Ascorbate (vitamin C) deficiency leads to low immunity, scurvy, and other human diseases and is therefore a global health problem. Given that plants are major ascorbate sources for humans, biofortification of this vitamin in our foodstuffs is of considerable importance. Ascorbate is synthesized by a number of alternative pathways: (i) from the glycolytic intermediates D-glucose-6P (the key intermediates are GDP-D-mannose and L-galactose), (ii) from the breakdown of the cell wall polymer pectin which uses the methyl ester of D-galacturonic acid as precursor, and (iii) from myo-inositol as precursor via myo-inositol oxygenase. We report here the engineering of fruit-specific overexpression of a bacterial pyrophosphatase, which hydrolyzes the inorganic pyrophosphate (PPI) to orthophosphate (Pi). This strategy resulted in increased vitamin C levels up to 2.5-fold in ripe fruit as well as increasing in the major sugars, sucrose, and glucose, yet decreasing the level of starch. When considered together, these finding indicate an intimate linkage between ascorbate and sugar biosynthesis in plants. Moreover, the combined data reveal the importance of PPI metabolism in tomato fruit metabolism and development.
of strong evidence. The AsA concentration remains the same in wild type and MoOX overexpression lines (Endres and Tenhaken, 2009).

Formation of GDP-D-mannose is the initial step in the D-Man/L-Gal pathway, which is synthesized from D-mannose-1P via GDP-mannose pyrophosphatase (Conklin et al., 1999) (Figure A1). This reaction generates inorganic pyrophosphate (PPI) as by-product. In plants, PPI plays a central role not only as by-product of activation and polymerization steps (Sonnewald, 1992; Geigenberger et al., 1998; Rojas-Beltran et al., 1999; Farre et al., 2001), if not as an energy donor per se (Stitt, 1998; Lopez-Marques et al., 2004). PPI is generally removed by inorganic pyrophosphatases, which hydrolyze PPI to orthophosphate (Pi). Heterologous expression of the Escherichia coli pyrophosphatase in an untargeted manner, conferring cytosolic localization of the encoded protein, showed an important role in the partitioning between sucrose (Suc) and starch (Sonnewald, 1992; Farre et al., 2001; Lee et al., 2005). In contrast, expression of the E. coli pyrophosphatase targeted to the plastid displayed only minor changes in metabolites levels (Farre et al., 2006). A transient down-regulation of plastid-targeted soluble pyrophosphatase in Nicotiana benthamiana, revealed an important role in photosynthesis as well as in the regulation of water exchanges under mild drought stress (George et al., 2010).

In this study, we generated Solanum lycopersicum cv MoneyMarker lines, which overexpress the gene encoding the inorganic pyrophosphatase from E. coli in an untargeted manner under the control of a fruit specific promoter. This strategy resulted in increased vitamin C levels up to 2.5-fold in ripe fruit as well as in the major sugars, sucrose, and glucose, yet decreasing the level of starch. When considered together, these finding indicate an intimate linkage between ascorbate and sugar biosynthesis in plants.

RESULTS

GENERATION OF B33-PPi OVEREXPRESSION TOMATO LINES

To assess the effect of over expression of a pyrophosphatase from E. coli (Sonnewald, 1992) in tomato fruit we introduced this gene in the sense orientation under the control of the patatin B33 promoter (Jelitto et al., 1992). This promoter has been shown to be ripening-specific promoter in tomato fruit (Frommer et al., 1994; Centeno et al., 2011). An initial screening was carried out on the basis of pyrophosphatase activity of ripe tomato fruits (data not shown). This screen allowed the identification of four lines displaying considerably elevated activity (L9, L28, L29, and L39), which were taken to the next generation. Eight T2 plants per line were grown in the greenhouse and young leaves (3 weeks old plants) as well as fruits at green (35 days after pollination; DAP) and red (60 DAP) stages were harvested. Assay of alkaline pyrophosphatase activity revealed that the selected lines displayed considerable increase in activity in red fruit (Figure 1). To ensure that this increase of target enzyme activity was restricted to fruits, the activity of the enzyme was additionally tested in young leaves where they were unaltered (10.8 ± 0.2; 11.1 ± 0.5; 11.4 ± 0.4; 10.4 ± 0.3; 10.5 ± 0.5 µmol min⁻¹ g⁻¹ FW in wild type, L9, L28, L29, and L39, respectively; values are mean ± SE).

