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QQS orphan gene regulates carbon and nitrogen partitioning across species via NF-YC interactions

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The allocation of carbon and nitrogen resources to the synthesis of plant proteins, carbohydrates, and lipids is complex and under the control of many genes; much remains to be understood about this process. QQS (Qua-Quine Starch; At3g30720), an orphan gene unique to Arabidopsis thaliana, regulates metabolic processes affecting carbon and nitrogen partitioning among proteins and carbohydrates, modulating leaf and seed composition in Arabidopsis and soybean. Here we examine the universality of QQS function in modulating carbon and nitrogen allocation by examining a series of transgenic experiments. We show that ectopic expression of QQS increases soybean protein independent of the genetic background and original protein content of the cultivar. Furthermore, transgenic QQS expression increases the protein content of maize, a C4 species (a species that uses 4-carbon photosynthesis), and rice, a protein-poor agronomic crop, both highly divergent from Arabidopsis. We determine that QQS protein binds to the transcriptional regulator AtNF-YC4 (Arabidopsis nuclear factor Y, subunit C4). Overexpression of AtNF-YC4 in Arabidopsis mimics the QQS-overexpression phenotype, increasing protein and decreasing starch levels. NF-YC4, a component of the NF-Y complex, is conserved across eukaryotes. The NF-YC4 homologs of soybean, rice, and maize also bind to QQS, which provides an explanation of how QQS can act in species where it does not occur endogenously. These findings are, to our knowledge, the first insight into the mechanism of action of QQS in modulating carbon and nitrogen allocation across species. They have major implications for the emergence and function of orphan genes, and identify a nontransgenic strategy for modulating protein levels in crop species, a trait of great agronomic significance.

Expression of the QQS transgene in the soybean cultivar Williams 82 causes a similar shift in composition, increasing protein and decreasing carbohydrate in leaf and seed, even though this organism does not have a polypeptide recognizable as QQS by sequence comparisons (3, 10). Protein gels show no visually detectable differences in accumulation of a particular polypeptide and no changes in the ratios of any of the major amino acids of proteins, indicating that, in soybean seeds, this increase in total protein is not due to a specific increase in storage proteins (3). The ability of QQS to affect soybean composition led us to investigate whether QQS interacts with a molecule or process in Arabidopsis that also exists in other plant species.

Here we report that the Arabidopsis orphan gene QQS increases protein content in multiple soybean lines with different protein levels, and also in rice and maize, two major agronomic crops that are highly divergent from Arabidopsis and are not high protein producers. Furthermore, we identify the protein NF-YC4 (nuclear factor Y, subunit C4) as a QQS-interacting protein, and provide direct evidence that NF-YC4 mediates plant composition. This understanding of how QQS functions uncovers a new node in the network that determines plant partitioning of precious carbon and nitrogen resources, and informs the current view on the evolutionary significance of newly formed (orphan) genes.

Results and Discussion

QQS Functions Across Varieties of Soybean with High or Low Protein Content. We determined more broadly the effect of ectopic expression of QQS in plants. First, we chose to evaluate whether QQS acts only in Williams 82, a single variety of soybean with a

Significance

Each species contains a subset of genes that are uniquely present in that species; the functions and origins of the vast majority of these “orphan genes” are not well-understood. Expression of the Arabidopsis QQS (Qua-Quine Starch; At3g30720) orphan transgene increases the level of protein in soybean lines with high and low protein and acts across flowering plants to increase the protein content of maize and rice. Our results begin to dissect the mechanism of QQS functions by identifying that it binds to the conserved transcription factor nuclear factor Y, subunit C4 (NF-YC4). Increased expression of NF-YC4 in Arabidopsis mimics the effects of increased expression of QQS. The ability to optimize protein productivity in plant-based foods would have far-reaching impacts on world health and sustainability.

