Journal of Experimental Botany, Vol. 72, No. 18 pp. 6490–6509, 2021
https://doi.org/10.1093/jxb/erab269 Advance Access Publication 8 June 2021

RESEARCH PAPER

Multiomics analyses reveal the roles of the ASR1 transcription factor in tomato fruits

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Received 24 January 2021; Editorial decision 3 June 2021; Accepted 5 June 2021

Editor: Fabrizio Costa, University of Trento, Italy

Abstract

The transcription factor *ASR1* (*ABA, STRESS, RIPENING 1*) plays multiple roles in plant responses to abiotic stresses as well as being involved in the regulation of central metabolism in several plant species. However, despite the high expression of ASR1 in tomato fruits, large scale analyses to uncover its function in fruits are still lacking. In order to study its function in the context of fruit ripening, we performed a multiomics analysis of *ASR1*-antisense transgenic tomato fruits at the transcriptome and metabolome levels. Our results indicate that ASR1 is involved in several pathways implicated in the fruit ripening process, including cell wall, amino acid, and carotenoid metabolism, as well as abiotic stress pathways. Moreover, we found that *ASR1*-antisense fruits are more susceptible to the infection by the necrotrophic fungus *Botrytis cinerea*. Given that ASR1 could be regulated by fruit ripening regulators such as FRUITFULL1/FRUITFULL2 (FUL1/FUL2), NON-RIPENING (NOR), and COLORLESS NON-RIPENING (CNR), we positioned it in the regulatory cascade of red ripe tomato fruits. These data extend the known range of functions of ASR1 as an important auxiliary regulator of tomato fruit ripening.

Keywords: ASR1, *Botrytis cinerea*, fruit ripening, fruit ripening regulators, fungal susceptibility, metabolomics, red ripe tomato, transcriptomics.

Introduction

Fruit ripening is a complex process that integrates changes in multiple traits including colour, flavour, texture, and central metabolite content (Shinozaki *et al.*, 2018). The mechanisms underlying the transcriptional regulation of genes involved in fruit ripening comprise a network of hormones, transcription factors and epigenetic modifications (Chen *et al.*, 2020).

Despite the importance of transcription factors in the modulation of fruit ripening, only a small fraction of the 1845 transcription factors estimated to be present in the tomato genome (Rohrmann *et al.*, 2011; Jin *et al.*, 2017) has been functionally studied so far (Chen *et al.*, 2020). Thus, there is a need to increase the knowledge on these regulators, especially given that they are believed to be strong candidates for altering complex traits in crop plants (Century *et al.*, 2008; Rabara *et al.*, 2014; H. Wang *et al.*, 2016).

ASR1 is a transcription factor belonging to the *ASR* (*ABA, STRESS, RIPENING*) gene family. The *ASR* genes are broadly distributed in the Plantae kingdom, being found both in angiosperms and gymnosperms (Hong *et al.*, 2002; Dóczi *et al.*, 2005; Frankel *et al.*, 2006; Philippe *et al.* 2010; Fischer *et al.*, 2011; Henry *et al.*, 2011; Virlouvet *et al.*, 2011), but unexpectedly absent in Arabidopsis (Carrari *et al.*, 2004). Most of the studies with ASR1 have centred on its role during stress due to its high expression under drought, salt, and osmotic stresses, with these studies being carried out in several species, including tobacco (Kalifa *et al.*, 2004b; Jha *et al.*, 2012; Hu *et al.*, 2013), maize (Riccardi *et al.*, 1998; Virlouvet *et al.*, 2011; J. Zhang *et al.*, 2019), rice (Vaidyanathan *et al.*, 1999; Kawasaki *et al.*, 2001; Yang *et al.*, 2004; Philippe *et al.*, 2010), plantain (Liu *et al.*, 2010; Dai *et al.*, 2011), banana (Henry *et al.*, 2011), lily (Yang *et al.*, 2005), *Salicornia brachiata* (Jha *et al.*, 2012) and wheat (Hamdi *et al.*, 2020). A more limited number of functional studies have demonstrated that the amount of ASR1 is directly related to abiotic stress responses, such as those in tobacco seedlings under saline stress (Kalifa *et al.*, 2004b), maize under drought stress (Virlouvet *et al.*, 2011) and rice under aluminium, cold, salt and drought stresses (Joo *et al.*, 2013; Arenhart *et al.*, 2016; Park *et al.*, 2020). The role of ASR in biotic stress has, however, been less explored and is largely based on observations of changes in gene expression following infection. For example, *ASR* genes are up-regulated in plantain and apple upon infection with the fungi *Fusarium oxysporum* f. sp. *cubense* and *Alternaria alternata* f. sp. *mali,* respectively (Liu *et al.*, 2010; Huang *et al.*, 2016), while *ASR1* expression is increased upon infection with *Botrytis cinerea* and powdery mildew in tomato fruits (Jia *et al.*, 2016). Indeed, to our knowledge, functional studies on the role of ASR in biotic stress are restricted to the study of Li *et al.* (2018), who found that the overexpression of *OsASR2* enhances the resistance to *Xanthomonas oryzae* pv. *oryzae* and *Rhizoctonia solani* in rice.

Besides its role in stress responses, the ASR family is known to have a role in central metabolism. A grape ASR, known as MSA (MATURATION, STRESS, ABA), recognizes specific sites in the regulatory regions of the *HT1* fruit hexose transporter (Çakir *et al.*, 2003). Accordingly, transgenic potato, and tobacco plants with altered expression of ASR1 have alterations in the concentrations of glucose and hexose transporters (Frankel *et al.*, 2007; Dominguez *et al.*, 2013). ASR1 is also implicated in the regulation of the amino acid content in species such as potato and maize (Frankel *et al.*, 2007; Virlouvet *et al.*, 2011). This feature of ASR is especially interesting, given that central metabolism, at least partially, determines tomato fruit quality (Klee and Giovannoni, 2011).

In tomato (*Solanum lycopersicum*), the ASR family is composed of five paralogs, namely, *ASR1* to *ASR5* (Iusem *et al.*, 1993; Amitai-Zeigerson *et al.*, 1994; Rossi *et al.*, 1996; Frankel *et al.*, 2003; 2006; Fischer *et al.*, 2011). The paralog ASR1 was the first one to be described (Iusem *et al.*, 1993), and has since been the focus of studies on the ASR family. ASR1 is expressed throughout the tomato plant (including seeds, leaves, stems, and fruits), under stress conditions, and following ABA treatment in tomato leaves and fruits (Iusem *et al.* 1993; Amitai-Zeigerson *et al.*, 1995; Maskin *et al.*, 2001; 2007; 2008; Golan *et al.*, 2014; Jia *et al.*, 2016). *ASR1* expression increases during tomato fruit development, reaching its maximum values in the turning and red ripe stages (Golan *et al.*, 2014; Jia *et al.*, 2016). Besides, tomato plants that overexpress *ASR1* have an enhanced tolerance to drought stress (Golan *et al.*, 2014). More recently, Jia *et al.* (2016) studied the role of ASR1 in the crosstalk between ABA and sucrose to regulate fruit ripening in tomato and strawberry.

Tomato has also served as the main system to uncover the dual molecular mechanism by which ASR1 acts. ASR1 is located in both the cytosol and the nucleus (Kalifa *et al.*, 2004a; Ricardi *et al.*, 2012). Konrad and Bar-Zvi (2008) found that SlASR1 could act as a chaperone-like protein in the cytosol protecting proteins from denaturation during cycles of freezing and thawing. Concomitantly, a nuclear location of ASR1 coupled with its capacity to bind to DNA (Kalifa *et al.*, 2004a) suggested its role as transcription factor. Ricardi *et al.* (2014) identified some of the genes directly regulated by ASR1 in tomato leaves under drought stress by employing chromatin immunoprecipitation followed by high throughput sequencing (ChIPseq) strategy. Intriguingly, genes associated with cell wall and transport (like aquaporins) were revealed to be the main targets of ASR1.

Despite the available information on the role of ASR1, there is still a lack of knowledge on the pathways in which ASR1 is involved during tomato fruit maturation on a large scale. The high expression of ASR1 in tomato fruits, especially during the red ripe stage (Golan *et al.*, 2014; Jia *et al.*, 2016), together with its potential roles in pathways related to fruit quality, like central metabolism (Çakir *et al.*, 2003; Frankel *et al.*, 2007; Virlouvet *et al.*, 2011; Dominguez *et al.*, 2013) and stress tolerance (Golan *et al.*, 2014), makes ASR1 an interesting subject matter. We used a combined strategy of transcriptomics and metabolomics analyses on *ASR1*-antisense tomato fruits with the aim of unveiling the role of ASR1 in red ripe tomatoes, since metabolism in this stage has a direct impact on the fruit quality for consumption (Giovannoni, 2007). The results show that ASR1 is involved in many pathways related to fruit maturation, like cell wall metabolism, photosynthesis, abiotic stress responses, fungal susceptibility, and amino acid metabolism. Besides, an analysis of previous studies on fruit regulators has

revealed that ASR1 could be regulated by FRUITFULL1/FRUITFULL2 (FUL1/FUL2), NON-RIPENING (NOR), and COLORLESS NON-RIPENING (CNR), among others. Altogether, our results show that ASR1 has an important role in tomato fruit maturation.

Materials and methods

Plant growth

All the experiments, including the selection of the transgenics and their evaluation together with the wild type plants, were performed under controlled greenhouse conditions (80% relative humidity; 200 mmol PAR $s^{-1}m^{-2}$; 16 h of light/8 h of darkness). Fruits were sampled when they reached complete maturity (red ripe stage; 50 days post-anthesis) in all the experiments and the mesocarps were kept at −80 °C for further use.

Generation of transgenic lines

The 348 bp coding region of the tomato *ASR1* gene (GenBank U86130.1) was cloned in antisense orientation into the multiple cloning site of the vector pBINAR (Liu *et al.*, 1990), which contains the Cauliflower Mosaic Virus (CaMV) 35S promoter, the octopine synthase (*ocs*) terminator and the NPTII (Neomycin phosphotransferase II) selection gene. The construct was transferred into tomato (*Solanum lycopersicum* 'Money Maker') cotyledons by means of *Agrobacterium tumefaciens* using a transformation protocol adapted from Nunes-Nesi *et al.* (2005). Emerging shoots were excised and selected on MS (Murashige and Skoog) medium containing kanamycin (100 mg l^{-1}). Once plants had rooted, they were transferred to soil in the greenhouse for subsequent selection. Control plants (WT) of the same tomato cultivar were transformed with an empty vector simultaneously.

