Central Metabolic Responses to Ozone and Herbivory Affect Photosynthesis and Stomatal Closure

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Plants have evolved adaptive mechanisms that allow them to tolerate a continuous range of abiotic and biotic stressors. Tropospheric ozone (O$_3$), a global anthropogenic pollutant, directly affects living organisms and ecosystems, including plant-herbivore interactions. In this study, we investigated the stress responses of *Brassica nigra* (wild black mustard) exposed consecutively to O$_3$ and the specialist herbivore *Pieris brassicae*. Transcriptomics and metabolomics data were evaluated using multivariate, correlation, and network analyses for the O$_3$ and herbivory responses. O$_3$ stress symptoms resembled those of senescence and phosphate starvation, while a sequential shift from O$_3$ to herbivory induced characteristic plant defense responses, including a decrease in central metabolism, induction of the jasmonic acid/ethylene pathways, and emission of volatiles. Omics network and pathway analyses predicted a link between glycerol and central energy metabolism that influences the osmotic stress response and stomatal closure. Further physiological measurements confirmed that while O$_3$ stress inhibited photosynthesis and carbon assimilation, sequential herbivory counteracted the initial responses induced by O$_3$, resulting in a phenotype similar to that observed after herbivory alone. This study clarifies the consequences of multiple stress interactions on a plant metabolic system and also illustrates how omics data can be integrated to generate new hypotheses in ecology and plant physiology.

Under natural conditions, plants are exposed continuously to abiotic and biotic stresses. When studied in the laboratory, individual stresses trigger a variety of molecular, cellular, and physiological responses (Rejeb et al., 2014; Suzuki et al., 2014). However, the way plants protect themselves in nature cannot be predicted on the basis of responses to individual stresses alone, because combined stresses may elicit antagonistic, neutral, or synergistic effects (Rizhsky et al., 2002, 2004; Mittler, 2006; Pandey et al., 2015). Thus, there is increasing interest in plant responses to multiple stress conditions. However, while several studies have examined multiple abiotic factors (Suzuki et al., 2014), biotic factors usually are limited to pathogen infection (Sharma et al., 1996; Xiong and Yang, 2003; Anderson et al., 2004; Prasch and Sonnewald, 2013; but see Atkinson and Urwin, 2012; Atkinson et al., 2013).

Global warming encompasses a range of interrelated abiotic phenomena, including increases in the Earth’s average temperature and changes in the atmosphere’s content of greenhouse gasses such as methane, carbon dioxide (CO$_2$), and ozone (O$_3$). Burning fossil fuels releases nitrogen oxides and hydrocarbons, which in the presence of sunlight react to form tropospheric O$_3$, the most significant atmospheric pollutant in terms of phytotoxicity (Ludwikow and Sadowski, 2008; Renaut et al., 2009; Van Dingenen et al., 2009). Depending on the duration and intensity of the exposure, O$_3$ can induce a range of responses that are typically deleterious to plant fitness, disturbing photosynthetic processes, energy and carbon metabolism, cellular detoxification, and transpiration (Bagard et al., 2008; Dizengremel et al., 2008; Fares et al., 2010; Goumenaki et al., 2010;
Salvatori et al., 2015). While acute exposure to high O$_3$ levels can rapidly induce cell death and chlorosis (Ashmore, 2005; Kangasjärvi et al., 2005; Vainonen and Kangasjärvi, 2015), negative yield responses are not always correlated with the severity of symptoms in leaves, and exposure to O$_3$ can affect metabolic processes before any visible injury (Long and Naidu, 2002; Dizengremel et al., 2009; Sawada and Kohno, 2009; Pinto et al., 2010).

Global climate change also is predicted to increase damage to plants caused by herbivory, via both direct effects on the herbivore behavior and survival and indirect effects on the host plant condition (Bale et al., 2002; Fuhrer, 2003; Ditchkoff et al., 2009; Lindroth, 2010; Khaling et al., 2015). Negative effects of herbivory and defoliation on plant fitness include the systemic down-regulation of photosynthesis and reduced CO$_2$ assimilation (Zangerl et al., 2002; Hui et al., 2003; Ralph et al., 2006; Tang et al., 2006). Thus, to protect against herbivores, plants allocate resources between growth and defense (Koricheva, 2002; Schwachtle and Baldwin, 2008; Firn and Jones, 2009; Havko et al., 2016). Brassicaceae plants fine-tune their defenses by inducing phythohormone signaling, which activates cross talk between the jasmonic acid (JA) and ethylene (ET) pathways in response to leaf chewing by herbivores (De Vos et al., 2005; Pieterse et al., 2012). As a specialist herbivore of Brassicaceae, the large white butterfly Pieris brassicae is an important pest of Brassica nigra (wild black mustard). P. brassicae oviposition on the host plant is influenced by glucosinolates (sulfur- and nitrogen-containing glucosides) that function as chemical cues for the butterfly (Fahey et al., 2001; Petersen et al., 2002; Halkier and Gershenzon, 2006; Textor and Gershenzon, 2008). Young caterpillars primarily feed on mature leaves of B. nigra, but after the third instar, they move to fresh tissues with higher glucosinolate content, such as flowers and buds (Smalleyganje et al., 2007). Therefore, descriptions of plant-herbivore interactions frequently emphasize specialized (secondary) metabolism (Simmonds, 2003; van Dam et al., 2004; Poelman et al., 2010; Boeckler et al., 2011; Lof et al., 2013; Onkokesung et al., 2014). However, there is an extensive functional overlap between the growth- and defense-related aspects of plant metabolism, and reconfiguration of the plant’s ability to adapt to sequential abiotic and biotic stresses. In a later experiment, we measured photosynthesis and gas exchange to assess the impact of the stresses on B. nigra physiology. These findings confirmed a negative effect of P. brassicae herbivory on the plant’s ability to regulate stomatal closure and transpiration in response to O$_3$.

RESULTS

P. brassicae was subjected to three stress scenarios: O$_3$ fumigation at 70 nL L$^{-1}$ for 5 d (O); herbivore feeding by first instar P. brassicae caterpillars for 24 h (P); and exposure to O$_3$ followed by herbivory (OP). Leaf samples were shared for omics analyses (Fig. 1, experiment 1). The transcriptomics screening was based on Arabidopsis (Arabidopsis thaliana) CATMA version 4 whole-genome microarrays (Sclep et al., 2007), while the metabolomics screening combined gas chromatography-mass spectrometry (GC-MS), liquid chromatography-mass spectrometry (LC-MS), and GC-MS analysis of volatile organic compounds (VOCs) collected via dynamic headspace sampling (Supplemental Data Sets S1 and S2). The omics data were first examined separately and then integrated via network and Gene Ontology (GO) analyses. Analysis of the molecular and metabolic responses prompted the development of a model suggesting that the regulatory dynamics associated with O$_3$ are opposed to those induced by herbivory. This hypothesis was tested in a follow-up experiment (Fig. 1, experiment 2) that measured physiological variables including chlorophyll levels, photosynthesis rates, and gas exchange for the same stress conditions (O, P, and OP) and during long-term (16-d) O$_3$ exposure (OL).

Transcriptome Responses

Hierarchical cluster and GO analyses of 970 differentially expressed genes ($P < 0.05$; Fig. 2, A and B) separated the O$_3$ stress treatment (O) from the herbivore stress (P) and the sequential stress (OP). The ontological category exhibiting the strongest response...
after GO enrichment was energy metabolism, including photosynthesis and the mitochondrial electron transport chain (ETC; Fig. 2A and B). Overall, changes in gene expression induced in O were highly homogenous (Pearson’s coefficient, \( r = 0.6-0.8 \)), whereas changes induced in P and OP treatments exhibited a weaker intracluster correlation (\( r = 0.2-0.5 \); Fig. 2C). MapMan pathway analysis confirmed that the responses for each treatment were consistent across several biological levels (Fig. 2D), and Venn diagrams based on log2 fold changes of 0.585 or greater (for \( P < 0.05 \)) indicated a dominance of up- and down-regulated genes in O, with the least impact in OP (Fig. 2E).

Genes involved in light harvesting and carbon assimilation were down-regulated in all stress scenarios but particularly in O (Fig. 2D and F), which also resulted in up-regulation of the nonphotochemical quenching gene \( NPQ1 \) (Fig. 2F). Other primary processes, such as amino acid and carbohydrate metabolism, generally showed opposite patterns of regulation for O and herbivory (Fig. 2D and G). In the sequential treatment (OP), \( B. \ nigra \) activated defense responses that resembled a response to herbivore stress alone (P; Fig. 2, H and I): lipoxygenases \( LOX2 \) and \( LOX3 \) (Felton et al., 1994; Haltitschek and Baldwin, 2003), the trypsin inhibitor \( WSCP \) (Zavala and Baldwin, 2004; Boex-Fontvieille et al., 2015), and the mitogen-activated protein kinase \( MPK3 \) (Pitzschke and Hirt, 2009). Notably, genes involved in stress responses and phytohormone signaling were differentially regulated between O3 stress alone and the herbivore treatments (e.g. \( WRKY5, MYC2, \) and \( ERF2 \)), and only in O were genes associated with abiotic stress responses induced, such as drought (MYB44), senescence (EIN3), and phosphate starvation (\( RNS1 \) and \( PT2/PHT1.4 \); Fig. 2, H and I).