Author contributions: L.L. and E.S.W. designed research; L.L., W.Z., Y.Z., H.Y., B.T., Z.W.A., D.J., R.L., D.O., and M.P.S. performed research; L.L., W.Z., X.Z., C.D., D.N., M.G.S.-F., Y.Y., and E.S.W. analyzed data; and L.L. and E.S.W. wrote the paper.

The authors declare no conflict of interest.

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moderate protein content, or whether the effect of *QQS* is more general. To do this, we introduced the *QQS* transgene into five elite soybean lines with varied seed protein contents by crossing *QQS*-expressing (*QQS-E*) Williams 82 soybeans (as the pollen donor) with each of these lines, selecting *QQS*-containing plants, and allowing them to self-pollinate. The self-pollinated offspring of the F2 (second filial) and F3 generations of the crosses containing the *QQS* transgene were similar to their respective segregating siblings in morphology, development, flowering time, seed shape and size, and seed weight per plant (Fig. 1A and Fig. S1B). However, *QQS* transgene expression increased seed protein content in each elite soybean line, regardless of the initial protein level in that line (Fig. 1B and Fig. S1B). Specifically, expression of *QQS* increased protein content in seeds of the F3 generation by 8–10% in line IA1022, 4–7% in lines IA2079 and IA2102, and 6–10% in lines IA2053 and IA3022, compared with the respective segregating WT (wild type) siblings. Seed oil content was similar or slightly decreased, and seed fiber was decreased (Fig. 1B).

**QQS Functions in Monocot Plants to Regulate Plant Composition.** Mono- and dicotyledonous plants have physiological differences in how seed reserves are partitioned. Typically, dicots accumulate significant amounts of protein and oil in their cotyledons, whereas monocots store starch and protein primarily in the endosperm and a small amount of oil in the germ (15). To test whether *QQS* could function across angiosperm lineages, we introduced the *QQS* transgene into the C3 monocot (a monocot that uses only 3-carbon photosynthesis) rice (cultivar Kitaake). Rice is the primary staple crop for over half of the world’s people, with global rice consumption at about 480 million metric tons per y (16). High in starch and gluten-free, rice contains only about 4–5 g of protein per cup serving (7.1–7.9% protein on a dry-weight basis). The vast majority of rice grown globally and in the United States is used for human consumption, generally as a milled grain. Independent transgenic lines of rice plants (Kitaake) expressing the *QQS* gene were grown in a growth chamber under long-day (LD) conditions of 16 h light/8 h dark. Plants were visually and developmentally similar to non-transgenic lines (Fig. 2A and Fig. S2A); however, starch staining of the T2 (second generation progeny of the transformants) plants from 10 independent events indicated a decreased leaf starch. Three independently transformed lines bearing the *QQS* transgene and their respective WT sibling controls were chosen for further study. Determinations were made of plant height, panicle number per plant, seed number per panicle, seed weight, and leaf and seed composition (Fig. 2A and Fig. S2A). Leaf and seed starch was decreased, and leaf and seed protein was increased, in rice *QQS-E* mutants compared with controls. Individual plants of siblings or mutants were identified by both herbicide resistance and PCR screening of leaf genomic DNA. The middle third of the second leaf from the primary tiller of 30-DAP T3 plants and the mature T4 seeds from three independent transformation events (lines *QQS-E* 3-1, 30-2, and 33-3, 10 plants per line, and a total of 10 sibling plants) were analyzed in triplicate. All leaf harvests were made just before the end of the light period. All data in bar charts show mean ± SEM; *n* = 3 independent transformation events. Student's *t* test was used to compare *QQS-E* and controls; **P < 0.01.

