Arabidopsis NAC Transcription Factor JUNGBRUNNEN1 Exerts Conserved Control Over Gibberellin and brassinosteroid Metabolism and Signaling Genes in Tomato

Sara Shahnejat-Bushehri1,2†, Annapurna D. Allu1,2*,†, Nikolay Mehterov††, Venkatesh P. Thirumalaikumar1,2, Saleh Alseekh2, Alisdair R. Fernie3, Bernd Mueller-Roeber1,2* and Salma Balazadeh1,2*

1 Institute of Biochemistry and Biology, University of Potsdam, Potsdam, Germany, 2 Max Planck Institute of Molecular Plant Physiology, Potsdam, Germany

The Arabidopsis thaliana NAC transcription factor JUNGBRUNNEN1 (AtJUB1) regulates growth by directly repressing GA3ox1 and DWF4, two key genes involved in gibberellin (GA) and brassinosteroid (BR) biosynthesis, respectively, leading to GA and BR deficiency phenotypes. AtJUB1 also reduces the expression of PIF4, a bHLH transcription factor that positively controls cell elongation, while it stimulates the expression of DELLA genes, which are important repressors of growth. Here, we extend our previous findings by demonstrating that AtJUB1 induces similar GA and BR deficiency phenotypes and changes in gene expression when overexpressed in tomato (Solanum lycopersicum). Importantly, and in accordance with the growth phenotypes observed, AtJUB1 inhibits the expression of growth-supporting genes, namely the tomato orthologs of GA3ox1, DWF4 and PIF4, but activates the expression of DELLA orthologs, by directly binding to their promoters. Overexpression of AtJUB1 in tomato delays fruit ripening, which is accompanied by reduced expression of several ripening-related genes, and leads to an increase in the levels of various amino acids (mostly proline, β-alanine, and phenylalanine), γ-aminobutyric acid (GABA), and major organic acids including glutamic acid and aspartic acid. The fact that AtJUB1 exerts an inhibitory effect on the GA/BR biosynthesis and PIF4 genes but acts as a direct activator of DELLA genes in both, Arabidopsis and tomato, strongly supports the model that the molecular constituents of the JUNGBRUNNEN1 growth control module are considerably conserved across species.

Keywords: Arabidopsis, tomato, fruit, growth, transcription factor, gibberellic acid, brassinosteroid, DELLA proteins
INTRODUCTION

Gibberellins (GAs) and brassinosteroids (BRs) play important roles in the regulation of plant growth and development. Because of their biological relevance, many key aspects of the metabolism and signaling of both phytohormones have been revealed in the past (Depuydt and Hardtke, 2011; Bai et al., 2012). GAs control processes like for example seed germination, leaf expansion, shoot elongation, flowering, fruit set and growth (Olszewski et al., 2002; Yamaguchi, 2008; Mutasa-Göttgens and Hedden, 2009; Seymour et al., 2013). Similarly, BRs function as growth-promoting hormones mediating many aspects of plant growth and development including the stimulation of growth through cell division and cell elongation, and the adaptation to environmental cues (Clouse, 2011). Mutants deficient in BR biosynthesis or perception exhibit various degrees of phenotypic similarity to GA-deficient mutants including dwarfism, short petioles and hypocotyls, curled dark-green leaves, and delayed flowering (Clouse and Sasse, 1998; Fleet and Sun, 2005).

With respect to the later steps of GA biosynthesis, GA 20-oxidase (GA20ox) and GA 3-oxidase (GA3ox) are particularly important for controlling the levels of bioactive GAs (Phillips et al., 1995; Xu et al., 1995; Yamaguchi et al., 1998). The bioactive GAs produced by GA3ox enzymes are perceived by a soluble GA receptor, GIBBERELLIN-INSENSITIVE DWARF1 (GID1) (Ueguchi-Tanaka et al., 2005). The binding of GA to GID1 triggers the destruction of DELLA proteins by the ubiquitin-proteasome pathway, or their inactivation through a degradation-independent mechanism (Ariizumi et al., 2008, 2013; Ueguchi-Tanaka et al., 2008). DELLA are major transcriptional growth repressors (Martí et al., 2007; Bai et al., 2012). In the absence of active GAs, DELLA accumulate allowing them to interact with the transcription factors (TFs) PHYTOCHROME-INTERACTING FACTOR4 (PIF4) and BRASSINAZOLE-RESISTANT1 (BZR1) to inhibit their DNA-binding ability; this restricts cell elongation controlled by the two TFs thereby resulting in reduced plant growth (De Lucas et al., 2008; Bai et al., 2012).

Gibberellins and DELLA have also been reported to control reproductive organ size and fruit development in several species (Martí et al., 2007; Dorcée et al., 2009; Fuentes et al., 2012). In tomato (Solanum lycopersicum), overexpression of SIDELLA (which is identical to SIG1) suppresses fruit development by inhibiting cell expansion (Martí et al., 2007).

