Running title: Molecular networks of fruit ripening mutants

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Systems biology of tomato fruit development: combined transcript, protein and metabolite analysis of tomato transcription factor (nor, rin) and ethylene receptor (Nr) mutants reveals novel regulatory interactions

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ABSTRACT

Tomato (*Solanum lycopersicum*) is an established model to study fleshy fruit development and ripening. Tomato ripening is regulated independently and cooperatively by ethylene and transcription factors, including non-ripening (*NOR*) and ripening-inhibitor (*RIN*). Mutations of *NOR*, *RIN* and the ethylene receptor Never-ripe (*Nr*), which block ethylene perception and inhibit ripening have proven to be great tools for advancing our understanding of the developmental programs regulating ripening. In this study, we present systems analysis of *nor*, *rin* and *Nr* at the transcriptomic, proteomic and metabolomic levels during development and ripening. Metabolic profiling marked shifts in the abundance of metabolites of primary metabolism which lead to decreases in metabolic activity during ripening. When combined with transcriptomic and proteomic data, several aspects of the regulation of metabolism during ripening were revealed. First, correlations between the expression levels of a transcript and the abundance of its corresponding protein were infrequently observed during early ripening, suggesting that post-transcriptional regulatory mechanisms play an important role in these stages; however, this correlation was much greater in later stages. Second, we observed very strong correlation between ripening-associated transcripts and specific metabolite groups, such as organic acids, sugars and cell wall related metabolites, underlining the importance of these metabolic pathways during fruit ripening. These results further revealed multiple ethylene associated events during tomato ripening providing new insights into the molecular biology of ethylene-mediated ripening regulatory networks.
INTRODUCTION

Fruit ripening is a complex developmental program involving the coordinated regulation of numerous metabolic pathways that influence colour, flavour, aroma and texture. Many of these attributes enhance fruit nutritional value and attractiveness, thereby promoting consumption and seed dispersal (Liu et al., 2004; Goff and Klee, 2006) by offering important dietary minerals, vitamins, fibres and antioxidants to seed dispersing organisms.

Insights into the genetic mechanisms that mediate fruit ripening related processes, such as cell wall metabolism, pigment synthesis, and sugar metabolism, have resulted from studies that collectively span a wide range of plant species, and indicate that they are broadly conserved (Seymour, 1993; Carrari and Fernie, 2006; Fait et al., 2008; Moing et al., 2011; Zhang et al. 2011). However, tomato (Solanum lycopersicum) has emerged as the primary experimental model to study the development and ripening of fleshy fruits (Giovannoni, 2004; Fernandez et al., 2009). This reflects its economic importance and many favorable genetic characteristics, such as small genome size (950 Mb), a relatively short life cycle and routine transient and stable genetic transformation.

The many studies of the development and maturation of tomato fruits have resulted in the identification of specific genes that participate in ripening (Vrebalov et al., 2002; Manning et al., 2006; Giovannoni, 2007; Matas et al., 2009; Wang et al., 2009; Chung et al., 2010; Nashilevitz et al., 2010; Karlova et al., 2011; reviewed in Giovannoni, 2007 and Matas et al., 2009). Similarly, much has been learnt about the substantial changes in both primary and secondary metabolism that accompanies tomato fruit ripening though much of this data has not been directly related to regulatory events (Carrari and Fernie, 2006) and often target specific pathways. On a more comprehensive scale, tomato development has also been examined at the levels of transcriptome (Alba et al., 2005; Lemaire-Chamley et al., 2005; Carrari et al., 2006; Vriezen et al., 2008; Wang et al., 2009), proteome (Saravanan and Rose, 2004; Faurobert et al., 2007; Page et al. 2010) and metabolome characterization (Roessner-Tunali et al., 2003; Carrari et al., 2006; Fraser et al., 2007; Moco et al., 2007). However, to date, relatively few analyses combine multilevel approaches (Carrari et al., 2006; Armengaud et al., 2009; Enfissi et al., 2010; Zamboni et al.,
A number of important advances in our understanding of the mechanisms that regulate ripening have also come from the characterization of monogenic tomato mutants, including ripening-inhibitor (rin), non-ripening (nor), colourless non-ripening (Cnr), green-ripe (Gr), green flesh (gf), high pigment 1 (hp1), high pigment 2 (hp2) and never-ripe (Nr) (Lanahan et al., 1994; Mustilli et al., 1999; Vrebalov et al., 2002; Liu et al., 2004; Barry and Giovannoni, 2006; Manning et al., 2006; Barry et al., 2008). Although the complete network of ripening regulatory pathways remains to be resolved, cloning of many of the underlying genes has provided insights into their respective roles and hierarchical relationships (Giovannoni, 2004). The rin and Cnr loci encode a MADS-box and SPBP transcription factor, respectively, and are necessary regulators of ripening (Vrebalov et al., 2002; Manning et al., 2006). The Gr gene is suggested to interact with components of the fruit-specific ethylene response (Barry and Giovannoni, 2006), whilst the Nr mutation has been characterized as an ERS-like ethylene receptor, which is impaired in the ability to bind ethylene (Lanahan et al., 1994). The high pigment mutations influence the role of light in ripe fruit pigmentation via tomato orthologs of the DEETIOLATED (DET1) and DAMAGED DNA BINDING PROTEINS1 (DDB1) genes originally defined in Arabidopsis (Mustilli et al., 1999; Liu et al., 2004). The gf gene encodes a senescence associated STAY-GREEN protein, indicating a role for plastids in both manifesting and regulating ripening phenotypes (Barry et al., 2008).

The identification and characterization of such mutants provide an opportunity to dissect the complex networks of ripening-related pathways at multiple levels. In this study, we have examined three dominant ripening mutants of tomato, nor, rin, and Nr along the developmental and ripening periods at the transcriptomic, proteomic and metabolomic levels in order to further extend the analysis carried out by Alba et al. (2005). Use of these specific mutants helps define transcriptional activity as transcriptionally-regulated and ethylene-response sub-transcriptomes. Transcriptional analysis was carried out using the tomato cDNA microarray (TOM1; (Alba et al., 2005), microarray containing ESTs, proteomic data were obtained using isobaric tag labeling (iTRAQ; (Wise et al., 2007) and metabolomics data were obtained by GC-
MS as per Carrari et al. (2006). The combined results are discussed in the context of current models of ripening and development.

RESULTS

Experimental Design

The experiments described here were designed to span tomato fruit ripening in cultivar Ailsa Craig which occurred under greenhouse conditions over a period of 57 days from fruit set to a fully ripe stage. Tomato fruit developmental can be divided in four major phases: cell differentiation, division, expansion and ripening (Gillaspy et al., 1993). As described by Alba et al. (2005), these phases correspond to wild type (WT) fruits between 7-27 days after pollination (DAP) for the first two phases, to the first visible carotenoid accumulation (42 DAP) in the third phase, and to full red colour development (57 DAP) in the ripening phase. Pericarp of anthesis tagged and equivalent age fruits of the rin, nor and Nr isogenic mutants were harvested (one biological replicate was considered as individual fruits from different plants). We note that at stages following Mature Green (39 DAP) the mutant fruit diverge in their development from wild type as they are indeed substantially (Nr) or nearly completely (rin and nor) ripening inhibited. Changes resulting from the selected mutations may result in delayed effects which could be further compounded by occurring in the context of an older fruit where developmental context is altered due to normal progression of activities not impacted by the mutations employed. Thus while an alternative to comparing identically aged fruit would be to compare identical physiological stages such as breaker or red ripe, rin and nor never achieve comparable stages and Nr in the Ailsa Craig background does not achieve red ripe (and Breaker is delayed). We have thus selected comparison of identically staged fruits as this approach allows for comparison of changes in the context of a developmental parameter (age post pollination) that can be measured accurately in all genotypes.