FRUIT SIZE AND YIELD

Fruit size and weight per fruit were determined in red fruit (60 DAP). All four lines exhibited a significantly lower weight per fruit (Figure 2A) as well as smaller fruit size in three of the four lines (L9, L28, and L29; Figure 2B). The total fruit number was, however, essentially unaltered (Figure 2C).

PYROPHOSPHATE AND INORGANIC PHOSPHATE LEVELS

Having determined that the transformants displayed the desired alteration in enzyme activity, we next evaluated pyrophosphate levels themselves. For this purpose, pericarp tissues of red fruit at 60 DAP and young leaves (3 weeks old plants) were harvested and pyrophosphate levels were determined taking care to observe all control procedures required to minimize the influence of contaminants (Farre et al., 2001). These analyses revealed significant decreases in pyrophosphate levels in all lines ranking from 25 for L39 to 55% for L29 in red tomato fruit (Table 1). However, as anticipated both from the specificity of expression of the transgene and the lack of change in the activity no changes in pyrophosphate levels were observed in leaves (Table 1). Relatively consistent changes were also seen in the level of inorganic phosphate in red fruit. Inorganic phosphate level increased in three transgenic lines (L9, L28, and L39; Table 1).

METABOLITE PROFILING OF GREEN AND RED FRUITS OF THE B33-PPi LINES

In order to further characterize the effects of the reduction of pyrophosphate content, we next applied and established gas chromatography (GC)-MS-based metabolite profiling method (Osorio et al., 2012) to pericarp tissue derived from green and red fruits. Surprisingly, at the green stage the metabolite profiles of the transgenic lines were remarkable similar to those of the WT (Figure 3). However, similar analysis in red stage revealed important changes in the levels of several few metabolites (Figure 4). In all four lines Suc was significantly increased in red tomato fruit by up to 2.5-fold in comparison to WT (Figure 4). This increase in Suc was accompanied by increases in Glc in three lines but
no significant changes in Fru (only L9 showed slight decrease). The changes in these sugars were coupled to a decrease in starch content (Table 2) as well as an increase in the total soluble solids content (Brix) in all transgenic red fruit (6.2 ± 0.3; 7.4 ± 0.2; 8.4 ± 0.4; 6.9 ± 0.2 for the wild type, L9, L28, L29, and L39, respectively). To better understand these metabolic alterations, we next measured the AGPase in the red B33-PPi fruits. We observed a decrease in this activity in all transformants with the exception of the L39. However, the activation stage of this enzyme was invariant (Table 3).

Interestingly, a strong increase in metabolites related to ascorbate biosynthesis, such as dehydroascorbic acid (all four lines), myo-inositol (all four lines), galacturonic acid (lines L28 and L39) (Figure 4) as well as a substantial increase of ascorbate (approximately between 2 and 3-fold) were observed (Figure 5).

Additionally, the transformants revealed an increase in two amino acids Ala (L28 and L39), and Asp (L28, L29, and L39) as well as a reduction in putrescine (L9, L28, L39) (Figure 4).