Brassinosteroids are synthesized from campesterol via two pathways, the early and the late C-6 oxidation pathways (Fujioka and Sakurai, 1997). DWARF4 (DWF4) encodes a cytochrome P450 protein that catalyzes sterol C-22 α-hydroxylation, a rate-limiting step in BR biosynthesis (Choe et al., 1998, 2001; Kim et al., 2006; Divi and Krishna, 2010). The BR signal is perceived by a membrane-bound receptor kinase, BRASSINOSTEROID INSENSITIVE1 (BRI1). BRs bind to BRI1 and initiate a signal transduction pathway which ultimately results in the dephosphorylation and thereby stabilization of BZR1 and BR1-EMS SUPPRESSOR1 (BES1) (Wang et al., 2002; He et al., 2005; Yin et al., 2005), two TFs regulating BR-responsive genes to promote growth (Sun et al., 2010; Clouse, 2011; Yu et al., 2011). BR and GA signals are integrated through the direct interaction of BZR1 and BES1 with DELLA, and they enhance each other's signaling through inactivation and degradation of DELLA proteins, respectively (Bai et al., 2012; Gallego-Bartolomé et al., 2012; Li et al., 2012). In the presence of BRs, active forms of BZR1 and BES1 attenuate the transcriptional activity of DELLA. On the other hand, in the absence of GA, accumulation of DELLA and their binding to BZR1 or BES1 prevents their dephosphorylation and thus results in their inactivation. BR-deficient and -insensitive mutants have, e.g., been identified in Arabidopsis thaliana, pea (Pisum sativum), tomato (Lycopersicon esculentum), and rice (Oryza sativa) (Clouse and Sasse, 1998; Bishop, 2003; Vert et al., 2005; Clouse, 2011). BR biosynthesis pathways appear to be conserved between Arabidopsis, tomato, and pea (Banco, 2002). Molecular genetic manipulation of BR biosynthesis and signaling has been used to improve crop yield and stress tolerance (Divi and Krishna, 2009). It has been shown that BR mutants in rice and barley have increased grain yield and productivity due to erect leaves and reduced plant height (Chono et al., 2003; Morinaka et al., 2006). Controlling plant architecture and height to obtain miniature ornamental plants has been achieved by co-suppression of genes controlling GA and BR biosynthesis pathways (Xie et al., 2014). Several studies have shown that BRs are required for normal fruit development. For example, BR levels are high in developing fruits of tomato (Montoya et al., 2005). Furthermore, tomato fruit ripening is accelerated by application of BRs, but is delayed by treatment with the BR biosynthesis inhibitor brassinazole (Vidya Vardhini and Rao, 2002; Lisso et al., 2006). In tomato, the BR-deficient dwarf mutant shows delayed flowering and fruit ripening. Dry mass content, starch and sugar levels are reduced in dwarf tomato fruits, but are rescued by BR application, indicating that BRs are required for normal fruit development in this species (Lisso et al., 2006).

We recently identified the Arabidopsis thaliana NAC transcription factor JUNGBRUNNEN1 (JUB1; in the following called AtJUB1) as a further element of the GA/BR control network. More specifically, AtJUB1 suppresses the expression of GA3ox1 and DWF4, two central genes of GA and BR biosynthesis, respectively, by directly binding to their promoters. Furthermore, AtJUB1 directly activates the expression of the DELLA encoding genes GAI and RGL1. The repression of the phytohormone biosynthesis genes and the activation of the DELLA genes leads to an accumulation of DELLA proteins in AtJUB1 overexpressers and growth characteristics typical of low-GA and low-BR mutants, mediated by the inhibition of cell elongation (Shahnejat-Bushehri et al., 2016). AtJUB1 further inhibits cell elongation by directly suppressing the expression of PIF4 (Shahnejat-Bushehri et al., 2016).

Here, we report that overexpression of AtJUB1 in tomato results in GA and BR deficiency phenotypes similar to those observed in Arabidopsis AtJUB1 overexpressers. Furthermore, we show that AtJUB1 directly regulates the expression of tomato genes orthologous to GA3ox1, DWF4, GAI, and PIF4 from Arabidopsis. Our study thus reveals considerable similarity of the GA/BR-related gene regulatory network controlled by JUB1 across species.
MATERIALS AND METHODS

General
Tomato orthologs of Arabidopsis genes were identified using the PLAZA 3.0 database\(^1\) (Proost et al., 2015). Gene annotations were done using the PLAZA 3.0 and Sol Genomics\(^2\) database, and information taken from the literature. Quantitative real-time polymerase chain reaction (qRT-PCR) primers were designed using QuantPrime (Arvidsson et al., 2008); only primer pairs giving high PCR amplification efficiencies tested on at least 20 different RNAs isolated from diverse tomato tissues were chosen for the experiments performed here. Sequences of primers used for qRT-PCR and chromatin immunoprecipitation-polymerase chain reaction (ChIP-PCR) are given in Supplementary Table S1.

Plant Material and Growth Conditions
To generate AtJUB1-OX tomato plants, S. lycopersicum cv. Moneymaker was transformed with the 35S:AtJUB1-GFP construct previously reported (Wu et al., 2012) by Agrobacterium-mediated transformation. Seeds were germinated on full-strength Murashige-Skoog (MS) medium containing 2% (w/v) sucrose in a plant growth cabinet at 22°C, at a 16/8 h light (100–120 mmol photons m\(^{-2}\) s\(^{-1}\))/dark cycle. Two-week-old seedlings were transplanted to soil (potting and quartz sand; 2:1 [v/v]) and grown in the greenhouse at 25°C under a 16/8 h light (500 mmol photons m\(^{-2}\) s\(^{-1}\))/dark cycle, in individual pots (18 cm diameter). After transfer to soil, plants were fertilized with 5 g/pot Composial Perfekt (15% N, 5% P\(_2\)O\(_5\), 20% K\(_2\)O, 2% MgO, 7% S; Manna, Duesseldorf, Germany); two further fertilizations (with each 4 g/pot) followed when the first flowers opened, and when the first stage B fruits appeared.

