PCP
PLANT & CELL PHYSIOLOGY

Regular Paper

Natural Variation among Arabidopsis Accessions in the Regulation of Flavonoid Metabolism and Stress Gene Expression by Combined UV Radiation and Cold

Elisa Schulz[1,4], Takayuki Tohge[1,5], J. Barbro Winkler[2], Andreas Albert[2,6], Anton R. Schäffner[3], Alisdair R. Fernie[1], Ellen Zuther [1,*] and Dirk K. Hincha[1,†]

[1]Max-Planck-Institut für Molekulare Pflanzenphysiologie, Am Mühlenberg 1, Potsdam 14476, Germany
[2]Research Unit Environmental Simulation, Institute of Biochemical Plant Pathology, Helmholtz Zentrum München, German Research Center for Environmental Health, Ingolstädter Landstr. 1, Neuherberg 85764, Germany
[3]Institute of Biochemical Plant Pathology, Helmholtz Zentrum München, German Research Center for Environmental Health, Ingolstädter Landstr. 1, Neuherberg 85764, Germany
[4]MetaSysX GmbH, Am Mühlenberg 11, Potsdam 14476, Germany
[5]Graduate School of Biological Science, Nara Institute of Science and Technology (NAIST), Ikoma, 630-0192 Japan
[6]Deutsches Patent- und Markenamt, Zweibrückenstr. 12, München 80331, Germany
†We dedicate this paper to the memory of our colleague Dirk K. Hincha who passed away during the preparation of this manuscript.
*Corresponding author: E-mail, zuther@mpimp-golm.mpg.de.
(Received 22 July 2020; Accepted 20 January 2021)

Plants are constantly exposed to stressful environmental conditions. Plant stress reactions were mainly investigated for single stress factors. However, under natural conditions plants may be simultaneously exposed to different stresses. Responses to combined stresses cannot be predicted from the reactions to the single stresses. Flavonoids accumulate in *Arabidopsis thaliana* during exposure to UV-A, UV-B or cold, but the interactions of these factors on flavonoid biosynthesis were unknown. We therefore investigated the interaction of UV radiation and cold in regulating the expression of well-characterized stress-regulated genes, and on transcripts and metabolites of the flavonoid biosynthetic pathway in 52 natural Arabidopsis accessions that differ widely in their freezing tolerance. The data revealed interactions of cold and UV on the regulation of stress-related and flavonoid biosynthesis genes, and on flavonoid composition. In many cases, plant reactions to a combination of cold and UV were unique under combined stress and not predictable from the responses to the single stresses. Strikingly, all correlations between expression levels of flavonoid biosynthesis genes and flavonol levels were abolished by UV-B exposure. Similarly, correlations between transcript levels of flavonoid biosynthesis genes or flavonoid contents, and freezing tolerance were lost in the presence of UV radiation, while correlations with the expression levels of cold-regulated genes largely persisted. This may indicate different molecular cold acclimation responses in the presence or absence of UV radiation.

Keywords: *Arabidopsis thaliana* • Cold acclimation • Flavonoids • Natural genetic variation • Stress tolerance • UV radiation.

Introduction

In their natural environments, plants are constantly exposed to a multitude of biotic and abiotic factors that may result in damage and may elicit stress responses to mitigate such damage and increase stress tolerance. In the past, research to elucidate stress tolerance mechanisms has largely focused on the effects of single stress factors. While such a reductionist approach has proven fruitful in the discovery of molecular mechanisms underlying plant stress adaptation, plants under natural conditions are usually exposed to more than one stress factor at the same time. A classical case of such a 'companion stress' scenario is the co-occurrence of heat and drought stress (see Mittler 2006 for reviews; Lawas et al. 2018b), where lack of water for transpirational cooling induces heat stress under otherwise nondamaging temperature conditions (Grill and Ziegler 1998; Lawas et al. 2018a). Such combined stresses are more damaging to plants than the single stresses (Suzuki et al. 2014). In addition, transcriptomic reactions to combined stresses cannot be predicted from the reactions to the single stresses, making the molecular reaction to companion stress a unique and specific response (Rizhsky et al. 2002, 2004, Prasch and Sonnewald 2013, Rasmussen et al. 2013, Georgii et al. 2017).

A potential stress factor that is always present during the daylight hours is UV radiation (both UV-A and UV-B) that may therefore under natural conditions interact with any additional stress factor, such as heat or cold, which plants may be exposed to. The depletion of the stratospheric ozone layer and the concomitant increase in UV intensity has triggered a significant amount of research into the effects of UV on plant performance and physiology (Rozema et al. 1997). It is well-established that UV-B radiation can lead to cell damage related to the

Plant Cell Physiol. 0(0): 1–13 doi:10.1093/pcp/pcab013, Advance Access publication on 5 February 2021, available online at https://academic.oup.com/pcp

generation of reactive oxygen species (ROS) (Foyer et al. 1994) and to direct effects on DNA stability (Britt 1995, Landry et al. 1997). With the characterization of UVR8 as a UV-B receptor (Kliebenstein et al. 2002), the molecular basis for the adaptation of plants to UV-B was discovered (see Heijde and Ulm 2012, Jenkins 2014 for reviews). Likewise, cryptochromes and phototropins sense UV-A in addition to blue light (Gyula et al. 2003, Chen et al. 2004). Since plants have evolved under constant, low levels of UV radiation, the resulting constitutive adaptation makes this exposure nonstressful (see Jenkins 2017 for a recent review), at least under otherwise benign conditions. This nevertheless leaves the possibility unexplored that the UV-A and/or UV-B signaling pathways may interact with signaling pathways triggered by other abiotic stresses and thereby modulate transcriptional and metabolic downstream reactions. This has to some extent been explored by comparing the effects of drought stress and UV-B on plants (see Bandurska et al. 2013 for a review). In the case of mild stresses, pretreatment with drought or UV-B may enhance subsequent tolerance to the other stress treatment. Conversely, combined salt and UV-A + B stress resulted in slightly lower fresh and dry weight of bell pepper plants than either of the single stresses (Ellenberger et al. 2020), while in poplar DNA lesions due to UV-B radiation were reduced when plants were simultaneously salt stressed (Ma et al. 2016). However, no detailed transcript and metabolite analyses have been published under such conditions.

One metabolic pathway where it is likely that different stressors interact is flavonoid biosynthesis. In the present study, we have therefore investigated this using nondamaging levels of UV-A and in addition UV-A + B and cold (4°C) in a full-factorial design (UV-A at 20°C; UV-A at 4°C; UV-A + B at 20°C; UV-A + B at 4°C; see **Supplementary Fig. S1** for a schematic depiction of the experimental design). It is well-established that flavonoid accumulation is induced by exposure to both UV-A and UV-B, albeit through different signal transduction pathways depending on different photoreceptors, namely cryptochromes and UVR8, respectively (Christie and Jenkins 1996). The accumulated flavonols act as a 'sun screen' in the epidermis, absorbing both UV-A and UV-B radiation, thus protecting the metabolically active parenchyma cells from radiation damage (Burchard et al. 2000, Emiliani et al. 2013). Mutant plants lacking flavonoids are more sensitive to UV-B and display increased oxidative damage compared to wild-type plants (Li et al. 1993, Landry et al. 1995).

Likewise, cold exposure increases flavonoid content in leaves, leading to increased UV-B tolerance (Bilger et al. 2007). In Arabidopsis and in most other plant species from temperate climates, low, nonfreezing temperatures result in cold acclimation, i.e. an increase in freezing tolerance (Levitt 1980, Steponkus 1984). Cold acclimation is accompanied by a massive reprogramming of gene expression, protein, metabolite and lipid composition (see Guy et al. 2008, Thomashow 2010 for comprehensive reviews; Hincha et al. 2012, Knight and Knight 2012, Ding et al. 2019). A central role in this process is played by the *CBF1*, *2* and *3* genes and their downstream target *COR* genes (Thomashow 1999, Shinozaki et al. 2003, van Buskirk and Thomashow 2006, Medina et al. 2011). However, with the exception of COR15A/B (Artus et al. 1996, Steponkus et al. 1998, Thalhammer et al. 2014), the functional role of the encoded proteins in cold acclimation is unknown.