Subsequently, transcriptome analysis was carried out using two-colour hybridizations of the TOM1 array, which contains 12,899 different EST clones representing ~8,500 tomato genes, using mRNA prepared from each stage. Proteomic data from three ripening stages (39, 42, 52 DAP) were obtained using the iTRAQ labelling technique. A total of 48 primary metabolites were analyzed by GC-
TOF-MS in fruits harvested between 27 DAP to 57 DAP for nor and rin, and from 42 DAP to 57 DAP for Nr as described in the Materials and Methods section.

**Transcriptomic profiling**

We investigated differential transcript accumulation using two-colour simultaneous hybridizations of the TOM1 array during tomato development for WT Nr, nor, and rin (ten time points: 7, 17, 27, 39, 41, 42, 43, 47, 52, 57 DAP. The transcript data for Nr has previously been published (Alba et al., 2005); however, it is included here for comparative purposes and in the broader context of the orthogonal data sets. It is important to note in interpreting our results that Nr in the Ailsa Craig background retains partial ethylene sensitivity and thus partial ripening occurs (Yen et al., 1995).

The PageMan (Usadel et al., 2006) and MapMan (Usadel et al., 2005) mapping files, described in Materials and Methods, were used to study the development and ripening of Nr, nor, and rin by identifying significantly over-represented functional groups, in comparison to the corresponding WT control samples, on the basis of Fisher’s exact tests and Wilcoxon tests for each category. This facilitated the analysis of the global activation and/ or repression of metabolic pathways and gene regulatory networks of the pericarp. We identified metabolic pathways that were enriched during these processes including several which would be anticipated based on previous studies, including hormone metabolism (Supplemental Fig. S1). The individual gene responses can be viewed in MapMan (Supplemental online file).

Visual inspection revealed the categories with either a qualitatively similar or different response between the mutants and the control. The general down-regulation of photosynthetic light reactions during the studied stages of Nr is immediately apparent, suggestive of reduced photosynthesis and reduced carbon assimilation in the fruit. In addition, this mutant is characterized by an induction of genes associated with the sucrose (Suc)-to-starch transition being most prominent at 42 DAP for genes related to sucrose degradation and at 17 DAP onwards for genes associated with starch synthesis. In rin, transcripts related to starch synthesis appear to exhibit a biphasic response, with a large induction occurring relatively early followed by a secondary increase (43 and 52 DAP). Moreover, these transcripts appear to be up-regulated in nor at 27 and 41 DAP (Supplemental Fig. S1). These results point to the importance of hexoses derived from degradation of Suc for starch synthesis during
early tomato development and at the onset of ripening, and are consistent with a recent study highlighting the importance of transitory starch in normal fruit development (Centeno et al., 2011).

A spectrum of genes/proteins involved in cell wall restructuring and disassembly (such as endo-β-1,4-glucanases from glycosyl hydrolase (GH) family, expansins, xyloglucan transglucosylase hydrolases (GH16) and a range of pectinases are canonically associated with fruit development and ripening (Rose and Bennett, 1999; Rose et al., 2004b; Brummell, 2006). In our study, a number of genes associated with cell wall degradation were down-regulated during ripening in nor and rin, whilst those putatively involved in reversible wall loosening and assembly were down-regulated at 27 and 41 DAP in Nr, consistent with the previously observed depolymerization of cell wall components at the onset of storage mobilization (Amor et al., 1995; Reiter, 2008) (Supplemental Fig. S1). At the onset of ripening (42 DAP), genes associated with amino acid synthesis were expressed at higher levels including the Asp-derived Met pathway in nor, rin and Nr (Supplemental Fig. S1). In contrast, we could infer that amino acid degradation metabolism is generally repressed in all three mutants, and the phytoene synthase-1 gene is down-regulated at the 42 DAP stage (breaker) in nor and 47 DAP in rin but one day before of breaker (41 DAP) for Nr (Supplemental Fig. S1).

The expression of genes associated with hormone biosynthetic pathways were altered in Nr and rin consistent with the lack of climacteric ethylene production and activity in these non-ripening fruits. During ripening, transcripts for genes involved in biosynthesis of ethylene were expressed at high levels in Nr as well as was observed in rin, but almost no change was observed in the same time-frame for nor suggesting nor activity my be upstream of rin in the regulatory hierarchy. Besides the well characterized participation of ethylene in the control of ripening in climacteric fruits (Giovannoni, 2004; Alba et al., 2005; Giovannoni, 2007), other hormones have more recently been demonstrated to play important roles (Marti et al., 2007; Chaabouni et al., 2009; Yang et al., 2010). These data indicate that a highly complex interactive hormonal network likely regulates fruit ripening. In Nr, transcripts for genes involved in the biosynthesis of auxin and jasmonate were up-regulated at 42 DAP (breaker stage) and 52 DAP, respectively, whilst nor was characterized by a down-regulation of abscisic acid (ABA) metabolism at the end of ripening (57 DAP) (Supplemental Fig. S1). Interestingly, transcripts associated with ethylene perception and signal
transduction changed relatively little but were significantly down-regulated during ripening in *nor* (see Supplemental online file and Supplemental Fig. S1).

We additionally observed differential expression of transcripts involved in protein synthesis which were down-regulated during all ripening phases in *Nr* but were up-regulated during earlier developmental in *nor* and also during the period that would correspond to normal ripening in *rin*. Moreover, genes involved in protein degradation were up-regulated in *Nr* (Supplemental Fig. S1).

Whilst the changes described above suggest considerable alterations in the programs influencing early development and ripening in the mutants, a linear analysis on a time-point by time-point basis may over exaggerate the differences between the genotypes. We therefore re-analysed the data such that gene expression at any time-point was compared, within genotypes only, to the first harvested time-point (Fig. 1; Supplemental Table 1). These data revealed a general down-regulation of photosynthetic light reactions during developmental and ripening in WT and the *rin* mutant (Fig. 1), and similarly in *nor* and *Nr*, but only from 39 DAP stage onwards. In addition the *Nr* mutant showed up-regulation of these genes in the first two developmental stages (17 and 27 DAP) (Fig. 1). The Calvin-Benson cycle was apparently down-regulated at the 39 and 41 DAP stages during normal tomato fruit development (Fig. 1A) but across all studied stages in *rin* (Fig. 1C). A similar pattern of changes was observed from the 39 DAP stage in *Nr*, which also showed up-regulation in the two earliest stages (17 and 27 DAP) (Fig. 1C). The same down-regulation was observed for *nor* in later stages (43, 47 and 57 DAP) (Fig. 1B).

The transcript analysis suggested that starch synthesis was down-regulated from 17 DAP stage onwards during normal fruit development and ripening with a similar pattern of changes seen in *rin* and *Nr*. However, in *Nr* this trend was only significant from the 39 DAP stage onwards (Figs. 1C and D). On the other hand, *nor* only showed this behaviour in later ripening stages (43, 52, and 57 DAP) (Fig. 1B). Another process related to sugar metabolism – the degradation of sucrose – was suggested to be up-regulated in all three mutants, but with differing temporal patterns: from 42 DAP (breaker) in *Nr*, 27, 39 DAP in *rin*, but only in the last ripening stage (57 DAP) in *nor* (Fig. 1B-D). Again, the more pronounced impact of *nor* on starch and sucrose metabolism is consistent with an earlier or more global role in ripening for *nor* than *rin* or *Nr*. 


Another well characterized ripening related process is the activation of alcohol hydrogenase (ADH) (Carrari and Fernie, 2006). Analysis of the ADH associated transcripts suggested a similar pattern in nor, rin and WT but that only occurred at 43 DAP (Fig. 1B) in nor, at 39, 42, and 47 DAP in rin (Fig. 1C) and in all ripening stages in WT (Fig.1A). We also observed that TCA metabolism was up-regulated across the later stages in Nr (43, 47, and 57 DAP) but down-regulated in rin and nor mutants at this time point (Fig. 1B-D).

