A comparative transcriptomics and eQTL approach identifies SlWD40 as a tomato fruit ripening regulator

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Abstract

Although multiple vital genes with strong effects on the tomato (Solanum lycopersicum) ripening process have been identified via the positional cloning of ripening mutants and cloning of ripening-related transcription factors (TFs), recent studies suggest that it is unlikely that we have fully characterized the gene regulatory networks underpinning this process. Here, combining comparative transcriptomics and expression QTLs, we identified 16 candidate genes involved in tomato fruit ripening and validated them through virus-induced gene silencing analysis. To further confirm the accuracy of the approach, one potential ripening regulator, SlWD40 (WD-40 repeats), was chosen for in-depth analysis. Co-expression network analysis indicated that master regulators such as RIN (ripening inhibitor) and NOR (nonripening) as well as vital TFs including FUL1 (FRUITFUL), SINAC4 (NAM, ATAF1,2, and CUC2 4), and AP2a (Activating enhancer binding Protein 2 alpha) strongly co-expressed with SlWD40. Furthermore, SlWD40 overexpression and RNAi lines exhibited substantially accelerated and delayed ripening phenotypes compared with the wild type, respectively. Moreover, transcriptome analysis of these transgenics revealed that expression patterns of ethylene biosynthesis genes, phytoene synthase, pectate lyase, and branched chain amino transferase 2, in SlWD40-RNAi lines were similar to those of rin and nor fruits, which further demonstrated that SlWD40 may act as an important ripening regulator in conjunction with RIN and NOR. These results are discussed in the context of current models of ripening and in terms of the use of comparative genomics and transcriptomics as an effective route for isolating causal genes underlying differences in genotypes.

Received November 19, 2021. Accepted March 28, 2022. Advance access publication May 4, 2022

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Introduction

Given that seed dispersal is of major ecological and evolutionary importance for all plants and the fact that fleshy fruit plays a vital role in this process, fruit ripening assumes a central importance in the plant life-cycle. It is well documented that hundreds of genes display altered expression during this process (Karlova et al., 2011; Osorio et al., 2011), and that metabolism also undergoes concurrent dramatic shifts to form fruit quality (Carrari and Fernie, 2006). As one of the most important appearance qualities, the accumulation of carotenoids, when combined with naringenin chalcone tailed yellow peel, forms the reddish color of tomato fruits (Solanum lycopersicum) (Ballester et al., 2010; Fernandez-Moreno et al., 2016; Zhu et al., 2018). Among the carotenoid biosynthesis pathway, phytoene synthase (PSY) is the key rate-limiting enzyme of the whole pathway; it catalyzes two molecules of GGPP to form the colorless phytoene. Subsequently, under the catalysis of a series of enzymes, phytoene undergoes dehydrogenation and isomerization reactions to form lycopene, which is the dominant carotenoid of tomato fruit (Bird et al., 1991; Bartley and Scolnik, 1993; Cazzonelli and Pogson, 2010; Chen et al., 2019). In addition, the texture of fruits is affected by the modification of cell walls and pectate lyase (PL), which hydrolyzes pectin and is the most substantially cell wall gene contributor to this process identified to date (Yang et al., 2017). Besides appearance and textural qualities, another important quality aspect is taste, which has been attributed to the sugar/organic acid ratio, and volatile and secondary metabolite accumulation. The key genes underlying the levels of these metabolites have been uncovered by a range of quantitative trait loci (Fridman et al., 2004; Tieman et al., 2006; Schauer et al., 2008; Centeno et al., 2011) and genome-wide association studies (Sauvage et al., 2014; Tieman et al., 2017; Ye et al., 2017; Gao et al., 2019).

Moreover, the considerable metabolic changes are coordinated and mediated by transcription factors (TFs) and epigenome dynamics on the metabolic structural genes’ expression (Centeno et al., 2011; Rohrmann et al., 2011; Giovannoni et al., 2017; Lu et al., 2018; Li et al., 2020). Over the last 50 years, several mutants, such as ripening-inhibitor (rin), nonripening (nor), Never ripe (Nr), and Colorless nonripening (Cnr) mutations, have been identified as severely impacting the tomato ripening process (Tigchelaar, 1973; Lanahan et al., 1994; Vrebalov et al., 2002; Manning et al., 2006). Among these mutants, rin is the one of the most famous ripening delaying mutants substantially lacking the ethylene burst and hindering the color change and softening processes, which results from the repression of the ripening inhibitor-macrocalyx (RIN-MC) chimeric protein (Robinson, 1968; Vrebalov et al., 2002; Ito et al., 2017). The integrated analysis of chromatin immunoprecipitation (ChiP)-chip and transcriptome indicated that RIN can directly induce the expression of the key ripening-related structural and regulator genes, ACS2/4, SGR1, PSY, Cel2, EXP1, PAL1, CaH, LoxC, AAT1, CNR, NOR, AP2a, and itself (Fujiwasa et al., 2012, 2013; Irfan et al., 2016). Furthermore, the transcriptional behavior of 1,000 TFs has been established during tomato ripening (Rohrmann et al., 2011) and gene regulator networks have been modeled on the basis of these data (Rohrmann et al., 2012). The stronger acting ripening genes mentioned above were, in contrast, identified in mutant screens intent on isolating strong mutants in order to enhance tomato shelf-life (Tigchelaar, 1973; Lanahan et al., 1994; Vrebalov et al., 2002; Manning et al., 2006). While recent years have resulted in the identification of many additional genes with ripening consequences (Li et al., 2021; Shi et al., 2021), it is probable that additional contributors to this complex process remain to be found. As such, further genome-wide analysis is required to mine the regulator affecting this process and provide more comprehensive knowledge on this process.