Quantitative Real-Time PCR
Total RNA from frozen powdered tissue (200 mg fruit, or 100 mg leaf) was isolated using Trizol reagent (Life Technologies). cDNA synthesis and qRT-PCR were performed as described (Caldana et al., 2007; Balazadeh et al., 2008). Prior to cDNA synthesis, RNA was treated with DNase (Ambion) to eliminate genomic DNA contamination. Purity of DNase-treated RNA was assessed for qRT-PCR experiments. Three \(\mu\)g of quality-checked RNA was processed for transcriptome profiling using GeneChip Tomato Genome Arrays (Affymetrix). Probe preparation and microarray hybridizations were performed by ATLAS Biolabs (Berlin, Germany). Data analysis was performed as in Balazadeh et al. (2010). Expression of differentially expressed genes (transgenic versus wild type) was reassessed by qRT-PCR using RNA isolated from plants of two independent biological experiments. Microarray expression data are available from the NCBI Gene Expression Omnibus (GEO) repository\(^3\) under accession number GSE87507.

Profiling of Primary Metabolites by GC–MS
Fruit materials were extracted in 100% methanol at 70°C for 15 min. After centrifugation, the resultant supernatant was dried under vacuum, and the residue was derivatized for 120 min at 37°C (in 50 \(\mu\)l of 20 mg ml\(^{-1}\) methoxamine hydrochloride in pyridine) followed by a 30 min treatment at 37°C with 50 \(\mu\)l of N-methyl-N-(trimethylsilyl)-trifluoroacetamide (MSTFA). The gas chromatography–mass spectrometry (GC–MS) system used was a gas chromatograph coupled to a time-of-flight mass spectrometer (Pegasus III, Leco). An autosampler system (PAL) injected the samples. Helium was used as carrier gas at a constant flow rate of 2 ml s\(^{-1}\) and gas chromatography was performed on a 30 m DB-35 column. The injection temperature was 230°C and the transfer line and ion source were set to 250°C. The initial temperature of the oven (85°C) increased at a rate of 15°C min\(^{-1}\) up to a final temperature of 360°C. After a solvent delay of 180 s mass spectra were recorded at 20 scans s\(^{-1}\) with m/z 70–600 scanning range. Chromatograms and mass spectra were evaluated by using Chroma TOF 1.0 (Leco) and TagFinder 4.0 software (Roessner et al., 2001; Schauer et al., 2005).

RESULTS

Tomato Plants Overexpressing AtJUB1 Exhibit Morphological Characteristics of GA- and BR-Deficient Mutants
To understand the potential role of the JUNGBRUNNEN1 TF in tomato, we generated transgenic plants (S. lycopersicum L. cv. Moneymaker) overexpressing AtJUB1-GFP (Wu et al., 2012) under the control of the Cauliflower Mosaic Virus

\(^1\)http://bioinformatics.psb.ugent.be/plaza/
\(^2\)https://www.solgenomics.net/
\(^3\)https://www.ncbi.nlm.nih.gov/geo/
transgenic lines and determined the level of AtJUB1 (Wu et al., 2012). We obtained more than 20 independent GFP fusion protein is fully functional when expressed in plants (Figures S1A,B). Accumulation of AtJUB1-GFP fusion protein in nuclei of AtJUB1-OX tomato leaves was confirmed by confocal microscopy (Figure 1C). AtJUB1-OX lines displayed distinct morphological changes compared to the Moneyemaker wild type (Figure 2). Strong AtJUB1 overexpressors (line OX2) had smaller shoots than wild type and developed smaller leaves (Figures 2A,B), while growth was not much reduced in plants moderately overexpressing AtJUB1, including OX1 and OX3 (not shown). Furthermore, AtJUB1-OX plants developed curly and dark-green leaves, a phenotype not observed in the wild type (Figure 2C). Additionally, overexpression of AtJUB1 in tomato delayed flowering, similar to Arabidopsis (Shahnejat-Bushehri et al., 2016). AtJUB1-OX tomato plants flowered when the main shoot had ∼13 leaves beneath the first inflorescence compared to ∼9 leaves in the wild type (Figure 2E). At the productive stage, AtJUB1-OX plants had smaller flowers and fruits than the wild type (Figures 2F,G), and fruit development and ripening were delayed. Fruits of AtJUB1-OX plants reached the mature green stage (MG) 4 days later than wild type tomatoes (measured as days after pollination) and ripening of AtJUB1-OX fruits was delayed by ∼6 days (Figure 2H). Upon visual inspection, AtJUB1-OX fruits showed reduced pigmentation at the breaker+7d (B+7) stage compared to wild type fruits at the same stage (not shown). Taken together, many of the growth phenotypes observed in AtJUB1-OX tomato plants were similar to those of Arabidopsis plants overexpressing AtJUB1, suggesting that JUB1 controls a similar set of growth-related genes in both species possibly including genes affecting hormone metabolism and signaling (Shahnejat-Bushehri et al., 2016).