Selection of transgenic lines

Twenty-three antisense T_0 plants were screened based on *ASR1* expression in their leaves by Northern blot analysis using a riboprobe, as described in Frankel *et al.* (2007). Three antisense lines (AS5, AS17 and AS18) were selected according to the amount of *ASR1* expression shown in the Northern blots, propagated, and transferred to the greenhouse for further characterization. *ASR1* expression in mature fruits (red ripe stage, 50 days post anthesis) of each of these T_1 lines was checked by qRT–PCR. Five biological replicates per line were evaluated. Total RNA of mature fruits was extracted by TRIzol (Invitrogen, USA) according to the manufacturer's instructions. RNA samples were treated with DNaseI (Invitrogen, USA). cDNA was obtained using reverse transcriptase (M-MLV, Invitrogen, USA), RNase inhibitor (RNAseOUT, Invitrogen, USA) and random primers (Invitrogen, USA) according to the manufacturer's specifications. qRT–PCR was performed with a master mix (QUANTITECT SYBR GREEN PCR KIT, Qiagen, USA) in a thermocycler ABI 7500 (Applied Biosystems, USA). All the primer sequences used are listed in Supplementary Table S1. The reference gene was *18S RIBOSOMAL RNA* (*18S rRNA*), and was chosen after the evaluation of five candidate genes that included *UNKNOWN EXPRESSED PROTEIN* (*EXPRESSED*; SGNU346908/ Solyc07g025390), *SAND* (*SP100, AIRE-1, NUCP41/75, DEAF-1*; SGN-U316474/ Solyc03g115810), *CLATHRIN ADAPTOR COMPLEXES MEDIUM SUBUNIT* (*CAC*; SGN-U314153/ Solyc08g006960), *TAP42-INTERACTING PROTEIN* (*TIP41*; SGN-U321250/Solyc10g049850) and *18S rRNA* (X98800). The list of candidate genes was chosen based on a study by Expósito-Rodríguez *et al.* (2008). The evaluation was performed with three different programs, namely BestKeeper (Pfaffl *et al.*, 2004), geNorm (Vandesompele *et al.*, 2002) and NormFinder (Andersen *et al.*, 2004). The results are presented in Supplementary Table S2. The Ct (cycle threshold) values and efficiencies of the qRT–PCRs were obtained with the LinRegPCR software (Ruijter *et al.*, 2009). The ratios were calculated according to Pfaffl (2001). The statistical analysis of the data was performed by the permutation test using the fgStatistics software (Di Rienzo, 2009a). Experimental conditions used in the qRT–PCRs based on MIQE requirements are summarized in Supplementary Table S3. The expression of the *ASR1* paralogs, namely *ASR2*, *ASR3*, *ASR4* and *ASR5*, was also measured by qRT–PCR (primer sequences in Supplementary Table S1). Between 3–5 biological replicates per line were evaluated. It was not possible to design primers to differentiate *ASR3* from *ASR5* since their sequences are 96% similar (Fischer *et al.*, 2011); so they were measured together.

Microarray analysis of mature fruits

Total RNA of mature fruits was extracted with Trizol® (Invitrogen, USA) according to the manufacturer's specifications. Pools of RNA from two mature tomato fruit mesocarps from three different plants per line were prepared. RNA samples were checked for their integrity on an Agilent 2100 bioanalyzer (Agilent Technologies, USA). The preparation of the biotinylated cRNA was done with 100 ng of total RNA using the MessageAmp Premier Amplification Kit (Ambion, USA) according to the manufacturer's instructions. The hybridizations were performed on the GeneChip Tomato Genome Arrays® from Affymetrix® (USA). The washing and staining steps were performed in a Gene-Chip® Fluidics Station 450 (Affymetrix). The scanning was done with the Affymetrix® GeneChip® Scanner 3000 7G. Data normalization, annotation and statistical analyses were performed with the Robin application (Lohse *et al.*, 2010). The normalization method was RMA (Robust Multichip Analysis; Irizarry *et al.*, 2003) and the statistical analysis method was Rank Product (Breitling *et al.*, 2004; Hong *et al.*, 2006) with $P < 0.05$ considered to be statistically significant. The P values were corrected for multiple testing using the approach designed by Benjamini and Hochberg (1995). Statistically significant normalized data are shown in Supplementary Tables S4; S5. The functional annotation of genes was performed using MapMan (Thimm *et al.*, 2004) through Robin (Lohse *et al.*, 2010). The databases TAIR (https://www.arabidopsis.org/) and Sol Genomics Network (http://www.solgenomics.net/) were used to annotate the genes. The enrichment analysis of the functional categories in the differentially expressed genes of the *ASR1*-antisense lines was done with the Fisher's exact test using PageMan (Usadel *et al.*, 2006). The summary of the experimental conditions used in the microarray analyses based on MIAME requirements is shown in Supplementary Table S6.

Construction of the gene correlation network and cluster analysis

The differentially expressed genes (DEGs) were used to construct a gene network by applying partial correlations employing a shrinkage estimator, based on the Ledoit-Wolf lemma for the DEG covariance matrix (Schäfer and Strimmer, 2005). The construction was done with the GeneNet application through the GeNeCK web server (https://lce.biohpc.swmed.edu/geneck/) using the default parameters (M. Zhang *et al.*, 2019). After the network was built, clusters were searched using the MCODE plugin (Bader and Hoguer, 2003) in Cytoscape (Shannon *et al.*, 2003). The employed parameters for the cluster search were the default ones, except for the node score cutoff that was set to 0.25.

Metabolite measurements in mature fruits

The characterization of metabolites in mature fruits was analysed using GC-MS. Six biological replicates per line were analysed. The extractions and runs were performed following the protocols developed by

Roessner *et al.* (2000) and Lisec *et al.* (2006), respectively. The metabolite extractions were done with approximately 250 mg of frozen tissue with ribitol (0.2 mg ml^{-1}) as an internal standard. FAMES (fatty acid methyl esters) were used to calculate the retention times. The samples were run in splitless mode in a GC 6890N gas chromatographer (Agilent Technologies, Germany) coupled to a Pegasus III time-of-flight mass spectrometer (LECO Instruments, USA) with an AS2000 autosampler (PAL Agilent, USA). The mass spectra were recorded at 20 scans per second with a scanning range of 70–600 m/z. Identification of the compounds was performed with the TagFinder 4.0 software (Luedemann *et al.*, 2008) using the Golm Metabolome Database (Kopka *et al.*, 2005; Schauer *et al.*, 2005). Due to saturation of the glucose and sucrose peaks in the GC-MS runs, these compounds were measured by the enzymatic method, as described in Fernie *et al.* (2001), assessing five biological replicates per line. β-carotene and lycopene were extracted with a mix of hexane:ethanol 3:4 and measured spectrophotometrically according to Heredia *et al.* (2009). Between three to four biological replicates of each line were analysed.

Mapping of the metabolic and transcript data onto MapMan metabolic maps

The averaged metabolite and transcript profiles of the *ASR1*-antisense lines AS5 and AS17 were mapped onto metabolic maps using MapMan (Thimm *et al.*, 2004). The mapped transcripts were statistically significant in at least one of the two lines, while the mapped metabolites were statistically significant in both lines.

Validation of the microarray results by qRT–PCR

The validation of the results obtained in the microarrays was performed in independent experiments by qRT–PCR. Total mRNA of red ripe fruits was extracted with Trizol® (Invitrogen, USA) according to the manufacturer's guidelines. Between three to five biological replicates of lines AS5, AS17 and WT plants were analysed. The cDNA was prepared using Invitrogen reagents, as mentioned above. The list of the employed primers is in Supplementary Table S1. The reference gene was *18S rRNA* and was chosen from a set of genes, as described above (Supplementary Table S2). The qRT–PCR Cts and efficiencies were obtained with the LinRegPCR software (Ruijter *et al.*, 2009). Statistical analysis of the data was performed by the permutation test with the fgStatistics software (Di Rienzo, 2009a). The experimental conditions used in the qRT–PCRs based on MIQE requirements are listed in Supplementary Table S3.

Infections of tomato fruits with Botrytis cinerea

Conidia of *Botrytis cinerea* CECT2100 (Instituto Nacional de Tecnología Agropecuaria, Argentina) were collected from 10-day-old PDA (potato dextrose agar) plates, quantified with a Neubauer chamber, and adjusted to 4×10^{-6} conidia ml^{-1} (low content inoculum). Infection experiments followed the protocols published by Verhagen *et al.* (2010) and Blanco-Ulate *et al.* (2013). Briefly, five red ripe tomato fruits of each line were surface-sterilized with 10% bleach and washed with sterilized water. Fruits were wounded by making four incisions along the equator and 5 µl of the conidia suspension was applied to the wounds. The conidia suspensions were homogenized by agitation during the application process to ensure an equal application on the fruits. All the fruits were incubated in a growth chamber under controlled conditions (23 °C, relative humidity 85%). After 96 h, the diameter of the infection on the wounds was measured, and the values for each fruit were averaged. The lesion size was used as a measure of disease severity, as suggested by Soltis *et al.* (2019) and Silva *et al.* (2021).

Statistical analyses

The plants were randomly placed in the greenhouse. In all the experiments, the mean and the SE are shown for each treatment. All the analyses were done with the Infostat software (Di Rienzo, 2009b) employing the analysis of variance (ANOVA) followed by the Duncan's test (except for the qRT–PCRs and microarray experiments, which are explained above). In all data analyses, the significance level was $P < 0.05$.

Results

Generation and selection of ASR1*-antisense tomato lines*

An *ASR1*-antisense construct carrying the 35S promoter, the octopine synthase (*ocs*) terminator and the NPTII (Neomycin phosphotransferase II) selection gene (Fig. 1A) was used to transform tomato cotyledons through *Agrobacterium*. Twenty-three T_0 lines were regenerated and leaf *ASR1* expression was assessed by Northern blot (Fig. 1B; the unedited blots are shown in Supplementary Fig. S1). Three antisense lines (AS5, AS17 and AS18) with reduced *ASR1* expression were selected (Fig. 1B) and selfed. *ASR1* expression in mature fruits of the T_1 plants was subsequently analysed by qRT–PCR and confirmed to be reduced in the three selected lines (Fig. 1C). All subsequent experiments were performed with these T_1 plants. The expression of the *ASR1* paralogs *ASR2*, *ASR3/5* and *ASR4* was simultaneously evaluated in these fruits (Fig. 1C). *ASR2* expression was reduced in the three AS lines ($P < 0.05$), while *ASR3/5* and *ASR4* expression did not show statistically significant ($P > 0.05$) differences in the AS lines compared with WT plants. The percentage of similarity between *ASR1* and *ASR2* is 82% (Frankel *et al.*, 2006), suggesting that the antisense construct most likely targets *ASR2* as well.

