Expression Quantitative Trait Loci Analysis of Two Genes Encoding Rubisco Activase in Soybean

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Rubisco activase (RCA) catalyzes the activation of Rubisco in vivo and plays a crucial role in photosynthesis. However, until now, little was known about the molecular genetics of RCA in soybean (Glycine max), one of the most important legume crops. Here, we cloned and characterized two genes encoding the longer α-isoform and the shorter β-isoform of soybean RCA (GmRCAa and GmRCAβ, respectively). The two corresponding cDNAs are divergent in both the translated and 3’ untranslated regions. Analysis of genomic DNA sequences suggested that the corresponding mRNAs are transcripts of two different genes and not the products of a single alternatively splicing pre-mRNA. Two additional possible α-form RCA-encoding genes, GmRCA03 and GmRCA14, and one additional β-form RCA-encoding gene, GmRCA11, were also isolated. To examine the function and modulation of RCA genes in soybean, we determined the expression levels of GmRCAa and GmRCAβ, Rubisco initial activity, photosynthetic rate, and seed yield in 184 soybean recombinant inbred lines. Correlation of gene expression levels with three other traits indicates that RCA genes could play an important role in regulating soybean photosynthetic capacity and seed yield. Expression quantitative trait loci mapping revealed four trans-expression quantitative trait loci for GmRCAa and GmRCAβ. These results could provide a new approach for the modulation of RCA genes to improve photosynthetic rate and plant growth in soybean and other plants.

Photosynthesis is a major target for improving crop productivity, and considerable research has been carried out to select and breed for genotypes with a superior photosynthetic rate (P_{N}) (Sinclair et al., 2004). In higher plants, photosynthesis is usually limited at the step of CO₂ assimilation as catalyzed by Rubisco (Hartman and Harpaz, 1994; Spreitzer and Salvucci, 2002). The activity of Rubisco is regulated by complex mechanisms in vivo. Numerous studies have shown that Rubisco can be maintained in an active state by the continued action of a second protein called Rubisco activase (RCA; Portis, 2003). The activities of RCA are thought to be key regulation points for photosynthesis under different environmental stress conditions (Crafts-Brandner and Salvucci, 2000; Pollock et al., 2003). Plants expressing reduced levels of RCA exhibit decreased levels of P_{N} and / or growth (Mate et al., 1996; Eckardt et al., 1997; He et al., 1997), and those with very low or no RCA expression cannot survive in atmospheric CO₂ (Somerville et al., 1982; Salvucci et al., 1985, 1986; Mate et al., 1993; von Caemmerer et al., 2005). These results make modulation of RCA an attractive experimental goal for the improvement of CO₂ fixation rates and, ultimately, crop productivity.

RCA is an AAA+ (ATPases associated with a variety of cellular activities) protein that functions like a molecular chaperone (Sanchez de Jimenez et al., 1995), catalyzing the activation of Rubisco in vivo by the ATP-dependent removal of various inhibitory sugar phosphates (Portis, 2003). Based on many mutagenesis studies of RCA and/or Rubisco, Portis et al. (2008) described a model for the mechanism of RCA action as follows. First, RCA is bound to Rubisco through electrostatic and other forces, including amino acid regions 89 to 94 on Rubisco and amino acid regions 311 to 314 on RCA. Second, ATP hydrolysis promotes movement of the C-terminal sensor-2 domain (including amino acid region 311–314) of RCA, with the Arg residue in the sensor-2 domain possibly establishing this coupling. Third, due to the interaction established at amino acid positions 89 to 94 and probably elsewhere, the N-terminal domain of Rubisco moves accordingly, which could break the interactions between Glu-60 in the N-terminal domain and probably establish new ones with Glu-60 in the C-terminal domain. These results suggest that RCA could be a potential target for improving crop productivity through genetic modification.
of Rubisco, Lys-334 in loop 6, and the bound sugar phosphate. Finally, loop 6 becomes free to move out of the active site, and the bound sugar phosphate is free to dissociate. In this way, RCA frees the active sites of Rubisco for spontaneous carbamoylation by CO$_2$ and metal binding and activates the Rubisco holoenzyme. Activated Rubisco catalyzes the carboxylation of ribulose 1,5-bisphosphate to form two molecules of 3-phosphoglycerate under ample concentrations of CO$_2$ (Portis et al., 2008).

In most plants studied so far, two forms of RCA (α- and β-isoforms, with molecular masses of 45–46 kD and 41–43 kD, respectively) are present, and they differ only at the C terminus (Salvucci et al., 1987; Portis, 2003). Unlike the β-isoform, the α-isoform holds a C-terminal extension that contains the redox-sensitive Cys residues (Zhang and Portis, 1999; Zhang et al., 2002; Salvucci et al., 2003; Portis et al., 2008). The number of RCA-encoding genes varies depending on the plant species. One RCA gene exists in spinach (Spinacia oleracea), Arabidopsis (Arabidopsis thaliana), rice (Oryza sativa), and wheat (Triticum aestivum; Werneke et al., 1988; To et al., 1999; Law and Crafts-Brandner, 2001); two in barley (Hordeum vulgare), cotton (Gossypium hirsutum), and maize (Zea mays; Rundle and Zielinski, 1991; Salvucci et al., 2003; Ayala-Ochoa et al., 2004); and at least three in tobacco (Nicotiana tabacum; Qian and Rodermel, 1993). In plants such as spinach, Arabidopsis, rice, and barley (rcaA gene), alternative splicing of RCA transcripts results in two isoforms of RCA (Werneke et al., 1989; Rundle and Zielinski, 1991; To et al., 1999). However, other RCA genes are not alternatively spliced. For example, the cotton RCA α- and β-isoforms are encoded by two different genes (Salvucci et al., 2003), and a second RCA gene (rcaL) in barley encodes only the β-isoform of RCA (Rundle and Zielinski, 1991). Interestingly, sequence analysis of RCA cDNAs from maize, tobacco, bean (Phaseolus vulgaris), cucumber (Cucumis sativus), and mung bean (Vigna radiata) suggested that these species might only express the β-isoform RCA (Portis, 2003).

