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ABSTRACT

Tomato fruit ripening is a complex metabolic process regulated by a genetical hierarchy. A subset of this process is also modulated by light-signaling, as mutants encoding negative regulators of phytochrome signal transduction, show higher accumulation of carotenoids. In tomato phytochromes are encoded by a multi-gene family, namely *PHYA, PHYB1, PHYB2, PHYE* and *PHYF*, however, their contribution to fruit development and ripening has not been examined. Using single phytochrome mutants- *phyA, phyB1* and *phyB2* and multiple mutants- *phyAB1*, *phyB1B2* and *phyAB1B2*, we compared the on-vine transitory phases of ripening till fruit abscission. The *phyAB1B2* mutant showed accelerated transitions during ripening with shortest time to fruit abscission. Comparison of transition intervals in mutants indicated a phase-specific influence of different phytochrome species either singly or in combination on the ripening process. Examination of off-vine ripened fruits indicated that ripening specific carotenoid accumulation was not obligatorily dependent on light and even dark incubated fruits accumulated carotenoids. The accumulation of transcripts and carotenoids in off-vine and on-vine ripened mutant fruits indicated a complex and shifting phase-dependent modulation by phytochromes(s). Our results indicate that in addition to regulating carotenoid levels in tomato fruits, phytochrome(s) also regulate the time required for phase transitions during ripening.

INTRODUCTION

Tomato fruit ripening is a fascinating process involving well-orchestrated coordination of several regulatory steps which transform the green, unpalatable fruit to a fleshy aromatic red fruit, rich with carotenoids and other phytonutrients. The above process involves initiation of multiple genetic and biochemical pathways, the regulation of which has been intensively investigated during last three decades. These studies have indicated the existence of a molecular hierarchy in regulating the diverse metabolic and cellular responses during fruit ripening. The analysis of naturally occurring tomato mutants impaired in ripening such as *rin* and *cnr* revealed a genetic hierarchy determining initiation of ripening, as fruits of these mutants failed to undergo characteristic changes in color and aroma. The positional cloning of above mutant loci indicated that *RIN* and *CNR* loci encode for transcription factors belonging to MADS and SBP box family respectively and act as master regulators of ripening (Seymour *et al.* 2013). In addition, other transcription factors such as HB-1, a HD-ZIP homeobox protein (Lin *et al.* 2008) and TAGL1 (Vrebalov *et al*. 2009; Itkin *et al.* 2009) have been shown to be necessary for ripening. Many of the above transcription factors such as RIN are considered to be master regulators of ripening as RIN influences ripening even in non-climacteric fruits like strawberries (Seymour *et al.* 2011). Recently developmental modification of epigenome in tomato has also emerged as an additional regulatory step influencing fruit ripening (Zhong *et al.* 2013).

Downstream of genetic regulation of ripening by transcription factors, the climacteric rise of ethylene also plays an important role and mutants defective in perception of ethylene such as *Nr* fail to ripen normally even after exposure to exogenous ethylene. The inhibition of ethylene emission by antisense suppression of genes involved in ethylene biosynthesis such as *ACS2* (Oeller *et al.* 1991) or *ACO1* (Hamilton, Lycett & Grierson 1990) inhibited ripening, whereas suppression of ethylene receptor genes such *LeETR4* and *LeETR6* elevated ethylene

production and accelerated tomato ripening (Kevany *et al.* 2007; Tieman *et al.* 2000). The climacteric regulation of ripening may be assisted by other hormones such as ABA, as exogenous ABA application promoted ethylene synthesis and ripening and inhibition of ABA synthesis delayed tomato fruit ripening and softening. It has been suggested that onset of tomato ripening is initiated by ABA and role of ethylene is exerted at later stage of ripening (Zhang, Yuan & Leng 2009). In addition to climacteric regulation of ripening by ethylene, an independent nonclimacteric pathway also influences ripening process. Hormones such as auxin plays an important role in fruit development and exogenous application of auxin delays fruit ripening and induces parthenocarpic fruit sets (Jones *et al.* 2002). Jasmonate-deficient tomato mutants showed reduced lycopene levels and exposure to jasmonate vapor restored lycopene formation. Similarly jasmonate vapor stimulated lycopene formation in *Nr* mutant of tomato suggesting an ethylene-independent action of jasmonates (Liu *et al.* 2012). In addition to hormones, metabolites like sugar have also been shown to play a role in fruit development and ripening (Sagar *et al.* 2013; Jia *et al.* 2013).

Though tomato ripening is principally regulated by genetically determined hierarchy of regulatory genes accompanied with coordinated changes in hormonal levels, several studies have indicated that in addition to contributing to fruit photosynthesis, light also modulates fruit ripening, particularly the accumulation of pigments. In recent years, use of photomorphogenic mutants has greatly aided the identification of signaling partners regulating light mediated specific developmental responses. In tomato naturally occurring high pigment mutants such as *hp1* and *hp2* show exaggerated light responsiveness, higher anthocyanin levels during vegetative development and higher carotenoid levels in ripe fruits (Azari *et al.* 2010; Kilambi *et al.* 2013). Molecular genetic analysis of these mutants revealed that *hp1* and *hp2* loci are encoded by homologs of *DDB1* and *DET1* genes of Arabidopsis

respectively, acting as negative regulators in light mediated signaling pathway (Azari *et al.* 2010).

Plants perceive light via photoreceptors sensing specific spectral regions namely phytochromes, cryptochromes, phototropins (Möglich *et al.* 2010) and recently discovered UVR-8 (Rizzini *et al.* 2011). Examination of effectiveness of spectral quality indicated that red light stimulated carotenoid synthesis in ripening tomato fruits can be reversed by far-red light suggesting the involvement of phytochrome (Khudairi & Arboleda 1971; Thomas & Jen 1975). In vivo spectrophotometric examination of phytochrome using red/far-red light mediated photoreversible changes showed the presence of functional phytochrome in tomato fruits and its level declined with progress of ripening (Jen, Norris & Watada *et al.* 1977). Alba, Cordonnier-Pratt & Pratt (2000) showed that fruit-localized phytochrome stimulated carotenoid formation as tomato fruits harvested at mature green stage on red light exposure accumulated higher levels of lycopene during subsequent fruit development. In addition to phytochrome, cryptochrome also influences fruit ripening, as tomato plants overexpressing *CRY2* displayed phenotypes similar to *hp* mutants with enhanced anthocyanin and chlorophyll levels in leaves and increased lycopene level in fruits (Giliberto *et al.* 2005).

Tomato phytochromes are encoded by a small multi-gene family and they include *PHYA*, *B1*, *B2*, *E* and *F* (Hauser *et al.* 1995). Expression of all the five phytochrome genes was observed in tomato fruits, of these *PHYB2* and *PHYF* showed high expression and *PHYE* showed low expression (Hauser, Pratt & Cordonnier-Pratt 1997). Though tomato mutants disrupted in function of *PHYA*, *PHYB1*, and *PHYB2* are available, the studies using mutants impaired in specific phytochrome genes were mostly restricted to study the role of phytochromes on vegetative development of tomato (van Tuinen *et al.* 1995 a&b; Kerckhoffs *et al.* 1997; Weller *et al.* 2000). Among the transgenic tomato plants overexpressing homologous *PHYA, PHYB1* and *PHYB2*, the *PHYB2* overexpressor showed a strong

inhibition of stem elongation and a strong amplification of the R-HIR (Husaineid *et al.* 2007), indicating that during seedling deetiolation *PHYB2* is more dominant than *PHYB1* in tomato.

Though phytochrome influences fruit ripening (Alba *et al.* 2000), the role of specific phytochrome species on fruit development and ripening has not been examined. Given the existence of multiple phytochrome species, it is likely that fruit development and ripening may be influenced by a specific phytochrome species or different phytochromes may act together to regulate the above response. In order to delineate role of different phytochrome species, we used the *phyA, phyB1* and *phyB2* mutants and combinations of these mutants to examine the participation of different phytochrome species in the regulation of fruit development and ripening. We show that in addition to modulation of carotenoid formation, phytochromes also influence time intervals marking different phases of fruit ripening.

MATERIALS AND METHODS

Plant materials

The tomato (*Solanum lycopersicum*) cultivars, MM (Moneymaker) and breeding line GT, and phytochrome mutants - *phyA*, *phyB1*, *phyB2*, *phyAB1*, *phyB1B2*, and *phyAB1B2* were obtained from R. Kendrick and M. Koornneef, University of Wageningen, The Netherlands. The genetic background of these phytochrome mutants has been described previously (Appenroth *et al.* 2005, Supporting Information Table S1). Tomato seeds were surface sterilized with 4% (v/v) sodium hypochlorite and seedlings were grown on coconut peat (Sri Balaji Agro Services, India) for 3 weeks. Thereafter the seedlings were transplanted to pots filled with soil and plants were grown in greenhouse at Hyderabad under natural photoperiod (12-14 h day, 10-12 h night) at 28±1°C during day and ambient temperature (14-18°C) in night.

Fruit development and light treatments

To maintain uniformity in the experiments, the fruits formed on first two trusses were only used for all experiments. The fruit development was monitored from the day post-anthesis (DPA) through different stages of ripening on vine: mature green, breaker, red ripe till fruits abscised from the plants. The transition between different stages of ripening was visually monitored by the changes in fruit color. To precisely determine the age of fruits, the flowers were tagged at anthesis and this time point was considered as zero DPA. The increase in the diameter of fruits from anthesis to red ripe stage was determined by using a digital Vernier caliper in horizontal direction. For off-vine experiments, fruits were harvested at MG stage after attaining respective chronological age and were incubated under red light (R; λ_{max} 650 nm, 5 Lmole m^{-2} sec^{-1}), far-red light (FR; λ_{max} 760 nm, 15 Lmole m^{-2} sec^{-1}), or kept in complete darkness (D) for 21 days at 25±1^{o}C. The light emitting diodes that were used to

generate R or FR were purchased from Kwality Photonics, Hyderabad, India and Roithner Laser Technik, Vienna, Austria respectively. The light intensity was measured using a quantum photometer (SKYE, UK).

Determination of ethylene evolution

The ethylene emission from harvested fruits was measured using a previously described procedure (Santisree *et al.* 2011; Kilambi *et al.* 2013). For every fruit sample, amount of ethylene emitted in the headspace was measured at least in duplicate and more than 5 independent biological replicates were analyzed for every developmental stage and light treatment.

Estimation of pigments

The fruits were harvested at different stages of development and also at the end of incubation under different light treatments. These were flash frozen in liquid nitrogen and stored at -80°C. Lycopene and β-carotene were extracted from fruits and estimated using a previously described procedure (Kilambi *et al.* 2013). Total chlorophyll content of mature green fruits were extracted by homogenizing fruit tissue in 80% (v/v) acetone as described in Arnon *et al.* (1949).

Sugar content

Sugar content of fruits at MG and RR stages, and also after exposure of fruits to different light treatments was measured using a pocket refractometer (ATAGO, Japan). Entire pericarp tissue of the fruit was homogenized using mortar and pestle and 300 Ll of homogenate was layered on the optical lens of the refractometer. The sugar content was recorded as °Brix. One unit of °Brix is approximately 1% (w/v) reducing sugars and soluble solids as measured by refractometer (Tieman *et al.*1992). For every fruit sample ($n \geq 5$), two parallel measurements were carried out.

Fruit firmness

The firmness of fruits was measured using a digital firmness tester Durofel (Agro-Technologie, France). The firmness was measured at equatorial plane of fruits at two diametrically opposite positions. A minimum of 5 fruits at each of the developmental stages and at the end of light treatments were used for measuring firmness.

RNA extraction and gene expression analysis

Total RNA was extracted from the pericarp tissue of fruits in two biological replicates at different developmental stages (for on-vine experiments) and at the end of light treatments (for off-vine experiments) using TRI Reagent (Sigma) according to the manufacturer's instructions. Genomic DNA contamination in the isolated RNA was eliminated by incubating with RNAse-free DNAse (Promega) as per manufacturer's protocol. RNA concentration and quality was determined spectrophotometrically using a ND-100Nanodrop UV-Vis spectrophotometer. cDNA was prepared from 2 Lg of RNA using Invitrogen cDNA synthesis kit (SuperScript III; Life Technologies/Invitrogen, USA). Quantitative Real-time PCR (qRT-PCR) was carried out in a 7300 Fast Real Time PCR system (Applied Biosystems). The transcript abundance was measured in 10 Ll volume of the SYBR Green PCR Master Mix (Takara, Japan) containing cDNA corresponding to 5 ng of total RNA with gene-specific primers (Kilambi *et al.* 2013). For every biological replicate two technical replicates were analyzed for transcript level quantification. The ΔCt value was calculated by normalizing Ct values of each gene to the mean expression of both internal control genes (*β-actin* and *ubiquitin3*). [ΔCt value = (Ct value of gene)-(mean Ct value of control genes)]. The real time data were subjected to hierarchical clustering using the ΔCt value. For ease of data visualization, the scale was inverted to facilitate direct correlation to enhanced (green/black) or reduced transcript (red) expression. Primers for *PSY1, PSY2, PDS, ZISO, ZDS, CYCB,*

LCYB, *RIN*, *ACS2*, *ACO1*, *COP1*, *PHYA*, *PHYB1*, *PHYB2*, *β-actin* and *ubiquitin3* were designed using primer3 software (for primer details, see Supporting Information Table S2).

Data analysis and hierarchical clustering

In view of near genetical identity of MM and GT cultivars all on-vine and off-vine data obtained in this study were compared with both wild types. In order to compare two means, we used the student's t-test at the 5% level. Several means were compared by one-way ANOVA test. Because of the large number of data, we did not indicate the results of these tests but mentioned differences in the text only in case of significant differences. Hierarchical clustering based on the unweighted pair group method with arithmetic mean was performed using PermutMatrix software version 1.9.3 (Caraux & Pinoloche 2005). The differences obtained in lycopene, *β*-carotene levels; transcript profiles; ethylene, Brix and firmness measurements between wild types and phytochrome mutants either during on-vine or off-vine study were visualized in the form of heat-maps.

