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The Glucosinolate Biosynthetic Gene *AOP2* Mediates Feed-back Regulation of Jasmonic Acid Signaling in *Arabidopsis*

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ABSTRACT

Survival in changing and challenging environments requires an organism to efficiently obtain and use its resources. Due to their sessile nature, it is particularly critical for plants to dynamically optimize their metabolism. In plant primary metabolism, metabolic fine-tuning involves feed-back mechanisms whereby the output of a pathway controls its input to generate a precise and robust response to environmental changes. By contrast, few studies have addressed the potential for feed-back regulation of secondary metabolism. In *Arabidopsis*, accumulation of the defense compounds glucosinolates has previously been linked to genetic variation in the glucosinolate biosynthetic gene *AOP2*. *AOP2* expression can increase the transcript levels of two known regulators (*MYB28* and *MYB29*) of the pathway, suggesting that *AOP2* plays a role in positive feed-back regulation controlling glucosinolate biosynthesis. We generated mutants affecting *AOP2*, *MYB28/29*, or both. Transcriptome analysis of these mutants identified a so far unrecognized link between *AOP2* and jasmonic acid (JA) signaling independent of *MYB28* and *MYB29*. Thus, *AOP2* is part of a regulatory feed-back loop linking glucosinolate biosynthesis and JA signaling and thereby allows the glucosinolate pathway to influence JA sensitivity. The discovery of this regulatory feed-back loop provides insight into how plants optimize the use of resources for defensive metabolites.

Keywords: feed-back regulation, jasmonates, JA signaling, glucosinolates, *Arabidopsis*

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INTRODUCTION

All organisms must coordinate a phenomenal array of highly interconnected metabolic processes to efficiently obtain and use resources. This coordination of metabolism is particularly critical to maximize a plant's survival in dynamically changing environments. A key component in the regulation of primary plant metabolism is the fine-tuning provided by feed-back mechanisms (Grant, 2006; de Kraker et al., 2007; Smith and Stitt, 2007; Kauss et al., 2012). These feed-back regulatory processes whereby the output controls a system's input are essential to generate a precise and robust response within any interconnected process (Freeman, 2000; Yi et al., 2000; Orrell et al., 2006; Lankau, 2007). In contrast, secondary metabolism is predominantly viewed to be under forward transcriptional regulation with minimal analysis of potential feed-back regulation. As secondary metabolites are biosynthesized from primary metabolites, feed-back regulation from secondary to primary metabolisms is likely to play an important role in the optimization of plant metabolism. A recent

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analysis within polyphenol metabolism in *Arabidopsis* has suggested the potential for plants to use unknown feed-back regulatory processes to coordinate growth and lignin deposition (Bonawitz et al., 2014).

Key defensive secondary metabolites within *Arabidopsis* are the amino acid-derived glucosinolates, produced predominantly from tryptophan (indole glucosinolates) and chain-elongated methionine (short- or long-chained aliphatic glucosinolates) (Sønderby et al., 2010b). These compounds help to defend *Arabidopsis* against both insect and microbial attackers (Chew, 1988; Brader et al., 2001; Stotz et al., 2001; Tierens et al., 2001; Hopkins et al., 2009). Although all classes of glucosinolates are constitutively present in all *Arabidopsis* tissues (Petersen et al., 2002; Brown et al., 2003), higher levels can be induced by herbivores (Mewis et al., 2005, 2006; Kim and Jander, 2007; Bidart-Bouzat and Kliebenstein, 2008) or upon treatment with jasmonates (Brader et al., 2001; Kliebenstein et al., 2002; Mikkelsen et al., 2003; Jost et al., 2005). Recent studies have linked this constitutive and inductive transcriptional regulatory control to a clade of six R2R3 domain MYB transcription factors and the three basic helix-loop-helix (bHLH) transcription factors MYC2 (bHLH006), MYC3 (bHLH005), and MYC4 (bHLH004) (Gigolashvili et al., 2007a, 2007b; Hirai et al., 2007; Sønderby et al., 2007; Gigolashvili et al., 2008b; Sønderby et al., 2010a; Schweizer et al., 2013; Frerigmann and Gigolashvili, 2014).

In contrast to the beneficial contribution to biotic defense, the accumulation of the amino acid-derived defense compounds glucosinolates has been shown to be metabolically costly using both modelling and empirical studies (Zust et al., 2011; Bekaert et al., 2012). Thus, higher levels of glucosinolates can have both beneficial and detrimental effects on plant fitness (Giamoustaris and Mithen, 1995; Mauricio, 1998; Lankau, 2007; Bidart-Bouzat and Kliebenstein, 2008). These contradictory effects require that the accumulation of these chemical defenses be tightly controlled. Within the modelling and empirical studies, the modelling studies predicted a much larger growth cost of glucosinolate accumulation then was empirically measured in defined mutants lacking glucosinolates (Giamoustaris and Mithen, 1995; Mauricio, 1998; Lankau, 2007; Bidart-Bouzat and Kliebenstein, 2008). One explanation for this contradiction is that *in vivo* glucosinolate accumulation may be regulated differently than implied in the modelling studies. Because the flux model was built to mimic glucosinolate levels, it inherently mimics the transcriptional regulation of the glucosinolate pathway under steady state conditions. The difference from the empirical studies thus indicates that there may be other regulatory processes such as feed-back regulation that can alleviate the costs of glucosinolate production under basal conditions.

Another line of evidence suggesting a potential for feed-back regulation in the glucosinolate pathway came from studies of naturally occurring *Arabidopsis* accessions that differ in their aliphatic glucosinolate structures. This glucosinolate profile variation can be partially explained by genetic variation in the *GS-AOP* locus encoding the two 2-oxo acid-dependent dioxygenases AOP2 and AOP3 (Kliebenstein et al., 2001b). AOP2 and AOP3 convert short-chained aliphatic methylsulfinylalkylglucosinolates to alkenyl- and hydroxyalkylglucosinolates, respectively. Genetic variation at *AOP2* is also linked to increased glucosinolate accumulation (Kliebenstein et al., 2001a; Wentzell et al., 2007). Furthermore, expression of *AOP2* from *Brassica oleracea* in the *Arabidopsis* accession Col-0, which lacks a functional *AOP2*, results not only in the accumulation of alkenyl glucosinolates, but also in a two-fold increase in total aliphatic glucosinolate content (Li and Quiros, 2003; Wentzell et al., 2007). Analysis of transcriptome changes in these *AOP2* expressing lines indicated that *AOP2* positively regulates glucosinolate accumulation by increasing the transcript levels of most biosynthetic genes in the pathway and two known transcription factors, i.e. the R2R3 domain MYB transcription factors *MYB28* and *MYB29* (Wentzell et al., 2007). Based on these findings, *AOP2* has been suggested to play a role in a positive feed-back loop controlling aliphatic glucosinolate biosynthesis via an unknown mechanism (Wentzell et al., 2007; Burow et al., 2010).

