INTRODUCTION

Fleshy fruit are important components of human and animal diets with a critical role in the reproductive development of plants. Fruit biology research has shed light on basic fruit development and ripening in addition to avenues to improve fruit and produce quality. Many fruit develop from carpels (true fruit) or other floral-associated tissues (false or accessory fruit). Accordingly, MADS box genes have been shown to play important roles in carpel and fruit development. As examples, AGAMOUS (AG) clade genes AG, SHATTERPROOF1 (SHP1), and SHP2 from Arabidopsis thaliana and their putative orthologs TOMATO AGAMOUS1 and TOMATO AGAMOUS-LIKE1 (TAGL1) in tomato (Solanum lycopersicum) are required for carpel identity and fruit development, dehiscence, or ripening (Liljegren et al., 2000; Pruett et al., 1994; Vrebalov et al., 2009). A-class MADS box genes, such as FRUITFUL in Arabidopsis and TDR4 in tomato, act together with SHP/TAGL1 to regulate fruit dehiscence and fruit ripening, respectively (Ferrándiz et al., 2000; Bemer et al., 2012), SEPALLATA genes SEP1, SEP2, and SEP3 in Arabidopsis show functional redundancy in normal petal, stamen, and carpel development, whereas the RIPENING-INHIBITOR (RIN)-MADS gene in tomato plays an important role as a master regulator of fruit ripening (Vrebalov et al., 2002; Zhong et al., 2013). Several additional classes of transcription factors also have defined functions in Arabidopsis and tomato carpel and fruit tissues, including KNOX, BHLH, RAVER, HB, HD-2 Zip, YABBY, SBP, and AP2/ERF (reviewed in Giovannoni, 2004; Østergaard, 2009; Klee and Giovannoni, 2011), suggesting complex and layered regulation during carpel/fruit development. Screens for such regulators have by no means been saturated and additional players remain to be identified.

Fruit are typically sink tissues accumulating high-energy reduced carbon compounds derived and transported from leaf source tissue. Photosynthesis occurs in green fruit, and up to 20% of fruit photosynthetic tissue is endogenously derived (Hetherington et al., 1998). Fruits from numerous species develop as green photosynthetic tissues (Blanke and Lenz, 1989; Gillaspy et al., 1998). Fruits from numerous species develop as green photosynthetic tissues (Blanke and Lenz, 1989; Gillaspy et al., 1998). Fruits from numerous species develop as green photosynthetic tissues (Blanke and Lenz, 1989; Gillaspy et al., 1998). Fruits from numerous species develop as green photosynthetic tissues (Blanke and Lenz, 1989; Gillaspy et al., 1998). Fruits from numerous species develop as green photosynthetic tissues (Blanke and Lenz, 1989; Gillaspy et al., 1998). Fruits from numerous species develop as green photosynthetic tissues (Blanke and Lenz, 1989; Gillaspy et al., 1998). 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end) of the ripe fruit. This uneven color phenotype is lost in the loss of function uniform ripening (u) mutant. U encodes a GOLDEN2-LIKE (GLK) transcription factor, Sl-GLK2, belonging to the GARP subfamily of the myb transcription factor superfamily in plants (Powell et al., 2012). The founding gene resides at the maize (Zea mays) Golden2 locus and subsequent functional characterization in several species (Arabidopsis, maize, rice [Oryza sativa], sorghum [Sorghum bicolor], and the moss Physcomitrella patens) revealed conserved plastid development functions of typically two GLK genes in most characterized plant genomes (Rossini et al., 2001; Fitter et al., 2002; Yasumura et al., 2005; Bravo-Garcia et al., 2009; Nakamura et al., 2009; Wang et al., 2013) that are necessary for normal plastid development (Waters et al., 2008, 2009). The u mutation is widely selected for in tomato varieties, as it results in uniform fruit ripening, which is attractive to consumers and facilitates the determination of optimal timing for destructive harvest, which is common for processing cultivars. The normal GLK2 gene is expressed in the fruit in a latitudinal gradient and with higher expression in the stem end (top) of the fruit conferring the green shoulder phenotype. Overexpression of tomato GLK2 resulted in uniformly dark green immature fruit, while cosuppression mimicked the uniform mutant, confirming that GLK2 is U (Powell et al., 2012). Tomato GLK1 function has not been described.

The green shoulder phenotype suggests a gradient of developmental regulation along the latitudinal (top-bottom) axis of the fruit. Such developmental gradients are described in both animal and plant systems. Examples include anterior-posterior axis patterning during Drosophila melanogaster embryogenesis (Berleth et al., 1988; Lehmann and Nüsslein-Volhard, 1991), while in plants, the spatial pattern of auxin specifies apical-basal axis formation during embryogenesis (Friml et al., 2003). The development of the apical-basal axis of the Arabidopsis gynoecium has also been hypothesized to involve auxin gradients (Nemhauser et al., 2000; Balanzá et al., 2006). Transcription factors also influence patterning of the Arabidopsis gynoecium (Østergaard, 2009; Trigueros et al., 2009), though the impact of regulatory gene expression gradients has not been described in tomato or other fleshy fruits beyond the prior characterization of GLK2 (Powell et al., 2012).

Here, we describe functional characterization of tomato GLK1 and expand our prior characterization of GLK2. Our results suggest functional equivalence of the GLK1 and GLK2 peptides in tomato, though differential transcription of each gene results in leaf phenotypes for GLK1 and fruit phenotypes for GLK2.

![Figure 1.](Image 110x213 to 493x433)

**Figure 1.** Expression of GLK2 and GLK1 in Normal and Transgenic Tomato Tissues.

**(A)** Expression of GLK2 as determined by hybridization of radiolabeled GLK2 probe to gel blot of RNA from immature green fruit tissues showing enhanced GLK2 transcript levels in dark-green fruit (overexpression) and reduced or degraded GLK2 transcripts in "mimicked" u fruit (cosuppression) from 'Ailsa Craig' (U/U) or 'M82' (u/u) transformed with 35S:SiGLK2. Each lane represents tissue from an independent transgenic line.