RESULTS

Phytochrome mutations accelerate tomato fruit ripening

Post-anthesis the development of tomato fruit undergoes four distinct phases, the expansion of fruit till mature green stage (MG), followed by the onset of the ripening visualized as breaker stage (BR), ripening of the fruits to red ripe stage (RR) and finally fruit abscission (FA) from the vine. Given that photoreceptors influence several facets of plant development, we examined whether the mutations in different phytochrome species influenced the process of fruit expansion. It is believed that tomato fruits attain the final size at MG stage followed by onset of ripening. Examination of diameter of fruits of wild types and mutants revealed that the fruits nearly attained 90% of their size by MG stage (Fig. 1). However post-MG stage, the fruits continued to expand, albeit at a slow pace and reached a plateau by RR stage. The growth of phytochrome(s) mutant fruits was nearly parallel to wild types excepting *phyAB1B2*mutant fruits which were smaller in size (Fig. 1b).

In contrast to fruit expansion, phytochrome(s) influenced the time duration from anthesis to FA. The time interval from anthesis to FA was nearly the same for wild type progenitors viz GT and MM (Fig. 2a). Barring *phyA* and *phyB2* mutants, other phytochrome mutants showed time intervals significantly different from that of wild types (Fig. 2a). Compared to wild types, *phyB1, phyAB1, phyB1B2,* and *phyAB1B2* mutants showed accelerated ripening as evident by shortened duration to FA. Among these mutants, the *phyAB1B2* showed most accelerated fruit ripening with fruit abscission nearly 21 days earlier than the wild types. The time duration to FA in other mutants was in the following order with decreasing duration *phyB1B2>phyB1>phyAB1* (Fig. 2a). The reduction in duration to FA ranging from 9-21 days in *phyB1B2, phyB1, phyAB1, phyAB1B2* mutants compared to wild types indicates an influence of phytochrome on duration of ripening.

We next examined whether different phytochrome(s) specifically influence a particular stage of fruit development (Fig. 2b-e). One of the earliest landmarks of fruit development is attainment of MG stage, post which fruit metabolism shifts to ripening. While wild types as well as the *phyB1* mutant attained MG stage at 36 DPA, *phyA*, *phyB2*, *phyAB1* and*phyB1B2* mutants required significantly longer time to attain MG stage (Fig. 2b). However, consistent with accelerated fruit ripening observed in *phyAB1B2* mutant (Fig. 2a, f), the attainment of MG stage in this mutant was accelerated. The transition from MG to breaker stage in wild types was attained in 14 days; however, in *phyA* mutant, time taken to

phytochrome mutants showed an accelerated transition from MG to the breaker stage. The transition interval to breaker stage was significantly shortened in *phyAB1* and *phyAB1B2* mutants (Fig. 2c,f). The mutation in different phytochromes also accelerated the transition from breaker to RR stage, with *phyB2* mutant requiring shortest time span to reach RR stage (Fig. 2d,f). Examination of time interval required for fruit abscission after attainment of RR stage revealed that *phyA* mutant fruits required time period similar to wild types (Fig. 2e,f). Consistent with reduction in duration in earlier two stages of ripening, all other phytochrome mutants showed accelerated fruit drop than the respective controls, with *phyB1* and *phyAB1* mutants showing shortened duration to FA. An exception was *phyB2* mutant, where after attainment of RR stage, fruits remained on vine for prolonged duration before FA compared to other mutants/wild types (Fig. 2e,f).

Phytochrome(s) influence carotenogenesis in a complex interactive/counteractive manner

A distinctive feature of tomato ripening is change in the coloration of fruits from green to red, wherein reduction in chlorophyll level is associated with an increase in the carotenoids level. At MG stage, the fruit skin of phytochrome mutants was light green compared to dark green

skin of wild types (Fig. 3a). Basically the pale green color was restricted to fruit skin and longitudinal sections of mutant and wild types fruits showed nearly identical color in pericarp. However, pericarp of *phyA* and *phyAB1* mutants was paler, while *phyAB1B2* mutant exhibited an intermediate phenotype (Fig. 3a). The observed pale green color in above mutants also correlated with the reduced chlorophyll levels in these fruits (Fig. 3b). During transition to RR stage though fruits of single and multiple phytochrome mutants developed characteristic red color attributed to accumulation of lycopene, however, the visually perceptible differences in fruit coloration between different mutants and wild types persisted (Fig. 4a).

To quantify the influence of different phytochrome mutations on carotenogenesis during ripening, the levels of two principal carotenoids viz β-carotene and lycopene were monitored by HPLC in on-vine ripened fruits harvested at MG and RR stages (Fig. 4b,c). The difference in carotenoid levels was first examined at MG stage in mutants and the wild types (Fig 4b,c). Both β-carotene and lycopene were present in the pericarp of MG wild type fruits at basal levels and were nearly the same in the fruits of the two wild types (Fig 4b,c). Comparatively the level of lycopene in MG fruits of *phyA* and *phyB1* mutants was significantly higher than the respective wild types MM and GT (Fig. 4b,d). Because different phytochrome species may interact with each other resulting in physiological effects different from that of single mutants, we also analyzed two double mutants (*phyAB1*, *phyB1B2*) and one triple mutant (*phyAB1B2*). The lycopene level in MG fruits of the double mutants (*phyAB1*, *phyB1B2*) was similar to that of the single phytochrome mutants. In contrast to lycopene levels, comparison of β-carotene levels in MG fruits of different single and multiple phytochrome mutants with wild types fruits did not reveal any significant differences, with the exception of *phyA* and *phyAB1B2* mutants that showed lower levels of β-carotene in MG fruits (Fig. 4c,e).

The transition from MG to RR stage of ripening significantly enhanced the accumulation of lycopene than the β-carotene levels. In wild type fruits the lycopene level increased almost 10-fold, whereas the ß-carotene level increased only by 1.5-3 fold (Fig. 4b,c). Comparison of lycopene levels in phytochrome mutant fruits at RR stage showed that while lycopene level was higher in the *phyB1* mutant, it was lower in *phyA* mutant than in the respective wild types (Fig. 4b,d). In the RR stage, only the *phyAB1* mutant showed slightly but significantly enhanced lycopene content compared to the single mutants, *phyA* and *phyB1*. Contrastingly, the β-carotene levels in all single phytochrome mutants were higher than in the respective controls (Fig. 4c,e). The β-carotene content in the RR stage, however, was lower in the *phyB1B2* and *phyAB1B2* mutants than in the related single mutants (Fig. 4c,e).

Above analysis of on-vine ripened fruits indicated the involvement of phytochromes in the modulation of carotenoid levels during ripening. However, on-vine ripened fruits are exposed to natural daylight which also activates other photoreceptors such as cryptochromes, phototropins and UVR8. To specifically distinguish the role of phytochromes on the above process, the mutant and wild type fruits were harvested at MG stage and were exposed to red light (R) or far-red light (FR) and respective controls were incubated in the darkness (D) for 21 days (Off-vine treatments; Fig.5). The above duration was chosen considering that on-vine ripened*phyA* mutant fruits required 21 days to reach RR stage from MG stage. Comparison of the accumulation of lycopene at the end of this incubation period revealed diverse patterns. Foremost light was not obligatorily required for pigment accumulation as fruits incubated in D showed increase in lycopene level (Fig. 5a,b). In D-incubated fruits, the increase in lycopene level comprised of three distinct groups consisting of little increase in *phyAB1, phyAB1B2, phyB1B2* mutants and high increase in *phyB1, phyB2* mutants with intermediate level in wild types and *phyA* mutant (Fig. 5b,h). Contrastingly differences between mutant

lines and wild types were not apparent in R-incubated fruits where the lycopene level was nearly equal (Fig. 5c,i). However, lycopene level in FR-incubated fruits showed little increase in *phyAB1* mutant, intermediate increase in *phyB2*, *phyA* and *phyB1B2* mutants and high increase in wild types and *phyB1* and *phyAB1B2* mutants (Fig. 5d,j).

Compared to lycopene, D-, R- or FR-incubated fruits showed much lower stimulation in β-carotene level (Fig. 5e-g). Among different treatments FR elicited much higher stimulation of β-carotene level than D and R. Comparison between different mutants revealed a lower stimulation in the β-carotene content in *phyAB1B2* in R-incubation and *phyB1B2* and *phyAB1* mutants in FR-incubation, whereas *phyB1* mutant showed most conspicuous increase in β-carotene levels under all three incubation conditions (Fig. 5e-g, k-m).

Phytochromes influence *PSY1* and *CYCB* transcript levels in an opposing manner at MG and RR stage

The transition from MG to RR stage in tomato fruits is associated with transformation of chloroplasts to chromoplasts. Several studies indicated that during this transition, a chromoplast-specific carotenogenic pathway is initiated. The pathway mainly differs from chloroplast-specific pathway in two enzymes, namely, phytoene synthase1 (PSY1) and chromoplast specific lycopene β-cyclase (CYCB), while phytoene desaturase (PDS) and ζ-carotene desaturase (ZDS) enzymes are common to both pathways. It is presumed that up-regulation of PSY1 and CYCB enzymes is associated with the inhibition of chloroplast-specific phytoene synthase2 (PSY2) and lycopene β-cyclase (LCYB) enzyme activities. A study by Schofield & Paliyath (2005) indicated that during fruit ripening phytoene synthase activity in tomato is influenced by phytochrome at post-transcriptional level. Similarly, ζ-carotene isomerase (ZISO) also plays an important role in accumulation of carotenoids (Chen, Li, & Wurtzel 2010). To decipher the relative contribution of phytochromes towards

carotenoid synthesis, we examined the transcript levels of *PSY1*, *PSY2*, *PDS*, *ZDS*, *ZISO*, *CYCB*, and *LCYB* at both MG and RR stages in the phytochrome mutants and the wild types.

Analysis of transcript levels of *PSY1* in on-vine ripened fruits of phytochrome mutants revealed a very interesting pattern (Fig. 6a). In MG fruits, while wild types had higher levels of *PSY1* transcript, mutation in phytochrome(s) genes as a single or in combination caused a decline in *PSY1* transcript level. The most severe decline in *PSY1* transcript was observed for *phyA* mutant, whereas in other single or multiple mutants the decline was less severe. In contrast to MG stage, at RR stage the level of *PSY1* transcript declined in wild types, whereas in single or multiple mutants of phytochromes accumulation of*PSY1* transcript was enhanced. This contrasting regulation indicated a shift in the role of phytochrome(s) on *PSY1* transcript accumulation during transition from MG to RR stage of ripening. Surprisingly at RR stage, the fruits of *phyAB1B2* mutant showed reduced *PSY1* level similar to wild types indicating opposing function of single phytochromes versus multiple phytochromes during transition to RR stage (Fig. 6a).

The fruits ripened under D, R, or FR revealed a complex interaction between phytochromes (Figure 6a). In D-incubated fruits, while *phyB1*, *phyAB1*, *phyAB1B2* mutants showed inhibition of *PSY1* transcript accumulation, the fruits of *phyA* mutant showed increased*PSY1* transcript levels than the wild types. In R-incubated fruits, mutations in *phyAB1*, *phyB1B2* inhibited accumulation of *PSY1* transcript, while mutations in *phyAB1B2* rather promoted *PSY1* transcript accumulation (Fig. 6a). As expected, *phyA* mutant fruits incubated in FR showed severely reduced level of *PSY1* transcript (Fig. 6a). Interestingly, barring stimulation of *PSY2* transcript levels in D-incubated fruits of *phyAB1* mutant (Fig. 6b), no specific influence of any other phytochrome mutation was perceptible on transcript levels of *PSY2* indicating that regulation of *PSY1* transcript level is the prime target of light during carotenogenesis in tomato fruits.

Examination of *ZISO* transcript in fruits revealed that in MG stage, the levels of *ZISO* transcript were drastically low in wild types as well as in phytochrome mutants (Fig. 6a). At RR stage the levels of transcripts were lower in both wild types and all phytochrome mutants except *phyA*, *phyB2* and *phyAB1B2* mutants wherein higher stimulation of *ZISO* transcript accumulation was observed. There was no perceptible effect of phytochrome mutation on transcript levels of *ZDS* and *PDS* in MG and RR stages excepting downregulation of *ZDS* level in RR fruits of *phyA* mutant (Supporting Information Fig. S1).

In detached fruits incubated in D, mutations in phytochrome species, *phyA*, *phyB2* and *phyAB1* stimulated *ZISO* transcript accumulation, while a combination of *phyB1B2* or *phyAB1B2*inhibited *ZISO* transcript accumulation (Fig. 6a). In fruits incubated under R, except *phyAB1*, and *phyAB1B2*, other phytochrome mutations stimulated *ZISO* transcript levels. Interestingly, in FR incubated fruits of *phyA* mutant, the *ZISO* transcript level was severely reduced, similar to that observed for *PSY1* transcript levels, while *phyB1*, *phyAB1*, *phyB1B2*mutations stimulated its transcript level (Fig. 6a). Similar to on-vine ripened fruits, the phytochrome(s) mutations did not influence the *ZDS* and *PDS* transcript levels in detached fruits except upregulation of *PDS* and *ZDS* in *phyAB1* mutant in D-incubated fruits (Supporting Information Fig. S1).