**Expression of E. Coli Pyrophosphatase in Tomato Fruits Leads to Alteration in Ascorbic Acid Biosynthesis**

Since some related ascorbate biosynthesis metabolites as well as ascorbate were modified in the red B33-PPi tomato fruits, we next evaluated if ascorbate biosynthesis and/or recycling were altered in these fruits. For this purpose we examined the different AsA biosynthetic pathways. First, we analyzed the transcript levels of some genes in the D-Man/L-Gal pathway. The expression of the two GDP-L-galactose phosphorylase (GGP) genes, a key point for the control of ascorbate pathway (Dowdle et al., 2007; Laing et al., 2007; Bulley et al., 2012) was up-regulated in all transgenic lines as well as the L-galactono-1,4-lactone dehydrogenase (GaLDH) gene, while the L-galactose dehydrogenase (GDH) gene showed a significant decrease only in one line (L9) (Figure 6). The higher activity of the last enzyme in this pathway, GaLDH, corroborated that D-Man/L-Gal pathway was up-regulated in red ripened B33-PPi fruits (Table 3). Second, related with the D-galacturonic acid pathway, we observed an increase in the level of its precursor, galacturonic acid, in two lines (Loewus and Kelly, 1961; Agius et al., 2003). However, the enzyme activities of the last two enzymes of this pathway, D-galacturonate reductase (GalUR) and aldonolactonase, were unaltered in these fruits (Table 3). Third, we observed that the myo-inositol level was altered in all transformants and considering that myo-inositol has been proposed as a precursor of ascorbate (Lorence et al., 2004), we determined the total myo-inositol oxygenase activity in red fruits (Table 3). Intriguingly, a significant decrease in the total myo-inositol oxygenase activity was observed in all transgenic lines (Table 3).

Additionally, we also determined the gene expression of three monodehydroascorbate reductase (MDHAR) and two dehydroascorbate reductase genes (DHAR) found in tomato. Both are involved in ascorbate recycling pathway. Interestingly, all transformats displayed a significant increase in MDHARI, MDHAR2,
and MDHAR3 transcript abundances (Figure 7). This result was in agreement with an increase in the MDHAR activity (Table 3). In contrast, we did not observe changes in the expression of the DHAR1 and DHAR2 genes (Figure 7).

**DISCUSSION**

Until now, the breeding of tomato has been dominated by a focus on traits that benefit the grower, such as yield, plant and fruit size, and storage characteristics (Schuch, 1994; Giovannoni, 2006; Cong et al., 2008). As a result, there has been a loss of consumer quality traits such as flavor and nutritional value, and this has focused recent interest on the molecular genetics of such traits (Giovannoni, 2001; Causse et al., 2002, 2004; Fraser et al., 2009; Mounet et al., 2009; Enfissi et al., 2010; Centeno et al., 2011; Morgan et al., 2013). The accumulation of a range of soluble metabolites is important for both flavor and nutrition. In this paper, we characterized the consequences of over-expressing an E. coli pyrophosphatase gene under fruit-specific promoter. This manipulation had a broad impact on fruit development and ripening, emphasizing both the important role of pyrophosphatase in ascorbate and starch biosynthesis.

**EFFECT OF INCREASING PYROPHOSPHATASE ACTIVITY ON STARCH AND SUGARS METABOLISM**

Detailed analysis of sugars level revealed that starch content decreased while the major sugars, Suc and Glc increased in red ripe B33-PPI fruit. These data support the contention that active starch accumulation is an important contributory factor in determining the soluble solids content of mature fruit (Schaffer and Petreikov, 1997; Baxter et al., 2005). Here, we demonstrated that alterations in PPI metabolism have a strong effect on sugars metabolism and, hence, influence agronomic yield. Intriguingly, the data presented here are analogous to those previously described for transgenic potato plants in which higher PPI levels increased starch accumulation and decrease the level of Suc (Fernie et al., 2001a; Geigenberger et al., 2001), and decreased levels have been associated with lower starch biosynthetic rates (Geigenberger et al., 2001).

Different studies concerning starch metabolism in potato and tomato have suggested that AGPase activity plays an important role in its regulation (Geigenberger et al., 1999; Sweetlove et al., 1999). Regulation of the AGPase reaction has been very well characterized for several years. This enzyme is sensitive to allosteric
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**FIGURE 4 | Primary metabolite levels in the receptacle of WT and B33-PPi lines at red stage.** Data are normalized to the mean value of WT at the G stage. Values are means SE of six replicates. Asterisks indicate significant differences by t-test ($P < 0.01$) of the transgenic lines compared with WT at red stage.

