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Hormone-regulated defense and stress response networks contribute to heterosis in *Arabidopsis* F1 hybrids

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Plant hybrids are extensively used in agriculture to deliver increases in yields, yet the molecular basis of their superior performance (heterosis) is not well understood. Our transcriptome analysis of a number of *Arabidopsis* F1 hybrids identified changes to defense and stress response gene expression consistent with a reduction in basal defense levels. Given the reported antagonism between plant immunity and growth, we suggest that these altered patterns of expression contribute to the greater growth of the hybrids. The altered patterns of expression in the hybrids indicate decreases to the salicylic acid (SA) biosynthesis pathway and increases in the auxin [indole-3-acetic acid (IAA)] biosynthesis pathway. SA and IAA are hormones known to control stress and defense responses as well as plant growth. We found that IAA-targeted gene activity is frequently increased in hybrids, correlating with a common heterotic phenotype of greater leaf cell numbers. Reduced SA concentration and target gene responses occur in the larger hybrids and promote increased leaf cell size. We demonstrated the importance of SA action to the hybrid phenotype by manipulating endogenous SA concentrations. Increasing SA diminished heterosis in SA-reduced hybrids, whereas decreasing SA promoted growth in some hybrids and phenocopied aspects of hybrid vigor in parental lines. *Pseudomonas syringae* infection of hybrids demonstrated that the reductions in basal defense gene activity in these hybrids does not necessarily compromise their ability to mount a defense response comparable to the parents.

biomass | hybrid vigor | salicylic acid | auxin | plant defense

Hybrid F1 plants are used in agriculture because of their superior performance compared with parental lines in traits such as growth rate, biomass, biotic and abiotic stress resistance, and yield. Increased yield and phenotypic uniformity apply only to the F1 hybrid and not to subsequent generations. An understanding of the molecular basis of hybrid vigor (heterosis) may enable the development of strategies to select better parental combinations, maintain the F1 vigor over successive generations, translate aspects of heterosis into nonhybrid plants, and increase growth and yield enhancements of hybrids.

Arabidopsis (*Arabidopsis thaliana*) provides a system to study heterosis and different combinations of parents yield different levels of hybrid vigor (1, 2). We have previously studied the development of F1 hybrids from crosses between C24, Landsberg *erecta* (L*er*), Columbia (Col), and Wassilewskija (Ws) accessions. These F1 hybrids show increased vegetative biomass and seed yield, but differ in their patterns of heterotic growth (3). The differences occur in various developmental traits throughout the growth cycle from embryogenesis through to reproductive architecture and seed production. A key phenotypic difference is the extent of increased cell size and cell number that generate the larger leaves of the hybrids (3). Developmental differences between hybrids support the idea that the heterotic phenotype can arise through different combinations of altered activity of a number of biological processes (3, 4).

The heterotic phenotype depends on the expression behavior of genes, with changes in gene expression in the hybrid occurring as a consequence of transinteractions between the two parental genomes and epigenomes. Whole-transcriptome studies of F1 hybrids have been performed in a number of plant species, including *Arabidopsis*, rice (*Oryza sativa*), maize (*Zea mays*), and wheat (*Triticum aestivum*) (4–6). Although the depth of analysis varies and interpretations are complex, transcriptional changes in hybrids are generally associated with processes involving energy production, metabolic rates, stress response, senescence, and hormone signaling.

We examined the transcriptomes of several *Arabidopsis* hybrids that differ in their heterotic growth (3), anticipating being able to identify both common and unique changes in regulatory pathways associated with the different patterns of heterotic growth. We found that all of the hybrids have transcription profiles implying increased stress tolerance and suppression of defense-related pathways as major altered processes, although the exact patterns of change differ between the hybrids. Given the competition between plant immunity and plant growth for resource allocation (7, 8), the reduction in basal defense levels could be important for generating heterosis. Substantial changes in the auxin [indole-3-acetic acid (IAA)]- and salicylic acid

Significance

Hybrids are extensively used in agriculture to deliver increases in crop yields, yet the molecular basis of their superior performance (heterosis) is not well understood. We report that some *Arabidopsis* F1 hybrids show changes to salicylic acid- and auxin-regulated defense and stress response gene expression. These changes could be important for generating the greater growth of some hybrids given the antagonistic relationship between plant growth and defense responses. Hybrids showing different levels of heterosis have changes in the salicylic acid- and auxin-regulated pathways that correlate with differences in the enhanced leaf growth. The larger leaves, and thus greater capacity for energy production, support the increased growth vigor and seed yields of the hybrids.

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Data deposition: The data reported in this paper have been deposited in the Gene Expression Omnibus (GEO) database, www.ncbi.nlm.nih.gov/geo (accession no. GSE64475).

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(SA)-regulated networks were observed in the hybrids, accounting for the changes in stress and defense response gene expression and possibly for the greater growth of the hybrids. Gene expression patterns consistent with increased auxin concentrations were found in most of the hybrids correlating with the common heterotic phenotype of greater leaf cell numbers. The SA network is reduced in some of the hybrids and is related to increased cell expansion and larger plants. We showed that by manipulating *in planta* SA concentrations, we could reduce or increase heterosis in some hybrids and promote hybrid vigor-like phenotypes in the parents.

