Arabidopsis flower specific defense gene expression patterns affect resistance to pathogens

Luisa Ederli1, Adam Dawe2*, Stefania Pasqualini1, Mara Quaglia3, Liming Xiong2 and Chris Gehring2*

1 Department of Chemistry, Biology and Biotechnology, University of Perugia, Perugia, Italy
2 Division of Biological and Environmental Sciences and Engineering, King Abdullah University of Science and Technology, Thuwal, Saudi Arabia
3 Department of Agricultural, Food and Environmental Sciences, University of Perugia, Perugia, Italy

INTRODUCTION

Successful plant sexual reproduction relies on many factors including optimal time of flowering. Consequently, mechanisms have evolved that integrate environmental signals such as light and temperature, with endogenous developmental signals such as autonomous and gibberellin-dependent pathways to regulate flowering time (Simpson and Dean, 2002). However, plants exposed to adverse environmental conditions can activate the flowering program prematurely. Stress factors that are able to promote flowering include pathogen attacks, high levels of ultraviolet light, poor nutrition and drought stresses (Martinez et al., 2004). It is also observed that salicylic acid (SA) regulates flowering in Arabidopsis, likely acting as a link between stress responses and the regulation of reproductive development (Martinez et al., 2004). SA-mediated floral promotion appears to be regulated through sumoylation and involves chromatin modifications (Jin et al., 2008). Indeed, SA-dependent regulation of chromatin modification through histone replacement mechanisms may be responsible for maintaining a concomitant repressive state of both systemic acquired resistance to pathogens and transition to flowering in Arabidopsis (March-Diaz et al., 2008). However, the molecular mechanisms of SA-dependent flowering induction remain elusive and little attention has been given to the accumulation of SA in floral organs nor indeed to the function of elevated SA levels, e.g., in the response to microbial pathogens (e.g., Thomma et al., 1998; Noutoshi et al., 2012).

Given that the flower is the organ of sexual reproduction, it is reasonable to expect that this organ has evolved effective morphological structures and mechanisms to protect itself from pathogens. There is growing interest in morphological structures, compounds and mechanisms of biotic defense of the flower (e.g., Rodrigues Marques et al., 2014). However, despite the fact that...
work in the Arabidopsis model system does offer the obvious advantages of access to a complete genome and transcriptome data as well as a large mutant collection, defense studies in Arabidopsis flowers have remained scarce, not least because of the comparatively small organ size.

It is somewhat surprising that hardly any attention has been given to the role of the sepal in the defense of plants against pathogens. Exceptions are an early report that states that in tobacco a pathogenesis-related protein (PR-1) accumulates in the sepal (Lotan et al., 1989), and more recently, PR-5 promoter activity was observed in tobacco sepal tips (Kenton et al., 2000). There is no report in the literature that would suggest a role of the sepal in the defense against pathogens and this may well be due to the small size of the floral tissue which in the past has limited molecular and biochemical analyses.

In Arabidopsis thaliana the sepals are modified green leaf-like organs that enclose the developing flower. They form the outermost whorl - the calyx - of the flower. Early flower development is divided into 12 stages beginning with the initiation of a floral buttress on the flank of the apical meristem (stage 1) and ending with the rapid extension of the petals to the height of the medial stamen (stage 12) (Smyth et al., 1990). The sepal primordia arise in stage 3 and outgrow the flower primordium (stage 4). Petal and stamen primordia appear at stage 5 and end up completely enclosed by the sepals (Smyth et al., 1990). The Arabidopsis flower organs are arranged in concentric whorls as four sepals, four petals, six stamens and two fused carpels (Bosinger and Smyth, 1996). A distinct feature of the sepals is that they contain cells of vastly different sizes, notably the polyploid giant pavement cells that have arisen through endo-reduplication (Roeder et al., 2010), performing karyokinesis but not cytokinesis. While the function of these pavement cells remains unclear, it has been speculated that they may play a role in the defense against insect predators, prevent water stress, and improve the mechanical properties of the organ (Traas et al., 1998), albeit by unspecified mechanisms.

Here we make use of the Arabidopsis model system to perform a comparative system analysis (Meier and Gehring, 2008; Meier et al., 2010) of the sepal and petal transcriptome with a view to gain insight into aspects of organ specific defense responses against pathogen attack. In addition, we also measure SA in the flower and describe responses of the flower to both biotroph and necrotroph pathogens. Finally, we propose that the sepal with its specific morphological characteristics functions not only as a mechanical but also (bio-)chemical defense shield for the developing reproductive organs.

MATERIALS AND METHODS

PLANT MATERIAL

Arabidopsis thaliana L. Heynh. wild-type Columbia (Col-0) and transgenic nahG plant which is defective in the SA accumulation (Lawton et al., 1995) were used in this study. Seeds were surface-sterilized first in 70% (v/v) ethanol and then in 7% (v/v) sodium hypochlorite with 0.2% (w/v) Triton X-100 for 8 min at room temperature under a sterile laminar flow hood. Seeds were rinsed three times with sterile distilled water and re-suspended in 500 μL sterile distilled water. Plants were grown in soil (Patzer Einheitserde, Manna Italia, Bolzano, Italy) in 10 cm pots in a growth chamber with a 14-h photoperiod, a photosynthetic photon fluence rate of 120 μmol m⁻² s⁻¹, day/night air temperatures of 22°C/20°C, and a relative humidity of 60–75%. The plants were watered by sub-irrigation. All seeds were treated at 4°C for 2 days before moving to the growth environment. For all analyses leaves were sampled from 4 week old plants, whereas sepals and petals were taken from completely open flowers corresponding to development stage 14–15 (Smyth et al., 1990) of 6–7 week old plants.