**Metabolome Responses**

A multivariate analysis of the metabolome profile explained 65% of the total metabolic variation and 95% of the treatment effects (partial least squares-discriminant analysis [PLS-DA]; Fig. 3A; Supplemental Fig. S1; Supplemental Table S1). Both O3 and herbivore stress caused a shift from the basal metabolic state of the untreated plants, inducing unique profiles as single treatments in O and P. However, the effect of herbivory largely overshadowed that of O3 in the sequential treatment OP, as indicated by the swarm overlap with P (Fig. 3A). Despite the importance of several metabolite pools in the multivariate model (Fig. 3B; Supplemental Table S2; 70 compounds for variable importance for projection [VIP] scores > 1), single univariate effects were confirmed only for a subset of these (Fig. 3C; ANOVA posthoc Tukey’s tests).

Opposite effects of O3 and herbivory were observed on pools of primary metabolites (sugars, amino acids, and organic acids), which increased in O and decreased in P and OP (Fig. 3B; Supplemental Table S2). However, a common response to these treatments was a 3-fold induction of \( \gamma \)-aminobutyric acid (GABA; Fig. 3, B and C), which partly correlated with its precursor \( \alpha \)-ketoglutarate (Fig. 3B). Most noticeably, glycerol levels in O increased to 155% of the steady-state value (Fig. 3, B and C) but were restored in the sequential treatment OP. A similar trend was observed for ethanolamine, another metabolite of the glycerophospholipid pathway and a component of lipid membranes (Fig. 3C). Phenolic compounds such as flavonols and cinnamic acid ester derivatives (Lin et al., 2011; Shao et al., 2014) increased in all treatments.

Glucosinolate levels were stable or reduced (with glucoraphanin the only exception), whereas VOCs...
Figure 2. Transcriptome responses in B. nigra under multiple O₃ and herbivory stress treatments: O₃ stress (O), herbivory by P. brassicae (P), and both stresses sequentially (OP). A, Heat map showing statistically significant (P ≤ 0.05) changes in the expression of 970 genes in different samples from the O, P, and OP treatments, relative to control plants. Red and blue indicate up- and down-regulation, respectively. The subfigure at right shows the GO enrichment of specific biological processes in each cluster visible in the heat map, ranked (I–III) according to the relative abundance of genes associated with the biological process in question within the cluster. The color scheme used here matches that used in B. B, All of the GO processes that were significantly enriched in all the gene clusters, ordered by their relative abundance (for all 970 genes). C, Heat map of r as calculated for each sample pair from the corresponding hierarchical clustering; darker shades of blue indicate stronger correlations. D, MapMan
were emitted upon herbivore damage (P and OP), including glucosinolate derivatives and green leaf volatiles (GLVs; Fig. 3, Band C; Supplemental Table S2). Two unidentified secondary compounds, described previously by Khaling et al. (2015; i.e. 421 [M-H]², mass-to-charge ratio [m/z] 485.13, and 381 [M-H]², m/z 349.15), also were induced, confirming their importance for both O and OP (Fig. 3C).

Omics Integrative Network Correlation Analysis

Transcriptomics and metabolomics profiles were integrated in a scale-free correlation network (Fig. 4; for topology, see Supplemental Fig. S2). The network was dominated by assortative high-degree nodes (hubs), with major hubs involved in processes such as stress signaling, cellulose biosynthesis, chloroplast activity, and stomatal regulation (Table I). Several primary metabolites clustered around the central region of the network, while distinct modules of secondary metabolites clustered at the periphery (i.e. glucosinolates, flavonol glucosides, hydroxycinnamic acid derivatives, and VOCs; Fig. 4). Most glucosinolates were connected to CYP71, a cytochrome involved in herbivore-induced responses and nitrile formation (At5g25120/At5g25180; Bennett et al., 1993; Irmisch et al., 2014). Glucobrassicin and neoglucobrassicin (indolics) clustered directly with VOCs (nitriles and GLVs) and correlated positively with biological functions (bins) showing the effect of the stress treatments on gene expression relative to amino acid metabolism, biotic and abiotic stress, cell wall biosynthesis, photosynthesis, and the Calvin cycle. E, Venn diagrams (MapMan generated) comparing genes up- and down-regulated in response to the treatments for log2 fold-change thresholds greater than 0.585 (corresponding to P ≤ 0.05). F to I, Univariate analysis relevant to the regulation of central metabolism and stress responses: processes of photosynthesis and light harvesting (F), carbohydrate metabolism (G), biotic and abiotic stress (H), and response and signaling (I). Relative transcript accumulation is reported as log2 values. Significance relative to the controls (zero level) is reported as Student’s t test values as follows: *, P ≤ 0.05; **, P ≤ 0.01; ***, P ≤ 0.001; and ****, P ≤ 0.0001. Error bars indicate s.e. Treatments are indicated by color: O3 stress (O) in white, herbivory by P. brassicae (P) in gray, and sequential treatment (OP) in black.
WRKY40 (involved in indolic glucosinolate biosynthesis and GLV emissions; Schön et al., 2013; Mirabella et al., 2015) as well as WRKY46 and CYP707A3 (both involved in abscisic acid [ABA] metabolism; Saito et al., 2004; Liu et al., 2012; Geilen and Böhmer, 2015; Fig. 4C). The herbivory response (P) linked to glycerol via four nodes, including two genes coding for flavin monooxygenases, NOGC1 (At1g62580) and FMO (At1g12200), which negatively correlated with glycerol ($\rho = -0.86$, $P < 0.001$) and positively correlated with each other ($\rho = 0.85$, $P < 0.001$; Fig. 4, A and B). Because MYB44 linked to the same module and negatively correlated with NOGC1 ($\rho = -0.91$, $P < 0.0001$), MYB44 positively correlated with glycerol ($\rho = 0.68$, $P = 0.006$; Fig. 4B). Notably, both MYB44 and NOGC1 are involved in the osmotic stress response and the regulation of stomatal closure.

Responses Relative to Energy and Glycerol Metabolic Networks

Genes associated with energy metabolism (including both the photosystems and the mitochondria) were the most heavily affected by the stress treatments (Fig. 2, A and B). A PLS-DA model for these energy genes (which were functionally connected in AraNet; Fig. 5A) strongly separated the stress treatments O, P, and OP, explaining 54% of the variation in gene expression and 99% of the treatment effects (Fig. 5B; Supplemental Table S4). In response to O$_3$ stress, the mitochondrial ETC complex II succinate dehydrogenase subunit SDH1-1 (At5g66760) and the mitochondrial superoxide dismutase MSD1 (At3g10920) were both up-regulated (Fig. 5C; Supplemental Table S5). In the general model for expression of all 970 genes (Fig. 5, D and E; Supplemental Tables S6 and S7), SDH1-1 again strongly described the effect of O$_3$ (O and OP) and was positively correlated with ALDH7B4 (At1g54100; $\rho = 0.65$, $P = 0.005$; Fig. 5, E and F), an aldehyde dehydrogenase involved in glycerol metabolism. Visualisation of the entire glycerolipid pathway in the Kyoto Encyclopedia of Genes and Genomes (KEGG)/KapPA-View4 confirmed the up-regulation in O of ALDH7B4 (At1g54100; $P < 0.05$), which, together with ALDH7B4, reversibly converts glycerate into glyceraldehyde and glycerol (for paths, see Supplemental Figs. S6 and S7). Interestingly, both AKR4c10 and ALDH7B4 are involved in oxidative...
Up-regulated in O compared with OP (fatty acid catabolism; El-Kouhen et al., 2005) was particularly the triacylglycerol lipase LIP1. In O, the triacylglycerol lipase LIP1 was strongly up-regulated by the activity of eight genes (VIP score > 1): GLR2.9, COR413, NOGC1, CML41, GH9B8, M3, NOGC1/FMO, and M3. Moreover, the glycerol 3-phosphate (G3P) metabolism (G3P shuttle), photosynthesis (chlorophyll biosynthesis, sulfolipids, and photosystem stabilization) processes highlighted the connection between glycerol, G3P shuttle, photosynthesis, sulfolipids, and photosystem stabilization. New functional links between these processes (Figs. 2, 3, 5, 6), possibly in connection with the osmotic stress response and stomatal regulation (Fig. 4). To better describe the system transitions during the stress treatments (O, P, and OP), we performed a comparative correlation analysis (Steuer, 2006) between the regulation of glycerol, G3P shuttle (GPDHc1/SDP6), mitochondrial ETC (SDH1), and stomatal closure (NOGC1/FMO; Fig. 7, A and B), while GO network analyses (AraNet and GeneMANIA) provided biological insights into potential functional relationships between these processes (Fig. 7, C and D). In AraNet, GO functional associations were predicted for genes involved in glycerol metabolism that most strongly responded to the dynamics of the sequential treatment (Fig. 5). Overall, the network was enriched in processes of fatty acid biosynthesis, mitochondrial metabolism (G3P shuttle), photosynthesis (chlorophyll biosynthesis, sulfolipids, and photosystem stabilization), response to phosphate starvation, and stomatal closure (Table II). New functional links between these processes highlighted the connection between glycerol metabolism (particularly via the G3P shuttle; GPDHc1 and SDP6) and central energy metabolism (e.g. the pentose phosphate shunt, NADPH regeneration, glycolysis, ATP synthesis, and mitochondrial ETC; Fig. 7C). In AraNet, SDP6 was linked to SDH1-1 (Fig. 7C), and GeneMANIA specifically predicted that the corresponding proteins interact and are coexpressed (Fig. 7D). In keeping with this result, we found that SDH1-1 and SDP6 were strongly coexpressed during O3 stress in O (ρ = 0.97, P = 0.005) and during herbivore stress in P (ρ = 0.95, P = 0.01; Fig. 7A). Up-regulation of