**Table 2 | Starch content in the B33-PPi red tomato fruits (60 DAP).**

|       | WT      | L9       | L28       | L29       | L39       |
|-------|---------|----------|-----------|-----------|-----------|
| Starch (nmol g$^{-1}$ FW$^{-1}$) |         |          |           |           |           |
| Fruit | 438.3 ± 9.5 | 354.2 ± 13.1 | 316.4 ± 16.9 | 275.5 ± 20.5 | 384.6 ± 27.7 |

Values are presented as the mean ± SE of 6 biological determinations. The values that are significantly different by t-test from the wild type are set in bold type ($P < 0.05$).

regulation, being inhibited by inorganic phosphate and activated by 3PGA (3-phosphoglycerate) (Sowokinos, 1981; Sowokinos and Preiss, 1982). Additionally, it has been demonstrated to be transcriptionally regulated by sugars, nitrate, phosphate and trehalose-6-phosphate (Müller-Röber et al., 1990; Nielsen et al., 1998; Kolbe et al., 2005; Michalska et al., 2009). Moreover, it has been described that AGPase is also redox regulated (Tiessen et al., 2002; Centeno et al., 2011; Osorio et al., 2013) with malic acid potentially being a key component in this process at least in photosynthetically active tissues (Szecowka et al., 2012).

In this study, a strong correlation was found between starch concentration and AGPase activity in red ripe B33-PPi stage. Additionally, we observed that redox-state of AGPase was not altered in these transgenic fruit. This observation described here have lead us to propose that the activity of this enzyme was modified due to either a change in the rate of sugar influx into the tomato fruit and/or in the lower PPi levels found in these transgenic fruits.

**INCREASED ACTIVITY OF PYROPHOSPHATASE CORRELATES WITH INCREASED ASCORBATE CONTENT IN TOMATO FRUIT**

There is a large potential for improving ascorbate content in food products by means of both genetic engineering and breeding. The exploitation of the large natural variation in ascorbate content in many fruit crops gives the opportunity of improving their nutritional value by classical breeding. The generation of linkage maps and the conduction of quantitative trait loci (QTL) analysis allow the identification of genomic regions associated with ascorbate content (Davey et al., 2006; Stevens et al., 2007; Zorrilla-Fontanesi et al., 2009).
et al., 2011). Such QTL analyses therefore increase our knowledge of the molecular mechanism by which ascorbate is regulated in plants.

The strategy to improve the amount of ascorbate by genetic engineering has been based on the up-regulation of genes encoding for enzymes of the biosynthetic or recovery pathways. In general, plants transformed with genes from different pathways have shown variable increases in ascorbate content in different plant tissues (Agius et al., 2003; Chen et al., 2003; Tokunaga et al., 2005; Eltayeb et al., 2007; Badejo et al., 2008, 2009; Bulley et al., 2009, 2012; Hemavathi et al., 2009; Qin et al., 2011; Zhang et al., 2011; Cronje et al., 2012). Mean increases in ascorbate content were usually two- to three-fold i.e., similar to those reported here. Other approaches have used genes that do not encode enzymes of the ascorbate biosynthetic pathway in plants. Thus, the ectopic expression of a rat L-gulonolactone oxidase, a gene involved in the synthesis of ascorbate in animals, produced an increase of about 7-fold in lettuce (Jain and Nessler, 2000). Similar levels were observed in potato plants ectopically expressing a bacterial pyrophosphorylase or a yeast invertase (Farre et al., 2008).

In this study, a strong correlation was displayed between the cellular PPI and ascorbate levels (up to 2.5-fold increase in red ripe transgenic fruits) and it was demonstrated that this was mechanistically linked to pyrophosphatase activity as previously was observed in potato tuber overexpressing a bacteria pyrophosphatase with a plastid targeting sequence (Farre et al., 2006). Additionally, an increase in some intermediates of ascorbate biosynthesis such as dehydroascorbate, galacturonate, and myo-inositol were also observed.