Unlike *PSY1* and *PSY2* genes, where only *PSY1* transcript is strongly influenced by different phytochromes, in tomato fruits transcript levels of both *LCYB* and *CYCB* are regulated by phytochrome (Fig. 6). In MG fruits similar to influence on *PSY1* transcript, mutations in *phyB2*, and *phyAB1* inhibited accumulation of *CYCB* transcript, while at RR stage mutations in *phyB2*, *phyAB1*, *phyB1B2* and *phyAB1B2* stimulated *CYCB* transcript accumulation (Fig. 6a). Interestingly, for *LCYB*, a shifting pattern of *phyAB1* influence was observed, where at MG stage the *phyAB1* mutation strongly inhibited accumulation of *LCYB* transcripts, but at RR stage it stimulated the accumulation of *LCYB* transcripts (Fig. 6b).

In D-incubated fruits, mutation in *phyAB1* stimulated accumulation of both *LCYB* and *CYCB* transcripts (Fig. 6a,b). In addition, in D-incubated fruits, *phyB1B2* mutation too stimulated accumulation of *CYCB* transcript. In contrast, *phyAB1B2* mutation inhibited *LCYB* transcript accumulation in D-incubated fruits (Fig. 6b). In R-incubated fruits, no specific influence of phytochrome mutation on *LCYB* transcript was observed, however, *phyA* mutation stimulated the accumulation of *CYCB* transcript (Fig. 6a,b). Interestingly, FR-incubated fruits showed no influence on *CYCB* and *LCYB* transcript levels though these fruits accumulated higher β-carotene than D- or R-incubated fruits (Fig. 6a,b). Considering that the MADS box transcription factor RIN is obligatory for initiation of tomato ripening, we also examined whether the mutations in phytochromes in turn influenced the expression of *RIN* gene. Remarkably no significant influence of phytochrome mutations on *RIN* expression was found in MG and RR stage (Supporting Information Fig S2).

Loss of Multiple Phytochromes Enhances Climacteric Emission of Ethylene

Several physiological evidences have indicated that light acting via phytochromes regulates ethylene biosynthesis during de-etiolation of seedlings. The red light exposure of etiolated bean (Vangronsveld, Clijsters, &Van Poucke 1988) and wheat (Jiao, Yip & Yang 1987) seedlings led to reduction in ethylene synthesis. A role of phytochrome interacting factor 3 (PIF3) has been observed for ethylene-induced hypocotyl elongation in Arabidopsis seedlings in light (Zhong *et al.* 2012). In view of interactions between ethylene and phytochrome action, effect of phytochrome(s) mutation on ethylene formation was analyzed during fruit ripening. In the wild types and the phytochrome mutants, ethylene levels increased by several folds during the transition from the MG to the RR stage consistent with the climacteric rise in ethylene emission during ripening. In on-vine ripened fruits, the ethylene emission was nearly similar in wild types and phytochrome mutants, except *phyB1*, *phyAB1* and *phyAB1B2* mutants which showed higher levels of ethylene synthesis at RR stage (Supporting

Information Fig. S3). In off-vine ripened fruits incubated under D, R or FR the ethylene emission was nearly similar, barring reduced emission in R-incubated *phyB1B2* and *phyAB1B2* mutants, and high ethylene emission in FR-incubated *phyAB1* mutant (Supporting Information Fig. S3b-d). Interestingly FR-incubated *phyAB1B2* mutant fruits showed very high ethylene emission (Supporting Information Fig. S3d) which might be related to observed accelerated ripening of these fruits (Fig. 2a). Examination of transcripts of *ACS2* and *ACO1* genes encoding the enzymes regulating ripening-specific ethylene biosynthesis indicated lack of correlation between quantum of ethylene emission and above transcript levels (Supporting Information Fig. S3).

We next examined whether phytochrome influenced any other ripening parameters such as sugars and firmness of fruits as well as regulation of their own transcript levels. The examination of transcript levels of *PHYA, PHYB1* and *PHYB2* genes in single and multiple mutants of phytochrome and wild types indicated that mutation in the phytochrome genes did not drastically influence the transcript levels of phytochrome(s) both in on-vine and off-vine ripened fruits. A lone exception was D-incubated fruits of *phyAB1* and *phyB1B2* mutants where *PHYA, PHYB1 and PHYB2* transcript levels were higher than the wild types (Supporting Information Fig. S4). Similar to phytochrome transcripts, no specific influence of phytochrome(s) mutations was observed in *COP1* transcript levels, a gene considered to be major regulator of light-mediated signaling in plants excepting up regulation in D-incubated fruits of *phyA* and *phyAB1* mutants (Supporting Information Fig. S5).

The mutations in different phytochrome(s) apparently had no influence on the BRIX content of the fruits as evident by nearly similar values in both on-vine as well as off-vine conditions (Supporting Information Fig. S6).The transition from MG to RR stage of ripening was associated with decrease in firmness of fruits of wild types and mutants (Supporting Information Fig. S7a). In D-incubation *phyA* and *phyAB1* mutant fruits were less firm,

whereas in R-incubation, fruits of *phyAB1* mutant were less firm and *phyB2* and *phyAB1B2* mutants were firmer (Supporting Information Fig. S7b,c). Consistent with higher ethylene emission from *phyAB1B2* mutant fruits in FR-incubation, these fruits were also less firm than other mutants and wild types (Supporting Information Fig. S7d).

DISCUSSION

Fruit ripening is a complex genetically programmed process leading to changes in aroma, color, texture and nutritional composition of fruits. Though, the ambient environment of fruits also influences ripening, the studies concerning environmental regulation are largely confined to influence of temperature on ripening (Lurie 2006; Qu *et al.* 2009). While several studies have indicated the role of light on accumulation of pigments such as carotenoids and anthocyanins in the fruits, limited information is available on the effect of light on fruit development and size. Physiological (Alba *et al.* 2000), spectrophotometric (Jen *et al.* 1977) and gene expression (Hauser *et al.* 1997) studies have indicated the presence of functional phytochrome(s) in fruits. However, the role of specific phytochrome species on fruit development and pigment accumulation during ripening has not been explicitly examined. The studies using specific phytochrome mutants have largely focused on deetiolation of tomato seedlings overlooking their role in regulating fruit quality (van Tuinen *et al.* 1995a&b; Kerckhoffs *et al.* 1997; Weller *et al.* 2000; Husaineid *et al.* 2007). So far the relative contribution of different phytochrome species on fruit ripening is not available and forms the core of present study.

In tomato, the growth of wild types and phytochrome(s) mutant fruits was nearly parallel, except *phyAB1B2* mutant fruits, which were smaller in size. Influence of the phytochrome(s) was more perceptible on time required to attain MG stage and subsequent transitory phases of ripening. Excepting *phyB1* mutant, mutations in other phytochrome(s) prolonged the time interval to attain the MG stage, indicating a role for phytochrome(s) in the regulation of fruit development. Faster attainment of MG stage in *phyAB1B2* mutant highlighted that this control is exerted in a complex manner and deficiency of multiple Phytochromes hastens the process of ripening. The acceleration of ripening is clearly manifested as shorter time intervals to attain BR and RR stages. Excepting *phyA* mutant,

other mutants showed faster transition to BR stage. Seemingly multiple phytochrome(s) contribute to this phase, as evident from the shorter interval to attain BR in double and triple mutants of phytochromes.

The studies on tomato phytochrome mutant(s) seedlings grown in white light have indicated that phyB1 and phyB2 together mediate hypocotyl elongation and greening of seedlings, while single mutants do not have a perceptible phenotype (Weller *et al.* 2001). In contrast to this, our study highlights distinct functions of phyB1 and phyB2 on different phases of tomato fruit ripening. Compared to *phyB1* mutant, *phyB2* mutant fruits showed accelerated transition from BR to RR stage. A diametrically opposite action of phyB1 and phyB2 was observed on time interval from RR stage to the abscission of fruits from vine. The above process was accelerated in *phyB1* mutant, while in *phyB2* mutant fruit abscission was considerably prolonged compared to wild types. The observed shifting and opposing function of phytochromes(s) during different phases of ripening highlight that plants fine tune the ripening process or its phases by contribution from specific or combination of phytochrome species.

The reduced chlorophyll levels in *phyA, phyAB1* and *phyAB1B2* mutants indicated a role for *phyA* on stimulating chlorophyll biosynthesis in developing fruits (Castelfranco & Beale 1983). In MG fruits, the levels of lycopene were significantly higher in *phyA* and *phyB1*mutant fruits including double mutants, *phyAB1* and *phyB1B2*. It can be therefore assumed that prior to initiation of ripening the fruit-localized phyA and phyB1 inhibit the accumulation of lycopene till MG stage of fruit development (Figure 4B). Contrastingly, the level of β-carotene in MG fruits is exclusively determined by the action of phyA, as β-carotene level was reduced only in the fruits of *phyA* mutant.

The transition from MG to RR stage of fruit development is also accompanied by the transformation of chloroplasts to chromoplasts (Kilambi *et al.* 2013). Interestingly this transition also shifts the relative function of phytochromes on carotenoid formation at RR stage compared to MG stage. The transition period is associated with phyA mediated stimulation of lycopene formation, whereas phyB1 inhibits the lycopene formation. Consistent with this, RR stage fruits of *phyA* and *phyB1* mutant show lower and higher accumulation of lycopene respectively. Likewise conversion from lycopene to β-carotene in RR fruits appears to be stimulated by phyA, phyB1 and phyB2, as mutants in these phytochromes exhibited higher β-carotene levels. In entirety different phytochrome species modulate chlorophyll/carotenoid formation at MG and RR stage of ripening in a multifarious fashion.

To reveal the specific contribution of phytochrome(s), and to rule out the contribution of other photoreceptors, the fruits harvested at MG stage were exposed off-vine to R or FR with control fruits incubated in D. Of interest was the observation that both wild types and phytochrome mutant fruits incubated in D turned red in color indicating that light is not essential for carotenogenesis in fruits. Our results are consistent with Alba *et al.* (2000) who also observed carotenogenesis under D in fruits harvested at MG stage. It is plausible that the carotenogenesis during ripening is not necessarily dependent on light. A solitary study carried out by Cheung, McNellis & Piekos (1993) supports this possibility, where post-anthesis confinement of tomato flowers in total darkness, yielded white fruits at MG stage and red fruits on ripening. The development of white fruits discounts the possibility of any residual light reaching the developing fruits, as light is mandatory for the conversion of protochlorophyll to chlorophyll in higher plants. Therefore it can be presumed that the carotenogenesis during tomato ripening is not obligatorily dependent on light.

Consistent with more orange hue in their fruit skin, FR-incubated fruits of mutants exhibited lower lycopene and higher β-carotene levels indicating a specific effect of FR on conversion of lycopene to β-carotene. The absence of influence of single phytochrome mutants on β-carotene level in D-, R- or FR-incubated fruits suggests redundancy in phytochrome action. Consistent with the above redundancy, action of phytochromes on β-carotene level becomes perceptible only in combination of two or more phytochrome mutations, as multiple mutants show modestly or significantly reduced β-carotene levels. In contrast to β-carotene, phytochrome influences lycopene formation in more complex manner. Nearly similar levels of lycopene in R-incubated fruits of wild types and all mutants negated any specific role for phytochrome(s) on lycopene formation in red light. In D-incubated fruits, only double or triple phytochrome mutations significantly reduced the lycopene levels. In contrast, in FR-incubated fruits, while *phyB1* and *phyAB1B2* mutants showed lycopene level like wild types, other single and double mutants showed reduced lycopene level. Taken together the above results indicate that while multiple phytochromes in unison modulate β-carotene levels, the regulation of lycopene levels by phytochrome(s) is more complex and dependent on light environment such R, FR and D.

Onset of tomato fruit ripening is accompanied by a major shift in carotenoid biosynthesis pathway at two steps involving replacement of PSY2 by PSY1 for phytoene formation and replacement of LCYB with CYCB for the conversion of lycopene to β-carotene. Consistent with this shift, no variance is discernible in *PSY2* transcript levels in wild types and phytochrome mutant fruits, excepting D-incubated *phyAB1* mutant fruits. Although PSY1 activity is critically needed for the accumulation of carotenoids, the expression of *PSY1* transcript considerably varied at different stages of ripening and also among the phytochrome mutants. In general, on-vine ripened fruits of the phytochrome mutants showed low *PSY1* transcript levels at MG stage and high levels at RR stage, whereas

PSY1 transcript levels in wild types followed a totally opposite pattern. Apparently phytochrome's action on *PSY1* transcript accumulation shifts from promotive at MG stage to inhibitory at RR stage of ripening. The variations in *PSY1* transcript levels in mutants also do not correlate with carotenoid levels in fruits. Though, fruit-localized phytochromes regulate carotenoid synthesis (Alba *et al.* 2000), it is not accompanied with corresponding increase in *PSY1* transcript levels (Schofield *et al.* 2005). In a recent study on *hp1* mutant, a negative correlation between transcripts of carotenoid pathway genes including *PSY1* and carotenoid contents in the fruit was also observed, reiterating the posttranscriptional regulation of carotenogenesis (Kilambi *et al.* 2013). Taken together our results suggest that the observed changes in *PSY1* transcript levels in phytochrome mutants cannot be correlated to carotenoid accumulation in tomato fruits.

Consistent with the principal role of CYCB in carotenogenesis, the transcript levels of *LCYB* were nearly 10-fold less than *CYCB* levels in the fruit. Moreover, like *PSY2* transcripts, the *LCYB* transcripts were at nearly similar levels in phytochrome mutants and wild types. Remarkably, the levels of *CYCB* transcripts were upregulated in *phyB2* and other double or multiple phytochrome mutants at transition from MG to RR stage. Ostensibly, a combination of multiple phytochromes constrains *CYCB* transcript accumulation during transition to RR stage. The mutations in multiple phytochromes relieve this constraint by upregulating the *CYCB* levels. Similar to *CYCB* regulation, *ZISO* upregulation is also constrained by coaction of multiple phytochromes; consequently *ZISO* transcript levels were higher in single phytochrome mutants and were strongly upregulated in *phyAB1B2* mutant at RR stage. However the observed upregulation of *CYCB* transcripts was not consistently correlated with β-carotene levels and only infrequently observed in respective phytochrome mutants. The absence of strong correlation between transcript levels and metabolite levels are often observed in tomato ripening indicating regulation at post-transcriptional and post-

translational level (Kilambi *et al.* 2013; Enfissi *et al.* 2010). Interestingly there was no specific influence of phytochrome mutations on transcript levels of *PDS* and *ZDS*, encoding intermediary enzymes in carotenoid biosynthetic pathway. The lack of any specific influence of phytochrome(s) on *RIN* expression is consistent with role of *RIN* as a master regulator of ripening, with other factors such as light acting downstream of *RIN*.