RCA gene expression seems to be tissue specific in all higher plants examined. It occurs almost only in green parts of the plant and is developmentally regulated by leaf age and light (Watillon et al., 1993; Liu et al., 1996). For example, circadian oscillations of RCA mRNA levels have been detected in tomato (Solanum lycopersicum), apple (Malus domestica), Arabidopsis, and rice (Martino-Catt and Ort, 1992; Watillon et al., 1993; Liu et al., 1996; To et al., 1999). Changes in mRNA levels may result from either transcriptional or posttranscriptional regulation or both (Chen and Rajewsky, 2007). A nuclear run-on analysis showed that the rhythmic oscillation is controlled at the transcriptional level in Arabidopsis, in which RCA mRNA synthesis is correlated with RCA mRNA accumulation (Pilgrim and McClung, 1993).

For some years, quantitative trait locus (QTL) analysis has been performed to detect the determinants of important agronomic or physiological traits (Mouille et al., 2006; Cui et al., 2008; Tisne et al., 2008), providing valuable information for gene discovery and crop improvement. Diversity in gene expression is one of the mechanisms underlying phenotypic diversity among individuals. Therefore, analysis of determinants of candidate gene expression not only helps in understanding the mechanisms for phenotypic variation but also provides an approach to improve phenotypes via the modulation of gene expression. With advances in gene expression profiling, an approach named “genetical genomics” has been put forward to identify the determinants of gene expression (Jansen and Nap, 2001). This approach treats mRNA expression levels as quantitative traits in a segregating population and maps expression QTLs (eQTLs) that control expression levels in vivo. For almost any gene analyzed in a segregating population, eQTL analysis can identify the genomic regions influencing its expression level. The genetical genomics approach has been employed for identifying eQTLs regulating gene expression (Potokina et al., 2006; Sladek and Hudson, 2006).

Soybean (Glycine max) is one of the most important legume crops and a typical allotetraploid (Shoemaker et al., 2006). However, to date, the only information concerning RCA in soybean is the existence of two RCA isoforms detected by immunoblotting (Salvucci et al., 1987). In this study, we cloned and characterized two soybean RCA genes, GmRCAα and GmRCAβ, encoding the longer α-isoform and the shorter β-isoform of RCA, respectively. We also isolated three soybean RCA-like genes and analyzed their phylogenetic relationships to GmRCAα and GmRCAβ. Correlation analysis of RCA gene expression level, Rubisco initial activity, PN, and seed yield in a set of soybean recombinant inbred lines (RILs) showed that RCA gene expression levels could affect photosynthetic capacity and plant growth, and eQTL mapping revealed four trans-eQTLs for GmRCAα and GmRCAβ. The existence of multiple family members and different expression patterns of RCA genes, in combination with long-term genome duplication, provide interesting information about soybean evolution. Taken together, these data provide new information for the modulation of RCA genes to improve PN and ultimately seed yield in soybean and other plants.

RESULTS

Characterization of GmRCAα and GmRCAβ cDNAs

The cDNAs of GmRCAα and GmRCAβ contained a complete open reading frame (ORF) and partial 3′ untranslated sequences. The predicted proteins encoded by GmRCAα and GmRCAβ contained 478 and 443 amino acids, with calculated molecular masses of 52.29 and 48.63 kD, respectively (Fig. 1). The first 58
amino acids at the N terminus of both proteins were predicted to be chloroplast transit peptides (chlorop version 1.1 server; http://www.cbs.dtu.dk/services/ChloroP/). Thus, the predicted mature proteins encoded by GmRCAa and GmRCBb contained 420 and 385 amino acids, with calculated molecular masses of 46.76 and 43.14 kD, respectively (Fig. 1). The deduced protein sequence of GmRCAa contained a 36-amino acid extension at the C terminus (Fig. 1, in boldface), including two Cys residues that are known to be involved in redox regulation (Zhang and Portis, 1999; Salvucci et al., 2003). Two conserved ATP-binding domains, GGKGQGKS and LFIND (Shen and Ogren, 1992), were identified at amino acid positions 169 to 176 and 229 to 233, respectively, in soybean RCA; here, numbers correspond to the amino acid positions in the complete GmRCAa protein sequence (Fig. 1). According to studies in spinach and tobacco (Shen et al., 1991; van de Loo and Salvucci, 1998), Lys-175 in the first domain of soybean RCA correlates with Rubisco activation and ATPase activities, and Asp-233 in the second domain of soybean RCA is necessary for the precise coordination of the γ-phosphate and, therefore, for subunit aggregation.

In addition to the 36-amino acid extension at the C terminus of the α-isoform, the two RCA isoforms differ at 41 positions, including eight residues in the putative transit peptides (Fig. 1). Obvious differences in the 3′ untranslated regions of GmRCAa and GmRCBb cDNAs were also observed, although only partial sequences were obtained (Fig. 2). The divergence in both the ORF and 3′ untranslated regions of the cDNA sequences encoding RCA α- and β-isoforms suggests that the two forms of soybean RCA are encoded by different genes.