Transcriptional profiles of the ASR1*-antisense fruits show altered expression of genes in the photosynthesis, cell wall, stress, and DNA functional categories*

To gain insight into the functions of ASR1 in mature fruits, the transcriptional profiles of mature fruits of the lines AS5 and AS17 in comparison to those of WT plants were examined via use of the Affymetrix GeneChip® Tomato Genome Arrays (Fig. 2). A principal component analysis of the microarray data showed that the expression profiles of the *ASR1*-antisense lines differed from those of the WT plants (Fig. 2A). The high level of variance observed among individuals of the AS lines could be due to differences in the number of copies of the construct in the individuals or to the variability in the silencing produced by the antisense construct (Butaye *et al.*, 2005). The expression of 53 genes was increased in both antisense lines (Fig. 2B; Supplementary Table S4). Most of these were genes in the functional category of encoding enzymes, with the others being related to photosynthesis, stress, cell

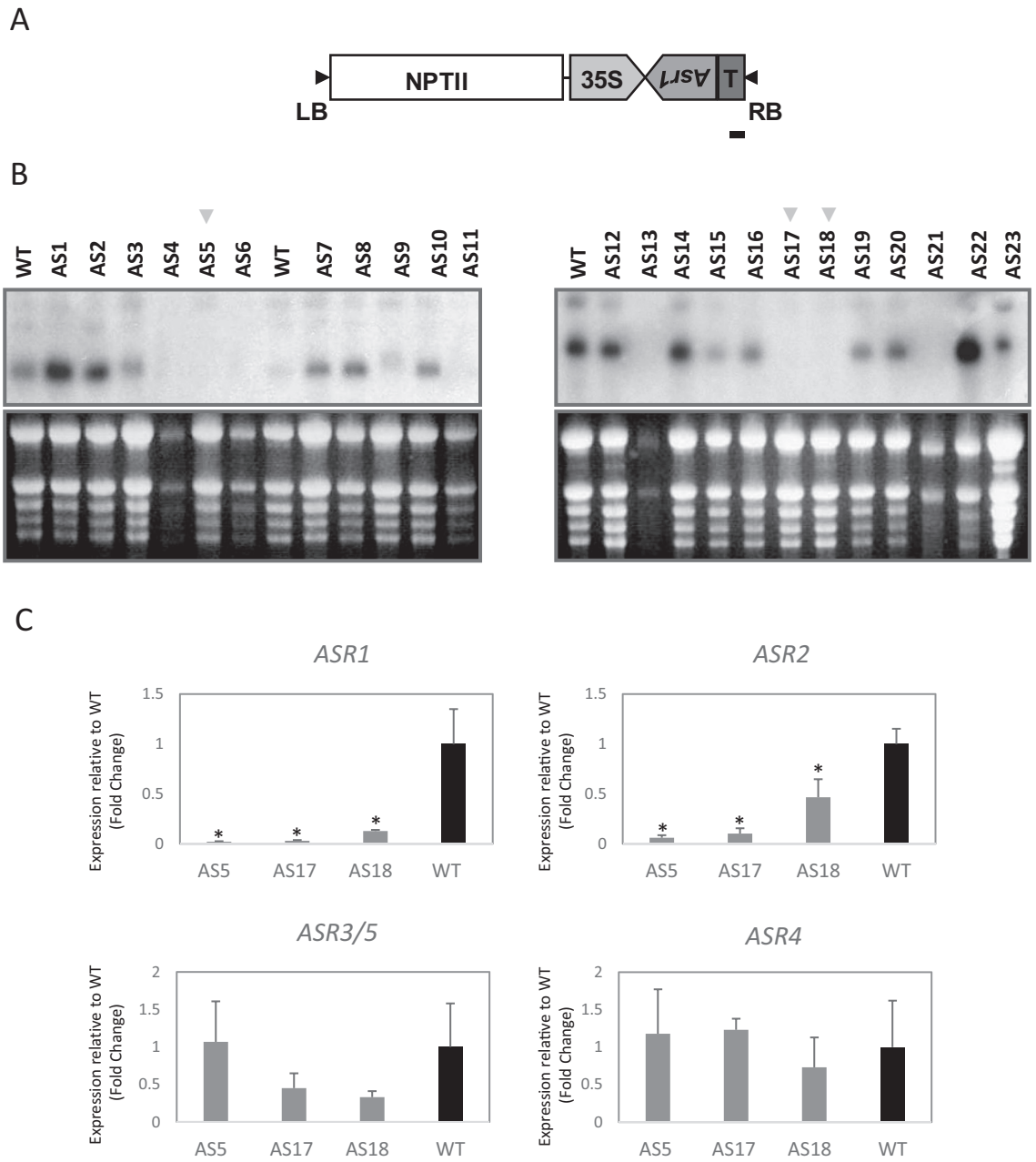

Fig. 1. Selection of the *ASR1* antisense lines and *ASR1* expression. (A) Scheme of the construct used for plant transformation. The size bar indicates 100 bp. (B) *ASR1* expression in leaves of tomato *ASR1*-antisense (AS) T_0 transgenic lines and control lines (WT) by Northern blot analysis. In each panel the Northern blot (above) and a 1.5% mRNA agarose gel (below) are shown. The grey arrows indicate the AS lines selected for further analyses. (C) *ASR1* and its paralogs (*ASR2*, *ASR3/5* and *ASR4*) expression analysis by qRT–PCR in mature fruits of *ASR1*-antisense (AS) T_1 transgenic lines and control (WT) plants. Asterisks in the qRT–PCR measurements indicate statistically significant differences by the permutation test ($P < 0.05$). The error bars represent the SE (n=3–5).

wall metabolism and RNA (Fig. 2C). In addition, 36 and 56 genes were up-regulated only in AS5 or AS17, respectively (Fig. 2B; Supplementary Table S4). Although the identities of these genes were different, most of them belonged to the same functional categories (Fig. 2C). On the other hand, most of the 29 genes showing down-regulated expression in both AS lines belonged to the enzyme, cell wall metabolism and DNA categories (Fig. 2B, C; Supplementary Table S5). Thirty-five genes were significantly down-regulated (P<0.05) in only a single line (the number was the same in AS5 and in AS17), with these genes belonging to the enzyme, RNA and hormone categories (Fig. 2B, C; Supplementary Table S5).

Three functional categories showed significant enrichment in the differentially expressed genes (DEGs): photosynthesis

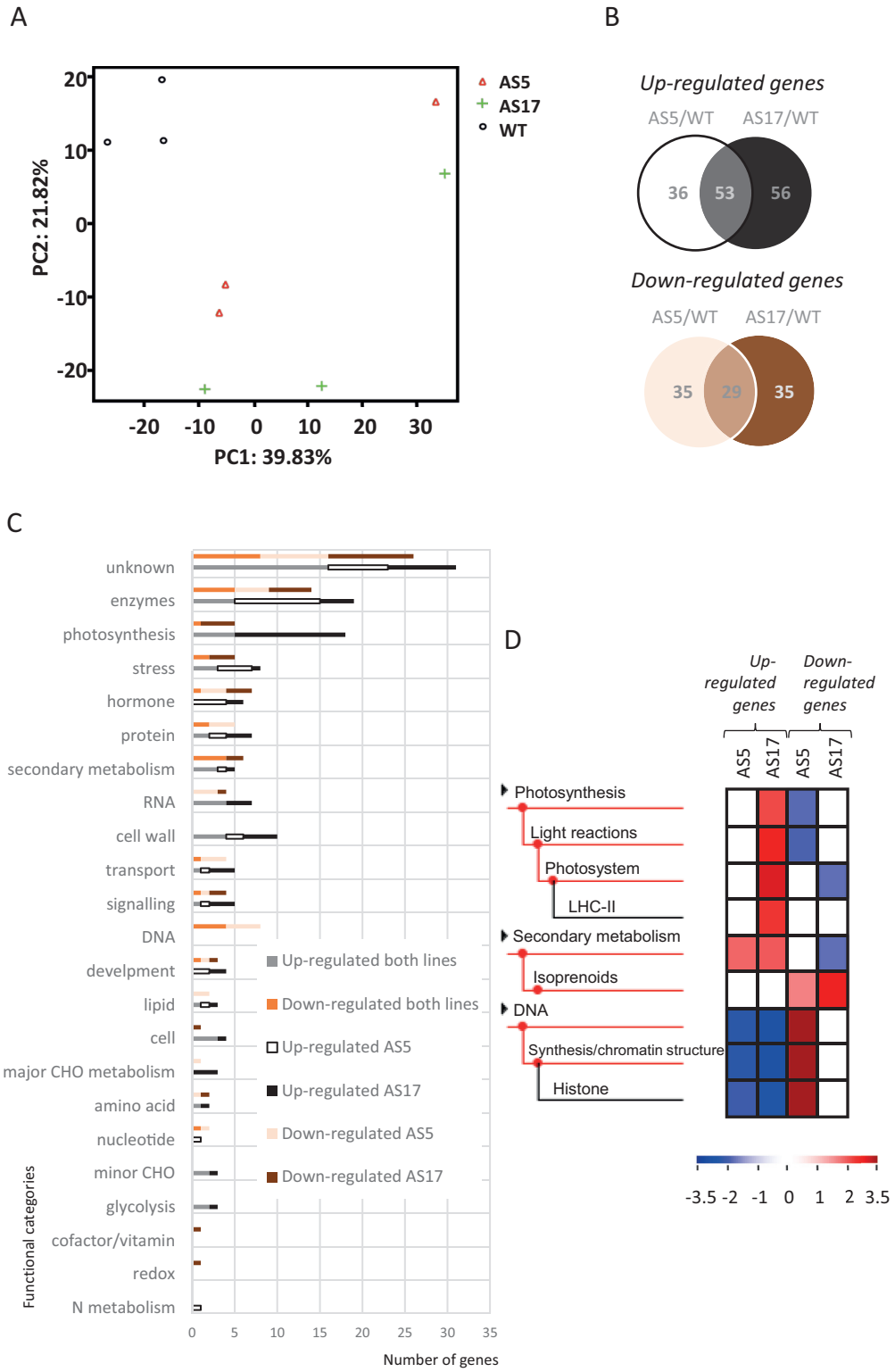

Fig. 2. Transcriptome analysis of the *ASR1*-antisense and WT mature tomato fruits. The analyses were performed with Chip Tomato Genome Arrays from Affymetrix. (A) Principal component analysis of the microarray standardized data of control plants (WT, shown as circles) and the *ASR1*-antisense lines, AS5 (shown as triangles) and AS17 (shown as plus signs). (B) Venn diagrams representing the number of transcripts differentially expressed in the antisense lines AS5 and AS17 in comparison to the control (WT) plants. Statistical test: Rank Product, $P < 0.05$ (n=3). (C) Number of up-regulated or down-regulated genes in the AS lines in comparison to control plants (WT) that belong to different MapMan functional categories. Grey: common up-regulated genes in both AS lines. White: up-regulated genes in line AS5. Black: up-regulated genes in line AS17. Medium orange: common down-regulated genes in both AS lines. Light orange: down-regulated genes in line AS5. Dark orange: down-regulated genes in line AS17. (D) Enrichment analysis of functional categories in the differentially expressed genes of the *ASR1*-antisense lines. The data were subjected to a Fischer´s exact test in PageMan (Usadel *et al.*, 2006). The graph shows the over- and underrepresented pathways coloured in red and blue, respectively. Cut-off: 1.