Table I. Major gene hubs in the B. nigra network for multiple stress response to O3 and herbivory (Fig. 4)

<table>
<thead>
<tr>
<th>Arabidopsis Genome Initiative No.</th>
<th>Gene Symbol</th>
<th>Gene Name</th>
<th>GO Processa</th>
<th>Cellular Locationb</th>
</tr>
</thead>
<tbody>
<tr>
<td>A1g427740</td>
<td>–</td>
<td>Yippee family putative zinc binding</td>
<td>Unknown</td>
<td>Nucleus</td>
</tr>
<tr>
<td>A1g16590</td>
<td>CSLA1</td>
<td>Cellulose synthase-like A01</td>
<td>Glycyl transferase</td>
<td>Golgi, cytosol</td>
</tr>
<tr>
<td>A1g504440</td>
<td>–</td>
<td>Unknown function (DUF1997)</td>
<td>Unknown</td>
<td>Chloroplast</td>
</tr>
<tr>
<td>A1g12390</td>
<td>–</td>
<td>Cornichon protein (guard cells)</td>
<td>Signal transduction</td>
<td>Plasma membrane</td>
</tr>
<tr>
<td>A1g29100</td>
<td>GLR2.9</td>
<td>Glutamate receptor2.9</td>
<td>Ca2+ homeostasis</td>
<td>Plasma membrane, Golgi, endoplasmic reticulum</td>
</tr>
<tr>
<td>A1g350830</td>
<td>COR413</td>
<td>Cold acclimation WCOR413-like</td>
<td>Unknown</td>
<td>Plasma membrane</td>
</tr>
<tr>
<td>A1g299100</td>
<td>LHCBl.2</td>
<td>Light-harvesting chlorophyll-binding1.2</td>
<td>Photosynthesis</td>
<td>Chloroplast</td>
</tr>
<tr>
<td>A1g22900</td>
<td>MUC10</td>
<td>Mucilage-related10</td>
<td>Galactosyltransferase</td>
<td>Golgi, trans-Golgi</td>
</tr>
<tr>
<td>A1g32990</td>
<td>GH9B8</td>
<td>Glycosyl hydrolase9B8</td>
<td>Cellulose biosynthesis</td>
<td>Extracellular (cell wall)</td>
</tr>
<tr>
<td>A1g549100</td>
<td>–</td>
<td>TBP-binding protein</td>
<td>RNA polymerase II (TF)</td>
<td>Nucleus</td>
</tr>
<tr>
<td>A1g162580</td>
<td>NOGC1</td>
<td>Nitric oxide-dependent guanulate cyclase</td>
<td>Stomatal closure</td>
<td>Chloroplast, cytosol</td>
</tr>
<tr>
<td>A1g407470</td>
<td>CML41</td>
<td>Calmodulin-like41</td>
<td>Signaling, Ca2+ binding</td>
<td>Chloroplast</td>
</tr>
<tr>
<td>A1g582700</td>
<td>M3</td>
<td>ABC transporter mitochondrial3</td>
<td>Mo-cofactor biosynthesis</td>
<td>Mitochondria, chloroplast</td>
</tr>
<tr>
<td>A3g24100</td>
<td>–</td>
<td>SERF (uncharacterized)</td>
<td>Unknown</td>
<td>Nucleus</td>
</tr>
<tr>
<td>A3g107040</td>
<td>–</td>
<td>Unknown protein</td>
<td>Unknown</td>
<td>Chloroplast</td>
</tr>
<tr>
<td>A1g220390</td>
<td>GST18</td>
<td>Glutathione S-transferase18</td>
<td>Amino acid biosynthesis</td>
<td>Cytoplasm, cytosol</td>
</tr>
</tbody>
</table>

aGO biological processes and/or molecular functions as reported by The Arabidopsis Information Resource database (https://www.arabidopsis.org).  
bCellular localization confirmed with the ePlant visualization tool (BAR; University of Toronto; http://bar.utoronto.ca/∼dev/eplant).

and osmotic stress tolerance during abiotic and biotic responses (Kotchoni et al., 2006; Missihoun et al., 2014; Sengupta et al., 2015).

On the basis of genes annotated for the GO enzyme substrate glycerol- (Supplemental Table S8), which were functionally connected in AraNet (Fig. 6A), a PLS-DA could separate the treatments O from P and OP, explaining 28% of the variation in gene expression and 60% of the treatment effects (Fig. 6B; Supplemental Table S9). In an orthogonal projection of latent structures-discriminant analysis (OPLS-DA), the single and sequential O3 treatments (O and OP) could be divided further by the activity of eight genes (VIP score > 1): LIP1, GPDHc1, SDP6, PLT5, PDAT, DGD1, SQD2, and PAD4 (Fig. 6, C and D; for model statistics, see Supplemental Table S10). Particularly, the triacylglycerol lipase LIP1 (At2g15230; fatty acid catabolism; El-Kouhen et al., 2005) was up-regulated in O compared with OP (P < 0.05; Fig. 6, E and F), whereas the sulfolipid synthase SQD2 (At5g12200; biosynthesis of photosynthetic membrane components) was significantly down-regulated in O (P = 0.02) but not in OP (Fig. 6, E and F; KEGG/KaPPA-View4 pathways are shown in Supplemental Figs. S6 and S7). Moreover, the glycerol 3-phosphate (G3P) dehydrogenases GPDHc1 and SDP6 (At2g41540 and At3g10370; Shen et al., 2006) were both up-regulated in O and negatively affected by herbivory in OP (P < 0.05; Fig. 5E). Pathway visualization in KEGG/KaPPA-View4 (Fig. 6F) showed that GPDHc1 and SDP6 (located in the cytosol and on the mitochondrial membrane, respectively) constitute the G3P shuttle that is responsible for transporting reducing equivalents to the mitochondrial ETC via dihydroxyacetone phosphate recycling (Shen et al., 2003, 2006; Quettier et al., 2008).

Predictive Interactions of GO Functional Networks

During the shift between O3 and herbivore stress, B. nigra actually regulated energy and glycerol metabolic processes (Figs. 2, 3, 5, 6), possibly in connection with the osmotic stress response and stomatal regulation (Fig. 4). To better describe the system transitions during the stress treatments (O, P, and OP), we performed a comparative correlation analysis (Steuer, 2006) between the regulation of glycerol, G3P shuttle (GPDHc1/SDP6), mitochondrial ETC (SDH1), and stomatal closure (NOGC1/FMO; Fig. 7, A and B), while GO network analyses (AraNet and GeneMANIA) provided biological insights into potential functional relationships between these processes (Fig. 7, C and D). In AraNet, GO functional associations were predicted for genes involved in glycerol metabolism that most strongly responded to the dynamics of the sequential treatment (Fig. 5). Overall, the network was enriched in processes of fatty acid biosynthesis, mitochondrial metabolism (G3P shuttle), photosynthesis (chlorophyll biosynthesis, sulfolipids, and photosystem stabilization), response to phosphate starvation, and stomatal closure (Table II). New functional links between these processes highlighted the connection between glycerol metabolism (particularly via the G3P shuttle; GPDHc1 and SDP6) and central energy metabolism (e.g. the pentose phosphate shunt, NADPH regeneration, glycolysis, ATP synthesis, and mitochondrial ETC; Fig. 7C). In AraNet, SDP6 was linked to SDH1-1 (Fig. 7C), and GeneMANIA specifically predicted that the corresponding proteins interact and are coexpressed (Fig. 7D). In keeping with this result, we found that SDH1-1 and SDP6 were strongly coexpressed during O3 stress in O (ρ = 0.97, P = 0.005) and during herbivore stress in P (ρ = 0.95, P = 0.01; Fig. 7A). Up-regulation of
**Energy metabolism**

SDH1-1 persisted in the sequential OP treatment (Fig. 7B), but its coexpression with SDP6 was reduced ($\rho = 0.48$, $P = 0.07$; Fig. 7A). Glycerol metabolism was also associated with osmotic stress, stomatal closure, and the ABA response via interactions of SDP6 with the flavin monooxygenases NOGC1/FMO and SRE1/ABA2 (At1g52340; Nambara et al., 1998; Fig. 7C). NOGC1 and FMO were negatively correlated with glycerol accumulation during O$_3$ stress in O (especially FMO; $\rho = -0.91$, $P = 0.03$) but not in OP (Fig. 7, A and B). Moreover, SDP6 interacted with water-glycerol protein channels (NIP aquaglyceroporins, vacuole BETA-TIP, and GAMMA-TIP3) and with the phosphate transporter PHT2;1 (via NOGC1/FMO; Fig. 7D).

