Molecular evolution of glutamine synthetase II: Phylogenetic evidence of a non-endosymbiotic gene transfer event early in plant evolution

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Abstract

Background: Glutamine synthetase (GS) is essential for ammonium assimilation and the biosynthesis of glutamine. The three GS gene families (GSI, GSII, and GSIII) are represented in both prokaryotic and eukaryotic organisms. In this study, we examined the evolutionary relationship of GSII from eubacterial and eukaryotic lineages and present robust phylogenetic evidence that GSII was transferred from γ-Proteobacteria (Eubacteria) to the Chloroplastida.

Results: GSII sequences were isolated from four species of green algae (Trebouxiophyceae), and additional green algal (Chlorophyceae and Prasinophytae) and streptophyte (Charales, Desmidiales, Bryophyta, Marchantiophyta, Lycopodiophyta and Tracheophyta) sequences were obtained from public databases. In Bayesian and maximum likelihood analyses, eubacterial (GSIIB) and eukaryotic (GSIIE) GSII sequences formed distinct clades. Both GSIIB and GSIIE were found in chlorophytes and early-diverging streptophytes. The GSIIB enzymes from these groups formed a well-supported sister clade with the γ-Proteobacteria, providing evidence that GSIIB in the Chloroplastida arose by horizontal gene transfer (HGT). Bayesian relaxed molecular clock analyses suggest that GSIIB and GSIIE coexisted for an extended period of time but it is unclear whether the proposed HGT happened prior to or after the divergence of the primary endosymbiotic lineages (the Archaeplastida). However, GSIIE genes have not been identified in glaucophytes or red algae, favoring the hypothesis that GSIIB was gained after the divergence of the primary endosymbiotic lineages. Duplicate copies of the GSIIB gene were present in Chlamydomonas reinhardtii, Volvox carteri f. nagariensis, and Physcomitrella patens. Both GSIIB proteins in C. reinhardtii and V. carteri f. nagariensis had N-terminal transit sequences, indicating they are targeted to the chloroplast or mitochondrion. In contrast, GSIIB proteins of P. patens lacked transit sequences, suggesting a cytosolic function. GSIIB sequences were absent in vascular plants where the duplication of GSIIB replaced the function of GSIIB.

Conclusions: Phylogenetic evidence suggests GSIIB in Chloroplastida evolved by HGT, possibly after the divergence of the primary endosymbiotic lineages. Thus while multiple GS isoenzymes are common among members of the Chloroplastida, the isoenzymes may have evolved via different evolutionary processes. The acquisition of essential enzymes by HGT may provide rapid changes in biochemical capacity and therefore be favored by natural selection.

Background

Glutamine synthetase (GS: E.C. 6.3.1.2) catalyzes the ATP-dependent formation of Gln from Glu and NH₄⁺ and is considered one of the oldest functioning enzymes [1,2]. The GS gene superfamily includes three distinct classes, GSI, GSII and GSIII, each differing in molecular size and number of subunits in the holoenzyme [3,4]. The distribution of the three classes is variable within the three domains of life and instances of multiple GS isoenzymes from different families functioning in the same organism are not uncommon in both euobacteria and eukaryotes [3,5-8]. These observations suggest the gene families arose early and prior to the divergence of the prokaryotes and eukaryotes [9-11].
characterized inlogenetic analyses incorporating the two GSII isoforms sol and chloroplasts within these organisms [21,22]. Phy-

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dicots [19,20].

tion event that preceded the divergence of monocots and
isoenzymes in angiosperms evolved via a gene duplica-
and cytosolic isoenzymes support the hypothesis that the
chloroplast. The isoenzymes are nuclear encoded, and in
vascular plants but not in other lineages within the
supergroup. In general, vascular plants express multiple
GS isoenzymes that are localized to either cytosol or
mitochondrial and eukaryotic lineages. The identification of GSII genes in the plant
symbiont Bradyrhizobium japonicum lead Carlson and
Chelm [12] to hypothesize that the gene evolved via HGT
from vascular plants to bacteria. However, this hypothe-
asis was not supported by subsequent phylogenetic analy-
esis [11,13], which established distinct eukaryotic (GSII_E)
and eubacterial (GSII_B) clades.