There is large natural variation in both nonacclimated and cold-acclimated freezing tolerance in Arabidopsis accessions, which is also evident at the levels of compatible solutes, such as several sugars, and *COR* gene expression (Zuther et al. 2012). Such natural variation is also present in leaf flavonoid content and in the expression levels of genes encoding transcription factors and enzymes involved in this pathway under both conditions (Biswas and Jansen 2012, Ilk et al. 2015, Schulz et al. 2015). The content of some flavonoids is significantly correlated with plant freezing tolerance after cold acclimation (Korn et al. 2008, Schulz et al. 2015) and investigations with various biosynthetic mutants revealed a functional role of flavonoids in Arabidopsis freezing tolerance and cold acclimation (Schulz et al. 2016). However, these investigations were all conducted in the absence of UV radiation during plant growth and cold acclimation, which would, however, be present when plants cold acclimate under natural conditions in autumn.

In the present study, we investigate the interaction of UV and cold in regulating the expression of well-characterized stress-regulated genes, and on transcripts and metabolites of the flavonoid biosynthetic pathway in a panel of 52 natural accessions of *Arabidopsis thaliana* that differ widely in their freezing tolerance (Zuther et al. 2012) and flavonoid content (Schulz et al. 2015).

Results

Both temperature and UV exposure influence expression of genes related to cold stress and flavonoid metabolism

To identify transcriptional responses of known stress-regulated genes and of genes encoding transcription factors and enzymes involved in flavonoid biosynthesis to both UV and cold, we determined the expression levels of selected genes in 52 natural Arabidopsis accessions (**Supplementary Table S1**) by qRT-PCR. We then used principal component analysis (PCA) to evaluate whether the expression patterns of stress-responsive and flavonoid biosynthesis genes showed variation in response to the different treatments (**Fig. 1**). Plants grown in the present study were exposed to either only UV-A or to UV-A + B at either 20°C or 4°C. In addition, we have included data from an earlier study (Schulz et al. 2015), where plants were grown in the total absence of UV radiation, under nonacclimating (20°C; NA) and cold acclimating (4°C; ACC) conditions. In this case, the freezing tolerance of the plants was directly determined using an electrolyte leakage assay (Zuther et al. 2012). While we are aware that growth conditions between the present and the previous study differed in more than just UV exposure, we believe that this is still a valid comparison of gene expression and flavonoid content in response to cold in this broad panel of accessions.

In the case of stress-regulated genes, plants grown at 20°C were clearly separated from plants grown at 4°C in the presence of both UV-A and UV-A + B (**Fig. 1a**). These data were fitted to

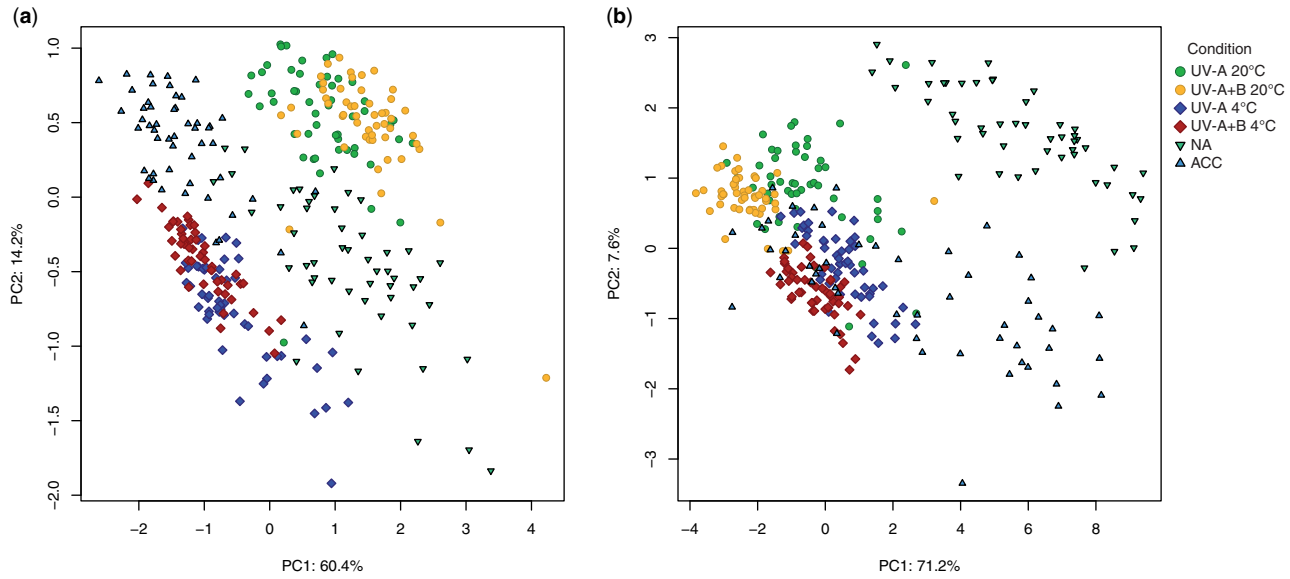

Fig. 1 PCA scores plots of (a) PC1 and PC2 of the expression of stress-regulated genes and (b) of the expression of flavonoid biosynthesis genes of 52 accessions grown in the presence of UV-A at 20°C (green), UV-A + B at 20°C (yellow), UV-A at 4°C (blue) and UV-A + B at 4°C (red) in environmental simulation chambers, or in the absence of any UV treatment at 20°C in a greenhouse (NA) or at 4°C in a climate chamber (ACC) (Zuther et al. 2012, Schulz et al. 2015). qRT-PCR data ($2^{-\Delta Ct}$) are $\log_{10}$ median transformed averages of three replicates consisting of two individual plants each in the present study and of two technical replicates of a pool of five plants for the published studies. Numbers on axes represent the percentage of the total variance explained by the indicated PC.

a PLS-DA model (**Supplementary Fig. S2a**) identifying the gene expression of *COR15a*, *COR15b* and the core clock gene *PPR5* as the variables with the highest importance (VIP) for Component 1 (**Supplementary Table S1**). While the influence of UV-A + B treatment at both 20°C and 4°C on separation is not strong, plants grown in the absence of any UV radiation at either temperature (NA, ACC) were separated from plants exposed to either only UV-A or UV-A + B. A very slight separation of UV-A + B treated from only UV-B-treated samples can be seen along PC2 in a PCA that excluded the data from plants grown in the absence of UV radiation with the separation direction being different for sample from either 20°C or 4°C (**Supplementary Fig. S3a**).

For the expression of flavonoid biosynthesis genes, the main factor of separation was the exposure to UV radiation along PC1 (71.2% of the total variance), with plants exposed to UV-A or UV-A + B clearly separated from those not exposed to any UV (NA, ACC) and with the UV exposed plants partially separated into those grown in the absence or presence of UV-B (**Fig. 1b**). This separation was mainly driven by the VIPs TRANSPARENT TESTA genes *F3H/TT6*, *F3′H/TT7*, *CHS/TT4 and UGT89C1/F7RT* involved in the first steps of flavonoid biosynthesis (**Supplementary Table S1**) in the respective PLS-DA model (**Supplementary Fig. S2b**). The latter separation was more obvious when the PCA was performed without the data from plants grown in the absence of UV (**Supplementary Fig. S3b**). Plants exposed to 20°C or 4°C in the presence of UV-A or UV-A + B were separated along PC3 (8.5% of the total variance).

Combining the transcript data of the present study from all 52 investigated accessions (**Supplementary Table S2**), the expression of most cold-regulated genes increased significantly when comparing plants grown at 20°C and 4°C, irrespective of the UV-B treatment (**Fig. 2a**, **Supplementary Table S3**). This is in agreement with our previous data on cold-induced genes in the absence of any UV exposure (Zuther et al. 2012) with the notable exceptions of *COR47*, which showed significant cold induction in the previous study. The anti-oxidant-related genes *sAPX* (stromal ascorbate peroxidase), *PrxQ* (peroxidase Q) and *2CPA* (2-Cys peroxiredoxin A), and the genes *ELIP1/2* [early light-induced protein 1/2 (Meyer and Kloppstech 1984)] showed significant upregulation in the cold, irrespective of UV-B treatment except for *sAPX* which was also induced by UV-B (**Fig. 2**).

The only exception to the general trend of cold induction among the selected genes was *ERCC1*, encoding an excision repair endonuclease subunit (Hefner et al. 2003), which was strongly downregulated upon cold exposure of the plants (**Supplementary Fig. S4**; **Fig. 2a**).