With the aim of investigating the regulatory role of *AOP2* in aliphatic glucosinolate biosynthesis and deciphering its interplay with *MYB28* and *MYB29* in the glucosinolate regulatory network, we introduced the *AOP2* from *B. oleracea* into different *myb* knockout mutants in the Col-0 background. We show that the *AOP2* feed-back mechanism for aliphatic glucosinolate accumulation depends on the presence of *MYB28* and *MYB29*. Transcriptome analysis of mutants affecting *AOP2*, *MYB28/29*, or both identified an unrecognized link between *AOP2* and jasmonic acid (JA) signaling that was independent of *MYB28* and *MYB29*. Using *AOP2* expression lines, we could show that the introduction of this gene into *Arabidopsis* leads to an altered sensitivity to exogenous JA. Our findings describe *AOP2* as a biosynthetic gene from a JA-inducible defense pathway that feed-back regulates JA signaling and thereby its own pathway and potentially links to other pathways controlled by JA.

RESULTS

AOP2 Depends on *MYB28* to Increase Levels of Aliphatic Glucosinolates

Expression of the enzymatically functional *AOP2* from *B. oleracea* in *Arabidopsis* Col-0, a natural AOP2 knockout, increased transcript levels of the transcription factors *MYB28* and *MYB29* and glucosinolate accumulation (Wentzell et al., 2007). Based on this observation, we hypothesized that *AOP2* increases glucosinolate levels via the *MYB28* and *MYB29* transcription factors. To test this, we crossed 35S:*AOP2* plants to *myb28-1* and *myb29-2* single knockouts and analyzed leaf glucosinolate accumulation in homozygous progeny obtained from the same maternal lineage (Figure 1). Introduction of *AOP2* to Col-0 led to a higher accumulation of short- and long-chained aliphatic glucosinolates. By contrast, no significant changes in aliphatic glucosinolate levels were detected upon *AOP2* over-expression in the *myb28-1* knockout background indicating that *AOP2* requires *MYB28* to up-regulate the biosynthesis of both short- and long-chained aliphatic glucosinolates (Figure 1A and 1B).

While *MYB28* is considered a major transcriptional activator of aliphatic glucosinolate biosynthesis, *MYB29* has been described as playing a lesser role in regulating basal glucosinolate levels (Gigolashvili et al., 2008a; Sønderby et al., 2010a). Accordingly, *AOP2* expression in the *myb29-2* knockout resulted in higher accumulation of short-chained aliphatic

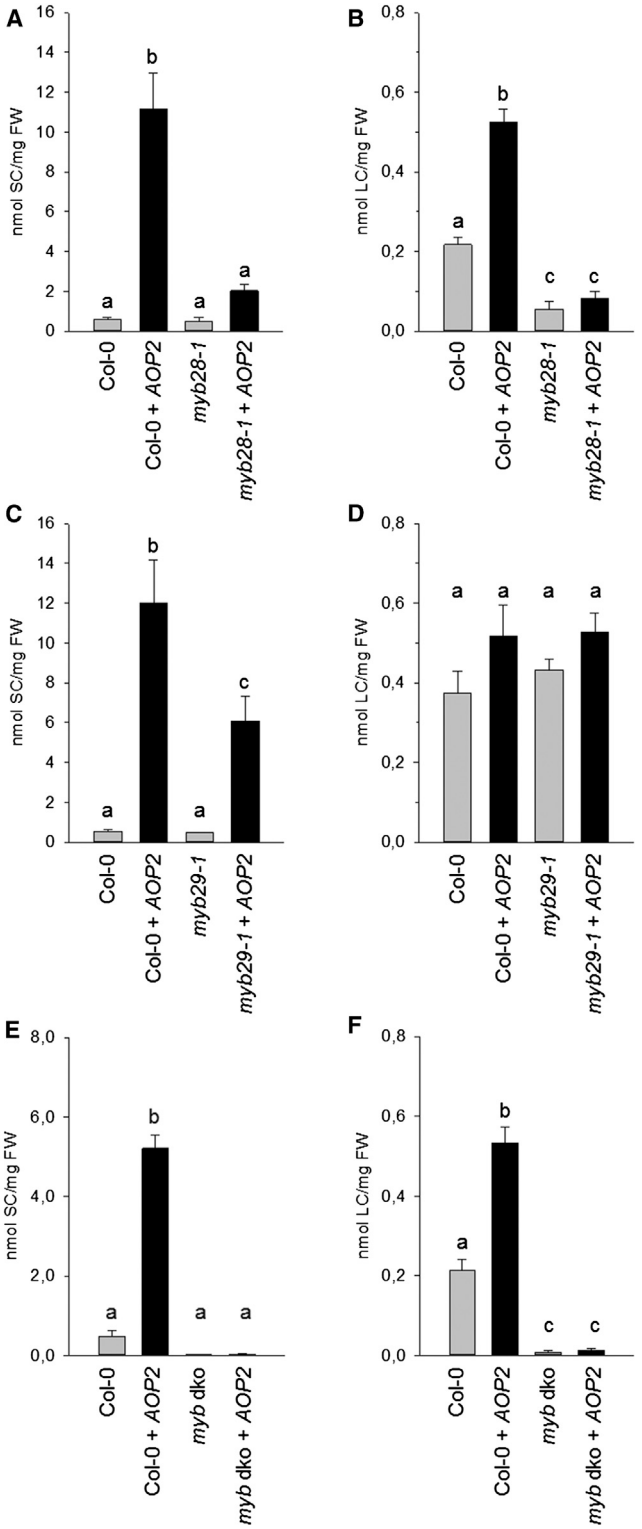

Figure 1. Effect of Constitutive *AOP2* Expression in *myb28-1*, *myb29-2* Single Knockout Mutants, and in the *myb28-1 myb29-1* dko.

(A and B) *myb28-1* single knockout mutants.

(C and D) *myb29-2* single knockout mutants.

(E and F) *myb28-1 myb29-1* dko.

Glucosinolates were extracted from rosette leaves of 21- to 24-day old plants and quantified as desulfo-glucosinolates by HPLC. Gray bars represent control plants not expressing *AOP2*, black bars 35S:*AOP2* lines. Data are shown as means (+SE) obtained in at least two independent experiments (n = 12–47). Different letters indicate significant differences between genotypes (ANOVA, $P < 0.05$, see Supplemental Tables 1, 2, and 4). Levels of individual glucosinolates can be found in Supplemental Tables 1, 2, and 4, short-chained aliphatic glucosinolates; LC, long-chained aliphatic glucosinolates; FW, fresh weight.

glucosinolates compared with the *myb29-2* knockout and the wild-type (Figure 1C). Short-chained aliphatic glucosinolate over-accumulation upon *AOP2* expression in the *myb29-2* knockout was significantly lower than in the wild-type background. *MYB29* therefore contributes to the regulatory role of *AOP2* but with a lower magnitude than *MYB28*. Wild-type and *myb29-2* knockout controls segregated out of this cross were found to accumulate relatively high levels of long-chained aliphatic glucosinolates, which were not found to be further increased by AOP2 expression (Figure 1D).