The supergroup Archaeplastida [14], consisting of
Glaucophyta, Rhodophyceae and Chloroplastida, harbors
members of GSII gene family that are well characterized
in vascular plants. The isoenzymes are nucleic encoded, and in
most angiosperms a single nuclear gene encodes the
chloroplast isoenzyme, while a small nuclear gene family
encodes multiple cytosolic isoenzymes that are expressed in
tissue-specific and developmentally-regulated patterns
[15-18]. Previous phylogenetic analyses of chloroplast
and cytosolic isoenzymes support the hypothesis that the
isoenzymes in angiosperms evolved via a gene duplica-
tion event that preceded the divergence of monocots and
dicots [19,20].

Biochemical studies of green algae provided the first
evidence that, as observed in vascular plants, multiple
GSII isoenzymes are expressed and localized to the cyto-
sol and chloroplasts within these organisms [21,22]. Phy-
genetic analyses incorporating the two GSII isoforms
classified in Chlamydomonas reinhardtii [23] uncov-
ered an unusual disparity between the two enzymes [20].
The cytosolic GSII sequence clustered with the vascular
plants while the plastid sequence branched more basally
and appeared to associate with the eubacterial sequences.

Here we examined the evolutionary relationship of the
GSII gene family and use increased taxonomic sampling
in Chloroplastida to determine if the basally branching,
eubacterial-like GSII_B was broadly distributed. GSII sequences were obtained from four members of the
Trebouxioiphyceae (Chlorophyta) by PCR amplification
using degenerate and gene specific primers. Additional
GSII sequences for members of the green algae (Chloro-
phyta and Prasinophytae) and streptophytes (Mesostigma, Charales, Desmidiales, Bryophyta, March-
antiophyta, Lycophodiophyta and Tracheophyta) were
obtained from publicly available databases, including
genome and EST projects. We also increased taxonomic
sampling within Eubacteria (to date, GSII genes have not
been reported from Archaea). GSII_E and GSII_B sequences
were identified in members of the green algae and early-
diverging streptophytes. Phylogenetic analyses provide
support for the hypothesis that GSII_E was gained in the
Chloroplastida from the Eubacteria via a HGT event after
the divergence of primary photosynthetic groups.

Results and Discussion

Amplification of GSII genes

Complete GSII mRNA sequences were obtained from
Pseudochlorella sp. CCAP211/1A, Chlorella luteoviridis,
Auxenochlorella protothecoides, and Prototheca zopfii. A
GSII sequence was also obtained for Pseudochlorella sp.
CCAP211/1A that included 912 bp of the ORF and all of
the 3’UTR. GenBank accession numbers and characteristics
of the transcripts obtained in this study are summa-
rized in Table 1.

Eukaryotic GSII phylogeny

Phylogenetic analyses of GSII amino acid sequences
resulted in a well-resolved tree. Assuming the root of the
tree lies outside the major eukaryotic clade, there was a
clear separation of the eukaryotic (GSII_E) and eubacterial
(GSII_B) enzymes (Figure 1). Within the eukaryote clade,
the opisthokonts (fungi+animals) and photosynthetic
eukaryotes formed separate groups (Figures 1 and 2). The
GSII_E proteins from streptophytes, chlorophytes, rhodo-
phytes, and chromalveolates formed distinct clades, each
with strong to moderate support. The position of the heter-
okont sequences within this clade is consistent with
previous analyses that provide evidence that GSII_E in heter-
okonts arose via endosymbiotic gene transfer [11]. Sequences from Chlorophyta (green algae, including rep-
resentatives of the Chlorophyceae and Trebouxiophy-
ceae) diverged from a basal node within the
photosynthetic clade and the Chloroplastida (eukaryotes
with chlorophylls a and b) were not monophyletic. How-
ever, the deeper nodes within the photosynthetic eukary-
otic clade were not well supported and thus the
branching pattern within the clade is unresolved (Figure
2). The streptophyte GSII_E sequences formed two major
groupings; one group contained protein sequences tar-
gested to the chloroplast of angiosperms and the other
contained protein sequences of non-vascular and vascu-
lar plants that are targeted to the cytosol. Multiple GSII_E
genes were also observed in the gymnosperms (Pinus
spp.) but to date, these appear to function in the cytosol
and evidence of plastid targeted isoenzymes is lacking
[24].
Evidence for the HGT of GSII

The GSII clade comprised sequences from eubacteria and some members of the Chloroplastida (green algae, liverworts, and mosses; Figures 1 and 3). The Chloroplastida sequences formed a single clade nested within the eubacterial sequences and branching within the clade was similar to predicted organismal phylogenies [25].