Results

Differentially Expressed Genes in *Arabidopsis* F1 Hybrids Are Enriched for Genes That Are Expressed at Different Levels in the Parents. *Arabidopsis* hybrids generated by crosses between the C24, L*er*, and Col accessions exhibit different levels of hybrid vigor (3). By 15 d after sowing (DAS), the hybrids show differences in growth traits of the cotyledons and leaves compared with each other and with their parents and continue to develop more vegetative heterosis. We analyzed the transcriptomes of each reciprocal hybrid at 15 DAS and compared them to the transcriptomes of the parents at the same developmental stage. There were between 5,300 and 5,963 genes (~23% of expressed genes at this stage) that differed in expression levels between each parental pair (DESEQ $P \leq 0.01$; FDR ≤ 0.1; Fig. S1 *A–C*). In the hybrids, 1,741 (C24/Ler), 2,103 (C24/Col), and 1,416 (Col/Ler) genes (slash denotes both reciprocal combinations), representing 6–8% of expressed genes, had expression levels that deviated from the average of the parental levels [midparent value (MPV); DESEQ $P \leq 0.01$; FDR ≤ 0.1; Fig. S1 *D–F*; Dataset S1, Table S1] and are therefore genes that potentially generate the heterotic phenotype. Down-regulation was the more prevalent outcome with almost two thirds of the differentially expressed genes in the hybrids showing a level of expression below MPV (Fig. S1 *G–I*). Between 42% and 60% of the genes up-regulated from MPV, expressed at a level that also exceeded the higher-expressing parent, whereas between 29% and 41% of genes down-regulated from MPV expressed at levels below the lower-expressing parent (DESEQ $P \leq 0.01$; FDR ≤ 0.1; Fig. S1 *G–I*). Most (~92%) of the remaining genes differentially expressed from MPV had expression levels equivalent to those in one of the parents (DESEQ $P > 0.01$; FDR > 0.1; Fig. S1 *G–I*). The differentially expressed genes across the three hybrids represented 3,335 unique genes, of which ~45% were common to at least two of the hybrids (Dataset S1, Table S2). Genes that differed in expression levels between the parents of the hybrid were overrepresented (3–5×) among the genes differentially expressed in the hybrids (Fig. S1 *J–L*).

Gene Regulatory Networks Involved in Defense and Stress Responses Are Altered in the F1 Hybrids. We presumed that only a subset of the differentially expressed genes contribute to the heterotic phenotype and that important heterosis-promoting expression changes may be more readily identified by examining changes at the level of pathways. An enrichment analysis of the differentially expressed genes in each hybrid identified 199 (C24/Ler), 162 (C24/Col), and 138 (Col/Ler) Gene Ontology (GO) terms in the biological process category (hypergeometric test, $P < 0.05$; Benjamini–Yekutieli FDR < 0.1; Dataset S1, Table S3*A*). We used REViGO (reduce and visualize gene ontology) (9) to cluster functional and semantic redundancies across these lists of significantly overrepresented GO terms (Dataset S1, Table S3*B*). The resulting amalgamation produced a simplified list of between 18–43 GO biological process terms associated with the differentially expressed genes in the hybrids (Table 1). Responses to abiotic stimuli and stresses and biotic defense responses were the most prominent and significantly overrepresented biological processes altered in the hybrids. Collectively, these two processes accounted for more than 50% of the GO biological process terms identified in the up-regulated genes and 78% of the GO biological process terms identified in the down-regulated genes in the three hybrids (Table 1). A similar association is seen in the initial output of GO terms where abiotic stress and biotic defense responses featured frequently among the 25 most significantly overrepresented gene ontologies (on average 14/25 for up-regulated genes and 20/25 for down-regulated genes; Dataset S1, Table S3*A*).

Responses to abiotic stimuli and stresses occurred in all three hybrids in both up- and down-regulated genes, but more so in the former, accounting for between 18% and 35% of the identified biological processes (Table 1). The more significantly overrepresented GO terms within the abiotic stimuli and stress grouping are associated with responses to changes in carbohydrate levels, mitigation of stress imposed metabolic changes, control of circadian rhythms, and sensing of nutrient status (Fig. 1*A*; Dataset S1, Table S3*A*). Enrichment of the terms associated with these processes is consistent with the hybrids showing greater energy production and a broader tolerance to environmental conditions than parental lines (10). Among the up-regulated genes with known roles in plant growth included key regulators of the circadian clock, such as *TOC1* (*TIMING OF CAB EXPRESSION 1*), *GI* (*GIGANTEA*), *PCL1* (*PHYTOCLOCK1*) and *PRR5* (*PSEUDO-RESPONSE REGULATOR 5*), previously shown to be changed in hybrids and implicated in generating heterosis through alterations to carbohydrate production (3, 11, 12). Other up-regulated genes in all three hybrids included sugar transporters (Fig. 1*B*), which can promote growth when overexpressed (13, 14), as well as *GLUTAMINE-DEPENDENT ASPARAGINE SYNTHASE 1* (*ASN1*), Class II *TREHALOSE-6-PHOSPHATASE SYNTHASE* genes (*TPS8* to *11*), and *LEA*/dehydrin genes (Fig. 1*B*), whose elevated expression supports plant growth under cold, salinity, or drought stress (15–17). The genes responding to nutrient levels and starvation that were down-regulated in all three hybrids are involved in transcriptional responses to *in planta* phosphate and sulfate concentrations (Fig. 1*C*) (18, 19). The down-regulation of these starvation-induced genes to below low-parent levels in the hybrids is a transcriptional state that has been related to greater plant growth (18, 19).

