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Anthocyanins on demand

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Transcription factor mediated control of anthocyanin biosynthesis in vegetative tissues

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N.S.O. and R.K. performed most of the experiments with the help of M.H., X.S., E.J., I.B. and C.D.S., A.J.v.D. analysed the data, J.B., R.D.H. and D.B supervised the project, N.S.O., R.K. and J.B. wrote the article together with the input from all the authors.

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SUMMARY

Plants accumulate secondary metabolites to adapt to environmental conditions. These compounds, here exemplified by the purple coloured anthocyanins, are accumulated upon high temperatures, UV-light, drought and nutrient deficiencies, and may contribute to tolerance to these stresses. Producing compounds is often part of a more broad response of the plant to changes in the environment. Here we investigate how a transcription-factor mediated program for controlling anthocyanin biosynthesis also has effects on formation of specialized cell structures and changes in the plant root architecture. A systems biology approach was developed in tomato for coordinated induction of biosynthesis of anthocyanins, in a tissue- and development independent manner. A transcription factor couple from Antirrhinum that is known to control anthocyanin biosynthesis was introduced in tomato under control of a dexamethasone-inducible promoter. By application of dexamethasone, anthocyanin formation was induced within 24h in vegetative tissues and in undifferentiated cells. Profiles of metabolites and gene expression were analysed in several tomato tissues. Changes in concentration of anthocyanins and other phenolic compounds were observed in all tested tissues, accompanied by induction of the biosynthetic pathways leading from glucose to anthocyanins. A number of pathways that are not known to be involved in anthocyanin biosynthesis were observed to be regulated. Anthocyanin-producing plants displayed profound physiological and architectural changes, depending on the tissue, including root branching, root epithelial cell morphology, seed germination and leaf conductance. The inducible anthocyanin-production system reveals a range of phenomena that accompany anthocyanin biosynthesis in tomato, including adaptations of the plants architecture and physiology.
INTRODUCTION

Anthocyanins are abundant vacuolar pigments derived from the phenylpropanoid pathway and are produced in many different plant species. Depending on the pH and their chemical modifications, anthocyanins can change colour from red to purple and blue. Selecting for petal colour in ornamental plants has been the subject of extensive research (Sasaki and Nakayama, 2015). This research has revealed many different enzymes involved in chemical modifications such as glycosylation, methylation and acylation of anthocyanins. While anthocyanins in flowers and fruits are known to function as attractants for pollinators and vectors for seed dispersal, the role of anthocyanin accumulation under stress in vegetative tissues is probably linked to the scavenging of reactive oxygen species (Gould, 2004). In tomato, anthocyanins are predominantly found in stem and hypocotyl tissues, as a result of stress conditions (Roldan et al., 2014).

Anthocyanins are powerful antioxidants and, as part of human diet in seeds, fruit and leaves are proposed to have health promoting properties (Bassolino et al., 2013; Martin et al., 2011), for reviews. It has been shown that the consumption of anthocyanins can lower the risk of cancer, diabetes and cardiovascular diseases (Zafra-Stone et al., 2007); (He and Giusti, 2010); (Tsuda, 2012); (Butelli et al., 2008). To be able to breed for fruits and vegetables that are rich in anthocyanins, it is important to understand both their biosynthesis and functions in plants. By expressing two transcription factor genes, *Rosea1* (*ROS1*) and *Delila* (*DEL*), isolated from *Antirrhinum majus* flowers, under control of the tomato *E8* promoter, which is expressed during fruit ripening, tomato plants were engineered that carry purple fruits (Butelli et al. 2008). These purple tomato fruits, that are otherwise isogenic to red fruits, have been essential for defining health claims for anthocyanins (Martin et al., 2011; Martin et al., 2013).

ROS1/DEL tomato fruits are enriched with anthocyanins which predominantly include delphinidin 3,5-diglycosides and are acylated with hydroxycinnamic acids (Butelli et al., 2008). Overexpression of *ROS1/DEL* transcription factors in tomato led to the induction of expression of a number of genes homologous to known genes from the anthocyanin pathway in *Arabidopsis* and petunia (Butelli et al., 2008). In tomato, the role of some of these genes in anthocyanin biosynthesis has been confirmed using mutants disrupted in the *FLAVONOID 3-HYDROXYLASE* (*F3H*) gene (Maloney et al., 2014) and the *DIHYDROFLAVONOL 4-REDUCTASE* (*DFR*) gene (Goldsbrough et al., 1994). The tomato anthocyanin-specific O-methyltransferase (*SIOMT*) was identified by a transcriptional analysis of a tomato seedling system, in combination with an interfering RNA strategy (Gomez Roldan et al., 2014). A next step in the understanding of anthocyanin biosynthesis in tomato should focus on the dynamic coordination and gene regulation of the anthocyanin pathways in time, and its integration within plant developmental programs.

Transcription factors (TF) that regulate anthocyanin biosynthesis have been identified in many plant species (Petroni and Tonelli, 2011). A complex of three TFs (MBW), including an R2R3-Myb type TF, a basic helix-loop-helix type TF (bHLH) and a WD repeat TF (WDR), was shown to control anthocyanin accumulation (Xu et al., 2015) and in some cases other flavonoids, in many plant species, including *Arabidopsis*, maize and petunia (Albert et al., 2014). In tomato, two highly homologous Myb TFs, *ANT1* and *AN2*, have been shown to be involved in the regulation of anthocyanin biosynthesis (Mathews et al., 2003); (Zuluaga et al., 2008). R2R3-Myb proteins, such as *ROS1* and bHLH proteins such as *DEL*, serve as transcriptional activators of anthocyanin biosynthesis (Broun, 2005). In contrast, *CAPRICE* (*CPC*), a R3-type Myb TF, serves as a negative regulator of anthocyanin biosynthesis in *Arabidopsis*. *CPC* inactivates the MBW protein complex by competing with R2R3-MYB binding to a bHLH TF, while being unable to activate transcription (Tomina, et al., 2008). Related Myb and bHLH TF complexes can control other biosynthetic processes, such as glucosinolate biosynthesis (Freigenn et al., 2014).

Interestingly, specific aspects of cellular differentiation such as root hair and trichome differentiation are also regulated by MBW complexes (reviewed in (Broun, 2005); (Xu et al., 2015); (Tominaga-Wada et al., 2013). For example, in *Arabidopsis* an MBW complex including *WEREWOLFE* (*WER*), *GLABRA3* (*GL3*) and *Transparent Testa Glabra* (*TTG*) controls the transcription of *GLABRA2* (*GL2*), a TF, which acts on root and trichome developmental programs
(Rerie et al., 1994); (Bernhardt et al., 2005). Recently it was suggested that MBW complexes controlling secondary metabolism may have evolved from similar MBW complexes that regulate more ancient gene networks for differentiation of cell types (Chezem and Clay, 2016).

Systematic transcriptomics and metabolomics analysis have been employed to obtain a more holistic view of the regulation of the anthocyanin biosynthetic program. Such studies have been done both on tomato seedlings in which anthocyanin formation is induced by nutrient stress (Roldan et al., 2014) and on purple ROS1/DEL fruits (Tohge et al., 2015). In these studies, networks of genes and metabolites were analysed combining data from different tissues and cell types. Genes were identified that encode putative anthocyanin-modifying enzymes and transporters. However, to put anthocyanin biosynthesis in a context beyond biosynthetic genes, one needs to make observations on transcription networks and metabolite profiles which are independent of nutrient stress or developmental changes, and with high resolution in time. To achieve this, a uniform and tightly-controlled system for steering the anthocyanin biosynthetic program is needed.