The Effects of *AOP2* and *MYB28* on Short-Chained Aliphatic Glucosinolates Are Additive

The elevated levels of aliphatic glucosinolates in plants over-expressing either *AOP2* or *MYB28* have been shown to be accompanied by increased transcript levels of the majority of the biosynthetic genes in the pathway (Sønderby et al., 2007; Wentzell et al., 2007). This suggests that the positive regulatory effect of *AOP2* on aliphatic glucosinolate biosynthesis could be explained by up-regulation of *MYB28*, which then transcriptionally up-regulates the biosynthetic genes. To further investigate the interplay between *AOP2* and *MYB28* in the regulation of glucosinolate biosynthesis, we generated lines over-expressing both genes. Analysis of leaf glucosinolates revealed that over-expression of both genes leads to higher short-chained aliphatic glucosinolate accumulation than each transgene alone (Figure 2). In this set of experiments, there were no observed effects of *AOP2* and *MYB28* on long-chained aliphatic glucosinolates suggesting that this phenotype is more environmentally sensitive (Figure 2). Together with the knockout analysis, the data show that *AOP2* acts upstream of *MYB28* to regulate short-chain aliphatic glucosinolate accumulation. Furthermore, wild-type levels of *MYB28* are a limiting factor in the ability of *AOP2* to increase the accumulation of aliphatic glucosinolates.

AOP2 Cannot Trigger Aliphatic Glucosinolate Accumulation in the Absence of Both *MYB28* and *MYB29*

While *myb28 myb29* double knockout plants have been shown to be devoid of aliphatic glucosinolates (Sønderby et al., 2007; Beekwilder et al., 2008), production of aliphatic glucosinolate is not strictly dependent on MYB28 and MYB29. Aliphatic glucosinolates can be induced in the *myb28 myb29* double knockout background either upon over-expression of MYB76 or upon feeding damage caused by *Mamestra brassicae* caterpillars (Beekwilder et al., 2008; Sønderby et al., 2010a). Thus, we introduced the full-length *B. oleracea AOP2* to the *myb28-1 myb29-1* double knockout to test if *AOP2* can act on regulatory factors that induce aliphatic glucosinolate biosynthesis independently of *MYB28* and *MYB29*.

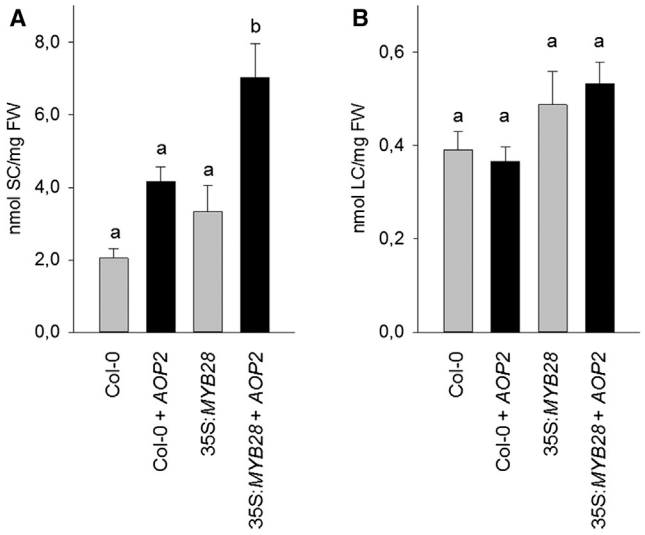

Figure 2. Effect of Constitutive *AOP2* Expression in 35S:*MYB28* Plants.

(A) Short-chained aliphatic glucosinolates (SC).

(B) Long-chained aliphatic glucosinolates (LC).

Glucosinolates were extracted from 23- to 25-day old plants and quantified as desulfo-glucosinolates by HPLC. Gray bars represent control plants not expressing *AOP2*, black bars 35S:*AOP2* lines. Data are shown as means (+SE) obtained in two independent experiments (n = 13–36). Different letters indicate significant differences between genotypes (ANOVA, $P < 0.05$, see Supplemental Table 3). Levels of individual glucosinolates can be found in Supplemental Table 3. FW, fresh weight.

As previously found, *AOP2* over-expression in the wild-type background led to increased accumulation of short- and long-chained aliphatic glucosinolates as well as indole glucosinolates (Figure 1E and 1F). The effect on indole glucosinolates is less consistent suggesting that it is more environmentally dependent (Supplemental Table 4). In the absence of *MYB28* and *MYB29*, *AOP2* was unable to induce a change in glucosinolate accumulation. This agrees with the observation that *AOP2* does not increase *MYB76* transcript levels in the Col-0 background (Wentzell et al., 2007). Together, this shows that the *AOP2*-mediated increase in glucosinolate levels is completely dependent on the presence of a functional *MYB28* or *MYB29.*

AOP2 Causes Distinct Changes in Glucosinolate-Related Transcripts Independently of *MYB28* and *MYB29*

To test for regulatory effects of *AOP2 expression* in the absence of both MYB28 and MYB29, we analyzed transcript profiles of wild-type, 35S:*AOP2*, *myb28-1 myb29-1* dko, and 35S:*AOP2* in the *myb* dko background. Consistent with the glucosinolate profile of the *myb28-1 myb29-1* dko (Figure 3), transcript levels of nearly all genes involved in aliphatic glucosinolate biosynthesis were reduced compared with wild-type (Figure 3). In contrast to a previous study, which found a slight but consistent increase in most aliphatic glucosinolate biosynthesis genes (Wentzell et al., 2007), *AOP2* expression led to altered transcript levels of a distinct subset of glucosinolate biosynthetic genes in the *myb* dko background in this experiment. This discrepancy between the two studies could be due to differences in the growth conditions. As glucosinolate biosynthesis undergoes diurnal rhythm, the direct comparison of both data sets is moreover precluded because the experiments were harvested at different times of the day (Kerwin et al., 2011; Huseby et al., 2013). Both studies, however, agree that introduction of *AOP2* into *Arabidopsis* alters the transcript accumulation of some aliphatic glucosinolate biosynthetic genes.

Remarkably, expression of *AOP2* changed the expression levels of some genes in the pathway both in the presence (wild-type background) and the absence of MYB28 and MYB29 (*myb28-1 myb29-1* dko background). This is despite the absence of detectable aliphatic glucosinolates in any lines lacking both MYB28 and MYB29. For example, transcript levels of *GGP1*, *CYP83B1*, and *SOT16* were up-regulated by *AOP2* independently of MYB28 and MYB29 (Bak et al., 2001; Hansen et al., 2001; Naur et al., 2003; Piotrowski et al., 2004; Geu-Flores et al., 2011) (Figure 3). Individual genes involved in secondary modification of glucosinolate side chains, i.e. *CYP81F4* and *GS-OX2* (Li et al., 2008; Kai et al., 2011; Pfalz et al., 2011), showed reduced expression levels in 35S:*AOP2* lines independent of *MYB28* or *MYB29*. These five transcripts regulated by *AOP2* in the absence of *MYB28* and *MYB29* were not changed in the *myb28-1 myb29-1* dko indicating that *AOP2* can bypass *MYB28* and *MYB29* to regulate specific glucosinolate biosynthetic genes. As this occurs in the absence of detectable levels of aliphatic glucosinolates, this MYB28/29-independent regulatory function may not require the AOP2-catalyzed formation of alkenyl glucosinolate metabolites.