Biological processes related to biotic defense responses were the most prevalent GO terms associated with the differentially expressed genes in the hybrids (Table 1); they accounted for between 58% and 65% of the REViGO summarized terms for the down-regulated genes, with all three hybrids having enrichments in many of the same basal processes (Fig. 1*A*). Included

Table 1. REViGO amalgamated GO biological processes associated with the F1 differentially expressed genes

	C24/Ler		C24/Col		Col/Ler	
Biological processes	Up-regulated	Down-regulated	Up-regulated	Down-regulated	Up-regulated	Down-regulated
Abiotic stress response	8 (22%)	7 (18%)	7 (35%)	8 (19%)	6 (33%)	13 (32%)
Biotic defense response	12 (32%)	24 (60%)	7 (35%)	28 (65%)	0 (0%)	18 (44%)
Stress and defense response	2 (5%)	1 (3%)	3 (15%)	1 (2%)	3 (17%)	1 (2%)
Other	15 (41%)	8 (20%)	3 (15%)	6 (14%)	9 (50%)	9 (22%)
Total no. of terms	37	40	20	43	18	41

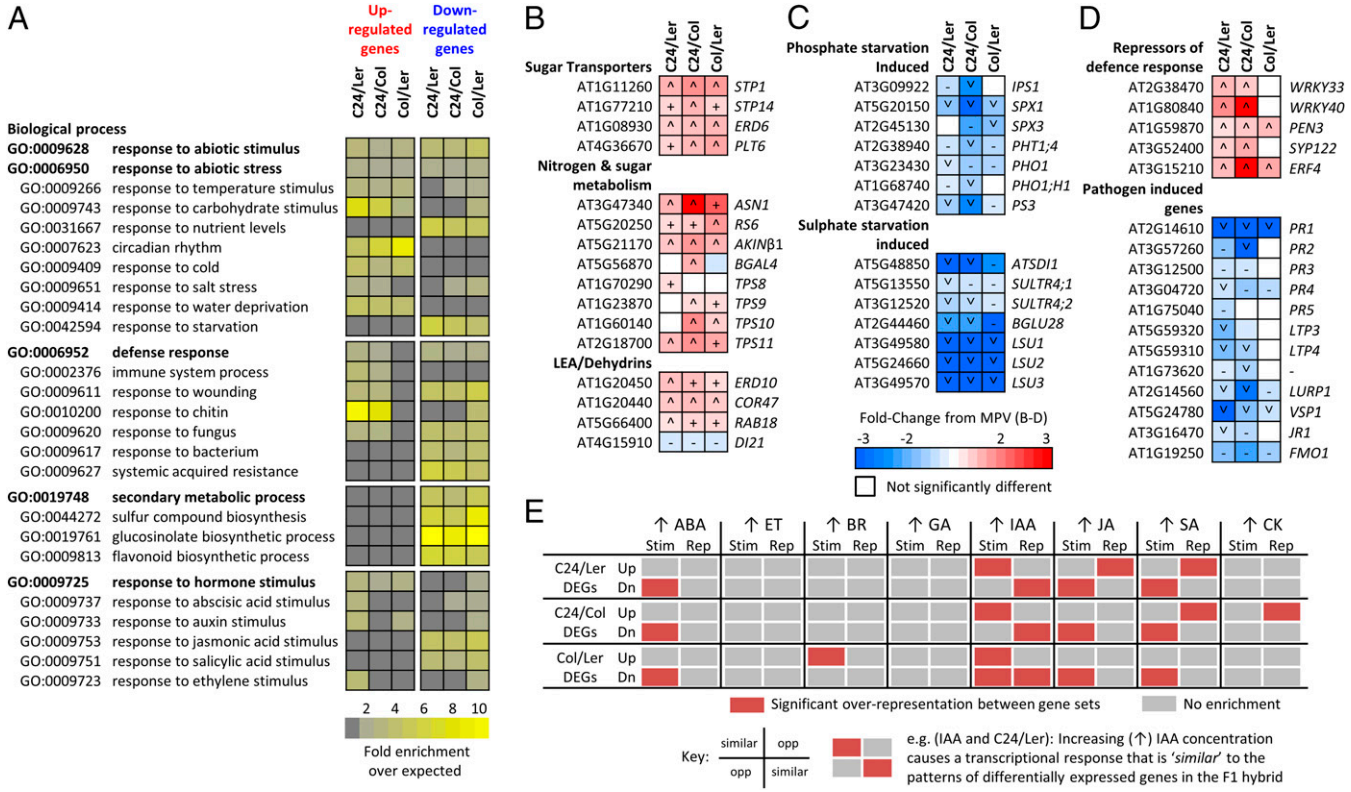

Fig. 1. Biological processes and regulatory networks associated with the differentially expressed genes (DEGs) of the F1 hybrids. (*A*) Prominent GO terms enriched in the up- and down-regulated DEGs of the C24/Ler, C24/Col, and Col/Ler hybrids ($P \leq 0.01$; FDR ≤ 0.1; for full GO listing, see Dataset S1, Table S3*A*). The intensity of the yellow shading indicates the relative fold-enrichment over the number of entries expected by chance. (*B–D*) Examples of differentially expressed genes within the abiotic stress and abiotic stimuli responses (*B* and *C*) and defense response GO categories (*D*). The genes are categorized into subprocesses: red shading, up-regulation; blue shading, down-regulation with the relative fold-change in expression specified by the shading intensity. Expression level changes in the hybrid relative to parental levels are also indicated: ^, up-regulated above high parent; ˅, down-regulated below low parent; +, up-regulated to high-parent level; –, down-regulated to low-parent level; shaded with but with no symbol are between MPV and one parent. (*E*) Identification of altered hormone-regulated networks in the hybrids. Identifying overrepresentations (Fisher's $P < 0.001$) of hormone-regulated genes (AtGenExpress, columns) among the DEGs of the F1 hybrids (rows). Up, up-regulated; Dn, down-regulated genes of the F1 hybrids; genes stimulated (Stim) or repressed (Rep) by increases (↑) in hormone concentration through exogenous application; ABA, abscisic acid; ET, ethylene; BR, brassinosteroid; GA, gibberellin; IAA, auxin; JA, jasmonic acid; SA, salicylic acid; CK, cytokinin. Each overrepresentation analysis between a hybrid and a given hormone response expression profile is a four-way comparison. An example of how to interpret the results is provided below the matrix.

among the down-regulated defense response genes are several known to be induced by pathogen attack (Fig. 1*D*), including *PATHOGENESIS-RELATED GENE 1* to *5* (*PR1* to *PR5*), *LIPID TRANSFER PROTEIN 3* and *4* (*LTP3* and *4*), *LATE UP-REGULATED IN RESPONSE TO HYALOPERONOSPORA PARASITICA* (*LURP1*), *VEGETATIVE STORAGE PROTEIN 1* (*VSP1*), *JASMONATE RESPONSIVE 1* (*JR1*), and *FLAVIN-DEPENDENT MONOOXYGENASE 1* (*FMO1*). Defense response genes were overrepresented in the up-regulated genes of only the C24/Ler and C24/Col hybrids (Table 1; Fig. 1*A*) and included genes known to function as repressors of plant defense, including *WRKY33*, *WRK40*, *PENETRATION 3* (*PEN3*), *SYNTAXIN OF PLANTS 122* (*SYP122*), and *ETHYLENE RESPONSIVE ELEMENT BINDING FACTOR 4* (*ERF4*) (Fig. 1*D*).

