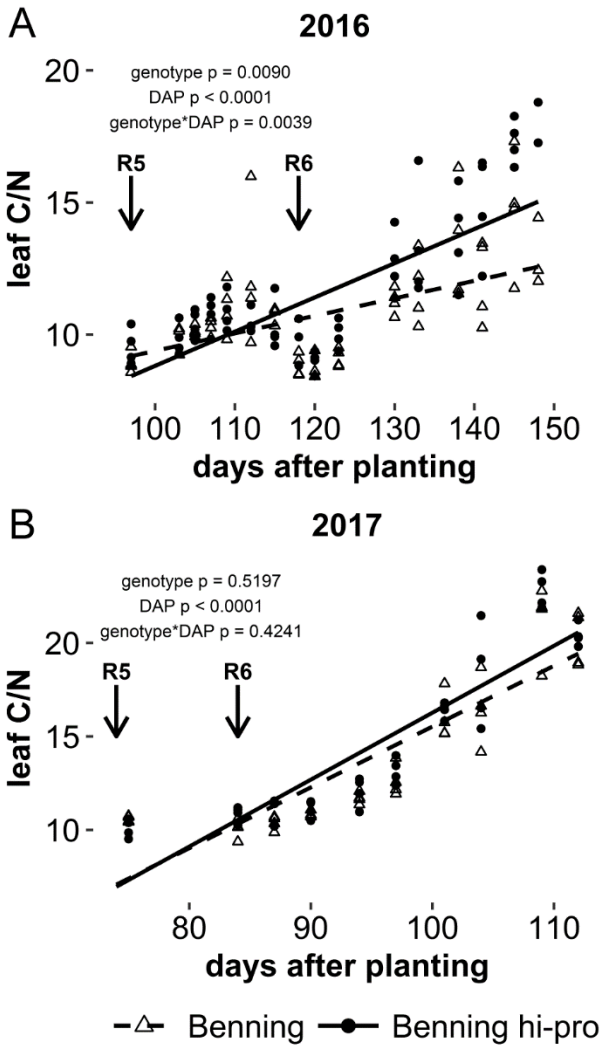
Genotype	**Leaf area index**	**Aboveground biomass**	**Aboveground N**	**Leaf + stem N**	**Reproductive N**
	———	———	g m^{-2}	———	———
Benning	4.484	638.2	19.9	11.2	8.6 **ab**
Benning hi-pro	4.359	595.7	18.2	9.8	8.4 **ab**
NC-Raleigh	4.065	505.9	15.6	9.0	6.6 **a**
Danbaekkong	3.757	617.8	19.7	8.3	11.1 **b**
p-value	0.3835	0.2060	0.2328	0.2304	**0.0108**

621

Figure 3. Individual seed weight, N, and seed C/N during seed fill for Benning and Benning hi-pro grown in the field. Significant pairwise differences (adjusted $p < 0.05$) between the genotypes within days are indicated by asterisks. Points and error bars are means ± standard error.



628 Figure 4. Regression analysis of leaf C/N ratio during R5 and R6. Dashed and solid lines are
629 regressions for the rate of N depletion relative to C in Benning and Benning hi-pro. Arrows
630 indicate the observed start of developmental stages. *P*-values indicate significance of the
631 difference between slopes for the genotypes.



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