**Fig. 1.** Soybean plants expressing the Arabidopsis *QQS* gene have increased protein content. (A) Visual phenotype, developmental patterns, and seeds of *QQS-E* mutant lines and their seeds were similar to those of the controls. Morphology of 30- and 90-d-after-planting (DAP) growth chamber-grown plants of transgenic line *QQS-E* 3-1 and segregating sibling controls is shown; T4 seeds from three independent transformation events (lines *QQS-E* 3-1, 30-2, and 33-3) are shown; see also Fig. S2. (B) Leaf and seed starch was decreased, and leaf and seed protein was increased, in rice *QQS-E* mutants compared with controls. Individual plants of siblings or mutants were identified by both herbicide resistance and PCR screening of leaf genomic DNA. The middle third of the second leaf from the primary tiller of 30-DAP T3 plants and the mature T4 seeds from three independent transformation events (lines *QQS-E* 3-1, 30-2, and 33-3, 10 plants per line, and a total of 10 sibling plants) were analyzed in triplicate. All leaf harvests were made just before the end of the light period. All data in bar charts show mean ± SEM; *n* = 3 independent transformation events. Student's *t* test was used to compare *QQS-E* and controls; **P < 0.01.
by about 6%. In contrast, protein was increased by 10% in leaves and 18% in mature seeds.

We also investigated the effect of QQS transgene expression in another monocot, the C4 species (a monocot that initially uses 4-carbon photosynthesis to concentrate CO₂ in maize). In these studies, a hybrid, transformable, maize line, HI-II, was used to determine whether QQS alters resource partitioning and effects compositional changes in maize kernels. Maize forms the basis for much of the US agricultural economy; the United States is the major exporter of maize worldwide, comprising ~11% of all its agricultural exports (www.ers.usda.gov/topics/crops/corn.aspx). In addition, maize is the most widely grown crop in the world, and provides the major staple food for populations in Latin America, eastern and southern Africa, and southern Asia. Thus, understanding the control of metabolic resource partitioning in this species is an important step toward improving the nutritional value and protein content of a crop that billions of people depend upon for sustenance.

Maize plants expressing QQS (T0 generation in the Hi-II background) were backcrossed to an inbred line, B73, avoiding a selfed hybrid Hi-II that would segregate and likely result in variation in seed composition. A major advantage of B73 is that its genome is sequenced; however, B73 is not readily transformable. The resultant QQS-E maize plants had indistinguishable morphology and seed development from their segregating sibling controls (Fig. 3A). However, QQS expression decreased starch content in mature kernels by 2–4%, increased oil content by 3–4%, and increased protein content by 10–20% (Fig. 3B).

**Ectopic Expression of QQS Does Not Alter Leaf Photosynthetic Rate.** QQS down-regulation does not affect starch degradation in Arabidopsis; rather, the increased starch content is due to increased starch biosynthesis (10). The effects of QQS on starch and protein content are similar in leaves and seeds. In leaves, protein may provide a stable capacity to produce more resources, whereas starch is a transiently accumulated carbon resource that is used for the night. One potential mechanism for the observed QQS-induced changes in protein and starch content is an alteration in leaf photosynthetic rate. To evaluate whether this might be the case, we measured the photosynthetic rate in soybean and maize leaves using five plants from each of two independent transformation events and their respective WT siblings grown under LD conditions. Photosynthetic rates of soybean plants were indistinguishable among the QQS-E transgenic lines and their segregating WT siblings (Fig. S3). Also, no significant difference was detected between photosynthesis of QQS-E maize plants and their segregating WT siblings (Fig. S3). This indicates that change in carbon and nitrogen allocation as a result of the ectopic expression of QQS is not likely associated with an increase in the rate of photosynthesis.

**QQS Interacts with NF-YC4.** QQS protein has no sequence similarity to any known functional domains, which might have provided a clue as to the mechanism by which it regulates carbon and nitrogen allocation; likewise, it does not contain any domain of unknown function that is computationally recognizable as conserved among proteins. The universal ability of QQS expression to impart a high-protein phenotype in other plants is consistent with the hypothesis that QQS confers its activity by interacting with a cellular protein or other molecule that is conserved across multiple species. To investigate a possible QQS–protein interaction, we conducted a yeast two-hybrid screening using QQS as bait against a CDNA library from Arabidopsis seedlings. Arabidopsis NF-YC4 (AtNF-YC4; At5g63470) was identified as a potential QQS interactor. The QQS–AtNF-YC4 interaction was supported by yeast two-hybrid pairwise reciprocal studies (Fig. S4 A and B).