In the *Brassicaceae,* pathogen defense responses are frequently associated with the production of secondary metabolites such as indole glucosinolates, camalexin, and flavonoids. Genes involved in processes associated with the biosynthesis of these compounds are overrepresented among the down-regulated genes in all three hybrids (Fig. 1*A*).

Changes to biotic defense responses were the principal themes associated with the transcriptional changes occurring in the F1 hybrids. The patterns of altered defense-related gene expression imply that the hybrids have a decreased basal defense response, this being more pronounced in the C24/Ler and C24/Col hybrids. Given the antagonistic relationship between plant immunity and plant growth (7, 8), the lower level of defense-related metabolism could be significant for generating the greater growth of the hybrids.

The F1 Hybrids Show Alterations to Hormone-Regulated Genes and Metabolic Pathways Associated with Defense Responses. Hormones are important regulators of plant growth and of responses to pathogen challenges. GO analysis showed that the hybrids have expression changes, both increased and decreased, in genes involved in hormone pathways (Fig. 1*A*). A comparison of the differentially expressed genes in the hybrids against datasets showing changes in gene expression following hormone treatment (AtGenExpress) (20) allowed a more detailed analysis of hormone-regulated genes beyond the limitation of the GO curations (21, 22) and enabled identification of directional responses in gene expression. Subsets of the hormone-regulated genes identified from among the genes differentially expressed in the hybrids were found to respond to more than one hormone (Dataset S1, Table S4). Such cross-talk has the potential to incorrectly implicate changes to a hormonal pathway in the hybrids; to avoid this we used genes principally regulated by one hormone which identified auxin (IAA), jasmonic acid (JA), and SA as the hormone-regulated pathways most likely to be altered in the hybrids (Fig. 1*E*). The data showed that increased IAA

concentration stimulates genes that are overrepresented among the up-regulated genes in each of the three F1 hybrids (Fig. 1*E*). Conversely, there is an overrepresentation of genes repressed by IAA in the down-regulated genes of each hybrid (Fig. 1*E*). This enrichment pattern suggests all three hybrids have an increased auxin transcriptional response. In the case of SA, the C24/Ler and C24/Col hybrids show an enrichment pattern, suggesting that these two hybrids have a decreased SA transcriptional response (Fig. 1*E*). For JA, only C24/Ler indicated a reduction in JA transcriptional response (Fig. 1*E*). Similar conclusions regarding changes to the IAA, SA, and JA networks applied in an additional 13 other array datasets tested against the C24/Ler hybrid (Fig. S2).

Genes differentially expressed among the C24, Ler, and Col parents show that the processes altered in the hybrids are differently emphasized in the parental lines (Fig. S3*A*). SA levels and biotic defense responses are high in C24, and associate with genes expressed at lower levels in Col and Ler. Genes associated with flavonoid biosynthesis are highest in Ler, and those involved in indole-derived defense compounds and auxin metabolism are lowest in Col.