The finding that *AOP2* feeds into regulatory networks that bypass *MYB28* and *MYB29* is further supported by the negative regulatory effect of *AOP2* on genes involved in the enzymatic activation of glucosinolates upon tissue damage (Figure 3), i.e. myrosinase-binding protein 1 (*MBP1*) and nitrile-specifier proteins (*NSP1*, *NSP3*, *NSP4*) (Capella et al., 2001; Burow et al., 2009). In contrast, these four *AOP2*-regulated genes involved in glucosinolate activation are not regulated by *MYB28* and *MYB29*. Thus, *AOP2* must be affecting an additional regulatory network (Hirai et al., 2007; Sønderby et al., 2007, 2010a).

AOP2 Feed-Back Regulates JA Biosynthesis and Signaling on the Transcriptional Level

One candidate for the additional regulatory network targeted by *AOP2* came from an observed increase in transcript levels of *MYC2* (*bHLH006*), known to regulate indole glucosinolate biosynthesis and other JA-responsive genes (Lorenzo et al., 2004; Dombrecht et al., 2007). This prompted us to further investigate the regulatory effect of *AOP2* on JA biosynthesis and signaling. Twelve of 25 JA biosynthetic and metabolic genes showed *AOP2*-induced changes in expression level with *LOX3*, *LOX4*, *AOC1*, *AOC3*, *OPR3*, *CYP94B3*, and *CYP94C1* (Sanders et al., 2000; Stintzi and Browse, 2000; Stenzel et al., 2003; Kandel et al., 2007; Koo et al., 2011; Chauvin et al., 2013) being most strongly up-regulated by *AOP2* (Figure 4 and Supplemental Table 6). Likewise, *AOP2* over-expression was sufficient to generate significantly higher transcript accumulation for eight members of the jasmonate ZIM-domain (JAZ) protein family that serve as key regulators of JA signaling (Chini et al., 2007; Thines et al., 2007). The *MYB28*/*MYB29* genotype had no significant effect on the transcript accumulation of any JA-related gene

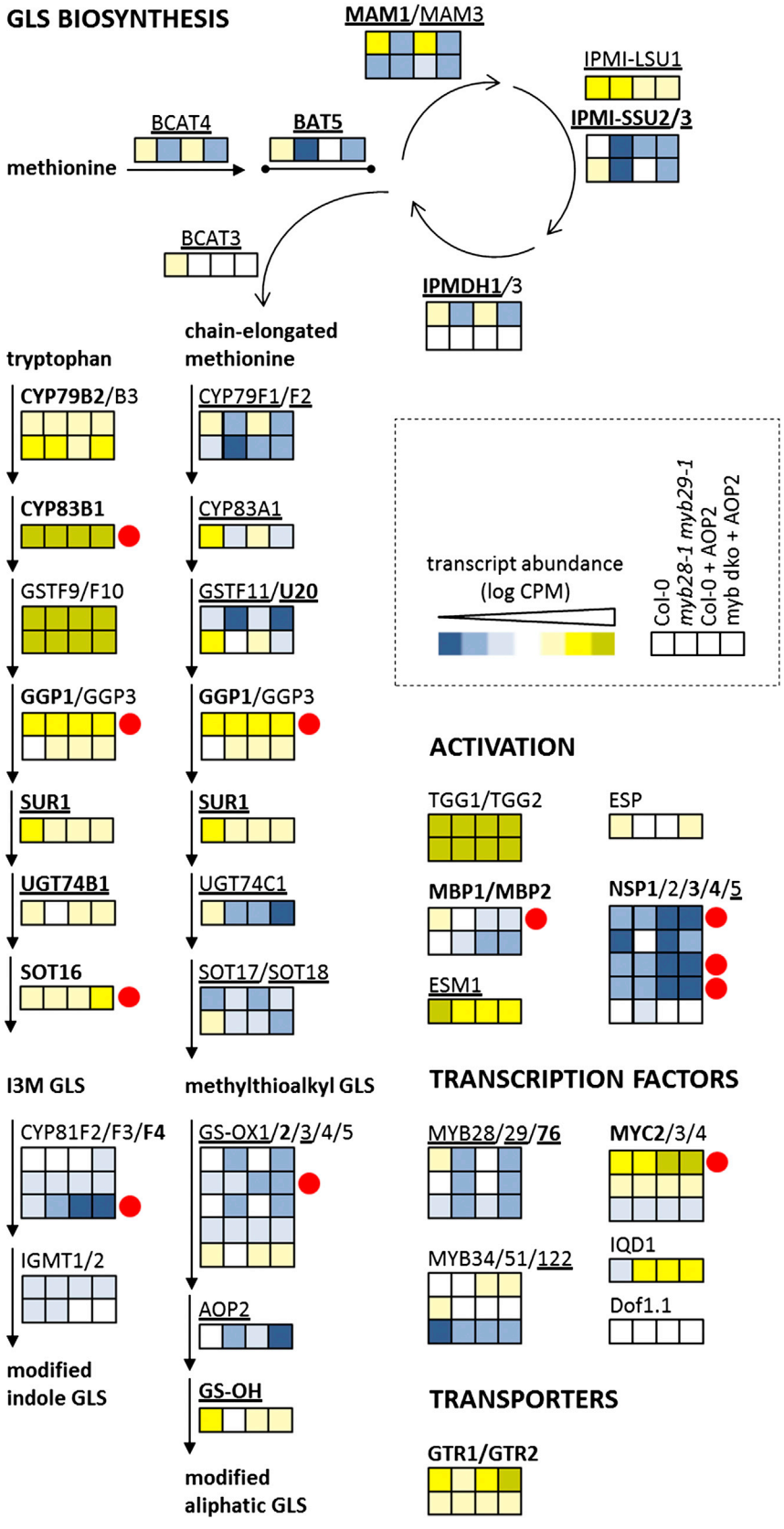

Figure 3. Transcript Levels of Genes Involved in Glucosinolate Biosynthesis, Activation, Regulation and Transport.

Average transcript levels are shown for Col-0, *myb28-1 myb29-1* dko, and lines constitutively expressing *AOP2* in these two backgrounds. Different colors represent different transcript levels in log CPM from −12 (dark blue) to ≥9 (dark yellow). Underlined gene names depict transcripts altered in lines devoid of MYB28 and MYB29, while bold letters indicate changes in transcript levels in lines expressing *AOP2* (ANOVA, FDR-adjusted $P < 0.05$). Red circles depict transcript levels significantly altered by *AOP2* independent of MYB28 and MYB29 ($P > 0.05$ for AOP2-MYB interaction). Gene names and transcript levels are as listed in Supplemental Table 5. CPM, counts per million. GLS, glucosinolate.