Genes associated with pectin degradation (pectate lyase and polygalacturonase) are clearly up-regulated during normal ripening in tomato fruit, as expected (Fig. 1A), but this pattern was delayed in Nr consistent with the residual ethylene response and partial ripening (up-regulated in 47, 52, and 57 DAP; Fig. 1D) and not observed in the other two mutants (Fig. 1B-C). Several genes related to reversible wall modification were up-regulated at the 42 DAP stage in the wild type while nor showed no such changes. In rin, these genes were, however, up-regulated at the 43 and 57 DAP stages (Fig. 1C) but in Nr only at 57 DAP (Fig. 1D).

The transcript pattern suggested that synthesis of aromatic amino acids was down-regulated from 39 DAP stage onwards in WT (Fig. 1A) whilst the associated genes showed the same behaviour at later stages in the three mutants (Fig. 1B-D) (43, 52 and 57 DAP in nor; 47 and 52 DAP in rin; 57 DAP in Nr. Interestingly, in WT, Ser, Gly and Cys degradation was up-regulated at the 47 DAP stage during WT fruit development (Fig. 1A) and throughout all stages of development in Nr, but not in rin or nor (Fig. 1B-D).

At the onset of ripening (42 DAP), transcripts associated with isoprenoid and carotenoid metabolism were upregulated in normal ripening (Fig. 1A), whereas in both nor and rin, this was apparent at earlier stages (Fig. 1B and C). In contrast, in Nr, this pattern was observed in later stages (52 and 57 DAP, again consistent with the partially ripening phenotype), exemplified by phytoene synthase-1, which was down-regulated in earlier development 27 and 41 DAP; Fig. 1D). As was expected from previous studies (Alba et al., 2005; Carrari and Fernie, 2006), ethylene metabolism was altered in all three mutants. In the WT fruit we observed an induction of ethylene biosynthesis at 42 DAP (breaker stage), with similar pattern of changes seen in Nr (Fig. 1A and D). Additionally, in the first developmental stages (17 and 27 DAP), a down-regulation of genes related to ethylene signal transduction was apparent (Fig. 1A). In rin, the up-regulation was also displayed at the same stage as
WT (42 DAP), but subsequently continued throughout ripening until 52 DAP as in \( Nr \) (Fig. 1C), but in \( nor \) the induction of ethylene biosynthesis was delayed (Fig. 1B). In addition, the \( Nr \) mutant displayed apparent up-regulation of jasmonate metabolism during ripening process (from 43 to 57 DAP stages).

During development of WT tomato fruit, we observed a down-regulation of polyamine synthesis in the earlier stages (27 and 39 DAP) and also at a later stage (47 DAP) (Fig. 1A) while \( Nr \) showed up-regulation late in development (57 DAP; Fig. 1D) and no significant changes were observed in \( rin \) and \( nor \) (Fig. 1B and C).

A general up-regulation of transcription regulatory genes was observed at 27 DAP and also from 42 DAP (breaker stage) until 57 DAP during normal tomato fruit development (Fig. 1A). In \( rin \), this up-regulation was only significant at 43 DAP and the same behaviour was observed in \( Nr \) mutant but again in later stages (52 and 57 DAP; Fig. 1D). However, in \( nor \) the only obvious difference was a down-regulation at 17 DAP (Fig. 1B). Interestingly, the transcription factor family APETAL2 (AP2/EREBP), which one of member (\( AP2a \)) has been described as a ripening related repressor of ethylene response (Chung et al., 2010), was substantially up-regulated in \( nor \) (Fig. 1B) at the time point equivalent to the onset of ripening in WT(42 DAP).

It is also worth noting that general down-regulation of genes involved in biosynthesis of ribosomal proteins occurred in both wild type and in \( rin \) (Fig. 1A and C). In \( nor \) and \( Nr \), these genes showed the same patterns during ripening (43, 52, and 57 DAP for \( nor \) and 52, 57 DAP for \( Nr \)). However, these genes were up-regulated in earlier stages (17, 27, and 39 DAP for \( nor \) and 17, 27, 39, 41, and 42 DAP for \( Nr \)) (Fig. 1B and D).

**Metabolic profiling**

To follow the repertoire of metabolic changes that occur during tomato development in the three mutants, we carried out extensive metabolic profiling for primary metabolism using an established GC-MS method (Fernie et al., 2004). For the analysis, we selected several stages that span develop and ripening for \( nor \) and \( rin \) (27, 39, 41, 42, 43, 47, 52, and 57 DAP) and from 42 DAP stage onwards for \( Nr \). Carrari et al. (2006), presented an exhaustive metabolic study of fruit development and ripening in the Moneymaker tomato cultivar,) which shows similarities and also differences in metabolite behaviour compared to the cultivar used in the present
study (Ailsa Craig). In the previous study, it was reported that Suc decreased strongly during the first developmental stages, until 28 DAP, and in parallel Glc and Fru accumulated in an essentially linear manner. However, these changes were not observed in Ailsa Craig (Fig. 2). Instead, Suc levels decreased during the transition to ripening (41, 42, and 43 DAP) and later increased (52 and 57 DAP). Glc and Fru also exhibited a reduction during the development-ripening transition (41 and 42 DAP) and increased in the same stages as Suc (Fig. 2). We observed that the levels of Glc, Fru and Suc, were reduced in the three mutants, across the time course. Although starch degradation has not been subject to detailed characterization in tomato pericarp tissue, it would thus appear that tomato fruit, like Arabidopsis leaves (Niittyla et al., 2004), are largely reliant on the hydrolytic pathway of degradation.

We were also able to detect the levels of a total of eleven amino acids across all genotypes. The three mutants and the wild type all revealed marked alterations during development and ripening (Fig. 2). Two main patterns of amino acid accumulation were apparent: (i) those increasing during ripening, and (ii) those decreasing exclusively during ripening. Group (i) was dominated by Phe, Asp, Ile, Glu and Ala while group (ii) consisted of Gaba, ß-Ala, Thr, Ser, Gly and Val. A comparison of these patterns with those observed by Carrari et al., (2006) in Moneymaker, revealed that the two cultivars display strikingly similar shifts in amino acid levels. That said, a few cultivar-specific differences were apparent such as a decrease in Ala during development in Moneymaker (Carrari et al., 2006) but not in Ailsa Craig (Fig. 2). Interestingly, when comparing with the metabolic shifts displayed by the mutants, we observed that in all three mutants, Asp, Glu, and Ala which were characterized by an increase on ripening in WT, were slightly increase or unaltered during earlier development and ripening (Fig. 2). Moreover, during ripening, nor accumulated considerable amounts of ß-Ala and GABA, whereas rin and Nr showed the opposite behaviour at the same temporal stages. Additionally, rin and Nr showed lower level of threonine compared to wild type while it was unaltered in nor.

During normal Ailsa Craig fruit maturation, the TCA cycle intermediates, citrate, isocitrate, succinate, and malate were highly variable, but generally displayed the same behaviour as observed in Moneymaker (Carrari et al., 2006). During ripening, we observed a strong increase in the level of succinate. In contrast, malate and isocitrate strongly decreased. All three mutants displayed no alteration in their levels of malate and succinate across the studied period. Furthermore, levels of
citrate and isocitrate changed significantly only in nor, which displayed the same behaviour, albeit to a less dramatic extent compared to wild type (Fig. 2).

Cell wall related metabolites, namely, mannose, galactose, rhamnose, ribose, arabinose, xylose and galacturonic acid increased during the normal tomato ripening. This trend is consistent with earlier work which indicated the particular importance of degradation of pectin-derived arabinan and galactans during fruit ripening (Sakurai and Nevins, 1993). The levels of these sugars were, however, unaltered in all stages in the mutants, which is again consistent with the characteristic post-harvest physiology of these mutants as they all show substantially reduced pectin degradation (Hobson et al., 1983).