In accordance with the spectrophotometric detection of phytochrome in tomato fruit (Jen *et al.* 1977), expression of phytochromes A, B1 and B2 transcripts was observed both at MG and RR stages of ripening. Phytochrome *per se* does not influence its own expression as transcript levels of respective phytochromes in mutants and wild types were nearly similar. Likewise there was no specific influence on expression of *COP1* gene, which could be related to ethylene emission or carotenoid formation. Unlike de-etiolating seedlings where phytochrome downregulates formation of ethylene along with the opening of hypocotylar hook, there is no specific action of phytochrome on ethylene evolution during ripening, excepting that *phyAB1B2* mutant emitted more ethylene at RR stage and on FR-incubation. Though the transcript levels of *ACS2* were upregulated in RR fruits, the increased levels were not specifically related to stimulation of ethylene emission. Nearly similar BRIX values in fruits of phytochrome mutants and wild types suggest that during ripening the formation of primary metabolites like sugars are not coupled to light regulated signaling. Likewise, though the firmness of fruits declines from MG to RR stage or incubation in D, FR or R, it was not correlated with a specific phytochrome mutation(s).

Phytochromes are omnipresent photoreceptors in all parts of plants including fruit and thus it is expected that they also influence fruit development and ripening. Our study highlights that during ripening; phytochromes function to maintain a dynamic equilibrium between at least two different processes, that is, chronological development of fruits and modulation of carotenoid formation. The analysis of phytochrome(s) mutant highlighted that

a phase-specific action of single or a combination of phytochrome species modulates the duration of ripening phases. In addition, phytochromes also restrain the faster development and ripening of fruit and hence triple mutant of phytochrome shows accelerated ripening compared to wild types. In summary our studies indicated that phytochrome(s) may act as a timekeeper to regulate the time interval required for transition between different phases of fruit development and ripening.

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Figure legends

Figure 1. Time course of increase in fruit diameter from anthesis till fruit abscission. (a) Time course for wild types and single phytochrome mutants. (b) Time course for double and triple phytochrome mutants. The diameter of developing fruits of wild types (MM and GT) and phytochrome (s) mutants was recorded using digital Vernier caliper (n≥5±SE).

Figure 2. Chronological development of tomato fruits post-anthesis. (a) DPA from anthesis to fruit abscission (FA). (b) From anthesis to MG stage. (c) From MG to BR stage. (d) From BR to RR stage. (e) From RR to FA. **(**f**)** Influence of phytochrome(s) mutation on duration of ripening phases. Green circle - increased duration, Red circle- decreased duration, Open circle- no effect. The letter above/below the grid box indicates the respective phytochrome mutation. Time interval [days post anthesis (DPA)] to attain specific ripening phases was visually monitored in on-vine ripened wild types (MM and GT) and phytochrome(s) mutant fruits (n≥ 5±SE). In Figure (b-e) the values indicated on Y axis are DPA required for transition from one phase to next phase and the dashed lines denote corresponding DPA values of wild types.

Figure 3. Chlorophyll levels in MG green fruits of tomato and phytochrome(s) mutants. (a) The pictures represent the wild type and mutant fruits harvested at MG stage. Top- Intact fruits, Bottom- Longitudinal section of fruits. (b) The level of chlorophyll in MG fruits. The reduced levels of chlorophyll in *phyA* and *phyAB1* mutant fruits is reflected as pale green
745 color in fruits of respective mutants (a). (c) Influence of phytochrome(s) mutation on chlorophyll levels. Green circle- increase in level, Red circle- decrease in level, Open circle- no effect. The letter above/below the grid box indicates the respective phytochrome mutation. The wild types (MM and GT) and phytochrome(s) mutant fruits were harvested at MG or RR stage and chlorophyll level was estimated (n≥5±SE).

Figure 4. Carotenoids accumulation in on-vine ripened tomato fruits. (a) The pictures represent the wild type and mutant fruits harvested at RR stage. Top- Intact fruits, Bottom- Longitudinal section of fruits. (b-c) The level of lycopene (b) and β-carotene (c) in MG and RR stage fruits. The scale at the bottom shows range (Lg/g FW) of lycopene (b) or β-carotene (c) accumulation in fruits. Note the difference in relative accumulation of lycopene and β-carotene in fruits. (d-e) Influence of phytochrome(s) mutation on lycopene (d) and β-carotene (e) levels. Green circle- increase in level, Red circle- decrease in level, Open circle- no effect. The letter above/below the grid box indicates the respective phytochrome mutation. The wild types (MM and GT) and phytochrome(s) mutant fruits were harvested at MG stage or RR stage and carotenoid levels were monitored by HPLC (n≥3-5±SE).

Figure 5. Carotenoids accumulation in off-vine ripened tomato fruits. (a) The representative pictures of wild type and mutant fruits were taken at the end of incubation period in D, R and FR. (b-g) The level of lycopene (b-d) and β -carotene (e-g) in fruits at the end of incubation period in D (b, e); in R (c, f); in FR (d, g). The scale at the bottom (b-g) shows range of lycopene or β-carotene accumulation in fruits. (h-m) Influence of phytochrome(s) mutation on lycopene (h-j) and β-carotene (k-m) levels in D (h, k); in R (i, l); in FR (j, m). Green circle- increase in level, Red circle- decrease in level, Open circle- no effect. The letter above/below the grid box indicates the respective phytochrome mutation. The wild types (MM and GT) and phytochrome(s) mutant fruits were harvested at MG stage and were incubated either in darkness (D) or in red light (R) or in far-red light (FR) for 21 days (n≥3-5±SE).

Figure 6. The carotenoids biosynthetic pathway depicting relative transcripts levels of enzymes catalyzing β-carotene formation from GGDP. Left- On-vine ripened fruits harvested at MG and RR stage; Right- Fruits were harvested at MG stage and incubated in D or R or FR for 21 days. (a) The transcript levels of *PSY1, ZISO* and *CYCB* in on-vine (left) and off-

vine ripened fruits (right). (b) The transcript levels of *PSY2*, and *LCYB* in on-vine (left) and off-vine ripened fruits (right). The vertical scale on left side of heat-map shows relative range of transcript accumulation. The grid box below the respective heat-map displays the influence of phytochrome(s) mutation on transcript levels. Green circle- increase in transcript level, Red circle- decrease in transcript level, Open circle- no effect. The letter above/below the grid box indicates the respective phytochrome mutation. The transcripts were quantified in the wild types and phytochrome(s) mutant fruits using real-time PCR. The transcripts were expressed after normalization with two internal controls, β-actin and ubiquitin as described in methods. Dotted arrows indicate multiple steps in the pathway. The relative expression of transcript levels is presented as heat-map on either side of pathway. For ease of expressing the ΔCt values were inverted to facilitate direct correlation to enhanced (green) or reduced transcript (red) expression. GGDP: geranyl geranyl diphosphate, *PSY1*: phytoene synthase1 (chromoplast-specific), *PDS*: phytoene desaturase (Supporting Information Fig S1), *ZISO*: ζ-carotene isomerase, *LCYB*, lycopene β-cyclase; *CYCB*, chromoplast specific lycopene β-cyclase.

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Figure S1. The relative transcript levels of*PDS*and*ZDS*genes in tomato fruits. Left- On-vine ripened fruits harvested at MG and RR stage; Right- Fruits were harvested at MG stage and incubated in D or R or FR for 21 days. The details regarding the analysis and representation of transcript levels were similar to that described in legends of Figure 6.

Figure S2. The relative transcript levels of*RIN*gene in tomato fruits. The first panel on left shows*RIN* transcript levels in on-vine ripened fruits harvested at MG and RR stage. The remaining three panels show transcript levels in fruits incubated in D or R or FR for 21 days after harvesting at MG stage. The details regarding the analysis and representation of transcript levels were similar to that described in legend of Figure 6. The horizontal scale below heat-map shows relative range of transcript accumulation.

Figure S3. Ethylene emission and transcript levels of*ACS2*and*ACO1*genes from tomato fruits. The first panel on left shows ethylene emission or *ACS2* and *ACO1* transcript levels in on-vine ripened fruits harvested at MG and RR stage. The remaining three panels show ethylene emission or *ACS2* and *ACO1*transcript levels in fruits incubated in D or R or FR for 21 days after harvesting at MG stage. The details regarding the analysis and representation of ethylene emission or transcript levels were similar to that described in legend of Figure 6. The horizontal scale below heat-map shows relative range of ethylene emission or transcript accumulation.

Figure S4. The relative transcript levels of*PHYA*,*PHYB1*and*PHYB2*genes in tomato fruits. The first panel on left shows *PHYA, PHYB1* and *PHYB2* transcript levels in on-vine ripened fruits harvested at MG and RR stage. The remaining three panels show *PHYA, PHYB1* and *PHYB2* transcript levels in fruits incubated in D or R or FR for 21 days after harvesting at

MG stage. The details regarding the analysis and representation of transcript levels were similar to that described in legend of Figure 6. The horizontal scale below heat-map shows relative range of transcript accumulation.

Figure S5. The relative transcript levels of*COP1*gene in tomato fruits. The first panel on left shows*COP1* transcript levels in on-vine ripened fruits harvested at MG and RR stage. The remaining three panels show transcript levels in fruits incubated in D or R or FR for 21 days after harvesting at MG stage. The details regarding the analysis and representation of transcript levels were similar to that described in legend of Figure 6. The horizontal scale below heat-map shows relative range of transcript accumulation.

Figure S6. The relative values of BRIX in wild types and phytochrome(s) mutant fruits. The BRIX value was determined from tomato fruits using a refractometer. The first panel on left shows BRIX levels in on-vine ripened fruits harvested at MG and RR stage. The remaining three panels show BRIX levels in fruits incubated in D or R or FR for 21 days after harvesting at MG stage. The details regarding the analysis and representation of BRIX levels were similar to that described in legend of Figure 6. The horizontal scale below heat-map shows relative range of BRIX.

Figure S7. Changes in firmness of wild types and phytochrome(s) mutant fruits. The fruit firmness was determined from tomato fruits using Durofel. The first panel on left shows firmness levels in on-vine ripened fruits harvested at MG and RR stage. The remaining three panels show firmness levels in fruits incubated in D or R or FR for 21 days after harvesting at MG stage. The details regarding the analysis and representation of firmness levels were similar to that described in legend of Figure 6. The horizontal scale below heat-map shows relative range of firmness.

Table S1. Genetic background of phytochrome mutants used in the present study.

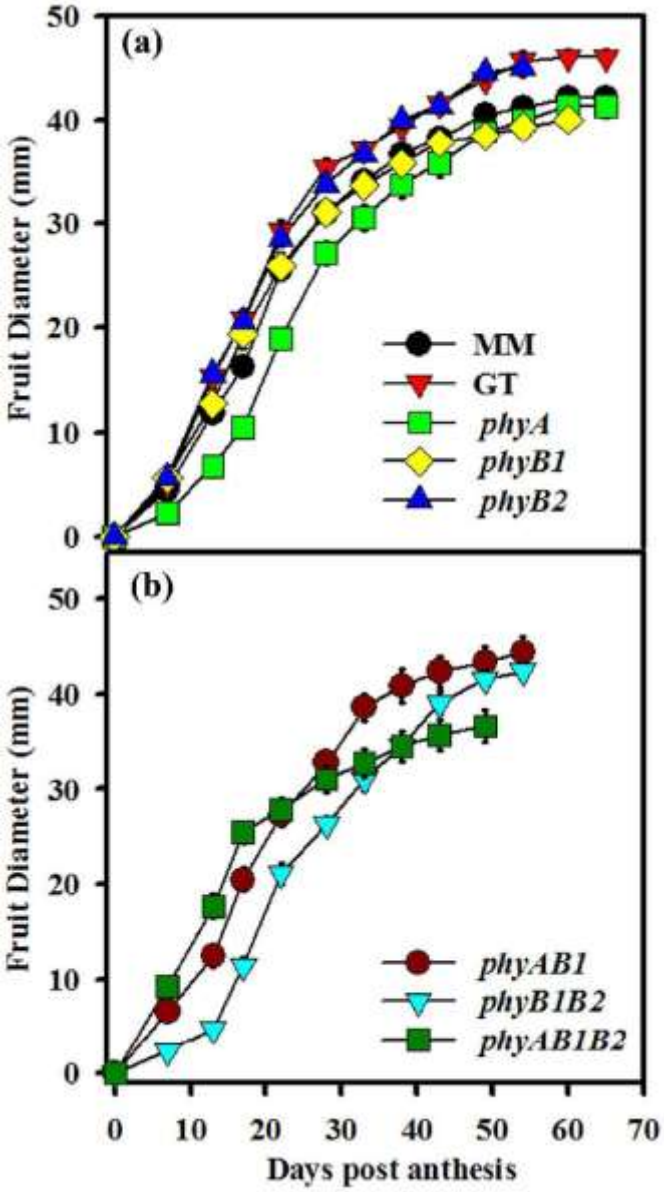


Figure 1.Time course of increase in fruit diameter from anthesis till fruit abscission. (a) Time course for wild types and single phytochrome mutants. (b) Time course for double and triple phytochrome mutants. The diameter of developing fruits of wild types (MM and GT) and phytochrome (s) mutant was recorded using digital Vernier caliper (n≥5±SE).