GSII sequences are not broadly represented among eubacteria but were identified in members of the Bacteriodes/Flavobacteria/Cytophaga, Planctomycetes, Verrucomicrobia, Actinobacteria, and the α- and γ-Proteobacteria (Figure 3; Additional files 1 and 2). The Chloroplastida GSII was sister to γ-Proteobacteria with strong (Bayesian posterior probability = 1.0) to moderate support (likelihood bootstrap support = 70%). The γ-Proteobacteria + Chloroplastida GSII clade was sister to the Actinobacteria, but this association was not strongly supported. The α-Proteobacteria GSII sequences were not related to the γ-Proteobacteria + Chloroplastida GSII clade, which makes the possibility of GSII gain via mitochondrial endosymbiosis unlikely. The α-Proteobacteria GSII were nested within the Verrucomicrobia and thus, we cannot exclude the possibility of an HGT event within the α-Proteobacteria lineage that obscures the mitochondrial origin of the GSII gene in the Chloroplastida. However, the lack of detection of GSII in genomes of other eukaryotic lineages reduces the likelihood of a mitochondrial origin. In addition, EST and genome analyses of other photosynthetic eukaryotes (Glaucophyta, Rhodophyceae and Chromalveolates) and extant cyanobacteria [26,27], have not uncovered GSII sequences, reducing the possibility that GSII was acquired via plastid endosymbiosis. Thus, we propose that GSII in the Chloroplastida arose via a HGT from γ-Proteobacteria early in plant evolution.

GSII sequences are not broadly distributed among eubacterial lineages and to date, within γ-Proteobacteria, only the genera represented in our analyses have annotated GSII sequences deposited in GenBank. Assuming the GS superfamly evolved prior to the divergence of the three domains of life [9-11], the distribution of GSII sequences suggests the gene has been lost in several lineages of Eubacteria and the Archaea. The analysis of GSII may become more robust as additional eubacterial GSII become available through genome sequencing projects. However, gene loss may make the identification of the true donor of GSII to the Chloroplastida difficult.

An alternative explanation for the limited distribution of GSII among the eubacteria is that the gene was transferred to the eubacteria from an eukaryotic donor. The possibility of an HGT from Chloroplastida to the γ-Proteobacteria is not supported by our phylogenetic analyses as it implies that the eubacterial sequences would nest within the GSII clade; which has not been observed in our phylogenetic analyses. Eukaryote to eubacterial HGT might be supported if GSII were found in diverse lineages of eukaryotes. Further investigation of GSII diversity in the eukaryotic lineages not represented in our study (e.g., Rhizaria, Excavata and Amoebozoa) will contribute to our understanding of the distribution and evolution of GSII. Given the data at hand, however, the hypothesis that GSII arose in the Chloroplastida via HGT remains the most parsimonious.

Table 1: Summary of the GSII sequences characterized in the present study

| Taxa | Sequences Obtained | Accession Number | Length (bp) | ORF (bp) | Amino Acids | ORF 5' UTR | 3' UTR |
|------|--------------------|------------------|-------------|----------|-------------|------------|--------|
| GSIIE Sequences | | | | | | | |
| Pseudochlorella sp. CCAP211/1A (2) | Four complete sequences of GSIIE | GQ465769 | 1486 | 1137 | 378 | 63.32 | 53.19 | 55.12 |
| Chlorella luteoviridis UTEX 28 | | GQ465770 | 1675 | 1146 | 381 | 56.20 | 46.27 | 46.17 |
| Auxenochlorella protothecoides | | GQ465771 | 1621 | 1161 | 386 | 67.96 | 58.18 | 67.28 |
| Prototheca zopfii ATCC16527 | | GQ465772 | 1632 | 1158 | 385 | 69.26 | 71.54 | 72.42 |