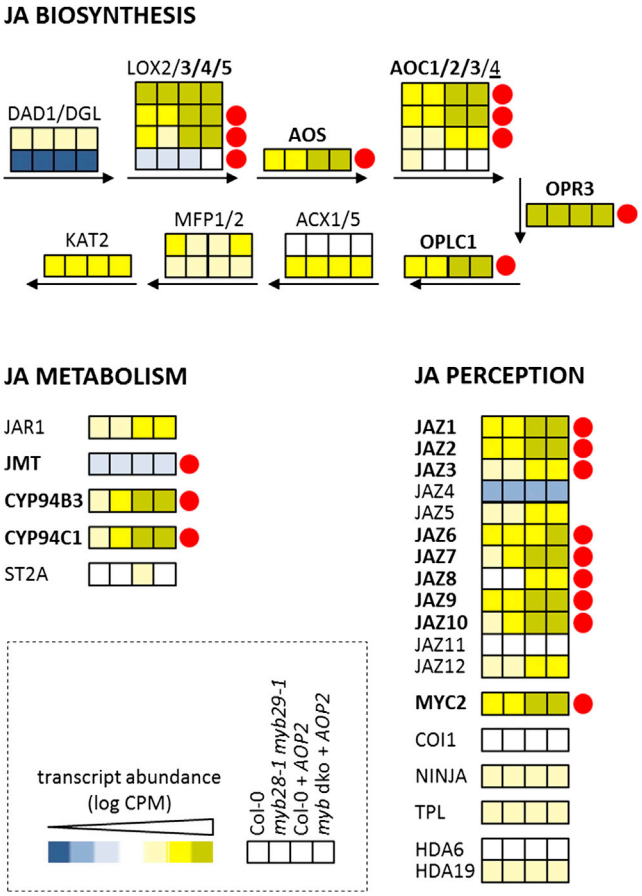

Figure 4. Transcript Levels of Genes Involved in JA Biosynthesis, Metabolism, and Perception.

Average transcript levels are shown for Col-0, *myb28-1 myb29-1* dko, and lines constitutively expressing *AOP2* in these two backgrounds (Col-0+AOP2 and *myb* dko+*AOP2*). Different colors represent different transcript levels in log CPM from −12 (dark blue) to ≥9 (dark yellow). Underlined gene names depict transcripts altered in lines devoid of MYB28 and MYB29, while bold letters indicate changes in transcript levels in lines expressing *AOP2* (ANOVA, FDR-adjusted $P < 0.05$). Red circles depict transcript levels significantly altered by *AOP2* independent of MYB28 and MYB29 ($P > 0.05$ for AOP2–MYB interaction). Gene names and transcript levels are as listed in Supplemental Table 6. CPM, counts per million, JA, jasmonic acid.

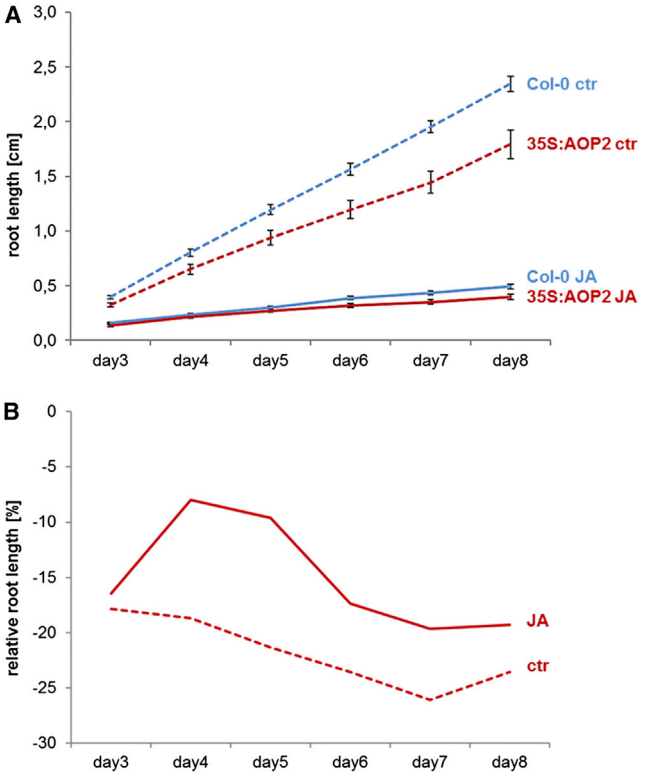

Figure 5. Root Growth of Wild-Type and 35S:*AOP2* Seedlings on JA.

(A) Data are shown as means (+SE) obtained in three independent experiments. Solid lines, seedlings grown on MS plates containing JA (blue, wild-type, total $n = 28$; red, 35S:*AOP2*, total $n = 23$); dashed lines, seedlings grown on control plates without JA (ctr; blue, wild-type, total $n = 24$; red, 35S:*AOP2*, total $n = 22$).

(B) Root growth of 35S:*AOP2* seedlings shown relative to the wild-type on MS medium supplemented with JA (solid line) and MS control plates (dashed line). For relative root growth of *myb28-1 myb29-1* dko and the *myb28-1 myb29-1* dko+*AOP2* seedlings, see Supplemental Figure 1 and Supplemental Table 7.

showing that these effects are specific to *AOP2* and independent of *MYB28* and *MYB29*. This *AOP2*-to-JA link was found even in the absence of glucosinolate substrates for the AOP2 enzyme (35S:*AOP2* vs. *myb28-1 myb29-1* dko + 35S:*AOP2*; Figure 4 and Supplemental Table 6). This suggests that the enzymatic activity within the glucosinolate pathway is not critical for *AOP2*'s ability to feed-back regulate JA biosynthesis and signaling components on the trancriptional level.

The effect of *AOP2* expression on JA pathway transcript accumulation led us to hypothesize that the genotypes over-expressing *AOP2* may have an altered sensitivity to JA. To test this hypothesis, we germinated seeds from wild-type, 35S:*AOP2*, *myb28-1 myb29-1* dko, and 35S:*AOP2*/dko on media with and without exogenous JA and measured the root length daily after 3–8 days. On control plates without JA, the roots of seedlings expressing *AOP2* in the Col-0 wild-type background were shorter (Figure 5A, dashed lines). Relative to wild-type seedlings on control plates, the *AOP2*-expressing seedlings were about 20%–25% shorter. Interestingly, when grown on JA, the root length of *AOP2*-expressing seedlings relative to the wild-type was increased (Figure 5B). These seedlings showed significantly decreased sensitivity to exogenous JA that was strongest on days 4 and 5 but significant at least to day 8, the end of the experiment. As predicted from the transcriptomics, the *MYB28*/*MYB29* genotype had no effect on sensitivity to JA in the growth media and no effect on the JA–*AOP2* interaction (Table 1). Despite the altered JA sensitivity in *AOP2*-expressing seedlings, JA levels were not significantly changed (Figure 6). Thus, *AOP2* leads to altered JA sensitivity in Col-0 seedlings by an unknown mechanism that is independent of *MYB28*/*MYB29* and thereby of glucosinolate precursor availability.