The responses of these stress-regulated genes to UV-B were more variable than the responses to cold and differed strongly between 20°C and 4°C (**Fig. 2a**, **Supplementary Fig. S4**) and also among the accessions (**Supplementary Fig. S5**). While most of the genes, with the exception of *ELIP1* and *ERCC1*, were unaffected or downregulated by UV-A + B exposure at 20°C, the cold-induced genes, with the exception of those encoding CBF transcription factors, were further upregulated by UV-B at 4°C. *ELIP1* and *ERCC1* were both significantly upregulated by UV-A + B compared to UV-A at 20°C, but unchanged (*ELIP1*) or downregulated (*ERCC1*) by UV-A + B at 4°C. Together, these results suggest strong and often antagonistic regulation by cold and UV-B for all investigated stress-related genes.

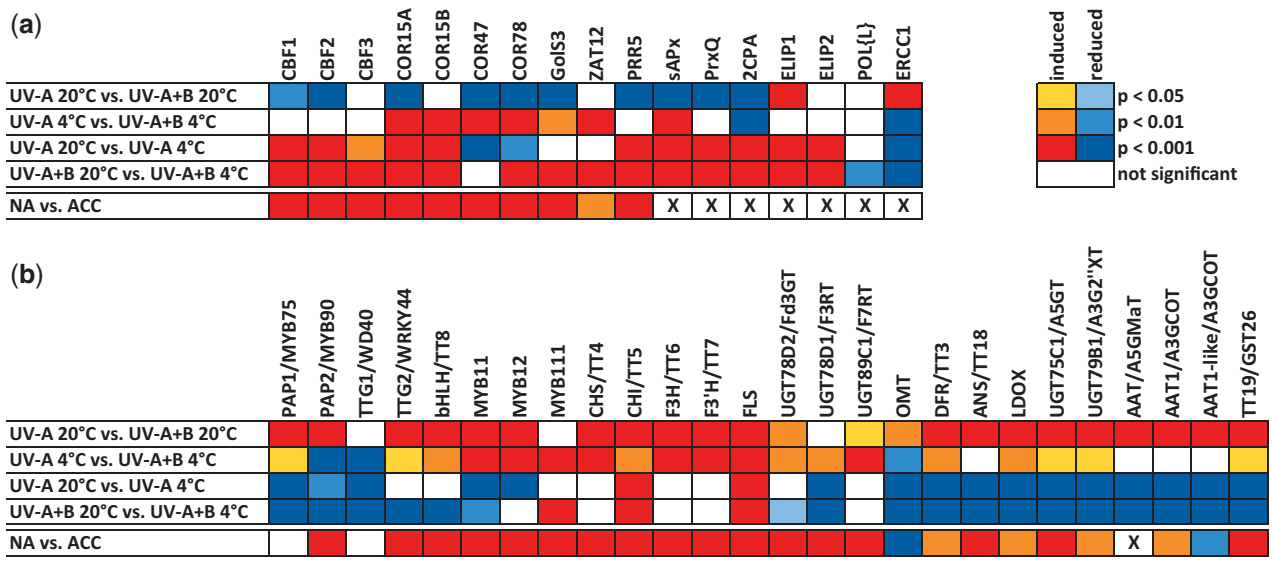

Fig. 2 Significant differences in the expression of stress-responsive genes (a) and flavonoid biosynthesis genes (b) across all 52 accessions between the indicated conditions of the environmental simulation experiment. For comparison, significant differences in the expression of the same genes between nonacclimated (NA) and cold-acclimated (ACC) conditions (Zuther et al. 2012, Schulz et al. 2015) are shown as well. An X indicates that this gene was not included in the latter studies. The significance levels (FDR *P*-values) determined by *t*-tests (two-sided with unequal variance) were corrected for multiple testing errors by the Benjamini–Hochberg procedure and are color coded according to the direction of the change as indicated in the legend. All *P*-values are shown in **Supplementary Table S3**.

We further determined the expression levels of 26 genes encoding transcription factors and enzymes involved in Arabidopsis flavonoid biosynthesis (**Supplementary Table S2**). With the exception of three genes (*TTG1*, *MYB111*, *UGT78D1*), they were all significantly upregulated at 20°C by UV-A + B (**Fig. 2b**, **Supplementary Table S3**). At 4°C, the upregulation of some anthocyanin biosynthesis genes by UV-A + B was no longer significant (*ANS*, *AAT*, *AAT1*, *AAT1-like*) and the genes encoding the transcription factors PAP2 and TTG1 were downregulated. Strikingly, while we had observed an upregulation of almost all flavonoid biosynthesis genes at 4°C in our previous study in the absence of UV-A radiation (Schulz et al. 2015 and **Fig. 2b**), all anthocyanin biosynthesis genes and several flavonoid-related genes were massively downregulated at 4°C in the presence of UV-A, or showed no significant changes in expression, under either UV-A or UV-A + B treatment (**Fig. 2b**, **Supplementary Fig. S6**). This was in fact true for almost all investigated accessions (**Supplementary Fig. S5**).

The content of flavonoids in the 52 investigated Arabidopsis accessions was also strongly influenced by temperature and UV treatment (**Supplementary Table S4**). PCA indicated that differences in flavonoid profiles were largest among plants grown in the absence or presence of any UV radiation separated by PC1, explaining 64.8% of the total variance in the data (**Fig. 3**). Likewise, PC2 (14.8% of the total variance) separated plants grown at 20°C from those grown at 4°C under no UV, UV-A or UV-A + B conditions. The particular effect of UV-A + B treatment can be seen in the PCA without data from plants grown in the absence of UV light (**Supplementary Fig. S7**), where data from plants grown in the presence of UV-A at

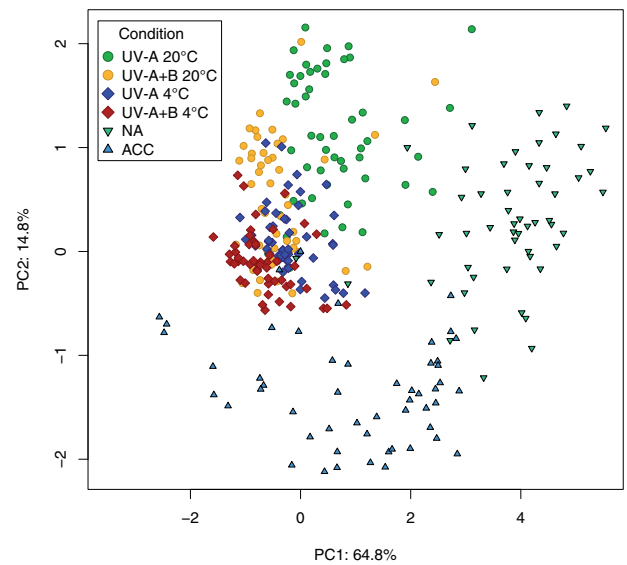

Fig. 3 PCA scores plot of flavonoid composition of 52 accessions in the presence of UV-A at 20°C (green), UV-A + B at 20°C (yellow), UV-A at 4°C (blue) and UV-A + B at 4°C (red) in environmental simulation chambers, or in the absence of any UV treatment at 20°C in a greenhouse (NA) or at 4°C in a climate chamber (ACC) (Schulz et al. 2015). Data are $\log_{10}$ median transformed averages. Numbers on axes represent the percentage of the total variance explained by the indicated PC.