**AtJUB1 Directly Regulates GA- and BR-Related Genes in Tomato**

Previously, we reported that AtJUB1 extends longevity, dampens intracellular H$_2$O$_2$ level, and enhances tolerance to various abiotic stresses partly through direct transcriptional regulation of DREB2A, an important TF involved in the response to various abiotic stresses (Sakuma et al., 2006a,b; Wu et al., 2012). Furthermore, AtJUB1 restricts growth and primes plants for stress tolerance in a DELLA-dependent manner by regulating a complex transcriptional module composed of key components of GA and BR pathways in Arabidopsis, AtJUB1 directly suppresses the expression of GA3ox1 (GA biosynthesis), DWF4 (BR biosynthesis), and PIF4 (regulation of cell elongation), but activates the expression of the GAI and RGA DELLA genes (Shahnejat-Bushehri et al., 2016). Considering the growth phenotypes observed for the AtJUB1-OX tomato plants (see above), we tested whether similar genes are targets of AtJUB1 in tomato. First, we checked the expression of tomato orthologs of Arabidopsis DWF4, GA3ox1, GAI, and RGL1 in AtJUB1-OX tomato leaves and in fruits at the MG, B and B+7 stages. As shown in Figure 3A, expression of the DWF4 tomato orthologs Solyc02g085360 (denoted SIDWF4-1 in the following) and Solyc04g080650 (SIDWF4-2) was not altered in fruits of AtJUB1-OX fruits at all stages tested, but was strongly reduced

(CaMV) 35S promoter. We previously showed that the JUB1-GFP fusion protein is fully functional when expressed in plants (Wu et al., 2012). We obtained more than 20 independent transgenic lines and determined the level of AtJUB1 expression in some of them by qRT-PCR in leaves (Figure 1A; lines OX1, OX2, and OX3) and fruits of different developmental stages (Figure 1B; OX2; thereafter called AtJUB1-OX). The formation of full-length AtJUB1 transcript in transgenic tomato plants was confirmed by PCR analysis of cDNA of leaves and fruits, using primers specific for AtJUB1 (Supplementary Figures S1A,B). Accumulation of AtJUB1-GFP fusion protein in nuclei of AtJUB1-OX tomato leaves was confirmed by confocal microscopy (Figure 1C). AtJUB1-OX lines displayed distinct morphological changes compared to the Moneyemaker wild type (Figure 2). Strong AtJUB1 overexpressors (line OX2) had smaller shoots than wild type and developed smaller leaves (Figures 2A,B), while growth was not much reduced in plants moderately overexpressing AtJUB1, including OX1 and OX3 (not shown). Furthermore, AtJUB1-OX plants developed curly and dark-green leaves, a phenotype not observed in the wild type (Figure 2C). Additionally, overexpression of AtJUB1 in tomato delayed flowering, similar to Arabidopsis (Shahnejat-Bushehri et al., 2016). AtJUB1-OX tomato plants flowered when the main shoot had ∼13 leaves beneath the first inflorescence compared to ∼9 leaves in the wild type (Figure 2E). At the productive stage, AtJUB1-OX plants had smaller flowers and fruits than the wild type (Figures 2F,G), and fruit development and ripening were delayed. Fruits of AtJUB1-OX plants reached the mature green stage (MG) 4 days later than wild type tomatoes (measured as days after pollination) and ripening of AtJUB1-OX fruits was delayed by ∼6 days (Figure 2H). Upon visual inspection, AtJUB1-OX fruits showed reduced pigmentation at the breaker+7d (B+7) stage compared to wild type fruits at the same stage (not shown). Taken together, many of the growth phenotypes observed in AtJUB1-OX tomato plants were similar to those of Arabidopsis plants overexpressing AtJUB1, suggesting that JUB1 controls a similar set of growth-related genes in both species possibly including genes affecting hormone metabolism and signaling (Shahnejat-Bushehri et al., 2016).

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in leaves of mature plants compared to wild type. Similarly, expression of the tomato orthologs Solyc06g066820 (denoted SlGAI-like) was slightly and strongly upregulated, respectively, in MG, B and B+7 fruits of AtJUB1-OX plants, consistent with the reduced size of the fruits. Expression of SlGA3ox1-2 was decreased in MG and B+7 fruits compared to wild type fruits of the same stage. Interestingly, expression of the tomato DELLA genes Solyc11g011260 (SlGAI) and Solyc01g009840 (SlGAI-like) was slightly and strongly upregulated, respectively, in MG, B and B+7 fruits of AtJUB1-OX plants, consistent with the reduced size of the fruits. Expression of SlGA3ox1 and SlGAI-like was not altered in leaves of AtJUB1-OX plants relative to wild type. Expression of Solyc07g043580 (SlPIF4), the positive regulator of cell elongation, was strongly suppressed by AtJUB1 in MG and B, but not affected in B+7 fruits or leaves of AtJUB1-OX plants.