DISCUSSION

Glucosinolate profiles change dynamically in response to internal and external signals (Petersen et al., 2002; Brown et al.,

Day		Plate	Treatment	AOP2	MYBs	AOP2×MYBs	Treatment×AOP2	Treatment×MYBs	Residuals
	Df	**4**	**1**	**1**	**1**	**1**	**1**	**1**	**78**
3	SS	0.106	0.732	0.063	0.090	0.000	0.011	0.022	0.282
	F	7.328	202.340	17.287	24.790	0.066	2.975	6.215	
	P	**<0.001**	**<0.001**	**<0.001**	**<0.001**	0.798	0.089	**0.015**	
4	SS	0.421	4.302	0.153	0.184	0.000	0.105	0.041	0.886
	F	9.259	378.688	13.455	16.160	0.013	9.242	3.637	
	P	**<0.001**	**<0.001**	**<0.001**	**<0.001**	0.909	**0.003**	0.060	
5	SS	0.595	11.832	0.361	0.102	0.020	0.204	0.018	1.567
	F	7.400	589.072	17.979	5.095	0.997	10.163	0.880	
	P	**<0.001**	**<0.001**	**<0.001**	**0.027**	0.321	**0.002**	0.351	
6	SS	0.823	20.794	0.531	0.142	0.100	0.164	0.060	3.362
	F	4.772	482.461	12.308	3.298	2.315	3.796	1.393	
	P	**0.002**	**<0.001**	**0.001**	0.073	0.132	0.055	0.242	
7	SS	1.159	34.267	0.798	0.156	0.270	0.287	0.137	3.949
	F	5.722	676.926	15.767	3.078	5.333	5.676	2.701	
	P	**<0.001**	**<0.001**	**<0.001**	0.083	**0.024**	**0.020**	0.104	
8	SS	1.931	56.100	0.915	0.328	0.280	0.319	0.279	6.071
	F	6.202	720.767	11.757	4.211	3.598	4.095	3.587	
	P	**<0.001**	**<0.001**	**0.001**	**0.044**	0.062	**0.046**	0.062	

Table 1. Results of ANOVA Testing of Plate, Treatment (±JA), AOP2 (Presence and Absence of the AOP2 Transgene), MYBs (Presence and Absence of MYB28/29), AOP2×MYBs Interaction, Treatment×AOP2 Interaction, and Treatment×MYBs Interaction for Significant Impact on Root Growth.

For means and SEs, see Supplemental Table 7. F, *F* value; P, the statistical significance of each term across the different days (numbers in bold indicate *P* values <0.05); SS, type III sums-of-squares.

2003); (Hirai et al., 2004; Falk et al., 2007; Hopkins et al., 2009; Huseby et al., 2013). The regulatory network controlling glucosinolate biosynthesis must therefore allow for integration of various signals, both internal and external. This fine-tuning of the pathway likely requires feed-back regulation. Previous findings had suggested that *AOP2* mediates feed-back regulation of glucosinolate biosynthesis via *MYB28* and *MYB29* (Wentzell et al., 2007). In agreement with this hypothesis, expression of *AOP2* from *B. oleracea* in the *myb28-1 myb29-1* dko did not result in accumulation of detectable levels of aliphatic glucosinolates (Figure 1). Thus, *AOP2* functions to regulate glucosinolate accumulation in a feed-back loop that involves the *MYB28*/*MYB29* transcription factors.

Transcriptomic analysis of plants expressing *AOP2* in the *myb28-1 myb29-1* dko background revealed an additional regulatory role for *AOP2* bypassing *MYB28* and *MYB29*. Transcript levels of numerous genes involved in JA biosynthesis and signaling were increased by *AOP2* irrespective of the *MYB28*/*MYB29* genotype (Figure 4). Likewise, *AOP2* alone altered the transcript levels of a distinct subset of genes involved in indole glucosinolate biosynthesis, side chain modification, and activation of glucosinolates (Figure 3). Most of these genes show strongly reduced transcript levels in the *myc234* triple knockout indicating that they are JA-responsive (Schweizer et al., 2013). Thus, *AOP2* has a regulatory effect on JA-mediated glucosinolate biosynthesis different from the transcriptional regulators *MYB28/29/76* and *MYB51/34/122*, which ascertain coordinated expression of all genes required for aliphatic and indole glucosinolate biosynthesis, respectively (Sønderby et al., 2010a; Frerigmann and Gigolashvili, 2014). Instead, *AOP2* can fine-tune glucosinolate biosynthesis and activation through feed-back regulation of JA signaling independently of *MYB28* and *MYB29*.

One of the JA-related transcripts changed by *AOP2* was the bHLH transcription factor *MYC2*, a master switch in the regulatory network coordinating JA responses and furthermore critical for the crosstalk with the signaling pathways of other phytohormones (Lorenzo et al., 2004; Dombrecht et al., 2007; Kazan and Manners, 2013). In concert with the two related transcription factors, *MYC3* (*bHLH005*) and *MYC4* (*bHLH004*), *MYC2* has recently been shown to be indispensable for the formation of both indole and aliphatic glucosinolates (Schweizer et al., 2013; Frerigmann et al., 2014). The triple knockout mutant *myc234* is nearly devoid of glucosinolates and shows strongly reduced expression of glucosinolate biosynthetic genes including *AOP2* (Schweizer et al., 2013; Frerigmann et al., 2014).

By contrast, *AOP2* expression levels are unchanged in the *myb28-1 myb29-1* dko that accumulates only trace amounts of aliphatic glucosinolates and shows highly reduced transcript levels of the genes in that pathway (Sønderby et al., 2010a). Interestingly, both MYB28 and MYB29 were shown to bind the *AOP2* promoter in a yeast-1-hybrid study, while MYB76, MYC2 and MYC4 did not (Li et al., 2014). Thus,

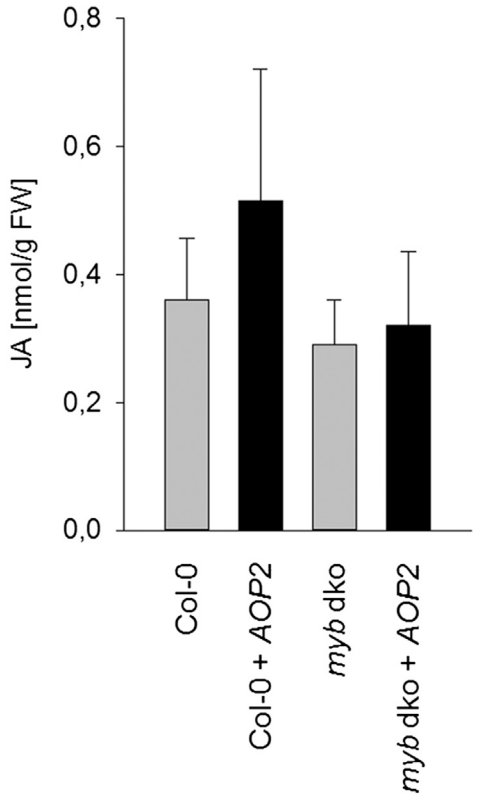

Figure 6. JA Levels in 4-day Old Seedlings Grown on MS Medium.

Gray bars represent control plants not expressing *AOP2*, black bars 35S:*AOP2* lines. Data are shown as means (+SE) obtained in three independent experiments (total $n = 9$). There were no significant differences between genotypes (ANOVA, $P > 0.05$). FW, fresh weight.