**(B)** Expression of GLK1 as determined by hybridization of radiolabeled GLK1 probe to gel blot of RNA from immature green fruit and leaf tissues showing enhanced transcript levels in dark-green fruited plants (overexpression [OE]) or reduced and degraded transcripts in pale-leaved plants (cosuppression [CS]) from 'Ailsa Craig' (U/U) or 'M82' (u/u) transformed with 35S:SiGLK1.

**(C)** Expression of GLK1 as determined by hybridization of radiolabeled GLK1 probe to gel blot of RNA from leaf tissues showing reduced or degraded transcripts in pale leaf plants (cosuppression) from 'Ailsa Craig' (U/U) transformed with 35S:SiGLK1.

**(D)** Relative expression levels of GLK1 and GLK2 in wild-type 'Ailsa Craig' (U/U) leaves as determined by qRT-PCR. Expression levels were normalized for internal control (18S) and amplification efficiency. Error bars represent ±, and data from a minimum of three biological replicates were used to determine each sample and ± value presented.
Interestingly, overexpression of either GLK1 or GLK2 affects chloroplast development in fruit and enhances nutritional and quality metabolites in ripe fruit, while overexpression of either has no impact on leaves. Repression of GLK1 results in plastid deficit in leaves. We also showed that GLK fruit quality enhancement can be combined with the hp1 light signaling mutation to further elevate chlorophyll and carotenoids in fruit, suggesting promising combinatorial gene targets for influencing nutritional value of tomato and additional fruit species. Finally, we demonstrate transcriptome gradients throughout the latitudinal axis during tomato fruit development, consistent with the observed uneven gradient of ripening displayed by tomato and many fleshy ripening fruits, and revealing additional molecular complexity in ripening control. The fact that the ripening gradient occurs in the absence of GLK2 activity (i.e., in the u/u uniform mutant) indicates that while GLK2 activity impacts ripe fruit phenotype and quality characteristics, it operates separately from the bottom–top ripening gradient.

**RESULTS**

**GLK1 and GLK2 Are Functionally Similar but Differentially Expressed, Resulting in Tissue-Specific Outcomes**

We previously showed that tomato u is due to a nonsense mutation in GLK2 (Powell et al., 2012). As is the case in other plant species (Arabidopsis, maize, and rice) (Rossini et al., 2001; Fitter et al., 2002; Nakamura et al., 2009), there are two GLK genes in tomato, GLK1 and GLK2. In Arabidopsis, the two genes have redundant functions in regulating a set of light-harvesting complex and chlorophyll biosynthetic genes (Waters et al., 2009). In tomato, elevated chlorophyll in the shoulder is positively regulated by GLK2, which is more highly expressed at this end of the fruit (Powell et al., 2012). To test the function and specificity of GLK1 and GLK2 activities, we generated constructs to overexpress each gene independently in tomato plants (both U/U and u/u genotypes) and driven by the 35S

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**Figure 2.** Fruit and Leaf Phenotypes of Transgenic Plants Overexpressing or Cosuppressing GLK1 or GLK2.

(A) Immature green fruit (20 DPA) from ‘Ailsa Craig’ (U/U) and ‘M82’ (u/u) containing 35S:SlGLK1 or 35S:SlGLK2 with overexpression (OE) or cosuppression (CS) of GLK1 or GLK2.

(B) Leaves from ‘Ailsa Craig’ (U/U) and ‘M82’ (u/u) cosuppressing GLK1 and GLK2. From left to right: ‘M82’ (u/u) cosuppression GLK1 leaf, ‘M82’ (u/u) leaf, ‘Ailsa Craig’ (U/U) cosuppression GLK1 leaf, ‘Ailsa Craig’ (U/U) cosuppression GLK2 leaf, and ‘Ailsa Craig’ (U/U) leaf.
Figure 3. Chlorophyll and Chloroplast Phenotypes of Immature Green Fruit and Leaves Expressing 3SS:SlGLK1 or 3SS:SlGLK2.

(A) Total chlorophyll levels from three sectioned segments along the latitudinal axis of immature green fruit (15 DPA) \( (n = 5) \) of ‘Ailsa Craig’ (U/U) and ‘Craigella’ (u/u). ‘Craigella’ is a near-isogenic line of ‘Ailsa Craig,’ except for the u locus. *P value t test < 0.05. Error bars are se. FW, fresh weight.

(B) and (C) Total chlorophyll levels from leaves (B) and immature green fruit (C) \( (n = 8) \) of different genotypes and transgenic plants expressing 3SS:SlGLK1 or 3SS:SlGLK2. AC, ‘Ailsa Craig;’ OE, overexpression; CS, cosupression. **P value t test < 0.001. Error bars are se.

(D) Transmission electron microscopy images of immature green fruit chloroplasts from ‘M82’ (u/u) overexpressing GLK1 or GLK2. Bars = 1 μm.

(E) Left: Horizontal cross-sectioned optical microscopy images of immature green fruit from ‘M82’ (u/u) and ‘M82’ overexpressing GLK2. Middle: Number of thylakoids per granum (sample size \( n = 20 \)). Right: Number of chloroplasts per cell (sample size \( n > 15 \)) from immature fruit tissues of ‘M82’ (u/u) overexpressing GLK1 or GLK2. **P value t test < 0.001. Error bars are se.
promoter. RNA gel blot analysis (Figure 1) indicates upregulation of each gene in the respective transgenic lines. Overexpression of either GLK1 or GLK2 using the 35S promoter results in darker green unripe fruit along the entirety of the latitudinal axis (Figure 2A). Dark-green and uniform coloration was consistent with significantly elevated chlorophyll levels (Figure 3A) and increased expression of the GLKs throughout the fruit as a result of 35S promoter activity (Figure 3E). In wild-type tomato fruit, only GLK2 is expressed at high levels and more so in the top than the bottom of the fruit, resulting in the green shoulder phenotype. GLK1 is expressed in the fruit but at very low levels compared with GLK2 (Figure 1B). In leaves, both genes are
expressed, though GLK1 is expressed at higher levels (Figure 1D).