(light reactions, photosystems and light-harvesting complex II -LHC II), secondary metabolism (isoprenoids), and DNA (synthesis/chromatin structure and histones)-related genes were over- or under-represented either in the up- or down-regulated pools (Fig. 2D). Interestingly, while photosynthesis and secondary metabolism-related genes were over-represented in the up-regulated pool, DNA-related genes were under-represented. The opposite was true in the case of down-regulated genes.

To gain further insight into the structure of the microarray data, we constructed a correlation network of the DEGs followed by a search of the most densely connected regions. Genes belonging to these highly dense regions could have a higher relationship among them, e.g. higher co-regulation. In total, four highly interconnected regions or clusters were detected in the network (Supplementary Fig. S2; Table S7). Cluster 1 included genes mainly related to photosynthesis, hormone metabolism and transcription regulation, e.g. *ONE-HELIX PROTEIN 2 (OHP2)*, *ETHYLENE FORMING ENZYME (EFE)*, and *C-REPEAT-BINDING FACTOR 4 (CBF4/DREB1D)*. Cluster 2 included genes mainly related to biotic stress (*PATHOGENESIS-RELATED 3* and *4, PR3* and *PR4*), hormone metabolism (*ETHYLENE RESPONSE FACTOR 1, ERF1*) and protein degradation (*PEPTIDASE M48*). Cluster 3 included genes mainly related to cell wall, DNA and amino acid metabolism, e.g. *EXPANSIN A15* (*EXPA15*), *HISTONE H3.2* and *PROLINE OXIDASE*. Cluster 4 included genes mainly related to enzymatic reactions, e.g. *CYTOCHROME P450* (*CYP707A1*), *UDP-GLYCOSYLTRANSFERASE* and *FARNESYL DIPHOSPHATE SYNTHASE 2* (*FPS2*).

Altogether, these results demonstrate an important role for ASR1 in the transcriptional regulation of genes belonging to different functional categories such as photosynthesis, cell wall and DNA metabolism/organization/regulation, and stress. Moreover, results from the enrichment analyses (Fig. 2D) suggest that photosynthetic, isoprenoid and DNA-related genes appear to display an ASR1-dependent coordinated regulation.

ASR1 is involved in amino acid and isoprenoid metabolism in mature fruits

Given that the role of ASR1 in central metabolism has been established in several species (reviewed by Dominguez and Carrari, 2015), the metabolite profile of mature fruits was next analysed by GC-MS (Fig. 3; Supplementary Table S8). A hierarchical clustering analysis of these data showed that the WT fruits cluster apart from the *ASR1*-antisense ones (shown in the columns of the heatmap in Fig. 3). Metabolite clustering analysis showed five groups according to their fold-change levels (shown in the rows of the heatmap in Fig. 3), with a clear predominance of amino acids in the groups with the largest changes. Clusters a, b, and c included the amino acids 4-hydroxyproline, methionine, valine, isoleucine, and leucine, which were increased in all the AS lines. Branched chain amino acids (BCAA; leucine, isoleucine, and valine) have already been linked to altered ASR expression in maize kernels (Virlouvet *et al.*, 2011). Cluster d only included one metabolite, succinate, which was decreased in the three AS lines. Cluster e comprised most of the identified metabolites, but only two were significantly changed ($P<0.05$); citrate, an intermediate of the Krebs cycle, was increased in all the AS lines, while glycerol, associated with lipid metabolism, was reduced in lines AS17 and AS18. This cluster also contained the only aromatic amino acid that could be detected, phenylalanine, whose concentration remained unchanged in the AS lines.

Notably, glucose and sucrose were not altered as measured by the enzymatic method (Supplementary Table S9). This suggests that ASR1 might not be involved in sugar metabolism in tomato fruits, at least under the conditions applied in our experimental set-up. This is different from the results published in other species such as *Vitis* (Çakir *et al.*, 2003), potato (Frankel *et al.*, 2007) and tobacco (Dominguez *et al.*, 2013). Thus, it would appear that ASR1 mainly regulates amino acid metabolism in tomato fruits.

The enrichment of the transcriptome of the *ASR1*-antisense fruits in isoprenoid pathway genes (Fig. 2D) prompted us to measure the most abundant isoprenoids of the tomato fruits, β-carotene, and lycopene (Klee and Giovannoni, 2011). While the former was decreased in lines AS5 and AS17, the latter was significantly decreased ($P<0.05$) in line AS5 (Table 1).

Integrated transcriptome-metabolome analysis shows that ASR1 regulates cell wall-related transcripts and amino acid content

To know whether the altered transcripts and metabolites in the mature fruits of the *ASR1*-antisense plants belong to the same pathways, the averaged data of transcripts (Fig. 2; Supplementary Tables S4 and 5) and metabolites (Fig. 3; Table 1; Supplementary Table S8) of lines AS5 and AS17 were graphically displayed by means of MapMan (Thimm *et al.*, 2004; Fig. 4). The graphs show transcripts that were significantly changed ($P<0.05$) in at least one line, and the metabolites that were significantly changed ($P<0.05$) in both lines on an overview map of the metabolism (Fig. 4A) and on a secondary metabolism map (Fig. 4B).

Given that fruit ripening is tightly linked to cell wall metabolism (Forlani *et al.*, 2019) and since ASR1 directly regulates some cell wall-related genes in tomato leaves (Ricardi *et al.*, 2014), we further focused our attention on simultaneous alterations in transcriptional and metabolic pathways associated with cell walls (Fig. 4A). This functional category showed several altered transcripts, including a cellulose synthase-like G1, two expansins (*EXPANSIN A15*- found in cluster 3 in the gene network, Supplementary Fig. S2- and *EXPANSIN A3*) and a pectate lyase (*PL*)-encoding gene. Increases in the transcript levels of the expansins and *PL*, involved in cell wall loosing (Marowa *et al.*, 2016; Forlani *et al.*, 2019; T. Zhang *et al.*,

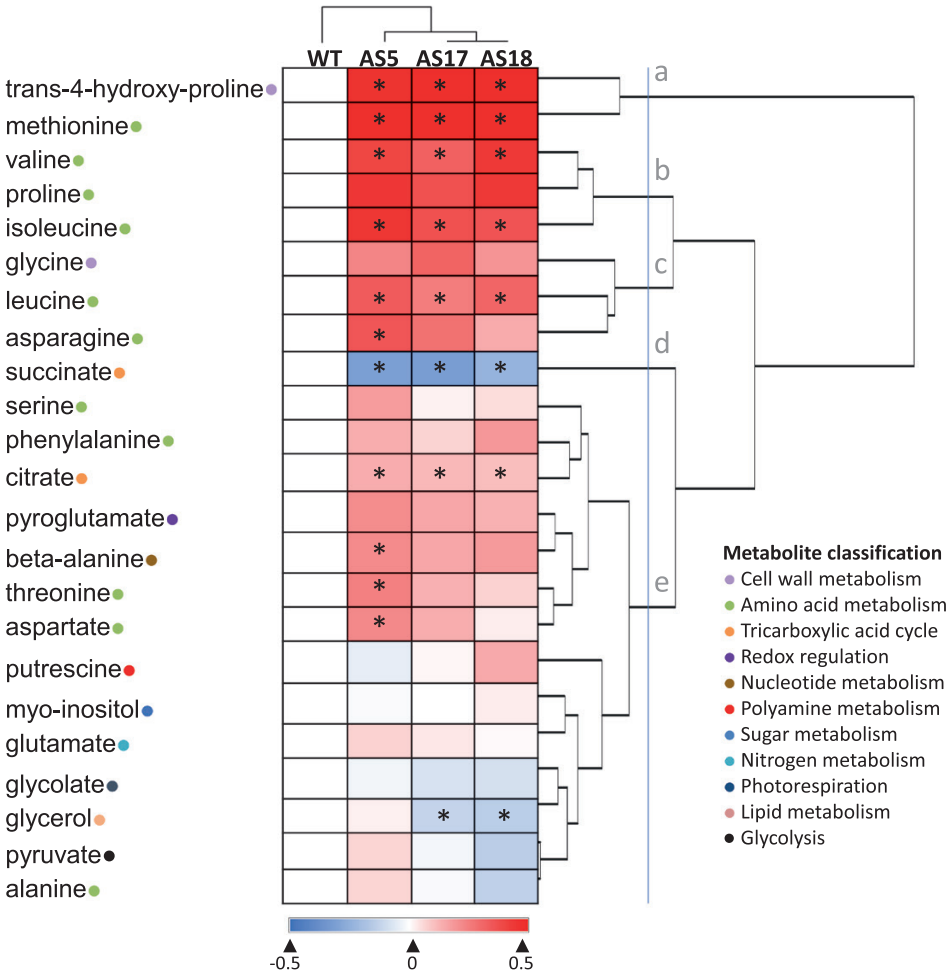

Fig. 3. Metabolite content of the *ASR1*-antisense and WT mature fruits measured by GC-MS. The heatmap represents the logarithmically transformed ratio of the metabolites in mature fruits of *ASR1*-antisense lines in comparison to control plants (WT). A two-dimension hierarchical clustering of the metabolite fold-change levels shows the treatment separation (columns) and the variable separation (rows). Method of the clustering: average linkage; data standardization: Euclidean distance; the vertical blue line indicates the selected cut-off for the variable separation, and the grey letters indicate the different clusters. The colour of the circles represents the MapMan classification of the metabolites. Asterisks represent significant differences between transgenic lines and control plants by ANOVA (Duncan; *P*-value< 0.05; *n*=6).

Table 1. Beta-carotene and lycopene content in *ASR1*-antisense mature tomato fruits relative to WT fruits measured by spectrophotometry.

	β-carotene	Lycopene
AS5	**0.43±0.01**	**0.4±0.01**
AS17	**0.48±0.09**	0.59±0.03
WT	1±0.11	1±0.17

The values represent the average ± SD. Bold letters indicate statistically significant differences by ANOVA (Duncan test, $P < 0.05$; n=3–4).

2019c) and fruit softening (Uluisik *et al.*, 2016; Uluisik and Seymour, 2020), respectively, were further confirmed by qRT–PCR in an independent experiment (Fig. 5A; Supplementary Table S10). Along with these changes in transcript levels, alterations in 4-hydroxyproline, an amino acid abundantly found in cell walls containing hydroxyproline-rich O-glycoproteins (HRGPs; Kavi Kishor *et al.*, 2015), were also detected (Figs 3; 4A). Moreover, expression of a cell wall signalling gene (Navarro *et al.*, 2004), belonging to the leucine-rich repeat (LRR) family, was also increased in the AS lines (Fig. 5A; Supplementary Table S10). Altogether, these results suggest that ASR1 has an impact on fruit cell wall metabolism through its effect on the transcript levels of the above-mentioned genes.