Time-series, species/accession specific or tissue specific and comparative transcriptomics studies have previously deciphered gene regulatory networks underlying plant developmental pathways allowing the identification of additional functional genes (Breschi et al., 2017; Chang et al., 2019; Batyrshina et al., 2020; Baranwal et al., 2021). For example, Bolger et al. (2014) compared the transcriptomes of M82 and Solanum pennellii, and identified 100 key candidate genes related to salt and drought stress. Additionally, transcriptomics studies on genetic mapping populations have defined expression QTLs (eQTLs) as genomic loci controlling variation in steady state levels of transcript between individuals (Sonderby et al., 2007) of what has subsequently become a well-characterized mapping population (Ofner et al., 2016; Szymanski et al., 2020). During tomato domestication, many phenotypes (such as the leaf structure and ripening process) of wild species, such as S. pennellii were under strong selection and were substantially different to that of in the cultivar species S. lycopersicum. Based on the eQTL analysis of the 76 introgression lines (ILs) from S. pennellii in the background of S. lycopersicum, Ranjan et al. (2016) identified important genetic regulators of leaf development on chromosomes 4 and 8. The above-mentioned studies demonstrate the power of comparative transcriptomics in combination with ILs; however, limited studies have been carried out using their integrated approach to mine the genes involved in tomato fruit ripening.

As a distant relative of the cultivated tomato S. lycopersicum, S. pennellii has many substantially different phenotypes with the cultivated tomato and one of these is the mature fruit morphology. The mature fruit of S. lycopersicum is red and soft while the mature fruit of S. pennellii is green and hard, which renders this pair the ideal parents to cross and illustrate the genetic landscape of fruit ripening. The core set of 76 S. pennellii ILs, which represent whole-genome coverage of S. pennellii in overlapping segments in the background of M82, have been widely used to identify the key genes of many traits such as yield and metabolic composition (Semel et al., 2006; Alseekh et al., 2013, 2015). In the present study, to identify key candidates regulating tomato fruit morphology under various conditions using an integrated approach to mine the genes involved in tomato fruit ripening.
fruit ripening, an integrated comparative transcriptomics and eQTL approach was taken utilizing *S. pennelli* ILs (Eshed and Zamir, 1995). We isolated 16 candidates and provided primary validation of eight of them as being involved in the ripening process via the virus-induced gene silencing (VIGS) method. Following this screen, one candidate, *SIW/D40*, was taken for further study. For this candidate stable RNAi and overexpression (OE) lines were generated and characterized. The OE of *SIW/D40* promoted ripening while its inhibition inhibited it. The co-expression networks, metabolome and transcriptome analysis indicated that *SIW/D40* acted as a positive regulator of tomato ripening with the key ripening TFs such as *RIN*, *NOR*, *AP2a*, and *SlWRKYs*. These results are discussed within the context of their implications regarding fruit ripening as well as with respect to the utility of genomic information in filling our knowledge gaps in important biological processes.

**Results**

Integrating comparative transcriptomics and eQTL mapping to mine for genes involved in tomato ripening

Our earlier work described a high-quality genome assembly of the parents of the *Solanum pennelli* IL population as well as identifying candidate genes involved in salt as well as drought stress tolerance (Bolger et al., 2014). Surprisingly, the open-reading frame sequence of most well-characterized ripening-related genes is identical between *S. pennelli* and *S. lycopersicum*. We therefore thought to try a comparative transcriptomics approach of the *S. pennelli* IL population since studies on fruit gene expression of a subset of the ILs has proven highly informative (Baxter et al., 2005; Alseekh et al., 2015) as well as in leaves (Chitwood et al., 2013). For this purpose, as an initial approach, transcriptome data for *M82* and *S. pennelli* mature fruits were sorted as follows: the total 34,727 genes in transcriptome sorted into two different data sets named as Lyco and Penn (Figure 1A) based on the ratio of their expression values. The Lyco data set contained genes that are highly expressed in *M82* (13,521), while the Penn data set contained genes that are highly expressed in *S. pennelli* (11,781) (Figure 1A). For the Lyco genes which we reasoned would be more likely to harbor genes underlying the "red" ripe phenotype of cultivated tomatoes, around 300 candidates could be narrowed down by using three independent filters. Firstly, we chose to focus on genes for which expression was at least five times higher in *S. lycopersicum* with respect to *S. pennelli*. Secondly, based on the transcriptome profiling of red ripe fruit from *S. lycopersicum* (*M82*) parent and a set of lines with distinct introgressed *S. pennelli* segments (http://red.bti.cornell.edu/cgi-bin/TFGD/array_data/home.cgi), large numbers of specific eQTL and nonspecific eQTL have been identified as the former definition that specific eQTL candidates are the candidates whose expression are sharply (exponentially) increased or decreased in its located IL compared with other 75 ILs while nonspecific eQTL candidates’ expression does not show such specificity and have expression in all ILs. With the specific eQTL and nonspecific eQTL information, the candidates of Lyco data set were classified into 119 specific and 223 nonspecific eQTLs (Figure 1B). For the Penn data set, around 105 specific and 202 nonspecific candidates were classified (Figure 1C). Here, the high number of nonspecific eQTL is attributed to the epistatic interactions between *S. pennelli* alleles and *M82* alleles or, alternatively, the presence of a large number of trans-eQTL as previously reported for leaf expression analysis (Chitwood et al., 2013) and fruit enzyme abundance analysis (Steinhauser et al., 2010). Previous studies have indicated that the candidates whose functional categories belong to transcription regulators, oxidase and cytochrome P450 may be involved in regulating tomato fruit ripening and secondary metabolism; therefore, we chose seven candidate genes that were of these three functional categories among the specific eQTL candidates.

In a parallel approach, given that TFs act as important regulators in fruit ripening, we also adopted a TF-centric approach (Figure 1D). From the total of 34,727 genes, candidates annotated as TFs and displaying more than five times higher expression in *S. lycopersicum* were selected. Next, eQTL mapping thinned the list to 127 candidates which were then arranged with respect to the ratio of their expression in *Breaker* + 10 to that in *Breaker* stage. Based on putative ortholog information (*Arabidopsis thaliana* and *S. lycopersicum*) and literature survey concerning their putative functions, a final set of 20 candidates was selected. Finally, on the basis of tissue specific expression and the *S. lycopersicum* to *S. pennelli* expression ratio, the 7 candidates from eQTL approach and 20 candidates from TF approach were narrowed down to the 16 potential candidates described in Supplemental Data Set S1.