Analysis of Genomic Fragments of GmRCAa and GmRCBb Genes

Soybean genomic DNA was analyzed to determine whether GmRCAa and GmRCBb mRNAs could arise from one alternatively spliced gene. Amplification of genomic DNA using gene-specific primers (Supplemental Table S1) resulted in PCR fragments of 3,533 bp for GmRCAa and 3,704 bp for GmRCBb. As shown in Figure 3, the exon, intron, and flanking sequences (including the 3′ and 5′ regions) of the GmRCAa genomic DNA were 1,434, 2,057, and 42 bp long, respectively, whereas those of the GmRCBb genomic DNA were 1,329, 2,175, and 200 bp. Alignment analysis (http://www.ncbi.nlm.nih.gov/spidey/) showed that the genomic sequences of GmRCAa and GmRCBb precisely matched their corresponding cDNA ORFs and contained six and five introns, respectively. BLAST analysis against the soybean genome sequence (http://www.phytozome.com/soybean.php) showed that GmRCAa and GmRCBb are located on chromosomes Gm02 and Gm18, respectively. These findings were consistent with the presence of two genes coding for RCA α- and β-isoforms in soybean.

Western-Blot Analysis Detected Two RCA Isoforms

Extracts from soybean and Arabidopsis leaves, Escherichia coli transformed with pET-30a expressing the ORF of GmRCAa or GmRCBb, E. coli transformed with empty pET-30a, and untransformed E. coli were sep-
arated by SDS-PAGE and probed with polyclonal Arabidopsis RCA antibodies. As shown in Figure 4, two polypeptides of approximately 47 and 43 kD were detected in Arabidopsis (lane 1), which is consistent with previous findings (Salvucci et al., 1987). Two polypeptides with similar molecular masses were also detected in soybean (lanes 4 and 5), which is consistent with the observations of Salvucci et al. (1987), but with a slight difference; we detected higher levels of the soybean RCA α-isoform than the β-isoform, whereas Salvucci et al. (1987) detected higher levels of the β isoform. As we used an anti-Arabidopsis RCA antibody and Salvucci et al. (1987) used an anti-spinach RCA antibody, this might reflect the affinity difference between the two antibodies. However, the difference in soybean genotypes or leaf developmental stages between these two studies cannot be excluded.

Figure 2. Partial sequences of translated and 3’ untranslated regions of GmRCAα and GmRCAβ cDNAs. Primer sequences used for real-time RT-PCR are indicated by arrows.

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Figure 3. Intron/exon structures of GmRCAα and GmRCAβ, and the nucleotide sequence of the sixth intron of GmRCAα. Gray boxes, Introns; black boxes, exons; white boxes, flanking sequences. Numbers refer to sequence lengths. The nucleotide sequence above the dotted line is contained in the sixth intron of GmRCAα; the underlined sequence is the ochre stop codon.

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As shown in Figure 4, both the recombinant proteins GmRCAα and GmRCAβ were immunoreactive to the anti-Arabidopsis RCA antibody (lanes 2 and 3), while the controls showed no specific bands (lanes 6 and 7). GmRCAα was approximately 4 kD larger than GmRCAβ. As both proteins contained an additional N-terminal His tag of 5.31 kD from the expression vector pET-30a, their molecular masses were larger than their native RCA α- and β-isoforms in soybean leaves (lanes 4 and 5). These results confirm that GmRCAα and GmRCAβ do encode the soybean RCA α- and β-isoforms, respectively.

Sequence Analysis of Three Additional RCA-Like Genes in Soybean

BLAST analysis of the GmRCAβ ORF sequence against the soybean genome sequence (http://www.phytozome.com/soybean.php) revealed three regions on chromosomes Gm03, Gm11, and Gm14 that may contain additional RCA genes. The corresponding cDNAs, tentatively designated as GmRCA03, GmRCA11, and GmRCA14, were cloned using genespecific primers (Supplemental Table S1) and sequenced. The calculated molecular masses of the predicted proteins encoded by GmRCA11, GmRCA03, and GmRCA14 were 48.67, 52.28, and 52.43 kD, respectively. This implies that GmRCA11 may encode the RCA β-isofrom and that GmRCA03 and GmRCA14 may encode the RCA α-isofrom.

The phylogenetic relationships among GmRCAα, GmRCAβ, GmRCA03, GmRCA11, and GmRCA14 were studied using the sequences encoding the deduced mature polypeptides (Fig. 5). GmRCA03 is the sister gene to GmRCAα, GmRCAβ, GmRCA11, and GmRCA14, which are grouped into another lineage in which GmRCAα and GmRCA14 form one sublineage and GmRCAβ and GmRCA11 form another. The latter relationship, however, is the only one that received strong bootstrap resampling support.

Correlation among Gene Expression, Rubisco Initial Activity, PN, and Seed Yield

Gene-specific primers (Fig. 2; Supplemental Table S1) were used to determine the expression levels of GmRCAα and GmRCAβ. The inherent variation in the expression of housekeeping genes makes the use of a proper endogenous reference gene indispensable for accurate normalization of mRNA samples. For reliable estimation of the relative expression level of a target gene, the amplification efficiency of reverse transcription (RT)-PCR for an endogenous reference gene should be equal to that for the target gene. In this study, the soybean tubulin gene was used as an endogenous reference to examine the sample-to-sample variation in the amount of cDNA (Potokina et al., 2006), since its amplification efficiency was similar to both GmRCAα and GmRCAβ (reflected by the slope of the lines shown in Fig. 6).