The rest of the significantly changed metabolites, which belong to the amino acid metabolism pathway (methionine, leucine, isoleucine, and valine) and the Krebs cycle metabolism (citrate, and succinate), were not accompanied by changes in the transcripts that regulate their synthesis or degradation (Fig. 4A). Interestingly, this suggests that the alterations of these metabolites in the AS lines originated at a level other than transcriptional regulation (i.e. protein and/or metabolic control levels).

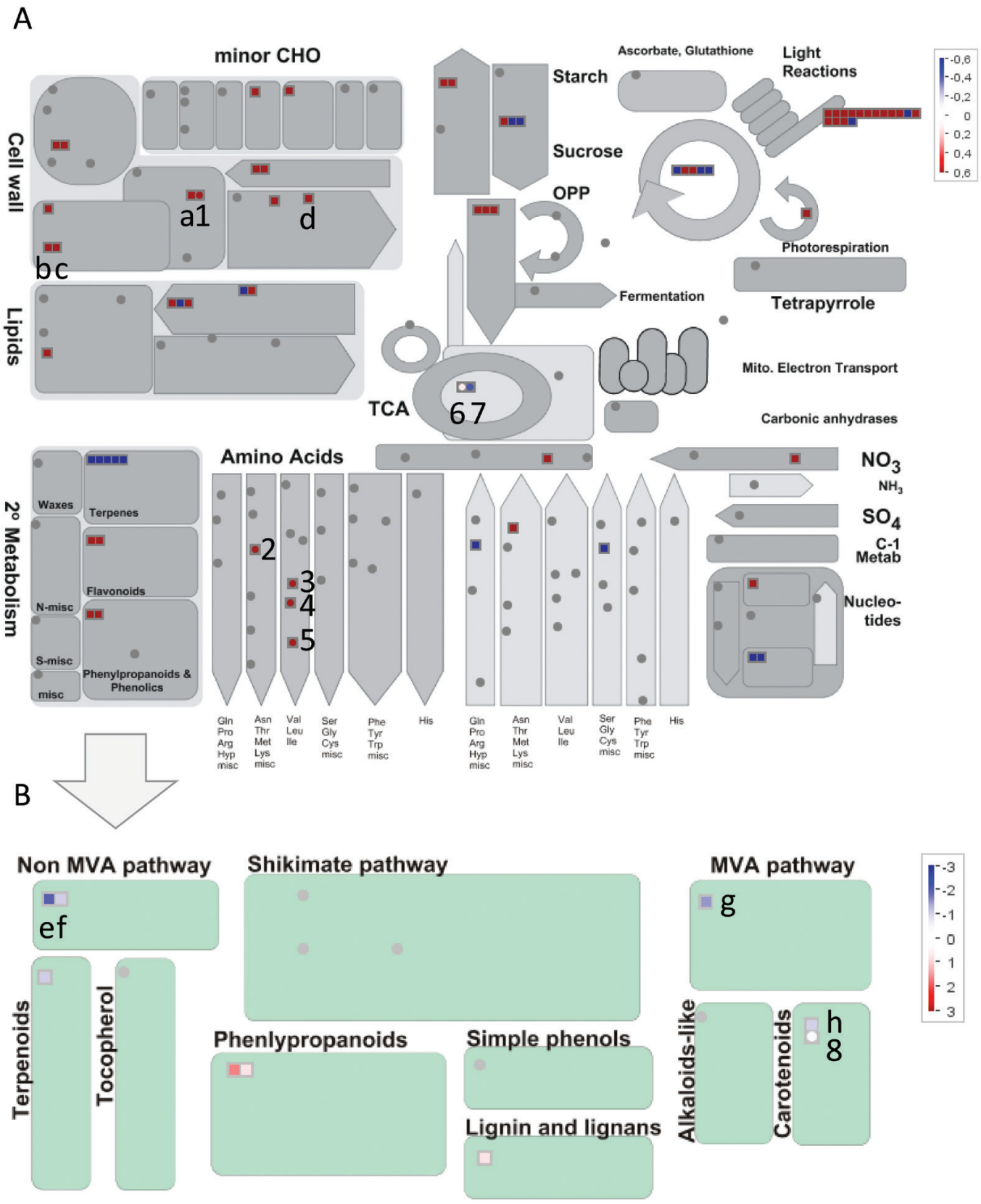

Fig. 4. Mapping onto a metabolism overview map (A) and a secondary metabolism map (B) of the relative amounts of transcripts and metabolites of the *ASR1*-antisense lines in comparison to control (WT) plants. The mapping of the averaged data of Figs 2, 3 and Table 1 for lines AS5 and AS17 was done with MapMan (Thimm *et al.*, 2004). Transcripts are represented with squares and metabolites with circles. Mapped transcripts (66 in A and

The reduced concentration of the isoprenoid compound β-carotene in lines AS5 and AS17 (Table 1) was accompanied by alterations in the expression of genes encoding GERANYLGERANYL REDUCTASE (*GGDR*), SOLANESYL DIPHOSPHATE SYNTHASE 1 (*SPS1*), FARNESYL DIPHOSPHATE SYNTHASE 2 (*FPS2*) and a FAD/NAD(P)- BINDING OXIDOREDUCTASE (Fig. 4B; Supplementary Table S5). The first three genes constitute important regulatory points of the pigment biosynthetic pathway (Quadrana *et al.*, 2013; Jones *et al.*, 2013). Besides, *FPS2* and the *FAD/NAD(P)-BINDING OXIDOREDUCTASE* were found in clusters 1 and 4, respectively, of the gene network (Supplementary Fig. S2; Supplementary Table S7).

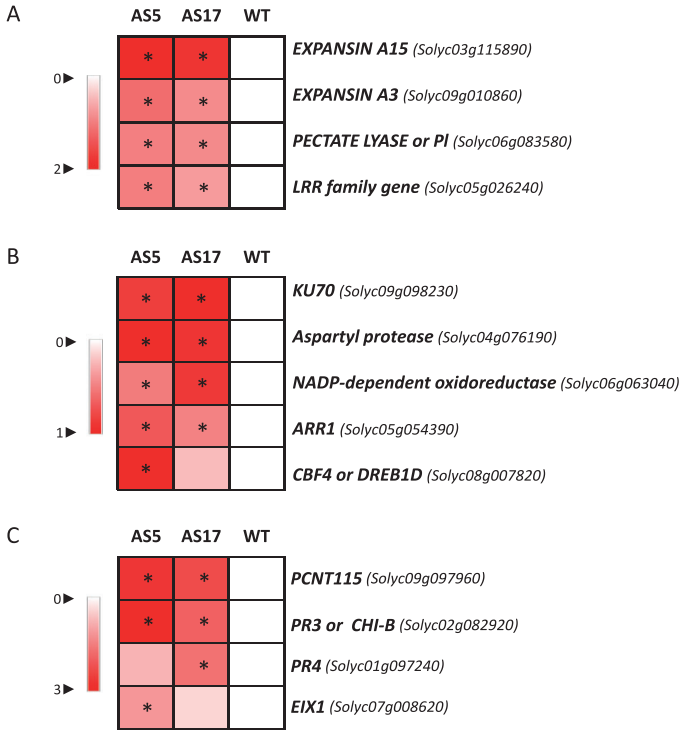

Fig. 5. Effect of ASR1 on cell wall and stress-related gene expression in mature tomato fruits. The heatmaps represent the mRNA level ratios in logarithmic scale of *ASR1*-antisense lines (AS) in comparison to control plants (WT) measured by qRT–PCR in an experiment independent from the microarray experiment. (A) Cell wall related genes. (B) Oxidative stress and abiotic stress related genes. (C) Biotic stress related genes. Asterisks indicate statistically significant differences by the permutation test ($P < 0.05$; n=3–5).

ASR1 modulates abiotic and biotic stress-related gene expression in mature tomato fruits

The cell wall structure and integrity are closely connected to environmental stress responses since cell walls contribute to the maintenance of the cell turgor pressure (Kesten *et al.*, 2017). Thus, we validated the expression of some of the oxidative and abiotic stress-related genes that were significantly altered (P<0.05) in the microarray experiment (Fig. 5B; Supplementary Table S10). The expression of a gene encoding a NADP-dependent oxidoreductase, which is involved in oxidative stress tolerance (Babiychuk *et al.*, 1995; Mano *et al.*, 2005), was increased in the AS lines (Fig. 5B). *KU70*, which encodes a subunit of the heterodimeric protein KU involved in several cellular processes including ABA-mediated heat responses (Liu *et al.*, 2008), was also increased in expression in both AS lines. Expression of the gene encoding ARABIDOPSIS RESPONSE REGULATOR 1 *(ARR1),* a regulator of the cytokinin-mediated drought response (Huang *et al.*, 2018), was increased in the AS lines. An aspartyl proteinase, whose expression is altered during oxidative stress (Giraud *et al.*, 2008), was also increased in the AS lines. The gene encoding the transcription factor C-REPEAT-BINDING FACTOR 4 *(CBF4)*, involved in the response to drought (Ding *et al.*, 2013; Guttikonda *et al.*, 2014) and present in cluster 1 in the gene network (Supplementary Fig. S2; Supplementary Table S7), was increased in expression in line AS5.

Because cell walls are also involved in the biotic stress response acting as the first barrier against pathogens (Kesten *et al.*, 2017) and because abiotic and biotic stress pathways are interconnected (Ali *et al.*, 2018; Ku *et al.*, 2018), we measured some biotic stress-related genes as well (Fig. 5C; Supplementary Table S10). Several defence-related genes were altered in the microarrays (Supplementary Table S11). PATHOGENESIS RELATED 3 (*PR3)* and PATHOGENESIS RELATED 4 (*PR4)* were found in cluster 2 in the gene network (Supplementary Fig. S2; Supplementary Table S7). *PR3* had increased expression in both lines while *PR4* had increased expression in line AS17 as measured by qRT–PCR (Fig. 5C). Both are chitinases (enzymes that hydrolyse glycosidic bonds in chitin, a component of fungal cell walls) involved in the defence against necrotrophic pathogens (De la Cruz *et al.*, 1992; Dai *et al.*, 2016). Besides, these two genes are induced by jasmonate and are considered as jasmonate signature genes (Ali et al., 2017; 2018). *PCNT115* encodes a NAD(P) dependent oxidoreductase altered during viral infections (Ascencio-Ibáñez *et al.*, 2008), which was increased in lines AS5 and ASR17.

11 in B) are statistically significant (P<0.05) in at least one of the two lines. Mapped metabolites (seven in A and one in B) are statistically significant in both lines (P<0.05). The scales on the top right of each panel represent the logarithmically transformed fold-changes of the transcripts or metabolites of *ASR1*-antisense lines in comparison to WT plants. Metabolites identified with numbers – 1: 4-hydroxyproline; 2: methionine; 3: leucine; 4: isoleucine; 5: valine; 6: citrate; 7: succinate; 8: β-carotene. Transcripts identified with letters – a: cellulose synthase-like G1 (Solyc00g030000); b: expansin A15 (Solyc03g115890); c: expansin A3 (Solyc09g010860); d: pectate lyase (Solyc06g083580); e: geranylgeranyl reductase (Solyc03g115980); f: solanesyl diphosphate synthase (Solyc12g015860); g: farnesyl diphosphate synthase (Solyc07g061990); h: FAD/NAD(P)-binding oxidoreductase (Solyc05g010180).