GSIIB Sequences

| Pseudochlorella sp. CCAP211/1A (1) | One complete sequence of GSIIB | GQ491030 | 1266 | 912 | 303 | 56.47 | n.d. | 47.93 |

Four complete sequences of GSIIE and a portion of one GSIIB were obtained from cDNA for the species listed above. NCBI (GenBank) accession numbers are given. Characteristics of the sequences in terms of nucleotide length (Length), size of open reading frame (ORF), and the length of the predicted amino acid sequences (Amino Acids) are presented. The % GC content of open reading frame (ORF) and the 5'and 3' untranslated regions (UTR) of each transcript are presented.
Figure 1 Evolutionary relationships among GSII enzymes from prokaryotes and eukaryotes. The phylogenetic analyses were based on 333 amino acid characters from 196 taxa. The 50% majority-rule consensus tree from the Bayesian analyses (48,49) is shown as inferred from 20,002 trees as described in the Methods. Nodes with BBP support > 0.95 are represented by thick lines. RAxML (50,51) bootstrap values are indicated for nodes recovered in both analyses. RAxML values are not indicated for terminal bifurcations. Eubacterial GSII E were used as the outgroup and considered monophyletic. The area of the triangles representing collapsed clades is not proportional to the number of taxa within the clade.
Figure 2 Evolutionary relationship of GSIIE genes from eukaryotes, terminal taxa expanded. Phylogenetic analyses are as described in figure 1. Nodes with BBP support > 0.95 are represented by thick lines. RAxML bootstrap values are indicated for major nodes. RAxML values are not indicated for terminal bifurcations. Sequences characterized in the present study are shown in bold.
Estimating the timing of the HGT

To estimate the relative and absolute timing of the HGT of GSII B, we used Bayesian relaxed molecular clock analyses [28]. Both the uncalibrated (Figure 4) and calibrated analyses (Additional file 3) show an overlap of the 95% highest density posterior node ranges of the origin of GSII B in the early-diverging Chloroplastida coinciding with the GSII E divergence in the opisthokonts and in the primary photosynthetic eukaryotes (Archaeplastida).

Our analyses indicate that GSII B and GSII E may have coexisted for an extended period of time and under this scenario, the putative timing of the HGT event from eubacteria to eukaryotes could be placed either prior to or after the divergence of the primary photosynthetic lineages. At present, there is no evidence of GSII B in genomes of red algae (Cyanidioschyzon merolae [29] and Galdieria sulphuraria [30,31]), or the glaucophyte Cyanophora paradoxa. We acknowledge that taxon sampling is not extensive within these two lineages and hence cannot exclude the possibility of the existence GSII B in these groups. However, given these limited data it is most parsimonious to assume that GSII B was acquired only by the Chloroplastida, early after the divergence from the Glaucophyta and Rhodophyceae (red algae).

The distribution of GSII B within Chloroplastida covers the major lineages of Chlorophyta (Chlorophyceae, Trebouxiophyceae, and Prasinophyceae; Additional file 1). In addition, a partial GSII B sequence was identified in a member of the Ulvophyceae (Acetabularia acetabulum; Additional files 2 and 4). Within Streptophyta, GSII B genes are present in Mesostigmatophyceae (Mesostigma viride; Additional files 2 and 4), Zygnemophyceae
GSII functions in the cytosol. It appears that the GSII B enzymes function in either the phytes, which is one of the oldest vascular plant lineages marked by the divergence of bryophytes and lycopodiophytes, or is one of the oldest vascular plant lineages [32].