20°C without UV-B were clearly separated from all other conditions, while additional exposure to UV-B at 4°C only had a minor influence on flavonoid composition. This is also evident from **Supplementary Fig. S8**, which shows that the $\log_2$-fold change in flavonoid content was only very small in this comparison, whereas the largest changes were observed between

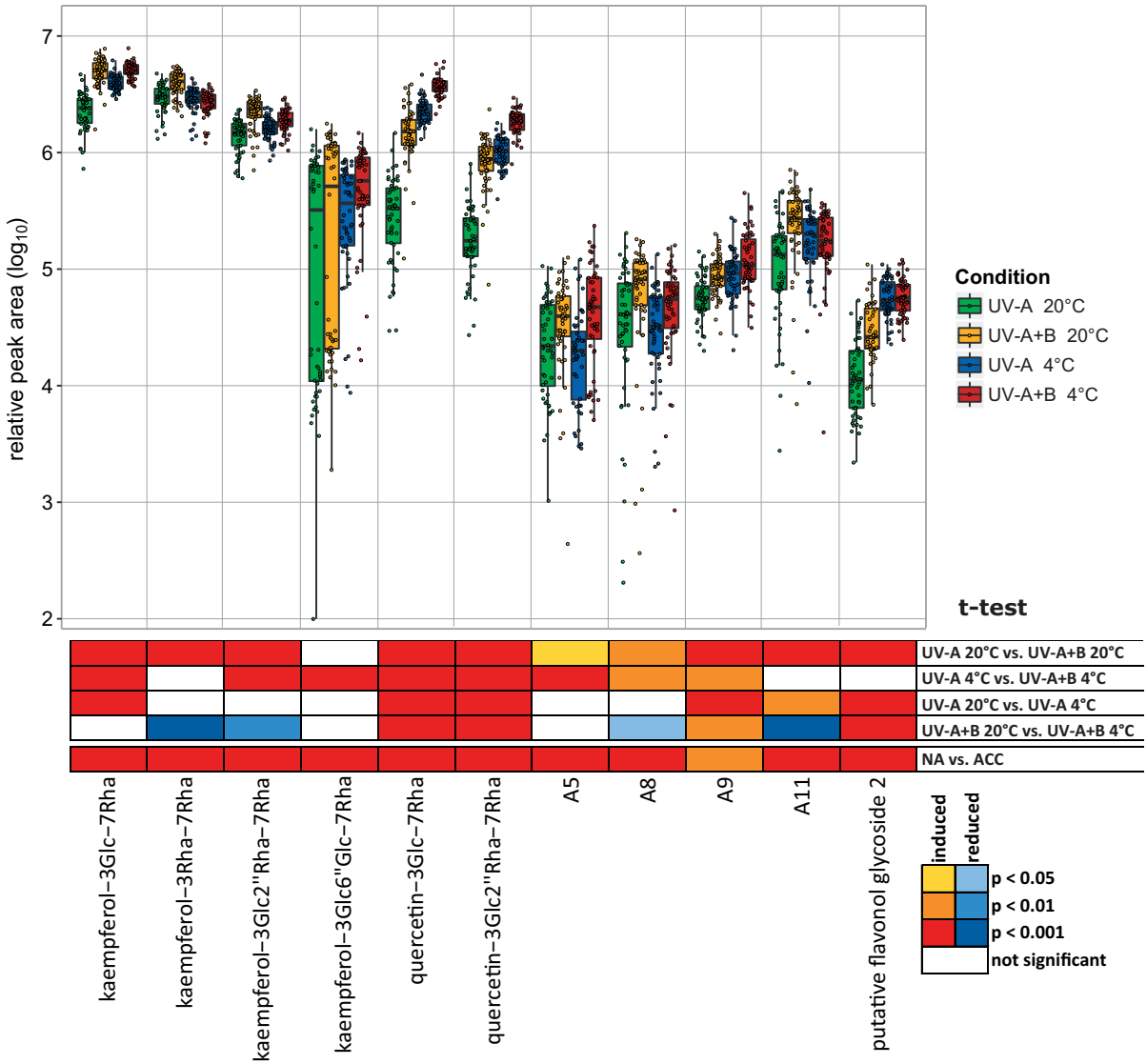

Fig. 4 Variability of flavonoid content in 52 *Arabidopsis* accessions under the indicated experimental conditions. Data represent the average of relative peak area of three biological replicates consisting of two individual plants per accession. Box plots represent the upper and lower quartile with black horizontal bars indicating the median. Whiskers indicate the highest and lowest data point within a 1.5-fold interquartile range. Significant differences in flavonoid content across all 52 accessions between the indicated conditions of the environmental simulation experiment are shown below the box plots. For comparison, significant differences in the content of the same flavonoids between nonacclimated (NA) and cold-acclimated (ACC) plants grown in the absence of UV radiation (Schulz et al. 2015) are shown as well. The significance levels (FDR *P*-values) determined by *t*-tests (two-sided with unequal variance) were corrected for multiple testing errors by the Benjamini–Hochberg procedure and are color coded according to the direction of the change as indicated in the legend. All *P*-values are shown in **Supplementary Table S4**.

the flavonoids of plants grown at 20°C with UV-A and those either grown for 2 weeks at 4°C with UV-A or at 20°C with UV-A + B. In a PLS-DA model fitted to the flavonoid data (**Supplementary Fig. S9**) quercetin-3Glc2″Rha-7Rha, kaempferol-3Glc-7Rha and quercetin-3Glc-7Rha contributed with the highest VIP scores to the separation of UV-A treated from UV-A + B treated samples (**Supplementary Table S1**).

Fig. 4 summarizes the flavonoid contents of all 52 accessions under the four different growth conditions. As already noted previously (Schulz et al. 2015), the flavonol kaempferol-3Glc6″Glc-7Rha (previously annotated as kaempferol-3GlcHex-7Rha; Ishihara et al. 2016) showed a very large range of content among the accessions, in particular at 20°C. As a consequence, no significant differences could be detected for this compound in any of the comparisons involving plants grown at 20°C (**Supplementary Table S4**). With this exception, all other flavonoids were significantly increased by UV-A + B radiation compared to UV-A at 20°C, and all except three (kaempferol-3Rha-7Rha, anthocyanin A11 and putative flavonol glycoside 2) by UV-A + B at 4°C (**Fig. 4**, **Supplementary Table S4**). The response to cold was more variable among the flavonoids, with six significantly increased and five unchanged compounds in the absence, and four significantly increased, four significantly decreased and three unchanged in the

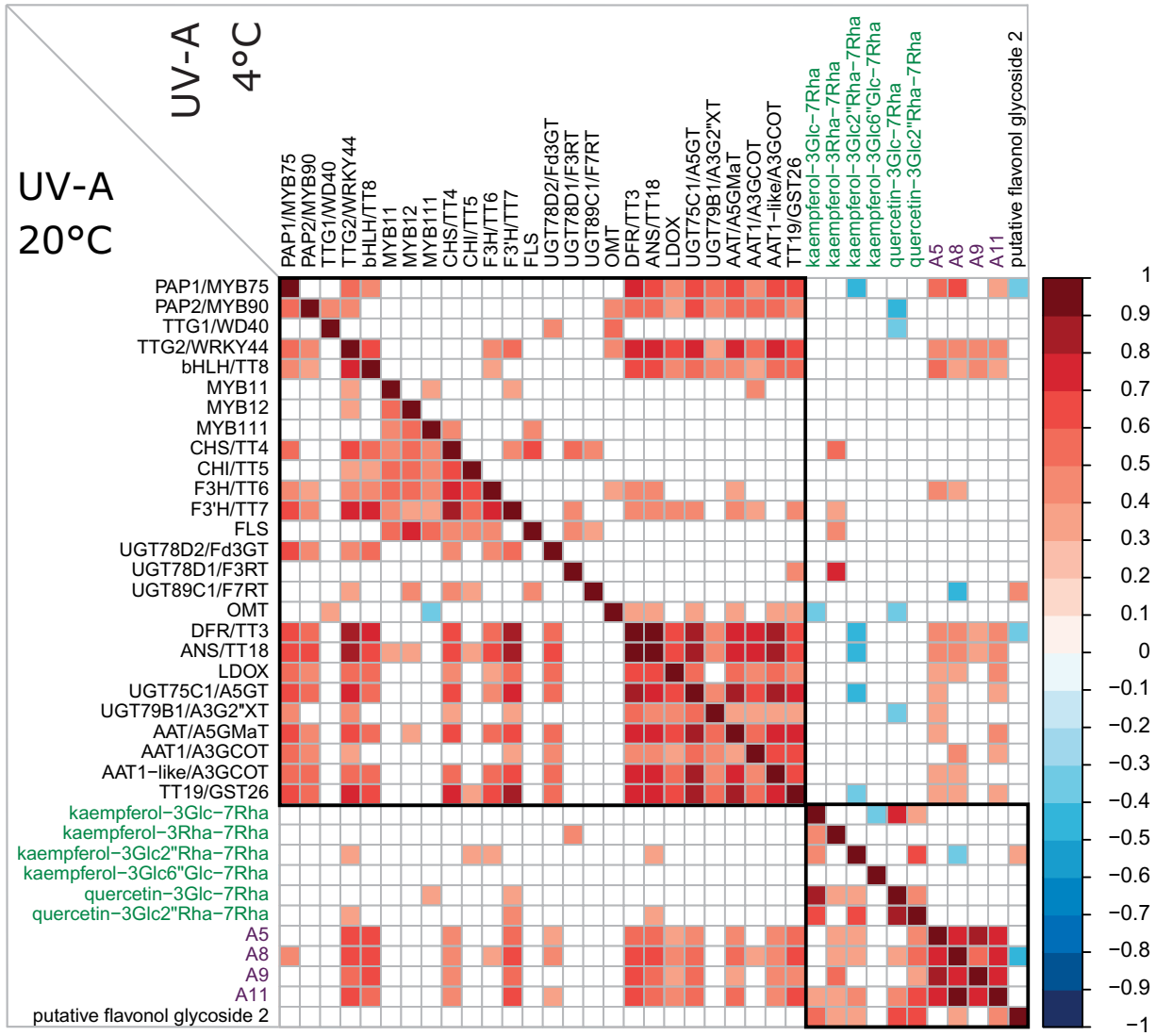

Fig. 5 Spearman correlation coefficients *r* for significant (FDR $P < 0.05$) pair-wise correlations between expression levels of flavonoid biosynthesis genes and the content of flavonoids at 20°C below and at 4°C above the diagonal. The plants were grown with UV-A, but without UV-B radiation. Blue indicates negative and red positive correlations. Correlations among transcripts or among metabolites are framed in black. All *r*- and *P*-values are shown in **Supplementary Table S5**.