### Physiological Measurements of Photosynthesis and Gas Exchange

Combined omics and network analyses highlighted the connection of glycerol and energy metabolism with the regulation of the osmotic stress response and stomatal closure. To assess the actual impact of O$_3$ and herbivory on B. nigra, we performed a second experiment where we measured phenotypic and physiological parameters for plants exposed to the stress treatments O, P, and OP as well as a long-term (16-d) O$_3$ stress treatment (OL; Fig. 8A). Although few individuals showed visible symptoms of early senescence and chlorosis, the chlorophyll content of the three youngest fully expanded leaves (L5–L7) decreased by 9.5% after 5 d of O$_3$ exposure ($P = 0.05$, $n = 20$; Fig. 8B). Herbivory alone did not directly affect chlorophyll levels, but plants exposed previously to O$_3$ in the sequential OP treatment had chlorophyll levels 13.8% lower than plants subjected to the P treatment ($P = 0.05$, $n = 10$; Fig. 8B). The deleterious effect of O$_3$ was further demonstrated by the long-term exposure (OL), in which plants exhibited strong symptoms of senescence and chlorosis, particularly on the central fully expanded leaves (L5–L7; Fig. 8A). Chlorophyll levels in the OL plants were consistently around 47.8% lower than those in control plants of the same age ($P < 0.05$, $n = 10$; Fig. 8B).

In B. nigra plants, 5 d of O$_3$ stress (O) also reduced photosynthetic activity and intracellular CO$_2$ levels and negatively affected stomatal conductance and leaf transpiration (Table III). After 16 d of exposure (OL), stomatal conductance decreased even further, but intracellular CO$_2$ levels increased. Twenty-four hours of herbivore stress by...
*P. brassicae* (P) did not affect photosynthesis but slightly reduced stomatal conductance and leaf transpiration compared with untreated controls. However, when plants were exposed to herbivory after O₃ stress in the sequential OP treatment, photosynthesis, stomatal conductance, and leaf transpiration all were reactivated. The sequential stress thus reversed the responses induced by either stress alone (Table III). Because stomatal regulation follows the circadian clock and decreases during the day, we verified these conductance measurements via steady-state porometry (Fig. 8, C and D). These measurements supported our initial findings, showing that conductance decreased in response to a single stress in the O (P = 0.07; Supplemental Fig. S10) and P (P = 0.02) treatments but that the sequential OP treatment induced stomatal reopening (P = 0.004; Fig. 8F).

**DISCUSSION**

Multiple stresses to plants may evoke unpredicted molecular responses with negative, neutral, or positive consequences for plant metabolism. We found molecular evidence of changes in the regulation of photosynthesis and mitochondrial activity during the responses of *B. nigra* to sequential O₃ stress and herbivory stress by *P. brassicae*. O₃ induced the suppression of photosynthesis and stomatal closure, but this response was redirected to higher photosynthetic activity after sequential exposure to herbivores. By using omics multivariate and network analyses, we identified glycerol metabolism as a central driver of this shift. As predicted by our combined omics models, stomatal conductance and gas exchange were enhanced after the sequential stress treatment, confirming the occurrence of a strategic change affecting photosynthetic activity and energy metabolism. This response to sequential stresses could not have been predicted from the individual stress responses alone.

**Effects of O₃ Stress on Photosynthesis and Stomatal Regulation**

High levels of O₃ negatively affect photosynthesis in plants (Bagard et al., 2008; Salvatori et al., 2015; Vainonen and Kangasjärvi, 2015). O₃ oxidizes thylakoid membranes in the chloroplasts, resulting in chlorophyll degradation and early leaf senescence (Bergmann et al., 1999; Ranieri...
et al., 2001; Goumenaki et al., 2010). However, even before symptoms of bleaching and chlorosis appear, as demonstrated in this study, the senescence process may be fully initiated with the down-regulation of chlorophyll and light-harvesting genes and the induction of the transcription factor involved in leaf senescence, EIN3 (Long and Naidu, 2002; Potuschak et al., 2003; Li et al., 2013). In addition to suppressing photosynthesis, chronic O₃ exposure above 40 nL L⁻¹ triggers a reactive oxygen species (ROS) signaling cascade that causes stomatal closure to prevent O₃ from entering the leaf. However, this process also limits CO₂ uptake (Bergmann et al., 1999; Ranieri et al., 2001; Booker et al., 2009; Castagna and Ranieri, 2009; Vahisalu et al., 2010; Settele et al., 2014). Our study confirmed a negative effect of O₃ on B. nigra stomatal conductance and leaf transpiration, which in the short term also led to lower levels of intracellular CO₂. After long-term exposure (70 nL L⁻¹ O₃ for 16 d), signs of bleaching and chlorosis in leaves became evident, whereas reduced stomatal conductance and low photosynthesis rates were associated with increased levels of intracellular CO₂, a condition that can reinforce stomatal closure (Paolelli and Grulke, 2005; Singh et al., 2009).

Figure 7. Components of the glycerol metabolic network and mitochondrial ETC under multiple O₃ and herbivory stress treatments. Comparative correlation analysis and predictive GO network interactions (AraNet and GeneMANIA) are shown. A, Comparative correlation analysis between glycerol, components of the G3P shuttle (GPDHC1 and SDP6), mitochondrial ETC complex II (SDH1-1), and flavin monooxygenases (NOGC1 and FMO). Treatments are O, P, and OP. Edges indicate ρ for positive (blue) and negative (red) correlations. All correlation values reported were significant (between P < 0.05 and P < 0.001), except the two correlations in the sequential (OP) treatment of glycerol/FMO (ρ = 0.85) and SDP6/SDH1 (ρ = 0.48), which were not significant. B, Average relative gene expression (log₂) and glycerol abundance for each treatment (O, P, and OP) and corresponding Student’s t test significance (*, P < 0.05 and **, P < 0.01) compared with the control conditions. C, Gene interactions predicted in AraNet for the glycerol metabolic network. Entry genes (in black squares) and emerging new members of a pathway are colored by GO categories after enrichment analysis (GOlorize/Cytoscape). Predicted interactions with members of the mitochondrial ETC complex II, SDH, are highlighted in the box. D, Gene interactions generated in GeneMANIA between query genes SDH1-1, SDP6, GPDHC1, NOGC1, and FMO. Node functions related the genes to processes of energy and mitochondria metabolism. Functional links indicated coexpression (purple), colocalization (blue), predicted interaction (orange), and shared protein domains (brown) between the network components.
O₃ stress also induced the expression of MYB44, a gene that may play a complex role in the responses to abiotic and biotic stresses such as drought and wounding (Baldoni et al., 2015). MYB44 overexpression in Arabidopsis mutants increases drought tolerance by modifying the regulation of stomatal closure (Jung et al., 2008). However, MYB44 also negatively regulates ABA responses (Jaradat et al., 2013; Li et al., 2014) involved in stomatal closure, leaf senescence, and ROS scavenging (Persak and Pitzschke, 2014). In our study, MYB44 expression correlated positively with glycerol levels and negatively with the expression of NOGC1, a nitric oxide-dependent guanylyl cyclase involved in stomatal closure via Ca²⁺ signaling (Mulaudzi et al., 2013). In addition, MYB44 correlated positively (ρ = 0.85, P = 0.001) with the chloroplastic lipid transfer protein LTPc1 (At2g10940), which together with LTPc2 (At2g45180) was up-regulated during O₃ stress but not herbivore stress (Supplemental Figs. S3 and S4; Supplemental Table S3). LTPs are known to be active in the transfer of glycerolipids between cell membranes (e.g. from chloroplasts to the endoplasmic reticulum; Xu et al., 2008). These results suggest a coordinated regulation of MYB44 and NOGC1 in response to O₃, which could serve as a feedback mechanism for ABA signaling and stomatal closure, potentially connecting central energy metabolism and glycerolipid pathways involved in osmotic stress responses.

### Importance of Glycerol and Energy Metabolism as Safety Valves

Glycerol metabolism allows plants to adapt to a range of environmental stresses. Spinach (Spinacia oleracea) leaves, for example, accumulate triacylglycerol derived from membrane galactolipids in response to O₃ fumigation (Sakaki et al., 1990). The regulation of glycerolipid pathways also is induced in Arabidopsis, wheat (Triticum aestivum), and saltbush (Atriplex spp.) in response to temperature stress, which results in diacylglycerol trafficking from the endoplasmic reticulum to the chloroplasts (Li et al., 2015). In Arabidopsis, heat, salt, and drought also induce triacylglycerol accumulation in the cytosol, an adaptation that enables structural remodeling of membrane lipids (Mueller et al., 2015).

The combined suppression of photosynthesis and reduction in chlorophyll levels observed in our study suggests that glycerol may originate from the O₃ stress-induced degradation of chloroplasts and glycolipid membranes. Increased glycerol levels also were correlated with changes in the expression of genes involved in stomatal closure and osmotic stress responses (MYB44, NOGC1, and FMO). Glycerol has osmoregulatory properties, and its accumulation in mutants that lack glycerol kinase enhances resistance to dehydration stress in Arabidopsis (Eastmond, 2004).