Here, we aimed to study the anthocyanin pathway in tomato and its associated cellular and developmental processes using such a tightly regulated transcription system. We developed, for the first time, an inducible dexamethasone-regulated switch, which can deliver, on-demand, anthocyanin accumulation in different tissues of the tomato cultivar MicroTom. We interrogated the transcriptional and metabolic networks associated with anthocyanin biosynthesis in different vegetative tissues of tomato, including undifferentiated totipotent callus cells. This study revealed new aspects of transcriptional regulation of anthocyanin accumulation in tomato plants, and linked it to epidermal cell fate, in particular in root tissues. We identified several targets of regulation by ROS1/DEL TFs including genes involved in epidermal cell fate determination, cuticle formation, auxin biosynthesis and transport as well as several transcription factors. These data can serve as a resource for the identification of genes involved in anthocyanin biosynthesis. As an example, we focussed on specific acyl transferases involved in the addition of hydroxycinnamic acids to the glycoside moieties of tomato anthocyanin in vivo. These data provide insight in the processes that may accompany anthocyanin biosynthesis, including physiological and architectural changes in tomato vegetative tissues.
RESULTS

Development of an inducible system for anthocyanin biosynthesis in tomato cv. MicroTom

Previous studies in tomato fruits have shown that substantial induction of anthocyanin biosynthesis can be achieved by ectopically expressing two transcription factors (ROS1/DEL) from snapdragon (Butelli et al., 2008; Tohge et al., 2015). These studies use the fruit specific promoter E8, which is regulated by ethylene and thereby is specifically activated during the breaker stage of fruit ripening.

To collect comprehensive information about the gene expression program and metabolic changes that specifically accompany anthocyanin biosynthesis in different tissues and at different time points during plant development, a tomato system was engineered in which ROS1 and DEL expression could be experimentally induced by exogenous application of dexamethasone (DEX). This DEX-inducible system was used as described previously (Aoyama and Chua, 1997). The cDNAs of ROS1 and DEL were both inserted behind synthetic promoters containing repeats of the upstream activating sequence of the yeast gal4 gene. These promoters were regulated by a DEX regulated chimeric transcription factor GVG, consisting of the DNA-binding domain of the yeast transcription factor GAL4, the transactivation domain of the herpes viral protein VP16, and the receptor domain of the rat glucocorticoid receptor (GR). Expression of the GVG was driven by the constitutive Arabidopsis UBQUITIN10 (UBQ10) promoter (Dijken et al., 2004).

The three cassettes (GAL4-ROS1; GAL4-DEL and UBQ10-GVG) were combined on a single plasmid as part of the same T-DNA (Fig. 1A). Transformation of this construct to S. lycopersicum Microtom yielded a number of transgenic calli, which were analyzed for presence of the transgenes by exposing them to DEX, upon which some of them turn purple (Fig. 1B). Some positive calli were maintained as undefined tissue, while others were regenerated into independent transgenic lines, ROS1/DEL lines 4, 8 and 11. Application of DEX to these plants, either by direct contact or by inclusion in the water supply to the soil, resulted in the formation of a purple colour in most of tested tissues (Fig. 1). This included vegetative tissues such as roots, stems and leaves (Fig. 1, C-D). In contrast, in none of the lines, formation of purple colour was observed in flowers or fruits upon application of DEX, even not when DEX was directly applied to these tissues.

It has been shown before that upon high accumulation of anthocyanins, anthocyanin vacuolar inclusions (AVIs) are formed inside the cell vacuoles in different plant species (Grotewold, 2006; Chanoca et al., 2015). The accumulation of such AVIs were also observed here in tomato leaf epidermal cells when anthocyanin formation was induced by DEX (Supplemental Fig. S1A).

ROS1 and DEL induced metabolites in tomato seedlings

When ROS1/DEL were expressed in the leaves of Nicotiana, a single anthocyanin and a range of non-flavonoid phenolic compounds (e.g. polyamine and nor-nicotine conjugates) were produced (Outchkourov et al., 2014), while in tomato fruit, ROS1/DEL expression lead to production of a set of complex anthocyanins and flavonoids (Butelli et al., 2008; Tohge et al., 2015). This suggests that the identity of ROS1/DEL regulated metabolites depends on the species or tissue, but at present there are no data available of the consequence of ROS1/DEL expression in tomato vegetative tissues. To test this, ROS1/DEL were induced in leaf and root tissue from ROS1/DEL line 4, and an untargeted metabolite analysis was performed. Seedlings were incubated with or without the addition of DEX for 24 hours, 5d and 14d, then extracted with methanol and analysed by liquid chromatography - photodiode array – mass spectrometry (LC-PDA-MS) (Fig. 2, A-C and Supplemental Fig. S1, B-C). A rapid induction of anthocyanins (absorbing at 520 nm) was observed within less than 24 hours after DEX application, in both roots and aerial parts of the seedlings (Fig. 2, A-C and Supplemental Fig. S1, B-C). MS analysis allowed the identification of seven anthocyanins (Fig. 2C, Supplemental Table S1), all of which had been previously identified in tomato hypocotyls and ROS1/DEL fruit (Roldan et al., 2014; Tohge et al., 2015). Both tissues contained the same anthocyanins, but at different ratios and with different kinetics. In roots, maximum induction of anthocyanins was reached within 24 hours, whereas in the aerial parts of the seedlings this was
reached only after 5 days. In the absence of DEX, roots did not contain detectable anthocyanins, while only minor amounts were found in the aerial parts of the seedlings.

To identify other metabolites regulated by ROS1/DEL induction, an untargeted analysis of the LC–MS data was performed. In both tissues, the anthocyanins and a number of related flavonoids were found to be the dominant compounds, while other compound categories were much less obviously represented. In root tissue, 63 metabolites were found to be more than two-fold upregulated by DEX treatment (Supplemental Table S1A). In addition to the seven anthocyanins, seven flavonols were also found to be induced in time by DEX. Furthermore, four dexamethasone-derived metabolites were observed as well, in addition to dexamethasone itself. Thirty metabolites were found to be more than two-fold downregulated by dexamethasone treatment. For most of those, no identity could be assigned, but some ferulic acid conjugates (e.g. feruloyl-quinic acid, feruloyl-tyramine and feruloyl octopamine) were observed. In shoots, 69 metabolites were consistently found to be more than two-fold upregulated by dexamethasone treatment. Most identifiable and major compounds (anthocyanins, flavonols, DEX metabolites) correspond to those observed in roots (Fig. 2, Supplemental Table S1B and Supplemental Fig. S1, D-G). DEX itself was not visible in shoot tissue, suggesting that it can only reach the aerial parts after conjugation. Seventeen metabolites were significantly downregulated by dexamethasone treatment, none of which could be identified.

A few tissue-specific changes were observed. For example, the root feruloyl conjugates found to be downregulated by DEX were not detectable in shoot tissue, even in the control samples. In shoots, chlorogenic acid increased upon dexamethasone treatment after 24h, and had increased further after 5 days and 14 days. In root tissue, an increase was only observed after 24h, while chlorogenic acid levels did not differ significantly from the control after 5 or 14 days (Supplemental Fig. S1, D-G). Thus, ROS1/DEL expression induced predominantly anthocyanin in tomato leaf and root tissue, in addition to a number of flavonoids, most of which were known to be induced in tomato fruit. In contrast to Nicotiana, no major changes in non-flavonoid metabolites could be observed in tomato root and shoot.

Transcriptome analysis in callus and roots
To obtain a detailed understanding of ROS1/DEL function in regulating secondary metabolism, we identified genes that are controlled by these TFs on a genome-wide scale in both undifferentiated and differentiated tissue. Studying callus tissue, consisting of basically uniform, non-differentiated cells, has the advantage that developmental programs will not influence the transcriptional response to ROS1/DEL expression. On the other hand, root tissue is highly differentiated and can respond rapidly to developmental cues, which will allow to address the interaction of ROS1/DEL controlled secondary metabolism with developmental processes. Therefore, transcriptional changes upon ROS1/DEL activation were studied in roots of ROS1/DEL line 4 and of three callus cultures from independent primary transformation events. Time points were selected based on the presence of the anthocyanin biosynthetic proteins, anthocyanin synthase (SIANS) and dihydroflavanol 4-reductase (SIDFR), which have both previously been shown to be induced by ROS1 and DEL (Butelli et al., 2008). Antisera were developed that recognize recombinant SIANS or SIDFR proteins and these were used to monitor expression of both proteins by western blot. SIANS was detectable as early as 3h after induction and was increased significantly after 24h in both callus and roots (Supplemental Fig. S2). The DFR protein was clearly detectable after 24h. Therefore, samples for transcriptome analysis were taken after 3h and 24h.