presence of UV-B. This is in contrast to our previous study that showed significant increases in all flavonoids upon cold treatment in the absence of any UV radiation (Schulz et al. 2015 and **Fig. 4**).

UV-B exposure uncouples gene expression from flavonoid content and both from freezing tolerance

Figs. 5, 6 show the results of correlation analyses between the contents of all transcripts and flavonoids at 20°C and 4°C in the presence of UV-A (**Fig. 5**) or UV-A + B (**Fig. 6**; see **Supplementary Table S5** for all *r*- and *P*-values). In all four cases, we observed tight correlations among all anthocyanins, on the one hand, and among all anthocyanin biosynthetic genes, on the other hand. In addition, there were strong positive correlations between the expression levels of the genes encoding the transcription factors PAP1, PAP2, TTG2 and bHLH, and the expression levels of all anthocyanin biosynthetic genes, indicating a tight transcriptional network regulating the expression of these downstream genes under all conditions. However, this regulation did not extend to the metabolite levels under all conditions. In particular, in the presence of UV-B there were hardly any significant correlations between transcripts and flavonoid levels (**Fig. 6**), while several correlations could be observed in the absence of UV-B (**Fig. 5**). A network analysis more clearly illustrates the correlations between expression of *PAP1*, *TTG2* and *bHLH* and all anthocyanins at UV-A 4°C, whereas only the last two were correlated with the levels of all anthocyanins at UV-A 20°C in the absence of UV-B (**Fig. 7**). In addition, expression of some genes encoding upstream flavonol biosynthetic enzymes that also feed into anthocyanin biosynthesis (*CHS*, *F3'H* and *Fd3GT*) were part of the anthocyanin correlation network under UV-A at 20°C (**Fig. 7**). Strikingly, these correlations were abolished by UV-A + B treatment (**Fig. 6**). In agreement with our previous results

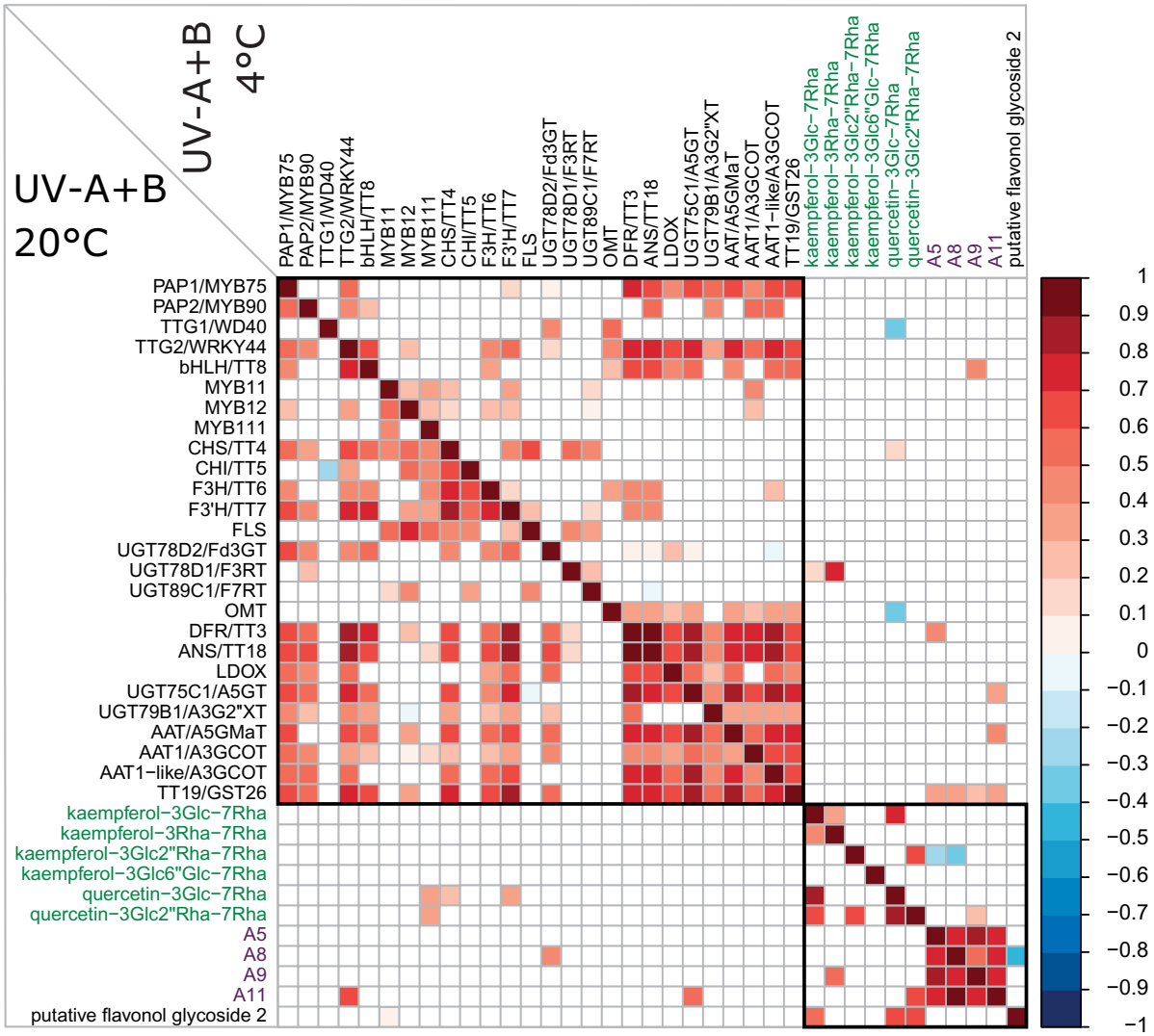

Fig. 6 Spearman correlation coefficients *r* for significant (FDR $P < 0.05$) pair-wise correlations between expression levels of flavonoid biosynthesis genes and the content of flavonoids at 20°C below and at 4°C above the diagonal. The plants were grown in the presence of UV-A + B. Blue indicates negative and red positive correlations. Correlations among transcripts or among metabolites are framed in black. All *r*- and *P*-values are shown in **Supplementary Table S5**.

(Schulz et al. 2015), we observed only very few correlations between the transcript abundance of either transcription factor or biosynthetic genes and flavonol content under any of the investigated conditions.

To gain further insight into the potential functional role of cold-induced genes and flavonoid metabolism during Arabidopsis cold acclimation, we correlated the transcript and metabolite levels under all conditions with the freezing tolerance (expressed as LT_{50}, the temperature where leaves had lost 50% of their electrolytes after a freeze-thaw cycle) of all 52 accessions determined with plants grown in the absence of any UV exposure (NA, ACC; Zuther et al. 2012). As a direct comparison, we included the corresponding correlation data from our earlier study with plants grown in the absence of any UV radiation (Schulz et al. 2015; **Fig. 8**; NA, ACC). In this previous study, we found a substantial number of significant correlations mainly for transcripts of the flavonoid biosynthetic pathway, but also for two flavonols and two anthocyanins. It is obvious from **Fig. 8** (see **Supplementary Table S6** for all *r*- and *P*-values) that all these correlations with freezing tolerance were lost when plants were grown in the presence of UV-A and/or UV-A + B.