SCANNING ELECTRON MICROSCOPY

Plant samples from whole soil grown plants were detached with a dissecting knife and immediately placed on a 6 mm-wide double adhesive and conductive tape (Canemco Inc., Quebec, Canada) that was pre-attached onto the specimen stage. The specimen was examined with a bench-top scanning electronic microscope (NeoScope JCM-5000, Jeol Ltd, Tokyo, Japan) and images were acquired using the software provided by the manufacturer.

FREE AND TOTAL SA EXTRACTION AND QUANTIFICATION

For SA quantification, four fully expanded leaves were harvested from 9 individual 4 week old plants of two independent cultivations. Sepal and petal SA quantification was carried out twice, sampling 4 mg for each replicate (approximately 80 sepals or petals). Plant material was quick-frozen with liquid nitrogen and stored at −80°C until processed for SA quantification and fungal DNA quantification. To perform SA extraction and quantification, leaf (500 mg FW) and sepal or petal (4 mg FW) samples were pulverized under liquid N₂ and homogenized in a mortar with 1.5 mL 90% (v/v) methanol in water. The homogenate was centrifuged at 11,000 g for 5 min and the extraction repeated with 0.5 mL 100% methanol. The recovery was evaluated by adding 2.5 μL of o-anisic acid (10 mg mL⁻¹) as internal standard in the first extraction mixture. All the data were corrected for SA recovery, which ranged from 85 to 100%. After the two extractions the supernatants were combined and the methanol: water mixtures were evaporated in a speed vacuum concentrator with heat (40°C) (Heto, Heto-Holten, Gydevang, Denmark). To avoid sublimation of SA during solvent evaporation 0.2 M sodium hydroxide was added to combined supernatants before concentration. The residue was suspended in 1 mL of 5% trichloroacetic acid (TCA), mixed by vortex for 10 min, and divided into two 0.5 mL aliquots. One aliquot was passed through 0.2 μm Millipore filters; then, the sample was partitioned with 1 mL of a 1:1 (vol/vol) mixture of ethyl acetate/cyclopentane containing 1% (vol/vol) isopropanol. The uppermost organic phase containing the free SA was then dried by using speed vacuum concentrator. The dried extract was suspended in 0.2 mL of the HPLC mobile phase [methanol: 2% aqueous acetic acid, 45:55 (v/v)] and free SA content was quantified by HPLC. The amount of total SA was quantified as follows: the TCA re-suspended aliquot after filtration through 0.2 μm Millipore filters was added to 1.25 mL of 8 M HCl and hydrolyzed for 1 h at 90°C to release SA from any acid-labile conjugated forms. The released free SA was then partitioned with 3.25 mL of a 1:1 (vol/vol) mixture of ethyl acetate/cyclopentane containing 1% (vol/vol) isopropanol. The
top organic phase was dried by using speed vacuum concentrator, resuspended in 0.2 mL of the HPLC mobile phase and analyzed by HPLC. Analysis of free SA was performed in HPLC (Jasco, Tokyo, Japan) by using a 5 μm C18 column (Luna, 150 mm x 4.6 mm; Phenomenex, Inc., Torrance, CA) with isocratic elution. The SA quantification was obtained with a spectrophotofluorometer detector (Jasco, Tokyo, Japan) using Ex = 209 nm and Em = 402 nm and SA concentrations were calculated using a linear range of calibration standards from 0 to 100 ng SA.

**GOLOVINOMYES CICHORACEARUM INOCULATION**

The inoculum of Golovinomyces cichoracearum (D.C.) V.P. Heluta (formerly Erysipe cichoracearum D.C.) (Quaglia et al., 2012) were maintained on tobacco plants cv Havana 425 and refreshed on new tobacco plants 10 days before use for Arabidopsis inoculation. Conidia were harvested from tobacco plants by irrigation with sterile deionized water added with 0.04% (v/v) of the surfactant Tween® 20 [10% (v/v) aqueous solution, Boehringer Mannheim, Germany]. The inoculum concentration was measured by hemocytometer and adjusted to 1 × 10⁶ conidia mL⁻¹. For leaf inoculation, 4 week old Arabidopsis plants were sprayed with the conidial suspension using a hand atomizer until run-off. Floral spray inoculation was carried out in the same manner on the whole attached inflorescence from 6 to 7 week old Arabidopsis plants. In addition, we also sprayed detached rosette leaves from 4 week old Arabidopsis plants and sepals and petals taken from flowers at stages 14 and 15 (Smyth et al., 1990). All samples were placed in Petri dishes (9 cm diameter) containing 1.2% sterilized water-agar (WA). Plants and plates were incubated in the growth chamber under the conditions described above and the plates were incubated in the growth chamber at the conditions described above. The spray inoculation technique allowed to obtain competitiveness of the fungus (forward 5′-TGATGTCAGGTGCTGCTG-3′ and reverse 5′-ACTCTCTTTAGGTCTTTTG-3′), or primer sequences specific for *G. cichoracearum* were derived from the ribosomal ITS region of the fungus (forward 5′-GGTTGTGTCGCCAGAGACC-3′ and reverse 5′-TGATGTCAGGTGCTGCTG-3′) as reported elsewhere (Chen et al., 2008). Cycling parameters were as follows: initial denaturation at 94°C for 2 min, followed by 28 cycles of 1 min at 94°C, 1 min at 58°C, 1.5 min at 72°C, and final extension at 72°C for 5 min. Serial dilutions of pure genomic DNA from *G. cichoracearum* and Arabidopsis were used to trace a calibration curve that was used to quantify plant and fungal DNA in each sample. Results are presented as a ratio between fungal and plant DNA in the leaf and sepal. Each data point represents the mean of two independent biological samples.