**Functional localization and GSII B gene duplication**

The Chloroplastida lineages that contain the GSII B gene also have a GSII E counterpart, which attaches to a basal node within the photosynthetic eukaryotes (Figure 1). Both the GSII B and GSII E genes are nuclear encoded and thus we identified the cellular location of each of the gene products based on the presence (organelle-localized) or absence (cytosol-localized) of N-terminal transit peptides using TargetP ver. 1.1 ([33], see Additional file 5). None of the early-diverging Chloroplastida GSII E enzymes contained transit peptides. In contrast, chloroplast transit sequences were identified in the GSII B protein sequences from Chlorella sp. NC64A, C. vulgaris and the streptophyte, Closterium peracerosum-strigosum-littorale ( Zygnemophyceae) but not in the moss (P. patens) or liverwort (M. polymorpha). Mitochondrial-targeting transit peptides were predicted in GSII B sequences from C. reinhardtii, Volvox carteri f. nagariensis and Scenedesmus obliquus (see Additional file 5). Previous work indicated that chloroplast transit sequences from C. reinhardtii shared features with both mitochondrial and higher plant chloroplast pre-sequences [34] and thus the prediction of a mitochondrial location of GSII B may not reflect its true functional localization. Alternatively, GSII B may be targeted to both the mitochondria and chloroplast, similar to what is observed for GSII E in leaves of some vascular plants [35,36]. While experimental evidence is required to confirm the cellular localization of the GSII B, it appears that the GSII B enzymes function in either the chloroplast or mitochondrion in the chlorophytes and early-diverging streptophytes (Closterium sp.) and that GSII B functions in the cytosol.

The GSII B gene is duplicated in C. reinhardtii, V. carteri f nagariensis and P. patens. The duplicated copies of GSII B in C. reinhardtii and V. carteri f nagariensis were nearly identical (90% and 95% identical, respectively) and present in the genome in a head-to-head orientation. Similarly, the GSII B genes in P. patens were 98% identical but do not appear to be in close genomic proximity.

Within our phylogenetic analyses (Figure 3), the duplicated GSII B of C. reinhardtii, V. carteri f. nagariensis and P. patens each formed separate clades, suggesting the genes evolved by independent duplication events. Alternatively, the GSII B genes in C. reinhardtii and V. carteri may have evolved via an early duplication within the Chlamydomonadales with subsequent gene conversion following the divergence of these lineages. The GSII B are differentially expressed in C. reinhardtii suggesting the need for maintenance of both the copies in the organism [37].

**GSII B loss and replacement of function**

In contrast to the expression of GSII E and GSII B genes in the early-diverging Chloroplastida, the chloroplast- and cytosolic-localized GSII enzymes in angiosperms are both members of the GSII B family and form two distinct clades in our phylogenetic analyses (Figures 1 and 2). As predicted in earlier studies [19], the genes encoding these enzymes arose via a recent gene duplication event with further expansion in the number of genes encoding cytosolic isoenzymes in several plant lineages (Figure 2, [38,39]). Since GSII B is absent from vascular plants, it appears that the chloroplast function of GSII B has been replaced by a gene duplication event in higher plants allowing for subsequent loss of the gene from this lineage. There is also an expansion of the GSII B gene family in gymnosperms (Figure 2), but the enzymes are all localized to the cytosol and the plastid targeted isoform appears to have been lost from this group. The expansion of the GSII B gene family coincides with the development of vascularization of land plants and maybe correlated with the partitioning of nitrogen assimilation between below and above ground tissue (see Additional file 3).

**Conclusions**

We have provided evidence of an ancient HGT event involving the gene for an essential enzyme, GSII. GSII has been well characterized at the molecular level in angiosperms but has been largely overlooked in the early-diverging plant lineages, which were addressed in the present study. Although recent comparative genomic analyses failed to identify bacterial genes in Chlamydomonas reinhardtii [40], our discovery of a eubacterial-like GSII in the chlorophytes and early-diverging streptophytes suggests that further exploration within these lineages is merited. The branching pattern within the monophyletic assemblage of the chlorophytes and early-diverging streptophytes is similar to other molecular and organismal phylogenies, suggesting the occurrence of a single HGT event. As a result, GSII B may be useful in...
Figure 4 Maximum clade probability chronogram from the BEAST analysis of the GSIIB and GSIIE amino acid sequence alignment. All lineages were allowed to evolve according to a relaxed molecular clock and WAG + Inv + Gamma model. Bars on nodes indicate the width of the 95% highest posterior density for each divergence time.
resolving taxonomic associations within and among green algal and early-diverging streptophyte lineages.