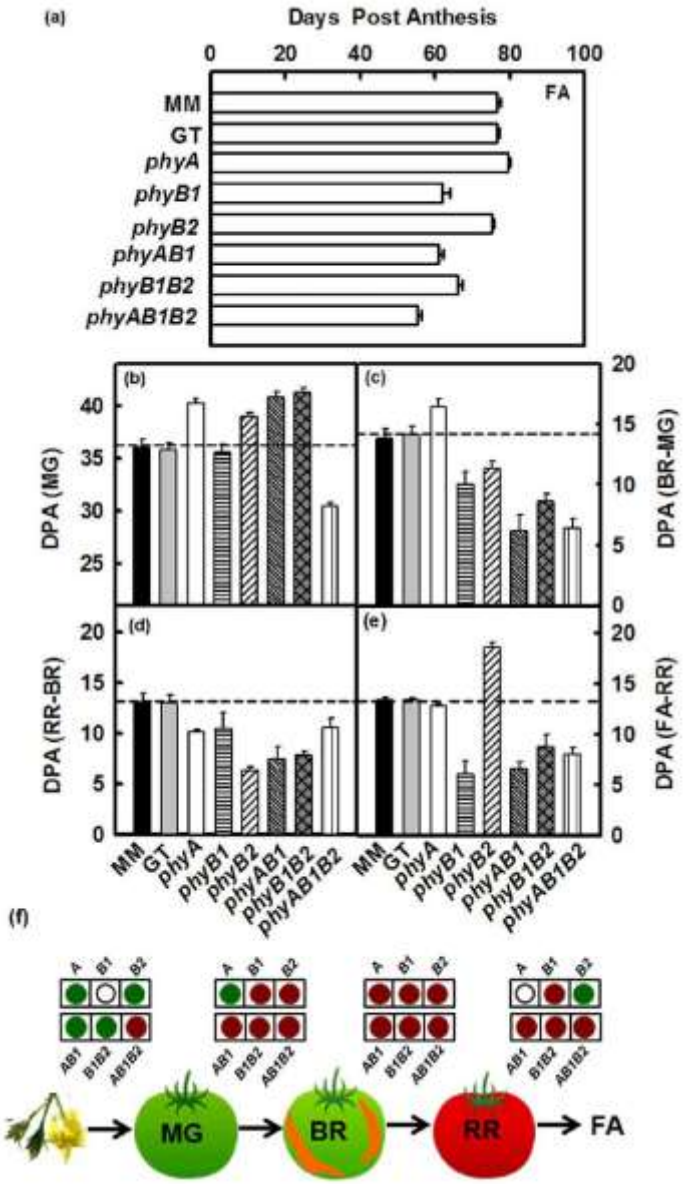


Figure 2. Chronological development of tomato fruits post-anthesis. (a) DPA from anthesis to fruit abscission (FA). (b) From anthesis to MG stage. (c) From MG to BR stage. (d) From BR to RR stage. (e) From RR to FA. (f) Influence of phytochrome(s) mutation on duration of ripening phases. Green circle- increased duration, Red circle- decreased duration, Open circle- no effect. The letter above/below the grid box indicates the respective phytochrome mutation. Time interval [days post anthesis (DPA)] to attain specific ripening phases was visually monitored in on-vine ripened wild types (MM and GT) and phytochrome(s) mutant fruits (n≥ 5±SE). In Figure (B-E) the values indicated on Y axis are DPA required for transition from one phase to next phase and the dashed lines denote corresponding DPA values of wild types.

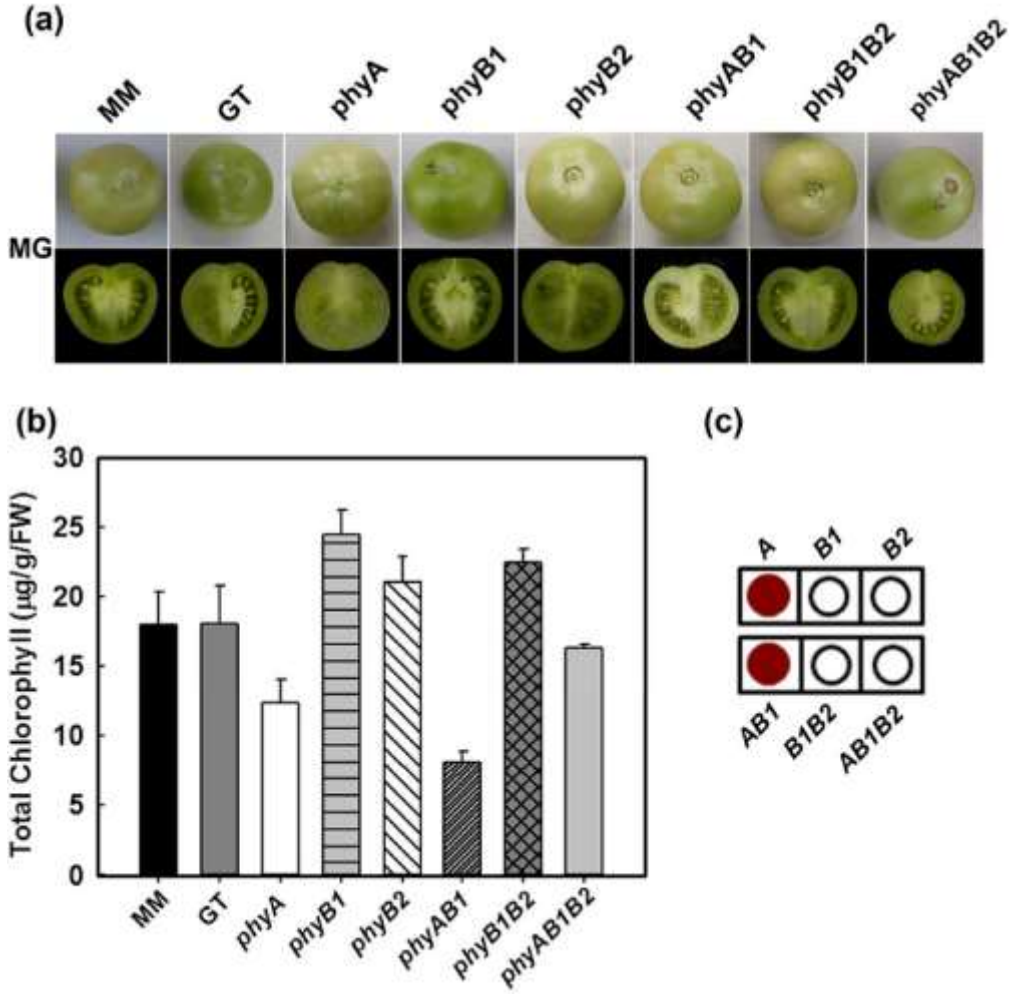


Figure 3. Chlorophyll levels in MG green fruits of tomato and phytochrome(s) mutants. (a) The pictures represent the wild type and mutant fruits harvested at MG stage. Top- Intact fruits, Bottom- Longitudinal section of fruits. (b) The level of chlorophyll in MG fruits. The reduced levels of chlorophyll in phyA and phyAB1 mutant fruits is reflected as pale green color in fruits of respective mutants (a). (c) Influence of phytochrome(s) mutation on chlorophyll levels. Green circle- increase in level, Red circle- decrease in level, Open circle- no effect. The letter above/below the grid box indicates the respective phytochrome mutation. The wild types (MM and GT) and phytochrome(s) mutant fruits were harvested at MG or RR stage and chlorophyll level was estimated (n≥5±SE).

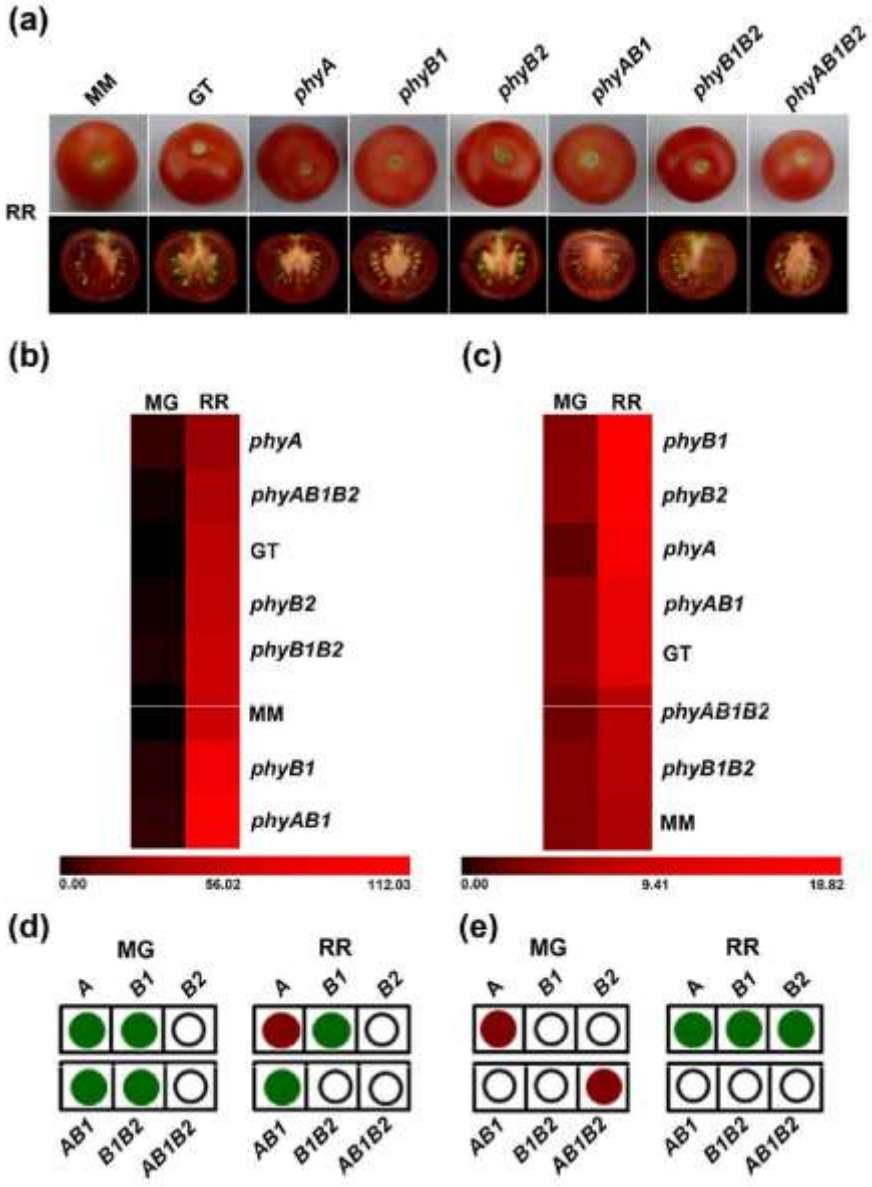


Figure 4. Carotenoids accumulation in on-vine ripened tomato fruits. (a) The pictures represent the wild type and mutant fruits harvested at RR stage. Top- Intact fruits, Bottom- Longitudinal section of fruits. (b-c) The level of lycopene (b) and β-carotene (c) in MG and RR stage fruits. The scale at the bottom shows range ('g/g FW) of lycopene (b) or β-carotene (c) accumulation in fruits. Note the difference in relative accumulation of lycopene and β-carotene in fruits. (d-e) Influence of phytochrome(s) mutation on lycopene (d) and β-carotene (e) levels. Green circle- increase in level, Red circle- decrease in level, Open circle- no effect. The letter above/below the grid box indicates the respective phytochrome mutation. The wild types (MM and GT) and phytochrome(s) mutant fruits were harvested at MG stage or RR stage and carotenoid levels were monitored by HPLC (n≥3-5±SE).