In vitro-grown callus, deriving from three different primary transformants (T0) and seedlings of line 4 (T2 generation) were transferred to fresh media with and without DEX and samples were collected at 3 and 24h post induction. RNA was extracted and cDNA was analysed by Illumina sequencing. Reads were mapped onto tomato gene models to which the ROS1 and DEL cDNA sequences were added, and gene expression data were calculated. In total, expression of 5295 genes significantly changed more than two-fold (n=3; FDR < 0.05) for at least one of the time points or tissues, relative to untreated materials. Genes that were affected by ROS1 and DEL induction across all tissues and time points formed only a small subset of these genes, as can be observed in the Venn diagrams in Fig. 3A. From a total of 5295 differentially-regulated genes, 220 were consistently
upregulated and 205 were consistently down regulated in both tissues, callus and roots, and at both
time points. This set of overlapping genes was used as the core of 425 genes affected by ROS1 and
DEL (Supplemental Table S2).

To obtain an overview of the functional implications of transcriptional changes mediated by
ROS and DEL induction, gene ontology (GO) annotations for the 425 consistently regulated core
genes were analyzed (Fig. 3B). This was done by comparing, for each GO category, its frequency
among the 425 core genes to its frequency among all annotated genes in the tomato genome. In this
way, GO categories that were overrepresented among the set of up-regulated or among the set of
down-regulated genes were obtained. Significantly overrepresented GO categories among the
upregulated genes were involved in the biosynthesis of phenylpropanoids, flavonoids and
anthocyanins, as well as responses to different types of known anthocyanin-related stresses such as
carbohydrate stimuli (Das et al., 2012). These functions obviously are relevant for the well-known role
of ROS1/DEL in anthocyanin biosynthesis. Interestingly, the upregulated genes were also enriched
for GO categories involved in lipid biosynthesis and epidermal cell specification. Among the genes
down-regulated by ROS1/DEL expression, GO terms involved in cell wall organization, root
morphogenesis and the differentiation of trichomes and epidermal cells were overrepresented. It is
remarkable that GO categories that are known to function in specific differentiated tissues (e.g. root
epidermis, leaf trichomes) were found to be consistently regulated by ROS1/DEL. Apparently, genes
with these functions are also regulated by ROS1/DEL in undifferentiated callus, where they have no
clear significance to the tissue.

Regulation of pathways leading to anthocyanins by ROS1 and DEL activation

The effect of ROS1 and DEL gene expression on the regulation of individual genes was
analysed. Already after 3h of induction, ROS1/DEL already strongly activated genes of the
anthocyanin pathway, both in callus and in roots (Table 1). In view of the observation that no
anthocyanins could yet be detected at these stages, this would indicate that these genes are directly
regulated by ROS1/DEL, and not by the presence of anthocyanins. Genes involved in converting
phenylalanine to polyphenols including anthocyanins mostly overlapped with those identified to be
upregulated in ROS1/DEL fruits, encoding enzymes and transporters from the pathway leading to
anthocyanins, flavonoids and chlorogenic acid (Butelli et al., 2008); (Tohge et al., 2015). Interestingly,
several genes from the phenylalanine biosynthetic pathways were also activated after 3 hours by
ROS1/DEL, including shikimate kinase (SK1), a key enzyme of the shikimate pathway towards
phenylalanine, and cystosolic pyruvate kinase and acetyl CoA carboxylase, involved in malonyl CoA
biosynthesis (Table 1 and Fig. 4). Transketolase, an important enzyme in the pentose phosphate
pathway, which converts glucose to supply erythrose 4-phosphate (the starting point of the shikimate
pathway) was initially down-regulated in roots, while being upregulated after 24h (Table 1). Likely this
indicates that the pentose phosphate pathway is not under direct regulation of ROS1 and DEL, but is
upregulated in the root when an enhanced supply of carbon into the shikimate pathway is needed.

Biosynthetic genes not known to participate in anthocyanin biosynthesis were also found to be
regulated by ROS1/DEL expression, including for instance, genes important for auxin homeostasis. A
number of auxin transporter genes (PINs and LAXs) were already found to be downregulated 3h after
DEX induction (Table S2). Gene homologous to Auxin-regulated Indole-3-acetic acid-amido
synthetase, GH3.4 (Liao et al., 2015) was up-regulated upon DEX addition (Table S2). GH3 genes in
plants have been shown to regulate auxin homeostasis levels by conjugating the excess of active IAA
(indole acetic acid) to an inactive form.

Regulation of a transcriptional network involved in epidermal differentiation

Upon upregulation of ROS1 and DEL, a considerable number of other transcription factors
were observed to be activated in both callus and roots (Table1 and Table S2). From the core of 425
genes a total of 27 genes were transcription factors, most of which (22) were upregulated. This
indicated the possibility that ROS1/DEL not only directly activate biosynthetic genes in the anthocyanin
pathway but also regulate or at least influence, a more complex transcriptional network. Interestingly,
among the ROS1/DEL-regulated TF genes, were several that are annotated with the GO-category epidermal cell fate. One of these genes is the tomato MIXTA-like TF, which is a key regulator of epidermal cell patterning and cuticle assembly in tomato fruit (Lashbrooke et al., 2015). Also, a homologue of Glabra2 (GL2), known to be involved in root and trichome developmental programs in Arabidopsis (Rerie et al., 1994);(Bernhardt et al., 2005), was 6-fold upregulated in callus and 30-folds in roots after DEX induction. Surprisingly, one of the most strongly up-regulated TFs (125 fold in roots) only 3h after DEX induction is homologous to the Arabidopsis root-hair regulator CPC.

Confirmation of the activation of root-morphology and auxin-related genes using quantitative real time PCR

Transcriptome analysis was based on a comparison of tissues from induced and non-induced plants from the same genotype, to avoid noise from genotype-related differences in the data. Gene expression changes relevant for root morphology and for auxin regulation, were validated to confirm their dependence on the expression of ROS1/DEL, and not to result from the application of DEX per se. qPCRs were performed for a subset of genes, comparing ROS1/DEL plants (T4 generation) to WT seedlings both of which were treated with DEX. The qPCR results confirmed the up-regulation of GH3.4 and GL2 and the downregulation of PIN6 and PIN9, observed from the transcriptomics results in roots treated with DEX for 3h and 24h (Fig. 3, C-D). Furthermore, we observed up-regulation of GH3.4 and GL2 in the aerial parts of seedlings incubated with DEX for 3 and 24h (Fig. 3C).

Activation of the tomato GL2 promoter by ROS1/DEL in Nicotiana

To confirm that the tomato GL2 homologue is a possible direct target of ROS1/DEL, a reporter transactivation assay was used. The tomato GL2 promoter was fused to a luciferase reporter gene (pGL2-LUC), which enables the visualization of GL2 promoter activation. N. benthamiana leaves were agro-infiltrated with pGL2-LUC alone, or in combination with 35S:ROS1 and 35S:DEL constructs (Supplemental Fig. S3, A-B). After 3 days post infiltration, the leaves were sprayed with luciferin and incubated for 1 more day, after which luciferase activity was measured. The pGL2-LUC construct alone resulted in very low luminescence (Supplemental Fig. S3, A-B) whereas the luciferase activity was strongly induced by co-infiltration of pGL2-LUC with ROS1/DEL, confirming that ROS1/DEL control GL2 expression also in leave tissue.

Tissue specific gene activation by ROS1/DEL

Besides the genes found to be regulated in both callus and root tissues and at both time points, a number of GO-categories was found to be overrepresented among genes regulated differentially in only one of the tissues (Supplemental Fig. S4A). This analysis was performed in the same way as described above for the set of 425 genes, but this time separately per tissue. Specifically for genes differentially regulated in callus, a set of genes involved in transcriptional regulation was observed. These genes consisted of a set of 18 TFs, from diverse TF families. These TFs are different from those identified to be regulated in all tissues (the core set of genes). Also the GO term “oxidoreductase activity” was found exclusively in callus; This category included genes involved in ethylene biosynthesis, such as five 1-aminocyclopropane-1-carboxylate oxidases (ACO), two of which (including ACO2) were up-regulated by ROS1/DEL in callus, while three were downregulated.