Formation of GDP-D-mannose is the initial step in the D-Man/L-Gal pathway of ascorbate biosynthesis, which is synthesized from D-mannose-1 phosphate via GDP-D-mannose. The D-Man/L-Gal pathway of ascorbate biosynthesis, which is synthetized from D-mannose-1 phosphate via GDP-mannose pyrophosphatase (Conklin et al., 1999; Keller et al., 1999)

| Enzymatic activities | WT | L9 | L28 | L29 | L39 |
|----------------------|----|----|-----|-----|-----|
| AGPase               | 6.65 ± 0.75 | 3.98 ± 0.73 | 4.65 ± 0.42 | 3.97 ± 0.89 | 6.38 ± 1.02 |
| AGPase activation state (Vsel/Vred) | 0.67 ± 0.08 | 0.58 ± 0.09 | 0.73 ± 1.04 | 0.63 ± 0.76 | 0.76 ± 0.06 |
| MDHAR                | 6.35 ± 0.31 | 12.34 ± 0.21 | 15.22 ± 0.46 | 11.10 ± 0.36 | 9.43 ± 0.42 |
| GalUR                | 15.65 ± 0.86 | 14.89 ± 0.75 | 16.24 ± 0.65 | 15.23 ± 0.54 | 16.33 ± 0.76 |
| Aldonolactonase      | 11.23 ± 1.34 | 10.34 ± 0.78 | 12.36 ± 1.06 | 13.23 ± 1.03 | 12.54 ± 0.87 |

Values are presented as the mean ± SE of six biological replicates. An asterisk indicates the values that were determined by the t-test to be significantly different (P < 0.05) from wild type.

**FIGURE 5** | Ascorbate of B33-PPI tomato lines. Ascorbic acid was determined in red tomato fruit (60 DAP). The values are presented as the mean ± SE of six biological replicates. An asterisk indicates the values that were determined by the t-test to be significantly different (P < 0.05) from wild type.

**FIGURE 6** | Expression of GGP, GaLDH, and GDH genes in red B33-PPI fruits. The abundance of GGP1 (acc. number Solyc06g073320), GGP2 (acc. number Solyc02g091510), GaLDH (acc. number Solyc10g079470), and GDH (acc. number Solyc01g106450) mRNAs were measured by quantitative RT-PCR, respectively. An asterisk indicates the values that were determined by the t-test to be significantly different (P < 0.05) from wild type.
Biosynthesis.

are required to understand the impact of sis pathways (T orabinejad et al., 2009), further investigation myo function that impacts both phatase enzyme from the D-Man/L-Gal pathway, has a dual previous study suggests that the L-galactose-1-phosphate phosphates as suggested by Lorence et al. (2004). Although, a myo suggesting that the increase in enhaken, 2009) leaf AsA being reported. Together with this reaction in the direction of ascorbate biosynthesis produces PPI as by-product. It is thus conceivable that the removal of PPI is favorable for ascorbate synthesis. Furthermore, the observed higher expression of GGPI, GG2 genes, a key point for the control of ascorbate pathway (Dowdle et al., 2007; Laing et al., 2007; Bulley et al., 2012), and GalDH, the last gene in the AsA biosynthesis pathway, corroborates that D-Man/L-Gal pathway is activated in the B33-PPI fruits in comparison to WT fruits.

We also evaluated if other alternative pathways of AsA biosynthesis were altered in these fruits. When looked at the D-galacturonic acid pathway, an increase in the level of its precursor, galacturonic acid, was observed. However, the enzyme activities that catalyze the two last steps in this pathway, GalUR and aldonolactonase, were not altered. Increasing myo-inositol production has also shown varied results, with both increased (Lorence et al., 2004) and unaffected (Endres and Tenhaken, 2009) leaf AsA being reported. Together with an increase in myo-inositol levels, we observed a decrease in the myo-inositol oxidase activity in the B33-PPI red fruits, suggesting that myo-inositol can act as a precursor for AsA biosynthesis as suggested by Lorence et al. (2004). Although, a previous study suggests that the L-galactose-1-phosphate phosphatase enzyme from the D-Man/L-Gal pathway, has a dual function that impacts both myo-inositol and AsA biosynthesis pathways (Torabinejad et al., 2009), further investigation are required to understand the impact of myo-inositol on AsA biosynthesis.

Also, our results revealed that ascorbate recycling pathway was altered in the B33-PPI red fruits since we found higher dehydroascorbic acid content and expression of the three tomato MDHAR genes.