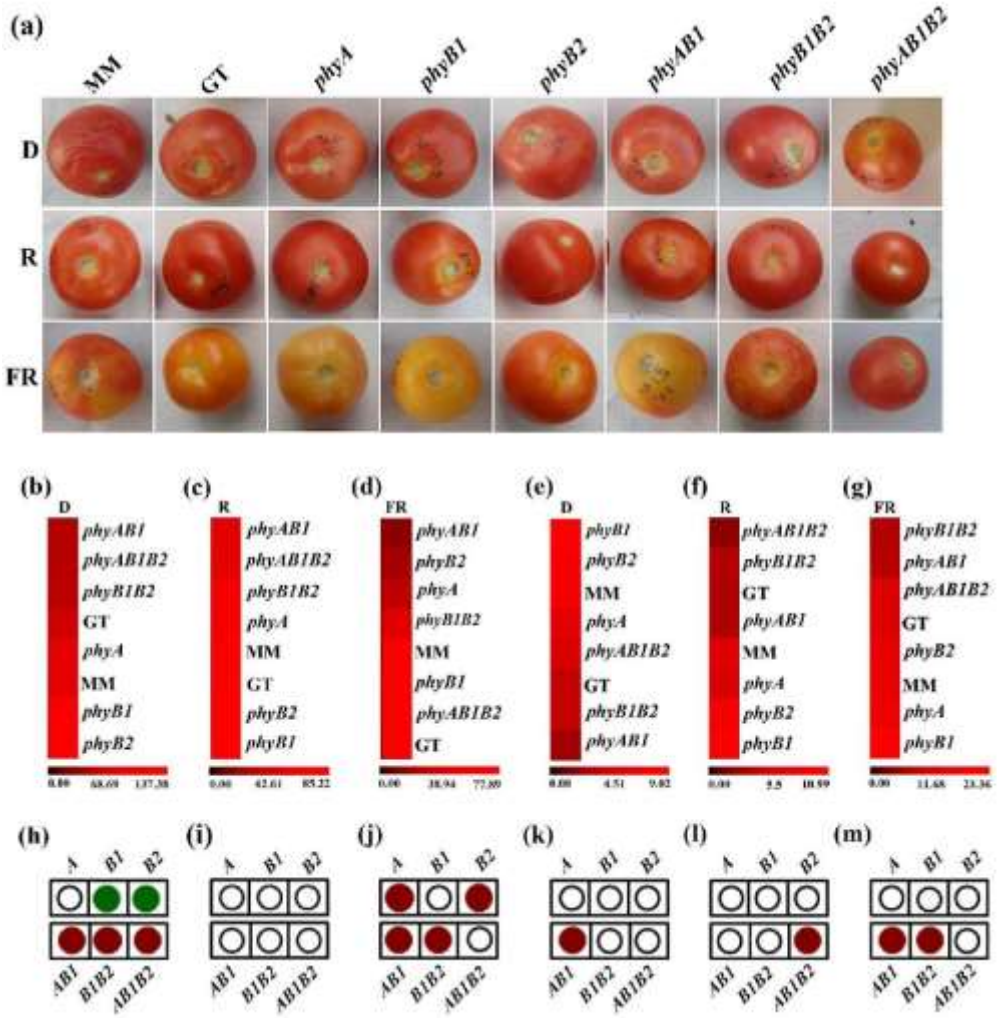
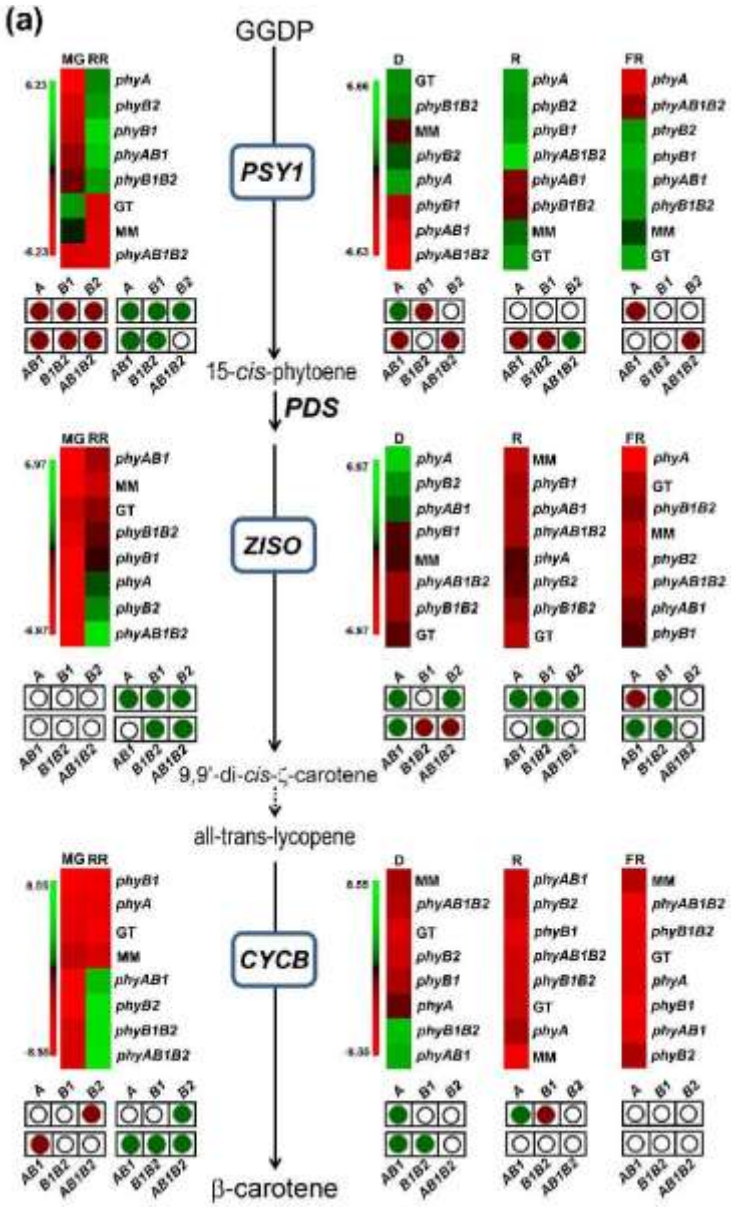


Figure 5. Carotenoids accumulation in off-vine ripened tomato fruits. (a) The representative pictures of wild type and mutant fruits were taken at the end of incubation period in D, R and FR. (b-g) The level of lycopene (b-d) and β -carotene (e-g) in fruits at the end of incubation period in D (b, e); in R (c, f); in FR (d, g). The scale at the bottom (b-g) shows range of lycopene or β-carotene accumulation in fruits. (h-m) Influence of phytochrome(s) mutation on lycopene (h-j) and β-carotene (k-m) levels in D (h, k); in R (i, l); in FR (j, m). Green circle- increase in level, Red circle- decrease in level, Open circle- no effect. The letter above/below the grid box indicates the respective phytochrome mutation. The wild types (MM and GT) and phytochrome(s) mutant fruits were harvested at MG stage and were incubated either in darkness (D) or in red light (R) or in far-red light (FR) for 21 days (n≥3-5±SE).


(a)
GGDP
PSY1
15-cis-phytoene
PDS
ZISO
9,9'-di-cis-ζ-carotene
all-trans-lycopene
CYCB
β-carotene
MG RR
D
R
FR
phyA
phyB2
phyB1
phyAB1
phyB1B2
GT
MM
phyAB1B2
A
B1
B2
AB1
B1B2
AB1B2

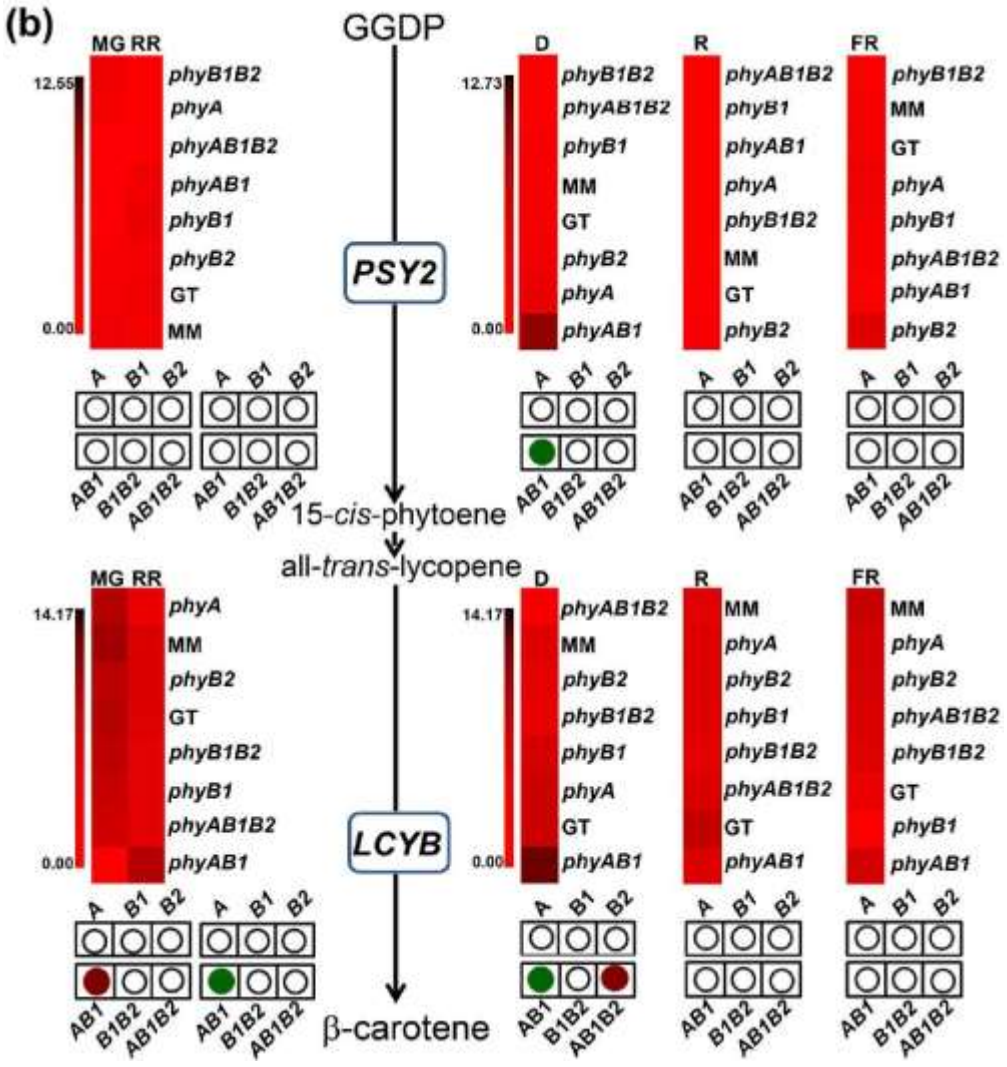
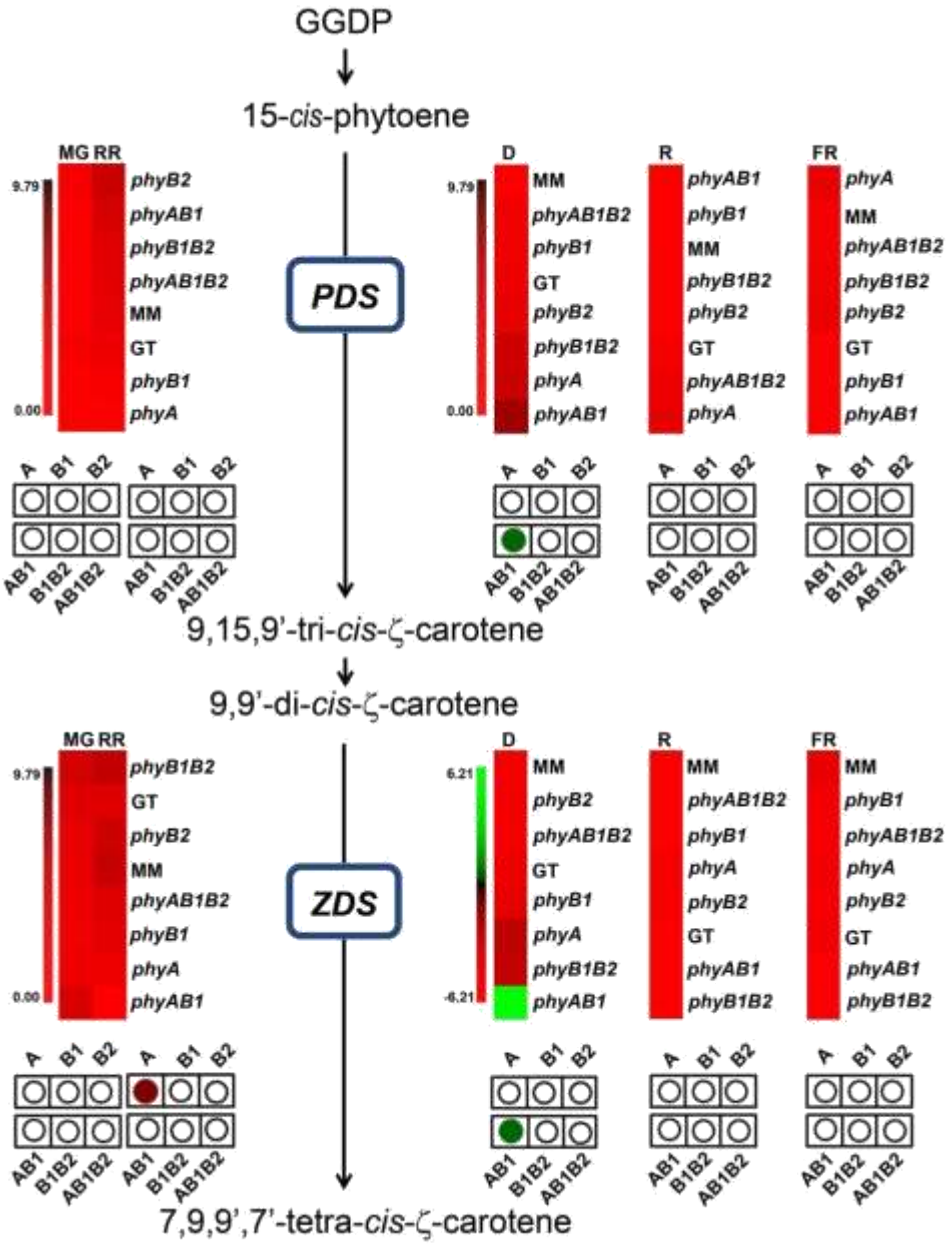
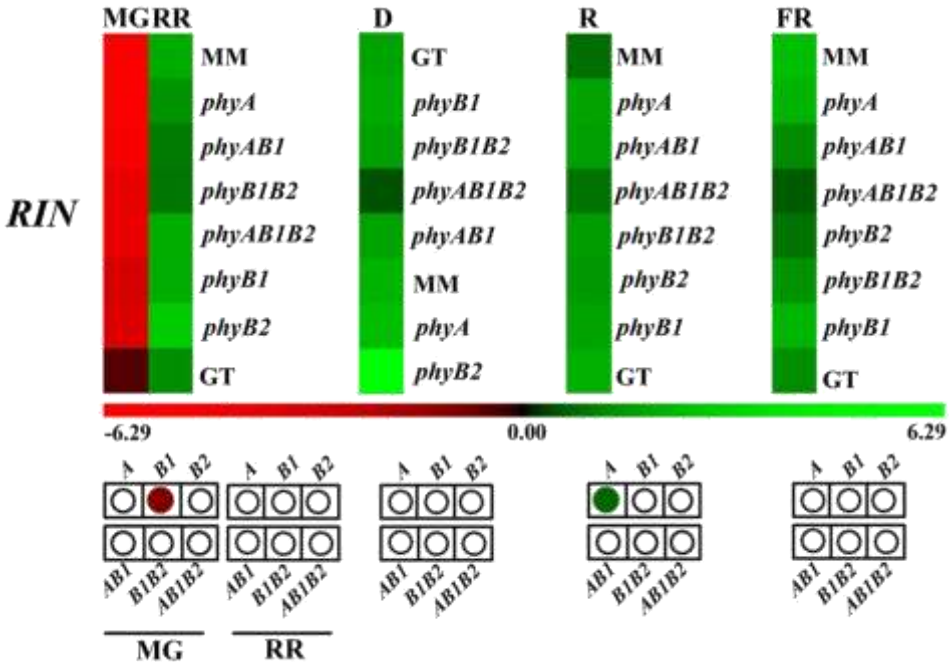