In roots, compared to callus, a larger set of GO categories was found to be overrepresented in genes that were differentially regulated by DEX-induced ROS1/DEL expression (Supplemental Fig. S4A and Supplemental Table S3). A number of these categories relate to lipid metabolism, for instance categories lipid transport and lipid localization and lipid metabolic processes. The regulated genes in these categories encode enzymes involved in cuticle polymerization (e.g. GDSL1/cutin deficient 1 which was 94 fold up-regulated in roots) (Girard et al., 2012); (Yeats et al., 2014) and several lipid transfer proteins. Interestingly, two of the highly up-regulated genes identified in both callus and roots correspond to LACS1 (long chain acyl-CoA synthase 1) and the tomato homologue of CER1, both of which have been predicted to be involved in cutin monomer synthesis (Lu et al., 2009); (Girard et al., 2012) (Table 1). Thus, the transcriptomics data suggest a positive link between anthocyanins biosynthesis and cuticular wax biosynthesis. Another GO category, antioxidant activity,
comprised five peroxidases, 4 of which were strongly downregulated upon ROS1/DEL expression in roots. In the GO category, response to biotic stimuli, we found three highly up-regulated genes (from 87 to 160 fold), which encode homologues of the birch pollen allergen Bet v 1 (Munoz et al., 2010), while other genes from the same protein family were downregulated by ROS1/DEL.

**Functional analysis of the ROS1 and DEL activated genes**

For functional analysis of genes regulated by ROS1 and DEL, a Virus Induced Gene Silencing (VIGS) system was used in combination with DEX induction of ROS1/DEL in Microtom seedlings. The efficiency of VIGS was tested by silencing the PHYTOENE DESATURASE (PDS) gene in parallel to the genes of interest. Silencing of PDS resulted in photo bleaching (Supplemental Fig. S5A), causing white patches and white leaves and is therefore easily detected visually (Velasquez et al., 2009); (Zheng et al., 2010). After 11 days, white patches and white leaves were observed, mainly in the newly formed leaves. After induction, by transferring seedlings to DEX-containing medium the white patches became bright purple (Supplemental Fig. S5A).

Among the genes that were highly upregulated by ROS1/DEL, were putative anthocyanin acyltransferases (AAT), which could potentially contribute to modification of the anthocyanins. Two AAT genes, Solyc08g068710, here referred to as AAT-1, and Solyc12g088170, referred to as AAT-2, were tested for their effect on anthocyanin biosynthesis. While AAT-1 silencing did not result in any significant changes in anthocyanin content or composition, the effects of AAT-2 were clearly visible in an LC-MS analysis (Supplemental Fig. S5B). Major tomato anthocyanins, such as petunidin 3-(trans-p-coumaroyl)-rutinoside-5-glucoside and delphinidin 3-(trans-p-coumaroyl)-rutinoside-5-glucoside were downregulated by silencing AAT-2, while delphinidin 3-rutinoside, lacking an acyl group, was strongly upregulated. Similarly, some less-abundant quercetin acylconjugates were strongly downregulated upon silencing of AAT-2 (Supplemental Fig. S5B). These metabolic changes support a role for this gene in acylation of anthocyanins as well as other flavonoids in tomato. The role of AAT-2 in acylation of anthocyanins was already supported by in vitro enzyme activity experiments, and overexpression of the gene in tobacco flowers (Tohge et al., 2015).

**Physiological and developmental effects of ROS1/DEL induction in different tomato tissues**

As observed from the RNA sequencing data and the qRT-PCR data, ROS1/DEL overexpression upregulates the expression of the tomato GL2 homologue (Fig. 3C and Table1). In Arabidopsis, GL2 has been reported to promote trichome development and to inhibit both root-hair formation (Ohashi et al., 2002); (Masucci et al., 1996) and stomata formation (Lin and Aoyama, 2012). We therefore hypothesized that ROS1/DEL overexpression, through upregulation of a homologue of GL2, would have similar effects on tomato. Therefore, several tissues were inspected for morphological perturbation.

To address the effect of ROS1/DEL overexpression on root morphology, 5d old seedlings of ROS1/DEL lines 4, 8 and 11, WT and EV (control) plants were transferred to tilted MS-agar plates with or without DEX. After 4d of growth, the newly-formed parts of the root were studied for root-hair length. The ROS1/DEL plants showed purple roots with much shorter root-hairs than plants grown on plates without DEX (Supplemental Fig. S6A). For ROS1/DEL line 4 and WT seedlings, root hair length was quantified (n=12), and was observed to be 10-fold reduced upon DEX induction (Fig. 5). Within one root, the intensity of the purple colour of the root appeared to correlate with a reduction in root-hairs (Fig. 5B). No significant change in root-hair length or density occurred when WT tomato plants were placed on DEX (Supplemental Fig. S6B). Also the number of lateral roots was quantified after 12 days on plates with and without DEX. While EV and WT plants did not display a significant difference in the number of lateral roots upon DEX induction, seedlings from ROS1/DEL lines 4, 8 and 11 developed significantly more (up to 2-fold) lateral roots on DEX, in comparison to plants transferred to medium without DEX (Fig. 5, C-D).

In leaf epidermis tissue, effects of ROS1/DEL induction on the morphology could not be observed, apart from the purple-coloured AVIs (see above). Upon prolonged exposure of 6-weeks old WT and ROS1/DEL plants to DEX for 5-10d, no changes in the number of trichomes or number of
stomata on newly-formed leaves could be observed using microscopy (Supplemental Fig. S5, A-B).

Also, no changes in chlorophyll levels were observed by spectral analysis (Supplemental Fig. S5D). Notably, leaf conductance in the DEX-induced \textit{ROS1/DEL} plants was reduced by ± 25% (Supplemental Fig. S5C). However no consistent differences in stomatal opening could be observed using a binocular microscope, indicating that transpiration rates are potentially lowered in these plants by other mechanisms.

Seed germination was strongly affected by \textit{ROS1/DEL} overexpression. While DEX itself hardly affects germination of control seeds, germination of \textit{ROS1/DEL} seeds of lines 4 and 11 was strongly delayed on DEX medium, compared to non-DEX medium (Supplemental Fig. S6C). When seedlings were germinated on non-inducing medium, and then transferred to DEX containing medium after 5d, no growth retardation upon \textit{ROS1} and \textit{DEL} activation was observed.

Combined these results strongly indicate that regulation of anthocyanin signalling is linked to various developmental programs, some of which are executed in a tissue specific manner.
In this work, we have engineered tomato plants with a DEX-inducible system for anthocyanin biosynthesis, to study the systems biology of a secondary metabolic pathway in tomato. This is the first time that such a system has been used in a crop species such as tomato. The use of DEX-mediated promoter control allows for the precise characterization of responses to regulatory genes, such as transcription factors, and is here used to drive expression of a well-known transcription-factor pair, ROS1 and DEL. In Arabidopsis, the DEX promoter system has mainly been deployed to study gene expression programs associated with organ development, e.g. by overexpression of TFs regulating flower formation (Kaufmann et al., 2010) or trichome patterning (Lloyd et al., 1994), and recently, also processes such as secondary cell wall formation (Li et al., 2016). Myb transcription factors such as ROS1 have hardly been studied before with such tight DEX inducible systems (Morohashi and Grotewold, 2009), probably because these TFs often do not tolerate the presence of C-terminal tags such as the glucocorticoid binding domain. An indirect induction system, using an artificial TF and artificial promoters, such as used here, avoids this limitation, and allows studying the activity of the native TFs. One could exploit such an inducible system to monitor specific responses of plants and plant organs to secondary metabolic pathways.

Expression of ROS1 and DEL in tomato is known to lead to the production of anthocyanins in fruits (Butelli et al., 2008), but was never addressed in root or callus tissues. In particular, purple coloration of tomato root is not known to occur in WT tomato plants. The DEX-inducible system as it was deployed here in tomato led to controlled anthocyanin production in leaves, stems, roots and undifferentiated callus within 24h of induction. It can be used in tissue culture, in whole plants that are grown in vitro or in soil. This allows us to monitor gene expression programs and metabolite profiles that are directly controlled by the ROS1/DEL TF pair, which will take place in all these tissues and conditions. In addition, one can observe tissue-specific responses at the transcriptional level which provide insights into the interactions between the ROS1/DEL-controlled processes, and the local, organ-specific physiological conditions and developmental programs. These interactions define the role of anthocyanins and their master regulators in the physiology and development of the tomato plant.