**VIGS analysis of candidate genes**

To provide preliminary analysis of the function of the candidate genes in tomato ripening, we carried out VIGS experiment using purple Microtom cv. tomato fruit which accumulate high amount of anthocyanin resulting from the introduction of *Del/Ros1* petunia (*Petunia hybridra*) TFs (Orzaez et al., 2009). Partial fragments of the 16 candidate genes were cloned into pTRV2-Ros/Del/GW vector. Around 10–15 fruits per plant were infected with agrobacterium carrying the respective VIGS vector. After silencing *Del/Ros1* (empty vector) in Microtom *Del/Ros1* fruits, there was depletion in purple pigments but not in lycopene content due to the silenced part accumulating less purple anthocyanin pigments and thereby being easy to discriminate from non-silenced (purple pigment rich) tissues (Figure 2). Phenotypes were scored visually after 15 d of infection for all the 16 validated candidates (Supplemental Data Set S1).

Red color of western tomato cultivars represents the accumulation of lycopene, which is an important indicator of tomato ripening. VIGS for the structural genes encoded by Solyc01g094080 and Solyc03g095900 displayed a red phenotype, indicating that these genes are not associated with...
Figure 1 Candidate gene filtration by integrating comparative transcriptomics and eQTL mapping. A, Pipelines for candidate gene filtration of eQTL approach. Filters are shown in bullet points. B, Heat map of relative expression level of filtered candidates. Lyco/Penn, Genes were sorted by ratio of expression value for *S. lycopersicum* and *S. pennellii*. ILs are arranged as per the number of chromosome (X-axis). Genes are arranged according to their Gene IDs (Y-axis). Regions of red or blue indicate that the gene expression is increased or decreased, respectively, over that of M82. Chr, chromosome. C, Heat map of relative expression level of filtered candidates. Penn/Lyco, Genes were sorted by the ratio of expression value for *S. pennellii* and *S. lycopersicum*. ILs are arranged as per the number of chromosome (X-axis). Genes are arranged according to their Gene IDs (Y-axis). Regions of red or blue indicate that the gene expression is increased or decreased, respectively, over that of M82. D, Pipelines for candidate gene filtration of TFs approach. Filters are shown in bullet points. Br, Break.

Figure 2 VIGS of empty vector (silencing of Del/Ros1) and SIWD40 in Microtom Del/Ros1 fruit.
lycopene biosynthesis or the pathways that fuel it. However, as the TFs regulating ripening are generally reported to hinder carotenoid biosynthesis, the yellowish phenotypes of Solyc11g010710 (ethylene response factors, ERF TF) and Solyc07g052700 (MADS-box TF, AGL66) VIGS fruits indicate that they may function as a ripening regulators in line with former studies that implicated SIERFs and MADS-box TFs in tomato fruit ripening (Wang et al., 2014; Liu et al., 2016; Supplemental Data Set S1 and Supplemental Figure S1).

Interestingly, the VIGS fruits of a transcription regulator, SIW/D40 (Solyc04g005020) also exhibited a yellowish phenotype. Given that ERF and MADS box family TFs are already well-known to be involved in tomato fruit ripening and that SIW/D40 was identified as a downstream target gene of RIN (Fujisawa et al., 2013), we selected SIW/D40 for in-depth analysis here (Figure 2).

Co-expression network and VIGS of SIW/D40 confirmed its role in tomato fruit ripening

In order to analyze the function of SIW/D40 on fruit ripening, we initially identified its potential regulators following cis-regulatory element analysis of the promoter of SIW/D40. This analysis indicated that the promoter contained several ethylene (AP2, B3, EIN3, and EIL) and ripening-related elements (C2H2, MADS, NF-YB, NF-YA, and NF-YC) in the 1-kb promoter region, which indicated that it may well be induced by the ripening and ethylene burst (Supplemental Figure S2). Moreover, the evaluation of publicly available expression data with tissue-specific expression analysis of SIW/D40 confirmed the hypothesis that SIW/D40 was only slightly expressed in the leaf, bud, flower, root, and young fruit but that its expression increases exponentially following mature green stage (Tomato Genome Consortium, 2012). Intriguingly, its expression in different cell types of the tomato fruit revealed that it is highly similar to that of the other known ripening regulators, such as RIN and NOR (Shinozaki et al., 2018).

Moreover, given that assembly of co-expression networks is an efficient method to identify the important interactions and relationship among different genes (Mutwil et al., 2011), available transcriptome data of different organ and fruit development stages were used to construct tomato co-expression network (Figure 3 and Supplemental Table S1; Tomato Genome Consortium, 2012). The co-expression sub-network containing SIW/D40 included 171 structure genes/regulators, which are involved in chlorophyll and carotenoid metabolism as well as tomato fruit ripening and cell wall metabolic pathways. Among the 171 genes, a total of 62 genes exhibited high co-expression phenotype (|Co-expression Coefficients| > 0.6, P < 0.05) with SIW/D40 (Supplemental Table S1). Consistent with the results of cis-regulatory element analysis, three MADS TFs, including RIN, two AP2s TFs, and one ARF TF were significantly positively co-expressed with SIW/D40. Moreover, another vital ripening-related TF, NOR, exhibited a co-expression coefficient of 0.86 with SIW/D40 (Supplemental Table S1). Besides the master ripening-related TFs, SIW/D40 also highly co-expressed with key carotenoid-related genes (PSY1), as well as ethylene (ACS4) and abscisic acid (NCED3) biosynthesis genes and cell wall modification genes (PL and PMEI) (Supplemental Table S1). All of these results indicate that SIW/D40 may act in concert with the better characterized ripening TFs to regulate the ripening processes, including those dependent on changes in pigmentation, hormone levels, and signaling and cell wall modification.

SIW/D40 affects the tomato fruit transcriptome

To confirm the accuracy of our approach and to assess in detail the function of SIW/D40 in the tomato ripening process, we chose the fruit specific patatin B33 promoter which has been widely used for fruit specific expression to carry out the stable transformation (Rocha-Sosa et al., 1989; Vallarino et al., 2020). T0 transformants of RNAi and OE lines were characterized by NPT-II-specific polymerase chain reaction (PCR). Real-time quantitative polymerase chain reaction (RT-qPCR) was also carried out using fruit samples from promising T0 transgenics to select high OE and knockdown lines to raise T1 generation (Supplemental Figure S3). Fruits from all generations were analyzed and phenotype was stable over T0 and T1 generations. Before the T1 generation plant transplant to soil, we also used the NPT-II-specific PCR to confirm that the plants are transgenic. Based on the expression of SIW/D40, two independent lines of RNAi (RNAi-1 and -2 lines) and OE (OE-1 and -2 lines) were chosen for subsequent experiments (Supplemental Figure S3).