AOP2 expression might require the formation MYC-MYB protein complexes *in vivo*. In turn, *AOP2* feeds into the regulatory network controlling *MYC2*, *MYB28*, and *MYB29* and thereby establishes a positive feed-back loop in the JA-mediated regulation of glucosinolate biosynthesis mediated by *AOP2* (Figure 7). As *AOP2* encodes a cytoplasmic enzyme, it is unlikely that the AOP2 protein directly or indirectly interferes with transcriptional gene activation in the nucleus. The direct target of *AOP2* as a regulator and the molecular mechanism underlying the regulatory function of this biosynthetic gene remain to be identified.

The observed changes in the transcript levels of genes involved in JA biosynthesis and signaling within the *AOP2* genotypes were accompanied by a decreased JA sensitivity in the *AOP2* plants (Figure 5). This effect of *AOP2* on root growth was independent of *MYB28* and *MYB29*. However, JA levels in the seedlings were not significantly altered (Figure 6) underlining the complexity of JA-mediated regulation *in vivo*. Nevertheless, feed-back regulation of JA signaling mediated by *AOP2* allows a gene within the glucosinolate biosynthetic pathway to influence JA sensitivity by an unknown mechanism. This shows that a downstream defense output, an enzyme in defense metabolism, can feed-back regulate the upstream signaling network that controls the defense output. It remains to be tested if this feed-back regulation is a broader property of plant defense networks.

The observation that the *AOP2* gene can alter JA sensitivity and glucosinolate gene expression in the *myb28 myb29* dko suggests that this regulatory activity is independent of the known enzyme activity. One possibility is that AOP2 has other, so far unknown enzymatic activities that mediate this regulatory function. There are, however, several arguments against this hypothesis. First, AOP2 is a newly evolved enzyme that arose via a tandem duplication and neo-functionalization within the *Arabidopsis* clade that has only ever been linked to glucosinolate metabolism and is thus not a conserved gene likely to have any ancestral function remaining (Wentzell et al., 2007; Kliebenstein et al., 2001a). Second, in metabolomics studies of lines that vary for the presence of the *AOP2* gene, there were no metabolites that showed similar variation that were not glucosinolate or precursors (Rowe et al., 2008; Chan et al., 2011; Joseph et al., 2013). This leaves two other possible explanations. First, it is possible that the residual trace level of aliphatic glucosinolates in the *myb28 myb29* dko may be sufficient for the regulatory process. Alternatively, there may be as yet unrecognized regulatory functions of the *AOP2* RNA or protein that remain to be identified. Further work is necessary to address the specific regulatory mechanisms of the *AOP2* regulatory feed-back mechanisms.

As *AOP2* expression significantly varies between naturally occurring accessions of *Arabidopsis* (Kliebenstein et al., 2001a; Wentzell et al., 2007), this regulatory feed-back loop linking *AOP2* expression and JA signaling suggests natural variation in JA-mediated regulation of glucosinolates as well as in glucosinolate-mediated tuning of JA sensitivity. This variation in fine-tuning of glucosinolate biosynthesis allows for the higher accumulation of glucosinolates in *AOP2*-expressing *Arabidopsis* accessions (Kliebenstein et al., 2001a; Burow et al., 2010). Natural variation in *AOP2* may moreover reflect differences in the JA regulatory network among *Arabidopsis* accessions both with regard to the input to and the output from JA signaling. Future efforts will aim to elucidate the upstream mechanistic basis allowing *AOP2*-mediated feed-back regulation of JA signaling. Identification of the specific mechanistic link between *AOP2* and JA signaling will provide new insight into how biological systems integrate internal and external signals to generate a specific phenotypic output in dynamically changing environments.

METHODS

Plant Material and Cultivation

The double knockout mutant *myb28-1 myb29-1* (*myb28-1* = *At5g61420*, line SALK_136312; *myb29-1* = *At5g07690*, line GABI_868E02) and the *MYB28* over-expression lines (35S:*MYB28*) have been described previously (Sønderby et al., 2007, 2010a). To introduce expression of the enzymatically functional *AOP2* allele of the *B. oleracea BoGSL-ALK* under the control of the 35S CaMV promoter (Li and Quiros, 2003), two independent homozygous 35S:*AOP2* lines were crossed to the homozygous *myb28-1 myb29-1* dko and to two independent homozygous 35S:*MYB28* lines. The F1 plants were self-fertilized and progeny in the F2 generation was genotyped by PCR on genomic DNA. For all experiments, F3 seeds were sown in a randomized design and

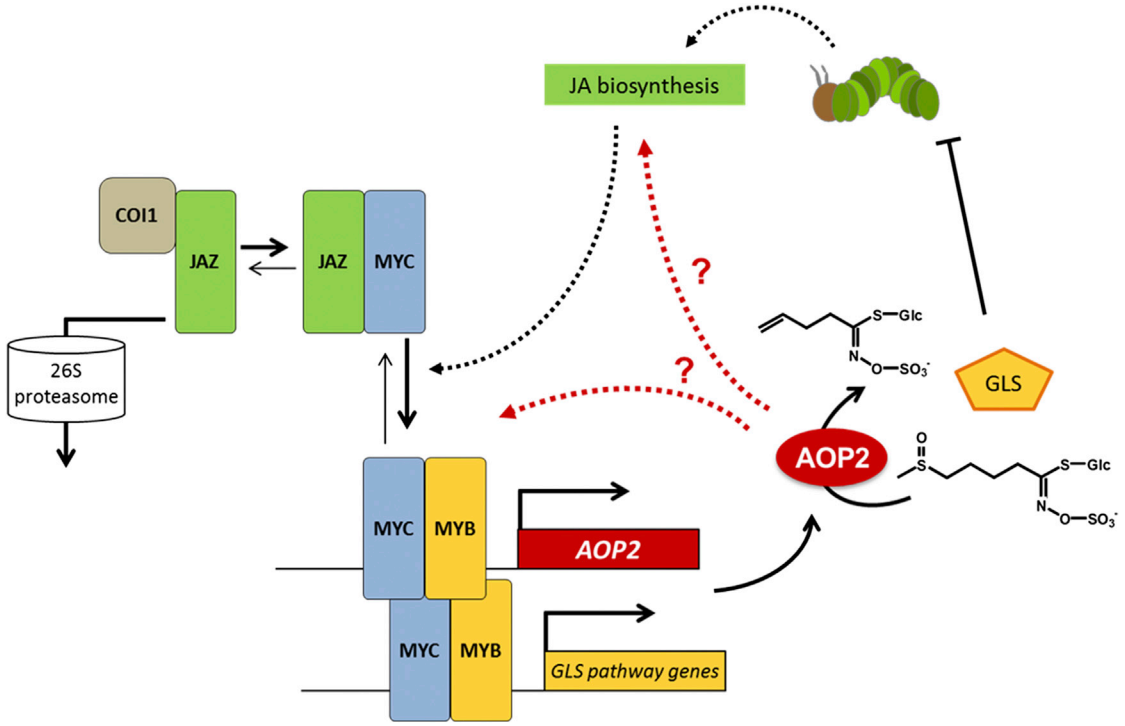

Figure 7. Proposed Regulatory Link between JA and Glucosinolate Biosynthesis.
Herbivory results in increased JA biosynthesis, which triggers increased degradation of JAZ proteins by the 26S proteasome and increased formation of protein complexes comprising MYC (MYC2, MYC3, MYC4) and MYB (MYB28, MYB29, MYB76, MYB34, MYB51, MYB122) transcription factors. Increased expression of glucosinolate pathway genes leads to higher glucosinolate levels. The glucosinolate biosynthetic gene *AOP2* mediates feed-back regulation by an unknown mechanism. GLS, glucosinolate; JA, jasmonic acid.

cold stratified at 4°C for at least 2 days before being moved to growth chambers. Plants were grown at 80–120 μE/(m^2 s), 16 h light, 21°C, 70% relative humidity.