Notably, the DEX-inducible system used in this study did not result in anthocyanin formation in tomato flower and fruit tissues. Clearly this provides a limitation to the applications of this system in tomato. Likely this observation relates to poor expression of the GVG TF from the UBI10 promoter in these tissues, since direct application of DEX to fruits and flowers also did not result in appreciable coloration. On the other hand, in the absence of DEX, no anthocyanins could be detected in roots, indicating that the regulation of the UBI10-GVG system is sufficiently tight to provide a no-expression condition, which is very useful for systems biology approaches.

**ROS1/DEL control anthocyanin-biosynthetic genes**

At the metabolite level, the response of tomato tissues to ROS1/DEL expression is visibly dominated by anthocyanins and related polyphenolic compounds. Although other metabolites were also consistently induced in all analysed tissues, their numbers are limited and less prominent in the metabolic profiles. Similarly, in tomato fruit, metabolite changes controlled by these TFs were found to be confined to anthocyanins and flavonols (Butelli et al., 2008; Tohge et al., 2015). Accordingly, the ROS1/DEL induced genes that encode biosynthetic enzymes for anthocyanins and their precursors largely correspond to those observed in these earlier transgenic studies.

Among the identified biosynthetic genes affected were two AAT-encoding genes that could putatively mediate the acylation of anthocyanins. The role of these genes was further explored using a VIGS approach in combination with DEX-induced anthocyanin biosynthesis. Tomato anthocyanins are derived from delphinidin 3-rutinoside, which is modified by methylation on the 3’ and 4’ position, glucosylated at the 5 position and acylated on the rhamnose (Butelli et al., 2008; Gomez (Roldan et al., 2014). Silencing the expression of one of the putative AATs lead to the accumulation of delphinidin 3-rutinoside in tomato leaves, demonstrating the role of this gene in anthocyanin acylation in tomato. Modifications such as methylation and 5-glucosylation apparently depend on rhamnose acylation,
since no anthocyanins lacking acyl groups but carrying methyl groups were observed. Also, only small amounts of delphinidin 3-rutinoside-5-glucoside could be observed upon silencing of AAT-2. Recently, it was shown that the SiFdAT1 gene, which corresponds to AAT-2, mediates acylation of cyanidin rutinoside in N. tabacum flowers (Tohge et al., 2015). The observed dependence of anthocyanin methylation and, to some extent, 5-glucosylation, on acylation is similar to the situation in Petunia, where the gf mutant, which localizes in an acyltransferase gene, produces anthocyanins that lack acyl-groups, 5-glucosides and methyl groups (Jonsson et al., 1984). Thus, the AAT-2 / SiFdAT1 is not only required for acylation of anthocyanins, but it is also necessary for their 5-glucosylation and O-methylation.

Interestingly, several tissue specific changes in gene expression were observed that can be related to anthocyanin biosynthesis and function. For example, a number of genes of the Bet v 1 family were observed to be highly regulated by ROS1/DEL overexpression only in roots. The Bet v 1 protein family encodes small lipocalin-like proteins with a hydrophobic core, which may contain polyphenolic compounds such as quercetin (Roth-Walter et al., 2014). A function of these proteins in anthocyanin accumulation was previously suggested from work in strawberry, where down-regulation of Fra a1, a BetV1 homolog, led to colourless fruits (Munoz et al., 2010).

A set of genes encoding peroxidases was found to be strongly downregulated only in roots, but not in callus. The role of these peroxidases is likely in controlling damage by scavenging reactive oxygen species (Davletova et al., 2005). Overexpression of ROS1/DEL in tomato fruit leads to a higher antioxidant capacity and control of the oxygen burst during fruit ripening (Zhang et al., 2013). Possibly the scavenging of reactive oxygen species by accumulating anthocyanins makes expression of peroxidases redundant, thus leading to their down-regulation. However, it remains unclear why such down-regulation is only observed in roots, and not in callus.

Regulation of epidermal programs

The DEX-controlled system for expression of ROS1/DEL apparently controls processes beyond anthocyanin biosynthesis. In fruits, effects of ROS1/DEL on ripening and glycoalkaloid accumulation have been observed (Tohge et al., 2015, Zhang et al., 2013). In the current work, substantial morphological changes were observed in roots, with regard to architecture and epidermal morphology, and in stomatal conductance. The transcriptomics analysis provides leads for mechanistic explanations of these phenomena. For instance, genes involved in auxin homeostasis and auxin flux, such as GH3.4, PINs and LAX (Liao et al., 2015) were already found to be strongly regulated 3h after DEX induction in root, callus and leaf tissue. Auxin is known to control formation of lateral roots and root hair elongation (Overvoorde et al., 2010); (Maloney et al., 2014). In addition, genes involved in epidermal cell fate regulation are strongly regulated by ROS1/DEL expression. Among those is a MIXTA-like TF gene, which was shown to regulate the epidermal cell patterning and cuticle assembly in tomato fruit (Lashbrooke et al., 2015). Genes known to act downstream of MIXTA-like were also observed to be strongly regulated by ROS1/DEL, for example genes involved in cuticular wax biosynthesis, such as CER1 and GDSL1-cutin deficient 1, and GL2. GL2 is known in Arabidopsis to play a role in root hair patterning (Girard et al., 2012); (Yeats et al., 2014), but its role in tomato has never been addressed. Now that these genes have been identified as putative actors on architecture and epithelial morphology in roots, more detailed functional studies are needed to elucidate their exact role in those processes.

Our results suggest that ROS1/DEL orchestrates anthocyanin biosynthesis by integrating and regulating a network of transcription factors, metabolic enzymes and transporters, growth, patterning, and hormonal pathways in tomato. Moreover, the presented data indicate that anthocyanin-regulating TFs like ROS1 and DEL can be linked to a more broader set of stress responses than solely anthocyanin accumulation. As with anthocyanins, the architectural changes in roots can also be related to stress due to nutrient availability and drought (Lopez-Bucio et al., 2003);(Kovinich et al., 2015). Also effects on cuticle-related gene expression and stomatal conductance could similarly be linked to protection against stress. One could interpret these results to hypothesize that MYB/bHLH transcription factor complexes share a number of programs that allow the adaptation of the plant to environmental changes. Thus, regulation of secondary metabolites such as anthocyanins appears as
an integral part of the plant’s adaptive repertoire, which also includes developmental and physiological programs. By using an inducible system for expression of such TF complexes, one can study these programs and their phenotypic consequences in different tissues, and provide novel leads for mechanisms which can be recruited by a plant for its survival and adaptation.

CONCLUSIONS

Anthocyanins are important in plants for protecting them against stress, and they are also important antioxidants in the human diet. Here a fully inducible system to make anthocyanins on demand is presented. With this we show that anthocyanin biosynthesis is integrated with changes in the architecture of the root.
MATERIALS AND METHODS

Plant Growth conditions

Tomato plants (Solanum lycopersicum) cultivars MicroTom and transgenic ROS1/DEL plants were grown in the greenhouse at ambient temperatures (>20°C) under natural light supplemented with artificial sodium lights, following a 16-h-light/8-h-dark cycle. Activation of ROS1/DEL seedlings by dexamethasone was done by placing seedlings on agar containing 10 mg/L dexamethasone. Dexamethasone was dissolved in ethanol at 10 mg/ml and diluted 1000x in the agar. As a negative control the same volume of ethanol was used. To increase ROS1/DEL activation, 3 mg/ml DEX was dripped with 10 µL drops on top of the agar near the plants. Induction experiments were done in round (base diameter 9 cm top 10.5 cm x 14 cm height) sterile plastic plant containers with a breathing strip.

For the VIGS experiments, 33d old plants were used with a maximum of 3 plants per pot supplemented with 200 mg/L cefotaxim and 50 mg/L vancomycin in the medium. Root development studies were done on large 24.5 x 24.5 cm plates with 0.6 cm of 1.3 % Duchefa daisin agar and 0.5x Duchefa MS medium. Five days after germination seedlings were transferred to plates with or without DEX. Only seedlings with intact roots were used. Plates were tilted between 45° and 60° and grown at 25°C.