Discussion

It is well-established that both cold and UV independently induce the accumulation of flavonoids and the expression of genes encoding transcription factors and enzymes involved in flavonol and anthocyanin biosynthesis (Christie et al. 1994,

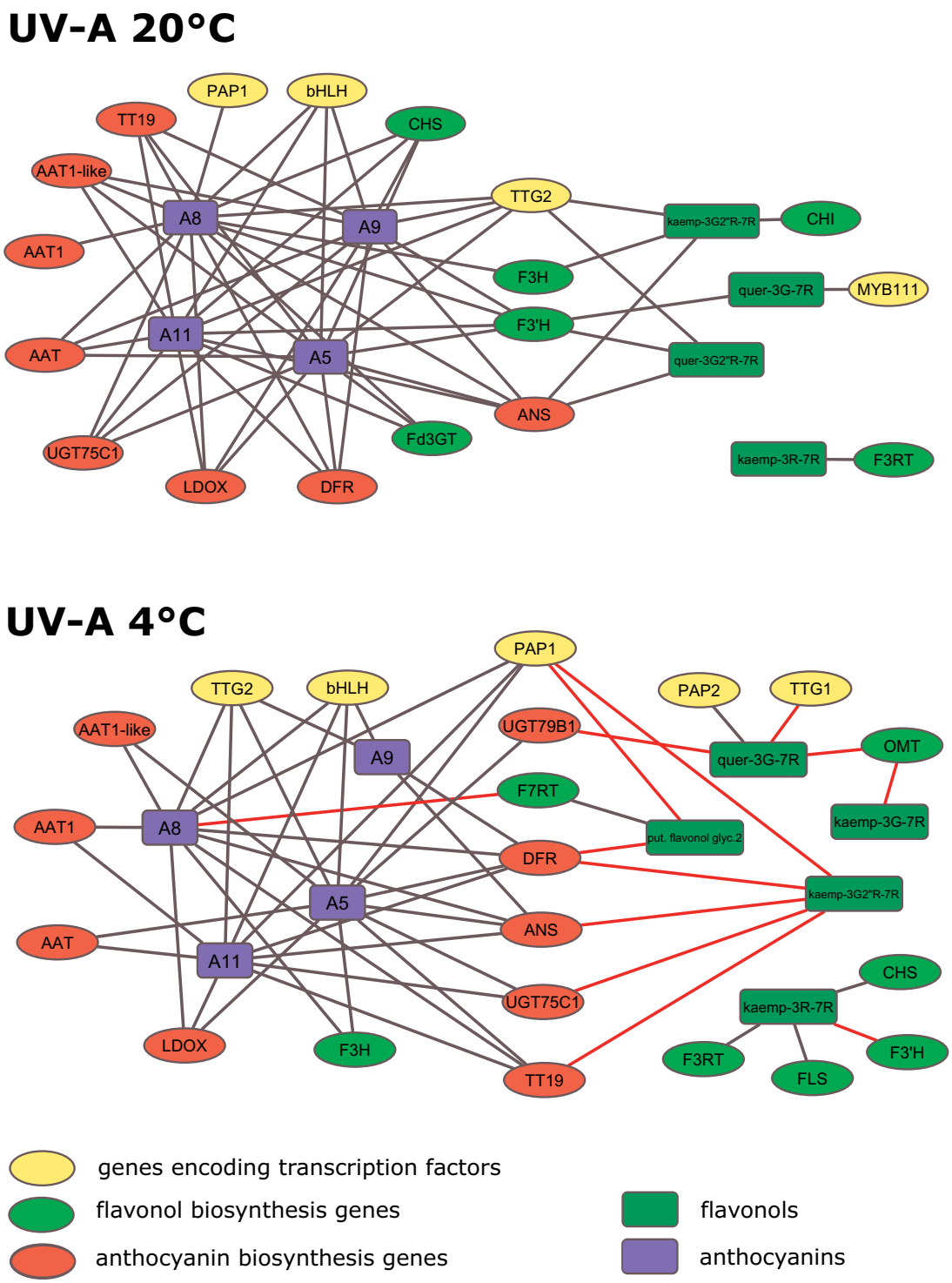

Fig. 7 Network of significant correlations ($P < 0.05$) between transcript levels of flavonoid biosynthesis genes and of flavonoid pool sizes for 52 accessions grown at 20°C (top) or at 4°C (bottom) with UV-A, but without UV-B exposure. Networks are based on Spearman correlations and only significant correlations (FDR $P < 0.05$) are shown. Positive correlations are shown by gray, negative correlations by red edges. kaemp, kaempferol; quer, quercetin, G, glucose, R, rhamnose; glyc, glycoside.

Christie and Jenkins 1996, Hannah et al. 2006, Schulz et al. 2015). However, the possible interaction between these environmental signals in the regulation of flavonoid metabolism and stress gene expression has not been explored previously. Although both cold and UV are generally considered as stress factors, they are both not damaging to many plant species, including Arabidopsis, unless temperatures become too low or radiation intensity too high (Stitt and Hurry 2002, Gitz and Liu-Gitz 2003, Hectors et al. 2007, Jenkins 2017). Instead, exposure may lead to acclimation, resulting in increased tolerance to freezing (Levitt 1980,

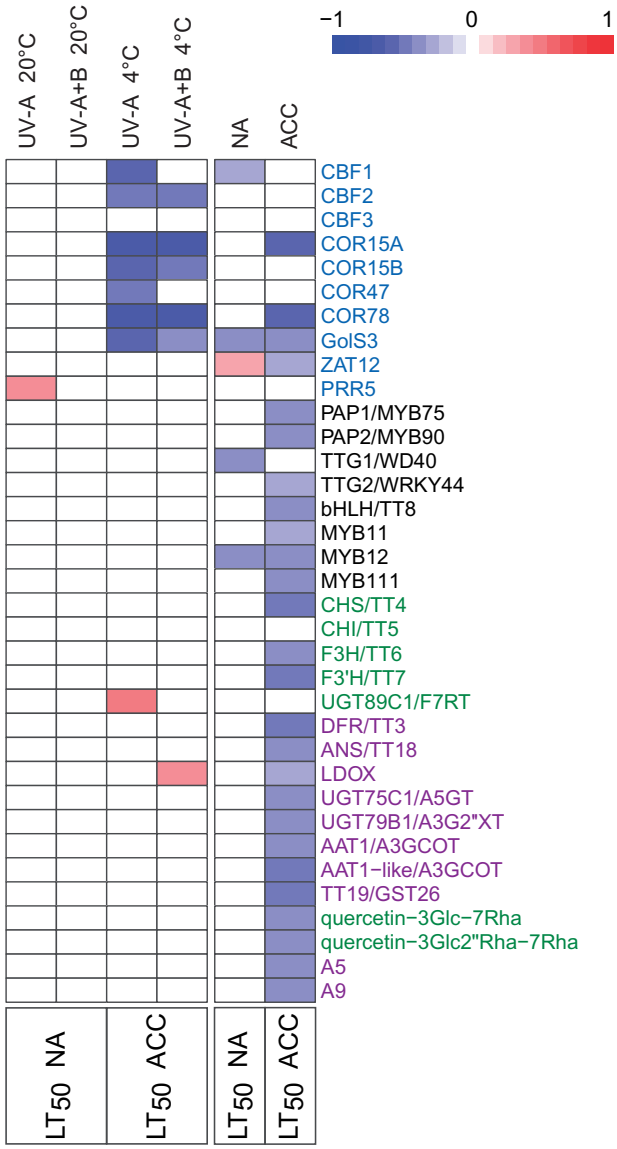

Fig. 8 Spearman correlation coefficients *r* for significant (FDR $P < 0.05$) pair-wise correlations between transcript abundance or flavonoid content and freezing tolerance expressed as LT_{50} (Zuther et al. 2012) for the 52 accessions common between the studies. LT_{50} was determined for nonacclimated (NA) and cold-acclimated (ACC) plants in the absence of UV radiation. Correlations with these LT_{50} values were calculated for the corresponding transcript and flavonoid data obtained under the same conditions (Schulz et al. 2015) or from the environmental simulation experiment reported in the present study using either UV-A or UV-A + B. Blue indicates negative and red positive correlations. Note that a negative correlation with LT_{50} means a positive correlation with freezing tolerance. All *r*- and *P*-values are shown in **Supplementary Table S6**.