**BOTRYTIS CINEREA INOCULATION AND LESION EVALUATION**

An isolate of *Botrytis cinerea* Pers. ex Fr. (Quaglia et al., 2011) was used essentially as described elsewhere (Muckenschnabel et al., 2002), conidia were harvested by irrigation with a sterile aqueous solution of 10 mM sucrose and 10 mM KH₂PO₄ added with 0.04% (v/v) of the surfactant Tween®20 from 10 days old colonies grown on Potato Dextrose Agar, at 21 ± 2°C, in the dark. The spore suspension was passed through two layers of cheese cloth and, after counting the numbers of spores with a hemocytometer, adjusted to the final concentration of 1 × 10⁶ conidia mL⁻¹. Inoculation with 5 mL of conidia in suspension was performed by spraying the conidia until run-off on 4 week old leaves or inflorescences of 6–7 week old Arabidopsis plants. This was performed on detached plant organs placed in Petri dishes (9 cm diameter) containing 1.2% WA. The plates were incubated in the growth chamber at the conditions described above and the high humidity was maintained by covering the plastic lid. Spray-inoculated plants were observed at 2 and 7 dpi for qualitative evaluation of the infection. On drop-inoculated detached leaves necrotic lesions area was determined at 3 dpi (Ferrari et al., 2007) and data were subject to one-way (genotype) analysis of variance (ANOVA). The means were compared using Duncan’s multiple range test at the 1% significance level.

**TRANSCRIPTOMICS ANALYSES**

Analyses were performed on sepal data (Voelckel et al., 2010) and publicly available microarray data from the AtGenExpress developmental series (Schmid et al., 2005) for flowers and pollen (GSE5632) and leaves (GSE5630) for sepal (stage 15, GSM131603-GSM131605), petal (stage 15, GSM131606-GSM131608), rosette (GSM131510-GSM131512),...
leaf 1+2 GSM131498-GSM131500) and senescent leaf (GSM131537-GSM131539) samples were downloaded from Gene Expression Omnibus (GEO, http://www.ncbi.nlm.nih.gov/geo/, Edgar et al., 2002; Barrett et al., 2011). Arrays were normalized using Robust Multi-Array Averaging (RMA) in the Bioconductor affylnGUI package (Smyth, 2004; Smyth et al., 2005; Wettenhall et al., 2006) before a linear fit model was applied and the following contrasts (differentials) calculated: sepal vs. petal, sepal vs. rosette, sepal vs. leaf (not shown), sepal vs. senescent leaf, petal vs. senescent leaf. The top 25 most up-regulated genes in each contrast were then selected according to descending B-statistics. GO analyses were performed on each up-regulated gene list using AgriGO (http://bioinfo.cau.edu.cn/agriGO/, Du et al., 2010). The data from the transcriptional analyses are in Table S1.

RESULTS

MORPHOLOGICAL CHARACTERISTICS OF THE ARABIDOPSIS THALIANA SEPAL

In Arabidopsis the sepals are green leaves, not dissimilar to upper shoot vegetative leaves, that enclose the developing reproductive organs. Scanning electron microscopy reveals that in the young developing buds, prior to the emergence of the petals, the leaves are bent and the upper edges are overlapping and appear tightly sealed (Figure 1A) effectively completely enclosing the reproductive organs. The pavement cells of the sepal show a degree of interdigitation (Figure 1B, inset), albeit less pronounced than in mature vegetative leaf cells. Separated by rows of ≥2 pavement cells of “normal” size, we find highly elongated giant cells with hardly any interdigitation typical for leaf epidermal cells (Staff et al., 2012). The sepal is also characterized by a high number of stomata (≥3/100 μm²) on the upper surface, which is directly exposed to the atmosphere, indicating high metabolic activity but also vulnerability to pathogens that can enter through the stomata (Melotto et al., 2008; Zhang et al., 2008) and may do so by forcing stomata open (Gottig et al., 2008). Bud burst is characterized by the breaking of the sepal seal and emergence of the petals (Figure 1B). The latter show small cells that are more elongated at the base and more globular at the tip of the growth axis and do not contain stomata. By this stage, the sepal seems to have largely lost the mechanical protective function and we note a loss of turgor and a decrease in cellular organization indicative for the onset of senescence (Warner et al., 2000).

INFERENCES FROM THE SEPAL TRANSCRIPTOME

In order to gain insight into the functions of the sepal, a systems level analysis of transcriptional data from different stages was undertaken (Meier et al., 2008, 2010; Meier and Gehring, 2008). Of the 25 most abundant transcripts at stage 12 (Table 1), a significant enrichment of genes in the gene ontology (GO) categories “Response to chemical stimulus” (RCS) and “Cellular metabolic process” (CMP) was noted. Furthermore, the SA UDP-glucosyltransferase (AT2G43820) involved in the formation of SA 2-O-β-glucoside (SAG) and its glucose ester (SGE) is one of the 10 most expressed genes at this stage. Dramatic increases in both these conjugates are considered a hallmark of the biotic defense program.

At stage 15 when the stigma extends above the anthers, we find that the 25 most highly expressed genes (Table 2) are enriched in the categories “Response to biotic stimulus” (RBS), “Response to chemical stimulus” (RCS), and “Response to other organisms” (ROO) and nine of the most highly expressed genes encode proteins that occur in the extracellular space. The functional enrichment is even more pronounced in the 10 most expressed genes (Table 2), with half of the encoded proteins being targeted to the extracellular space and three directly involved in biotic stress responses (GO:0009607). One of the genes not directly involved in stress responses is the Agamous-like 16 MADS-box encoding gene that is expressed in leaf, root and stem, with higher RNA accumulation in guard cells and trichomes. This protein is believed to have a role in stomatal lineage progression and stomatal development (Kutter et al., 2007) and is induced in response to ABA and by pathogens.