Generation of inducible ROS1/DEL transgenic Plants

The DEX inducible system pTA7002 (Dijken et al., 2004) was modified by placing the genes ROSEA1 and DELILA from A. majus under the control of a DEX activated fusion protein constitutively expressed by the UBIQUITIN10 promoter. Coding sequence of ROS1 and DEL from (Outchkourov et al., 2014) were amplified using the primers: XhoI_ROS, ROS_SpeI and XhoI_Del, Del_Spe (TableS4) and ligated into the pTA7002 (UBQ10) (Dijken et al., 2004) vector digested with XhoI and SpeI restriction enzymes to generate pTA7002(UBQ10)-ROS1 and pTA7002(UBQ10)-DEL constructs. Next the pTA7002(UBQ10)-DEL vector was modified to contain an extra multiple cloning site (pTA7002(UBQ10)DEL-MSC). Briefly pTA7002(UBQ10)-DEL was amplified by PCR using oligonucleotides: AsiSI_FW and MauBI_Rev (TableS4). The obtained PCR fragment was ligated into pTA7002(UBQ10)-DEL pre-digested with the same enzymes to generate pTA7002(UBQ10)DEL-MSC. A new PCR was conducted using the oligonucleotides MauBI_B_FW and ApaI_FW using pTA7002(UBQ10)-ROS1 as a template and the obtained PCR fragment was ligated in pTA7002(UBQ10)DEL-MSC digested with Apal and MauBI to generate pTA7002(UBQ10) ROS1/DEL. The resulting vectors (pTA7002(UBQ10) ROS1/DEL and pTA7002) were transformed into Agrobacterium tumefaciens strain AGL0 using electroporation and transformed to MicroTom wild-type plants as described before (Karlova et al., 2011; Bemer et al., 2012). Primary (transgenic) callus was obtained during the plant transformation procedure and was maintained on MS medium supplemented with 1.0 mg/l 2,4-dichlorophenoxyacetic acid and 0.1 mg/l kinetin. ROS1/DEL positive callus was selected by transferring part of the callus to DEX-containing medium and screening for formation of purple colour after 48h. Seed from T2 to T4 generations, self-pollinated transgenic plants were used for all experiments, including WT segregants from the same population. Seeds were sterilized in 1% bleach for 20 minutes and washed 2 times for 5 minutes in sterile water, dried on sterile filter paper for 10 minutes and sowed on 0.8% Duchefa Daisin agar containing 2.2 g/L Duchefa MS with vitamins. 25 seeds were used per sterile plastic rectangular container, Duchefa (base: 12 cm x 6.5 cm: top 8 cm x 14 cm: height: 7 cm) with a breathing strip in the lid. Containers with seeds were placed at 4°C overnight and grown for 5-9d at 25°C at 16h light/8h dark.

Generation of the constructs

Construction of pTRV2 plasmids for virus induced gene silencing

Fragments for VIGS were obtained from a frozen sample of purple tomato fruit (Butelli et al., 2008), which was used as a source of RNA. mRNA extraction was performed using the QIAGEN RNeasy® kit. cDNA was synthesized with the Iscript cDNA synthesis kit (BioRad). VIGS fragments were designed by using the VIGS tool from www.solgenomics.com. PCR fragments were obtained with
Phusion DNA polymerase (ThermoFisher). AAT VIGS fragments were amplified using primers AAT-2VIGSFw (300bp fragment) and AAT-2VIGSRev, AAT-1Fw and AAT-1Rev. The PDS-VIGS fragment was described previously (Romero et al., 2011). PCR products sizes were confirmed on 1% agarose and excised from gel. The PCR products and pTRV2 vector were digested with EcoRI and XhoI (NewEngland). Digested PCR products were purified from the gel. pTRV2 and VIGS fragments were ligated with T4 ligase for 3h at room temperature. The full length ORF of AAT-2 was obtained from genomic DNA with the primers AAT-2ORFFw and AAT-2ORFRev.

The PDS-VIGS fragment was described previously (Romero et al., 2011). PCR products sizes were confirmed on 1% agarose and excised from gel. The PCR products and pTRV2 vector were digested with EcoRI and XhoI. Digested PCR products were purified from the gel. pTRV2 and VIGS fragments were ligated with T4 ligase for 3h at room temperature. The full length ORF of AAT-2 was obtained from genomic DNA with the primers AAT-2ORFFw and AAT-2ORFRev. PCR products were gel purified and TOPO cloned into the pCR8/GW/TOPO-TA vector (Invitrogen). After sequence verification the ATT fragment was transferred by GATEWAY recombination to pK7WG2 to create p35S-AAT. The plasmids obtained were then introduced into Agrobacterium tumefaciens AGL0 as described before (Outchkourov et al., 2014). All the constructs were verified by sequencing (EZ-seq Macrogen). AGL0 harboring an empty pBINPLUS (pBIN) plasmid (Van Engelen et al., 1995) was used as a negative control.

**Constructs used for transactivation assay**

The GL2 promoter (pGL2, 1938bp) was amplified from tomato genomic DNA using the primers Gl2promoterFw and Gl2promoterRev (Table S4). The fragment was A-tailed and inserted into the pCR8/GW/TOPO-TA vector (Invitrogen). The sequence was verified and pGL2 was recombined into pGKGWG (containing the GFP reporter gene) and pGreen-LUC (containing the luciferase reporter gene, kindly provided by Dr. Franziska Turck) vectors (Adrian et al., 2010). Plasmids were transformed into A. tumefaciens strain AGL0 for plant infiltration.

**Virus induced gene silencing of in vitro tomato seedlings**

VIGS was based on the method described before (Gomez Roldan et al., 2014). Seedlings (15 per container) were raised in a container on 0.5xMS agar. The pTRV1, pTRV2, pTRV2-AAT1 and pTRV2-AAT2 vectors in A. tumefaciens were grown at 28 °C with shaking in LB medium containing 50 µg/ml kanamycin and 25 µg/ml rifampicin. After 24 h, culture aliquots were transferred to 1.6 mL YEB medium (0.5% beef extract, 0.1% yeast extract, 0.5% peptone, 0.5% sucrose, 2 mM MgSO4, 20 µM acetylsyringone, 10 mM MES, pH 5.6) plus antibiotics, and grown for 3 hrs. After this, the cells were washed twice in 10 mM MgCl2, 100 µM acetylsyringone, and resuspended in co-cultivation medium (0.25 x MS with vitamins pH 6, 0.1% sucrose, 100µM acetylsyringone, 0.005% Silwet L-77), and painted onto leaves of 9d old seedlings (T2 generation). After inoculation with Agrobacterium, seedlings were kept at 21°C and allowed to recover and grow for 24 days before DEX induction. Activation of ROS1/DEL by dexamethasone was done by placing seedlings on agar containing 10 mg/L dexamethasone for 3d, after which the seedlings were harvested for analysis.

**Trans-activation assays**

Agrobacterium clones were grown for 24 hours at 28°C in LB medium (10g/L tryptone, 5g/L Yeast extract, 10g/L NaCl) with antibiotics (kanamycin 50µg/ml or spectinomycin 100µg/ml and rifampicin 25µg/ml). The OD of the cultures was measured at 600nm and the bacteria were re-suspended in infiltration media (10mM MES buffer, 10mM MgCl2, 100µM acetylsyringone) to an OD of 0.5. After 3h incubation with rotation, leaves of 4-5 weeks old Nicotiana benthamiana plants were infiltrated as described before (Outchkourov et al., 2014). After 3 days Agro-infiltrated leaves were sprayed with luciferin (1mM) to inactivate accumulated luciferase. Next day the leaves were again sprayed with luciferin, and 5 minutes after the treatment the leaves were cut from the plant and measured with a cooled CCD camera. Leaves were places on a plastic tray in a box coated with aluminium foil to reduce noise in the pictures caused by cosmic rays. Measurements were made for 5 or 10 minutes. Emission of luminescence has a maximum at 560nm therefore a filter in the camera was used to block most other wavelengths. Analysis of the pictures was performed with ImageJ software. The measured intensity is proportional to the amount of luciferase produced. The intensity of selected areas was measured and values were processed using IPM SPSS Statistics 22.