**Coordinated changes of related metabolites**

In addition to the metabolite changes, we performed a combinatorial analysis of metabolites by subjecting all data points to pairwise correlation analysis. The nor, rin and Nr mutants showed a total of 520, 864, and 360 significant correlations ($P<0.05$), respectively. Off these, 450, 686, and 360 were positive ($r^2>0.70$) and 70, 178, and 36 were negative ($r^2<-0.70$), respectively. The full data set of correlation coefficients is presented in the heatmap shown in Fig. 3. To simplify the interpretation, metabolites are grouped by compound class. For the Nr mutant, the metabolite correlations were performed only in ripening stages (42, 47, 52 and 57 DAP) while for nor and rin the analysis were extended to 39, 41, 42, 43, 47, 52, 57 DAP. In both WT sets, all metabolites measured showed significant correlations to compounds outside of their compound class, as was previously observed in the Moneymaker cultivar (Carrari et al., 2006). The individual metabolites with the highest number of correlations were glycine and GABA, with 21 associations in nor; alanine, ribose and xylose with 28 associations in rin; β-alanine with 18 associations and rhamnose, arabinose and valine with 17 in Nr. In general, Nr and nor displayed far fewer correlations than rin or WT. Nr and nor had a considerable number of positive correlations between the levels of various amino acids at different stages of development as well as between amino acids and sugar phosphates, sugar alcohols and fatty acids, similar to those correlations previously described across development for the WT (Carrari et al., 2006; Do et al. 2010). That said, the correlations observed for the rin dataset were much more similar to those seen in WT (Carrari et al., 2006; Do et al. 2010), where cell wall related metabolites, organic acids and amino acids
were highly correlated. As stated above, the “core” metabolite correlations seen in all four genotypes are reminiscent of the metabolites defined to be reflective of development in our previous study of wild type tomato development (Carrari et al., 2006). Furthermore, the tight correlation of the amino acids is in keeping both with surveys of tomato pericarp metabolite levels in populations of introgression lines resulting from wide crosses of tomato (Carrari et al., 2006; Schauer et al., 2006; Do et al. 2010) and of diverse Arabidopsis genotypes (Sweetlove et al., 2008; Sulpice et al. 2010).

**Proteomic analysis**

Pericarp tomato proteins from wild type and three ripening stages (39, 42 and 52 DAP) in nor, rin and Nr mutants were treated and labelled using iTRAQ (Isobaric Tag for Relative and Absolute Quantitation) reagents and separated by strong cation exchange fractionation, prior to protein identification and quantification. This approach, in combination with nanoLC/ESI-MS/MS, resulted in the identification of 158 differentially expressed proteins (Supplemental Table 2 and Fig. 4). As anticipated, nor and rin are phenotypically similar in terms of fruit fail to produce climacteric ethylene and show responsiveness to ethylene at the molecular level (Lincoln and Fischer, 1988) and combine with the inability to induce ripening in either mutant via exogenous ethylene, defines a regulatory network in which ethylene regulates a subset of ripening genes either direct or in concert with developmental signals influenced by nor and rin gene product (Giovannoni, 2004). Based on this regulatory relationships between the nor and rin genes, a greater number of proteins displayed a similar pattern when comparing nor to rin than comparing nor to Nr, or rin to Nr (Supplemental Fig. S3). In general, we observed that across the three stages analyzed, fruits at the 52 DAP stage showed the highest number of differentially abundant proteins in the mutants compared to WT. This total can be sub-divided into the 123, 71, and 29 proteins that vary in abundance in nor, rin, and Nr mutants compared to WT, respectively. This stage also showed the highest number of common proteins (17) that were detectable in all three genotypes (Supplemental Fig. S3). Of these common proteins, some were related to stress (pathogenesis related protein, heat shock proteins, glutathione S-transferase like protein, catalase protein), cell wall metabolism (polygalacturonase), hormone biosynthesis (ACC oxidase), and secondary metabolism (flavonoid glucosyltransferase).
Given that the previously discussed transcript data was obtained from the exact same powdered pericarp samples, we next determined the number of the differential identified proteins for which corresponding transcript probes were represented on the TOM1 array. Of the 158 identified proteins, 133 had representative transcript probes on the microarray. Scatter plot analysis of the log2 transformed ratios showed the distribution of the corresponding mRNA-to-protein ratio (Fig. 4). At 39 and 42 DAP stages for all three mutants, almost all mRNA-to-protein ratios were concentrated at the centre of the plot (quadrant e), wherein protein and mRNA levels did not vary above 1.5 and 2 fold, respectively. Off centre, a total of 45 mRNA-to-protein ratios across all four genotypes were found in which both the mRNA and protein level exceeded this level of variation (Table I and Fig. 4). At the 39 DAP stage it was apparent that all three mutants had fewer mRNA-to-protein ratios outside the central quadrant. In *Nr* several ratios fell in quadrants b and h, indicating that protein levels were likely up- or down-regulated, but mRNA levels were unchanged. In contrast, *nor* and *rin* were characterized by significant down-regulation of Sn-1 and Sn-2 proteins in both protein and mRNA levels (accession numbers; Sn-1 CAA55812, Sn-2 CAA55813). At the 42 DAP we observed that mRNA-to-protein ratios also mainly fall in the quadrants b, e, and h. It, therefore, follows that only a few mRNA-to-protein ratios reflected significant changes at both transcript and protein levels. *Nr* displayed up-regulation at both levels for four proteins, polygalacturonase-2 (acc. number, P05117), pectinesterase (P14280), cysteine proteinase 2 (Q40143) and gibberellin 20 oxidase (XP_00251754). However, contrasting levels were also observed for one gene; the up-regulated expression of ACC-oxidase (acc. number, ABP68407) yet down-regulation of its protein abundance. *nor* and *rin* additionally shared the commonly up-regulated protein, SGRP-1 (acc. number, CAA73034) whose transcript was also significantly up-regulated.

The 52 DPA stage was characterized by a large number of mRNA-to-protein ratios falling in the quadrants a, c, g, and i, wherein mRNA-to-protein ratios are substantially different. Moreover, seven down-regulated proteins, which reflected significant down-regulation at the transcript level, were detected in common between *nor* and *rin*, namely; pyruvate decarboxylase (BAC23043), glutathione S-transferase-like (AAL92873), methionine sulfoxide reductase (P54153), polygalacturonase-2 (P05117), heat shock protein 83 (P51819), pyruvate decarboxylase (BAC23043) and alcohol dehydrogenase 2 (P28032). The last three on this list were similarly down-
regulated in Nr. In addition to these common abundant proteins, aquaporin-like protein (AALD30452) was also down-regulated in rin and oxygen-evolving enhancer protein 2 (P29795) in Nr mutant. Additionally, nor displayed up-regulation in both protein and transcript levels for Sn-1 (CAA55812) and Sn-2 (CAA55813), as did Nr. Moreover, rin showed up-regulation of two additional proteins, phosphoenolpyruvate carboxylase 2 (CAB65171) and ASR4 (AAY98032). Other up-regulated proteins which corresponded to up-regulated transcript expression were also detected in Nr such as pectinesterase 1 (P14280), pathogenesis-related protein P2 (P32045), lipoxygenase A (P38415) and lipoxygenase (AAB65766). Additionally, polygalacturonase 2 (P05117) protein and a small heat shock protein (AAD30452) were down-regulated but showed significant up-regulation at the transcript level in Nr and rin, respectively. Also, the up-regulated Sh-RNase (BAE92268) protein in Nr was down-regulated at the transcriptional level. In summary these results suggest a substantive degree of post-transcriptional regulatory activity during fruit maturation that has not been previously described in reports focused solely on transcript or protein analysis.