The Altered Auxin Response Pathway in the Hybrids. Changes in the auxin transcriptional response pathway include genes at the level of auxin biosynthesis. The majority of the genes were down-regulated in the hybrids (Fig. 2 *A* and *B*), which appears to contradict the increased auxin response expression profile seen in the three hybrids; this is not the case when the roles of these genes are considered. Auxin is derived mainly from tryptophan through several key intermediates (Fig. 2*B*) (23). The genes in the main biosynthetic pathway involving indole-3-pyruvic acid (24) were not altered in the three hybrids (Fig. 2 *A* and *B*). The changes occurred predominantly in the indole-3-acetaldoxime (IAOX) pathway, which is specific to the Brassicaceae. In addition to producing IAA, this pathway is responsible for the biosynthesis of the antimicrobial compounds, indole glucosinolates, and camalexin (23). Genes leading to the production of these secondary metabolite defense compounds were down-regulated in all three hybrids (Fig. 2 *A* and *B*). Also down-regulated were the *NITRILASE* genes (*NIT1*,*2*,*3*), which produce IAA from indole-3-acetonitrile (IAN; Fig. 2 *A* and *B*). Blocking production of indole-derived secondary metabolites leads to elevated levels of IAA via an accumulation of IAOX and subsequently an increased flow through the IAOX → indole-3-acetamide (IAM) → IAA route (25). Consistent with this occurring in the hybrids, *AMIDASE 1* (*AMI1*) was up-regulated in each hybrid (Fig. 2 *A* and *B*). In support of our findings, we identified similar alterations to the auxin biosynthesis pathway in an independent C24/Ler hybrid dataset (Fig. S3*B*; GSE34655; *Materials and Methods*) (26). The results imply that IAA production via the IAM route is enhanced in the hybrids as a consequence of decreased production of defense compounds, which relates to the increased auxin response in the hybrids.

In addition to changes in auxin biosynthesis, alterations to auxin response may come about by changes in auxin transport that affect localized auxin concentrations. Flavonoids are repressors of auxin transport (27, 28), and all three hybrids showed a down-regulation of genes involved in flavonoid biosynthesis (Fig. 2 *C* and *D*), implying a reduction in flavonoid levels in the hybrids and presumably increased rates of IAA transport compared with the parental lines; the latter is confirmed for C24/Ler (26). The down-regulation of flavonoid biosynthesis has broad

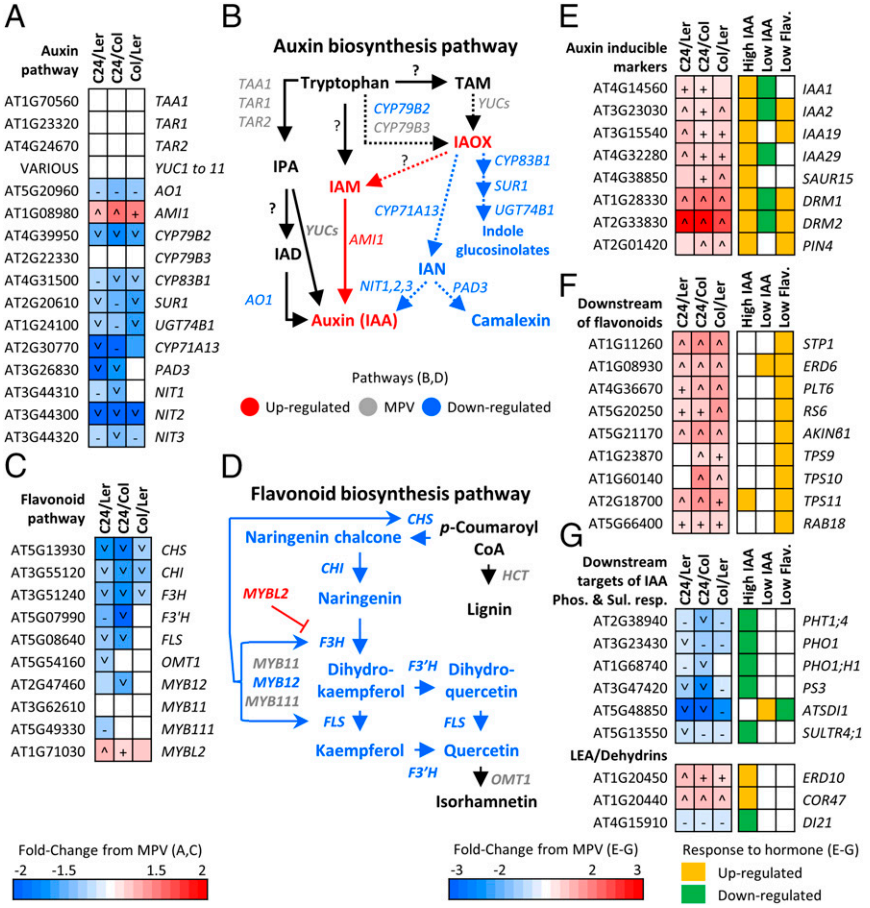

Fig. 2. Changes to the auxin and flavonoid pathways in the F1 hybrids. (*A*) Altered expression patterns of genes in the auxin biosynthesis pathway. (*B*) The auxin biosynthesis pathway showing routes for production of bioactive auxin (IAA) and secondary metabolites indole glucosinolates and camalexin. Gene names are color-coded to represent the change in expression between the hybrids, as are the biosynthetic products to reflect their expected change in levels. (*C*) Differential expression patterns of genes in the flavonoid biosynthesis pathway. (*D*) The flavonoid biosynthesis pathway. (*E* –*G*) Examples of auxin and flavonoid (*E*), flavonoid alone (*F*), and auxin alone (*G*) regulated genes that are differentially expressed in the hybrids. First three columns from the left show levels of differential expression in the hybrids; the next three columns show change in expression levels of the given gene in response to changes in auxin or flavonoid concentrations derived from publically available datasets (Fig. S2). Expression level changes in the hybrid relative to parental levels: ^, up-regulated above high parent; ˅, down-regulated below low parent, +, up-regulated to high-parent level; –, down-regulated to low-parent level; shaded with but with no symbol are between MPV and one parent.