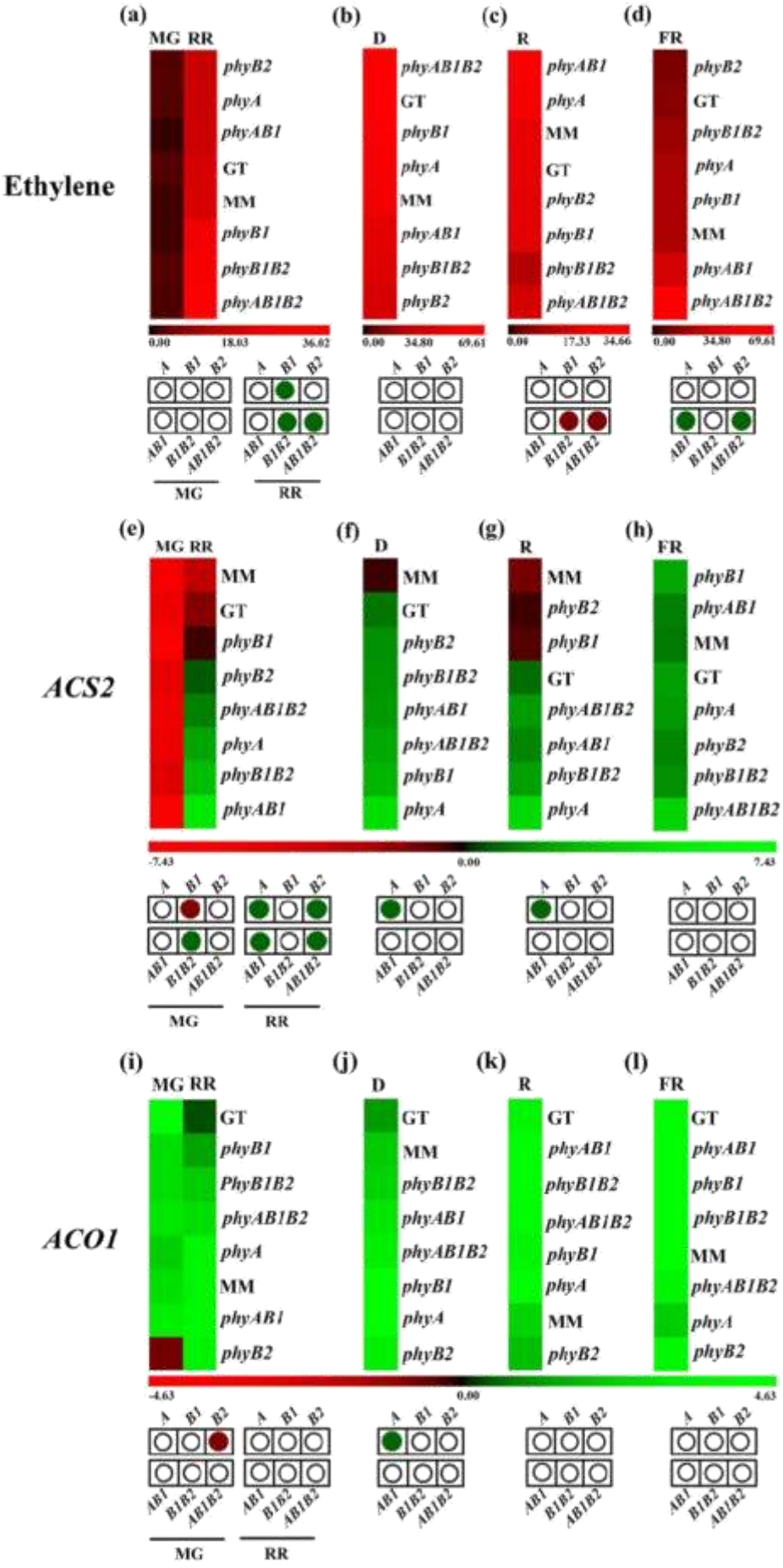
Figure 6. The carotenoids biosynthetic pathway depicting relative transcripts levels of enzymes catalyzing β-carotene formation from GGDP. Left- On-vine ripened fruits harvested at MG and RR stage; Right- Fruits were harvested at MG stage and incubated in D or R or FR for 21 days. (a) The transcript levels of PSY1, ZISO and CYCB in on-vine (left) and off-vine ripened fruits (right). (b) The transcript levels of PSY2, and LCYB in on-vine (left) and off-vine ripened fruits (right). The vertical scale on left side of heat-map shows relative range of transcript accumulation. The grid box below the respective heat-map displays the influence of phytochrome(s) mutation on transcript levels. Green circle- increase in transcript level, Red circle- decrease in transcript level, Open circle- no effect. The letter above/below the grid box indicates the respective phytochrome mutation. The transcripts were quantified in the wild types and phytochrome(s) mutant fruits using real-time PCR. The transcripts were expressed after normalization with two internal controls, β-actin and ubiquitin as described in methods. Dotted arrows indicate multiple steps in the pathway. The relative expression of transcript levels is presented as heat-map on either side of pathway. For ease of expressing the ΔCt values were inverted to facilitate direct correlation to enhanced (green) or reduced transcript (red) expression. GGDP: geranyl geranyl diphosphate, PSY1: phytoene synthase1 (chromoplast-specific), PDS: phytoene desaturase (not investigated), ZISO: z-carotene isomerase, LCYB, lycopene β-cyclase; CYCB, chromoplast specific lycopene β-cyclase.



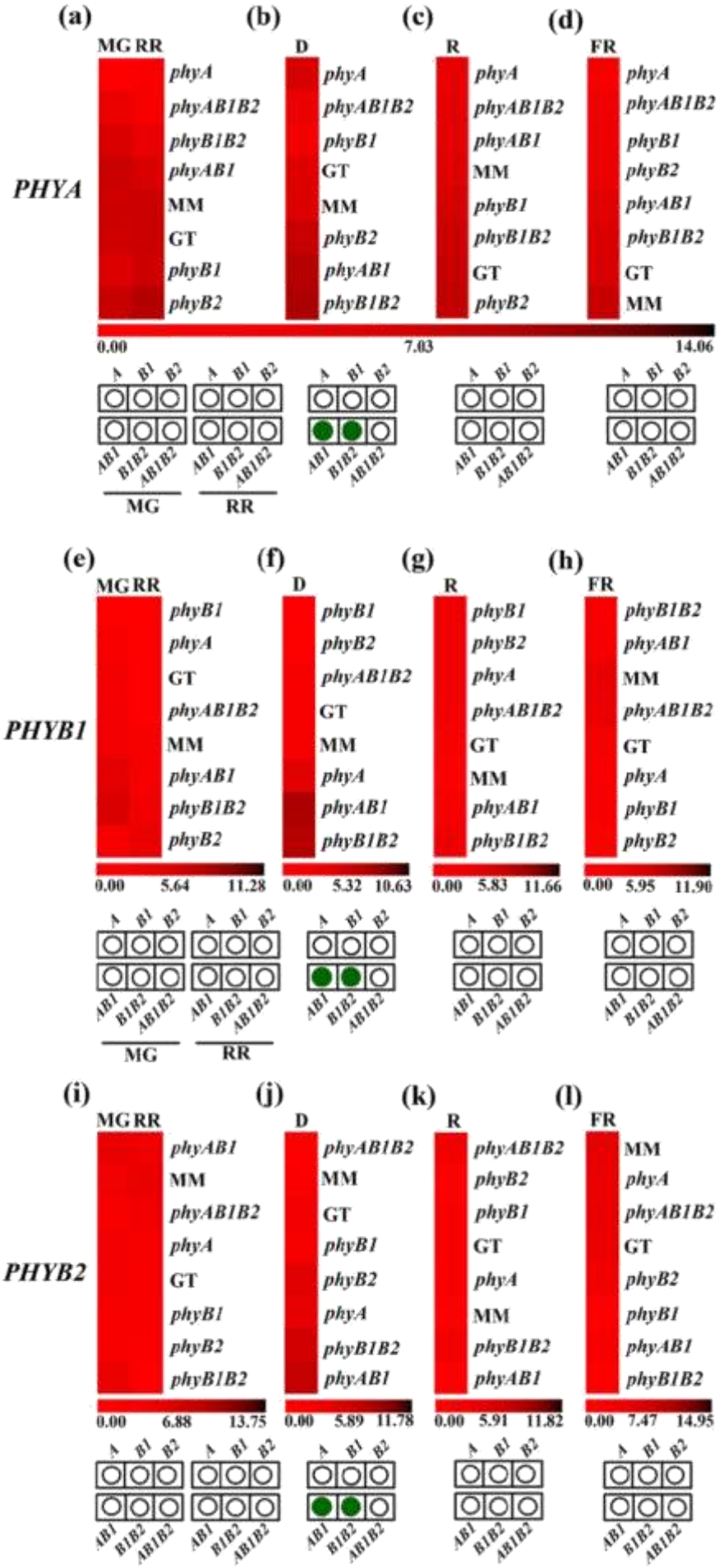
Supplemental Figure S1. The relative transcript levels of*PDS*and*ZDS*genes in tomato fruits.Left- On-vine ripened fruits harvested at MG and RR stage; Right- Fruits were harvested at MG stage and incubated in D or R or FR for 21 days. The details regarding the analysis and representation of transcript levels were similar to that described in legends of Figure 6.



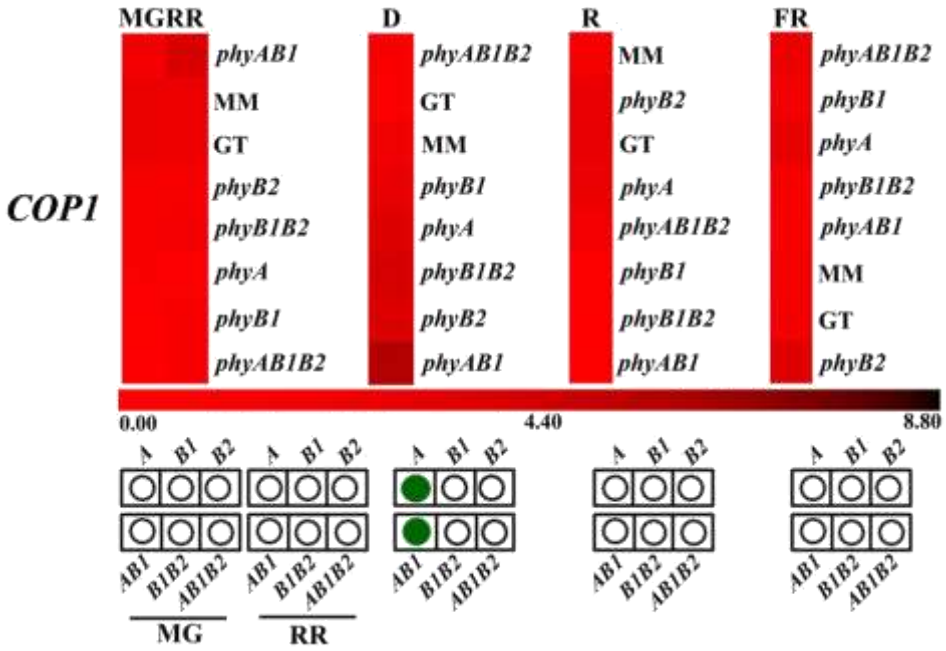
Supplemental Figure S2. The relative transcript levels of*RIN*gene in tomato fruits. The first panelon left shows *RIN* transcript levels in on-vine ripened fruits harvested at MG and RR stage. The remaining three panels show transcript levels in fruits incubated in D or R or FR for 21 days after harvesting at MG stage. The details regarding the analysis and representation of transcript levels were similar to that described in legend of Figure 6. The horizontal scale below heat-map shows relative range of transcript accumulation.