The fact that JUB1 regulates orthologous genes in Arabidopsis and tomato when overexpressed indicates that this TF obeys a conserved function in plants as part of a complex regulatory network associated with GA and BR pathways. To consolidate this conclusion we next analyzed the promoters of selected GA- and BR-related genes whose expression is affected by AtJUB1 overexpression (shown in Figure 3A and Supplementary Table S2), and found that SlDWF4-1, SlGA3ox1-1, SlGA3ox1-2, SlGAI and SlGAI-like harbor the conserved AtJUB1 binding site (Wu et al., 2012) in their 5’ upstream regulatory regions (Figure 3B). An AtJUB1 binding site is also present in the promoter of SlPIF4 (Figure 3B). To test whether AtJUB1 is able to bind to the selected putative target genes in tomato, we performed chromatin immunoprecipitation followed by qPCR using leaves of mature AtJUB1-OX plants. Binding of AtJUB1 was observed for all promoters tested, while no binding was detected for a promoter region lacking an AtJUB1 binding site (negative control) (Figure 3C). Taken together, our data indicate a direct transcriptional regulation of SlDWF4-1, SlGA3ox1-1, SlGA3ox1-2, SlGAI, SlGAI-like and SlPIF4 by AtJUB1 in vivo.

**Differentially Expressed Genes in AtJUB1 Overexpressing Tomato Fruits**

To identify genes globally affected by AtJUB1 during fruit development and ripening, we compared the transcriptomes of AtJUB1-OX and wild type fruits at MG and B+7 stages, using microarray analysis. We selected 92 genes that were differentially expressed in MG and/or B+7 fruits of AtJUB1-OX compared to wild type (at a 1.5-fold cut-off in increase or decrease of gene expression) and re-analyzed their expression in additional biological replicates by qRT-PCR, which confirmed altered expression of 60 genes (Figure 4 and Supplementary Table S3). The AtJUB1-regulated genes fall into different functional categories including TFs, cell wall-modifying enzymes, antioxidant enzymes, enzymes involved in flavonoid biosynthesis, and hormone-related genes. Among the TFs, RIPENING INHIBITOR (RIN; Solyc05g012020), a MADS-box TF with a key function in fruit ripening, was significantly downregulated in AtJUB1-OX tomato fruits at MG stage. Similarly, ETHYLENE RESPONSE FACTOR H15 (SIERF.H15; Solyc06g050520), which shows increased expression at the onset of ripening and together with other ERFs was suggested to be involved in regulating the ripening process (Liu et al., 2016), was downregulated in AtJUB1-OX fruits at MG stage. Furthermore, expression of cell wall-related genes including a pectin esterase family protein (Solyc06g051960), a cellulase
synthase (Solyc12g056580), and expansins (Solyc06g051800, Solyc02g088100, and Solyc05g007830) was reduced in AtJUB1-OX fruits. A group of genes associated with cellular oxidation and peroxidation processes, including several peroxidases (Solyc01g006300, Solyc03g080150, Solyc06g076630), were lower expressed in AtJUB1-OX fruits. Peroxidases are considered to affect fruit softening and ripening (Kumar et al., 2016). Expression of Solyc12g005350, encoding dihydroflavonol-4-reductase, a key gene in anthocyanin biosynthesis (Holton and Cornish, 1995), is slightly decreased in AtJUB1-OX fruits. Among hormone-related genes, expression of 1-aminoacyclopropane-1-carboxylate (ACC) synthase (ACS; Solyc08g081550) and ACC oxidases (ACO; Solyc02g036350, Solyc07g026650, Solyc11g072110) is either not altered or repressed in AtJUB1-OX fruits. These genes are known to be involved in ethylene biosynthesis and their expression is induced during fruit ripening (Barry et al., 2000). Moreover, Solyc02g037550 and Solyc02g082450, members of the auxin efflux carrier family, are significantly downregulated in AtJUB1-OX fruits at stages MG and B+7. These proteins are involved in the distribution of auxin, a growth hormone controlling many aspects of fruit development and ripening (Gillaspy et al., 1993; Srivastava and Handa, 2005). Next, we searched for the binding site of AtJUB1 in the promoters of the differentially expressed genes. The absence of the full AtJUB1 binding site in the 5′ upstream regulatory regions of the 60 genes differentially expressed between AtJUB1-OX and wild type suggests that AtJUB1 indirectly, e.g., in conjunction with hormonal networks, regulates their expression.

AtJUB1 Affects Metabolite Profiles in Tomato Fruits

Metabolite profiling was performed on fruits of AtJUB1-OX and wild type plants at three independent ripening stages: mature green (MG), breaker (B) and red ripe fruit (B+7). Metabolites were separated and quantified by GC–MS. In total, 54 metabolites were quantified including amino acids, organic acids, sugars and a few miscellaneous compounds. Figure 5 shows the metabolic changes in the AtJUB1-OX line in comparison to wild type, where the relative changes are depicted in blue (decreased) or red (increased) (complete data are given in Supplementary Table S4). Thirty-four metabolites out of 54 (~63%) were significantly increased in the overexpression line compared to the wild type, with similar trends being observed at all three ripening stages. In general, the content of amino acids increased.
FIGURE 4 | Quantitative real-time polymerase chain reaction analysis of AtJUB1-regulated genes in tomato fruits. Heat map showing differentially expressed genes in AtJUB1-OX fruits compared to wild type. Means of three experiments are shown; complete data are given in Supplementary Table S3. The log₂ fold change scale is indicated under the heat map. Transcription factors, hormone metabolism-related, cell wall-related and peroxidase categories are shown.
FIGURE 5 | Heat map showing the metabolite changes in fruits of AtJUB1-OX plants in comparison with wild type at three ripening stages. Mature green fruit (MG), breaker stage (B), and red ripe fruit (B+7) were analyzed. Red or blue indicate that the metabolite content is increased or decreased, respectively. Asterisks denote significant difference (p ≤ 0.01, Student’s t-test) in AtJUB1-OX compared to wild type; complete data are given in Supplementary Table S4.