Gene expression levels of GmRCAα and GmRCAβ, Rubisco initial activity, PN, and seed yield were measured in a set of RILs derived from soybean cv Nannong1138-2 and cv Kefeng No.1 (Supplemental Table S2). Correlation analysis among these five traits was performed (Table I). The expression level of GmRCAα showed a highly positive correlation with that of GmRCAβ, and the expression levels of both genes correlated positively with Rubisco initial activity, PN, and seed yield. The correlation between the expression of GmRCAβ and the other traits was higher than that of GmRCAα. Rubisco initial activity showed a significantly positive correlation with PN and seed yield, and PN correlated positively with seed yield.

eQTL and QTL Mapping

Expression levels of GmRCAα and GmRCAβ, Rubisco initial activity, PN, and seed yield in the RILs followed a continuous distribution and were consistent with quantitative genetic variation (data not shown). Subsequent QTL analyses were performed on the means of corresponding genetic effects of each RIL (Table II; Fig. 7). We detected 12 eQTLs or QTLs for all traits examined, and each trait was controlled by two to three eQTLs or QTLs dispersed among the chromosomes. Individual eQTLs or QTLs explained 4.24% to 12.58% of the total phenotypic variation (r²) of given traits, and one-third of eQTLs or QTLs had r² exceeding 10%. eQTLs or QTLs with positive and negative allelic effects were observed.

Figure 5. Phylogenetic analysis of mature proteins deduced from GmRCAα, GmRCAβ, GmRCA03, GmRCA11, and GmRCA14 genes. The tree was constructed by the neighbor-joining method using MEGA software (version 4.1). Numbers at branches represent percentage bootstrap support from 1,000 resampling replicates, and the scale bar indicates branch length in terms of substitutions per site.
identified, with a positive effect implying a higher value for the trait conferred by the allele from Kefeng No.1 and vice versa.

**QTLs for Rubisco Initial Activity, \( P_N \), and Seed Yield**

Two QTLs were detected for Rubisco initial activity, on LG-B2 and LG-D2, respectively (Table II; Fig. 7). The QTLs \( qRacB2.1 \) and \( qRacD2.1 \) explained 5.24% and 9.81% of the total phenotypic variance, respectively. Additive effect values indicated that Nannong1138-2 alleles were positive for both QTLs. QTL \( qRacD2.1 \) was possibly colocalized with QTL \( qPND2.1 \), as described below for \( P_N \), due to their overlapping confidence intervals on LG-D2 and their shared direction of additive effect with positive alleles from Nannong1138-2.

Three QTLs were detected for \( P_N \) on LG-C1, LG-D2, and LG-E (Table II; Fig. 7). These QTLs, \( qPNC1.1 \), \( qPND2.1 \), and \( qPNE.1 \), explained 6.97%, 9.35%, and 12.24% of the total phenotypic variance, respectively. Additive effects indicated that Kefeng No.1 alleles were positive for \( qPNE.1 \) but not for \( qPNC1.1 \) and \( qPND2.1 \). None of these QTLs was colocalized with any QTL described below for seed yield.

Three QTLs for seed yield, located on LG-C2, LG-G, and LG-O, were revealed (Table II; Fig. 7). These QTLs, \( qSYC2.1 \), \( qSYG.1 \), and \( qSYO.1 \), explained 8.32%, 4.24%, and 10.61% of the total phenotypic variance, respectively. Additive effects indicated that Kefeng No.1 alleles were positive for \( qSYG.1 \) and \( qSYO.1 \) but not for \( qSYC2.1 \). Of these QTLs, \( qSYC2.1 \) and \( qSYO.1 \) have previously been detected for soybean seed yield (Cui et al., 2008).

**DISCUSSION**

The mRNAs Encoding Soybean RCA- and \( \beta \)-Isoforms Are Transcripts of Separate Genes

In plant species studied so far, RCA proteins are present either as larger \( \alpha \)-isoforms and smaller \( \beta \)-isoforms or only \( \beta \)-isoforms (Portis, 2003). In plants such as Arabidopsis, spinach (Werneke et al., 1989), and rice (To et al., 1999), which have two RCA isoforms, the two forms are encoded by mRNAs produced from alternative splicing of the transcribed pre-mRNA from a single RCA gene. Cotton is so far the only known plant with two RCA isoforms encoded by different genes (Salvucci et al., 2003). In this study, we found that the mRNAs encoding soybean RCA \( \alpha \)- and \( \beta \)-isoforms were also transcribed from two separate genes.

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**Table 1.** Correlation coefficients and significance of correlations among GmRCA\( \alpha \) and GmRCA\( \beta \) expression levels, Rubisco initial activity, \( P_N \), and seed yield in a soybean recombinant inbred line

| Trait                | GmRCA\( \alpha \) Expression | GmRCA\( \beta \) Expression | Rubisco Initial Activity | \( P_N \)   |
|----------------------|-------------------------------|-------------------------------|--------------------------|-------------|
| GmRCA\( \beta \) expression | 0.645**                      |                               |                          |             |
| Rubisco initial activity | 0.266**                      | 0.364**                       |                          |             |
| \( P_N \)           | 0.156*                       | 0.208**                       | 0.178*                   | 0.348**     |
| Seed yield          | 0.176*                       | 0.259**                       | 0.219**                  | 0.348**     |
different RCA genes. This conclusion is supported by the following evidence: (1) the large difference in amino acid composition between GmRCAα and GmRCAβ (Fig. 1) could not be resolved by the current RCA transcript splicing mechanism observed in Arabidopsis, spinach, and rice; (2) although only partial 3′ untranslated regions of cDNAs were cloned and sequenced, GmRCAα and GmRCAβ showed considerable diversity in this region (Fig. 2); (3) the genomic sequences of GmRCAα and GmRCAβ showed only approximately 40% identity; (4) a BLAST survey against the soybean genome sequence revealed different chromosomal positions for GmRCAα and GmRCAβ; and (5) no common eQTL was detected for GmRCAα and GmRCAβ (Table II; Fig. 7).