Finally, *ETHYLENE-INDUCING XYLANASE 1* (*EIX1*), a gene related to biotrophic infections (Hok *et al.*, 2011) and found in cluster 4 in the gene network (Supplementary Fig. S2; Supplementary Table S7), showed increased expression in line AS17.

The expression of the genes measured by qRT–PCR shows the same trend as in the microarrays (Supplementary Tables S4; S5). The results clearly indicate that there is an alteration at the transcript level in the cell wall metabolism, and in the abiotic and biotic stress pathways in the *ASR1*-antisense tomato fruits. This is in accordance with previous findings in tomato leaves (Ricardi *et al.*, 2014), in tomato fruits (Jia *et al.*, 2016) and at the whole tomato plant level (Golan *et al.*, 2014).

ASR1-*antisense fruits are more susceptible to* Botrytis cinerea *infection*

The alterations detected in *PR3* and *PR4* expression (Fig. 5C), which are induced by the defence hormone jasmonate (Ali *et al.*, 2018), considered together with the alterations in expansins and pectate lyase (Fig. 5A) that contribute to the maintenance of the cell wall structure, point to an alteration in the susceptibility towards pathogens that are necrotrophic and profit from altered cell walls such as *B. cinerea* (Cantu *et al.*, 2009; Mbengue *et al.*, 2016). This pathogen is the causal agent of the grey mould disease, an important constraint for tomato production (Blanco-Ulate *et al.*, 2015). To test this hypothesis, we inoculated detached ripe fruits from the AS and WT plants with low content spore suspensions of this fungus. The relative lesion area upon infection with *B. cinerea* was significantly ($P<0.05$) larger in the AS17 and AS18 lines in comparison with the WT fruits (Fig. 6), clearly indicating that the silencing of *ASR1* resulted in fruits more susceptible to *Botrytis* infection.

Potential upstream regulators of ASR1

The transcriptional and metabolic alterations in the *ASR1*-antisense lines indicate that ASR1 is involved in modulating traits important for fruit quality in ripe fruits. Thus, in order to position ASR1 in the regulatory cascade of this ripening stage, we searched the literature for published experimental data on known fruit ripening regulators that could allow us to propose a potential link between those regulators and ASR1. We searched for the presence/absence of *ASR1* in ChIPseq experimental outputs and for changes in *ASR1* expression in transcriptomics analyses of transgenics/mutants for several regulators, including RIPENING INHIBITOR (RIN), FRUITFULL1/FRUITFULL2 (FUL1/FUL2), NON-RIPENING (NOR), COLORLESS NON-RIPENING (CNR), AGAMOUS-LIKE1 (TAGL1), PYROBACTIN RESISTANCE-LIKE 9 (PYL9), and PROTEIN PHOSPHATASE 2C3 (PP2C3; Supplementary Table S12). Transcriptional analyses performed on *rin* mutants and ChIPseq studies suggest that ASR1 is not directly regulated by RIN (Fujisawa *et al.*, 2012; 2014; Supplementary Table S12). On the contrary, RNAseq studies on *FUL1/FUL2*-suppressed

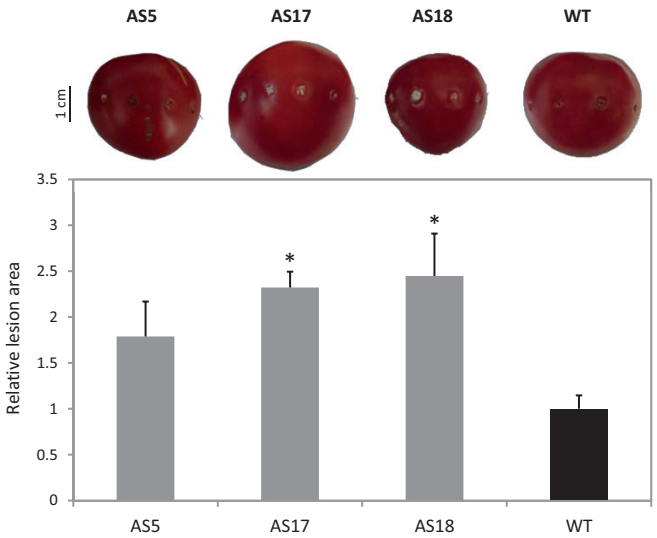

Fig. 6. Susceptibility of *ASR1*-antisense tomato fruits to *Botrytis cinerea*. Five detached tomatoes of each line were inoculated with 5 µl of a *B. cinerea* spore suspension (4×10^{-6} conidia ml^{-1}) on four wounds produced on the fruit surface. Bars represent the means of the diameters of the infection wounds relative to the WT fruits 96 h after the inoculation. The error bars represent the SE. Asterisks indicate statistically significant differences by ANOVA (Duncan test, $P < 0.05$; n=5).

lines showed decreased *ASR1* expression, while a ChIPseq experiment showed that *ASR1* could be a direct target of FUL1/FUL2 (Fujisawa *et al.*, 2014; Supplementary Table S12). These data imply that FUL1/2 could directly regulate *ASR1* in a RIN-independent manner. Furthermore, *ASR1* is down-regulated in *NOR*-silenced fruits, while it is up-regulated in *NOR* overexpressing tomatoes (Gao *et al.*, 2020; Supplementary Table S12), suggesting that NOR could also be involved in the regulation of *ASR1*. A transcriptomics analysis of a *CNR* knocked-out line also showed reduced *ASR1* expression (Gao *et al.*, 2019; Supplementary Table S12). On the contrary, studies related to other regulators like TAGL1 and PYL9 suggest that these proteins do not regulate ASR1 (Karlova *et al.*, 2011; Kai *et al.*, 2019) (Supplementary Table S12). The silencing of the ABA-related *PP2C3* in tomato fruit skin led to reduced expression of *ASR1* (Liang *et al.*, 2021; Supplementary Table S12), suggesting that *ASR1* could also be regulated by the ABA signalling pathway in fruits. None of these regulators were altered in our microarray analyses (Supplementary Tables S4; S5; data in GEO accession number GSE163738), except for CNR (Supplementary Table S5; probe 47). However, as they act in the earliest steps of the fruit ripening cascade (Karlova *et al.*, 2014), it is reasonable to assume that ASR1 acts downstream of these factors and does not directly regulate them.

Discussion

Transcriptomic and metabolic analyses performed in fruits from *ASR1*-antisense tomato plants allowed us to uncover the pathways in which this protein is involved. Enrichment

in photosynthesis, DNA and secondary metabolism categories was found in the DEGs (Fig. 2D). In line with these results, through ChIP-seq experiments we previously found that photosynthesis-related genes like *CHLOROPHYLL A-B BINDING PROTEIN* (Solyc07g063600) and *PHOTOSYSTEM II 5 KDA PROTEIN* (Solyc12g099650) are potential direct targets of ASR1 in tomato leaves (Dataset 2 in Ricardi *et al.*, 2014). Although the role of photosynthetic genes in mature fruits is not completely clear (Lytovchenko *et al.*, 2011), it seems likely that the regulatory role exerted by ASR1 on the photosynthetic pathway is conserved in both leaves and fruits. DNA-related genes (specifically histones) were also enriched in the DEGs of the *ASR1*-antisense fruits (Fig. 2D). This could be a consequence of either a direct or indirect regulation of histones by ASR1, although they were not found as targets in the above-mentioned ChIP-seq experiments (Ricardi *et al.*, 2014). However, considering that chromatin features related to histones can be altered by environmental stressors (Asensi-Fabado *et al.*, 2017), the decreased *ASR1* expression could result in a stressful condition for the fruits, and subsequently lead to histone alterations, given the role for ASR1 in stress (González and Iusem, 2014). The third enriched functional category was that involving isoprenoid pathway genes (Fig. 2D), which included *GGDR*, *SPS*, and *FPS* (Fig. 4B; Supplementary Table S5). The down-regulation of these genes in the AS lines caused significant reductions ($P<0.05$) in the fruit β-carotene concentrations (Table 1). This could be due to potential alteration of the isoprenoid geranylgeranyl diphosphate (GGPP), the main precursor for the carotenoid biosynthetic pathway in the chloroplasts (Lichtenthaler, 1999). GGPP also acts as a substrate for GGDR (Tanaka *et al.*, 1999) and SPS (Hirooka *et al.*, 2005), while it is one of the products of catabolism of farnesyl diphosphate, which in turn is produced by FPS (Takahashi and Ogura, 1981). Besides, it is believed that the transcriptional regulation of GGDR impacts directly on carotenoid accumulation in tomato fruits (Enfissi *et al.*, 2010; Quadrana *et al.*, 2013). Beyond the important role of fruit β-carotene on the fruit ripening program, it is a precursor for the synthesis of the tomato volatile compound β-ionone, which contributes to fruit flavour (Klee and Giovannoni, 2011). The volatile profile of *ASR1*-overexpressing tomato fruits differs from that of WT (Messina *et al.*, 2012), suggesting that ASR1 could impact fruit flavour through its effect on the isoprenoid pathway.

Our results also indicate that the main primary metabolic pathway in which ASR1 is involved in tomato fruits is the amino acid pathway (Fig. 3). A relationship between ASR and amino acid metabolism has been previously observed in potato (Frankel *et al.*, 2007), and maize (Virlouvet *et al.*, 2011). Branched chain amino acids are also precursors of tomato fruit volatiles (Klee and Giovannoni, 2011). This adds new evidence suggesting that ASR1 could be involved in shaping the fruit volatile metabolite profile. The lack of transcriptional changes in the amino acid pathway (Fig. 4A) suggests that the effect is taking place at the protein level. Similarly, Virlouvet *et al.* (2011) found decreased amounts of branched chain amino acids in *ASR1*-overexpressing maize leaves, that were explained by the decreased protein amounts of two isopropylmalate dehydrogenases (ZmIPMDH1 and ZmIPMDH2), whose transcript levels were not altered. These authors propose that the alterations in the amino acids could be produced by the ASR chaperone-like activity acting on different redox-regulated proteins.