GO network analyses further emphasized a connection between osmotic stress responses, glycerol metabolism, and central energy processes in chloroplasts and mitochondria. In the absence of CO₂ photons harvested during photosynthesis cannot be assimilated, so plants suppress photosynthesis and activate nonphotochemical quenching to protect cellular structures against excess excitation energy (Niyogi, 2000; Murata et al., 2007). Similarly, mitochondria are sinks for excess electrons generated during oxidative stress and oxidize reducing equivalents via respiration (Hoefnagel et al., 1998; Niyogi, 2000; Scheibe et al., 2005; Noctor et al., 2007; Nunes-Nesi et al., 2008). We observed that O₃ exposure increased the activity of the mitochondrial manganese superoxide dismutase MSD1 (Tsang et al., 1991; Martin et al., 2013; Fig. 5D), suggesting that mitochondria also play a role in the oxidative stress response to O₃. This hypothesis was further supported by the increased activity of the mitochondrial succinate dehydrogenase SDH1-1 and the G3P shuttle (GPDHe1/SDP6). In the mitochondrial ETC, SDH1-1 acts as the binding site for coenzyme Q in complex II (Huang et al., 2013), while the G3P shuttle is pivotal in supplying the

### Table II. Biological processes relative to the B. nigra glycerol metabolic network responsive to O₃ and herbivory

<table>
<thead>
<tr>
<th>Rank</th>
<th>GO Identifier</th>
<th>Biological Process</th>
<th>P*</th>
<th>Genes</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>GO:0006636</td>
<td>Unsaturated fatty acid biosynthesis</td>
<td>0.0001632</td>
<td>SQD2, LIP1, PDAT</td>
</tr>
<tr>
<td>2</td>
<td>GO:0006127</td>
<td>Glycerol phosphate shuttle</td>
<td>0.0002918</td>
<td>GPDHe1, SDP6</td>
</tr>
<tr>
<td>3</td>
<td>GO:0016117</td>
<td>Carotenoid biosynthesis</td>
<td>0.0003421</td>
<td>SQD2, LIP1, PDAT</td>
</tr>
<tr>
<td>4</td>
<td>GO:0019375</td>
<td>Galactolipid biosynthesis</td>
<td>0.0003564</td>
<td>DGD1, SQD2</td>
</tr>
<tr>
<td>5</td>
<td>GO:0015995</td>
<td>Chlorophyll biosynthesis</td>
<td>0.0004397</td>
<td>DGD1, SQD2, LIP1</td>
</tr>
<tr>
<td>6</td>
<td>GO:0046506</td>
<td>Sulfitolipid biosynthesis</td>
<td>0.0005835</td>
<td>DGD1, SQD2</td>
</tr>
<tr>
<td>7</td>
<td>GO:0016036</td>
<td>Phosphate starvation</td>
<td>0.0006321</td>
<td>DGD1, SQD2</td>
</tr>
<tr>
<td>8</td>
<td>GO:0019563</td>
<td>Glycerol catabolism</td>
<td>0.0008752</td>
<td>GPDHe1, SDP6, LIP1</td>
</tr>
<tr>
<td>9</td>
<td>GO:0042550</td>
<td>PSI stabilization</td>
<td>0.0008752</td>
<td>DGD1</td>
</tr>
<tr>
<td>10</td>
<td>GO:0019761</td>
<td>Glucosinolate biosynthesis</td>
<td>0.0009609</td>
<td>SQD2</td>
</tr>
<tr>
<td>11</td>
<td>GO:0006072</td>
<td>Glycerol-3-phosphate metabolism</td>
<td>0.001167</td>
<td>GPDHe1, SDP6</td>
</tr>
<tr>
<td>12</td>
<td>GO:0009247</td>
<td>Glycolipid biosynthesis</td>
<td>0.001458</td>
<td>DGD1, SQD2</td>
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<tr>
<td>13</td>
<td>GO:0019288</td>
<td>Isopentenyl diphosphate biosynthesis</td>
<td>0.001849</td>
<td>DGD1, SQD2</td>
</tr>
<tr>
<td>14</td>
<td>GO:0090332</td>
<td>Stomatal closure</td>
<td>0.002915</td>
<td>NOGC1</td>
</tr>
<tr>
<td>15</td>
<td>GO:0006071</td>
<td>Glycerol metabolism</td>
<td>0.00466</td>
<td>GPDHe1, SDP6</td>
</tr>
</tbody>
</table>

*GO term P < 0.01 calculated in AraNet as a hypergeometric test on all 27,416 gene entries in the Arabidopsis database.

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ETC with redox energy derived from NADH (McKenna et al., 2006; Shen et al., 2006; Berg et al., 2012; Mráček et al., 2013). Curiously, we found that some phosphate response genes (RNS1 and PT2) also were up-regulated in response to O₃ stress, and our GO network analyses linked glycerolipid metabolism to phosphate starvation (SQD2 and DGD1). Phosphate starvation symptoms are similar to those of O₃ stress, including down-regulation of photosynthesis, low CO₂ assimilation, photooxidation of membrane lipids (Hernández and Munné-Bosch, 2012).
membrane lipid remodeling with down-regulation of SQD2 (Jost et al., 2015), and transfer of digalactosyldiacylglycerol from chloroplasts to mitochondria (Jouhet et al., 2004).

Overall, the cumulative regulation of glycerol metabolism observed in our study may reflect a flux reconfiguration in B. nigra to sustain mitochondrial activity, which led to the accumulation of glycerol as an intermediate metabolite in the pathway (Kleijn et al., 2007; Morandini, 2015), membrane lipid remodeling with down-regulation of SQD2 (Jost et al., 2015), and transfer of digalactosyldiacylglycerol from chloroplasts to mitochondria (Jouhet et al., 2004).

**Sequential O₃ Stress and Herbivory Induce Abiotic and Biotic Cross Talk**

Herbivory by P. brassicae induced the down-regulation of photosynthesis and reduced carbon assimilation rates, although herbivore stress was less severe than O₃ stress. Chewing herbivores systemically reduce photosynthetic activity in damaged leaves and in neighboring tissues (Zangerl et al., 2002; Bilgin et al., 2010; Halitschke et al., 2011), whereas JA signaling and LOXs directly affect photosynthesis and ETC activity in chloroplasts (Nabity et al., 2013; Havko et al., 2016). The underlying mechanisms that suppress photosynthesis in favor of induced defense responses are not fully understood, but a tradeoff between growth and defense appears to determine how plants rearrange their metabolism and redirect primary resources to the production of specialized defensive compounds (Schwachtje and Baldwin, 2008; Tang et al., 2009; Meldau et al., 2012). Herbivory induced partial stomatal closure in B. nigra, although we did not observe any distinct up-regulation of MYB44, as would be expected after biotic stress and wounding (Jung et al., 2010; Persak and Pitzschke, 2013; Shim et al., 2013). Although O₃ stress by itself induced the up-regulation of MYB44 and stomatal closure, O₃ stress followed by herbivore stress caused the stomata to reopen. Consequently, the phenotype resulting from the sequential stresses resembled that observed after herbivore damage alone, with a relatively low stomatal conductance.

Sequential herbivory positively induced the expression of MYC2 (JA signaling) and negatively affected EIN3 (ET signaling) expression, which was induced by O₃. MYC2 and EIN3 are key integrators of plant abiotic and biotic stress responses (Abe et al., 2003; Anderson et al., 2004; Fujita et al., 2006; Dombrecht et al., 2007; Atkinson et al., 2013). In a mutually antagonistic interaction, JA-activated MYC2 represses the transcription of EIN3/EIL1, whereas the induction of EIN3/EIL1 reciprocally represses MYC2 and JA responses (Song et al., 2014, 2015; Zhang et al., 2014; Kim et al., 2015).

Similarly, ERF2, which is involved in ET signaling (Fujimoto et al., 2000) and positively regulates JA responses (McGrath et al., 2005; Pré et al., 2008), was down-regulated after exposure to O₃ stress alone but was induced during herbivore stress. This asymmetric regulation of the ABA and JA/ET pathways and the observed relationship with stomatal behavior suggest a specific cross talk that balances the metabolic responses to O₃ and herbivory in the sequential stress situation.

**O₃ and Herbivory Affect Central Metabolism in Opposite Ways**

In a previous study, we showed that the interaction between O₃ stress and herbivory in B. nigra induced changes in the plant’s distribution of secondary metabolites (glucosinolates and phenolics). Moreover, while O₃ fumigation at 120 nL L⁻¹ promoted feeding damage by P. brassicae, caterpillars that fed on the fumigated leaves exhibited reduced performance and fitness, with delayed development and lighter pupae (Khaling et al., 2015).

Here, we show that central (primary) metabolism also plays a pivotal role in the way plants respond to concurrent abiotic and biotic stressors. Both transcriptomics and metabolomics indicated that O₃ stress induced changes in carbon and nitrogen metabolism. Specifically, pools of central metabolites were expanded, while the activity of many genes involved in carbohydrate metabolism and cell wall biosynthesis was reduced, including that of F2KP (for Fru-2,6-bisphosphatase/phosphofructokinase), the central regulator of glycolysis and gluconeogenesis (Draborg et al., 2001; Nielsen et al., 2004; McCormick and Kruger, 2015; Fig. 5B). In addition, several genes involved in amino acid metabolism were up-regulated, suggesting an increased flux toward mitochondrial activity and nitrogen mobilization, both of which are cellular processes associated with senescence (Bouché et al., 2008; Li et al., 2006; Breeze et al., 2011; Debouba et al., 2013; Watanabe et al., 2013). Similar effects of O₃ stress on central metabolism have been
reported previously: changes in the activity and expression of genes and pathways involved in detoxification and redox balance (e.g. the aldehyde dehydrogenase ALDH, chloroplastic superoxide dismutase activity, and NADH regeneration) were linked to stomatal closure, decreased photosynthetic activity, and increased mitochondrial respiration (Dizengremel et al., 2009; Yendrek et al., 2015). However, when herbivory was applied as a second stress, all these initial effects of O₃ stress were reversed, resulting in decreased levels of sugars and amino acids and a reconfiguration of gene expression, including down-regulation of the amino acid transporter AAP2, which promotes nitrogen accumulation in siliques and seed development in Arabidopsis (Hirner et al., 1998; Ortiz-Lopez et al., 2000).