Glucosinolate Analysis

Glucosinolate were analyzed as desulfo-glucosinolates as previously described (Kliebenstein et al., 2001b). 96-well filter plates were charged with 45 mg of DEAE Sephadex A25 and 300 μl of water per well and equilibrated at room temperature for at least 2 h. The water was removed using a vacuum manifold (Millipore). Plant material was harvested in 300 μl of 85% MeOH (v/v) containing 5 nmol *p*-OH-benzyl glucosinolate extracted from seeds of *Sinapis alba* (SeedCom A/S, Vissenbjerg, Denmark) as previously described (Thies, 1979; Zrybko et al., 1997) as an internal standard. The tissue was homogenized with one stainless steel ball by shaking for 2 min at a frequency of 30/s on a Mixer Mill 303 (Retsch, Haan, Germany), centrifuged, and the supernatant was applied to the filter plates and absorbed on the ion exchanger by vacuum filtration for 2–4 s. Sephadex material was washed with 2× 100 ml of 70% methanol (v/v) and 2× 100 μl of water and briefly centrifuged before addition of 20 μl of sulfatase solution (1.25 mg/ml, sulfatase type H1, Sigma-Aldrich) on each filter. After incubation at room temperature overnight, desulfo-glucosinolates were eluted with 100 μl of water for 96-well filter plates and 250 μl for media samples. Media sample elute was lyophilized and resuspended in 50 μl of water. All samples were analyzed by HPLC on an Agilent HP1200 Series instrument equipped with a C-18 reversed phase column (Zorbax SB-Aq, 25 cm × 4.6 mm, 5 μm particle size, Agilent or Lichrospher RP18-5 25 cm × 4.6 mm, 5 μm particle size, Supelco) by using a water (solvent A)–acetonitrile (solvent B) gradient at a flow rate of 1 ml/min at 25°C (injection volume 45 μl). The gradient applied was as follows: 1.5%–7% B (5 min), 7%–25% (6 min), 25%–80% (4 min), 80% B (3 min), 80%–35% B (2 min), 35%–1.5% B (2 min), and 1.5% B (3 min). The eluent was monitored by diode array detection between 200 and 400 nm (2-nm interval). Desulfo-glucosinolates were identified based on comparison of retention times and UV absorption spectra with those of known standards (Reichelt et al., 2002). Results are given as nmol/(mg fresh weight) calculated relative to response factors (Fiebig and Arens, 1992; Brown et al., 2003). ANOVA was used to test for significant differences.

RNA Sequencing

To test for differential expression across the genotypes, they were planted in a randomized complete block design with three completely separate outgrowths. Two plants per genotype were used to make independent libraries per experiment providing six samples per genotype. RNA was isolated from individual plants and sequencing libraries created as previously described (Kumar et al., 2012).

The libraries were then sequenced at the Beijing Genome Institute. All reads were mapped against the *Arabidopsis thaliana* Ensembl (TAIR10) genome using TopHat v2.0.8 (Trapnell et al., 2009) and default settings. Mapped reads were counted using HTSeq (Anders et al., 2014) using the setting -m intersection-nonempty. Differential expression was analyzed with edgeR (Robinson et al., 2010) using a model that accounted for the genotype at *AOP2*, *MYB28/29*, and directly tested for an interaction of the genotypes. The model also included experiment to allow for this to be included in the statistical assessment. All *P* values were adjusted to a false discovery ratio (FDR) of 0.05 within edgeR using the factorial model and are presented along with the mean corrected counts per million per transcript per genotype (Supplemental Table 8).

Root Growth Assays

Seeds were sterilized and plated on 1× MS medium (Murashige & Skoog medium, M0221, Duchefa Biochemie BV) containing 2% (w/v) sucrose. For the JA treatment, (±)-jasmonic acid (J2600, Sigma-Aldrich) was added to a concentration of 50 μM. Thirty seeds were placed in a line 2 cm below the upper plate margin on each plate and cold stratified on the plate for 2 days. The experiment was carried out in two rounds of sowing with two plates per experimental round and treatment. All four genotypes were individually randomized on each plate. The seedlings were grown vertically in a Percival growth chamber at 80–120 μE/(m^2 s), 12 h light, 21°C, and 70% relative humidity. Plates were scanned on days 3–8 after placing them in the chamber. Root length was measured using the SmartRoot image analysis software (Lobet et al., 2011).

Jasmonic Acid Analysis

JA was extracted and analyzed as described previously (Stingl et al., 2013). The seedlings were sown three times in a random block design. Per experimental round, three pools of seedlings were harvested per combination of genotype and treatment (total $n = 9$). 10–20 mg of 5-day-old seedlings (pools of 15–20 seedlings) were snap frozen in liquid nitrogen and homogenized with two stainless steel balls by shaking for 2 × 30 s minimum at a frequency of 20/s on a Mixer Mill 303 (Retsch, Haan, Germany). 1 ml of cold ethyl acetate/formic acid (99:1 v/v) was added. The samples were mixed and then centrifuged at 16 000 *g* at room temperature for 10 min. The supernatants were transferred to new tubes and the solvents were removed by using a centrifugal vacuum evaporator at 30°C (30–40 min). The residues were dissolved in 80 μl of 5% acetonitrile in water (v/v), the samples were filtered, and stored at −20°C until analysis. The samples were analyzed by UHPLC/TQ-MS on an Advance™-UHPLC/EVOQ™ Elite-TQ-MS instrument (Bruker) equipped with a C-18 reversed phase column (Kinetex 1.7 u XB-C18, 10 cm × 2.1 mm, 1.7 μm particle size, Phenomenex) by using a 0.05% formic acid in water (v/v) (solvent A)–0.05% formic acid in acetonitrile (v/v) (solvent B) gradient at a flow rate of 0.4 ml/min at 40°C. The gradient applied was as follows: 2% B (0.5 min), 2%–30% (0.7 min), 30%–100% (0.8 min), 100% B (0.5 min), 100%–2% B (0.1 min), and 2% B (1.4 min). Compounds were ionized by electrospray ionization with a spray voltage of –3900 V, heated probe temperature 210°C, cone temperature 250°C. JA was monitored based on the MRM (–)209 > 59 [11V]. Results are given as nmol/(g fresh weight) calculated based on external standard curves.

SUPPLEMENTAL INFORMATION

Supplemental Information is available at *Molecular Plant Online*.

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