Another of the highly expressed genes encodes a leucine-rich receptor kinase (LRRK; AT1G35710). If we do a GO analysis on the top 25 expression correlated genes (r > 0.85) with AT1G35710, we observe that they are highly significantly enriched in the categories “Defense response” (GO:0006952; FDR = 9.8e−08) and “Response to salicylic acid stimulus” (GO:0009751; FDR = 7.6e−08). From this we inferred that the protein is
involved in responses against pathogen defense and this is again entirely consistent with its stimulus specific expression profile (Zimmermann et al., 2004).

Furthermore, a gene encoding a Band 7 family protein that is involved in N-terminal myristoylation, the modification of a protein with a hydrophobic 14-carbon fatty acid myristate that enables membrane attachment of soluble proteins, protein targeting and interactions and partitioning into specific membrane domains (Sorek et al., 2009) is also highly expressed. This is relevant since post-translational protein modifications including myristoylation have a role in pathogen-induced defense signaling (Stulemeijer and Joosten, 2008) and can both specifically activate (Nimchuk et al., 2000; Shan et al., 2000) or repress (Andriouts and Rathjen, 2006) defense signaling components.

Also in the group of the 10 most abundant sepal transcripts is AtPNP-A (AT2G18660) that encodes a Plant Natriuretic Peptide with a role in both responses to pathogens (Gottig et al., 2008; Meier et al., 2008) as well as photosynthesis and the regulation of cellular homeostasis (Garavaglia et al., 2010; de Jonge et al., 2012). In addition, both a chitinase (AT2G43570) and an aspartyl protease family protein (AT5G10760) are annotated as targeted to the extracellular region, apoplast.

The NIT4 expression profile appears to be highly development-and organ-specific, suggesting a critical role in the sepal. Expression is strongly down-regulated in response to methyljasmonate and strongly induced by Pseudomonas parasitica and Pseudomonas syringae, but not in penta mutants (loss-of-function in the gibberellic acid (GA) mutants GAI, RGA, RGL1, and RGL2, four DELLA genes) where expression is suppressed. The expression data would therefore support the idea that GA can signal through jasmonates in flower development (Cheng et al., 2009).

In a next step we analyzed the promoters of the highly expressed genes in the sepal. In the top 10 (stage 15) we find a significant enrichment ($P < 10^{-4}$) of the LS7 element (ACGTCATAGA). The cis-element is found in the promoter of the PR-1 gene and in the coronatine-induced gene (Table 2).

Previously the promoter of PR-1 has been reported to contain two putative TGA transcription factor-binding targets termed...
linker scan7 (LS7) and LS5 (Lebel et al., 1998), the former acting as positive regulator of PR-1 expression in response to 2,6-dichloroisonicotinic acid and SA. The TGA transcription factors regulate expression of PR genes through their interaction with the positive regulator NPR1 (non-expressor of PR-1). In particular, PR-1 expression was reported to be dependent on TGA factor recruitment to the LS7-containing PR-1 promoter in an SA- and NPR1-dependent manner (Johnson et al., 2003).

If we expand the promoter content analyses to the 100 most highly expressed sepal genes, we find significant enrichments for the I-box motif ($P < 10^{-4}$) and the W-box motif (TTGACC or TTTGACT; $P < 10^{-4}$) that is the target site for WRKY transcription factors which in turn have been implicated in the regulation of transcriptional re-programming associated with plant immune responses (Eulgem and Somssich, 2007). $NPR1$ expression is important for the activation of plant defense responses and WRKY encoding genes act upstream of $NPR1$ and promote its expression during the activation of plant defense responses, a mechanism entirely consistent with SA-induced expression of WRKY (Yu et al., 2001). In addition, it was noted that five WRKYs (AT2G30250, AT4G01720, AT2G23320, AT5G07100, and AT4G1550) are positively expression correlated ($r > 0.65$) with the Band 7 family protein that has a role in myristoylation which in turn is implicated in pathogen-induced defense signaling (Stulemeijer and Joosten, 2008).

Since pathogen defense is likely to be an essential function of the sepals, we were interested to see the induction profile of the isochorismate synthase ($ICS$) gene (AT1G74710) that is a key gene for SA biosynthesis in Arabidopsis. Between stages 12 and 15 it increased $>1.5$-fold. Remarkably, one of the highly expressed genes at the stages 12 and 15 encodes a SA UDP-glucosyltransferase (AT2G43820) that inhibits the accumulation of SA (Tables 1, 2). Recently, it has been demonstrated that activators of defense that inhibit SA glucosyltransferases (SAGTs) can indeed augment the pool of free SA and thus enhance plant resistance to pathogens (Noutoshi et al., 2012). It would therefore appear that the synthesis of SA is not only enhanced in the sepal but also very tightly controlled.

THE SEPAL, PETAL, AND ROSSETTE LEAF HAVE SPECIFIC TRANSCRIPTOMIC SIGNATURES

In order to gain further insight into unique sepal functions, we have undertaken to identify transcripts that are differentially expressed in the sepal as compared to the petal and/or rosette leaves. The comparison between the highly up-regulated genes in the sepal as compared to the petal revealed that in this group the top 25 differentially expressed genes are enriched in the categories “Response to stress” and “Catalytic activity” (Table 3 and Table S1). This is consistent with a specialized role in defense and in chemical defense in particular given that the latter depends heavily on catalytic activity essential for the production of flavonoids, phenolics, glucosinolates, terpenoids, and alkaloids (Kliebenstein, 2012). When the highly expressed sepal genes were compared to the rosette leaf transcriptome, a significant enrichment for proteins with a role in catalysis was observed (Table 4 and Table S1). Again, enhanced catalytic activity is an indication of chemical defense. Since the senescence response shares some similarity with the defense response, we also looked at highly up-regulated genes in the sepal rather than in the senescent leaf and found significant enrichments in the categories “Response to external stimulus” and “Response to endogenous stimulus” (Table 5 and Table S1). These responses are defined as any process that results in a change in the state or activity of a cell or organism, e.g., in terms of secretion or enzyme production as a result of an external stimulus. The result therefore is further support for a sepal specific metabolic response different to the one observed in leaf senescence. Finally, a similar comparison between the petal and the senescent leaf transcriptomes sees the categories “Cell wall” and “External encapsulating structures” enriched in the petal (Table 6 and Table S1).