Interestingly, a common response to both O₃ and herbivory was an increased abundance of the central metabolite GABA. In mitochondria, the GABA shunt delivers additional succinate and NADH to the ETC and provides an alternative route to amino acid metabolism (Bouché et al., 2003). During abiotic and biotic stresses, GABA concentrations can spike rapidly, but its function in plant responses remains unclear (Bouché and Fromm, 2004; Fait et al., 2008). Studies have shown that GABA is involved in stress signaling processes, from leaf senescence (Ansari et al., 2005) to plant communication with insects and microorganisms (Shelp et al., 2006; Michaeli and Fromm, 2015). Under conditions of oxidative stress and inhibited photosynthesis, GABA participates in ROS scavenging in support of normal growth and stress tolerance (Bouché et al., 2003; Dizengremel et al., 2012). GABA also has been proposed to regulate the carbon and amino acid metabolism of plants through interaction with GABA/Glu receptors (GLR) in concert with differential regulation of the ABA/ET signaling pathways (Lancien and Roberts, 2006; Forde and Lea, 2007). Under the conditions examined in this work, increased levels of GABA may contribute to the maintenance of central metabolism, oxidative stress responses, and/or the regulation of stomatal closure, possibly via GLR and Ca²⁺ signaling.

Figure 9. Summary of omics and physiological responses in B. nigra during sequential O₃ and herbivory stress treatments. The model links metabolome and transcriptome fluctuations to physiological responses of photosynthesis, CO₂ assimilation, and stomatal opening. Stress adaptation mechanisms are proposed (1–5). Blue, O₃ fumigation (5 d at 70 nL L⁻¹, 16 h per day); red, O₃ followed by P. brassicae (24 h, 30 first instar caterpillars). Up arrows indicate up-regulation (genes) or increase (metabolites), and down arrows indicate the opposite. 1 and 2, O₃ induces the abiotic stress responses of senescence (EIN3 and ERF2) and stomatal closure (MYB44), with feedback on NOGC1 and FMO. ABA and JA/ET cross talk integrates responses between O₃ and sequential herbivory (MYC2 and ERF2), with opposite effects on stomatal closure. 3 and 4, Photosystem suppression (LHCs) and non-photochemical quenching (NPQ1) in response to O₃ are linked to the regulation of glycerolipid metabolism (LPTs, LIP1, and SQD2). Glycerol derived from degraded chloroplast membranes enters the G3P shuttle (GPDHc1/SDP6) to sustain NAD⁺ recycling and mitochondrial activity (SDH1-1, MSD1, and GABA) as antioxidative stress mechanisms. A possible role of glycerol and sugars as osmolytes also is suggested. Sequential herbivory restores the glycerolipid pathway for alternative source-sink priorities (e.g. JA responses [LOXs/MYC2]). 5, GABA plays multiple roles in plant stress adaptation, regulating Ca²⁺ homeostasis, carbon-nitrogen metabolism, leaf senescence, ROS scavenging, and signaling of plant-insect interactions.
In conclusion, we propose the existence of a tolerance mechanism in B. nigra (Fig. 9) where glycerol and central energy metabolism play a central role in adaptation to the sequential stresses of O₃ exposure and herbivory, enabling B. nigra to prioritize the demands of stomatal osmoregulation and oxidative stress. The suppression of photosynthesis and the regulation of glycerol and mitochondrial metabolism during O₃ stress may dissipate excess energy to avoid the formation of oxidative radicals (Hoefnagel et al., 1998), combining fast NAD⁺ recycling with the maintenance of central metabolism and growth (Dizengremel et al., 2008, 2012). This hypothesis is supported by previous studies on the physiological functions of glycerol metabolism in NADH/NAD⁺ homeostasis (Shen et al., 2006; Quettier et al., 2008), osmotic stress responses (Biela et al., 1999; Eastmond, 2004; Chen et al., 2011; Geijer et al., 2012), and plant development (Hu et al., 2014). These pathways were redirected in the sequential treatment, during which glycerol resources may have been reallocated toward JA signaling and defense against herbivores (Turner et al., 2002; Kachroo et al., 2004; Havko et al., 2016). Alternatively, these reversed effects may represent a manipulation of the host’s metabolism by the herbivore (Karban and Agrawal, 2002), which can interfere with plant defense and water stress response genes (Reymond et al., 2000; Consales et al., 2012).

**MATERIALS AND METHODS**

**Plants**

Seeds from Brassica nigra plants collected from a natural population growing along the Rhine River in Wageningen were obtained from the Laboratory of Entomology of Wageningen University. The seeds were planted individually in plastic pots (9 × 9 × 9.5 cm) filled with a 3:1:1 mix of peat, potting compost, and sand. They were grown under greenhouse conditions at the University of Eastern Finland in Kuopio. The plants were watered intermittently with sprinklers for 5 h every day without chemical control for pests or diseases. The seedlings were fertilized twice per week with 0.1% Superex (nitrogen:phosphorus:potassium, 19:5:20). When the plants were 4 weeks old and had developed approximately seven leaves, they were taken to growth chambers (Weiss Bio 1300; Weiss Umwelttechnik) and subjected to the different treatments for 6 d.

**Treatments**

*B. nigra* plants were subjected to one of four treatments: exposure to ambient air with and without feeding by *Pieris brassicae* caterpillars (C and P) and O₃ fumigation with and without subsequent feeding by *P. brassicae* caterpillars (O and OP). The experiment was repeated five times (biological replicates), with each replicate including three plants per treatment. Two extra replicates were performed for the evaluation of gene expression after exposure to ambient air and *P. brassicae* feeding (seven replicates in total). In the follow-up experiment, *B. nigra* was again treated as in C, O, P, and OP and with additional long-term O₃ fumigation (OL).

**O₃ Fumigation**

The plants (12 in total, three per treatment) were moved to plant growth chambers. The chambers had been modified so that each had an independently controlled O₃ concentration. High-O₃ chambers were set to 70 nL L⁻¹ from 4 a.m. to 8 m and maintained at a basal O₃ concentration of 30 nL L⁻¹ for the remaining hours each day. This treatment was done to imitate natural diurnal variation in O₃ concentration. In ambient chambers, the O₃ concentration fluctuated between 15 and 20 nL L⁻¹. Chambers were maintained at a temperature of 23°C ± 3°C, relative humidity of 60% during the day and 80% at night, a photoperiod of 16 h of light/8 h of darkness, with a light intensity of 300 μmol m⁻² s⁻¹. The plants were watered daily. The above conditions were maintained for 5 d, after which the plants subjected to herbivore feeding were infested for 24 h. In the follow-up experiment, long-term O₃ fumigation was started on 3-week-old plants and continued for 16 d.

**Herbivore Feeding**

The large cabbage white butterfly, *Pieris brassicae* (Lepidoptera: Pieridae), was obtained from stocks at the Laboratory of Entomology, Wageningen University, and reared on Brussels sprouts plants (*Brassica oleracea var gemmifera ‘Brilliant’*) at the University of Eastern Finland under greenhouse conditions. Before the experiments, *B. nigra* adults were presented with *B. nigra* plants for oviposition, and first instar caterpillars were collected soon after hatching in a climate-controlled insect-rearing room with a temperature of 25°C ± 2°C, a photoperiod of 16 h of light/8 h of darkness, light intensity of 300 μmol m⁻² s⁻¹, and relative humidity of approximately 60%. For the herbivore treatment, a total of 30 first instar *P. brassicae* caterpillars were mounted on the three highest fully expanded leaves of each plant (10 caterpillars per leaf) and left to feed for 24 h. After 24 h (day 6), VOCs were collected and plant samples were harvested for metabolomics and transcriptomics analyses.

**Physiological Measurements of Photosynthesis and Gas Exchange**

The relative chlorophyll content of leaf tissues was determined by measuring the optical Aₘₐₜ (CCM-200 plus; Opti-science) of the three youngest fully expanded leaves (L₅–L₇). Photosynthetic and gas-exchange parameters (i.e. the carbon assimilation rate [μmol CO₂ m⁻² s⁻¹], intracellular CO₂ levels, stomatal conductance, and leaf transpiration [μmol water m⁻² s⁻¹]) were measured for about 1 h on one fully expanded leaf (L₆) per plant (n = 10) using a LI-COR gas analyzer (LI-6400). The leaf chamber parameters were set to mimic the ambient growth conditions, with block temperature at 24°C, relative humidity at 60%, CO₂ at 400 μL L⁻¹, and saturating light at 1,000 μmol m⁻² s⁻¹. In addition, leaf L₆ stomatal conductance was determined via steady-state porometry (SC-1; Decagon Devices), which measured the actual water vapor flux (μmol water m⁻² s⁻¹) from the leaf through the stomata and out to the environment.