In the case of overexpression constructs transformed into wild-type 'Ailsa Craig' (U/U), possessing functional alleles for both GLK1 and GLK2, we observed cosuppression (Napoli et al., 1990) in several transgenic lines (Figure 1). Cosuppression of GLK2 resulted in u-like fruit in that they are absent the green shoulder, but no discernible leaf phenotypes, whereas cosuppression of GLK1 resulted in pale leaves with reduced chlorophyll, but no notable differences in the fruit (Figure 2). Cosuppression of GLK1 in cultivar 'M82', a processing variety with the u/GLK2 mutation, also yielded a pale leaf phenotype and low chlorophyll levels as in the Ailsa Craig (U/U) wild type. Together, these data suggest that both GLK1 and GLK2 are functionally similar peptides, but their expression patterns effectively restrict GLK1 largely to leaf and GLK2 largely to fruit functions, respectively, in vivo.

Overexpression of GLKs Enhances Chloroplast Development and Nutritional Quality

We initially focused on characterizing the dark green fruit of GLK1 and GLK2 overexpression lines. Consistent with the function of GLK genes in other plant species, overexpression of GLKs increased chlorophyll content, chloroplast number, and thylakoid grana stacks in green fruit. Specifically, chlorophyll content increased 5- to 9-fold, chloroplast numbers 2.5- to 3-fold, and the observed numbers of grana stacks 3- to 4-fold (Figure 3). It is interesting to note that the changes in chloroplast number and grana stacks have roughly multiplicative effects on chlorophyll levels, which is expected as most chlorophyll resides in the grana thylakoid membrane. The starch levels in green fruit of overexpressing transgenic plants were also increased (Figure 4C) as was observed with overexpression of Arabidopsis GLK2 in tomato fruit (Powell et al., 2012). Because the chlorophyll content in leaves of GLK1- and GLK2-overexpressing lines was not substantially altered compared with control leaves (Figure 3B), the increase in transgenic fruit starch levels likely directly reflects increased fruit photosynthesis. In red tomato fruit, many desirable nutritional compounds are elevated, including not only carbohydrates/sugars, but also carotenoids and ascorbate (vitamin C) (Figure 4). Total soluble solids (°Brix, percentage w/w of soluble solids in solution) and sugar levels were significantly increased (20 to 40% and 40 to 60%, respectively), and total carotenoid levels were elevated 25 to 40% in both GLK1- and GLK2-overexpressing lines compared with the control 'M82' (u/u). Interestingly, in the red ripe pericarp tissue, β-carotene and lutein, the protective carotenoids usually found at high levels associated with the light-harvesting complexes of leaves, were also substantially increased (Figure 4A). We also note that the elevation of the predominant carotenoid, lycopene, is consistent with elevated plastid numbers and, thus, storage capacity, while the larger changes in β-carotene and lutein are more difficult to interpret as they reflect a much smaller proportion of the total carotenoid pool. One possibility is that this increase includes residual thylakoid-associated carotenoids from dark-green unripe stages of GLK-overexpressing fruit.

GLK2 Acts Independently of hp1/DDB1

Prior studies of tomato mutations reveal additional genes impacting fruit pigmentation, and we explored whether or not said genes might act in the same pathway as GLKs. For example, high pigment (hp) mutations including hp1, hp2, and hp3
display elevated green fruit chlorophyll and ripe fruit carotenoids as a result of mutations in light signal transduction genes DDB1(hp1) and DET1(hp2) (Mustilli et al., 1999; Liu et al., 2004) or an abscisic acid biosynthetic gene, zeaxanthin epoxidase, in the case of hp3 (Galpaz et al., 2008). To test for epistasis when combining different genes or mutations that have positive effects on tomato chloroplasts and associated nutritional value, we focused initially on the relationship of the well characterized HP1/DDB1 gene (Liu et al., 2004) and U/GLK2. hp1/hp1 u/u double mutant fruit display darker green color and higher chlorophyll content than u/u fruit (Figure 5A), suggesting independent and additive activities. The additive actions of DDB1 and GLK2 were supported when we characterized F2 plants of a cross between hp1/hp1 and the wild type (U/U) overexpressing 35S:SlGLK2, where both lines are in the same ‘Ailsa Craig’ genetic background. Homozygous hp1/hp1 plants with dark-green color throughout the fruit due to 35S:SIGLK2 activity had more chlorophyll in green fruit and carotenoid in red fruit than the other genotypic classes in the F2 population (Figure 5B). These observations indicate the additive effects of both genes on fruit quality traits and suggest the feasibility of combining elevated expression of GLKs with other genes (including DDB1/HP1 reduction) in improving fruit characteristics.

An additional transcription factor gene that has been characterized in tomato and resulted in dark-green immature fruit color and elevated chlorophyll levels when repressed is TAGL1 (Itkin et al., 2009; Vrebalov et al., 2009; Giménez et al., 2010). We measured the expression level of U/GLK2 in immature green fruit of TAGL1 suppressed (via RNA interference) lines and saw no changes in the expression level of GLK2 compared with the wild type (Supplemental Figure 1), suggesting that the dark-green color of fruit in these lines is not a direct result of changes in GLK2 expression level.

**GLKs Alters Expression of Fruit Photosynthesis and Chloroplast Development Genes**

To test the molecular consequences of overexpressing GLK1 or GLK2 in transgenic fruit, we compared the gene expression

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**Figure 6.** Overexpression of GLK1 or GLK2 Influences Similar Photosynthesis-Related Genes in Green Fruit.

(A) Venn diagram of upregulated genes unique to and shared between 35S:SILK1 and 35S:SILK2 immature green fruit (15 DPA) tissues.