Network analysis of transcript, metabolite and protein levels during ripening of nor and rin tomato fruits

As is typical of climacteric fruit, ripening in tomato is regulated to a substantial degree by the hormone ethylene. To better understand the ethylene regulatory network, we performed an association study of individual metabolites, a subset of transcript data and proteins in both the nor and rin mutants (Supplemental Data Set 2). For the integration analysis we selected three different stages which covered the ripening process in WT: 39 DAP (mature green, MG), 42 DAP (breaker, Br), and 52 DAP (ripe). The data sets used for the correlation comprised 48 metabolites from primary metabolism and a subset of transcripts showing significantly differential expression (178 transcripts for nor and 191 for rin mutants). The latter included those associated with cell wall metabolism, starch synthesis and degradation, redox stress, polyamine metabolism, transcription factors, fermentation metabolism, TCA cycle, hormone metabolism and signalling and secondary metabolism. All proteins showing significant differential abundance in the developmental stages studies were selected (154 proteins for nor and 121 for rin). We analysed co-responses between metabolic
pathway genes, proteins, and metabolites in each genotype (nor and rin) using a Pearson's correlation coefficient approach at a strict stringency threshold (P<0.01). In each correlation matrix (Fig. 5 and Supplemental Fig. S4), transcripts, proteins and metabolites are represented as nodes (different node shapes represent the different data sets: significant correlations are represented as links which connect the nodes). Network analysis emphasized links between both and within the various types of data. We built the network from each mutant with data relative to WT values (transcript, proteins, and metabolites) in exactly the same way. Interestingly, nor revealed markedly higher connectivity that rin (nor showed a total of 889 links between a total of 357 nodes; rin showed a total of 792 links between a total of 354 nodes) suggesting again that NOR may represent a higher level in the regulatory hierarchy as compared to RIN.

Based on our previous results, where a high number of common proteins was found between nor and rin in the ripe stage (52 DAP), we expected to have a highly significant overlap between the nor and rin networks. Surprisingly, taking into account the comparative network analysis of three ripening stages when comparing both networks (39, 42, and 52 DAP), we observed different regulation between transcripts, proteins, and metabolites within the three ripening stages (Fig. 5). Comparative network analysis also suggested that in nor associations between nodes were largely modular, while in rin we observed more robust associations between specific metabolites, namely malate, fumarate, glutamate, proline and glycine (Fig. 5B). The nor network analysis revealed two interesting clusters, one of which includes the Nr transcript (3.3.1.9; TOM1 ID; SGN-U590044) in cluster 1 in Fig. 5A) and another which includes RIN (8.2.16.2; TOM1 ID; SGN-U578471) in cluster 2 in Fig. 5A. Interestingly, in the nor network (cluster 1), the Nr transcript showed co-expression with hormones and cell wall metabolism regulators. The Nr transcript level (3.3.1.9; TOM1 ID; SGN-U590044) displayed positive correlations with lipoxygenase protein abundance, which is, in turn, positively correlated to lipoxygenase transcript levels (6.4.16.5; TOM1 ID; SGN-U578028). The Nr transcript also showed positive correlations to the pectin degradation related genes; pectate lyase (1.1.6.7; TOM1 ID; SGN-U585252) and polygalacturonase (1.4.12.4; TOM1 ID; SGN-U577423). In turn, these pectin degradation related genes (pectate lyase; 1.1.6.7; TOM1 ID; SGN-U585252 and polygalacturonase; 7.4.12.12; TOM1 ID; SGN-U577423) transcripts showed positive correlation hormones related genes, lipoxygenase (6.4.16.5; TOM1
ID; SGN-U578028) and ACC-oxidase (3.3.4.14; TOM1 ID; SGN-U578607). This small regulated cluster was clearly related to primary metabolism with all these nodes showing a negative correlation with a phosphoglycerate kinase protein. Moreover, the phosphoglycerate kinase protein displayed a positive correlation with photosystem I reaction subunit II and photosystem II proteins that are negatively correlated with the Nr transcript, as well as with cell wall related genes, and lipoxygenase protein.

The other interesting cluster in nor contains the rin transcription factor (TF) (8.2.16.2; TOM1 ID; SGN-U578471) (cluster 2 in Fig. 5A). Interestingly, the rin TF showed a positive correlation with another TF (8.4.16.5; TOM1 ID; SGN-U577950), ripening related TF, fruitfull-like MADS-box (TDR4) (Carrari et al., 2006); 3.4.19.21; TOM1 ID, SGN-U572851, homobox-leucine zipper protein HAT 22; 4.4.10.20; TOM1 ID, SGN-U584756, ethylene response factor (ERF2). Another interesting positive correlation was an ethylene-responsive transcriptional coactivator (ER24) (6.1.16.18; TOM1 ID; SGN-U566716) as well as others negative correlations with an alcohol dehydrogenase protein related to tomato ripening (Carrari et al., 2006), pathogenesis related protein 10 and the metabolite saccharinate.

DISCUSSION

Tomato has become the primary experimental model to study fleshy fruit ripening. While earlier studies focused on elucidating ethylene synthesis and signal transduction (Hamilton et al., 1991; Oeller et al., 1991; Picton et al., 1993; Lanahan et al., 1994; Wilkinson et al., 1995; Yen et al., 1995; Lashbrook et al., 1998; Barry et al., 2000; Tieman et al., 2001; Leclercq et al., 2002; Adams-Phillips et al., 2004a; Alba et al., 2005; Zhong et al., 2008) and cell wall modifying proteins. More recent targets include understanding ripening control upstream of ethylene action, ripening-related signal transduction systems and downstream metabolic networks. A wide range of studies have shed light on diverse aspects of ripening. The most obvious changes are the transition from partially photosynthetic to fully heterotrophic metabolism (Camara et al., 1995), marked shifts in cell wall composition (Rose et al., 2004a) and other metabolic changes (Giuliano et al., 1993; Fraser et al., 1994; Carrari and Fernie, 2006): all being under strict hormonal regulation of climacteric ripening (Lanahan et al., 1994; Wilkinson et al., 1995; Adams-Phillips et al., 2004a; Adams-Phillips et al., 2004b; Alba et al., 2004; Barry and Giovannoni, 2006;
Giovannoni, 2007; Chung et al., 2010). Together, these data have elucidated how the developmental and ethylene signals are transduced to mediate ripening (reviewed in Giovannoni, 2004, 2007; Matas et al., 2009).

**Ripening mutations provide tools to parse transcription and hormonal control**

The availability of well characterized genetic mutants in fruit development and ripening has promoted considerable research on tomato (Giovannoni, 2007). These include two ripening associated transcription factors, ripening-inhibitor (*rin*) which encodes a SEPALLATA MADS-box gene (Robinson and Tomes, 1968; Vrebalov et al., 2002), non-ripening (*nor*) (Genbank Accession AY573802; Tigchelaar et al., 1973) and a two-component HIS-kinase ethylene receptor, *ETR3* (never-ripe; *Nr*) (Wilkinson et al., 1995; Yen et al., 1995).

The RIN transcription factor is necessary to induce ripening-associated increases in respiration and ethylene concentration resulting in fruits that fail to complete normal ripening (Vrebalov et al., 2002; Giovannoni, 2007). Furthermore, fruits remain responsive to ethylene in that ethylene-responsive genes are induced by exogenous ethylene. The ripening regulator transcription factor NOR encodes a NAC-domain protein (Tigchelaar et al., 1973, J. Vrebalov and J. Giovannoni, unpublished). *nor* is phenotypically similar to *rin* in that *nor* fruit fail to produce climacteric ethylene yet show responsiveness to ethylene at the molecular level via ethylene-induced gene expression while remaining unripe in response to ethylene (Lincoln and Fischer, 1988). While *nor* and *rin* may well act together in a cascade to control ripening (Giovannoni, 2007), a number of molecular differences were observed when expression analysis of ripening-related genes in the presence and absence of exogenous ethylene, were examined. Specifically, in mature *nor* fruit transcripts corresponding to both phytoene synthase (*PSY1*) and the *E8* gene which are known to be up-regulated during normal ripening (Lincoln et al., 1987) are absent and expression of these genes is not restored by exposing the fruit to ethylene (Yen et al., 1995; Thompson et al., 1999). Similar behaviour was observed for the polygalacturonase transcript in presence and absence of ethylene (Yen et al., 1995; Thompson et al., 1999). However, in *rin*, the effect was not as strong as in *nor*, with *rin* displaying normal induction though reduced levels of expression of *PSY1* and *E8* in mature fruits (Knapp et al., 1989) which could be restored with exogenous ethylene application (Dellapenna et al., 1989). These data suggest that *nor* may have a more
global effect on ethylene/ripening-related gene expression than *rin* in that reduced expression in *rin* reflected the lack of ethylene in these fruit but the lack of expression in *nor* and the inability to restore expression with ethylene suggests a higher level of control. In the model proposed by Thompson et al. (1999), both genes operate upstream of crucial ripening activity and *nor* is upstream of *rin*, since ethylene/ripening-related gene expression appears to be more extensively inhibited in *nor*.