**Generation of anti ANS antibody**
ORFs of tomato DFR and ANS genes were PCR-amplified from a total cDNA isolated from purple tomato fruits (Butelli et al., 2008) using the oligonucleotides: tDFR_Fw, tDFR_Rev and tANS_Fw, tANS_Rev. The obtained PCR fragments were gel purified, digested with EcoRI-BglII (for DFR) and EcoRI-SalI (for ANS) restriction enzymes and ligated into the pACYCDuet-1 vector (Novagen). The newly prepared constructs were sequence verified and immobilized into E.coli BL21DE3 cells. Bacterial cultures at an optical density 600 nm of 0.8, were induced with 0.1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) and incubated overnight at 18°C. Soluble protein was extracted and Histagged proteins were purified on a Ni-NTA column (Qiagen). Subsequently, fractions containing tDFR and tANS4 protein were further purified over a Superdex 75 (GE Healthcare Life Sciences) 10/30 column in 100 mM Tris-HCl pH8 buffer. Protein purity of >95% as visible using SDS-PAGE was used for rabbit immunization at Eurogentec. Western blot analysis was done as described before (Outchkourov et al., 2014). Primary antibodies were diluted 1:1000 and secondary anti-Rabbit IgG (whole molecule)–Alkaline Phosphatase antibody produced in goat (SigmaAldrich A3687) was diluted 1:6000. After washing, signals from the blots were developed using substrate tablet BCIP®/NBT from SigmaAldrich.

LC-PDA-MS analysis

Samples of three biological replicates were used for analysis. Semi-polar compounds were extracted and analysed as described in Moco et al. (Moco et al., 2006). Tissues were snap-frozen, and subsequently ground to a fine powder using mortar and pestle. The powder derived from the tomato tissues was weighed exactly (90 - 110 mg dry weight), and was extracted using 10 volumes of 70% methanol solution acidified with 1% v/v formic acid. Samples were sonicated and filtered through a 0.45 µm filter before LC-MS analysis. Separation was achieved using a Luna C18 (2) pre-column (2.0 x 4 mm and an analytical column (2.0 x 150 mm, 100 Å, particle size 3 µm), both from Phenomenex (Torrance, CA, USA). Samples (5 µl) were injected and eluted using formic acid/water (1:1000 v/v; eluent A) and formic acid/acetonitrile (1:1000 v/v; eluent B) as elution solvents. The flow rate was set at 0.190 mL min\(^{-1}\) with the following linear gradient elution program: 5% B to 35% B over 45 min, with washing for 15 min to equilibrate before the next injection. The column temperature was maintained at 40°C. UV absorbance analysis was performed with a Waters 2996 photodiode array detector (range from 240 to 600 nm) and metabolite masses were detected using a LTQ Orbitrap XL hybrid MS system (Waters) operating in positive electrospray ionization mode heated at 300°C with a source voltage of 4.5 kV for full-scan LC–MS in the m/z range 100–1500. Acquisition and visualization of the LC–FTMS data were performed using Xcalibur software. The MetAlign software package (www.metAlign.nl) (Lommen, 2009) was used for baseline correction, noise estimation, and spectral alignment. Aligned masses were directly used for further analysis. Comparison and visualization of the main features of the LC–MS data were performed by loading the data matrix into GeneMaths XT 1.6 software (www.applied-maths.com). Metabolite intensities were normalized using log2 transformation and standardized using range scaling (autoscaling normalization).

RNA-sequencing and data analysis

Primary callus from three independent transformation events (T0 generation) was grown in vitro in MS medium supplemented with 1.0 mg/L 2,4-dichlorophenoxyacetic acid (2,4-D) and 0.1 mg/l kinetin. From line ROS1/DEL-04 (T2 generation), four week old seedlings were grown in ½ MS medium, both with 8% agar. Both calli and seedlings were placed on new medium with or without 10 mg/L dexamethasone. Dexamethasone was dissolved in ethanol at 10 mg/ml and diluted 1000x in the agar. As a negative control the same volume of ethanol was used. Triplicates of each induction timepoint – DEX, 3h+DEX and 24h+DEX of both tissues were taken for analysis giving in total 18 samples. Total RNA was extracted from 50 mg ground tissues using the RNEasy Plant Mini Kit from Qiagen according to the manufacturer’s instructions. Purified poly(A) RNA was used to produce libraries using a TrueSeq RNA library Prep Kit (Illumina) following the manufacturer’s instructions. Pooled libraries were sequenced on an Illumina HiSeq 2000 by WUR-Applied Bioinformatics (The Netherlands).
Differential expression was analysed by CLCBIO software, using tomato ITAG2.4 gene models (Tomato Genome, 2012). GO term analysis was performed using the tool available at http://bioinfo.bti.cornell.edu/tool/GO/GO_enrich.html (Boyle et al., 2004). This determines whether any GO terms annotated to a specified list of genes occur at a frequency greater than that would be expected by chance. It calculates a P-value using the hypergeometric distribution followed by Benjamin-Hochberg multiple testing correction, applying a False Discovery Rate cut-off of 0.1.

Gene expression analysis by quantitative PCR.
Total RNA was extracted using the RNeasy Plant Mini Kit from Qiagen. cDNA synthesis, and real-time quantitative PCR (qPCR) were performed as described before (Karlova et al., 2013). The primers used for qPCR are listed in Table S4.

Microscopy
Micro-Tom wild type and ROS1/DEL 7d old seedlings were transferred to plates (base: 11cm, height: 11cm) with or without DEX. Seedlings were placed ± 2 cm from the top of the plate in order to allow the roots to grow downwards: root length was marked. Plates were placed in an angle of 60° (16h light, 25°C). Microscopy was performed 3d after transferring the seedlings to the plates. Two pictures were made of five roots per treatment. Close-up root pictures to show root hair development were also made by placing the camera on a ZEISS stemi SV11 binocular by means of an adapter. From each picture the length of 10 root hairs was measured using ImageJ. In total, 20 root hairs per root and 100 root hairs per treatment were measured.

Scanning electron microscopy (SEM)
Small pieces of Micro-Tom Wild type and ROS1/DEL leaves were attached on a brass Leica sample holder with carbon glue (Leit-C, Neubauer Chemikalien, Germany). The holder was fixed onto the cryo-sample loading system (VCT 100, Leica, Vienna, Austria) and simultaneously frozen in liquid nitrogen. The frozen holder was transferred to the cryo-preparation system (MED 020/VCT 100, Leica, Vienna, Austria) onto the sample stage at -92 °C. For removal of frost contamination on the sample surface the samples were freeze dried for 5 min at -92°C and 1.3x10-6 mbar. After sputter coating with a layer of 20 nm tungsten at the same temperature the sample holder was transferred into the field emission scanning electron microscope (Magellan 400, FEI, Eindhoven, The Netherlands) onto the sample stage at -120°C. The analysis was performed with SE detection at 2kV and 6.3pA. SEM pictures were taken at a magnitude of 250x, and trichomes, hairs and stomata were counted on squares of 500x500μm.

Measurement of chlorophyll content with Pigment Analyzer
For non-destructive measurements of total chlorophyll levels, a CP Pigment Analyzer PA1101 (Control in Applied Physiology, Germany) was used according to the manufacturer’s instructions.

Measurement of stomatal conductance
Stomatal conductance (mmol H₂O m⁻² s⁻¹) was measured on the abaxial side of the leaf, using a Decagon leaf porometer SC-1 (Decagon devices, Pullmann WA, USA).

Pictures
Photos were made with a CANON Powershot G12.

Statistical analyses
Statistical analyses were performed using IBM SPSS Statistics 23. For root hair analysis an ANOVA was performed with genotype and DEX treatment in a model. The natural logarithm of the root hair length was calculated to obtain a normal distribution and values were used in the ANOVA (ref) and LSD test. For the luciferase assay background values were subtracted and log values were calculated.
from remaining luminescence values to achieve a normal distribution. Values were used for a LSD test.
SUPPLEMENTAL MATERIAL

Figure S1. Metabolite analysis of ROS1/DEL activated tomato plants.

Figure S2. Induction of ANS and DFR proteins upon DEX induction.

Figure S3. Direct activation of GL2 by ROS1/DEL and pleiotropic effects of anthocyanin induction.

Figure S4. Functional categories of genes significantly regulated by ROS1/DEL in a tissue specific manner.

Figure S5. Virus induced gene silencing of anthocyanin acyltransferase.

Figure S6. Phenotypic effects of anthocyanin induction in root tissue.

Figure S7. Phenotypic effects of anthocyanin induction in leaf tissue.

Table S1. Metabolite analysis of roots and shoots of ROS1/DEL activated tomato plants.