(B) GO term enrichment analysis of the overlapping upregulated genes. Terms are ranked by adjusted P values.
levels in the pericarp of immature green transgenic fruit with that in untransformed isogenic control pericarp using strand-specific mRNA sequencing (Zhong et al., 2011). Using cutoff criteria with an expression ratio of ≥2 and P < 0.05 between transgenic and control tissues, overexpressing 3SS:SlGLK1 or 3SS:GLK2 in u/u upregulated 324 and 352 genes, respectively. Consistent with microarray studies using Arabidopsis GLK genes (Waters et al., 2009), a large portion (>60%) of the upregulated genes are common to both transgenes (Figure 6). It is important to note that we observe higher expression of 3SS:SlGLK2 than 3SS:GLK1 in our transgenic lines, based on higher reads per kilobase of exon model per million mapped reads (RPKM) values and supported by higher chlorophyll levels in 3SS:SlGLK2 overexpression lines. The additional expression may partially explain the differences in upregulated genes between GLK1 and GLK2 overexpression in immature green pericarp tissues. Moreover, when using more stringent criteria for defining upregulated genes (ratio ≥ 5, P value ≤ 0.05), the percentage of shared upregulated genes increases to ~70%. Assessing Gene Ontology (GO) term enrichment in 3SS:SlGLK1 and 3SS:SlGLK2 overexpression fruit compared with the control ('M82' u/u) yielded similar outcomes for both transgenes (Figure 6; Supplemental Tables 1 and 2). A large portion of the common enriched terms relate to photosynthesis and chloroplast structure and include genes involved in the chlorophyll biosynthesis pathway, thylakoid membrane biogenesis, light-harvesting complexes, and starch metabolism (Figure 6). These changes in gene expression agree well with the phenotype of the fruit, where the chlorophyll content, chloroplast numbers, thylakoid grana stacks, and starch levels are significantly elevated.

Deploying the same methodology in ripe fruit tissues, however, revealed just a small number of genes that are upregulated despite the fact that both GLK1 and GLK2 are expressed at high levels (Supplemental Data Set 1). Together, these results suggest that tomato GLKs exert their primary influence on green fruit tissues even in cases of phenotypes that manifest in later stages of fruit development. Examples include the higher levels of ripe fruit carotenoids and sugars resulting from increased green fruit plastids and photosynthetic activity, respectively.

Overexpression of GLK1 and GLK2 Does Not Affect Overall Ripening Regulation

We questioned whether the early fruit development phenotypes of immature green fruit overexpressing 3SS:SlGLKs (i.e., dark-green color and high chlorophyll content) would impact the overall ripening process. We initially examined four GLK overexpression lines through three generations and observed no differences from the time of fruit set to the initiation of ripening (Figure 7B), although there were changes in carotenoid accumulation and coloration as the fruit matured (Figures 4 and 7B). Ethylene evolution rates were similar between the transgenic fruit and controls (Figure 7A). In the RNA sequencing (RNA-seq) data comparing gene expression levels in the red fruit of these transgenic lines with those of the control (Supplemental Data Set 3), there were no differences in the expression of genes known to coordinate ripening (RIN, NON-RIPENING [NOR], COLORLESS NON-RIPENING [CNR], and TAGL1) or ethylene biosynthesis and signaling. Together, these results suggest that GLK1 and GLK2 do not affect general ripening control systems.

Figure 7. Ethylene Evolution and External Color of 'M82' (u/u) Fruit Overexpressing GLK1 and GLK2 during Fruit Ripening.

(A) Ethylene levels as determined by gas chromatography in 'M82' (u/u) control fruit and transgenic fruit overexpressing GLK1 or GLK2 during development (n = 10).

(B) Fruit of 'Ailsa Craig' (U/U), 'M82' (u/u), and transgenic plants overexpressing GLK1 or GLK2. IM, immature green (15 DPA); MG, mature green; BR, breaker.
Figure 8. Gradient of Gene Expression along the Latitudinal Axis of Tomato Fruit.

(A) Top row: Wild-type U/U and mutant u/u tomato fruit at mature green (MG), breaker (BR), breaker + 1 d (BR+1), and breaker + 3 d (BR+3) stages. In BR and BR+1, color can be visually observed to “break” at the stylar end of the fruit. Second and third rows: Cross sections.

(B) Gradient expression levels of GLK2 as determined by qRT-PCR through three latitudinal sections of wild-type ‘Ailsa Craig’ (U/U) fruit during six stages of tomato fruit development.
including those involving ethylene and critical ripening transcription factors. Rather, they exert their influence on fruit development through phenotypes manifested from altered green fruit plastid activity and plastid numbers, including sugar and later carotenoid accumulation.

**The GLK2 Green Shoulder Phenotype Results from a Gradient of Expression through the Fruit**

Expression of GLK2 reflects a gradient of differential expression from the stem end to the blossom end of the fruit with expression varying 4- to 16-fold between the two ends (Figure 8B). Ectopic expression of either GLK gene minimized the repertoire of genes comprising this gradient and resulted in the immature fruit having a more uniform dark-green color (Figure 2), supporting the role of the GLK2 expression gradient as cause of the green shoulder phenotype in wild-type fruit. Interestingly, tomato cultivars with elevated anthocyanin content also demonstrate a higher concentration of metabolites at the shoulder/stem end of the fruit (Gonzali et al., 2009). Moreover, the ripening process, at least as defined by pigment accumulation, usually does not occur uniformly throughout the fruit but initiates at the stylar end (bottom) of the fruit (Figure 8A). These phenotypes suggest a gradient of gene expression along the latitudinal axis of the fruit that may extend beyond GLK2 and that has not been extensively characterized. Indeed, the fact that this ripening gradient occurs in the absence of functional GLK2 (u/u mutation; Figure 8A) confirms that GLK2 and ripening gradients act largely independently.

To specifically explore this phenomenon of gradient ripening, we deployed RNA-seq to characterize the transcriptome activity of genes in three latitudinal sections along the vertical axis of wild-type ‘Ailsa Craig’ (U/U) fruit pericarp: the stem end or the top, the middle and the stylar end (bottom) of the fruit, and through six stages of fruit development (10 d postanthesis [DPA]; 20 DPA, mature green, breaker, breaker + 5, and breaker + 10). Fruit were cut into five equal sections and the intervening two sections discarded to minimize developmental and gene expression overlap (Figure 8C). We focused our analysis on two specific gene expression profile classes: high at the stem end, low at the stylar end or vice versa, and with 2-fold or greater ratio differences between the two ends and adjusted P value ≤ 0.05. We identified tens to hundreds of genes exhibiting differential expression along the latitudinal axis in all examined fruit stages (Figures 8C and 8D; Supplemental Figure 2 and Supplemental Data Set 1).