In the overexpression line compared to the wild type. Among these the levels of proline, β-alanine and phenylalanine showed considerable increases being a maximum of 12-, 14- and 7-fold higher in the AtJUB1-OX line compared to wild type. However, the greatest change was observed in the gamma-aminobutyric acid (GABA) content which was approximately 34-fold higher in ripe fruit of the AtJUB1-OX line compared to wild type fruit at the same stage (B+7). In addition, a marked increase was observed in major organic acids, particularly for glutamic acid (12-fold), aspartic acid (4.8-fold) and pyroglutamic acid (3.6-fold). Among the detected disaccharides, the levels of trehalose and maltose were mostly changed. By contrast, relatively few metabolites decreased in the three ripening stages in the overexpression line compared to wild type with only succinic acid, galactinol and tyramine displaying this behavior, although urea, glycerol, raffinose, glyceric acid and quinic acids were less abundant in the overexpression line compared to wild type in red ripe fruit (B+7).

DISCUSSION

In this study, we characterized the biological function exerted by the Arabidopsis NAC transcription factor AtJUB1 (JUNGBRUNNEN1) when expressed in the heterologous plant tomato (S. lycopersicum L. cv. Moneymaker). Similar to Arabidopsis, overexpression of AtJUB1 in tomato resulted in reduced plant size, dark-green curled leaves, a delay in flowering, and reduced reproductive organ size compared to wild type (Shahnejat-Bushehri et al., 2016); in addition, fruit growth and ripening was delayed in AtJUB1-OX tomatoes. In Arabidopsis, AtJUB1 regulates various growth-related genes by directly binding to their promoters: it represses expression of GA3ox1 and DWF4, which encode key genes of GA and BR biosynthesis, respectively, and of PIF4, a growth promoting TF of the bHLH family. In contrast, AtJUB1 activates the expression of the DELLA genes GAI and RGA, which encode important repressors of growth. Collectively, the transcriptional control exerted by AtJUB1 leads to the characteristic GA- and BR-deficiency phenotypes observed for AtJUB1 overexpressors, which is associated with a reduction of the levels of bioactive GAs and BRs and an accumulation of DELLA proteins (Shahnejat-Bushehri et al., 2016).

To gain insights into the mechanisms through which AtJUB1 affects growth in tomato, we analyzed genes orthologous to AtJUB1 targets in Arabidopsis (GA3ox1, DWF4, GAI, RGL1, and PIF4) in different tissues of AtJUB1-OX tomato plants and found that expression of all genes was altered, like in Arabidopsis itself.

In Arabidopsis, GA3ox1 encodes a key enzyme involved in the biosynthesis of bioactive GAs, and it is expressed at relatively high levels at all stages of development (Mitchum et al., 2006); ga3ox1 knockout mutants display a semi-dwarf phenotype due to reduced levels of bioactive GAs (Talon et al., 1990; Chiang et al., 1995). Here, we observed that expression of the GA3ox1 tomato orthologs, SlGA3ox1-1 and SlGA3ox1-2, was down-regulated in leaves as well as in three fruit developmental stages.
(MG, B and B+7) of AtJUB1-OX plants compared to wild type. The reduced expression of the rate-limiting GA biosynthetic enzymes may lead to lower levels of GA, thus contributing to GA-deficient phenotypes in AtJUB1-OX tomatoes similarly to Arabidopsis plants overexpressing AtJUB1 (Shahnejat-Bushehri et al., 2016).

Furthermore, expression of SIDWF4-1 and SIDWF4-1, orthologs of the Arabidopsis DWF4 gene, was strongly reduced in leaves of AtJUB1-OX tomatoes, sharing this behavior with the repression of DWF4 by AtJUB1 in Arabidopsis (Shahnejat-Bushehri et al., 2016). DWF4 encodes a sterol C-22 α-hydroxylase that catalyzes a rate-limiting step in BR biosynthesis (Choe et al., 1998, 2001; Kim et al., 2006; Divi and Krishna, 2010); its reduced expression in tomato may contribute to the BR-deficiency growth characteristics observed.

Additionally, elevated expression was observed for the DELLA genes SIGAI and SIGAI-like in AtJUB1-OX fruits compared to wild type in the three developmental stages tested. While DELLA proteins are encoded by five genes in Arabidopsis (GAI, RGA, RGL1, RGL2, and RGL3) (Achard and Genschik, 2009), two DELLA, namely SIGAI (identical to SIDELLA; Marti et al., 2007) and SIGAI-like, are encoded by the tomato genome (as deduced from the PLAZA 3.0 web database; Proost et al., 2015), and were analyzed here. GA promotes plant growth by removing DELLA proteins, whereas at low GA levels DELLA accumulate and repress growth by directly inactivating several TFs including PIF4, a positive regulator of cell elongation (De Lucas et al., 2008; Feng et al., 2008; Arnaud et al., 2010; Leivar and Quail, 2011). In Arabidopsis, we found that AtJUB1 directly up-regulates DELLA genes thereby promoting their accumulation. Additionally, AtJUB1 represses the expression of PIF4, which further restricts cell elongation (Shahnejat-Bushehri et al., 2016). Similarly, reduced SIPIF4 expression was evident in MG and B stage fruits of AtJUB1-OX tomato plants compared to wild type. Activation of DELLA and repression of SIPIF4 by AtJUB1 may contribute to the smaller fruit size of AtJUB1-OX plants.