Several genes of bacterial origin have been identified in *Dictyostelium discoideum* and are thought to be advantageous to organisms living in soil [40]. More recently, Richards et al. [41] identified five genes in plants that appear to be of fungal origin and argue that two may have been advantageous for organisms colonizing a terrestrial environment. We propose that the acquisition of enzymes by HGT results in a more rapid change in enzymatic capacity or kinetic diversity than evolution of isoenzymes by gene duplication and subsequent specialization. Biochemical studies have suggested that GSII$_B$ has a lower affinity for NH$_4^+$ and Glu than GSII$_E$ [42], characteristics that would be advantageous for enzymes assimilating higher concentrations of NH$_4^+$ from environmental sources, NO$_3^-$ assimilation, or increased rates of photorespiration. Increased taxon sampling and an enlarged fossil age constraint dataset will allow for a more detailed examination of the timing of GSII gains and losses over geological history and coupled with major transitions in plant evolution.

**Methods**

**Algal cultures and sequencing**

Four members in the class Trebouxiophyceae were selected for GSII gene amplification. Cultures of *Pseudochlorella* sp. CCAP211/1A, *Chlorella luteoviridis*, and *Auxenochlorella protothecoides* were a gift from Dr. Peggy Winter (University of West Florida), and *Prototheca zopfii* was a gift from Dr. Drion Boucias (University of Florida). Cultures were grown axenically in ATCC medium 847, (*Pseudochlorella* sp. CCAP211/1A, *C. luteoviridis* and *A. protothecoides*) and in ATCC medium 28: Emmons' modification of Sabouraud's agar (*P. zopfii*). Cultures of *Chlorella* sp. CCAP211/1A, *Prototheca zopfii* was a gift from Dr. Drion Boucias (University of Florida). Cultures were grown axenically in ATCC medium 847, (*Pseudochlorella* sp. CCAP211/1A, *C. luteoviridis* and *A. protothecoides*) and in ATCC medium 28: Emmons' modification of Sabouraud's agar (*P. zopfii*).

All sequences were translated into amino acids in silico. Contigs were assembled using CodonCode Aligner (CodonCode Corporation, Dedham, MA). All sequences were translated into amino acids in silico.

**Phylogenetic analyses**

GSII sequences were retrieved from public databases as well as genome and EST projects using the GSII sequence from the diatom *Skeletonema costatum* (AAC77446) as query, or glutamine synthetase as a keyword. Subsequent queries with eubacterial GSII sequences did not retrieve any additional sequences. Complete information on taxa, database sources and accession numbers is provided in
Additional file 1. The initial alignment of amino acid sequences was done with the web based program CLUSTAL W, using default parameters [45], followed by manual adjustment using BioEdit Sequence Alignment Editor [46] and MacClade 4.08 [47]. The N- and C terminal ends of the proteins along with highly variable regions within the alignments were excluded in the phylogenetic analyses.

The final GSII alignment consisted of 196 taxa and 333 characters for Bayesian analysis. Trees were inferred by calculating Bayesian posterior probabilities using MrBayes 3.1.2 [48,49]. Two parallel runs, each with four chains (three heated and one cold) were run for 10^6 generations. The evolutionary models implemented in MrBayes3.1.2 were explored using a gamma distribution with proportion of invariable sites estimated from the data. Trees were sampled every 100 generations. Likelihood tree scores of two independent runs were plotted to estimate the point of convergence to a stable likelihood, and to determine the trees to be excluded via “burnin.” Bayesian posterior probabilities of the branches were calculated from trees from both the runs, totaling 20,002 trees. Trees remaining (10,000) after a burnin of 5001 for each run were used to compute a 50% majority-rule consensus.

Maximum likelihood (ML) based inference of the phylogenetic trees was done using the software RAxML 7.0.4 [50,51]. The analysis used a random starting tree and the rapid hill-climbing algorithm (i.e., option -f d in RAxML) and the WAG model of amino acid substitution were used. A random seed number was used to turn on rapid bootstrapping (-x) and 1000 bootstrap trees were generated by invoking -# 1000 and -x options in RAxML. A majority rule consensus tree was created in PAUP* 4.0 b [52]. The phylogenetic trees in figures 1, 2 &3 are the 50% majority rule consensus trees from the Bayesian analyses on which the RAxML bootstrap values have been indicated. The eubacterial GSII_E sequences were used as the monophyletic outgroup in the graphical representation of the phylogenies.