Ethylene is perceived by two-components His kinase ethylene receptors (Chang and Shockey, 1999) and a mutation in the receptor, *LeETR3* (*Never-ripe; Nr*) interferes with ethylene perception, and therefore, significantly delays fruit ripening (Lanahan et al., 1994; Wilkinson et al., 1995) and represents a tool to define the fruit ethylene-regulated transcriptome/proteome/metabolome/phenome. Previous transcriptomic, phenotypic and targeted metabolic analysis revealed that *Nr* influences fruit morphology, seed number, ascorbate accumulation, carotenoid biosynthesis and ethylene evolution (Alba et al., 2005). Here we exploited the *rin*, *nor* and *Nr* mutations to define systems under the influence of transcriptional (*rin*, *nor*) and ethylene (*Nr*) control.

**Systems analysis of ripening control using ripening mutations**

Here we utilized an extensive array of tools to characterize the various molecular entities (transcriptome, proteome, metabolome) of the cell (Alba et al., 2004; Fernie et al., 2004; Rose et al., 2004b) in the three ripening tomato mutants, *rin*, *nor* and *Nr* with the aim of better understanding transcriptional and ethylene dependent ripening control. Transcriptional analysis on the three ripening mutants at ten different developmental stages spanning fruit and ripening revealed how ethylene, via NOR, RIN and Nr influences the expression of hundreds of genes during development both prior to the onset of, and during ripening. These results corroborate the central role that ethylene plays in governing biochemical, physiological, and developmental processes throughout tomato development.

**Different regulation of ethylene-related genes in *nor*, *rin* and *Nr***

Pageman analysis of microarray data revealed significant changes in different functional gene categories across the three mutants supporting overlapping and independent regulatory effects. Regarding ethylene metabolism, and in agreement with previous studies (Wilkinson et al., 1995; Vrebalov et al., 2002), we observed that ethylene-related genes were differentially expressed in the three mutants and as
compared with WT. During the normal development, ethylene synthesis-related genes were up-regulated in the breaker stage (42 and 43 DAP) fruits and ethylene signal-transduction genes in late ripe stages (47 and 57 DAP). In Nr, ethylene synthesis-related genes were strongly up-regulated in a time frame consistent with the normal ripening process (42 DAP) as described by Alba et al., (2005). Interestingly, those genes showed similar up-regulation in rin but were unaltered in nor suggesting response to nor-specific activities. Downstream in the ethylene signalling cascade, ethylene signal-transduction was apparently unaltered across development and ripening in the three mutants. These data thus support the model postulated by Thompson et al. (1999) where nor is a higher order regulator that rin or Nr (ethylene). Recent chromatin precipitation (CHiP) analysis of RIN protein targets indicates that ethylene synthesis (ACC synthase 2 and 4) gene promoters directly interact with RIN (Fujisawa et al., 2011). This would suggest a linear order of NOR-RIN-NR activity.

Characterization of the fruit ethylene-ome demonstrates regulated metabolomic flux toward ethylene synthesis

Climateric ethylene biosynthesis includes conversion of Asp to Met, conversion of Met to ethylene, and the Met recycling pathway (Yang and Hoffman, 1984). Ethylene is synthesized from S-adenosylmethionide (SAM) by the sequential action of two ethylene-biosynthetic enzymes, namely, 1-aminocyclopropane-1-carboxylate (ACC) synthase and ACC oxidase. SAM also has the potential to participate in polyamine or ethylene biosynthesis, or both (Mattoo et al., 2007; Mattoo and Handa, 2008). From the transcriptomic and metabolomic data in this current study (Figs. 1 and 2), it is apparent that ethylene biosynthesis may predominate the flux through SAM. During normal tomato ripening at the initiation of ethylene biosynthesis, Asp, the precursor of Met, increased in addition to putrescine, one of the three major plant polyamines (Carrari et al., 2006). Asp and putrescine levels were higher in rin and Nr; however, the changes in Asp levels in the two mutants were far less dramatic than those observed in WT whilst the change in putrescine was more substantial. Interestingly, these changes in Asp and putrescine correlated with the up-regulation of ethylene synthesis-related genes in rin and Nr. In contrast, no significant changes in Asp and putrescine levels were observed when comparing nor to WT; which was mirrored by the lack of change in the expression of
ethylene synthesis-related genes in this genotype. These changes are in agreement with a previous study (Mattoo et al., 2007) in which detailed analysis of polyamine accumulation in transgenic tomato lines, deficient in the expression of enzymes of polyamine biosynthesis, revealed a shift in SAM flux concomitant with an increase of ethylene, and a decrease of Asp levels.

**nor, rin and Nr are displayed metabolic shifts**

Levels of malate, the precursor of Asp, decreased during ripening in WT, as previously reported (Carrari et al., 2006). Malate levels also decreased in *rin* and *Nr*, but the change observed in these mutants was less than that observed in WT. In contrast, malate content was unaltered during fruit development in *nor*. These data suggest that ethylene feedback-regulates its own synthesis not only at the transcriptional but also the metabolic level. Another general trend was a general up-regulation of genes related to sucrose degradation in *rin*, *Nr*, but not in *nor*, as well as down-regulation of aromatic amino acid synthesis related genes across the late stages of ripening among the three mutants. These changes were reflected in lower Glc and Fru levels, the major hexoses in tomato fruits, in the three mutants in comparison to WT. Levels of Phe, the only aromatic amino acid detected in this study, gradually increased during normal ripening and also in the three mutants, but to a lesser extent. Interestingly, aromatic amino acids can act as alternative respiratory substrates when carbohydrates are not abundant (Ishizaki et al., 2005). As such, this decline may reflect a partial reliance of the mitochondrial electron transport chain during later stages of fruit development when carbon demand is provided entirely by source leaves of the plants, as opposed to a partially autonomous supply of photoassimilates during early fruit development.

**Cell wall metabolism is affected in nor, rin and Nr**

One of the most evident ripening related changes in tomato fruit is the progressive and extensive loss of firmness, results in part from cell wall disassembly (Brummell, 2006; Vicente et al., 2007). Many reports have described modifications to pectic polysaccharides in primary cell walls and middle lamellae, which include the removal of methyl ester groups by pectin esterases, solubilisation of galacturonic acid (GalA)-rich polysaccharides and the loss of galactose (Gal) from pectic fractions (Pressey, 1983; Redgwell et al., 1997). The disassembly of hemicellulosic and pectin
cell wall polysaccharides is known to be severely reduced in rin fruit and apparently is diminished in nor, since they also have only limited softening (Seymour et al., 1987; Giovannoni et al., 1989; Dellapenna et al., 1990; Maclachlan and Brady, 1994). The cell wall associated transcript, protein and metabolite data in this current study corroborate and extend these previous results. At the transcript level, we observed up-regulation of the cell wall degradation related genes polygalacturonase and pectate lyase during ripening in WT, but not in equivalent stage nor and rin fruits, and the up-regulation of these genes was delayed in Nr consistent with the partial ripening response of the mutant. However, at the cell wall-related metabolite level, all three mutants displayed similar patterns during ripening: galacturonate, galactose, arabinose, xylose, ribose and rhamnose levels increased substantially during ripening in WT but no such changes were observed in nor, rin and Nr. These transcript and metabolite data therefore indicate that normal ripening related cell wall degradation is compromised in all three mutants and further suggests this is an ethylene influenced phenomenon. Moreover, at the protein level, it has is already shown that in cell wall preparations from normal tomato fruit during ripening, salt-soluble protein (cell wall related proteins) were increased (Hobson et al., 1983). However, polygalacturonase-solubilized protein from rin was less, and that from the Nr cell walls was more than that from normal wall preparations (Hobson et al., 1983). Similar results were observed in our data, in Nr, polygalacturonase and pectin esterase (cell wall degradation related proteins) were more abundant as compared to WT at breaker stage (42 DAP); however, the opposite behaviour was observed in the same stage in rin. Moreover, in late ripening stages (52 DAP) a reduction of these proteins was apparent in all three mutant reflecting that cell wall degradation is likely compromised in these mutants (Dellapenna et al., 1989; Giovannoni et al., 1989). Furthermore, we observed that the reduction in the level of polygalacturonase protein was stronger in nor and rin as compared to Nr at the 52 DAP stage suggesting that PG protein accumulation is influenced by both ethylene and additional transcriptional effects.