Steponkus 1984) and higher intensities of UV (Burchard et al. 2000, Emiliani et al. 2013), respectively. Likewise, the plants in our experiments did not show visible signs of stress damage under any of the applied treatments, although plants stop to grow at 4°C (Beine-Golovchuk et al. 2018) and grow more slowly under mild UV radiation (Hectors et al. 2007). Consequently, we also did not observe increases in transcript levels of well-characterized stress-induced genes, such as ROS-related genes, at 20°C when we compared plants grown under UV-A or UV-A + B. In fact, under these conditions, most of these genes showed significantly reduced expression after 11 d under UV-A + B, indicating that the plants had acclimatized to this condition. On the other hand, UV-A + B radiation led to an increase in the expression of almost all flavonoid biosynthesis genes and a corresponding accumulation of almost all flavonols and anthocyanins. It has been shown previously that the accumulation of flavonols is necessary for the acclimation of plants to withstand higher doses of UV-B radiation (Brown et al. 2005).

When comparing the effects of all treatments on the expression of stress-related genes, PCA indicated that cold had the strongest effect on the expression pattern across all accessions, while UV treatment had a much smaller effect. Interestingly, most of these genes were downregulated by UV-A + B at 20°C compared to UV-A, while they were upregulated at 4°C. Likewise, cold treatment in the presence of UV-A, with or without UV-B, triggered the induction of most of the genes that have previously been identified as cold induced in the absence of UV. This indicates that UV-B signaling was strongly influenced by temperature (20°C vs. 4°C), while the cold regulation of these genes was not substantially influenced by UV radiation. The molecular basis of the interaction between the cold and UV-B signaling pathways remains to be established.

The *CBF* genes and their down-stream *COR* genes are crucial for cold acclimation, including increased freezing tolerance (see Thomashow 2010 for a review). Quite strikingly, the expression of *COR47* and *COR78* was significantly reduced at 4°C in the presence of UV-A, while a significant increase in their expression was found when the plants were grown without UV treatment (NA vs. ACC; Zuther et al. 2012). Both genes belong to the CBF regulon, which comprises ~300 genes (Shinozaki et al. 2003, van Buskirk and Thomashow 2006). Since the cold induction of the *CBF* genes was still observed in the presence of UV-A, the altered cold regulation of the *COR47* and *COR78* genes must be due to a different, so far not identified, regulatory factor. Whether there are more genes in the CBF regulon that respond in a similar manner and whether that has an influence on plant cold acclimation and freezing tolerance remains to be investigated.

Similar to the *COR* genes, also the three genes encoding ROS defense proteins (*sAPX*, *PrxQ*, *2CPA*) and the two *ELIP* genes were significantly induced by cold in the presence of UV-A and UV-A + B. APX activity also showed an increase during an UV-B treatment in *Deschampsia antarctica* (Köhler et al. 2017) and was increased upon salt stress but not UV-B treatment in Populus, but increased with combined salt/UV-B (Ma et al. 2016). In the absence of any UV radiation, only *sAPX* is cold induced, while *PrxQ* and *2CPA* show no significant changes in expression, as determined by RNA-Seq in the accessions Col-0 and N14 (Zuther et al. 2019). This may indicate a stronger activation of ROS defenses in the cold when the plants are additionally challenged with UV, which may indicate a higher ROS production under the combined stress conditions. Interestingly, *ELIP2* showed an ~100-fold cold induction and was therefore more strongly cold induced than genes such as *COR15A*.

However, both *ELIP1* and *ELIP2* are also strongly cold induced at 4°C in the absence of UV (Zuther et al. 2019). It is currently unclear, whether the two proteins play a functional role in cold acclimation or UV protection. It has only been shown that inactivation of both genes has no detrimental effect on high-light tolerance of Arabidopsis at low temperature (8°C), but freezing tolerance or UV effects were not investigated (Rossini et al. 2006).

While the influence of UV radiation on flavonoid biosynthesis has been studied extensively, the interaction of UV with other abiotic stress factors has received much less attention. Only interactions of UV-B and drought stress have been investigated in any detail (Bandurska et al. 2013). It has been shown that the growth reduction observed under combined UV-B and drought is an additive effect of the reduction observed under the separate stresses (Comont et al. 2012). Furthermore, the higher tolerance of plants pretreated with UV-B before drought could be related with reduced water loss under drought conditions (Schmidt et al. 2000, Poulson et al. 2006) due to lower stomatal conductance and density after UV-B treatment (Gitz and Liu-Gitz 2003, Poulson et al. 2006). Since UV-B induces flavonoid accumulation, a protective role of increased flavonoid content, e.g. as antioxidants, has been proposed (Bandurska et al. 2013), but has, to the best of our knowledge, not been experimentally tested.

In the interaction between UV and cold, PCA and PLS-DA indicated that, unlike the stress-response genes, the expression of flavonoid biosynthesis genes and also of flavonoids was more strongly influenced by UV than by cold treatment. However, there were interesting interactions between the two conditions. While in the absence of UV radiation cold induced a general increase in the expression of flavonoid biosynthesis genes and of flavonols and anthocyanins (Schulz et al. 2015), the same genes were mostly downregulated at 4°C in the presence of UV-A and UV-A + B. On the other hand, changes in flavonoids were more variable, showing increases, decreases or no significant changes after cold exposure. At 20°C, all flavonoid biosynthesis genes and all flavonoids were strongly induced by UV-A + B compared to UV-A. This response was much weaker at 4°C, where the gene encoding the transcription factor PAP2 was even downregulated.

We did not observe any significant correlations between the expression levels of flavonol biosynthesis genes and flavonols at either 20°C or 4°C under UV-A with or without UV-B. This is in agreement with a previous analysis that also reported a lack of such correlations in the absence of any UV radiation (Schulz et al. 2015). On the other hand, we observed significant correlations between the expression of anthocyanin biosynthetic genes and anthocyanin content under UV-A in the absence of UV-B. These correlations were completely lost after UV-A + B treatment. Collectively, these observation indicate that UV-A has a massive influence on the cold regulation of both flavonoid biosynthesis genes and flavonoids, leading to a repression of the expression of genes that are cold induced in the absence of UV-A. On the other hand, cold reduces the UV-B response of flavonoid biosynthetic genes in the presence of UV-A, and abolishes any correlations between gene expression and anthocyanin content. This suggests that UV-B triggers specific post-translational regulatory mechanisms in anthocyanin biosynthesis that remain to be identified.

Exposure of Arabidopsis plants to low temperature triggers a multitude of changes in transcript, protein and metabolite content (see e.g. Shinozaki et al. 2003, Guy et al. 2008, Thomashow 2010 for reviews; Hincha et al. 2012), including an accumulation of flavonoids (Schulz et al. 2015) that ultimately result in increased freezing tolerance, although the quantitative contribution of any single factor is unknown. While it has been shown that visible light (i.e. photosynthetically active radiation) is necessary for cold acclimation (Wanner and Junttila 1999) and for the efficient cold induction of CBF regulon genes (Catala et al. 2011), comparable data for cold and UV have, to the best of our knowledge, not been published. However, the reduced expression of *COR* and ROS-related genes indicates that at 20°C UV-B radiation may result in reduced freezing tolerance. It is tempting to speculate that this may be an additional trigger to increasing temperatures in spring for plants to deacclimate and induce growth and development (Vyse et al. 2019). The cold induction of these genes, on the other hand, is very similar in the absence of UV, or in the presence of UV-A or UV-A + B, indicating functional cold acclimation under all investigated conditions. Obviously, further investigations will be necessary to test these hypotheses.

In the absence of UV radiation, the expression levels of several flavonoid biosynthesis genes and the content of two flavonols and two anthocyanins are significantly correlated with the freezing tolerance of Arabidopsis accessions after cold acclimation in the absence of UV (Schulz et al. 2015). In addition, the investigation of several flavonoid biosynthesis mutants showed the functional importance of at least some flavonols for cold acclimation (Schulz et al. 2016). Here, we showed that all correlations of gene expression and flavonoid content with freezing tolerance were lost when plants were grown in the presence of UV-A or UV-A + B. Of course, the LT_{50} values used for these correlation analyses were determined with plants grown in the absence of UV (Zuther et al. 2012) and it is possible that freezing tolerance itself is influenced by UV exposure. However, we found strong correlations between LT_{50} and the expression levels of most of the cold-induced genes that we investigated, with an overlap of three cold-induced genes (*COR15A*, *COR78*, *GolS3*) that showed significant correlations with freezing tolerance under all three 4°C treatment conditions, i.e. no UV, UV-A, UV-A + B. The persistence of these correlations indicates that the complete loss of correlations related to flavonoids may have biological significance. It suggests that under different environmental conditions, different classes of molecules may be more or less important for stress tolerance, with plants obviously balancing different transcriptional and post-transcriptional/post-translational responses that lead to new adaptation strategies that are not readily predictable from reactions to single stress factors. Our work provides a solid basis for further, more detailed molecular studies into the interplay between cold and UV signaling and its effects on freezing tolerance.