Prediction of functional localization of GSII_E and GSII_E protein sequences in early-diverging Chloroplastida
We used the web-based programs TargetP 1.1[33] and ChloroP 1.1 [53] to identify N-terminal translocation peptides in GSII_E and GSII_E proteins (see Additional file 5).

Estimation of divergence times
We estimated the divergence times using Bayesian approach implemented in BEAST 1.4.8 [28]. We did an un-calibrated and calibrated run. A relaxed molecular clock model of uncorrelated log normal distribution was used. For the un-calibrated analysis, a starting tree generated by RAxML 7.0.4 [50] was used as the input tree with the GS amino acid sequence alignment. For the calibrated analysis we set uniform priors on tmrca parameter. Fossil dates were used as minimum dates and were, as follows, Ascomycota, 400 MYA [54], Bilateria, 550 MYA [55] and streptophytes 475 MYA [56]. Secondary age constraints based on published estimates of divergence times were not used. We used the following models, WAG + Inv + Gamma with priors, birth death speciation on the tree. Markov Chain Monte Carlo was set to default 10 million with sampling at every 1000 generation, resulting in 10,000 trees. Convergence was assessed in Tracer v 1.4 [57] and the first three million samples were excluded as burnin. A maximum clade credibility tree was generated by analyzing the BEAST tree file in TreeAnnotator 1.4.6 [58]. This program determined the 95% highest posterior densities and estimated the node heights as mean heights.

| Table 2: Primers used for amplification of GSII genes from green algae |
|--------------------------|--------------------------|--------------------------|
| Gene    | Primer name | Direction | Sequence            |
| GSII_E | MossGS2-1F   | Forward    | 5’-TGGGTGTGATGTTGANGARGG-3’ |
|         | MossGS2-2R   | Reverse    | 5’-ATNCCGAAMTCTTCNCC-3’  |
|         | Green UNI 1-F | Forward    | 5’-CCRAITGGWSITTYGAYGGG-3’ |
|         | cpGSII(QGPFY)-R | Reverse    | 5’-CCRCARTARAAGGGICCYTGIGG-3’ |
| GSII_E | GALG GS F    | Forward    | 5’-TGC CCA TCC CCA ACA C - 3’ |
|         | GALG GS R    | Reverse    | 5’-TCT CGT GCT TCG TCG TCA GG - 3’ |
|         | GS2ChloroF   | Forward    | 5’-CCGG CTG CGA GCA GGA GTA CAC - 3’ |
|         | GS2ChloroR   | Reverse    | 5’-CCG AYC TGG WAC TCC CAC TGG - 3’ |

Sequences of degenerate primers are presented using IUBMB single letter codes. I represents inosine.
Acknowledgements
We thank Jenna Nguyen and Jacqueline Mitchell for their assistance with this project and David Hibbett for helpful discussions regarding the phylogenetic analyses. We also thank two anonymous reviewers and the editor for their constructive comments and recommendations. This research was supported by an NSF CAREER Award (IBN 0238426) to DLR.

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Abbreviations
GS: Glutamine synthetase; HGT: Horizontal gene transfer; BPP: Bayesian posterior probability;

Authors’ contributions
SG participated in the cloning and sequencing of GSII genes and assisted in writing the manuscript. MB participated in the execution and interpretation of the relaxed molecular clock analyses and assisted in the writing of the manuscript. AT was responsible for the cloning and sequencing of GSII genes and assisted in writing the manuscript. DLR conceived of the study, coordinated the research, and helped draft the manuscript. All authors read and approved the final manuscript.

Additional material
Additional file 1 GSII protein sequences used in the present study
GenBank accession numbers and JGI DOE scaffold and protein ID information for the GSII proteins are provided.

Acknowledgements
We thank Jenna Nguyen and Jacqueline Mitchell for their assistance with this project and David Hibbett for helpful discussions regarding the phylogenetic analyses. We also thank two anonymous reviewers and the editor for their constructive comments and recommendations. This research was supported by an NSF CAREER Award (IBN 0238426) to DLR.

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Received: 23 December 2009 Accepted: 25 June 2010
Published: 25 June 2010