At 10 DPA, many differentially expressed genes were related to photosynthesis and chloroplast structure, consistent with the high expression level of GLK2 in the top of the fruit. At breaker + 5 and breaker + 10 (both red ripe fruit stages), several genes involved in ethylene biosynthesis and additional ripening pathways showed differential expression (high at the top and low at the bottom; Supplemental Figure 3), including ACS1, ACS3, ACS5, ACS4, E4, and E8. Interestingly, at the breaker stage, there is no significant differential expression of these genes between the sections. This may be due to the fact that all are regulated by the gaseous and readily diffusible hormone ethylene.

Using Venn diagrams (Figure 8C), we describe genes that showed differential expression in all three green stages (Table 1), all red fruit stages (Table 2), and both green and red fruit stages (Table 3). To confirm the RNA-seq results, we also measured expression levels of selected genes by quantitative RT-PCR (qRT-PCR) that showed differential expression through multiple fruit stages, and the results agree well with the RNA-seq data (Figures 8B and 8D). As examples, in green fruit tissues, in addition to GLK2 showing differential expression in all green fruit stages as expected, there are also two genes of the light-harvesting complex (Solyc02g070990 and Solyc02g070950, both chlorophyll binding proteins) and one in chlorophyll biosynthesis (Solyc10g006900, protochlorophyllide reductase) that presumably reflect GLK2 responses. Indeed, most of the genes that show differential expression from the top to bottom of fruit in the green stages are also upregulated due to GLK2 overexpression (Table 1).

Interestingly, the macrocalyx MC-MADS gene and the tomato AP1 ortholog functioning in floral determinacy and sepal development (Vrebalov et al., 2002) also showed differential expression in these green stages. Fruit of the mc mutant display the normal green shoulder as in the wild type, and its repression yielded no discernible fruit phenotypes (Vrebalov et al., 2002) so its role in fruit development may be redundant with other of the many MADS box genes expressed in fruit. In all three stages of red fruit, the suc gene that hydrolyzes Suc to Glc and Fru in tomato red fruit (Kiani et al., 1996) showed high expression levels in the stylar end with low expression in the stem end. It is interesting to note that known ripening regulators like RIN, NOR, CNR, TAGL1, and AP2a in tomato do not display these gradient patterns; that is, there are no significant differences in expression levels between the sections during fruit development (Supplemental Figure 4), suggesting that other factors beyond these well described and necessary ripening regulators regulate the formation of the gradient during the ripening process in tomato.

A *knotted*-related transcription factor and three genes annotated as auxin-responsive genes showed differential expression throughout the six stages of fruit development analyzed. These three auxin-responsive genes are very similar to the NGATHA transcription factor family in *Arabidopsis* (Alvarez et al., 2009; Trigueros et al., 2009) that has been shown to affect gynoecium development. *knotted* transcription factors have been shown to

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**Figure 8.** (continued).

(C) Transcriptome analysis for differential gene expression through three latitudinal sections of wild-type ‘Ailsa Craig’ (U/U) fruit. Two expression profiles were used to define differential expression: expression levels (RPKM value) highest at stem end, lower at the middle part, and lowest at stylar end, or vice versa. Three green fruit stages (10 DPA, 20 DPA, and mature green) and three red fruit stages (breaker, breaker + 5, and breaker + 10) were represented in Venn diagrams to determine the shared differentially expressed genes among the different stages.

(D) Example of differential expression pattern of Solyc01g100510 (a *knotted* transcription factor), Solyc08g083400 (ARF/NGATHA homolog gene), and Solyc02g003910 (suc, invertase gene) as determined by RNA-seq (top) and confirmed by qRT-PCR (bottom).
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**Table 1. Genes Showing Differential Expression along the Three Latitudinal Sections of Tomato Fruit in All Three Green Stages**

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**Table 2. Genes Showing Differential Expression along the Three Latitudinal Sections of Tomato Fruit in All Three Red Stages**

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**DISCUSSION**

**Fruit but Not Leaf GLK Activities Are Limiting**

It has been suggested that GLK duplication predated and facilitated the evolution of C4 photosynthesis, as C4 species such as eudicots may have GLK activities that are not present in C3 species. This hypothesis is supported by the presence of GLK genes in the Arabidopsis genome and their putative involvement in leaf and shoot meristem development (Pautot et al., 2001; Ragni et al., 2008), but there is still no report on the effect of this class of transcription factor in fleshy fruit, making them interesting candidates for further functional characterization.

To more fully assess the differences in transcriptome activity resulting from the GLK2 gradient versus the ripening gradient (and to ascertain the degree of any regulatory overlap), we analyzed transcriptome data from the top versus bottom of mature green fruit in addition to comparing the bottom of mature green to the bottom of breaker fruit. The former differ substantially in GLK2 expression and are not ripe, so should represent GLK-mediated differences, while the latter have very low and similar GLK expression and are not ripe, so should represent GLK-locus for...
as maize and sorghum demonstrate differential mesophyll (GLK1) versus bundle sheath (G2) GLK expression (Wang et al., 2013). Our results extend this hypothesis further to suggest that the duplication facilitated additional regulatory fidelity of chloroplast development for C3 species bearing fleshy fruits.

Fruit are net sink tissues with photosynthesize from source tissues (leaves) transported to and largely responsible for their carbohydrate accumulation. Here, we show that green tomato fruit but not leaves can be elevated in terms of photosynthetic capacity through GLK manipulation. Specifically, overexpression of either GLK1 or GLK2 resulted in dark green tomato fruit with high chlorophyll and chloroplast levels in addition to more stacked thylakoid grana and elevated starch in the fruit. However, no significant changes were observed in leaves. GLK1 repression resulted in chlorotic leaves, demonstrating its importance in leaf plastid development, while GLK2 repression had no leaf phenotype. Elevated GLK activity also impacted mature fruit via increased sugar and carotenoid content but did not influence the rate or timing of ripening. These results indicate that leaf GLK activity is saturated, while the potential to increase fruit photosynthetic activity via GLK induction remains. Elevating GLK activity via transgenes or through natural diversity thus represents a strategy to manipulate fruit photosynthesis to enhance fruit crop quality without affecting non-fruit tissues.