**Sampling for Metabolomics and Transcriptomics**

At the time of sampling, the plants were 5 weeks old. Counting from the apex, the three youngest fully expanded leaves (L₅–L₇) of each of the three plants (altogether nine leaves from three plants per treatment) were cut at the petiole with a sharp knife and pooled together. These leaves were immediately wrapped in aluminum foil and flash frozen in liquid nitrogen. The leaves were stored at −80°C and later ground into a fine powder with liquid nitrogen using a mortar and pestle. Equal amounts of powder from each sample were express mailed on dry ice to the laboratories in Umeå (SE) and Lausanne (CH) for metabolomics and transcriptomics analyses, respectively. Each laboratory received subsamples of the same material.

**Metabolomics Analyses**

All metabolomics analyses were performed at the Umeå Plant Science Center-Swedish Metabolomics Center (UPSC-SMCC) in Umeå. Leaf tissue samples for LC-MS and GC-MS were prepared by extracting 10 to 12 mg of frozen sample using 1 mL of cold chloroform:methanol:water (20:60:20) containing 7.5 ng mL⁻¹ labeled salicylic acid-D₄ (m/z [M-H]⁻ 141.046) as an internal standard. A 3-mm tungsten carbide bead was then added to each vial, and the samples were agitated for 3 min at 30 Hz in a MM 301 Vibration Mill (Retsch). To separate the mixture from tissue debris and avoid contamination, the extracts were centrifuged at 20,800g for 10 min at 4°C, and 200 μL of the supernatant was separated and evaporated to dryness using a SpeedVac. For GC-MS analysis, samples were derivatized using 30 μL of methoxyamine (15 μg mL⁻¹ in pyridine) and agitated for 10 min before being left to react for 16 h at 25°C. Silylation was achieved using 30 μL of N-Methyl-N-(trimethylsilyl) trifluoroacetamide (MSTFA) for 1 h at 25°C. Finally, samples were diluted with 30 μL of heptane containing 15 ng μL⁻¹ methyl stearate (internal standard) and injected into the system. For LC-MS analysis, dried samples were redissolved in 10 μL of cold methanol and diluted with 10 μL of cold water before being injected into the system.
GC-MS

One microliter of the derivatized sample was injected splitless using a CTC Combi Pal autosampler (CTC Analytics) into an Agilent 6890 gas chromatograph equipped with a 10-m x 0.18-mm i.d. fused silica capillary column with a chemically bonded 0.18-μm DB 5-MS UI stationary phase (J&W Scientific). The injector temperature was 270°C, the purge flow rate was set to 20 mL min⁻¹, and the purge was turned on after 60 s. The gas flow rate through the column was 1 mL min⁻¹; the column temperature was held at 70°C for 2 min, then increased by 40°C min⁻¹ to 320°C, and held for 2 min. The column effluent was introduced into the ion source of a Pegasus III time-of-flight mass spectrometer (Leco). The transfer line and the ion source temperatures were set to 250°C and 200°C, respectively. Ions were generated by a 70-eV electron beam at an ionization current of 2 mA. Mass spectra were acquired in the mass range 50 to 800 m/z. The acceleration voltage was turned on after a solvent delay of 150 s. The detector voltage was set to 1,700 V.

LC-MS

For the analysis of secondary metabolites, samples were analyzed by ultrahigh-performance liquid chromatography-electrospray ionization/time-of-flight mass spectrometry (UHPLC-ESI-TOF-MS; Waters). The Acquity system was equipped with a 2.1- x 100-mm, 1.7-μm C18 UPLC column (reverse-phase column/nanoparticle stationary phase) held at 40°C. The liquid chromatography system was coupled to an LCT Premier time-of-flight mass spectrometer. Two microliters of each sample was injected and separated using a mobile phase containing a mix of solvents A (water + 0.1% formic acid) and B (acetonitrile + 0.1% formic acid). Gradient elution was performed using the following program, gradually increasing the proportion of solvent B over time: 0 to 4 min, 1% to 20% B; 4 to 6 min, 20% to 40% B; 6 to 9 min, 40% to 60% B; and 9 to 13.5 min, 95% B. The total running time for each sample was 19 min, with a flow rate of 500 μL min⁻¹. The source temperature was 120°C, cone gas flow was 10 L h⁻¹, desolvation temperature was 320°C, nebulization gas flow was 600 L h⁻¹, and capillary and cone voltages were set at 2.5 kV (negative ionization mode) and 35 V, respectively. Data were acquired in dynamic range enhancement mode every 0.1 s, with a 0.01-s interscan delay. The lock mass compound for accurate mass measurements (Leu enkephalin) was infused directly at 400 pg μL⁻¹ in 50:50 acetonitrile:water at 20 μL min⁻¹. The normal lock mass in dynamic range enhancement mode was the negative ¹³C ion of Leu enkephalin (m/z 555.263), and the extended lock mass was the normal negative ion (m/z 554.262). Mass spectra were acquired in centroid mode with an m/z range of 100 to 1,000, and the data threshold value was set to 3.

Orbitrap Tandem Mass Spectrometry

To verify the data acquired by UHPLC-ESI-TOF-MS, samples were reanalyzed for the determination of selected peaks of interest by ultra-HPLC-tandem mass spectrometry using linear ion traps (LTQ Orbitrap). Separation was performed on a Thermo Accela liquid chromatography system, equipped with a column oven (held at 40°C) and a Hypersil C18 GOLD column (2.1 x 50 mm, 1.9 μm; mobile phase as for UHPLC-ESI-TOF-MS), and analyzed by tandem mass spectrometry using an LTQ Orbitrap mass spectrometer (Thermo Fisher Scientific). External mass calibration was performed according to the manufacturer’s guidelines.

Data Processing and Identification

The GC-MS instrument was operated using the LECO ChromaTOF software (optimized for Pegasus HT; Leco). Retention time indexes (RIs) were calculated relative to an alkaline series (C8–C40). From the raw data, feature extraction and peak integration were all performed with Matlab, combining target analysis (a predefined list of retention time windows and m/z values) and automated peak deconvolution. Compounds were identified comparing RIs and mass spectra with the UPSC-SMC in-house database and with the public Golm Metabolome Database of the Max Planck Institute. For comparison with the Golm Metabolome Database, RIs measured on the 5% phenyl/95% dimethylpolysiloxane capillary column VARS (Golm Metabolome Database) were transferred to the DB-5 (10m) system of the UPSC-SMC (Strehmel et al., 2008; Hummel et al., 2010). Samples were normalized on the unit variance and no centering (UVN) scores of integrated areas for the internal standards (methyl stearate and salicylic acid-D4).

The UHPLC-ESI-TOF-MS instruments were operated with MassLynx version 4.1 software (Waters). Compounds from LC-MS analysis were compared with standards of glucosinolates (sinigrin, glucobrassicin, glucopnin, glucotropaeolin, glucorasturtin, and sinabin [Phytopian, Diehm & Neuburger]), data from the METLIN mass spectra depository, and additional literature references for glucosinolates (Clarke, 2010), hydroxynamic acid derivatives, and flavonol glucosides (Liu et al., 2011). Tandem mass data analysis from the Orbitrap were used to compare the MSn profiles and further confirm the identifications. Raw data were processed using Sieve and Matlab software for peak alignment and integration. The peak areas were normalized against that of the labeled internal standard of salicylic acid-D4 (m/z [M+H]⁺ 141.046).

VOCs Collection and Analyses

All VOCs collection and analyses were performed at the University of Eastern Finland. The plants were enclosed in glass jars, and VOCs were collected for 60 min via dynamic headspace sampling. Filtered air was fed into the glass jars at a rate of 250 mL min⁻¹ and pulled out at a rate of 200 mL min⁻¹ through stainless steel tubes filled with Tenax TA and Carbopack B adsorbents (150 mg each; mesh 60/80; Markes International). Samples were analyzed by GC-MS (Agilent 7890A GC and 5975C VL MSD). Trapped compounds were desorbed with an automated thermal desorber (TD-100; Markes International) at 250°C for 10 min, cryofocused at ~10°C, and then injected in split mode onto an HP-5 capillary column (50 m x 0.2 mm; film thickness, 0.35 μm) with helium as the carrier gas. The oven temperature was held at 40°C for 1 min, raised to 210°C at a rate of 5°C min⁻¹, and finally raised to 250°C at a rate of 20°C min⁻¹. The column flow was maintained at a rate of 1.2 mL min⁻¹. The compounds were identified by comparing the mass spectrum of an individual compound with the spectra of compounds in an external authentic standard and with compounds in the Wiley Library. Relative emissions were measured by peak integration (absolute rates expressed as nmol m⁻² h⁻¹ are reported in Supplemental Data Set S2).