In addition to the differential expression of GA-/BR-related genes in tomato upon AtJUB1 overexpression, the presence of the AtJUB1 binding site in their 5’ upstream regulatory regions and the presence of an AtJUB1 ortholog in the tomato genome and other plants (with in most case one predicted ortholog, see PLAZA 3.0) strongly argue for a JUB1 regulatory network that has a similar architecture and function in Arabidopsis and tomato, and likely other plants. In support of our hypothesis, ChIP-qPCR revealed a significant enrichment of promoter regions of all tomato GA- and BR-related gene orthologous to the AtJUB1 targets in Arabidopsis, while no enrichment was detected for a promoter region lacking an AtJUB1 binding site.

Additionally, we were interested in understanding the molecular mechanisms associated with the delay of fruit ripening in AtJUB1-OX plants. Toward this end, we performed transcriptome analysis of AtJUB1-OX and wild type fruits at MG and B+7 stages. Genes differentially expressed in AtJUB1-OX fruits compared to wild type encode TFs, genes involved in ethylene synthesis or signaling, cell wall modification, flavonoid biosynthesis, peroxidases, and others. Of note, expression of several ethylene-related ripening genes such as ACS (ACC synthase) and ACO (ACC oxidase), MADS-RIN, and SLERF.H15, among others, was repressed in AtJUB1-OX tomatoes. Tomato is a climacteric fruit that requires an ethylene burst for normal fruit ripening (Alexander and Grierson, 2002). In fruits of several species, including, e.g., tomato and melon, inhibition of key enzymes of ethylene biosynthesis (ACO and ACS) by antisense RNA leads to strong delay in ripening, demonstrating the requirement of ethylene in the process (Oeller et al., 1991; Picton et al., 1993; Ayub et al., 1996). With respect to TFs, the MADS-box protein RIPENING INHIBITOR (RIN) has been particularly well-characterized. It exerts its function in fruit ripening by directly regulating the expression of genes involved in ethylene production, lycopene accumulation, chlorophyll degradation, among several other physiological processes (Fujisawa et al., 2013). Ripening is completely abolished in the rin mutant (Vrebalov et al., 2002).

Apart from ACS and ACO, a PECTIN ESTERASE gene (Soly06g051960) is repressed in AtJUB1-OX fruits compared to wild type; similarly, several pectin esterase encoding genes are repressed in the rin mutant during fruit ripening (Kumar et al., 2016). The absence of the AtJUB1 binding site in the promoters of the ripening-related genes, however, indicates indirect regulation by AtJUB1; the details of the mechanisms that underlie this regulation are currently unknown.

With respect to metabolites, in general, similar significant changes were found in AtJUB1 Arabidopsis and tomato overexpressors: 67 and 63% of the metabolites detected were significantly different between wild type and overexpression lines, respectively. However, here (in tomato AtJUB1 overexpressors) the relative changes were much higher than what we previously reported in Arabidopsis AtJUB1 overexpressors (Wu et al., 2012). In tomato, 44 out of 53 metabolites were significantly increased and nine decreased at least in one developmental stage. On the other hand, in Arabidopsis AtJUB1 overexpressors 21 metabolites were significantly increased and 17 decreased compared to wild type (Table 1). Comparing the metabolite changes indicates that 20% of the metabolites (nine increased and two decreased) overlapped and were significantly different in AtJUB1 overexpression plants compared to wild type, in both, tomato and Arabidopsis. On the other hand, 17 metabolites (34%) found to be significantly different between overexpression

| Table 1 | Summary comparing the number of metabolites in Arabidopsis and tomato AtJUB1 overexpression lines. |
|----------|---------------------------------|
| Tomato   | Arabidopsis                     |
| Number of metabolites | 53 | 51 |
| Significantly increased | 44 | 21 |
| Significantly decreased | 9 | 17 |
| Significant overlap – increased | 9 |
| Significant overlap – decreased | 2 |
| Significantly changed in both, but in different directions | 17 |
and wild type plants showed different behaviors in tomato and Arabidopsis.

In tomato AtJUB1 overexpressor plants proline showed one of the most drastic increases in overexpression lines at all developmental stages, which increased up to 12-fold higher than in the wild type. We made a similar observation in Arabidopsis AtJUB1 overexpression plants where proline was increased up to twofold compared to wild type (Wu et al., 2012). Proline is known to be involved in the response to various environmental stresses. Among other metabolites that showed significant changes and similar trends to proline are trehalose, malic acid, and citric acid.