Materials and Methods

Plant material and growth conditions

We used 52 accessions of *Arabidopsis thaliana* as reported previously (Zuther et al. 2012, Schulz et al. 2015). Seeds were either kindly provided by the INRA, Versailles, France (http://publiclines.versailles.inra.fr/naturalAccession/index, last accessed: 15.11.2020) belonging to the set of 48 accessions of the Versailles Core collection or were propagated at the MPIMP, Potsdam, Germany as described previously (Hannah et al. 2006).

Plants were grown in environmental simulation chambers at the Research Unit Environmental Simulation (EUS), Institute of Biochemical Plant Pathology at the Helmholtz Centre Munich (see **Supplementary Fig. S1** for a flow chart of the growth conditions). Seeds were vernalized for 2 d at 4°C in the dark, subsequently sown on soil and kept for 7 d under long-day conditions (16/8 h, 20°C/18°C, day/night cycle) for germination, followed by 6 d under short-day conditions in the greenhouse (10 h/14 h, 20°C/18°C, day/night). Plants were transferred into environmental simulation chambers (PAR 370 μmol m^{-2} s^{-1}, UV-A 6 W m^{-2}, 20°C/18°C day/night, 70% relative humidity) and subjected for 9 d to short-day conditions (8 h/16 h light, day/night) and for 3 d to long-day conditions (16 h/8 h light, day/night). Then, six individual plants per accession were subjected to four different treatments in parallel simulation chambers for 11 d (until 36 days after sowing), respectively. As a control, one set of plants was kept under the previous conditions (PAR 370 μmol m^{-2} s^{-1}, UV-A 6 W m^{-2}, 16 h/8 h, 20°C/18°C day/night, 70% relative humidity, UV-A 20°C condition). The second set of plants was additionally treated with UV-B radiation for 13 h during the light phase (UV-A 6 W m^{-2}, UV-B$_{BE}$ 115 mW m^{-2}), termed UV-A + B 20°C condition. For the UV-A 4°C condition, plants were treated with lower PAR to avoid light stress (PAR 280 μmol m^{-2} s^{-1}, UV-A 6 W m^{-2}, 4°C/2°C day/night). The fourth set of plants was subjected to UV-A + B and 4°C conditions (PAR 280 μmol m^{-2} s^{-1}, UV-A 6 W m^{-2}, UV-B$_{BE}$ 115 mW m^{-2}, 4°C/2°C day/night). The UV-B treatment was started 1 d after the cold treatment to minimize plant stress. The biologically active UV-B radiation (UV-B$_{BE}$) was calculated as described by Caldwell (1971) using a normalization at 300 nm. The UV-B$_{BE}$ of 115 mW m^{-2} corresponds to a daily dose of UV-B of ~4.9 kJ m^{-2} d^{-1}.

Liquid chromatography - mass spectrometry (LC-MS) analysis of flavonoids

For the extraction of flavonols and anthocyanins a combined extraction protocol for primary metabolites, secondary metabolites and lipids was used (Giavalisco et al. 2009). In brief, 1 ml of precooled extraction mixture (−15°C) containing 25% (v/v) methanol and 75% (v/v) methyl-*tert*-butyl-ether (MTBE, HPLC grade), spiked with 0.5 μg ^{13}C sorbitol (Sigma Aldrich, Taufkirchen, Germany), 5 μg isovitexin (Extrasynthese, Lyon, France) and 0.5 μg 1,2-diheptadecanoyl-*sn*-glycerol-3-phosphocholine (Avanti Polar Lipids, Alabaster, AL) as internal standards was added to 100 mg of homogenized rosette material. Samples were incubated for 10 min at 4°C on a thermomixer and for an additional 10 min in an ultra-sonication bath at room temperature. Phase separation was induced by adding 500 μl of a mixture of water and methanol (3:1, ULC/MS grade) and subsequent centrifugation (14,000 × *g*, 5 min, 4°C) of the homogenized extracts. For determination of flavonoids, 20 μl of the lower polar phase containing primary and secondary metabolites were diluted 1:5 in 80 μl of the water methanol mixture (3:1) and stored at −80°C.

Five μl of the extracts were applied to an HPLC/photodiode array (PDA)/Electrospray Ionization (ESI)-Linear Ion Trap mass spectrometry (ITMS) system (Thermo Finnigan, San Jose, CA). Profiling of flavonoids by LC-MS was performed in positive and negative ion detection mode as described previously (Schulz et al. 2015). Peak identification and annotation were performed with Xcalibur (Thermo Finnigan) based on previously published reference data (Schulz et al. 2015, 2016, Tohge et al. 2016). Relative peak areas representing mass spectral ion currents were normalized to sample fresh weight and the peak area of the internal standard isovitexin. The average was calculated for the three replicates per accession and condition consisting of two individual plants, respectively. Description of all detected peaks is provided in **Supplementary Table S7**.

For comparison with the flavonoid data of the greenhouse and cold chamber experiment (NA, ACC) from Schulz et al. (2015), the raw data for the common 52 accessions and 10 flavonoid compounds of both experiments were normalized to the sum of all flavonoid compounds of each replicate.

Quantitative real-time PCR (qRT-PCR) analysis of gene expression

Quantitative RT-PCR was performed as described (Schulz et al. 2015). In brief, total RNA was extracted from leaf material using Trizol reagent (Invitrogen, Nidderau, Germany) and DNase treated (Ambion, Austin, TX, USA). First-strand cDNA was synthesized from 2.5 μg quality-proved total RNA using Superscript III reverse-transcriptase (Invitrogen). qRT-PCR was performed with an ABI PRISM 7900 HT 384-well plate Sequence Detection System (Applied Biosystems, Darmstadt, Germany). Reactions containing 2.5 μl 2 × SYBR Green Master Mix (Fast Power SYBR Green; Applied Biosystems), 0.5 μl cDNA (diluted 6-fold) and 2 μl of 0.5 μM primers were pipetted with an Evolution P3 pipetting robot (PerkinElmer, Zaventem, Belgium). The sequences of all primers, including those for quality checks, are given in **Supplementary Table S8**. Ct values for the genes of interest were normalized by subtracting the mean of four reference genes and averages of three biological replicates were calculated per accession and condition (**Supplementary Table S2**).

Data analysis

The statistical software R (**http://www.r-project.org**, (November 15, 2020, date last accessed)) was used to perform statistical analyses of the normalized data. Probabilistic PCA was carried out with the *pcaMethods* package and the function respPCA on log$_{10}$-transformed data centered to the median of all samples. Two-sided *t*-tests with unequal variance were performed to evaluate differences between the investigated treatments. Multiple testing correction was performed with the function p.adjust from the *stats* package according to the Benjamini and Hochberg procedure (Benjamini and Hochberg 1995). Spearman correlation analysis was computed with the function rcorr with the package *Hmisc* and multiple testing correction was performed as stated before. The package *corrplot* was used to visualize significant transcript-metabolite correlations and networks were built up on this with Cytoscape. Heatmaps were generated with the package *pheatmap* to visualize fold changes (FC) calculated for the accessions between the conditions in log$_2$ scale.

PLS-DA was performed according to Pérez-Enciso and Tenenhaus (2003) using the R-package mixOmics (Rohart et al. 2017). Data were log10 transformed and fitted into PLS-DA models with maximum or centroid (flavonoids) distance and a corresponding number of components. The model performance was calculated using a 5-fold cross-validation and 10 replicates. Classification error rates were calculated (0.1836 for stress-related genes, 0.1035 for flavonoid biosynthesis genes, 0.1746 for flavonoids). ROC curves and AUC values were generated (not shown). Sample plots were established with confidence ellipses for each class (confidence level set to 95%). VIP were calculated.

Supplementary Data

Supplementary data are available at PCP online.

Funding

We acknowledge the Max Planck Society for funding. E.S. was supported through a PhD fellowship from the Deutsche Bundesstiftung Umwelt.

Acknowledgments

We thank Kora Vyse for her excellent help with sample preparation.

Disclosures

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

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