**INCREASED ACTIVITY OF PYROPHOSPHATASE ALSO AFFECTS OTHER METABOLIC CHANGES**

When other areas of metabolism are considered some interesting observations are apparent. Interestingly, the total level of organic acids and amino acids were largely invariant in the B33-PPI lines in comparison with WT. The exception to this was that we observed an accumulation in two amino acids, namely Ala and Asp. The accumulation of Asp can be explained because it acts as precursor in the synthesis of Asn via asparagine synthase. This reaction produces PPI as by-product that can be removed via pyrophosphatase. Therefore, we expect a shift in the reaction equilibrium to favor the Asn synthesis direction. Although significant differences were not found in the levels of Asn, this may be due to a co-ordinate up regulation of its metabolism. The reason for the increase in alanine is less clear but may merely reflect the additional availability of glucose for glycolytic reactions.

**MORPHOLOGICAL EFFECTS ON B33-PPI FRUITS**

In addition to the increase in soluble solids content, the fruit of the transgenic lines were compromised in size. This observation was also largely to be expected, since several direct genetic studies (Zrenner et al., 1996; Sonnewald et al., 1997; Sturm and Tang, 1999) have implicated Suc mobilization as a key determinant of sink strength in a broad range of species. As much as 10% (w/v) Suc has been reported in the phloem of plants (Hayashi and Chino, 1990), and the presence of AsA in the phloem sap was confirmed by radiolabeling (Franceschi and Tarlyn, 2002). It was also reported that the presence of sugar within the plant acts as potent signal that promotes AsA biosynthetic gene expression (Nishikawa et al., 2005). Within tomato fruit itself, a positive correlation between Suc feeding and the expression level in some genes of the D-Man/L-Gal pathway was described (Badejo et al., 2012), supporting the view that transportation of sugars from source tissues affect the AsA content in sink tissues through the up-regulation of AsA biosynthesis pathway genes. Despite the wide changes in morphological parameters, metabolic changes in the transgenic fruit were, by and large, confined to sugar and AsA metabolisms. We believed that fruit growth is largely dependent on the relationship between import of photoassimilate and AsA intake and/or biosynthesis.

In summary, the results presented in this study provide direct evidence that the reduction in PPI content had strong effects on metabolism of sugar and ascorbate contents. Detailed analysis of starch metabolism revealed that this phenomenon was due to alteration in AGPase activity, caused by either a change in the rate of sugar influx into the tomato fruit and/or in the lower PPI levels found in these transgenic fruits. During ripening, the lack of accumulation of transitory starch was reflected by a decrease of soluble sugars. Moreover, we demonstrated that alterations in the level of PPI resulted in dramatic effect on ascorbate metabolism. These lines displaying low PPI content showed and increased flux to, and accumulation of, ascorbate. This occurred in spite
of increases the ascorbate level via D-mannose-1P and via GDP-mannose pyrophosphatase. Further investigation is required to define this control, especially in fruit such as tomato, where it may contribute to taste (sugars and organic acids) and nutritional value (ascorbate) of the fruit which are important in determining fruit quality.

MATERIALS AND METHODS

PLANT MATERIAL

The gene encoding a pyrophosphatase from *E. coli* (Sonnewald, 1992) was introduced in the sense orientation into the vector pBinAR between the patatin B33 promotor (Rocha-Sosa et al., 1989) and the octopine synthase polyadenylation signal. This construct was introduced into tomato (*Solanum lycopersicum*, L.) cv Moneymaker plants by an Agrobacterium-mediated transformation protocol, and plants were selected and maintained as described in the literature (Taubberger et al., 2000). An initial screening was carried out on the basis of pyrophosphatase activity. This screen allowed the identification of four lines, which were taken to the next generation.

METABOLITE DETERMINATIONS

PPI and Pi determination

PPI was extracted from tomato fruit by TCA/ether method (Jelitto et al., 1992). PPI was determined using the colorimetric PiPer Pyrophosphate assay kit (Invitrogen) according to the manufacturer’s specifications. All glassware was pretreated overnight with 0.1 M HCl to remove residual phosphate. PPI levels were determined by a sample blank with or without sPPase, and total Pi was calculated by comparison at 595 nm with a linear Pi standard curve.