THE FLOWER SHOWS ELEVATED LEVELS OF SALICYLIC ACID

Given that the transcriptome analysis showed that the SA-induced $PR-1$ was over expressed in sepals in stage 15 (Table 2), we were interested to discover if SA accumulates in sepals and petals. We assessed the total (free and sugar-conjugated) and free SA levels in leaves, sepals and petals of Arabidopsis Col-0. The glucosylated form makes the largest part of the total SA content. In the leaves it is 64%, in the sepal 62 and 79% in the petal (Table 4). It is noteworthy that free and total SA content were significantly higher in sepals as compared to leaves ($\approx$10-fold). In petals the free SA levels were similar to those in the sepals while the total SA

| Table 3 | Genes highly up-regulated at stage 15 sepals vs. petals. |
| Gene ID | GO cat. | Annotation |
|---------|---------|------------|
| AT4G17030 | RS CA | EG45-like domain containing protein 2 |
| AT4G25100 | RS CA | Superoxide dismutase [Fe] |
| AT1G02920 | RS CA | Glutathione S-transferase 11 |
| AT4G23600 | RS CA | Coronatine-induced, JA and ABA responsive |
| AT1G19680 | CA | Carbonic anhydrase, chloroplastic |
| AT2G43570 | CA | Chitin-binding, chitinase activity |
| AT3G13790 | RS CA | β-Fructofuranosidase, insoluble isozen. |
| AT4G23150 | RS CA | Cysteine-rich receptor-like protein kinase 7 |
| AT2G02930 | RS CA | Glutathione S-transferase 16 |
| AT2G37770 | RS | NADPH-dependent aldo-keto reductase |
| AT1G75040 | RS | Pathogenesis-related protein 5 (PR-5) |
| AT3G32110 | RS CA | Receptor-like protein 3, defense response |
| AT5G19440 | | Alcohol dehydrogenase, NAD activity |
| AT1G52200 | RS | Divalent metal ion transport |
| AT3G51600 | RS | Lipid transfer protein (PR-14) family |
| AT3G23570 | CA | α/β-Hydrolases superfamily protein, salt resp. |
| AT5G30120 | | Defense response to fungus |
| AT2G05380 | | Glycine-rich protein 3 |
| AT4G14365 | | XB3 ortholog 4, defense (zinc-finger protein) |
| AT5G23010 | CA | 2-Isopropylmalate synthase 3 (MAM1) |
| AT1G13080 | RS | Cytochrome P450 71B15 |
| AT3G57260 | RS CA | β-1,3-Glucanase |
| AT5G22600 | | Lipid-transfer protein |
| AT5G44580 | | Regulator of defense response (SAR) |
| AT2G26440 | CA | Pectinesterase/pectinesterase inhibitor 12 |

RS, Response to stress (GO: 0006950, FDR: 0.001); CA, Catalytic activity (GO: GO:0003824, FDR: 0.0023).
was considerably higher (Figure 2A). We also performed experiments to detect the levels of total and free SA in leaves and in floral organs of plants carrying the nahG transgene salicylate hydroxylase that converts SA to catechol. As expected, nahG plants accumulated just detectable quantities of total and free SA without any significant differences among the three organs examined (Figure 2).

### PLANT RESPONSES TO INFECTION WITH GOLOVINOMYCES CICHORACEARUM

The markedly higher SA content in Col-0 sepalas and petals compared to leaves might suggest a different defense reaction of these organs against biotrophic pathogens. To test this, Col-0 and SA-deficient nahG plants were inoculated with *G. cichoracearum*. At 4 days post-inoculation (dpi) all leaves of both genotypes were infected, as seen from the count of the Trypan blue stained surfaces (Figure 3A and Table 7). However, on SA-deficient nahG leaves, the pathogen produced significantly more conidiophores and conidia per colony than on the Col-0 leaves (Table 7). In contrast, at the same time (4 dpi) only on a very few sepalas did *G. cichoracearum* form colonies and they did not develop conidiophores and conidia. In general, the colony growth on sepalas appeared to be impaired in comparison to the leaf (Figure 3A). At 4 dpi, no colonies were detected on inoculated petals in both Col-0 and nahG genotypes (Figure 3). We also carried out the

| Gene ID       | GO cat.                  | Annotation                                         |
|---------------|--------------------------|----------------------------------------------------|
| AT2G38840     | Non-spec. lipid transfer prot., binds CAM |
| AT1G35310     | MLP-like protein, defense response |
| AT5G45890     | CA Senescence-assoc. gene 12 (Cys-type pep.) |
| AT2G37770     | CA NADPH-dependent aldo-keto reductase |
| AT3G13400     | CA Multicopper oxidase |
| AT1G68620     | CA Hydrolase superfamily protein |
| AT2G02990     | CA Ribonuclease 1 |
| AT4G24000     | CA Cellulose synthase G2 |
| AT4G23680     | CA Polyketide cyclase, lipid transport |
| AT1G02790     | CA Exopolygalacturonanase |
| AT4G15620     | CA UPF 497 membrane protein |
| AT3G27810     | CA MYB21, R2R3-MYB family |
| AT1G80160     | CA Lactosylglutathione lyase |
| AT5G15800     | CA Developmental protein SEPALLATA 1 |
| AT1G54570     | CA Acreatively-like protein, chloroplast |
| AT2G47030     | CA Pectinesterase 4 |
| AT4G33040     | CA Glutaredoxin-C6 |
| AT5G02880     | CA Unknown protein |
| AT1G61563     | CA Rapid Alkalisation Factor 8 |
| AT2G41380     | CA S-adenosyl-L-methyl, dep. methyl transferase |
| AT1G09500     | CA Alcohol dehydrogenase |
| AT1G65480     | CA Flowering locus T, promotes flowering |
| AT1G61680     | CA Linalool synthase, chloroplastic |
| AT4G39480     | CA Cytochrome p450, family 96 protein |
| AT5G07430     | CA Pectin lyase-like superfamily protein |

CA, Catalytic activity (GO: 0003824, FDR: 0.004).