To analyze fruit phenotype at the identical stage, fruits of each genotype were labeled upon anthesis and harvested for phenotyping, transcriptome, and metabolite profiling at mature green (MG, 34 DPA), breaker (Br, 37DPA), and pink (Pink, 45 DPA) stages of the wild type (WT). As seen in Figure 4, the development and ripening process were substantially hindered in the RNAi fruit while the ripening process was significantly accelerated in comparison to the OE lines. Moreover, especially at the Br stage of WT fruits, the size of RNAi fruits was significantly smaller than that of the OE and WT lines and the RNAi fruits were still at the mature green stage while the OE fruit were almost at the pink stage. The contents of chlorophylls and carotenoids, some of the most important parameters of fruit ripening, also confirmed the positive function of SIW/D40 on tomato ripening process: The degradation of chlorophylls and synthesis of the predominant carotenoid, lycopene, were significantly hindered in RNAi fruits but accelerated in the OE fruits (Figure 4B).

In order to estimate the effect of SIW/D40 on the global difference of gene expression during the different fruit developmental stages, we additionally analyzed the differentially expressed genes (DEGs) among the RNAi, OE lines, and WT fruit at MG, Br, and Pink stages. For this purpose, we used FPKM (fragments per kilobase per million mapped fragments) and identified genes with |log2 (fold change)| ≥ 1 and false discovery rate (FDR) (corrected P value) < 0.05 (Supplemental Table S2). Firstly, we checked the SIW/D40
expression among the different genotypes at the MG stage. Given the low expression level of *SlWD40* of WT fruit at MG stage, RNAi fruit did not exhibit significantly different expression from WT fruit. However, as the ripening process was initiated, the expression of *SlWD40* was significantly induced and its expression was remarkably lower in the RNAi fruit compared with that of WT fruit at Br and Pink stages without affecting the expression of other WD40 family genes (Supplemental Table S2). In the OE fruits, *SlWD40* expression was 5.64- and 5.71-fold higher than that of WT fruit at the MG stage. That said owing to the massive induction of the endogenous *SlWD40* expression, the OE effect of B33 promoter was concealed and *SlWD40* expression was not significantly different between the OE and WT fruit at the Br and Pink stages (Supplemental Table S2). These results were further confirmed by the principal component analysis (PCA) and cluster analysis based on the transcriptome data of different samples. RNAi samples were closely grouped with WT at MG stage but substantially separated samples at Br and Pink stages. Conversely, OE samples were clustered with WT sample especially at the Pink stage and subsequently separated from the WT sample at the MG stage.

**Figure 3** Co-expression network of *SIWD40* with tomato ripening pathway-specific genes. Well-characterized key regulators such as RIN (ripening inhibitor), NOR (nonripening), and FUL1 (FRUITFUL1) (labeled in blue) strongly co-expressed with *SIWD40*. ELIP, early light induced protein; NF-Y, nuclear factor Y; MADS, MADS domain protein.

**Figure 4** Photographs and pigments content of WT and T1 generation RNAi (lines 1 and 2) and OE (lines 1 and 2) lines at 28, 34 (MG), 37 (Br), and 45 (Pink) DPA fruits. A, Photographs of WT and transgenic *SIWD40* fruits. Images were digitally extracted for comparison. B, Chlorophylls and carotenoids of WT and transgenic *SIWD40* fruits. The values in each column are the mean of at least three biological replicates. Error bars indicate SD. The asterisks indicate statistically significant differences determined by the Student’s t test (two-tail): *P < 0.05; **P < 0.01. ND, not detected.
In order to further mine the important DEGs under the effect of SIWD40, we further analyzed the overlapping DEGs of OE-WT fruit at MG stage and RNAi-WT fruits at Br and Pink stage. The results indicate that 244 genes were stably downregulated in the OE-MG and upregulated in the RNAi Br and Pink stages, while 60 genes were stably upregulated in the OE-MG and downregulated in the RNAi Br and Pink stages (Supplemental Table S3). To further mine the functional categorization of DEGs, AgriGO v2.0 analysis tools (http://bioinfo.cau.edu.cn/agriGO/) by singular enrichment analysis has been used based on the conserved DEGs (Wang et al., 2017; Supplemental Table S4). Among the GO terms included in the “Molecular Function” category of the DEGs upregulated in the OE-MG and downregulated in the RNAi Br and Pink stages, the pathways that affected the lysis and enzyme activity, such as lyase activity (FDR = 0.0014), oxidoreductase activity (FDR = 0.011), and monoxygenase activity (FDR = 0.013), were enriched. Moreover, in the “Biological Process” category of DEGs downregulated in the OE-MG and upregulated in the RNAi Br and Pink stages, several cell-wall-related pathways were significantly enriched (Supplemental Table S4). Interestingly, in the SIWD40-OE lines, the expression of SIWRKY75 (Slyc05g015850.4.1), SIWRKY37 (Slyc02g021680.3.1), SIWRKY23 (Slyc01g079260.4.1), SIWRKY30 (Slyc07g056280.3.1), SIWRKY6 (Slyc02g080890.3.1), SIWRKY17 (Slyc07g051840.4.1), SIWRKY31 (Slyc06g066370.4.1), and SIWRKY79 (Slyc02g072190.4.1) was increased by 5.7-, 3.4-, 2.6-, 2.4-, 2.2-, 2.2-, and 1.8-fold, respectively. Moreover, since fruit size of SIWD40-RNAi fruits was smaller and IAA content directly affects organ size, we found that the expression of SIGH3-2 (gene regulating auxin homeostasis) was increased by three-fold in OE lines while the level of the same gene was decreased in RNAi lines by three- to six-fold.