We further confirmed the physical interaction between QQS and AtNF-YC4 by GST pull-down assays using purified recombinant fusion proteins (Fig. 4A). To identify which region of the AtNF-YC4 protein interacts with QQS, we expressed the fusion proteins containing different segments of AtNF-YC4 and used them in pull-down assays. The binding to QQS appeared to require the region from amino acids 73–162 of AtNF-YC4, corresponding to the location of the AtNF-YC4 histone fold-like domain. To evaluate whether QQS would bind generally to proteins containing histone fold-like domains, we tested QQS with AtNF-YB7 (At2g13570) in a pull-down assay. AtNF-YB7 contains a histone fold-like domain similar to that of AtNF-YC4; it is also predicted to be an AtNF-YC4 interaction partner (17). QQS did not bind to AtNF-YB7 in pull-down interaction partner (Fig. 4A), indicating that features unique to only some histone fold-like domains are likely to confer specificity for QQS binding.

Coexpression of QQS and AtNF-YC4 in tobacco leaf in vivo detected the presence of the QQS–NF-YC4 protein complex in the cytosol and in the nucleus (Fig. 4B and Fig. S4 C and D), indicating that the QQS–NF-YC4 interaction occurs in a cellular environment. This localization is different from that obtained in QQS–GFP expression studies, which indicates that the bulk of expressed QQS protein is in the cytosol (10). Coimmunoprecipitation (co-IP) assays using protein extracted from transgenic Arabidopsis plants stably overexpressing MYC-tagged QQS (QQS–TAP) further confirmed that NF-YC4 binds with QQS in vivo (Fig. 4C).

NF-YC is conserved across eukaryotes (18, 19), consistent with our hypothesis that QQS acts via a conserved protein. To investigate whether QQS indeed interacts with NF-YC from soybean, rice, and maize, we selected the soybean, rice, and maize species. To investigate whether QQS indeed interacts with NF-YC from soybean, rice, and maize, we selected the soybean, rice, and maize
and Os6g55640), and maize (GrmZm2g089812). Indeed, GST pull-down assays indicated that QQS interacted with each of these soybean, rice, and maize NF-YC4 homologs (Fig. 4D). These findings are consistent with the concept that expression of QQS confers a high-protein phenotype to soybean, rice, and maize via interaction with NF-YC4. One example of a small, conserved plant peptide binding with a transcriptional regulator to regulate flowering time is the interaction between the florigen FLOWERING LOCUS T (FT) and the bZIP transcription factor FD (20, 21). The QQS–NY-VC4 complex provides an example of a small orphan peptide–transcription factor interaction in plants that can affect metabolic composition. NF-YC4 protein acts in a heterotramer complex with NF-YA and NF-YB proteins to remodel nuclear architecture and to mediate transcription of a variety of CCAAT box-containing genes, few of which have been defined (18, 22). NF-YBs and NF-YCs contain histone-fold domains, whereas NF-YAs have a conserved 56-amino acid domain that incorporates a CCAAT-binding region (18). In the canonical model, an NF-YC and NF-YB heterodimer is formed in the cytosol, transported to the nucleus, and binds with NF-YA to generate the NY complex (23). This complex interacts with promoters containing CCAAT sequences and with other nuclear factors to regulate transcription and profoundly influence multiple developmental and stress- and disease-associated conditions (17, 19, 24). NF-Y, which also appears to play a more general role in plant development or morphology but did decrease starch content up to 15–20% compared with WT controls (Fig. S6B).