**Table 4 | Genes highly up-regulated at stage 15 sepal vs. rosette leaves.**

| Gene ID       | GO cat.                  | Annotation                                         |
|---------------|--------------------------|----------------------------------------------------|
| AT1G35310     | CA Senescence-assoc. gene 12 (Cys-type pep.) |
| AT2G38840     | CA NADPH-dependent aldo-keto reductase |
| AT1G91680     | CA Multicopper oxidase |
| AT5G59310     | CA Lipid-transfer prot. 4, abiotic stress |
| AT1G55260     | CA Lipid-transfer protein |
| AT5G24150     | CA Squalene monoxygenase 1, 1 |
| AT5G15800     | CA Developmental protein SEPALLATA 1 |
| AT3G27810     | CA MYB21, R2R3-MYB family |
| AT1G665480    | CA Flowering locus T, promotes flowering |
| AT4G15210     | CA Cytosolic β-amyrase |
| AT1G02205     | CA Production of stem epicuticular wax |
| AT1G61680     | CA Linalool synthase, chloroplastic |
| AT4G14690     | CA Early light-induced protein. ELIP |
| AT1G96120     | CA Apetala 1 |
| AT1G29670     | CA GDS-like lipase |
| AT1G24260     | CA MADs box transcription factor |
| AT4G39480     | CA Cytochrome p450, family 96 protein |
| AT2G02990     | CA Ribonuclease 1 |
| AT1G66120     | CA Butyrate metabolic process |
| AT4G23600     | CA Coronatine-ind., JA and ABA resp. |
| AT2G37770     | CA NADPH-dependent aldo-keto reductase |
| AT3G11480     | CA Methyltransferase for SA and benzoic acid |
| AT2G006850    | CA Xylogluc. endotransglycosylase/hydrol. |
| AT5G57560     | CA Xylogluc. endotransglycosylase/hydrol. |
| AT1G35140     | CA Xylogluc. endotransglycosylase/hydrol. |

REX, Response to external stimulus (GO: 0009605, FDR: 0.0017); REN, Response to endogenous stimulus (GO: 0009719, FDR: 0.0017).

**Table 5 | Genes highly up-regulated at stage 15 sepal vs. senescent leaves.**

**Table 7**
Table 6 | Genes highly up-regulated at stage 15 petals vs. senescent leaves.

| Gene ID   | GO cat. | Annotation                                         |
|-----------|---------|---------------------------------------------------|
| AT5G25460 | CW      | DUF 642, plant-type cell wall function            |
| AT1G61880 | CW      | Linalool synthase, chloroplastic                   |
| AT2G27810 | CW      | MYB 21, R2R3-MYB family                           |
| AT1G55260 | EXE     | Lipid-transfer protein                            |
| AT2G06850 | CW      | Xylogluc. endotransglucosylase/hydrolase           |
| AT1G29670 | CW      | GDSL-like lipase                                  |
| AT2G10940 | EXE     | Lipid-transfer protein                            |
| AT2G38540 | CW      | Non-spec. lipid transfer prot., binds CAM          |
| AT1G35310 | EXE     | MLP-like protein, defense response                |
| AT4G39480 |         | Cytochrome p 450, family 96 protein               |
| AT1G02205 |         | Putative cytochrome p 450                         |
| AT1G66120 |         | Production of stem epicuticular wax                |
| AT1G66120 |         | Butyrate metabolic process                        |
| AT3G4340  |         | Apetala3                                          |
| AT3G01980 |         | NAD(P)-bind. Rossmann-fold protein                |
| AT1G55260 |         | Chaperone DnaJ-domain superfam. prot.             |
| AT2G17880 |         | GDSL-like Lipase                                  |
| AT3G25460 | CW      | Unknown protein in the cell wall                  |
| AT5G62360 | CW      | Unknown protein in the cell wall                  |
| AT4G25830 |         | UFP0497 membrane protein                          |
| AT5G47550 |         | Cysteine proteinase inhibitor 5                   |
| AT1G12090 |         | Extensin-like protein (ELP)                       |
| AT1G24260 |         | Sepallata 3                                       |
| AT1G11850 |         | Unknown protein                                   |

CW, Cell wall (GO: 0005618, FDR: 4.5e-06); EXE, External encapsulating structure (GO: 0030312, FDR: 4.5e-06); *Not included in the AgriGO analysis.

In terms of isolate virulence, environmental conditions (such as temperature, humidity and light intensity) and host susceptibility. Here, visual assessment of inoculated plants at 7 dpi revealed chlorotic and necrotic lesions on nahG leaves and the leaf surfaces had the white powdery appearance caused by mycelia, conidiphores and conidia. At the same time, only small chlorotic lesions and limited conidiation were detectable on Col-0 leaves. In contrast, no disease symptoms or pathogen structures were detectable on flowers of both genotypes (Figure 5). On the other hand, our microscopic investigation (Figure 3) clearly showed that at 4 dpi the spray-inoculated conidia had not germinated or only just germinated on the surface of the floral organs, without development of fungal hyphae. The fungal DNA quantification confirmed an absence of growth of the fungus on Col-0 and nahG sepal tissues (Figure 4), which indicated that these reproductive structures are resistant to G. cichoracearum infection.