Transgenic overexpression of either GLK1 or GLK2 leads to elevated quality and nutrient compound levels in red ripe tomato fruit (e.g., increases in sugars, multiple carotenoids, and ascorbate [vitamin C]). High sugar levels in transgenic fruit overexpressing GLKs correlate with higher starch in green fruit that is metabolized to Glc and Fru in red fruit. Although carotenoid biosynthetic genes were not differentially expressed (Supplemental Data Set 2) in transgenic red fruit tissues compared with controls, the higher chloroplast numbers and carotenoids in green fruit presents a source of more carotenoids, as chloroplasts are converted to chromoplasts during fruit ripening, increasing the sink capacity for carotenoid accumulation (Lu and Li, 2008). Elevated fruit ascorbate levels in transgenic GLK fruit likely reflect a photoprotective response to quench hydroperoxides, by-products of photosynthesis, resulting from higher chlorophyll and chloroplasts in photosynthetic green fruit. From a practical perspective, fruit GLKs can be overexpressed without affecting non-fruit tissues, such as leaves, meaning that natural or transgenic variation resulting in elevated GLK expression can be employed as a means of positively impacting fruit quality through increased sugars, carotenoids, and organic acids, such as ascorbate, which are positively correlated with favorable flavor attributes (Baldwin et al., 2008; Klee and Tieman, 2013).

GLK and DDB1 Fruit Quality Effects Are Additive, Suggesting a Route to Amplified Fruit Quality Improvement

Several tomato hp mutants conferring elevated chlorophyll and carotenoid levels have been described (e.g., hp1, hp2, and hp3) and result from mutations in light signaling processes [DET1 (hp2) DDB1 (hp1)] or abscisic acid biosynthesis (hp3). Here, we show that GLK2 acts independently of HP1/DDB1, suggesting that different routes regulate plastid/chlorophyll and carotenoid accumulation in tomato fruit. While GLKs are transcription factors that positively regulate chlorophyll biosynthesis and accumulation of light-harvesting complexes in addition to chloroplast development in general, DDB1 and DET1 contribute to multiple developmental and response processes, including light signal transduction, chromatin modeling, and plastid biogenesis (Benvenuto et al., 2002; Liu et al., 2004). Comparing the numbers of genes differentially regulated in response to GLK2 overexpression (Supplemental Data Set 1) compared with perturbation of DET1 suggests much broader effects resulting from DET1 activity (Enfissi et al., 2010). Because DET1 and DDB1 are components of the same light signaling process and interact with each other (Schroeder et al., 2002), we anticipate a similar result if we were to cross the hp2 mutant with GLK2-overexpressing lines. As such, breeding combinations deploying alternate high activity alleles of GLKs with either high activity HP1 or HP2 alleles could be pursued toward optimized fruit quality. TAGL1 suppression results in both early (high chlorophyll content) and late (reduced carotenoid levels) ripening effects (Vrebavol et al., 2009), suggesting GLKs and TAGL1 as another combination of genes that could influence fruit quality. However, in this case, TAGL1 alleles with reduced activity during early fruit development but not during ripening would be required to positively impact fruit quality. Alternatively, targeted repression via an early fruit development promoter would be predicted to yield similar results.

**Figure 9.** H3K4me3 of the GLK1 Locus.

The plot shows the level of methylation (y axis) as determined by chromatin immunoprecipitation sequencing using a H3K4me3 antibody probe along the gene locus (x axis) in leaf and fruit tissues. BR, breaker stage. GLK1 is oriented 3’ to 5’, left to right, as shown.

**Table 3.** Genes Showing Differential Expression along the Three Latitudinal Sections of Tomato Fruit in All Six Stages

<table>
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<th>Gene ID</th>
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GLK2 Is a Component of the Latitudinal Gradient of Ripening

The mature tomato fruit is derived from the ovary/carpels of the gynoecium. In terms of tissue patterning, there are several axes of development and morphological variation in fruit: apical-basal, abaxial-adaxial, and medial-lateral (Østergaard, 2009). Here, we focused on the gradient of expression levels of genes in tomato fruit along the apical-basal or latitudinal axis that displays an obvious gradient of chlorophyll accumulation (defined by U/GLK2) and later ripening, which has not been characterized at the molecular level. Indeed, most reports on tomato (including our own) typically focus on whole-fruit pericarp characterization, without distinguishing between the phenotypic and physiological variation across the latitudinal gradient. To distinguish the transcriptome effects of GLK2 versus ripening gradients, and to establish whether genes in addition to GLK2 present expression gradients through the fruit, we sectioned developing fruit and assayed transcriptome activity by RNA-seq in the top, middle, and bottom fruit pericarp tissues. We showed that in each stage of development, there are numerous genes that exhibit expression gradients through the fruit, and the number of differentially expressed genes generally increases with fruit development. Analogous studies have been performed on developing maize leaves, consistent with the gradient of blade maturation (Li et al., 2010). Unlike the maize blade that displays a developmental gradient reflecting the synthesis of new cells from meristematic stem cells and a gradient across the blade effectively representing cell age and maturity, the fruit ripening gradient occurs in the context of a tissue that has long ceased cell division. Interestingly, we found several transcription factors that maintained gradient expression patterns during one or more fruit stages. These include members of the KNOX, WRKY, and RAVB3/NGATHA gene families (Tables 1 to 3). Homologous transcription factors have been shown to play roles in gynoecium development in Arabidopsis. For example, the knat1/brevipedicellus (bp) mutants produce downward rather than upward oriented siliques (Venglat et al., 2002), whereas KNAT2 and KNAT6 (Arabidopsis knotted-like genes) are restricted by KNAT1/BP and REPLUMLESS to promote correct inflorescence development (Pautot et al., 2001; Ragni et al., 2008). KNAT2 overexpression in Arabidopsis induced homeotic conversion of the nucellus into a carpel-like structure. Here, Solyc01g1005110, a tomato homolog of Arabidopsis KNAT2 and KNAT6, displayed high expression at the stem end of the fruit compared with the basal end and throughout development. It is noteworthy that knotted-like genes are usually active in the shoot apical meristem (Hake et al., 2004). The fact that fruit are developed from carpels and the stem end of carpels originate from floral receptacles containing the shoot apical meristem may explain the gradient expression of this knotted-like gene along the apical-basal axis. NGATHA gene family members also play roles in style and stigma development in Arabidopsis via a mechanism dependent on relative expression levels (Álvarez et al., 2009; Trigueros et al., 2009). The fact that the tomato homologs have higher expression levels at the stylar end derived from the tips of the carpels may indicate a similar gradient with roles during both gynoecium and later fruit maturation. It is noteworthy that previously described transcriptional regulators that are necessary for ripening, including RIN, CNR, and TAGL1, do not display gradients of expression, suggesting additional regulators are central to the ripening gradient.