Although the mRNAs encoding soybean RCA α- and β-isofoms were transcribed from two genes, we could not exclude the possibility that an alternative splicing mechanism for RCA mRNA might exist in soybean. First, the structure of GmRCAα was similar to that of the alternatively spliced RCA gene in other plants. GmRCAα genomic DNA had six junctions between introns and exons (Fig. 3). The sixth junction (counting from the beginning of the ORF) was interrupted by a 266-bp intron that separated the 36-amino acid extension from the rest of the coding region. About 10 bp beyond the splice junction was an ochre stop codon, which if read through in frame would generate a polypeptide of the approximate size of the β-isofom. This phenomenon has been observed in cotton (Salvucci et al., 2003). However, searching the soybean EST data (http://www.ncbi.nlm.nih.gov/) with the sequence covering the splice junction revealed no EST identities, and no cDNAs were amplified using the specific primers designed against the predicted alternatively spliced β-isoform (data not shown). This indicates that the GmRCAα gene transcript has a low probability of being alternatively spliced. Second, other putative α-isoform-encoding RCA genes, such as GmRCA03, might be capable of alternative splicing. Sequencing the N-terminal amino acids of the soybean α- and β-isoforms would be a direct way to understand the mechanism for producing RCA, since this region might differ between the polypeptides encoded by GmRCAβ and GmRCAα or other possible α-isoform-encoding RCA genes (M.E. Salvucci, personal communication).

### Expression Levels of RCA Genes Could Modulate Photosynthetic Capacity and Plant Growth

Much attention has focused on the function of RCA since its discovery. RCA may exert considerable control over photosynthesis (Portis, 2003; Salvucci and Crafts-Brandner, 2004; Kim and Portis, 2005; Kurek et al., 2007; Westphal et al., 2007; Hendrickson et al., 2008) and plant growth (Wu et al., 2006, 2007; Kurek et al., 2007), especially under heat stress. So far, studies investigating the effect of RCA on photosynthesis and/or plant growth have used rca mutants with reduced or increased levels of RCA (Pollock et al., 2003; Westphal et al., 2005; Salvucci et al., 2006; Wu et al., 2006, 2007, Salvucci, 2008). In our study, a new strategy was employed to investigate the effect of RCA on photosynthetic capacity and seed yield in soybean. Rubisco initial activity, PN, seed yield, and gene expression levels were measured in a set of soybean RILs to
examine the function of GmRCA\textsubscript{a} and GmRCA\textsubscript{b}. Like the cotton RCA genes (Salvucci et al., 2003), the soybean GmRCA\textsubscript{a} and GmRCA\textsubscript{b} genes appeared to be functionally equivalent to alternatively spliced RCA genes in other plants.

The significant correlation between the expression of both GmRCA\textsubscript{a} and GmRCA\textsubscript{b} and Rubisco initial activity, PN, and seed yield indicated that these genes could play a role in increasing photosynthetic capacity and seed yield (Table I). However, the correlation coefficients between gene expression and Rubisco initial activity, PN, and seed yield were relatively small (Table I). This was also reflected by the fact that no coincident QTL (eQTL) was found between gene expression levels and the other three traits examined (Table II; Fig. 7). The coincidence of QTLs for two traits, with allelic differences corresponding to the expected relationship between the traits, is strong evidence that the two traits are causally related (Thumma et al., 2001). Thus, we must conclude that factors other than GmRCA\textsubscript{a} and GmRCA\textsubscript{b} limit photosynthetic capacity and seed yield. One possible factor for our inability to detect coincident QTLs may be the nonstressing experimental conditions we used. In experiments with rca mutants under nonstressing conditions, a strong relationship between RCA levels and CO\textsubscript{2} assimilation rates was observed only when RCA levels were reduced to below about 30\% of wild-type levels (Mate et al., 1996; Eckardt et al., 1997; von Caemmerer et al., 2005); however, although at a high relative concentration, RCA correlated closely with Rubisco activity under heat stress (Salvucci et al., 2006; Salvucci, 2008). Another possible interfering factor could be the genetic background of the mapping population used in this study. As Rubisco initial activity, PN, and seed yield are all multifactorial traits, the relationships between them and RCA gene expression are expected to be complex and variable in different plant materials.

Figure 7. Summary of QTL (eQTL) locations detected. Markers are shown on the right of the linkage groups (chromosomes), as shown by Fu et al. (2006). QTLs (eQTLs) represented by bars are shown on the left of the linkage groups, close to their corresponding markers. The lengths of the bars are proportional to the confidence intervals of the corresponding QTLs (eQTLs), as shown in Table II.
eQTL Analysis Provides New Insights into the Modulation of RCA in Vivo

The correlation of a structural gene’s map position and its eQTL provides an indication of its regulation (Potokina et al., 2006). If the position of one gene and its eQTL are congruent, cis-regulation could be inferred, which means that the allelic polymorphism of the gene itself, or closely linked regulatory elements, directly impact the gene’s expression. Such a pattern was observed for the Ser carboxypeptidase I gene, Cxp1, where colocalization of the Cxp1 eQTL and structural gene provided circumstantial evidence that observed differences in gene expression levels are the result of cis-regulation (Potokina et al., 2006). In our study, the eQTLs for two soybean RCA genes do not colocalize with these two genes. A BLAST survey of the soybean genome sequence (http://www.phytozome.com/soybean.php) using the sequences of markers closely linked to the eQTLs showed that the four markers (sat267, satt385, sat_171, and satt648; Table II) linked to $qRbA2.1$ or $qRbI.1$ are located on chromosome Gm08 or Gm20, whereas GmRCAA is on chromosome Gm18. This result suggests that the observed differences in RCA gene expression could be the consequences of trans-regulation, which means that gene expression is mainly regulated by trans-acting factors. A similar phenomenon has been observed for a set of genes involved in the biosynthesis of lignin in Eucalyptus (Kirst et al., 2004). Most of these genes were significantly influenced by two eQTLs on linkage groups 4 and 9, whereas the structural genes were distributed throughout the entire genome.