Pi was determined in the TCA extracts with a colorimetric assay as described by Taussky and Shorr (1953).

Primary metabolic profiling

Metabolite extraction derivatization, standard addition, and sample injection for GC-MS were performed according (Osorio et al., 2012). Both chromatograms and mass spectra were evaluated using TAGFINDER (Luedemann et al., 2008).

Ascorbic acid determination

Ascorbic acid extraction and analysis were performed as described (Lima-Silva et al., 2012). Ascorbic acid content was determined by comparison with a linear ascorbic acid standard curve.

Starch determination

The level of starch in the tissues were determined exactly as described previously (Fernie et al., 2001b).

ENZYME ACTIVITIES

Alkaline pyrophosphatase activity

The protein extraction and enzyme activity were analyzed as described Farre et al. (2001).

AGPase

AGPase activity was measured in the pyrophosphorolysis direction with a spectrophotometric assay, as described Tiessen et al. (2002, 2003). Frozen tissues were homogenized in liquid N2 and approx. 100 mg was extracted rapidly (1 min) with 1 ml of extraction buffer (50 mM Hepes-KOH, pH 7.8, and 5 mM MgCl2) at 4°C. After centrifugation (30 s at 13,000 g at 4°C), 10 µl of the supernatant was used for the AGPase assay. The reaction was performed in a total volume of 200 µl containing 50 mM Hepes-KOH, pH 7.8, 5 mM MgCl2, 10 µM Glc-1,6-bisP, 0.6 mM NADP+, 2.5 mM Na-PPI, 1 unit/ml phosphoglucomutase, 2.5 units/ml Glc-6-P dehydrogenase, and a range of concentrations of ADP-Glc (0.4—1 mM) in the absence of Pi, with or without DTT (10 mM) for activation assay. Reactions were followed on line at 340 nm and were linear up to 30 min. The activation state of AGPase is defined as the ratio of Vsel (−DTT) to Vred (+DTT).

Myo-inositol oxygenase

Two-hundred mg of tissue was incubated for 30 min at 30°C in a buffer containing 100 mM KPO4 (pH 7.2), 2 mM L-cysteine, 1 mM ammonium ferrous sulfate hexahydrate, and 60 mM myo-inositol. The reaction was stopped by boiling for 10 min and denatured protein removed by centrifugation (20,000 g, 15 min). Glucuronic acid was determined at 540 nm before and after samples developed a pink color with addition of a 3-hydroxybiphenylphenol color reagent (van den Hoogen et al., 1998).

D-galacturionate reductase

One gram of samples were homogenized in liquid nitrogen and extracted with 50 mM sodium phosphate buffer, pH 7.2, containing 2 mM EDTA, 2 mM dithiothreitol, 20% glycerol and PVP. GalUR activity was measured by the decrease in absorbance at 340 nm at 25°C after the addition of 100 µl of crude enzyme extract to the assay mixture (1 ml) consisted of 50 mM phosphate buffer (pH 7.2), 2 mM EDTA, 0.1 mM NADPH, 30 mM D-galacturonic acid and 2 mM dithiothreitol. The GalUR activity in the crude enzyme extract was recorded as nmol of NADPH oxidized min-1 mg-1 protein (Agius et al., 2003).

Aldonolactonase

The activity was measured by the change in absorbance of *p*-nitrophenol through acidification at 405 nm essentially as described Ishikawa and Shigeoka (2008).

L-galactono-1,4-lactone dehydrogenase

Samples were prepared as described by Mieda et al. (2004) and assayed at 340 nm by measuring the reduction of NAD+ in a reaction mixture containing 0.5 mM NAD+, 1 mM L-Gal, and the enzyme extract. L-Galactono-1,4-lactone dehydrogenase activity was assayed by the reduction of cytochrome c resulting in an increase in absorbance at 550 nm in a reaction mixture containing 30 mM TRIS-HCl, pH 8.5, 1 mM sodium azide, 42 mM L-Gal, 0.1% Triton X-100, 1.05 mg/ml cytochrome c, and the extract in a final volume of 1 ml as described by Yabuta et al. (2000).
**MEASUREMENT OF FRUIT BRIX**

Ripe fruit tissue was homogenized with a razor blade, and the soluble solids (Brix) content of the resulting juice measured on a portable refractometer (Digita1es Refractometer DR6000; Krüss Optronic GmbH, Hamburg, Germany).