impacts on the transcriptomes of all three hybrids as indicated by the overrepresentation of flavonoid-regulated genes in an enrichment pattern consistent with a reduction in flavonoid levels (Fig. S4*A*). These effects of reduced flavonoid production on the hybrid transcriptome appear to be partly mediated through changes to localized IAA concentrations, as indicated by over half (~58%) of the flavonoid-regulated F1 differentially expressed genes also being regulated by auxin (Fig. S4*B*). Included among these coregulated genes are those highly responsive to changes in auxin concentration (i.e., typical auxin-inducible target genes). The activities of these genes in the hybrid matched changes in expression induced in nonhybrid parents by IAA application and flavonoid deficiency (Fig. 2*E*) and reflected the state of increased auxin response occurring in all three hybrids. Among the auxin-independent flavonoid-responsive genes were those involved in carbohydrate metabolism, transport, and responses to abiotic stresses identified in the GO analysis (Fig. 2*F*). However, despite influencing auxin transport, changes in flavonoid biosynthesis only accounted for ~28% of the auxin-regulated F1 differentially expressed genes (Fig. S4*B*), implying that the changes to auxin biosynthesis were the major influence altering auxin response in the hybrids. Among the F1 differentially expressed genes regulated by auxin but not flavonoids are the nutrient- and stress-responsive sulfur and phosphate starvation-responsive genes and *LEA*/dehydrin genes identified in the GO analysis; their patterns of differential expression in the hybrids matched the directional change in expression when auxin levels were experimentally increased in the nonhybrid parent (Fig. 2*G*).

The Altered Salicylic Acid Response in the Hybrids. As observed in the auxin pathway, altered SA response in the hybrids involved changes in gene expression at the level of hormone biosynthesis, but are limited to the C24/Ler and C24/Col hybrids. Of the two pathways of SA biosynthesis, the isochorismate (IC) pathway is the predominant route for both basal and pathogen induced SA production (29). Both *ISOCHORISMATE SYNTHASE 1* (*ICS1*) and *ICS2* were down-regulated in C24/Ler and C24/Col (Fig. 3 *A* and *B*). In the later steps of the IC pathway, *AVRPPHB SUSCEPTIBLE 3* (*PBS3*) is crucial for accumulation of SA (30) and was also down-regulated in the C24/Ler and C24/Col hybrids (Fig. 3 *A* and *B*). A small proportion of SA is produced through an alternate pathway involving four *PHENYLALANINE AMMONIA-LYASE* (*PAL*) genes (31). In all three hybrids, only *PAL1* showed changes in expression with levels at or below the low parent (Fig. 3 *A* and *B*). The data suggest that the reduced SA transcriptome response of C24/Ler and C24/Col hybrids occurs through reduced SA production. In support of our findings, a similar repression of SA biosynthesis genes was observed in the GSE34655 C24/Ler dataset (Fig. S3*B*).

In addition to the core SA biosynthesis genes, upstream regulators of SA biosynthesis showed repressors being up-regulated and activators down-regulated in C24/Ler and C24/Col, but not in Col/Ler (Fig. 3*C*). There is no difference in the expression of genes whose products directly perceive SA (Fig. 3*C*). Of the many downstream genes regulated by SA most were differentially expressed in the C24/Ler and C24/Col hybrids (Fig. 3 *D* and *E*). Of those that were differentially expressed in the Col/Ler hybrid, some showed an opposite pattern to those in the C24 hybrids (Fig. 3 *D* and *E*), including *ACCELERATED CELL DEATH 6* (*ACD6*) and *CYSTEINE-RICH RLK 4* (*CRK4*). SA-inducible genes that were down-regulated in the hybrids cover a large spectrum of SA targets ranging from pathogenesis-related genes to *WRKY* transcription factors, SA-dependent activators of cell death, and regulators of cell expansion (Fig. 3 *D* and *E*).