Others metabolic changes are showed by nor, rin and Nr

In addition to cell wall metabolism per se the closely related metabolic pathway leading to ascorbate biosynthesis has also been described in ripening tomato fruits (Carrari et al., 2006). We observed an up-regulation of ascorbate related
genes during ripening as well as ascorbate accumulation, as described previously (Alba et al., 2005), only in Nr. Interestingly, neither nor or rin showed changes in this pathway, suggesting that ascorbate metabolism is influenced by ethylene perception.

Phytoene synthase (PSY1) catalyzes the formation of phytoene, the first C40 carotene intermediate in carotenoid biosynthesis. This is an essential step in the production of the carotenoids, which give the fruit its red colour. Nr, nor, and rin, all showed some carotenoid production, although the synthesis of lycopene is reduced, delayed, or absent, depending on the mutation (Tigchelaar et al., 1973). These phenotypes can be probably explained by the different time frame of expression of PSY1. During normal tomato ripening, this gene was induced from breaker stage (42 DAP), however in nor and rin this up-regulation was at earlier stages (39 and 41 DAP, respectively) and Nr a later stage (52 DAP) again indicating complex ethylene modulation of PSY1 gene expression.

To date, several studies have indicated that levels of organic acids correlate strongly with genes associated with ethylene and cell wall metabolism associated pathways, underscoring the importance of these metabolic intermediates in ripening (Carrari et al., 2006; Centeno et al., 2011). In the current, study we report than in nor levels of all organic acids, mainly TCA cycle intermediates, were strongly affected across ripening when compared with WT, while in rin and Nr, relatively small changes were observed. These results support the hypothesis that TCA cycle intermediates are regulated at the transcriptional level (Carrari et al., 2006), although we cannot exclude the possibility that in plants, as in animals (He et al., 2004), these intermediates could play a key role in mediating retrograde-regulated gene expression (Zarkovic et al., 2005). Furthermore, the fact that organic acids were stronger affected in nor, a mutant in which ethylene biosynthesis and signalling are also strongly affected, based on our transcriptomic data, suggests that these metabolites are developmentally regulated at the level of ripening associated transcripts. This observation has important biotechnological implications given that the manipulation of organic acids in tomato ripening is relatively facile (Centeno et al., 2011).

**Protein variations as compared to transcriptomic data in nor, rin and Nr**

Transcriptomic profiling allowed the identification of hundreds of genes that were differently regulated during the ten stages of tomato fruit development among the
three mutants. While of course by no means comprehensive, in this study a total of 158 proteins showed variations in abundance during ripening or equivalent temporal stages of development in nor, rin and Nr when compared with WT. We also found that a similar number of proteins showed decreased or increased expression among the three mutants. The highest number of differently expressed proteins with respect to WT were at the ripe stage (52 DAP) among the three mutants. Moreover, nor and rin shared a greater number of common proteins, especially at 52 DAP (Supplemental Fig. S3). These data further support our conclusion that nor and rin act together in the ethylene signalling cascade. Of the 158 identified varying proteins, 133 corresponded to gene sequences that figure on the TOM1 microarray, allowing a comparison of ripening-related or genotypic differences in specific transcripts or cognate proteins. In this study, 32% (42 out of 133) of protein and transcript pairs decreased or increased in parallel during ripening. This correlation was particularly apparent at the late ripening stage (52 DAP). A high number of proteins which showed not correlations with the corresponding transcripts belonged to primary metabolism such as serine malate dehydrogenase, enolase, glyceraldehyde-3-phosphate dehydrogenase, acid invertase, hydroxymethyltransferase, glutamate decarboxylase, aspartate aminotransferase, suggesting the concept that post-transcriptional regulation plays an important role in primary metabolism gene expression, and highlighting the need for combined transcriptomic and proteomic analyses.

**Summary**

Network analysis revealed key candidate regulators that may play defining roles in tomato fruit ripening. This combination of transcriptome, proteome and targeted metabolite analysis has helped to refine the ethylene regulated transcriptome of tomato fruit and added to our knowledge the role of ethylene in both protein and metabolite regulation in tomato ripening. Furthermore, in this study, we were able to draw several important conclusions concerning transcriptomic/metabolic regulation during tomato ripening. First, our data support the contention that nor and rin act together in a cascade to control ripening (Giovannoni et al., 1995; Thompson et al., 1999) and also suggest that nor has a more global effect on ethylene/ripening related gene expression than rin which
indicates that nor operates upstream of rin. The data also support that rin and nor act upstream of Nr which would be logically anticipated given the necessity of rin and nor for ethylene synthesis. A potential genetic regulatory network centred on ethylene governing tomato fruit development and ripening is presented in Figure 6. Thirdly, metabolite abundance of specific compound classes such as TCA-cycle organic acids and cell wall related metabolites appear to be strictly controlled with specific compounds influenced by ethylene, transcriptional control or both. Based on integrated analysis of transcripts, proteins and metabolites we were able to identify areas of metabolism that seem to be of high importance to the ripening process such as hormones and cell wall metabolisms in ethylene perception. Therefore, the integrated analysis enables us to uncover additional information for the comprehensive understanding of biological events relevant to the metabolic regulation during tomato fruit development. There outputs will provide potential targets for engineering of metabolism to facilitate the controlled modulation of ripening in tomato fruit.

MATERIALS AND METHODS

Plant material and sampling

Tomato (Solanum lycopersicum cv. Ailsa Craig) and isogenic lines for nor, rin, and Nr mutation (cv. Ailsa Craig, backcross parent) were grown in the greenhouse at 26°C under 12 h supplemental lighting, followed by 12 h at 20°C. To collect stages prior to ripening, fruits were tagged 7 days post anthesis (DPA<1 cm fruit; (Giovannoni et al., 1989) and harvested at one of the following five time points: 7, 17, 27, 39 (Mature green; MG), 41 (Breaker-1; Br-1), 42 (Breaker; Br), 43 (Breaker+1; Br+1), 47 (Breaker+5; Br+5), 52 (Breaker+10; Br+10), and 57 DPA(Breaker+15; Br+15). The first signs of carotenoid accumulation on the external surface of the fruit were taken to define the breaker stage. To maximize developmental synchrony, harvested fruit were visually inspected externally and internally (e.g. size, shape, pigmentation, seed development, and development of locular jelly), and only fruits appearing developmentally equivalent were kept for the analysis. Transcriptome, proteome, and metabolomic analyses were performed in the same material. For Nr mutant was performed in three separate pools of fruits of 15-30 fruit each. All fruits were collected from four individual plants. For nor and rin mutants 8 to 10 individual fruits for each
time point as biological replicates were considered. These individual fruit replicates came from 10-15 rin, nor, and WT plants (all of which were grown in the greenhouse crop; the genotypes were randomly distributed in the greenhouse).

For transcriptome, proteome and metabolomic analyses eight, four and five biological replicates were used. After tissue selection, pericarp tissue was collected, then frozen in liquid nitrogen, and stored at -80°C until further analysis.