The molecular mechanism by which the QQS–NF-YC4 interaction alters carbon and nitrogen allocation remains to be determined. Because NF-YC4-OE (overexpression) increased the total carbon composition, we evaluated the ability of the QQS–NF-YC4 complex to affect composition in Arabidopsis. Under LD conditions, Arabidopsis plants overexpressing the AtNF-YC4 transgene looked similar to the WT controls (Fig. 5A). Overexpression of AtNF-YC4 decreased leaf starch accumulation by about 15% and led to a mean increase in leaf protein content of 17% compared with WT controls (Fig. 5B and C). These data suggest that AtNF-YC4 has a similar function in regulating carbon and nitrogen allocation. Arabidopsis QQS T-DNA KO mutants and AtNF-YC4 T-DNA KO mutants grew similarly to the WT controls, but QQS-KO mutants had increased leaf starch whereas AtNF-YC4-KO mutants did not show an obvious increase in leaf starch content at the end of the light cycle (Fig. S6A). This lack of increased-starch phenotype in AtNF-YC4-KO may be due to the redundancy of NF-YCs. Moreover, when OsNF-YC4-1 (Os3g14669) was overexpressed in Arabidopsis under LD conditions, it did not alter the plant development or morphology but did decrease starch content up to 15–20% compared with WT controls (Fig. S6B).

Taken together, the data are consistent with a model in which QQS acts in conjunction with AtNF-YC4 to alter the allocation of nitrogen and carbon (Fig. 6). We envision three possible mechanisms. QQS protein might simply facilitate translocation of NF-YC4 from the cytosol to the nucleus. Alternatively, NF-YC4 binding to histones might be enhanced due to QQS interaction. NF-YC4 is known to affect flowering and germination in Arabidopsis (28, 29). Furthermore, the α helix of the NF-YCs, which is required for dimerization to NF-YB/NF-YC, can also bind MYC and p53 (30). Thus, there is precedence for NF-YCs acting as a target for transcriptional regulatory factors in the nucleus. Finally, NF-YC4 might act simply to shuttle QQS into the nucleus, where QQS would then act on its own.

Overexpression of AtNF-YC4 or OsNF-YC4-1 in rice (Kitaake) decreased leaf starch content (Fig. S6C), similar to its effect in Arabidopsis. These rice NF-YC4-OE lines appeared identical to WT control plants (Fig. S6C). These data indicate that NF-YC4 can function across species to regulate plant metabolism and can function independently of QQS to alter plant composition. This is the first report, to our knowledge, showing that NF-YC4 plays a role in regulation of primary metabolism.

It has long been known that composition varies widely with environmental conditions (31), although the underlying mechanisms are little-understood. Despite its dissimilarity to any protein-coding
gene model in other (non-*A. thaliana*) species (10, 11). QQS expression is diversely patterned over time and space (10), is highly responsive to stresses (3, 10, 11, 32), and mediates compositional change (3, 10, 33). It is our hypothesis that many orphan genes arise because they confer a selective advantage to an organism by modulating an internal pathway in response to environmental perturbations, thus facilitating adaptation to a new environment (11). The emergence of “new” orphan genes provides an alternative “explosive” means for evolutionary adaptation. The data presented herein suggest that for QQS this is achieved through QQS interaction with a conserved protein complex. Taken together, the data indicate that QQS may represent a mechanism that promotes evolutionary survival during speciation across changing environmental challenges. These data may have broad implications for how orphan genes can function and affect biological processes.