**ANALYSIS OF GENE EXPRESSION BY QRT-PCR**

Total RNA was extracted according to Bugos et al. (1995) with minor modifications. Integrity of the extracted RNA was checked by electrophoresis under denaturing conditions after treating the RNA with RNase-free DNaseI (Roche). First-strand cDNA synthesis of 1 mg of RNA in a final volume of 20 mL was performed with Moloney murine leukemia virus reverse transcriptase, Point Mutant RNase H Minus (Promega), according to the supplier's protocol using oligo(dT) T19 primer.

Expression of the monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR), L-galactono-1,4-lactone dehydrogenase (GalDH), and L-galactose dehydrogenase (GDH) genes was analyzed by real-time qRT-PCR using the fluorescent intercalating dye SYBR Green in an iCycler detection system (Bio-Rad; http://www.bio-rad.com/). Relative quantification of the target expression level was performed using the comparative Ct method. The following primers were used: for analysis of MDHAR1 transcript levels (GenBank accession no. Solyc09g009390), forward, 5′-TCTAGCAGTTAATAAGTTGGTGAG-3′, reverse, 5′-ATTGGCTTTGCTTTTCAAGGTTG-3′; for MDHAR2 (GenBank accession no. Solyc02g086710), forward, 5′-TGGTTTGTGAATGTCCACGG-3′, reverse, 5′-TTGTTTGTAGTCTTACAGTCAA-3′ (Gilbert et al., 2009); for GGP1 (GenBank accession no. Solyc06g073320) forward, 5′-AGGGTGCAAACGAGCAAAATG-3′, reverse, 5′-ATGGCGTGGAGGTGTGACA-3′; for GGP2 (GenBank accession no. Solyc02g091510) forward, 5′-GTCCTTGGTTGGTGTGTAAT-3′, reverse, 5′-TGCAAAAAGTGTGCTAGTCT-3′. To normalize gene expression for differences in the efficiency of cDNA synthesis, transcript levels of the constitutively expressed elongation factor 1a of tomato (GenBank accession no. X14449) were measured using the following primers: forward, 5′-ACCACAGACTCTCCAGGAG-3′; reverse, 5′-CAT TGAACCAACATTGTTACACC-3′ (Zanor et al., 2009).

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Pyrophosphate influences tomato ripening metabolism

Osorio et al. (2015) investigated the effects of pyrophosphate on tomato fruit ripening. They found that pyrophosphate influences tomato ripening metabolism, leading to altered carbon partitioning and hormone transcript abundance. The co-regulation of oxidative response and hormone transcript abundance, along with reduced photosynthetic activity, indicated in their source leaves and both altered carbon partitioning and hormone transcript abundance. In tomato fruit, the pyrophosphatase display and hormone transcript abundance were reduced, indicating a potential role in the development of tomato fruit.

Specifically, the study revealed that pyrophosphate influences tomato ripening by regulating the expression of genes involved in the metabolism of pyruvate and the interconversion of pyruvate and cytosol. The study also highlighted the potential importance of pyrophosphate in the regulation of tomato fruit ripening, suggesting a novel role for pyrophosphate in plant metabolism.
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APPENDIX

FIGURE A1 | The proposed biosynthetic pathways for ascorbate in plants. L-Gal, L-galactose; L-GalL, L-galactono-1,4-lactone; L-Gul, L-gulose; L-GuL, L-gulono-1,4-lactone; D-Man, d-mannose; UDP, uridine diphosphate; GGP, GDP-L-galactose phosphorylase; GDH, L-galactose dehydrogenase; GaLDH, L-galactono-1,4-lactone dehydrogenase; GalUR, D-galacturonate reductase; MYOX, myo-inositol oxidase; MDHAR, monodehydroascorbate reductase; DHAR, dehydroascorbate reductase.