As we and many others have shown, RCA plays an important role in regulating plant photosynthesis and plant growth. Genetic engineering experiments have been carried out to improve RCA activity (Wu et al., 2006; Kurek et al., 2007). Can breeders modulate RCA gene expression by design? The GmRCAA and GmRCAH eQTLs we identified make it possible to improve RCA gene expression, and ultimately RCA activity and seed yield, by marker-assisted breeding methods such as QTL pyramiding, which is a process of assembling several QTLs for a specific trait from different loci to produce superior genotypes (Xu, 1997). However, our study constitutes only first-order knowledge about the genetic determinism of RCA expression levels in soybean. Considering that RCA activity decreases severely under heat stress (Crafts-Brandner and Salvucci, 2000; Salvucci and Crafts-Brandner, 2004) and that only those QTLs (eQTLs) detected in different materials and under multiple environments are the most valuable ones for breeding, further eQTL mapping of RCA genes in a range of soybean materials under different environments is warranted.

Genome Duplication and Evolution Might Have Led to Multifamily Members and Different Expression Patterns of RCA Genes in Soybean

Polyploidy is a crucial force in plant evolution, and many angiosperms have experienced one or more episodes of polyploidization (Adams and Wendel, 2005). Cotton (Salvucci et al., 2003) and soybean (this study) each have different genes encoding two RCA isoforms. Compared with old polyploid plant species such as rice (To et al., 1999) and Arabidopsis (Wernke et al., 1989), which contain one alternatively spliced RCA gene encoding two RCA isoforms, cotton and soybean experienced additional genome duplications within the past 5 million years (Adams and Wendel, 2005). Did the recent genome duplication result in two separate genes encoding two RCAs? The lack of information on soybean’s diploid progenitors (Shoemaker et al., 2006) makes it difficult to test this hypothesis directly in soybean. However, two separate genes encoding two RCA isoforms exist in cotton diploid species (Salvucci et al., 2003). In addition, Rundle and Zielinski (1991) showed that the alternatively spliced RCA gene (rcaA) in barley evolutionarily preceded the
second one (\textit{rcaB}, encoding the RCA \textit{\-}isoform). The former existed 150 million years ago when monocots and eudicots diverged, and the latter appeared only after this lineage split (Rundle and Zielinski, 1991). Based on this information, we hypothesize that the phenomenon of separate genes encoding two RCA isoforms in soybean might have been formed in an early whole genome duplication event. During this event, the primitive alternatively spliced \textit{RCA} gene might have duplicated, and following the long-term diploidization process (Doyle et al., 2008), the duplicates diverged to encode either the \textit{\alpha} - or \textit{\beta} -isoform of soybean \textit{RCA}. This hypothesis is supported by the argument that the diploid progenitor(s) of soybean underwent an early large-scale genome duplication event (Shoemaker et al., 2006).

A BLAST search against the soybean genome, combined with cDNA cloning, showed that soybean possibly contains five \textit{RCA} genes. Small families of \textit{RCA} genes have also been observed in tobacco (Qian and Rodermel, 1993). Both soybean and tobacco underwent a recent round of whole or segmental genome duplication within the last approximately 5 to 10 million years (Adams and Wendel, 2005). During long-term diploidization, levels of retention for gene duplicates might vary among different plant species; for example, \textit{LYK} gene duplicates are retained more often in legumes than in Arabidopsis or rice (Zhang et al., 2007). Therefore, the numbers of \textit{RCA} genes could also vary among recently polyploidized plant species. To form the current possible five \textit{RCA} genes, we postulate at least two rounds of duplication of \textit{RCA} genes in soybean. This is consistent with the argument that soybean has undergone at least two rounds of whole genome duplication, with disparate time estimates of approximately 14 and 44 million years ago (Schlueter et al., 2004) or approximately 4 and 16 million years ago (Blanc and Wolfe, 2004).

Duplicate genes may retain original gene function, subfunctionalize, neofunctionalize (i.e. obtain a new function), or be silenced (Wendel, 2000; Doyle et al., 2008). In this study, the significant correlation of \textit{GmRCA\textit{aa}} and \textit{GmRCA\textit{B}} expression levels with Rubisco initial activity, \textit{P}_{\text{\textit{N}}}, and seed yield suggests that \textit{\alpha} - and \textit{\beta} -isoforms of \textit{RCA} might play similar roles in soybean (Table I). However, eQTL mapping indicates that these two forms of \textit{RCA} might have differential expression patterns, since the expression levels of \textit{GmRCA\textit{aa}} and \textit{GmRCA\textit{B}} were controlled by different loci on different linkage groups (Table II). These data indicate that soybean \textit{RCA} genes might have undergone subfunctionalization, suggesting that ancestral functions were partitioned among duplicate genes (Doyle et al., 2008).

In this study, we only analyzed the expression levels of \textit{GmRCA\textit{aa}} and \textit{GmRCA\textit{B}}, although as mentioned, there may be at least five \textit{RCA} genes in soybean. Could the other three \textit{RCA} genes also affect photosynthesis and yield? What is the relative contribution of these genes to overall \textit{RCA} function in vivo? Multiple \textit{RCA} genes could result in finer control over protein expression (M.E. Salvucci, personal communication); if so, how does each of these genes respond to stresses? Future studies of the expression of all soybean \textit{RCA} genes under different environmental conditions could address these questions, providing better understanding of the functions and the evolution of \textit{RCA} genes in soybean.

**CONCLUSION**

\textit{GmRCA\textit{aa}} and \textit{GmRCA\textit{B}} encode the longer \textit{\alpha} -isoform and the shorter \textit{\beta} -isoform of soybean \textit{RCA}, respectively. Their mRNAs are transcribed from two separate genes, and their expression levels are controlled by different loci on different linkage groups. The correlation between gene expression levels and Rubisco initial activity, \textit{P}_{\text{\textit{N}}}, and seed yield suggests that \textit{RCA} genes could play an important role in regulating photosynthetic capacity and plant growth. Mapping analysis revealed four eQTLs acting in trans-mode for \textit{GmRCA\textit{aa}} and \textit{GmRCA\textit{B}}, which may be useful in future marker-assisted breeding. The multiple family members and different expression patterns of \textit{RCA} genes might be the consequences of long-term whole genome duplication and duplicate evolution in soybean.