Transcriptome analysis
Total RNA from tomato pericarp was extracted as described in Alba et al., (2004, 2005). cDNA synthesis, labelling, hybridization, washes, and scanning were conducted as described in (Alba et al., 2004). Glass slides containing arrayed tomato ESTs were obtained directly from the Centre for Gene Expression Profiling at the Boyce Thompson Institute, Cornell University synthesized in the Giovannoni lab as described in Alba et al., (2004). The tomato array (TOM1) contains approximately 12,000 unique elements randomly selected from cDNA libraries isolated from a range of tissues, including leaf, root, fruit, and flowers, and representing a broad range of metabolic and developmental processes. Nucleic acid sequence and annotation data pertaining to the TOM1 microarray are available via the Tomato Functional Genomics Database (http://ted.bti.cornell.edu). Data acquisition and filtering was conducted as in (Alba et al., 2005). The raw intensity values were normalized using Robin’s default settings for two-colour microarray analysis (Lohse et al., 2010). Specifically, background intensities were subtracted from the foreground values and subsequently a print tip-wise loess normalization (Yang et al., 2002) was performed within each array. To reduce technical variation between chips, the logarithmized red and green channel intensity ratios on each chip were subsequently scaled across all arrays (Yang et al., 2002; Smyth and Speed, 2003) to have the same median absolute deviation. Statistical analysis of differential gene expression was carried out using the linear model-based approach developed by (Smyth, 2004). The obtained P values were corrected for multiple testing using the strategy described by (Benjamini and Hochberg, 1995) separately for each of the comparisons made. Genes that showed an absolute log2 fold-change value of at least 1 and a P value lower than 0.05 were considered significantly differentially expressed. The log2 fold-change cutoff value was imposed to account for noise in the experiment and make sure that only genes that show a marked reaction are recorded. For visualization, the data were loaded
into MapMan (Usadel et al., 2009), which displays individual genes mapped on their pathways as false colour-coded rectangles. MapMan bins were assigned to each cDNA on the glass based on the SGN tomato unigene mapping. Wilcoxon rank sum tests were performed to test whether there were bins that were significantly and consistently behaving different than the other bins in the MapMan ontology using the built-in function in MapMan. The PageMan software package (Usadel et al., 2006), was then used to select and display biologically relevant details of these datasets. The transcript data for Nr has previously been published (Alba et al., 2005). The design of nor and rin microarrays were performed using RNA pool from different tissues (combination of tissues from different developmental stages of pericarp, leaf, and stem tissue) as a reference.

**Proteome Analysis**

Proteins were extracted from pericarp tissues of MG, Br, and Br+10 stages from four biological replicates of WT and the nor, rin, and Nr mutants. Samples were prepared according as described in (Isaacson et al., 2006). Extracted proteins were quantified using a modified Bradford method (Bradford, 1976). In order to confirm the quantification, 20 µg of each sample was loaded in 12% acrylamide gel and run under denaturing conditions. The gel was stained with 0.04% Coomassie Blue R-250 staining solution and analyzed using Image J software (Abramoff et al., 2004). Proteins preparation and identification were done as described in López-Casado et al., (2011). Proteins were labeled using iTRAQ Reagents Multiplex kit (Applied Biosystems, Foster City, CA) according to the manufacturer’s instructions and digested with trypsin. Labeled peptide mixtures were first analysed by MALDI-TOF MS (ABI 4700; Applied Biosystems) to confirm digestion and labeling. A strong cation exchange fractionation (SCX) LC using an Agilent 1100 HPLC with a UV detector (Agilent Technologies, Inc. Santa Clara, CA) was followed by nano liquid chromatography-tandem mass spectrometry (nLC-MS/MS). All nLC-MS/MS experiments were performed using an LC Packings Ultimate integrated capillary HPLC system equipped with a Switchos valve switching unit (Dionex, Sunnyvale, CA) connected in line to a hybrid triple quadrupole linear ion trap mass spectrometer (4000 Q Trap, ABI/MDS Sciex, Framingham, MA) and equipped with a Micro Ion Spray Head ion source. MS data acquisition was performed using Analyst 1.4.2 software (Applied Biosystems) in the positive ion mode for information dependent
acquisition (IDA) analysis. MS data were interrogated using the Paragon method (Shilov et al., 2007) using ProteinPilot™ software 2.0 (Applied Biosystems) and searched against a translated custom unigene database derived from RNA-seq (454 pyrosequencing; GS FLX, Roche, Indianapolis, Indiana, USA) database (Assembly2009_protein: ftp://ted.bti.cornell.edu/pub/tomato_454_unigene/). Only proteins showing a protein score higher than 95% (or 1.3 in ProteinPilot) with two or more unique peptides identified with a \( P \) value of \( \leq 0.05 \), and False Discovery Rate (FDR) <0.15 were further analyzed.

**Metabolome Analysis**

Metabolite analysis by GC-MS was carried out essentially as described by (Lisec et al., 2006) but with modifications specific for tomato (Schauer et al., 2006). The mass spectra were cross-referenced with those in the Golm Metabolome Database (Kopka et al., 2005). Analyses of primary metabolites were done in pericarp tissue from 27, 39, 41, 42, 43, 47, 52, and 57 DAP in lines for \textit{nor}, \textit{rin} mutations, and from 42, 47, 52, and 57 DAP from line \textit{Nr} mutation. Each sample point was analysed with five biological replicates.

**Data analysis and statistics**

Data normalization, visualization, Heatmaps, and correlation analysis based on Pearsson correlation were performed using R software (Gentleman, 1996). Visualization of transcripts-proteins-metabolites correlation was conducted using Cytoscape (Cline et al., 2007).
**Acknowledgements**
Support was provided in part through funds from the National Science Foundation (DBI-0606595) to JKR and (DBI-0923312) JJG. We thank Axel Nagel for online data deposition.

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

**Supplemental Figure S1.** A condensed PageMap display of changed pathways. The gene expression was represented as log2 fold changes between the wild type and each mutant at the different studied stages.

**Supplemental Figure S2.** A condensed PageMap display of altered pathways which were not represented in Figure 1.

**Supplemental Figure S3.** Venn diagrams of proteins that increased or decreased among the three mutants.

**Supplemental Figure S4.** Visualization of transcripts-proteins-metabolites correlation.

**Supplemental Table 1.** Raw data from Pageman analysis (Figure 1).

**Supplemental Table 2.** Total significantly detected proteins among the three mutant relative to WT

**Supplemental Data Set 1.** Transcriptomic data set.

**Supplemental Data Set 2.** Selected transcripts and proteins for network analysis.

**Supplemental Data Set 3.** Primary metabolite levels in WT, nor, rin, and Nr (metabolites not presented in Figure 2).

**Supplemental Data online.** MapMan (http://mapman.gabipd.org/web/guest/mapmanweb)
Literature cited


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**Figure legends**

**Figure 1. Expression analysis in WT, nor, rin, and Nr.**
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pathogenesis-related protein P2 (P32045); green-blue, lypoxygenase A (P38415); light grey, lypoxygenase (AAB65766); orange, oxygen-evolving enhancer protein 2 (P29795); yellow, alcohol dehydrogenase (P28032); dark blue, pyruvate decarboxylase (BAC23043); dark lilac, heat shock protein 83 (P51819); dark pink, methionine sulfoxide reductase (P54153); dark blue, glutathione S-transferase-like protein (AAL92873); brown, pyruvate decarboxylase (AAZ05069); grey-blue, phosphoenolpyruvate carboxylase 2 (CAB65171); light yellow, ASR4 (AAY98032).

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**Figure 6.** A potential genetic regulatory network centred on ethylene governing tomato fruit development and ripening.

**Table I.** Correlation between the expression ratios (mutant/WT). The relative change in abundance (mutant/WT) is shown in log2 scale from proteins from 39, 42, and 52 DAP tomato fruits. Bold values denote differences that were determined to be significant by Students $t$-test analysis ($P<0.05$) compared to WT samples harvested at the same stage.
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