Transcriptomics Analyses

Transcriptomics analyses were performed at the University of Lausanne. B. nigra leaves (3–6 g) were ground in liquid N₂, and total RNA was extracted, reverse transcribed, and processed according to a previously published procedure (Bodenhausen and Reymond, 2007). Labeled probes were hybridized onto CATMA version 4 microarrays containing 32,998 Arabidopsis (Arabidopsis thaliana) gene-specific tags and gene family tags (Sclep et al., 2007). Hybridization and scanning have been described previously (Reymond et al., 2004). Data analyses were carried out using an interface developed at the University of Lausanne (Gene Expression Data Analysis Interface; Liechti et al., 2010). Differentially expressed genes were identified by fitting a linear model for each gene and evaluating the fold change and moderated t statistic P values (Smyth, 2004). To address the issue of multiple comparisons, we used the false discovery rate method developed by Storey and Tibshirani (2003) and computed a q value. Because we employed Arabidopsis whole-genome microarrays to probe the expression of B. nigra genes, the number of genes that produced hybridization signals was clearly low and overall hybridization signal intensity was weaker than with Arabidopsis samples. Hence, we noticed that high false discovery rate values are estimated when the number of induced genes was relatively small. However, by comparing gene expression between experiments, genes with small P values in response to one treatment often had a small P value in another treatment. Thus, interexperiment comparison adds to data interpretation, and false discovery rate calculations might be too conservative in some cases. Therefore, we used an unadjusted P value of 0.05. False discovery rate values are indicated in Supplemental Data Set S1.

Statistical Analyses

Matrices for gene expression and metabolites were created in Excel. All steps of basic statistics (i.e. Pearson’s correlation, Student’s t test, ANOVA, and posthoc Tukey’s test) were performed with Excel and with Minitab 17 Statistical Software (Minitab; www.minitab.com). Other more specific analyses were performed with the open-source software R (https://www.r-project.org/) and RStudio (https://www.rstudio.com/) or with other software as mentioned below.

Multivariate Analyses

Gene expression and metabolite profiles were subjected to multivariate analysis using the SIMCA 14 software package (Umetrics). Supervised regression models such as PLS-DA and OPLS-DA were used to investigate the
variation in X variables (gene or metabolites), which were modeled for the Y explanatory variables corresponding to O₂ and herbivore treatments. The cumulative (c) variations in X and Y explained by the models are reported as R²X (c) and R²Y(c), respectively. Models were fit to the minimum number of latent variables corresponding to the highest value of predicted variation: Q²(c). Selection of important variables was based on the VIP score, considered significant if above the threshold of 1.

**Gene Expression Data Evaluation and Pathway Analyses**

Gene selection for the Student’s t test cutoff of P ≤ 0.05 was executed in Excel. The open-source software R and RStudio were used to create heat maps, hierarchical clustering, and correlation of gene expression profiles using the CRAN library packages and functions heatmap (gplots), hclust, and corrr, respectively. More specifically, gene expression values for each sample (up- or down-regulation) were represented graphically using the heatmap function, while rows (genes) and columns (samples) of the matrix were reordered in dendrograms following hierarchical clustering. The default function hclust was used with its method of complete linkage, which agglomerates clusters computing the largest distance between any object in one cluster and the other objects in the other clusters. Similarity between each sample group was further tested using the corrr function (default method of Pearson’s correlation). Thus, a correlation matrix was produced as a graphical display (i.e. the correlogram) with cells colored according to the respective correlation coefficient (p) for each paired sample comparison.

GO enrichment analysis of the gene clusters was performed with the Web-based tool Functional Classification SuperViewer of the University of Toronto (http://bar.utoronto.ca/ntools/cgi-bin/ntools_classification_superviewer.cgi), where GO categories for genes in a given cluster are normalized for the frequency in Arabidopsis, while bootstraps and so provide confidence intervals for the accuracy of the output (Provart and Zhu, 2003). P values of the hypergeometric distribution were used to select the significant functional classes. Only enrichments with significant P ≤ 0.05 were considered and reported in the graphs. The MapMan tool (Thimm et al., 2004; http://map.man.gabiipd.org/web/guest) was used to visualize the gene expression data set in the context of metabolic pathways or other processes represented in modules (bins) and to build the Venn diagrams for up- and down-regulated genes. In addition, we performed pathway analysis and interpretation of omics data using the free MetaboAnalyst software (H inamuna et al., 2019), which can also be used to confirm the network enrichment in glycerol and glycerolipid metabolic processes of the gene set selection (by enzyme substrate annotation; 76 genes), which was later used for multi-variate effects of the treatments. Results from the AraNet analysis were imported into Cytoscape and colored according to statistically overrepresented GO categories using the plugin GOlorize (Garcia et al., 2007; http://apps.cytoscape.org/apps/golorize). Another public Web server was used for the prediction of biological interaction, GeneMANIA (http://www.genemania.org/), which was also used as a Cytoscape plugin (Warde-Farley et al., 2010). The initial entry list consisted of five genes, CPDH61 (At2g41540), SDP6 (At5g10370), NOGCC (At1g22580), and FMO (At1g12200), constituting the core region of the glycerol network in prediction, and the predicted interaction with SDH1-1 (At5g67600). GeneMANIA extended this list to create a network of genes identified as having similar functions. The predicted gene interactions and their weights were estimated on the basis of Arabidopsis knowledge through genomics and proteomics data (e.g. coexpression and protein interaction), which were retrieved from GEO, BioGRID, Pathway Commons, and IDZ as well as organism-specific functional genomics data sets (Warde-Farley et al., 2010).

**Figure Layout and Editing**

Photoshop CS5.1 (Adobe) was used for editing the final graphic layouts of the figures.

**Accession Numbers**

Microarray data from the transcriptomics analysis were deposited in the ArrayExpress database (www.ebi.ac.uk/arrayexpress) under accession number E-MTAB-5030. Sequence data from this article can be found in the Arabidopsis Genome Initiative or GenBank/EMBL databases under the following accession numbers: At1g30570 (PSBW), At3g54890 (LHC1A), At1g19150 (LHC6), At2g34420 (LHB1B2), At2g40100 (LHC6B), At1g15820 (LHC1B), At1g06950 (FED A), At1g08550 (NPQ1), At1g07110 (F2Kp), At1g23500 (FBA1), At1g38970 (FBA2), At2g54050 (CFB1/CFE1), At2g25540 (CES4), At1g35980 (CSA401), At2g29800 (MUC109), At5g46700 (MPH51), At1g66760 (SDH1-1), At3g19920 (MSD1), At4g31800 (WKRXY18), At1g08040 (WKRKY40), At2g46400 (WKRKY46), At3g45100 (LOX2), At1g17420 (LOX3), At1g32640 (MYC2), At1g67300 (MYB44), At1g37630 (MYB91), At1g72290 (WSCP), At3g45600 (MPK3), At5g45340 (CFP707A3), At5g47220 (ERF2), At2g20700 (EIN3), At2g02590 (RNS1), At2g38940 (PT1), At2g38170 (CACX), At1g61180 (IAR1), At1g25980 (NOGC1), At1g12200 (FMO), At5g29740 (LRR-RRK), At1g29310 (SecY protein/seed1), At2g15230 (UPL1), At2g41540 (GPDH61), At3g10370 (SDP6), At3g18830 (PLT5), At3g16400 (PDAT1), At3g11670 (DG01), At5g12010 (SQ2D), At5g24300 (PAD4), At1g43100 (ALHD7B4), At2g37990 (AKR4C10), At2g10940 (LTP1), and At2g45180 (LTP2).

**Supplemental Data**

The following supplemental materials are available.

**Supplemental Figure S1.** Metabolomics: PLS-DA score plot latent variables.

**Supplemental Figure S2.** Integrative omics network analysis: topology.

**Supplemental Figure S3.** Chloroplastic lipid transfer protein (LTP1; At2g10940).

**Supplemental Figure S4.** Chloroplastic lipid transfer protein (LTP2; At2g45180).

**Supplemental Figure S5.** KEGG pathway analysis for glycerolipid metabolism (O).

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**Supplemental Figure S6.** KEGG pathway analysis for glycerolipid metabolism (OP).

**Supplemental Figure S7.** Glucosinolates: correlation analysis.

**Supplemental Figure S8.** Flavonols: kaempferol glucosides, correlation analysis.

**Supplemental Figure S9.** Flavonols: quercetin glucosides, correlation analysis.

**Supplemental Figure S10.** Stomatal conductance: effect of S d of O3 stress.

**Supplemental Table S1.** Metabolomics: PLS-DA model statistics.

**Supplemental Table S2.** Metabolomics: metabolite identities and PLS-DA VIP scores.

**Supplemental Table S3.** Gene correlations with MYB44 in the integrative network.

**Supplemental Table S4.** Transcriptomics: PLS-DA for the energy metabolic network.

**Supplemental Table S5.** Transcriptomics: gene identities and VIP scores for the energy network.

**Supplemental Table S6.** Transcriptomics: PLS-DA general model (797 genes).

**Supplemental Table S7.** Transcriptomics: gene identities and PLS-DA VIP scores.

**Supplemental Table S8.** Transcriptomics: genes in the glycerol metabolic network.

**Supplemental Table S9.** Transcriptomics: PLS-DA for the glycerol metabolic network.

**Supplemental Table S10.** Transcriptomics: OPLS-DA for the glycerol metabolic network.

**Supplemental Data Set S1.** Transcriptomics: microarray and selection (P < 0.05).

**Supplemental Data Set S2.** Metabolomics: identifications and integrated peak areas.

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