**MATERIALS AND METHODS**

**Plant Material and Plant Growth Conditions**

Soybean (\textit{Glycine max} ‘Kefeng No.1’) was used for gene cloning and western-blot analysis of \textit{RCA}. Plants were field grown under natural conditions at Nanjing Agricultural University. Sowing was carried out on May 28, 2007. Once the third euphylls had expanded, fully expanded leaves were collected and frozen immediately in liquid nitrogen, then stored at –80°C until further use.

A soybean RIL population derived from a cross between Kefeng No.1 and cv. Nannong138-2 was used to determine expression levels of \textit{RCA} genes, \textit{P}_{\text{\textit{N}}}, Rubisco initial activity, and seed yield. This population consists of 184 F7:11 lines derived via single-seed descent at the National Center for Soybean Improvement of China. The planting experiment was conducted under natural conditions at Jiangpu Experimental Station, Nanjing Agricultural University. Genotypes were grown individually in pots containing 3 L of soil in a completely randomized design with six replications (one pot per replication). Nine seeds were sown per pot, and 7 d after emergence, plants were thinned to one per pot. To control environmental effects on phenotypic evaluation, the RIL population was divided into three groups according to their maturity time observed in previous years (data not shown). Each group was sown at different times, so that when trait data were collected, all lines were at a similar growth stage. Sowing was carried out on May 8, 15, and 22, 2007. Nutrition and water were supplied sufficiently throughout the experiment to avoid potential nutrient and drought stresses. At the R6 stage of development, the mature upper third leaves were collected individually from three plants of each RIL in the morning (9:00–11:30 AM) on a sunny day, frozen immediately in liquid nitrogen, and stored at –80°C until further use.

**Isolation of Genomic DNA and Synthesis of cDNA**

Genomic DNA was extracted from young leaflets of soybean using the cetyl-trimethyl-ammonium bromide protocol as described by Weising et al. (1995). Total RNA was isolated from leaves using the RNeasy Plant Mini Kit (Qiagen) and was then treated with 10 units of RNase-free DNase I (TaKaRa).
First-strand cDNA was synthesized in a final volume of 20 μL containing 4 μL of 5% buffer, 1 μg of total RNA, 500 μM oligo(dT)6 primer, 10 units of avian myeloblastosis virus reverse transcriptase (Takara), 1 μL deoxyribonucleo-
tide triphosphates, and 20 units of RNase inhibitor (Promega).

Sequence Retrieval and Primer Design

Soybean RCA genes were identified using an in silico mRNA subtraction strategy. Homologue searches in the soybean EST database (http://www.ncbi.nlm.nih.gov/) and tentative consensus sequences database (http://compbio.dfci.harvard.edu/tgi/) were performed using the Arabidopsis (Arabidopsis thaliana) RCA gene (GenBank accession no. 818558) as a query. RCA-like EST and tentative consensus sequences were downloaded and assembled, and then resulting contigs were predicted. Consequently, two putative cDNAs with complete ORFs encoding RCA-like proteins in soybean were obtained and tentatively designated as GmRCAa (for the sequence with the longer ORF) and GmRCAb (for the sequence with the shorter ORF). After verifying the prediction of GmRCAa and GmRCAb in soybean by gene cloning and recombinant protein expression, we performed another round of homo-
logue searches against the soybean genome sequence using the GmRCAb ORF sequence as a query. This predicted three additional RCA-like genes, design-
ated as GmRCAa3, GmRCAa11, and GmRCAa14, located on chromosomes Gm03, Gm13, and Gm14, respectively. On the basis of sequence information, gene-specific primers (Supplemental Table S1) were designed for PCR ampli-
ification from cDNA and genomic DNA, expression of recombinant RCA protein, and/or real-time quantitative PCR analysis.

Cloning and Sequence Analysis of PCR Products

PCR products obtained from genomic DNA and cDNA prepared from leaves were separated on 1% agarose gels and purified with a gel extraction kit (HuaShun) according to the manufacturer’s protocol. The purified product was cloned into the pGEM-T vector (Promega) and sequenced (Invitrogen). Sequence analysis was performed using DNA MAN software (http://www.
lynnom.com), spidey software (http://www.ncbi.nlm.nih.gov/spidey/), and the ChloroP version 1.1 server (http://www.cbs.dtu.dk/services/ChloroP/). The M, of the predicted protein was calculated using the BioXM program (version 2.6; http://www.bio-soft.net/format/bioxm.html). A phylogenetic tree was constructed using the MEGA program (version 4.1; Kumar et al., 2008).

Expression of Recombinant RCA Proteins

GmRCAa and GmRCAb cDNAs were used as templates for PCR amplifi-
cation. PCR products were digested with BamHI and XhoI restriction enzymes and introduced into the pET-30a expression vector (Novagen). The resulting constructs were introduced into Escherichia coli strain BL21 (DE3) (Novagen). Expression of these two recombinant RCA isoforms was performed as described previously (van de Loo and Salvucci, 1996).

Leaf Protein Extraction

Soybean (Kefeng No.1) or Arabidopsis (ecotype Columbia, C14) leaf samples (40 mg) were ground with a mortar and pestle in liquid nitrogen until pulverized. Two milliliters of extraction buffer (20% [v/v] glycerol, 0.25% [w/v] bovine serum albumin, 1% [v/v] Triton X-100, 50 mM HEPES/ KOH [pH 7.5], 10 mM MgCl2, 1 mM EDTA, 1 mM benzamidine, and 1 mM phenylmethylsulfonyl fluoride) was added immediately. The slurry was centrifuged for 10 min at 12,000g at 4°C. Protein-containing supernatant was used to determine Rubisco initial activity. The same leaf protein extraction procedure was followed for western-blot analysis, except that 1 g of leaf per sample was used.