The gradient expression of tomato sucR is also noteworthy. The stylar end is the location of the first visual signs of ripening initiation and would be anticipated to be the site of initial conversion of starch and Suc to monosaccharides. Indeed, soluble solid (°Brix) levels are significantly higher at the stylar end compared with the stem end of red ripe tomato fruit (Supplemental Figure 6). Multiple ethylene biosynthesis and ethylene-responsive genes displayed gradient expression at the breaker + 5 and breaker + 10 stages when the fruit are most ripe, consistent with the fact that the stem end or top of the fruit displays ripening phenotypes later than the bottom.

Characterization of the two tomato GLK genes demonstrates the positive impact on fruit nutritional value stemming from more chlorophyll and chloroplasts in preripe green fruit and suggests a means to improve the nutritional quality of tomato and additional fruit species. We revealed that while GLK is not limited in leaf tissue, it is limited in fruit, confirming the potential for using transgenes or allelic diversity to increase fruit quality by elevating GLK activity. Previously described ripening regulators, such as the RIN MADS box gene, did not show gradient expression though a number of putative regulators previously uncharacterized in tomato are candidates for gradient control.

METHODS

Plant Materials

Tomato (Solanum lycopersicum) cultivars ‘Ailsa Craig’ (U/U) (LA2838A), ‘Craigella’ (u/u) (LA3247), ‘M82’ (u/u) (LA3475), hp1 (LA0279), and hp1/u (LA3377) were provided by the Tomato Genetics Resource Center, Davis, CA. Craigella harbors the u mutation and is nearly isogenic (five back-crosses) with Ailsa Craig. Plants were grown in greenhouses at the Boyce Thompson Institute for Plant Research, Ithaca, NY, with a 16-h-light (27°C) and 8-h-dark (19°C) cycle. Tomato fruit were tagged at 7 to 8 DPA when they were 1 cm in diameter and reached breaker stage at 37 to 38 DPA.

Expression Constructs and Transformation

The full-length sequences of GLK1 and GLK2 cDNA were cloned into pB121 (Clontech) and pBTEX (Lin et al., 2006) vectors digested with SmaI/Sacl and SmaI/SalI, respectively, under the control of the cauliflower mosaic virus 35S promoter. The fidelity of the construct was confirmed by DNA sequencing and transgenic ‘Ailsa Craig’ (U/U) and ‘M82’ (u/u) tomato plants were generated by Agrobacterium tumefaciens–mediated (strain LBA4404) transformation using previously described methods (Fillatti et al., 1987). Plants with transgene insertions were screened by PCR and DNA gel blot using cauliflower mosaic virus 35S-specific primers/probe. TAGL1 RNAi lines were described previously (Vrebalov et al., 2009).

RNA Gel Blot Analysis

Total RNA was extracted from frozen tissues and gel blot analysis were performed as described (Vrebalov et al., 2009) using 25 μg of total RNA per lane. pGEM-T Easy vectors containing GLK1 and GLK2 full-length cDNAs were used as template to generate GLK1- and GLK2-specific probes by PCR using primers GLK1F/R for GLK1 and GLK2F/R for GLK2 (Supplemental Table 3).
qRT-PCR Analysis
Quantitative real-time PCR was performed using the ABI PRISM 7900HT sequence detection system and the Power SYBR Green RNA-to-C\textsubscript{T} 1-Step kit (Applied Biosystems) following the manufacturer’s instructions. Quantification of gene expression was calculated using the relative standard curve method (Applied Biosystems). Gene-specific primers used in the qRT-PCR assay are listed in Supplemental Table 3. Standard errors were calculated based on a minimum of three biological replicates.

Soluble Solid, Sugar, and Starch Analysis
Soluble solids (°Brix) from fruit juice were measured with a digital refractometer (HR200; APT Co.). For sugar analysis, 200 mg of frozen red ripe tissue samples was ground in a mortar with a pestle in 2.5 mL of methanol/chloroform/water (12/5/3 [v/v/v]) and 2 mL of chloroform. Then, 0.6× volume water was added to create an aqueous phase to separate from the chloroform phase. After centrifugation, the water layer was saved and purified by anion and cation exchange resins to remove charged components. The neutral fractions were vacuum dried and rehydrated with water and analyzed by HPLC using a Waters SugarPak column as described (Turgeon and Medville, 1998). Starch quantification was determined using a starch assay kit (STA20; Sigma-Aldrich) following the manufacturer’s protocol.

Ethylene Measurement
Tomato fruit were placed in open 250-mL airtight jars at room temperature for 2 h to reduce harvest stress. The jars were then sealed, and after 2 h, 1-mL air samples were taken and analyzed by gas chromatography using a Hewlett-Packard 5890 series II with a flame ionization detector and an activated alunina column. Ethylene concentrations were calculated by comparison with a standard of known concentration and normalizing for fruit mass.

Carotenoid and Chlorophyll Analysis
Carotenoid and chlorophyll extraction and carotenoid quantification by HPLC were performed as previously described (Vrebalov et al., 2009). For chlorophyll, the dried down extracts were resuspended in 100% acetone, and the amount of chlorophyll was determined spectrophotometrically using published formulas (Lichtenthaler, 1987).