Additionally, given that SIWD40 was strongly co-expressed with RIN and NOR, the conserved DEGs of rin, nor mutants, and SIWD40 transgenic fruits were analyzed and 31 genes were found as conserved DEGs across the genotypes (Figure 5C and Supplemental Table S5; Fujisawa et al., 2012; Gao et al., 2020). Among the 31 genes, several important ripening-related genes, such as ACS4 for ethylene biosynthesis, PSY and Z-ISO for carotenoid biosynthesis, PL and PMEI for cell wall modification, INV for sugar metabolism, and branched chain amino transferase 2 (BCAT2) and THA1 for amino acid metabolism, were significantly downregulated in the SIWD40-RNAi, rin, and nor fruits and upregulated in the SIWD40-OE fruits, which indicates that SIWD40 may participate in some or all of the primary regulatory functions of these important regulators, such as RIN and NOR. As the transcriptome data indicated that SIWD40 exhibited...
substantially changed expression in the MG stage of two OE lines and Br and Pink stage of two RNAi lines compared with that of in the WT, to further confirm the expression difference, we checked the expression of *SIWD40* and other 17 ripening-related genes such as *RIN*, *NOR*, *PSY*, and *PL* in these samples. The results indicate that all of these genes were significantly upregulated in the two OE lines at the MG stage but significantly downregulated in the two RNAi lines at Br and Pink stages, which further confirm the positive function of *SIWD40* on tomato ripening with the strongly co-expressed TFs (Figure 6).

**Metabolome analysis of *SIWD40* transgenic fruits**

Given that metabolites are important parameters for estimating progression of the fruit ripening process (Carrari et al., 2006), we analyzed their levels in the different genotypes at a time concurrent to the MG, Br, and Pink stages of WTs. To obtain the global metabolome variation of different samples, a PCA analysis was carried out based on the primary metabolites, lipids, and secondary metabolites. The results indicate substantial differences among RNAi, OE, and WT fruits, which were particularly prominent at the Pink stage. RNAi pink fruits were closest to Br fruits and separated clearly from OE and WT fruits (Figure 7, A and B). Given that *SIWD40* was strongly co-expressed with *RIN* and *NOR*, we analyzed the overlapping differentially enriched primary metabolites between *SIWD40*-RNAi lines, *rin* and *nor* mutants at the Pink stage (Osorio et al., 2011). The results indicated that as the principal free amino acid that most contributes to the “umami” flavor of ripe tomato fruits, glutamate exhibited a conserved lower accumulation in the *SIWD40*-RNAi lines, *rin* and *nor* pink fruits (Figure 7C). In detail, given that several amino acids are increased and organic acid decreased during WT tomato ripening (Carrari et al., 2006), it is of interest that we observed that aspartic acid, glutamic acid, and tryptophan levels were significantly lower in both RNAi lines compared with those exhibited by the OE and WT pink fruits (Figure 8 and Supplemental Table S6). Moreover, nicotinic acid and glyceric acid levels were significantly higher in at least one RNAi line compared with those observed in OE and WT pink fruits (Figure 8 and Supplemental Table S6). Moreover, the former results indicated that the representative lipid components, triacylglycerols (TAGs) are significantly decreased in avocado fruit ripening (Rodriguez-Lopez et al., 2017). The same trends also have been found in the present study that the content of TAG 48:0, TAG 52:5, TAG 52:6, TAG 54:6, TAG 54:7, and TAG 54:8 was remarkably higher in the RNAi fruits than that of OE and WT pink fruits (Figure 9 and Supplemental Table S6). As the secondary metabolites (such as naringenin chalcone, naringenin hexose, and chlorogenic acid derivatives) dramatically accumulate during ripening and represent an important quality of fruit, the significantly higher and lower content of them in the OE and RNAi pink fruits compared with that of WT fruits, respectively, further confirmed the positive influence of *SIWD40* on the fruit ripening process (Figure 9 and Supplemental Table S6).

**Discussion**

We adopted a computational approach to mine key candidate genes involved in tomato fruit ripening by integrating comparative transcriptomics and eQTL analysis. In doing so, we identified 16 previously uncharacterized candidate genes for involvement in ripening. Among them, we chose to follow up on *SIWD40* since it was identified in the ChiP seq screen as a direct target gene of RIN and relatively little is known concerning its regulatory role (Fujisawa et al., 2013). Intriguingly, cross-tissue co-expression networks for *SIWD40* strongly suggest that it may act with the other important ripening regulators, such as *RIN* and *NOR* in affecting tomato fruit ripening, and the detailed analysis of DEGs and
differentially abundant metabolites between SlWD40 transgenic fruits further illuminate the important function of SlWD40 on tomato ripening process.

Integrating comparative transcriptomics with the eQTL approach to mine for ripening regulators
Several tomato genes with strong ripening phenotypes have been identified via mutagenesis-based breeding programs including rin, nor, NR, and Cnr (Tichelaar, 1973; Lanahan et al., 1994; Vrebalov et al., 2002; Manning et al., 2006). Moreover, the recombinant inbred line (RILs) population is also useful to identify key loci regulating quantitative traits including fruit ripening as well as aroma, color, and disease resistance (Kimbara et al., 2018). However, since RILs usually harbor more than one introgression, the possibility of the introgressed loci having beneficial/inhibitory interactions with the genes in the genetic background renders gene function elucidation more complex in RILs. In contrast, ILs are high resolution in that they normally carry only single introgressions and as such epistatic interactions masking single gene effects are largely minimized (Ofner et al., 2016). Here, we used available transcriptome data from red ripe fruit of S. lycopersicum (M82) parent and a set of lines with distinct introgressed S. pennellii segments (http://ted.bti.cornell.edu/cgi-bin/TFGD/array_data/home.cgi) and optimized a candidate gene filtration pipeline. Moreover, on the basis of the distance between QTL and the target transcript, eQTL can either be classified as cis-eQTL (where the gene encoding the transcript resides within the QTL interval) or trans-eQTL with both types being prominent in tomato (Rockman and Kruglyak, 2009; Zhu et al., 2018). Combining the gene filtration pipeline and the comparative transcriptomics, we filtered genes responsible for the red ripe phenotype of tomato fruits (i.e. genes highly expressing only in “red” ripe fruits). Since gene duplication and subsequent functional diversification creates novel metabolic pathways and regulation, we focused on TF families that were evolved by gene duplication and already reported to be regulating tomato fruit ripening (e.g. MADS box and basic helix loop helix (bHLH) TF families) (Hileman et al., 2006; Waseem et al., 2019). Out of the 16 candidate genes, 10 were found to be generated either through tandem or block duplication (https://bioinformatics.psb.ugent.be/plaza). In the VIGS experiment, green and yellow phenotypes have been obtained for the candidates Solyc11g010710 (AP2 like) and Solyc07g052700 (MADS TF, AGL66). Our previously published work implicated AP2a in regulating tomato fruit ripening via regulation of ethylene biosynthesis and signaling (Chung et al., 2010; Karlova et al., 2011). MADS box TFs such as SICMB1 (Solyc04g005320), TAGL1 (Solyc07g055920),
Figure 8  The scheme of major metabolic changes of the transgenic lines. The difference of sugar and amino acid-related metabolites between transgenic lines and WT fruit at MG (A), Br (B), and Pink (C) stage. Blue and red color depicts a decrease and increase in metabolic levels compared with the WT fruit samples, respectively.