Western-Blot Analysis of RCA Protein

Protein extracts were subjected to SDS-PAGE using a 12.5% acrylamide resolving gel (Mini Protein II System; Bio-Rad; Flign and Gregerson, 1986). Separated proteins were then transferred to polyvinylidene difluoride membrane, and nonspecific binding of antibodies was blocked with 5% nonfat dried milk in phosphate-buffered saline (pH 7.4) for 2 h at room temperature. Membranes were then incubated overnight at 4°C with polyclonal Arabidop-

sis anti-RCA antibodies (aA-18; sc-15864; Santa Cruz Biotechnology) diluted 1:3,000 in phosphate-buffered saline plus 1% nonfat milk. Immune complexes were detected using rabbit anti-goat IgG (H+L) horseradish peroxidase (BSO3503; Biovert World Technology). The color was developed with a solution containing 3,3’-diaminobenzidine tetrahydrochloride as the peroxidase sub-
strate, and membranes were scanned.

Trait Measurement and Data Collection

Pb of RIL families was measured using the LI-6400 portable photosynthesis system (LI-COR) on upper third leaves at the R6 development stage. Two plants from two replications were submitted for measurement per genotype. All measurements were made in the morning (9:00–11:30) to avoid high afternoon temperatures and high air vapor pressure deficit. The photon flux density within the cuvette was supplemented with a light-emitting diode lighting system set at 1,200 μmol m−2 s−1. Leaf temperature was kept at 25°C to 30°C. The large number of tested materials meant that two sets of LI-6400 portable photosynthesis systems were used.

Gene expression levels were determined using real-time RT-PCR assays using the ABI 7500 system (Applied Biosystems). Each reaction contained 50 ng of first-strand cDNA, 0.5 μL of 10 μmol L−1 gene-specific primers, and 10 μL of real-time PCR SYBR MIX (QPK-201; TOYOBO). Amplification condi-
tions were 95°C for 5 min followed by 40 cycles of 95°C for 15 s and 60°C for 60 s. The soybean endogenous reference gene tubulin was used as a control to test for PCR reaction. Two samples for each genotype were used to determine the relative expression levels of GmRCAa and GmRCAb. The results presented are means of the biological replicates for each genotype.

Rubisco initial activity was determined at 25°C using a rapid, nonradio-
active microplate-based method (Sulpice et al., 2007). Two samples for each genotype, each consisting of 40 mg of leaves from separate plants, were submitted for assay. The results presented are means of samples for each genotype.

Seed yield refers to seed weight per plant. These data were collected at maturity. Seeds of six plants per genotype were hand harvested and dried to constant weight, and seed weight was recorded as mean seed yield per plant.

Statistical Analysis and QTL (eQTL) Mapping

Expression level data of both GmRCAa and GmRCAb, Rubisco initial activity, Pb, and seed yield of the RIL population were analyzed using the SAS system 9.0 (for Windows). The mean values of the above traits for each RIL were calculated by SAS PROC MEANS and used for QTL (eQTL) analysis. Pearson phenotypic correlations among traits were calculated by SAS PROC CORR.

QTL (eQTL) analysis allows the genetic basis of variation of quantitative traits of interest to be dissected. Scoring every individual of a mapping population for the trait of interest and establishing a genetic linkage map for that population are two prerequisites for QTL (eQTL) detection. In this study, the mean values of seed yield, Rubisco initial activity, Pb, and seed yield of the RIL population were used as the calibrator on each RT-PCR plate. Two technical replicates of each reaction were performed, and data were analyzed using the ABI 7500 system version 1.40. Normalized expression for each line was calculated as ΔΔCT = –(ΔCt_target – Ct_tubulin_calibrator) – (ΔCt_target – Ct_tubulin_genotype).

Two samples from two plants of each genotype were used to determine the relative expression levels of GmRCAa and GmRCAb. The results presented are means of the biological replicates for each genotype.

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of 2.0 for declaring a QTL (eQTL) was employed. Low thresholds may not be useful in plant breeding programs but they have been shown to help in understanding relationships among traits (Thumma et al., 2001).

The maximum LOD score along the interval was taken as the position of the QTL (eQTL), and the region in the LOD score within 1 LOD unit of maximum was taken as the confidence interval. Additive effects of QTLs (eQTLs) detected were estimated from composite interval mapping results as the mean effect of replacing both Nanmeng1138-2 alleles at the locus of interest by Kefeng No.1 alleles. Thus, for a QTL (eQTL) to have a positive effect, the Kefeng No.1 allele must increase the trait value. The contribution of each identified QTL (eQTL) to total phenotypic variance (\( r^2 \)) was estimated by variance component analysis. QTL (eQTL) nomenclature was adapted as described previously (Cui et al., 2008): starting with “q,” followed by an abbreviation of the trait name, the name of the linkage group, and the number of QTLs (eQTLs) affecting the trait on the linkage group.

The GenBank accession numbers of the identified soybean RCA genes are GQ917180 (GmRCAa cDNA), GQ917181 (GmRCAa3 cDNA), GQ917182 (GmRCAa4 cDNA), GQ917183 (GmRCAa1 cDNA), GQ917184 (GmRCAb cDNA), GQ996944 (GmRCAa cDNA), and GQ996945 (GmRCAb DNA).

Supplemental Data
The following materials are available in the online version of this article.

Supplemental Table S1. Primer pairs used in this research.

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