**PLANT RESPONSES TO INFECTION WITH BOTRYTIS CINEREA**

To further investigate possible different responses of the floral organs and, in particular, of the sepal against necrotrophic pathogens, the Col-0 and SA-deficient nahG plants were spray-inoculated with *Botrytis cinerea* and the lesion development was measured. Visual assessment of spray-inoculated Arabidopsis plants at 2 dpi showed no symptoms on Col-0 and nahG leaves.

In contrast, sepal and petals of both genotypes were necrotic. Moreover, gray mycelia started to appear on the floral surface (Figure 6A). At 7 dpi, the gray fungal structures completely covered the inflorescence of both genotypes. At the same time, clear symptoms (chlorosis and rot) were detectable on both Col-0 and nahG leaves (Figure 6A) and no difference in susceptibility was noted between Col-0 and nahG plants (Figure 6A). The lack of a significant difference in susceptibility of Col-0 and nahG leaves to *B. cinerea* was confirmed by lesion area measurements on drop-inoculated detached leaves at 3 dpi (Figure 6B). Thus, in this system, in contrast to the responses against the biotroph, the susceptibility to the necrotroph *B. cinerea* showed no differences between flowers and leaves, and between the two genotypes, which suggests a limited role for SA in the *B. cinerea* defense response.

**DISCUSSION**

The first indication of the presence of PR proteins in the flower comes from work on tobacco and, perhaps most importantly, it was demonstrated that there is a pathogen-independent induction (Lotan et al., 1989) and that PR proteins in the sepal are induced as part of the developmental program. More recently it was shown that tobacco and petunia contain chemically distinct floral defensins, basic small proteins that can retard the growth...
of fungi, oomycetes, and gram-positive bacteria. These defense mechanisms are specifically induced during the early stages of flower development (Lay et al., 2003) and operate in the outermost cell layers of sepal, petals, anthers, and styles, where they presumably serve in the first line of defense against pathogens.

Given these observations we hypothesized that the sepal may serve not just as a physical but also as a chemical defense barrier that protects the developing reproductive organs. The sepal has a very distinct morphology that includes polyploid giant cells (Figure 1). The biological role of these giant cells of the sepal is still not clear, but we know that they are the result of endoreduplication (Roeder et al., 2010). Endoploidy is essential for normal development and physiology in many different organisms. There are cells that go into endoreplication as part of terminal differentiation to enable specialized function (Lee et al., 2009). Plants grow by increasing cell numbers, cell size or both. Since increased DNA content correlates with increased cell size, endoreplication is a highly efficient growth mode since it reduces the cell (and cell wall) surface to volume ratio. Thus, such a growth mode may be particularly desirable when rapid growth must occur or high metabolic activity is required (Inzé and De Veylder, 2006). It is generally assumed that endoreplication-associated growth is indicative for cell types that perform specific biological functions. It has recently been reported that in Arabidopsis increasing gene copy number by localized endoreduplication, mediated by MYB3R4 (AT5G11510), may serve as a mechanism to meet the enhanced metabolic demands imposed by, e.g., the pathogenic biotrophic fungus Golovinomyces orontii that depends entirely on the nutrient supply from the host (Chandran et al., 2010). Furthermore, there are numerous examples in other systems where specific cell types undergo endoreduplication and interestingly, it appears that these cell types often have roles in secretion and in some cases secretion of antimicrobial compounds (Reilly et al., 1994; Dai et al., 2010). For these reasons and based on the transcriptional profiles (Tables 1–6), we hypothesize that the sepal is in an heightened state of biochemical defense with many genes induced that encode enzymes that catalyze the production of defense components targeted for secretion into the extracellular space. In addition, we propose that the giant cells of the sepal are actually the metabolic factories that are synthesizing the defensive proteins as a preventive and/or protective measure. SA is a well-characterized molecule that regulates the activation and potentiation of plant defense responses (Vlot et al., 2009). However, the activation of defense responses is not the only regulatory role of SA. The first reported physiological responses to SA were the induction of thermogenesis through the activation of the mitochondrial alternative oxidase in Arum flowers (Raskin et al., 1987, 1989). Since then several other functions have been assigned to SA, including a regulatory role in flowering (Martinez et al., 2004), regulation of gene expression during leaf senescence (Morris et al., 2000) and regulation of cell growth by specifically affecting cell enlargement, endoreduplication and/or cell division. In addition, methyl SA is a volatile compound, and like other methyl esters (e.g., methyl benzoate, methyl cinnamate, methyl jasmonate) it is a widespread fragrant component in the plant kingdom contributing significantly to the floral scent output (Knudsen and Tollsten, 1993). Our data show very high constitutive levels of free and conjugated SA in both sepals and petals, whereas in pathogen-unchallenged leaves the SA content was very low. The high SA levels correlate well with the up-regulation of PRs protein encoding genes revealed by the transcriptome analysis.

We challenged leaves and inflorescence of Col-0 and nahG plants with the biotrophic fungus G. cichoracearum and the necrotrophic B. cinerea with the aim to clarify whether the defense program against these pathogens is enhanced in the inflorescence with respect to the leaves. When the leaves were infected with the fungus G. cichoracearum, the SA-deficient transgenic nahG plants were more sensitive than Col-0 and this is consistent with the SA-dependent resistance against the biotrophic pathogen in Arabidopsis leaves (Ellis et al., 2002). However, sepals...
Table 7 | Quantitation of *Golovinomyces cichoracearum* growth on Col-0 and nahG leaves and sepals.