Table S2. Gene expression profiles after DEX activation of ROS1/DEL TFs.

Table S3. GO categories only in roots. Functional categories of the UP- and Down-regulated tissue specific genes by ROS1/DEL

Table S4. Oligonucleotides used in this study.

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The authors have no conflict of interest. The authors would like to acknowledge, Bert Schipper for LC-MS measurements. The authors thank Prof. Cathie Martin for E8:ROS1/DEL tomato seeds.

ONE SENTENCE SUMMARY

A systems biology study reveals that anthocyanin biosynthesis in tomato vegetative tissue is accompanied by changes in the epidermis and architecture of the root.
FIGURES LEGENDS:

Figure 1. DEX inducible system for anthocyanin accumulation in tomato. (A) Schematic presentation of the constructs used in the study: Promoter of the *ubiquitin 10* gene from Arabidopsis drives the expression of a chimeric transcription factor consisting of the yeast GAL4 DNA recognition motive-VP16 activation domain and glucocorticoid binding domain (GR). *ROS1* and *DEL* expression is controlled by a separate cassettes driven by promoter with gal4 binding sites. (B) Representative primary callus before induction and two weeks after induction with DEX. (C) Representative seedlings of *ROS1/DEL* line 4, 5 days after DEX induction. (D) Plants of *ROS1/DEL* line 4 in soil two weeks after induction with DEX.

Figure 2. Metabolite analysis of *ROS1/DEL* activated tomato plants. Examples of chromatograms from LC-PDA-MS at 520 nm showing 7 peaks specific for anthocyanins in roots (A) and shoots (B) of tomato seedling, in the absence of DEX, or induced with DEX and sampled after 24 hours, 5 days and 14 days. (C) Core structure of the tomato anthocyanins; (D) Identity of the anthocyanins observed in (A).

Figure 3. Gene expression profiles after DEX activation of *ROS1/DEL*. (A) Venn diagrams showing the overlap of up- and down-regulated genes (p<0.05, fold 2) in the different tissues and time points. A core of 220 up- and 205 down-regulated genes were identified as tissue and time independent functional targets of *ROS1/DEL*. (B) Functional GO categories of the Up-regulated and Down-regulated genes. Bars represent p-values of GO categories which are significantly overrepresented (left) and underrepresented (right), comparing to all GO-annotated genes on the tomato genome. (C-D) Quantitative RT-PCR confirmation for *GH3.4*, *GL2* (C), *PIN6* and *PIN9* (D) gene expression (n=4 biological replicates; shown are mean values with standard deviations). *ROS1/DEL* line 4 seedlings (T4 generation) and WT seedlings were incubated with DEX for 3h and 24h. After that the roots and the shoots were analysed separately.

Figure 4. Genes regulated by *ROS1/DEL* in the anthocyanin pathway. Schematic overview of the anthocyanin biosynthetic pathway and the genes changing upon induction with DEX. More intense red colour indicates stronger upregulation.

Figure 5. Phenotypic effects of *ROS1/DEL* activation on root morphology. (A) Root hair length of *ROS1/DEL* line 4 plants and WT plants in the presence and absence of DEX. Shown are mean values (n=12) and standard deviation. (B) Sectors with less purple coloration have longer root hairs. (C) Influence of *ROS1/DEL* activation on the number of lateral roots in *ROS1/DEL* line 4. Plants were transferred to media with or without DEX 5 days after germination and scored for lateral roots after 12 days of DEX induction. Black dots are placed at the end of each lateral root. (D) Number of lateral roots in the absence and presence of DEX in WT MicroTom, EV control and *ROS1/DEL* lines 4, 8 and 11. Shown are mean number of lateral roots (n=7): asterisks indicate significant differences between induced and non-induced seedlings from the same line after 12 days of growth (students t-test: *: p<0.05; **: p<0.01). Error bars represent the standard deviation.
### Table 1. Genes regulated upon DEX induction.

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<th>Roots 3h</th>
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<td>Chalcone isomerase</td>
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<td>Soly11g066580</td>
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<td>Flavonoid-3'-monooxygenase</td>
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<td>1585</td>
<td>25118</td>
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<td>Dihydroflavonol 4-reductase</td>
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<td>251</td>
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<th>Fold-change in Roots (3h)</th>
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<td>Acyltransferase</td>
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**Pentose phosphate pathway**

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**Shikimate pathway**

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**Phenylpropanoid pathway**

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Shown are accession numbers (1st column), gene abbreviation (2nd column), putative function (3rd column), fold-change induction upon DEX application in callus after 3h (4th column), in roots after 3h (5th column), in callus after 24h (6th column) and in roots after 24h (7th column) and a reference for the function of the gene (8th column). Only genes for which a t-test indicates that expression upon DEX induction is significantly different from non-inducing conditions (t-test n=3; P≤ 0.05) are shown. More genes can be found in Supplemental Table S2.
REFERENCES


Chezem WR, Clay NK (2016) Regulation of plant secondary metabolism and associated specialized cell development by MYBs and bHLHs. Phytochemistry 131: 26-43


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Figure 1. DEX inducible system for anthocyanin accumulation in tomato. (A) Schematic presentation of the constructs used in the study: Promoter of the ubiquitin 10 gene from Arabidopsis drives the expression of a chimeric transcription factor consisting of the yeast GAL4 DNA recognition motive-VP16 activation domain and glucocorticoid binding domain (GR). ROS1 and DEL expression is controlled by a separate cassettes driven by promoter with gal4 binding sites. (B) Representative primary callus before induction and two weeks after induction with DEX. (C) Representative seedlings of ROS1/DEL line 4, 5 days after DEX induction. (D) Plants of ROS1/DEL line 4, 5 days after DEX induction.
Figure 2. Metabolite analysis of ROS1/DEL activated tomato plants. Examples of chromatograms from LC-PDA-MS at 520 nm showing 7 peaks specific for anthocyanins in roots (A) and shoots (B) of tomato seedling, in the absence of DEX, or induced with DEX and sampled after 24 hours, 5 days and 14 days. (C) Core structure of the tomato anthocyanins. (D) Identity of the anthocyanins observed in (A).
Figure 3. Gene expression profiles after DEX activation of ROS1/DEL. (A) Venn diagrams showing the overlap of up- and down-regulated genes (p<0.05, fold 2) in the different tissues and time points. A core of 220 up- and 205 down- regulated genes were identified as tissue and time independent functional targets of ROS1/DEL. (B) Functional GO categories of the Up-regulated and Down-regulated genes. Bars represent p-values of GO categories which are significantly overrepresented (left) and underrepresented (right), comparing to all GO-annotated genes on the tomato genome. (C-D) Quantitative RT-PCR confirmation for GH3.4, GL2 (C), PIN6 and PIN9 (D) gene expression (n=4 biological replicates; shown are mean values with standard deviations). ROS1/DEL line 4 seedlings (T4 generation) and WT seedlings were incubated with DEX for 3h and 24h. After that the roots and the shoots were analysed separately.
Figure 4. Genes regulated by ROS1/DEL in the anthocyanin pathway. Schematic overview of the anthocyanin biosynthetic pathway and the genes changing upon induction with DEX. More intense red colour indicates stronger upregulation.
Figure 5. Phenotypic effects of ROS1/DEL activation on root morphology. (A) Root hair length of ROS1/DEL line 4 plants and WT plants in the presence and absence of DEX. Shown are mean values (n=12) and standard deviation. (B) Sectors with less purple coloration have longer root hairs. (C) Influence of ROS1/DEL activation on the number of lateral roots in ROS1/DEL line 4. Plants were transferred to media with or without DEX 5 days after germination and scored for lateral roots after 12 days of DEX induction. Black dots are placed at the end of each lateral root. (D) Number of lateral roots in the absence and presence of DEX in WT MicroTom, EV control and ROS1/DEL lines 4, 8 and 11. Shown are mean number of lateral roots (n=7): asterisks indicate significant differences between induced and non-induced seedlings from the same line after 12 days of growth (students t-test: *: p<0.05; **: p<0.01). Error bars represent the standard deviation.


Chezem WR, Clay NK (2016) Regulation of plant secondary metabolism and associated specialized cell development by MYBs and bHLHs. Phytochemistry 131: 26-43


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