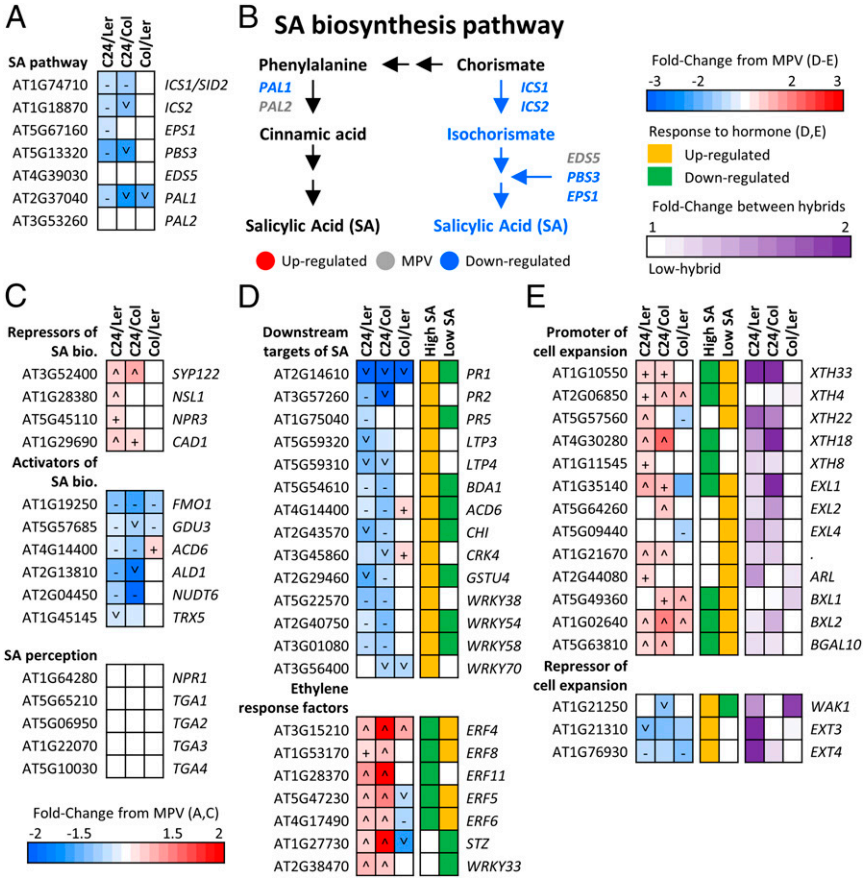

Fig. 3. Changes to the salicylic acid pathway in the F1 hybrids. (*A*) Differential expression patterns of genes in the SA biosynthesis pathway for the individual hybrids. (*B*) SA biosynthesis occurs via two routes, with the isochorismate pathway accounting for vast majority of SA production. Gene names are color-coded, representing the changes in gene expression seen in hybrids having C24 as a parent, as are the biosynthetic products to reflect their expected change in levels. (*C*) Differential expression of upstream regulators of SA biosynthesis. (*D* and *E*) Examples of downstream SA-regulated genes, including genes directly involved in controlling cell expansion. First three columns in *D* and *E* show levels of differential expression in the hybrids; next two columns show change in expression levels of the given gene in response to altered SA concentrations; last three columns in *E* show relative expression levels of cell expansion genes between the three hybrids. Expression level changes in the hybrid relative to parental levels: ^, up-regulated above high parent; ˅, down-regulated below low parent; +, up-regulated to high-parent level; – down-regulated to low-parent level; shaded with but with no symbol are between MPV and one parent.

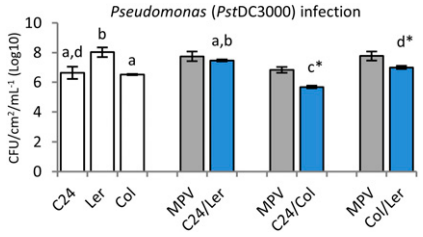

Fig. 4. F1 hybrids are no more susceptible than parental lines to *Pseudomonas syringae* infection. Number of bacteria extracted from leaves of parents and hybrids 3 d postinfection. Mean colony-forming units/cm^2 extracted from three biological replicates, each containing eight leaf discs. Error bars are SEM. Asterisk indicates below MPV (Student's *t* test $P < 0.01$). Letters above columns denote statistical comparisons among the samples, with different letters indicating statistical difference from the other samples (Student's *t* test $P < 0.05$).

Included among SA-repressed genes that were up-regulated in the C24/Ler and C24/Col hybrids are several *ETHYLENE RESPONSE FACTORS* (*ERF*) (Fig. 3*D*) that had changes in expression consistent with increased plant growth. *ERF6*, whose lack of expression results in reduced plant growth (32), activates stress tolerance genes such as *SALT TOLERANCE ZINC FINGER* (*STZ*) and *WRK33* (33), which were also up-regulated in the C24/Ler and C24/Col hybrids (Fig. 3*D*). Col/Ler showed an opposite pattern of expression for these genes (Fig. 3*D*). Increased *ERF4* represses JA signaling independent of changes to JA levels (34) and can promote vegetative growth (35), whereas *ERF11* down-regulates pathogen defense-related genes (36).

A number of SA-regulated genes controlling cell expansion were among the differentially expressed genes of the hybrids (Fig. 3*E*). Promoters of cell expansion were predominantly up-regulated and more highly expressed in C24/Ler and C24/Col compared with Col/Ler (Fig. 3*E*), consistent with these two hybrids showing greater cell expansion than Col/Ler (3).

Are the Hybrids More Susceptible to Pathogen Attack Than Parental Lines? The reduced basal level of defense gene expression that could be promoting the greater growth of the hybrids could also lead to the hybrids being more susceptible to biotic stress than their parental lines. We examined the hybrids for an altered response to *Pseudomonas syringae*, because infection by this bacterial pathogen is greater when SA response is reduced and IAA levels are increased (37). We found that following *P. syringae* infection, the hybrids had elevated expression of *BDA1* and *WRKY46* (two genes known to be induced by *P. syringae* infection; Fig. S4*C*) and accumulated fewer bacteria than their more susceptible parent. The levels of infection in C24/Col and Col/Ler were similar to the more resistant parent and for C24/Ler equivalent to both parents (Fig. 4). The results suggest that although there was a reduction in the basal level of defense response gene expression, the hybrids were not compromised in their inducible response and ability to resist at least this biotic challenge.