Figure 9  The difference of representative lipids and secondary metabolites of T1 transformants of RNAi and OE lines compared with WT fruits. The values in each column are the mean of at least three biological replicates. Error bars indicate SD. The asterisks indicate statistically significant differences determined by the Student’s t test (two-tail): *P < 0.05; **P < 0.01.
and the canonical TF RIN (Solyc05g012020) have previously been reported as positive regulators of tomato fruit ripening (Zhang et al., 2018). Therefore, VIGS for AP2 like and MADS box candidates (Solyc11g107010 and Solyc07g052700) acted as positive control supporting the efficacy of our approach. Moreover, among the 16 genes, VIGS for two bHLH TFs (Solyc03g044460 and Solyc12g098620) showed green and light red phenotypes (Supplemental Figure S1). Here, light red phenotype for Solyc12g098620 is in line with the recently published work by D’Amelia et al. (2019) in which the authors reported that this bHLH TF regulates carotenoid biosynthesis. Additionally, one of the candidates, Solyc12g010950 (alcohol dehydrogenase), obtained a whitish green fruit phenotype, and another candidate, SIWD40, obtained a yellowish fruit phenotype (Figure 2 and Supplemental Figure S1). While the link between the alcohol dehydrogenase and ripening is currently unclear, that for SIWD40 is not without precedence since it is a transcriptional regulator which has been linked to rip and nor in ChIP experiments but not characterized in detail in its own right.

The eQTL for SIWD40 can be defined as a cis-eQTL since SIWD40 is located in the region which has been introgressed by S. pennellii chromosomal architecture in the IL4-1 and IL4-1-1 and SIWD40’s expression is only dramatically lowered for IL4-1 and 4-1-1 (Supplemental Table S7). At the onset of fruit ripening process (MG stage), SIWD40 is moderately expressed in S. lycopersicum (RPKM mean value 31) but negligibly expressed in S. pennellii (RPKM mean value 0.17) and consistent with the ripening stage, the expression of SIWD40 was substantially induced in concert with fruit ripening. Considering the dramatically lowered SIWD40 expression in IL4-1 and 4-1-1 and substantial difference of the red/green colored fruits of S. lycopersicum and S. pennellii, respectively, we hypothesized that SIWD40 may participate in the S. lycopersicum ripening process. Furthermore, VIGS for SIWD40 in MicroTom inhibited normal red coloration. These results demonstrate the utility of our candidate gene filtration pipeline integrating comparative transcriptomics with eQTL in identifying candidate ripening genes.

**SIWD40 is an important regulator of tomato fruit ripening**

Among the eight genes which we validated by VIGS, the information concerning the role of the WD40 family in regulating tomato ripening is the most limited. Ripening function of this gene was further confirmed by the stable OE and RNAi transformation (Figures 4 and 5). WD40 proteins contain a signature WD (Trp-Asp) dipeptide and 40 amino acids in single repeats that then fold into four-stranded anti-parallel β-propeller sheets and are highly promiscuous interactors, being both platforms for protein-DNA and protein–protein interactions (Xu and Min, 2011; Mishra et al., 2012; Chen et al., 2022). In the canonical MYB–bHLH–WD40 protein complex, WD40 acts as a recruiter and stabilizer of the MYB and bHLH protein which enhances the regulation of the complex during anthocyanin biosynthesis (Ramsay and Glover, 2005; Zhang et al., 2014). In the present study, the green phenotype of fruits of SIWD40-RNAi lines clearly demonstrated a positive role of SIWD40 in tomato fruit ripening (Figure 4). Moreover, given that the global gene co-expression analysis is a powerful approach to identify the important interactions among different genes during the development of certain organs, it is important to note that the co-expression network of SIWD40 revealed links with RIN and NOR, while transcriptional analysis indicated they shared conserved regulated genes associated with ethylene (ACs) and carotenoid (PSY and Z-ISO) biosynthesis as well as cell wall degradation (PL and PMEI) and sugar (INV) and amino acid (BCAT2 and THA1) metabolism (Supplemental Table S5). Interestingly, based on available ChIP and transcriptome data, the RIN protein can directly bind to the promoter of SIWD40 and thereby increase its expression (Martel et al., 2011; Fujisawa et al., 2013).

Moreover, as an important TF family member involved in ethylene signal, AP2a belonging to the AP2/ERF superfamily has been shown to inhibit ethylene biosynthesis as well as positively regulate chlorophyll degradation and carotenoid biosynthesis in tomato (Wang et al., 2019). And in Setaria italica, SiAP2 can bind to the SIWD40 promoter to mediate abiotic stress responses (Mishra et al., 2012). In the present study, the promoter region of SIWD40 was found to harbor eight different AP2 TF binding sites and AP2a was significantly induced in SIWD40-OE-MG fruits while repressed in the WD40-RNAi-Breaker fruits (Supplemental Table S2). Additionally, it has been well-documented that several SIWRKYS regulate tomato fruit ripening and lycopene accumulation (Cheng et al., 2016; Wang et al., 2017). In our analysis, SIWD40 OE resulted in upregulation (by two- to five-fold) of a broad number of SIWRKYS (SIWRKY75, SIWRKY37, SIWRKY23, SIWRKY30, SIWRKY6, SIWRKY17, SIWRKY31, and SIWRKY79) in mature green fruits of OE lines. Moreover, SIWRKY17, which was shown to interact with RIN, SIERFB2, and SIERF7, is strongly co-expressed with ELIP2 and RIN in our analysis (Wang et al., 2017; Supplemental Table S1). All of these results indicate that SIWD40 might act as a junction point and facilitate binding of one or more above mentioned co-expressed TFs such as RIN, NOR, AP2a, and SIWRKYS to be involved in tomato ripening process.