Ascorbate Measurement
Ascorbate levels were determined spectrophotometrically based on the ΔA\textsubscript{265} following ascorbate oxidation as previously described (Alba et al., 2005).

Chloroplast Counting and Transmission Electron Microscopy
For plastid counting, immature outer fruit pericarp tissues were fixed in 3.5% glutaraldehyde solution for 1 h in the dark and then heated in 0.1 M Na\textsubscript{2}EDTA at 60°C for 10 to 15 min as previously described (Cookson et al., 2003). Numbers of chloroplasts were determined from separated cells using a Leica DMS500 epifluorescence microscope. For transmission electron microscopy, outer pericarp tissues were fixed in 2% glutaraldehyde in 0.05 M cacodylate buffer with picric acid for 2 h at 4°C and then rinsed three times for 10 min each in 0.05 M cacodylate buffer. Tissues were post fixed in 1% Os\textsubscript{2}O\textsubscript{4} for 1 h at 4°C, rinsed again with cacodylate buffer, and dehydrated in an ethanol series. Tissues were infiltrated with EmBed-812 resin, embedded in molds, and polymerized at 60°C for 48 h. Ultratricotomized sections were viewed with a FEI Tecnai T12 Twin transmission electron microscope.

Transcriptome Sequencing
Fruit were harvested at the stages indicated in the text from three biological replicates, and each replicate contained four to five combined fruit of the same developmental stage and from the same genotype. For gradient expression, fruit were equally cross-sectioned in five parts and pericarp tissues from the top (stem end), middle, and bottom (stylar end) parts were kept for RNA extraction. The two intervening sections were discarded. Total RNA from pericarp tissues was used to construct strand-specific RNA libraries as described (Zhong et al., 2011). Two or three biological replicates for each sample were sequenced using a HiSeq2000 sequencing system (illumina) according to the manufacturer’s instructions.

Chromatin Immunoprecipitation Sequencing
Chromatin immunoprecipitation sequencing and subsequent data analysis were performed as described (Zhong et al., 2013) using H3K4me3 antibody (Millipore).

RNA-Seq Data Processing and GO Enrichment Analysis
RNA-seq reads were first processed by removing barcode and adaptor sequences. The resulting reads were aligned to rRNA and tRNA sequences using Bowtie allowing up to three mismatches (Langmead et al., 2009) to remove potential contaminating reads. The resulting cleaned reads were then aligned to the tomato genome using Tophat, allowing one segment mismatch (Trapnell et al., 2009). Following alignments, raw counts of mapped reads for each tomato gene model were derived and then normalized to RPKM. Differentially expressed genes between transgenic and control tissue were identified using the DESeq package (Anders and Huber, 2010). To identify differentially expressed genes across the fruit gradient, the raw counts of RNA-seq expression data were first transformed using the getVarianceStabilizedData function in the DESeq package. The variance-stabilizing transformed expression data were then fed to the LIMMA package (Smyth, 2004), and F tests were performed. Raw P values were adjusted for multiple testing using the Benjamini-Hochberg procedure (Benjamini and Hochberg, 1995). Differentially expressed genes were then fed to the Plant MetGenMAP system to identify enriched GO terms (Joung et al., 2009).

Accession Numbers
Full-length cDNA sequences of tomato GLK1 and GLK2 can be found in the GenBank/EMBL data libraries under accession numbers JQ316460 and JQ316459, respectively. Transcriptome sequencing reads were submitted to the National Center for Biotechnology Information Sequence Read Archive (accession number SRA079879 for the GLK overexpression fruit tissues and accession number SRA080322 for the cross-sectioned fruit tissues).

Supplemental Data
The following materials have been deposited in the DRYAD repository (http://datadryad.org) under accession number doi: 10.5061/dryad.v154j.

Supplemental Figure 1. GLK2 Expression Level in the Wild Type and RNAi TAGL1-Repressed Green Fruit Tissues.

Supplemental Figure 2. Number of Genes Showing Differential Expression along Three Latitudinal Sections of Tomato Fruit Tissues during Fruit Development Stages.

Supplemental Figure 3. Gradient Expressions of Ethylene-Related Genes.
Supplemental Figure 4. Expression Levels of Known Ripening Regulators during Fruit Development along the Latitudinal Axis of Tomato Fruit.

Supplemental Figure 5. Expression Levels of GLK1 and GLK2 during Fruit Development along the Latitudinal Axis of Tomato Fruit.

Supplemental Figure 6. °Brix Level along the Latitudinal Axis of Tomato Red Fruit.

Supplemental Table 1. GO Term Enrichment of GLK1-Upregulated Genes.

Supplemental Table 2. GO Term Enrichment of GLK2-Upregulated Genes.

Supplemental Table 3. DNA Primer Sequences.

Supplemental Data Set 1. List of Genes Upregulated by GLK1 and GLK2 Overexpression in Immature Green (15 DPA) and Red (Breaker + 10) Fruit Tissue and List of Differentially Expressed Genes along Three Latitudinal Sections of Wild-Type ‘Ailsa Craig’ U/U Fruit Tissues during Tomato Fruit Development.

Supplemental Data Set 2. List of Differentially Expressed Genes between the Stem End (Top) versus Stylar End (Bottom) of Mature Green Fruit Tissues and List of Differentially Expressed Genes between the Stylar End (Bottom) of Mature Green Fruit Tissues versus Stylar End (Bottom) of Breaker Fruit Tissues.

Supplemental Data Set 3. Normalized RPKM Values of Transcriptomes from Fruit Transgenic Tissues Overexpressing Si-GLK1 or Si-GLK2 Compared with the Control ‘M82’ u/u.

Supplemental Data Set 4. Normalized RPKM Value of Transcriptomes from Three Latitudinal Sections of Wild-Type ‘Ailsa Craig’ U/U Fruit during Tomato Fruit Development.

ACKNOWLEDGMENTS

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AUTHOR CONTRIBUTIONS

C.V.N. and J.J.G. designed the research, C.V.N., J.T.V., N.E.G., and S.Z., performed the research, C.V.N., J.T.V., N.E.G., S.Z., Y.Z., Z.F., and J.J.G. analyzed the data. C.V.N. and J.J.G. wrote the article.

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