Another metabolite that had strongly increased levels in fruits of tomato AtJUB1 overexpressors compared to wild type fruits is GABA, which was 4-, 8-, and 34-fold higher in the MG, B and B+7 stages, respectively (Figure 5 and Supplementary Table S4). In wild type fruits GABA levels accumulate before the breaker stage, but shortly thereafter GABA is rapidly catabolized (Akihiro et al., 2008), most likely to support the high respiratory demand of later stages of fruit development. The fact that GABA content remains elevated in AtJUB1 overexpressors is in accordance with the delayed fruit ripening observed in these plants. The biological function of GABA in tomato fruits is not well-known, but may include the regulation of pH to prevent cellular acidification during fruit development when organic acids are synthesized, or help to maintain glutamate import from source organs by converting it to GABA (through the action of glutamate decarboxylase) (reviewed in Takayama and Ezura, 2015). The high accumulation of glutamate (9- and 12-fold in B and B+7 fruits, respectively; Supplementary Table S4) in AtJUB1 overexpressors compared to wild type supports this conclusion. Alternatively, GABA, which functions in defense against pests and pathogens, might protect the immature seeds in developing fruits (Takayama and Ezura, 2015).

Also strongly elevated in AtJUB1 overexpressor fruits throughout all developmental stages is β-alanine (10- to 14-fold higher than in wild type; Figure 5 and Supplementary Table S4). Typically, β-alanine decreases during tomato fruit ripening (Carrari et al., 2006); the fact that its level remains high in AtJUB1 overexpressors even at later stages of fruit development is also in accordance with the delayed ripening observed in these plants.

In addition, among the few metabolites showing a significant decrease in overexpression lines in tomato AtJUB1 plants, raffinose (0.4-fold) and galactinol (0.2-fold), these metabolites decreased up to 0.6-fold and 0.2-fold in Arabidopsis overexpression plants compared to wild type, respectively. In contrast to Arabidopsis AtJUB1 overexpression lines, some organic acids, in particularly glyceric acid and succinic acid, showed a significant decrease in the tomato AtJUB1 plants (0.7- and 0.2-fold, respectively) compared to wild type. Succinic acid is a breakdown product of GABA and indeed is mobilized to augment flux through the TCA cycle (Studart-Guimarães et al., 2007); the low level of succinic acid in the presence of high GABA concentration (see above) indicates a reduced conversion of GABA to succinic acid in AtJUB1 overexpressor fruits compared to wild type fruits.

An accumulation of GABA levels was previously observed in fruits of transgenic tomato plants in which expression of GABA transaminase (GABA-T) was suppressed by RNA interference. GABA-T converts GABA to succinic semialdehyde which is then further metabolized to succinic acid by succinic semialdehyde dehydrogenase. Interestingly, inhibition of GABA-T led to plant dwarfism, concomitant with the over-accumulation of GABA (Koike et al., 2013), similar to the metabolic and growth phenotypes we observed here for AtJUB1 overexpressors. In addition, the excessive accumulation of GABA may negatively affect cell elongation (Renault et al., 2011), leading to the dwarfed phenotype of plants strongly overexpressing JUB1.

Taken together, we show here that the NAC TF JUNGBRUNNEN1, which we previously reported to control key elements of the GA/BR metabolism and signaling network in Arabidopsis thaliana (Shahnejat-Bushehri et al., 2016), exerts a similar control over orthologous genes in tomato. A key observation of our study is that in both plants, AtJUB1 negatively controls the expression of growth promoting genes (GA3ox1, DWF4, and PIF4), while it positively regulates the expression of growth repressing genes (DELLAs). This finding suggests that other, as yet unknown elements of the JUNGBRUNNEN1 growth control module are considerably conserved between species. Interestingly, JUB1 is a single-copy gene in most sequenced plant genomes, including dicots and monocots, with only few exceptions; e.g., two AtJUB1 orthologs are reported by PLAZA 3.0 for maize (Zea mays), poplar (Populus trichocarpa) and soybean (Glycine max), while three orthologs are reported for field mustard (Brassica rapa). The multiple regulatory roles that JUB1 exerts on central components of GA- and BR-related metabolism and signaling, which is key to the control of plant growth, suggests that JUB1 activity is finely tuned during growth and in different organs. Future research should focus on identifying further control elements of the JUB1 module in Arabidopsis and other species.

AUTHOR CONTRIBUTIONS

SB and BM-R conceived the idea for the study and supervised the work. SB and BM-R wrote the manuscript, with contributions from SS-B and AA. SS-B, AA, and NM performed the phenotype analysis; VT performed the confocal imaging; AA, NM, and VT performed the expression profiling; SS-B performed the ChiP-PCR experiments. SA and AF performed the metabolite profiling and analyzed the respective data.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fpls.2017.00214/full#supplementary-material

FIGURE S1 | AtJUB1 transcript in transgenic tomato plants. (A) Presence of full-length AtJUB1 transcript (arrow) in leaves of transgenic plants Ox1, Ox2 and Ox3, confirmed by PCR using primers specific for AtJUB1. (B) Presence of AtJUB1 transcript in fruits and leaves of AtJUB1-OX (≡ Ox2) tomato plants (arrow). Samples were taken from fruits of different developmental stages (MG, B, B+7). As expected, no AtJUB1 transcript is detected in wild type tomato plants (WT).

TABLE S1 | List of primers.

TABLE S2 | Expression of GA- and BR-associated genes.

TABLE S3 | Gene expression in tomato fruits.

TABLE S4 | Primary metabolites.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Author/s:
Shahnejat-Bushehri, S; Allu, AD; Mehterov, N; Thirumalaikumar, VP; Alseekh, S; Fernie, AR; Mueller-Roeber, B; Balazadeh, S

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