Besides the important ripening-related TFs, hormone signaling also plays a vital role in the ripening process. As an important regulator of auxin-ethylene homoeostasis which affects fruit ripening (Kumar et al., 2012; Sravankumar et al., 2018), the expression of SIGH3-2 (Solyc01g107390) increased in SIWD40-OE lines by three-fold while decreased in RNAi lines by three- to six-fold (Figure 6 and Supplemental Table S2). Combined with the induction and repression effect of ACS4 in the SIWD40-OE and -RNAi fruit, respectively, these results collectively indicate that SIWD40 may also affect the tomato ripening process through the regulation of the...
ripening-related hormone homoeostasis. However, considerably further experimentation will be required in order to test this hypothesis.

Conclusion
Our study aimed at better understanding the molecular mechanisms underlying tomato fruit ripening. Comparative transcriptomics of small green fruited wild species with red fruited *S. lycopersicum* alongside eQTL mapping allowed the identification of key candidate genes involved in tomato fruit ripening. Utilizing co-expression networks alongside detailed metabolome and transcriptome analysis indicated that *SlWD40* has a positive impact on tomato ripening process and suggest that it may act in concert with strongly co-expressed TFs such as RIN, NOR, AP2a, and SlWRKYs (Figure 10). Beyond these insights into ripening, we believe our study also acts as a proof-of-concept study whereby the transcriptome of phenotypically divergent wild relatives, alongside eQTL mapping, can be used to identify causal genes underlying trait variance.

Materials and methods
Narrowing down candidate genes involved in fruit ripening
For M82 and *S. pennellii* (Penn) fruit, RNA-seq data are available (Bolger et al., 2014). Moreover, RNA-seq data for *S. pennellii* ILs fruit were also available (http://ted.bti.cornell.edu/cgi-bin/TFGD/array_data/home.cgi). Starting with the RNA-seq data for M82 and *S. pennellii* fruit (Bolger et al., 2014) all 34,727 genes in the transcriptome were sorted in two ways. Firstly, the ratio of their expression value in M82 relative to that in *S. pennellii* and secondly, by ratio of their expression value of *S. pennellii* to that of M82 in order to get genes that are highly expressed in M82 and *S. pennellii*, respectively. Candidates from these two lists were further filtered by using three different criteria namely (1) that the relative fold change (FC) in the expression was at least > 5, (2) the presence of an eQTL for the gene, and (3) the presence of a functional annotation. Subsequently, these candidates were sorted into specific and nonspecific eQTL based on their expression in respective IL. Here, specific and nonspecific eQTL were defined based on the expression of a particular *S. pennellii* candidate in a specific IL or several ILs, respectively. Specific eQTL candidates were then focused and classified based on their function. For promoter analysis, the 1-kb upstream sequence from the start codon of *SlWD40* was retrieved from the SGN tomato genome web browser and the cis-regulatory elements were analyzed using PlantCARE (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/) and PlantPAN 2.0 (http://plantpan2.itps.ncku.edu.tw/index.html) web tools.

*SlWD40* co-expression network construction with tomato ripening pathway genes
We listed 171 target genes involved in carotenoid biosynthesis, tomato fruit ripening, and cell wall metabolic pathways and their regulation. Some of these genes were well characterized. For all 171 genes, expression values were extracted from Tomato Genome Consortium (2012). The R script written by Contreras-Lopez et al. (2018) was used to calculate correlation values and P values, both positive and negative correlation values were calculated and cytoscape was used to visualize network (Shannon et al., 2003).

Virus-induced gene silencing
Vector construction, infiltration, and fruit harvesting procedures were performed as previously described (Orzaez et al., 2006, 2009). Briefly, an approximately 300-bp fragment of the candidate gene was amplified from tomato M82 fruit cDNA using gateway compatible primers and recombined into the GATEWAY vector pDONR207 (Invitrogen, http://www.invitrogen.com/) by the BP reaction following the manufacturer’s protocol to generate an entry clone. An error-free entry vector was confirmed by sequencing and then

*Figure 10* Proposed schematic overview of network of regulatory factors controlling tomato fruit ripening.
recombined with the pTRV2-Ros/Del/GW destination vector using an LR reaction to produce the expression clones pTRV2-Ros/Del/GW-Respective Gene ID. Agrobacterium tumefaciens strain GV3101:pMPP90 was then transformed with sequenced expression vectors by electroporation. In order to infiltrate fruit for VIGS, purple MicroTom tomato was used and agroinfiltration was performed as previously described (Alseekh et al., 2015).

Development of OE and RNAI lines
The sequence encoding Solyc04g005020 was amplified from S. lycopersicum cv. Moneymaker (MM) cDNA by using gene-specific primers and inserted into the pDONR207 by attB recombination to generate entry clone. Primer sequences are provided in Supplemental Table S8. An error-free entry clone was confirmed by sequence analysis before recombination into destination vector B33BinAR for fruit-specific OE and named as B33BinAR_SWD40. Additionally, artificial miRNA (amiRNA) cassette was designed for Solyc04g005020. For this, Solyc04g005020 cDNA sequence was used as target sequence, employing the WMD3 program (http://wmd3.welgelworld.org/cgi-bin/webapp.cgi) to design corresponding amiRs. An overlapping PCR (polymerase chain reaction) strategy was employed with in-hand precursor DNA, following the WMD3 protocol (http://wmd3.welgelworld.or/downloads/CloningofartificialmiRNAs.pdf). The pre-amRs obtained from overlapping PCR (using the athmir-319a backbone) were cloned into the pENTR/D-TOPO vector and the clones were confirmed by DNA sequencing. Subsequently, these sequences were cloned into B33BinAR via Asp718 and BamHI digestion and cohesive end ligation. Primer sequences are provided in Supplemental Table S8. This and other final LR plasmids were then introduced into A. tumefaciens strain GV2260 by electroporation and subsequently submitted for transformation into MM plants using the leaf disc transformation method (McCormick et al., 1986).