In contrast to the results that show the involvement of ASR1 in the amino acid pathway, we have found no evidence either at the transcriptional or metabolic level that ASR1 is involved in sugar metabolism or transport in tomato fruits (Fig. 2, Supplementary Table S9). Jia *et al.* (2016) found that *Sl*ASR could trans-activate the expression of a hexose transporter gene in tomato fruits, although they did not show whether *ASR1*-altered fruits had altered hexose concentrations. Despite these results, our ChIP-seq experimental data do not support a direct regulation of these types of genes by ASR1 (Ricardi *et al.*, 2014). In this sense, the role of ASR1 in tomato fruit sugar metabolism differs from what is described for some other *Solanaceae* species like potato tubers (Frankel *et al.*, 2007) and tobacco leaves (Dominguez *et al.*, 2013), suggesting that the effect of ASR on sugar metabolism is tissue- and species-specific. However, in accordance with Jia *et al.* (2016), our results support a role for ASR1 in tomato fruit cell wall metabolism (Figs 2; 3; 4A; 5A). Direct regulation by ASR1 of genes involved in leaf cell wall degradation (e.g. pectin esterases, beta-1,3-glucano hydrolases) and cell wall formation (e.g. cellulose synthase) was also found in our previous work (Ricardi *et al.*, 2014). This suggests that ASR1 has a dual role in the cell wall metabolism (degradation and formation) and that its final effect on cell walls would depend on several signals regulating ASR1. Moreover, a relationship between carotenoids and cell wall formation has been described in tomato fruits (Diretto *et al.*, 2020). Considered altogether, these studies suggest that the involvement of ASR1 in the regulation of tomato fruit cell walls could influence the cell wall remodelling processes that occur during fruit ripening (Shinozaki *et al.*, 2018).

ASR1 is also extensively known to be involved in abiotic stress responses in several tissues and species (reviewed by González and Iusem, 2014). Thus, not surprisingly, our data show that several genes belonging to the abiotic stress pathway are altered in *ASR1*-antisense tomato fruits (Figs 2; 5B; Supplementary Tables S4; S5). ASR2 could be contributing to the effect since its expression is also altered in the fruits (Fig. 1C) as well as under stress conditions and by ABA treatment (Maskin *et al.*, 2001; Golan *et al.*, 2014). Ricardi *et al.* (2014) found that drought-related genes, like aquaporins, are direct targets of ASR1 in tomato leaves. During the development and ripening of fruits a high number of genes described as being involved in the stress response, are expressed as part of the fruit ripening program; these include *HEAT-SHOCK*

COGNATE 70 (*HSC70), CATALASE* (Zegzouti *et al.*, 1999), *HEAT SHOCK PROTEIN 21* (*HSP21;* Neta-Sharir *et al.*, 2005), *SUPEROXIDE DISMUTASE* and *LATE EMBRYOGENESIS ABUNDANT-like (LEA-like*) genes (Srivastava *et al.*, 2010). This suggests that the effect of ASR1 on the stress pathway could also contribute to tomato fruit development. It is worth noting that ASR1 has a positive role during abiotic stress, improving the response of the plants to the stressors (González and Iusem, 2014). However, the effect of ASR1 on abiotic stress-related genes in the context of fruit ripening could be different from its effect on abiotic stress responses, considering that the measured transcripts were increased in the AS lines (Fig. 5B). The continuous generation of reactive oxygen species (ROS) during fruit maturation requires the expression of antioxidant enzymes (Decros *et al.*, 2019). Indeed, while some oxidoreductases were altered in the transgenic lines (Fig. 5B; Supplementary Tables S4; S5), several other studies support the role of ASR during oxidative stress (Kim *et al.*, 2012; Feng *et al.*, 2016; L. Wang *et al.*, 2016).

The results presented in this work show that silencing *ASR1* leads to reduced tolerance to the necrotrophic pathogen *Botrytis cinerea* in tomato fruits (Fig. 6). This is the first time that the relationship between ASR1 and fungal susceptibility has been clearly demonstrated, given that previous reports only indicated that *ASR1* expression increases upon fungal infection in plantain (Liu *et al.*, 2010), tomato (Jia *et al.*, 2016) and apple (Huang *et al.*, 2016). *PR3* and *PR4*, signature genes of the jasmonate pathway (Ali *et al.*, 2018), and other defence-related genes (Fig. 5C; Supplementary Table S11), were altered in the AS lines, indicating an alteration in the defence pathway. The susceptibility phenotype (Fig. 6) could not be explained by the expression fold-changes of PR3 and PR4, since they show increased expression (Fig. 5C). However, the ethylene-responsive genes *ETHYLENE RESPONSE FACTOR 1* (*ERF1)* was reduced in expression in the microarrays (Supplementary Table S11). ERF1 activity confers resistance to *B. cinerea* (Knoester *et al.*, 1998; Berrocal-Lobo *et al.*, 2002); thus, its reduced expression in the AS lines could be contributing to the observed susceptible phenotype. It is also possible that unknown defence-related direct targets of ASR1 exist. Our previous work found two potential direct targets of ASR1 related to defence in tomato leaves, a disease resistance gene (*R* gene; Solyc05g008070) and a glucan endo-1,3-beta-glucosidase (Solyc05g054440), involved in fungal defence (Dataset 2 in Ricardi *et al.*, 2014). This suggests that ASR1 could potentially act as a transcription factor regulating defence-related genes. In line with this, ASR2 was found to regulate a rice defence-related gene called *Os2H16s* in leaves (Li *et al.*, 2018). Since *ASR2* expression is reduced in the analysed fruits (Fig. 1C), this suggests that it could contribute to the control of defence-related transcripts as well. However, the fact that cell wall-related genes are altered in the fruits (Figs 2; 5A) and that cell wall synthesis and remodelling enzymes have been proven to be direct targets of ASR1 in tomato leaves (Ricardi *et al.*, 2014) suggest that the effect could be secondary to a direct role of ASR1 in cell walls. Moreover, red ripe tomato fruit susceptibility to *B. cinerea* is determined by susceptibility factors that outweigh the defence responses (Silva *et al.*, 2021). The most important susceptibility factors that exist in tomato fruits are cell wall degrading enzymes, especially pectate lyase (Silva *et al.*, 2021), but also polygalacturonase and expansins (Cantu *et al.*, 2008). Reduced expression of these cell wall-related genes has been linked to reduced susceptibility to *B. cinerea* (Cantu *et al.*, 2008; Silva *et al.*, 2021). This strongly suggests that the increased pectate lyase and expansin expression found in the AS lines (Fig. 5A) contribute significantly to the observed phenotype. Besides, an alteration in the cell wall structure usually triggers the immune response (Bacete *et al.*, 2018; Hou *et al.*, 2019; Gallego-Giraldo *et al.*, 2020). This could explain why *PR3* and *PR4* expression is increased in the antisense lines (Fig. 5C). Thus, the increased susceptibility of the fruits could be due to alterations in the cell walls and the decreased *ERF1* expression, despite the possibly activated jasmonate pathway. Interestingly, *nor* and *cnr* mutants are differentially affected by *B. cinerea*, which indicates that *B. cinerea* relies on certain ripening pathways to establish infection (Cantu *et al.*, 2009; Silva *et al.*, 2021). The fact that ASR1 could participate in the NOR and CNR cascades (Supplementary Table S12) together with the increased susceptibility to this pathogen shown by the AS lines (Fig. 6) might indicate that *ASR1* would be involved in the regulation of the host conditions that are involved in the outcome of the interaction between the fruit and *B. cinerea*. Although *B. cinerea* is known for promoting the ripening process (Cantu *et al.*, 2009), more studies are needed to understand whether *B. cinerea* profits from the *ASR1* suppression to accelerate the ripening process. On the other hand, crosstalk occurs between abiotic stress and biotic stress pathways (Fujita *et al.*, 2006; Rejeb *et al.*, 2014; Ali *et al.*, 2018). ABA, for instance, is a modulator of the drought and salinity stress responses, and of plant immunity (Pieterse *et al.*, 2012). This suggests that the biotic stress pathway alteration in the *ASR1*-antisense fruits could be due to the alterations in the abiotic stress pathway as well.

The results obtained in this work point to a role of ASR1 in the determination of tomato traits associated with fruit ripening. Thus, we constructed a model showing how ASR1 could be positioned in this process (Fig. 7). We incorporated into the model data on potential ASR1 upstream regulators (Supplementary Table S12) and ASR1 downstream pathways previously reported, along with the new information presented here.

Ethylene is the most relevant hormone acting in the climacteric fruit ripening process (Fenn and Giovannoni, 2021). Several important regulators of the fruit ripening process have been linked to this hormone (Karlova *et al.*, 2011), including FUL1, FUL2, CNR and NOR. The role of FUL1/FUL2

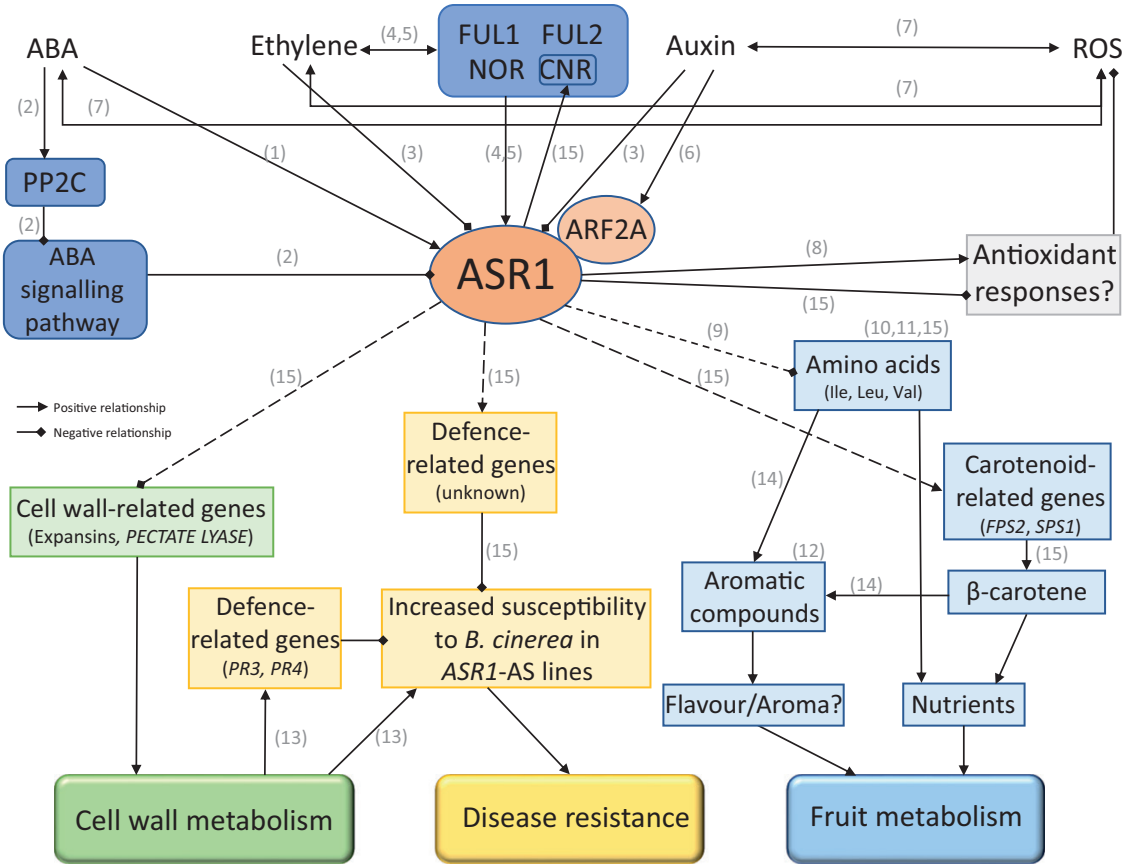

Fig. 7. Model of the pathways in which ASR1 is involved in red ripe tomato fruits. Thick dotted lines indicate potential direct regulation by ASR1. Fine dotted lines indicate potential regulation by ASR1 chaperone-like activity. The numbers indicate the studies from which this model was constructed. (1) Iusem *et al.* (1993) (2) Liang *et al.* (2021) (3) Jia *et al.* (2016) (4) Fujisawa *et al.* (2014) (5) Gao et al. (2019; 2020) (6) Breitel *et al.* (2016) (7) Kumar *et al.* (2016) (8) Kim *et al.* (2012) (9) Konrad and Bar-Zvi (2008) (10) Virlouvet *et al.* (2011) (11) Frankel *et al.* (2007) (12) Messina *et al.* (2012) (13) Bacete *et al.* (2018) (14) Klee and Giovannoni (2011) (15) Results from this study.