In addition to the enigma surrounding how metabolic partitioning is controlled, the regulation of seed composition has major practical implications. Low protein intake contributes to mental retardation, stunting, susceptibility to disease, wasting diseases, and sometimes death in hundreds of millions of children each year (34, 35). Because plants provide over 60% of human dietary protein and are the major source of protein for many of the world’s at-risk populations (36), increasing protein content in staple crop plants could greatly impact human health. Furthermore, the negative environmental impact of using animal-based foods as a protein source is substantial: About 100 times more water and 11 times more energy are required to produce an equivalent amount of animal-based protein compared with plant-based protein (37). Breeding efforts often fail to produce high-protein varieties without sacrificing yield or protein quality (1). Historically, it has been difficult to uncouple seed protein content from that of carbohydrate and lipid; furthermore, the partitioning of carbon and nitrogen into storage compounds is considered a multigenic trait (1). Here we reveal a piece of this molecular puzzle. Our data indicate that QQS increases protein accumulation and decreases carbohydrate accumulation at least in part via its interaction with NF-YC4 protein. This QQS effect manifests itself irrespective of total protein content, and extends to rice and maize, two monocot species that diverged from *A. thaliana* about 150 million y ago (38). Because NF-YC4 is conserved across eukaryotes, the results open a potential nontransgenic strategy to create high-protein crops via targeted mutagenesis approaches such as transcription activator-like effector nucleases (TALENs) or clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein-9 nuclease (Cas9) technologies. More broadly, this demonstration that an orphan gene from one species can interact with a metabolic network of another species via a conserved protein suggests previously unidentified approaches to investigating the modulation of complex traits.

**Materials and Methods**

**Constructs and Transformation.** The 355::QQS and 355::NF-YC4 fusion constructs were made respectively by cloning the amplified full-length coding sequences of QQS and NF-YC4 into binary vectors pB2GW7 as described (3). The genes are expressed under the control of the constitutive cauliflower mosaic virus (CaMV) 35S promoter. Constructs were introduced into *Agrobacterium tumefaciens* strain GV3101 for transformation of *A. thaliana* ecotype Columbia (Col-0) (3, 10, 39), and A. *tumefaciens* strain EHA101 for transformation of soybean (*Glycine max*) cultivar Williams 82 (3), rice (*Oryza sativa*) cultivar Kitaake, and maize (Zea mays) hybrid Hi-II at the Iowa State University (ISU) Plant Transformation Facility (PTF) (agon-www.agron.iastate.edu/ptf). The transformed plants were delivered from the PTF at the T1 generation (soybean) or T0 generation (rice and maize).

**Fig. 5.** Overexpression of *AtNF-YC4* increases protein content in *Arabidopsis*. (A) *AtNF-YC4-OE* plants had a similar visual phenotype to the WT control plants. (B) Effect of overexpressing *AtNF-YC4* on starch by starch staining assay (3). (C) Effect of *AtNF-YC4* overexpression on starch and protein content by quantitative determination of starch and protein. Bar charts show mean ± SEM; n = 3 replicates with 3 (for leaf starch) or 10 (for leaf protein) plants each. Student’s t test was used to compare protein and starch composition in WT and *AtNF-YC4-OE* lines. *P < 0.05, **P < 0.01.* See Fig. S5 for information about *AtNF-YC4* knockout and NF-YC4-OE mutants. Plants were grown in soil in a growth chamber under LD conditions and harvested at 20 DAP at the end of the light period.

**Fig. 6.** Model of QQS-induced change in composition. The working hypothesis is that cytosolic QQS forms a complex with NF-YC4, moves to the nucleus, and modulates transcription of target genes. The molecular mechanism is as yet to be determined. QQS might increase translocation of NF-YC4 to the nucleus and/or the nuclear QQS–NF-YC4 complex might enhance NF-YC4 activity/NF-Y transcription factor complex activity. Alternatively, NF-YC4 might simply shuttle QQS into the nucleus and release it, and QQS would itself have activity. The shifts in expression of targeted genes would result in altered composition with increased protein and decreased starch. Examples of conditions that induce QQS expression (11) are included to reinforce the intimate link between QQS expression and environmental perturbations.
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Materials and Methods

Additional Methods. Plant selection, crossing, and growth, plant harvest and molecular analysis, photosynthesis measurement of soybean and maize plants, yeast two-hybrid, protein expression and purification, pull-down asay, commonprecipitation assay, bimolecular fluorescence complementation assay, phylogenetic inference, and statistical design and analysis are in SI Materials and Methods.

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