Plant material and growth conditions
Transgenic plants for each genotype were selected on kanamycin containing MS medium (50 mg L⁻¹). SNN and MM (WT) were germinated on MS medium without kanamycin. Both transgenic lines and WT were selected and transferred to soil pots for cultivation under long-day conditions (16-h/C1/1986). Both transgenic lines and WT were selected and transferred to soil pots for cultivation under long-day conditions (16-h/C1/1986).

Transcriptome analysis
Two biological replicate samples from two independent plants of each genotype of MG, Br, and Pink stages have been harvested. Total RNA was extracted using the NucleoSpin RNA Plant kit (Macherey-Nagel) and sent to the Novogene Company (Beijing, China) for Illumina HiSeq PE150 sequencing. The cDNA library was constructed following the manufacturer’s recommendations and then purified to remove the low-quality sequences. The clean data are available in Zenodo (https://zenodo.org/) (doi:10.5281/zenodo.5525948 and 10.5281/zenodo.5525946). The RNA seq data were analyzed using LSTrAP (Proost et al., 2017). The clean reads of each sample were aligned to the Tomato Genome version SL4.0 and Annotation ITAG4.0 (ftp://ftp.solgenomics.net/tomato_genome/annotation/ITAG4_0_release/). The DEGs between transgenic fruit and WT fruit were identified under the parameter of FC ≥ 2 and FDR < 0.05.

Metabolic profiling
Fruit pericarp samples were harvested, immediately frozen in liquid nitrogen, and stored at −80°C until further analysis. Samples were then powdered by using retsch mill at 30 L·s⁻¹, for 30 s. Extraction of pigments, primary metabolites, lipid, and secondary metabolites was performed as described previously (Salem et al., 2016). In brief, 500 μL of the upper lipid and pigments containing phase was dried in a SpeedVac concentrator and resuspended in 250 μL acetonitrile: 2-propanol (7:3, v/v) solution. Two microliters of the solution were analyzed by the Waters Acquity ultra-performance LC system coupled with Fourier transform MS in positive ionization mode. Moreover, 150 and 300 μL of the polar phase were dried in a centrifugal vacuum concentrator for primary and secondary metabolite profiling. The primary metabolite pellet was resuspended in 40 μL of methoxyaminohydrochloride (20 mg·mL⁻¹ in pyridine) and derivatized for 2 h at 37°C. Afterward, 70 μL of N-methyl-N-[trimethylsilyl] trifluoroacetamide was added containing 20 μL·mL⁻¹ fatty acid methyl esters mixture as retention time standards. The mixture was incubated for 30 min at 37°C at 400 rpm. A volume of 1 μL of this solution was used for injection. The gas chromatography–mass spectrometry system comprised a CTC CombiPAL autosampler, an Agilent 6890N gas chromatograph, and a LECO Pegasus III time of flight mass spectrometry (TOF-MS) running in EI+ mode. The secondary metabolite pellet was resuspended in 200-μL 50% (v/v) methanol in water and 2 μL was injected on RP high strength silica T3 C18 column using a Waters Acquity UPLC system. The analysis workflow included peak detection, retention time alignment, and removal of chemical noise following the method of Salem et al. (2016). For metabolites and transcriptome data processing, the PCA and heat map analysis were performed by MetaboAnalyst 5.0 (https://www.metaboanalyst.ca/).

RT-qPCR analysis
Total RNA was extracted from fruit using TRIzol reagent (Invitrogen, Waltham, MA, USA). The first-strand cDNA synthesis was carried out as the manufacturer’s instructions of PrimeScript RT Reagent Kit with gDNA Eraser (Takara, Shiga, Japan). RT-qPCR was performed using an ABI Prism 7900 HT real-time PCR system (Applied Biosystems/Life Technologies, Darmstadt, Germany) in 384-well PCR plates. The RT-qPCR data were analyzed using the 2−ΔΔCt analysis method according to Bustin et al. (2009) and all primers are listed in Supplemental Table S8.
**Statistical analysis**

Student’s paired t test was performed to assess whether the differences between different genotypes were statistically significant. The asterisks indicate statistically significant differences determined by the Student’s t test (two-tail): *P < 0.05; **P < 0.01.

**Accession numbers**

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers SIW40, Solyc04g005020.

**Supplemental data**

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

- **Supplemental Figure S1.** VIGS phenotype of candidate genes.
- **Supplemental Figure S2.** Promoter analysis of SIW40 for the presence of ethylene (C2H2, AP2, EIN), auxin, and MADS-box binding-related cis-regulatory elements.
- **Supplemental Figure S3.** Genotyping of the SIW40 of T0 transformants.
- **Supplemental Table S1.** Co-expression network of SIW40 with tomato ripening pathway specific genes.
- **Supplemental Table S2.** Transcriptome profiling of SIW40 transgenic fruits.
- **Supplemental Table S3.** The overlapped DEGs of SIW40 OE and RNAi fruit.
- **Supplemental Table S4.** Functional categorization of DEGs of SIW40.
- **Supplemental Table S5.** The overlap DEGs of SIW40, rin, and nor mutants.
- **Supplemental Table S6.** Metabolite profiling of SIW40 transgenic fruits.
- **Supplemental Table S7.** Expression of SIW40 in ILs.
- **Supplemental Table S8.** Primer sequences used in this study.

**Supplemental Data Set S1.** Finalized potential candidates from both eQTL and TF approaches and their VIGS phenotypes.

**Acknowledgments**

We thank Dr Youjun Zhang and Regina Wendenburg from Max-Planck-Institut für Molekulare Pflanzenphysiologie for useful discussion and experiment assistance.

**Funding**

F.Z. acknowledges funding of The Key R&D Program of Hubei Province (2021BBA095) and the National Natural Science Foundation of China (32002102) and the work in Fernie Lab was supported by the Deutsche Forschungsgemeinschaft in the framework of Deutsche Israeli Project FE 552/12-1. In addition, S.A. and A.R.F. acknowledge funding of the PlantASYST project by the European Union’s Horizon 2020 Research and Innovation Programme (SGA-CSA No. 664621 and No. 739582 under FPA No. 664620). S.S.J. acknowledges funding by ICAR, India, in the form of ICAR-International Fellowship.

**Conflict of interest statement.** None declared.

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