is associated with the general regulation of the carotenoid pathway (Fujisawa *et al.*, 2014). NOR has been associated with lycopene, cell wall and several other metabolic pathways (Gao *et al.*, 2019). CNR is related to carotenoid and cell wall regulation (Eriksson *et al.*, 2004; Manning *et al.*, 2006). Previous studies showed that tomato fruits with altered expression of *FUL1/2*, *CNR* or *NOR* have altered *ASR1* expression, and that FUL1/2 could be direct regulators of ASR1 (Fujisawa *et al.*, 2012; 2014; Gao *et al.*, 2019; 2020; Supplementary Table S12). These data, together with the fact that *FUL1/2* and *NOR* are not altered in the transcriptomics analyses of the *ASR1*-AS fruits (Supplementary Tables S4; S5), suggest that ASR1 acts downstream of FUL1/2 and NOR, and that it is activated by them (Fig. 7). On the contrary, *CNR* shows altered expression in the microarrays (Supplementary Table S5; probe 47), suggesting that CNR and ASR1 could have a feedback regulation. We propose that FUL1/2 and CNR partially regulate the carotenoid pathway through their action on *ASR1*. The effect of ASR1 on the cell wall pathway in this study (Fig. 5A) seems to be opposite to that of NOR and CNR (Eriksson *et al.*, 2004; Gao *et al.*, 2020), suggesting that ASR1 could be part of a regulatory or compensatory mechanism of the NOR and CNR action on cell walls. This idea is also strengthened by the fact that *ASR1* transcription seems to be inhibited by ethylene (Jia *et al.*, 2016), which in turn is a positive regulator of NOR and CNR (Fujisawa *et al.*, 2013).

ABA is positively associated with tomato fruit ripening and can stimulate ethylene emissions (Fenn and Giovannoni, 2021). ABA activates *ASR1*-controlled responses by direct interaction with abscisic acid-responsive element (ABRE) motifs in the promoter of *ASR1* (Iusem *et al.*, 1993; Hong *et al.*, 2002; Joo *et al.*, 2013; Fig. 7). Studies on the role of the ABA signalling pathway in tomato fruit ripening are limited, but silencing the negative ABA pathway regulator PP2C3, a phosphatase belonging to the group A of protein phosphatases 2C, in tomato fruit skin led to reduced expression of *ASR1* (Liang *et al.*, 2021; Supplementary Table S12). This suggests that *ASR1* expression could be inhibited by the ABA signalling pathway, as opposed to the effect of ABA on *ASR1* expression. The effect of ABA on cell walls (Fenn and Giovannoni, 2021) seems to be similar to the role of ASR1 observed in this study (Fig. 5A), which suggests that the effect of ABA on cell walls could be

explained, at least partly, by ASR1. However, when the level of *ASR1* silencing is higher than in the current study [e.g. through VIGS (Virus-induced Gene Silencing)], the effects on cell wall-related transcripts seem to be opposite (Jia *et al.*, 2016). This could mean that some of the effects of ASR1 depend on its relative abundance. In a wild-type tomato fruit different amounts of ASR1 could be a consequence of different hormonal ratios, implying that the final action of ASR1 is a consequence of the fine control exerted by the different hormones and their signalling pathways.

Crosstalk occurs between ethylene/ABA with the auxin pathway during the fruit ripening process (Fenn and Giovanonni, 2021). The AUXIN RESPONSE FACTOR 2A (ARF2A) is a component of the auxin signalling whose expression induces fruit ripening in an ethylene-dependent manner (Hao *et al.*, 2015; Breitel *et al.*, 2016). Breitel *et al.* (2016) proved that ARF2A can physically interact with ASR1 through two-hybrid and bimolecular fluorescence complementation (BiFC) assays (Fig. 7). The role of this association is not known, but the authors proposed that it could be related to the fine tuning of the sensitivity of the fruit tissue to hormones, especially ethylene. Alternatively, ARF2A could inhibit the action of ASR1 since ARF2A acts as a negative regulator. For its part, auxin treatment on tomato fruits inhibits *ASR1* transcription (Jia *et al.*, 2016), while it induces *ARF2A* transcription (Breitel *et al.*, 2016), suggesting that ASR1 and ARF2A co-regulate each other.

ROS are known signalling molecules that regulate several processes, including hormonal activity during fruit ripening (Kumar *et al.*, 2016). ASR1 could also be involved in this process through its antioxidant activity and its capacity to control antioxidant responses, since it has been described to be both positively (Kim *et al.*, 2012; Feng *et al.*, 2016; L. Wang *et al.*, 2016) and negatively (Fig. 5B) related to oxidative stress tolerance (Fig. 7). Although not explicit in Fig. 7, ROS are involved in other processes like pathogen susceptibility (Barna *et al.*, 2012), which suggests that ASR1 could also have an effect on ROS through its role in the oxidative stress pathway.

As discussed earlier, the role of ASR1 on cell walls, defence, abiotic stress, and metabolism could be performed by two mechanisms: as a transcription factor and as a chaperone-like protein (Konrad and Bar-Zvi, 2008; Kim *et al.*, 2012; Ricardi *et al.*, 2014; Wetzler *et al.*, 2018). The previous discussion on the ASR1 downstream pathways in tomato fruits is summarized in Fig. 7. ASR1 exerts both positive and negative regulation on the parameters associated with maturation under our experimental conditions: the effects on β-carotene suggest a positive regulation (Table 1), while the effects on genes associated with the cell wall (Fig. 5A), biotic stress (PR3 and PR4; Fig. 5C), and the BCAA content (Fig. 3) suggest a negative regulation. This could indicate that ASR1 is acting both as an activator and as a repressor, as has already been proposed for other transcription factors and regulators (Boyle and Després, 2010; Bonaccorso *et al.*, 2012; Feng and Lu, 2017). Potential ASR1 co-activators and co-repressors during fruit ripening are largely unknown, except for ARF2A (Breitel *et al.*, 2016). The final effect of ASR1 on a certain pathway probably depends on the hormone balance and the presence of other co-activators and co-repressors controlling its action at a given time, which would explain why ASR1 has been observed to have opposite effects on the same pathway.

To summarize, the role of hormones on the ASR1 pathway suggest a complex regulatory mechanism: while auxin and ethylene inhibit *ASR1* expression, ABA stimulates it. The ethylene-associated regulators (NOR, FUL1/2, CNR) appear to activate ASR1, unlike the ABA signalling pathway (PP2C3). Besides, ARF2A, with whom ASR1 physically interacts, is postulated as an auxin-related positive regulator of the ethylene pathway (Breitel *et al.*, 2016). Thus, the picture that emerges is that ASR1 is a transcription factor associated with hormonal crosstalk in fruit ripening, with possible implications in the ABA, ethylene and auxin pathways, which exerts effects on several ripening traits that affect fruit quality.

Supplementary data

The following supplementary data are available at *JXB* online.

Fig. S1. Unedited Northern blots shown in Fig. 1.

Fig. S2. Cluster finding in gene network constructed with DEGs in the AS lines.

Table S1. Primers employed in the qRT–PCRs.

Table S2. Choice of a reference gene for the qRT–PCRs with three different programs.

Table S3. Experimental conditions used in quantitative real time PCRs (qRT–PCRs) based on MIQE requirements.

Table S4. Identification of the probes of the Affymetrix GeneChip® Tomato Genome Arrays with increased expression.

Table S5. Identification of the probes of the Affymetrix GeneChip® Tomato Genome Arrays with reduced expression.

Table S6. Experimental conditions used in the microarray analyses based on MIAME requirements.

Table S7. Clusters found in the gene network displayed in Supplementary Fig. S2.

Table S8. Ratio of metabolite contents in mature fruits of *ASR1*-antisense lines in comparison to control plants (WT) measured by GC-MS.

Table S9. Glucose and sucrose content in mature fruits of *ASR1*-antisense plants measured by the enzymatic method.

Table S10. Gene expression ratios in mature fruits of the *ASR1*-antisense lines (AS) in comparison to control plants (WT) measured by qRT–PCR.

Table S11. Differentially expressed genes associated with defence and biotic stress in the AS lines in comparison to WT plants found in the microarrays (Supplementary Tables S4; S5).

Table S12. Summary of the behaviour of *ASR1* in transgenic lines, or mutants of regulators of tomato fruit ripening, and in chromatin immunoprecipitation (ChIP) assays.

Acknowledgements

This work was partially supported by grants from INTA (Instituto Nacional de Tecnología Agropecuaria, Argentina), CONICET (Consejo Nacional de Investigaciones Científicas y Técnicas, Argentina), and ANPCYT (Agencia Nacional de Promoción Científica y Tecnológica, Argentina). PGD is a recipient of a CONICET fellowship. TD was a recipient of a CONICET fellowship. GC, SAs and FC are members of CONICET. SAl and ARF are supported by the European Union's Horizon 2020 research and innovation program, project PlantaSYST (SGA-CSA No. 739582 under FPA No. 664620). FC´s laboratory received a grant from the European Union Horizon 2020 Research and Innovation Programme (Grant Agreement Number 679796).

Author contributions

PGD performed most of the experiments and analysed the data; PGD and TD performed the bioinformatics analyses; SAl ran the GC-MS samples; PGD and MI analysed the GC-MS data; GC and SA performed the *B. cinerea* experiments; PGD, ARF and FC designed the experiments; PGD and FC wrote the manuscript, which was revised and approved by all the authors.

Data availability

Raw and total normalized microarray data are available in the Gene Expression Omnibus (GEO) repository at the National Center for Biotechnology Information (NCBI), Accession number GSE163738. The rest of the data supporting the findings of this study are available in the main body of the manuscript or in the Supplementary data, or can be directly requested from the corresponding author.

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