Ethylene
ACS2
ACO1
(a)
MG RR
phyB2
phyA
phyAB1
GT
MM
phyB1
phyB1B2
phyAB1B2
0.00 18.03 36.02
(b)
D
phyAB1B2
GT
phyB1
phyA
MM
phyAB1
phyB1B2
phyB2
0.00 34.80 69.61
(c)
R
phyAB1
phyA
MM
GT
phyB2
phyB1
phyB1B2
phyAB1B2
0.00 17.33 34.66
(d)
FR
phyB2
GT
phyB1B2
phyA
phyB1
MM
phyAB1
phyAB1B2
0.00 34.80 69.61
A B1 B2
AB1 B1B2 AB1B2
MG
RR
(e)
MG RR
MM
GT
phyB1
phyB2
phyAB1B2
phyA
phyB1B2
phyAB1
-7.43 0.00 7.43
(f)
D
MM
GT
phyB2
phyB1B2
phyAB1
phyAB1B2
phyB1
phyA
(g)
R
MM
phyB2
phyB1
GT
phyAB1B2
phyAB1
phyB1B2
phyA
(h)
FR
phyB1
phyAB1
MM
GT
phyA
phyB2
phyB1B2
phyAB1B2
(i)
MG RR
GT
phyB1
PhyB1B2
phyAB1B2
phyA
MM
phyAB1
phyB2
-4.63 0.00 4.63
(j)
D
GT
MM
phyB1B2
phyAB1
phyAB1B2
phyB1
phyA
phyB2
(k)
R
GT
phyAB1
phyB1B2
phyAB1B2
phyB1
phyA
MM
phyB2
(l)
FR
GT
phyAB1
phyB1
phyB1B2
MM
phyAB1B2
phyA
phyB2

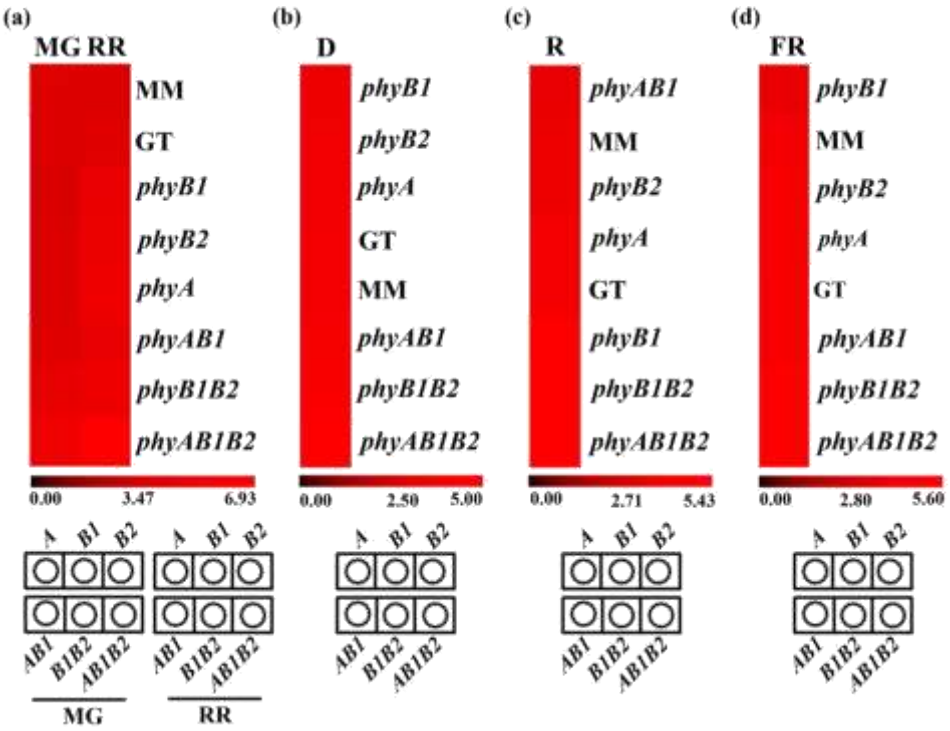
Supplemental Figure S3. Ethylene emission and transcript levels of*ACS2*and*ACO1*genes from tomato fruits. The first panel on left shows ethylene emission or *ACS2* and *ACO1* transcript levels in on-vine ripened fruits harvested at MG and RR stage. Theremaining three panels show ethylene emission or *ACS2* and *ACO1*transcript levels in fruits incubated in D or R or FR for 21 days after harvesting at MG stage. The details regarding the analysis and representation of ethylene emission or transcript levels were similar to that described in legend of Figure 6. The horizontal scale below heat-map shows relative range of ethylene emission or transcript accumulation.


(a)
(b)
(c)
(d)
PHYA
MG RR
D
R
FR
phyA
phyAB1B2
phyB1B2
phyAB1
MM
GT
phyB1
phyB2
0.00
7.03
14.06
A B1 B2
AB1 B1B2 AB1B2
MG
RR
(e)
(f)
(g)
(h)
PHYB1
0.00 5.64 11.28
0.00 5.32 10.63
0.00 5.83 11.66
0.00 5.95 11.90
(i)
(j)
(k)
(l)
PHYB2
0.00 6.88 13.75
0.00 5.89 11.78
0.00 5.91 11.82
0.00 7.47 14.95

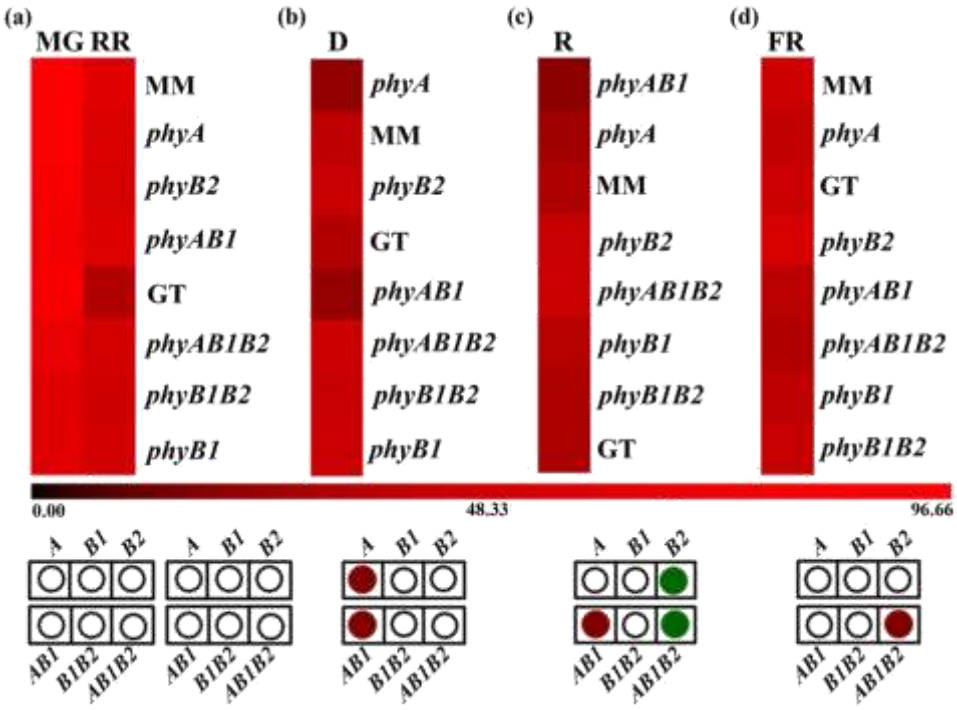
Supplemental Figure S4. The relative transcript levels of*PHYA*,*PHYB1*and*PHYB2* genes in tomato fruits. The first panel on left shows *PHYA, PHYB1* and *PHYB2* transcript levels in on-vine ripened fruits harvested at MG and RR stage.The remaining three panels show *PHYA*, *PHYB1* and *PHYB2* transcript levels in fruits incubated in D or R or FR for 21 days after harvesting at MG stage. The details regarding the analysis and representation of transcript levels were similar to that described in legend of Figure 6. The horizontal scale below heat-map shows relative range of transcript accumulation.



Supplemental Figure S5. The relative transcript levels of*COP1*gene in tomato fruits. Thefirst panel on left shows *COP1* transcript levels in on-vine ripened fruits harvested at MG and RR stage. The remaining three panels show transcript levels in fruits incubated in D or R or FR for 21 days after harvesting at MG stage. The details regarding the analysis and representation of transcript levels were similar to that described in legend of Figure 6. The horizontal scale below heat-map shows relative range of transcript accumulation.



Supplemental Figure S6. The relative values of BRIX in wild types and phytochrome(s)mutant fruits. The BRIX value was determined from tomato fruits using a refractometer. The first panel on left shows BRIX levels in on-vine ripened fruits harvested at MG and RR stage. The remaining three panels show BRIX levels in fruits incubated in D or R or FR for 21 days after harvesting at MG stage. The details regarding the analysis and representation of BRIX levels were similar to that described in legend of Figure 6. The horizontal scale below heat-map shows relative range of BRIX.



Supplementary Figure S7.Changes in firmness of wild types and phytochrome(s)mutant fruits. The fruit firmness was determined from tomato fruits using Durofel. The first panel on left shows firmness levels in on-vine ripened fruits harvested at MG and RR stage. The remaining three panels show firmness levels in fruits incubated in D or R or FR for 21 days after harvesting at MG stage. The details regarding the analysis and representation of firmness levels were similar to that described in legend of Figure 6. The horizontal scale below heat-map shows relative range of firmness.

Supplement Table S1. Genetic background of phytochrome mutants used in the present study.

Phytochrome mutant	**Genetic background**	**Reference**
phyA (*fri*)	Moneymaker (MM)	Pratt *et al.* (1997)
phyB1 (*fri*)	TMV-resistant breeding line of a MM type (GT)	Kerckhoffs *et al.* (1996)
phyB2	MM &GT	Kerckhoffs *et al.* (1999)
phyAB1 (*fri*, *tri*)	MM & GT	Kendrick *et al.* (1997)
phyB1B2	Mixed background from *phyA* and *phyB1*	Kerckhoffs *et al.* (1999); Weller *et al.* (2000)
phyAB1B2	Mixed background from *phyA* and *phyB1*	Weller *et al.* (2000)

Kendrick R.E., Kerckhoffs L.H.J., Van Tuinen A. & Koorneef M. (1997) Photomorphogenic mutants of tomato. *Plant Cell & Environment***20**, 746-751.

Kerckhoffs L.H.J., Kelmenson P.M., Schreuder M.E.L., Kendrick C.I., Kendrick R.E., Hanhart C.J., Koornneef M., Pratt L.H. & Cordonnier-Pratt M.M. (1999) Characterization of the gene encoding the apoprotein of phytochrome B2 in tomato, and identification of molecular lesions in two mutant alleles. *Molecular and General Genetics* **261**, 901-907.

Kerckhoffs L.H.J., van Tuinen A., Hauser B.A., Cordonnier-Pratt M.-M., Nagatani A., Koorneef M., Pratt L.H. & Kendrick R.E (1996) Molecular analysis of tri mutant alleles in tomato indicates the Tri locus is the gene encoding encoding the apoproteins of phytochrome B1. *Planta***199**, 152-157.

Pratt L.H., Cordonier-Pratt M.M., Kelmenson P.M., Lazarova G.I., Kubota T. & Alba R.M. (1997) The phytochrome gene family in tomato (*Solanum lycopersicum* L.). *Plant, Cell & Environment***20**, 672– 677.

Weller J.L., Schreuder M.E.L., Smith H., Koornneef M., & Kendrick R.E. (2000) Physiological interactions of phytochromes A, B1 and B2 in the control of development in tomato. *The PlantJournal* **24**, 345-356.

Supplement Table S2. Primers used in the quantitative real time PCR analysis

Gene	Forward primer (5′ to 3′)	Reverse primer (5′ to 3′)	Product Size (bp)
PSY1	CAGCCTTAGATAGGTGGGAAAAT	CTGAATGGCTGAATATCAACTGG	121
PSY2	AATTCCGAGGTCTCATACGG	CCTTTCCACATCGAATTCCT	110
PDS	TATCATCAACGTTCCGTGCT	TATCGGTTTGTGACCAGCAT	122
ZISO	AGAGCGTGCTTTTCGTGTATTG	ATTGCCATAACTGCACTCCATC	107
ZDS	TCCAAAAGGGCTATTTCCAC	TTGATCCAAGAGCTCCACAG	115
CYCB	GGGTAATGAGCCATATTTAAGGG	TCCAACGACTCTCTGAGGTA	92
LCYB1	TCCTGGCCTGCGTATAGATG	TCCAACGACTCTCTGAGGTA	148
RIN	ATGGCATTGTGGTGAGCAAAG	GTTGATGGTGCTGCATTTTCG	147
ACS2	AAGCTTAACGTCTCGCCTGGAT	AGCGCAATATCAACCGTTCCAT	101
ACO1	AAGAGGCAGAGGAAAGTACACA	GGATCACTTTCCATTGCCTTCA	130
COP1	GTGATTGCCCCTGTTGTTCT	AACAGGGGATGCAGTTTTTG	118
PHYA	TAGCATTGCAGGGGAAAGAG	CCCACAACATTATCCCGAACA	133
PHYB1	AGCCCTCACTGCAAGTTTCT	TTGCACAGGGTTGATGTAGG	111
PHYB2	GTCCTCACATCCGTTCCAAT	GCTGTTGAAGAGGCGAGTTT	131
β-ACTIN	GTCCCTATTTACGAGGGTTATGC	CAGTTAAATCACGACCAGCAAGATT	108
UBIQUITIN 3	GCCGACTACAACATCCAGAAGG	TGCAACACAGCGAGCTTAACC	110

Phytoene synthase 1 (*PSY1*, Solyc03g031860.2.1), *Phytoene synthase 2* (*PSY2*, Solyc02g081330.2.1), *Phytoene desaturase* (*PDS*, Solyc03g123760.2.1), *ζ-carotene isomerase*, (*ZISO*, AK326152.1), *ζ-carotene desaturase* (*ZDS*, Solyc01g097810.2.1), *Lycopene β-cyclase* (*CYCB*, Solyc06g074240.1.1), *Lycopene-β-cyclase* (*LCYB1*, Solyc04g040190.1.1), *Ripening Inhibitor* (*RIN*, Solyc05g012020.2.1), *1-aminocyclopropane-1-carboxylate synthase 2* (*ACS2*, Solyc01g095080.2.1), *1-aminocyclopropane-1-carboxylate oxidase* (*ACO1*, Solyc07g049530.2.1), *Constitutive photomorphogenic1* (*COP1*, *Solyc12g005950*), *Phytochrome A* (*PHYA*, Solyc10g044670.1.1), *Phytochrome B1* (*PHYB1* Solyc05g053410.2.1), *Phytochrome B2 (PHYB2*, Solyc01g059870.2.1), *β-actin* (FJ532351.1) and *ubiquitin3* (X58253.1)