| Genotype | Examined leaves | Leaves with colonies (%) | Total colonies | Conidiophores/Colony | Conidia/Colony |
|----------|-----------------|--------------------------|---------------|----------------------|---------------|
| Col-0    | 24              | 100                      | 98            | 0.64 ± 0.13<sup>a</sup> | 2.13 ± 0.61<sup>a</sup> |
| nahG     | 24              | 100                      | 284           | 1.94 ± 0.22<sup>b</sup> | 5.40 ± 0.71<sup>b</sup> |

| Genotype | Examined sepals | Sepals with colonies (%) | Total colonies | Conidiophores/Colony | Conidia/Colony |
|----------|-----------------|--------------------------|---------------|----------------------|---------------|
| Col-0    | 60              | 6                        | 5             | 0                    | 0             |
| nahG     | 60              | 5                        | 6             | 0                    | 0             |

Leaves and sepals of Col-0 and nahG plants were sprayed with a conidial suspension of *G. cichoracearum*. Leaves were sampled from 4 week old plants, whereas sepals and petals were taken from 6 to 7 week old plants at flower stages 14–15. Samples were stained with Trypan blue at 4 dpi. Experiments were repeated 3 times with similar results. Conidiophores and conidia were counted on randomly selected single fungal colonies on a total of 24 leaves and 60 sepals per genotype. Data represent the mean ± SE. Different letters indicate statistically significant differences using Duncan’s multiple range test (*P* ≤ 0.01).

and petals of Col-0 and nahG are both resistant to the biotroph with no or very few colonies forming on petals and sepals, respectively. Moreover, *G. cichoracearum* did not develop conidiophores and conidia on sepals. Since sepals and petals of Col-0 and SA-deficient plants were resistant to *G. cichoracearum*, we argue that in these organs the resistance to biotrophic pathogen is not exclusively or critically dependent on SA but depends on constitutive up-regulation of stress-responsive genes in flower. This interpretation is supported by the transcriptional analysis that shows a high induction of genes encoding proteins with a role in responses to chemical stimuli in the sepal (stage 12) and genes encoding proteins with a role in responses to biotic and chemical stimuli (stage 15). A comparison between the highly up-regulated genes in the sepal as compared to those in the petal reveals an enrichment in the categories “Catalytic activity” and “Response to stress” again point to an organ-specific and enhanced defense program (Table 3). NahG plants do not accumulate SA or camalexin (Nawrath and Metraux, 1999). However, there are many reports that show PRs activation after pathogen infection (Nawrath and Metraux, 1999; Govrin and Levine, 2002) thus suggesting that also in nahG mutant flowers there is a constitutive activation of defense mechanisms able to protect the flower against biotrophic pathogen infection. This point remains to be further elucidated and will be resolved when the transcriptome of nahG sepals becomes available.

Given the short lifespan of sepals and petals, and the possibility of senescence-related defense responses, we compared the sepal and petal transcriptomes to the transcriptome of senescing leaves (Tables 5, 6). In the case of the sepal, we noted enrichment in genes encoding proteins with catalytic activity (Tables 5...
and Table S1) and in the case of the petal, an overrepresentation of genes encoding proteins with a role in cell wall function and external encapsulating structures (Tables 6 and Table S1). These results point to specific functions beyond the leaf senescence program and are conceivably indicators of enhanced functional and structural defense components. Additionally, the Arabidopsis petal with its absence of photosynthesis (Pyke and Page, 1998) is hardly particularly attractive to biotrophic pathogens.

In contrast to the response to the biotrophic pathogen, the response to the necrotroph B. cinerea showed no difference between the flower and the leaves. In addition, the B. cinerea phenotype of nahG was indistinguishable from Col-0 suggesting a limited role for SA in the interaction between B. cinerea and Col-0 in Arabidopsis. Contrary to previous reports (Govrin and Levine, 2002; Ferrari et al., 2003), in our experiments nahG plants had resistance to B. cinerea that was comparable to Col-0 plants as previously reported (Thomma et al., 1998; Véronese et al., 2004; Abuqamar et al., 2006). These discrepancies may be caused by differences in the Botrytis strain or the methods of inoculation. In conclusion, the same B. cinerea disease susceptibility of leaves and flowers and Col-0 and nahG plants suggests that neither constitutive stress-responsive gene induction in sepals nor SA accumulation can prevent infections by this necrotrophic pathogen. On the other hand, induced defense responses to pathogens are mediated by multiple signal transduction pathways. While SA-mediated defenses are prominent against biotrophic pathogens, jasmonate/ethylene signaling exerts a major influence on plant response to necrotrophic pathogens such as Botrytis cinerea (Thomma et al., 1998, 1999). In addition, elicitors released from the cell wall during pathogen infection and genes involved in the biosynthesis of secondary metabolites play an important role in determining the enhanced resistance against B. cinerea through a signaling pathway activated by pathogen-associated molecular pattern molecules and, therefore, independently of SA, JA, and ET (Ferrari et al., 2007). Furthermore, it has been demonstrated that B. cinerea can induce multiple defense responses in Arabidopsis resembling to hypersensitive response (HR) (Govrin and Levine, 2002), but contrary to the effect on biotrophic pathogens, HR facilitates rapid growth and spread of this necrotrophic pathogen.

In summary, we report that the sepal and petal express a distinct set of genes that encode proteins with a role in defense against pathogens. This tissue specific transcriptional program is reflected in the enhanced host responses, in particular to biotrophic pathogens. We also propose that the giant cells in the sepal are the metabolic factories that provide the chemical defense shield and we are currently planning to experimentally test this hypothesis.

**Author Contributions**

CG, SP, LE, MQ, and LX conceived and designed the experiments. LE and MQ performed the experiments. AD and CG performed the bioinformatics analyses. LE, SP, AD, and CG analyzed the data. CG and SP wrote the paper.

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**Supplementary Material**

The Supplementary Material for this article can be found online at: http://www.frontiersin.org/journal/10.3389/fpls.2015.00079/abstract

**Table S1 | Sepal gene expression at different stages.**

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