Changes to the IAA, Flavonoid, and SA Pathways Correlated with the Development of Heterotic Increases in Leaf Cell Size and Cell Number. Changes to the IAA, flavonoid, and SA biosynthesis pathways were examined in an additional set of hybrids, generated by crossing the Wassilewskija (Ws) accession with C24, Ler, or Col accessions (3). Quantitative RT-PCR (qRT-PCR) analysis of key biosynthetic and response genes indicated that glucosinolate, camalexin, and flavonoid biosynthesis were down-regulated in all of the Ws hybrids (Fig. S5*A*). Increases in expression of the IAA biosynthesis gene *AMI1* occurred in Ws×Ler and Ws×Col, but not in C24×Ws hybrids (Fig. S5*A*), which had a reduction in SA biosynthesis and responses (Fig. S5*A*).

The changes in IAA, flavonoid, and SA responses across the six hybrids between C24, Ler, Col, and Ws were compared against the heterotic phenotypes of increased leaf cell size and cell number previously characterized for these hybrids (3). A greater IAA response occurred in those hybrids showing increased leaf cell number, whereas a reduced SA response was associated with larger photosynthetic leaf cell size promoted by the C24 parent and is associated with the larger sized hybrids (Fig. 5).

Changing *in Planta* SA Concentrations Affected the Growth of Parents and Levels of Heterosis in Hybrids. We decided to focus on the altered SA response in the hybrids because the changes to this pathway correlate with increased leaf cell expansion and the larger hybrids. In another set of sowings (independent of those used for the mRNA-seq data), qRT-PCR showed expression levels of the SA biosynthesis genes *ICS1* and *PBS3*, along with SA downstream markers *BDA1*, *PR1*, and *PR2*, were high in C24 and decreased in the hybrids having C24 as a parent (Fig. S5*A*), as seen in our RNA-seq data. Consistent with our mRNA-seq transcriptome profiles, LC-MS hormone quantification of these plants revealed that C24 has substantially higher endogenous levels of SA than the other parental lines (Fig. 6*A*). Hybrids involving C24 showed concentrations of SA reduced below MPV and equivalent to the non-C24 hybrids (Fig. 6*A*). The reduced SA concentrations and SA responses in the C24 hybrids were present by 7 DAS (Fig. S5*B*), suggesting that the SA decrease occurred early in development. Differences between the measured vs. expected midparent SA concentrations in the hybrids at 15 DAS correlated well with heterosis values for mature (~45 DAS) rosette size (Fig. 6*B*), with the differences in SA concentrations between parents at 15 DAS possibly serving as a predictor for maximum vegetative heterosis (Fig. 6*C*). In determining the concentrations of SA, the extraction procedure also allowed abscisic acid (ABA) and JA concentrations to be assayed. No difference was observed in the levels of these two hormones in the hybrids (Fig. S6*A*) in agreement with our transcriptome profiles that indicated no change in the biosynthesis of these hormones.

We explored the relationship between SA concentration and heterosis by manipulating *in planta* SA. Increased SA concentration and stimulation of the SA response pathway were achieved by either a foliar spray of a SA mimic [BTH (benzothiadiazole), which has a superior dissolving capability and

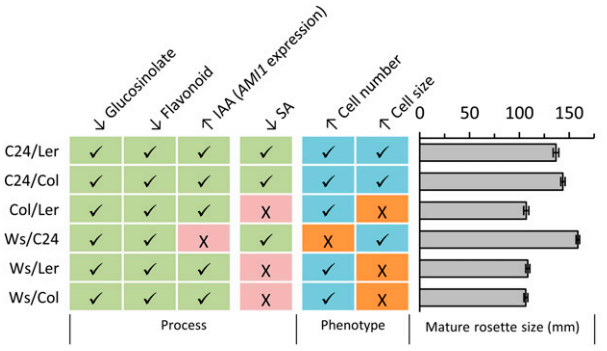

Fig. 5. Changes to specific hormone-regulated pathways in the hybrids coincide with the heterotic traits of increased leaf cell size and leaf cell number in the hybrids. *AMI1* expression (increased auxin levels) correlates with cell number in all of the six combinations of hybrid offspring. Decreased SA production and response occurs in hybrids showing increases in cell size and is associated with the larger rosette diameter of the hybrids. Transcriptome profiles (this study); cell size and cell number phenotypes (3).

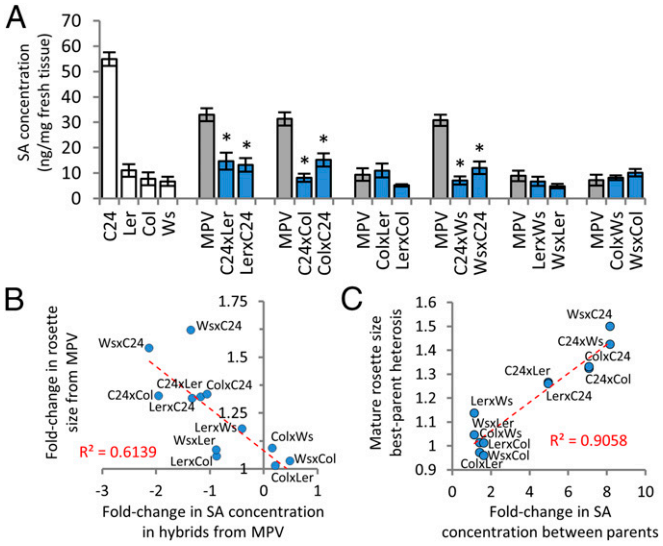

Fig. 6. Endogenous SA levels in parents and hybrids correlate with levels of heterosis. (*A*) LC-MS quantification of endogenous SA concentrations in aerial tissue of 15-d-old parental and hybrid lines. Asterisk indicates below MPV (Student's *t* test $P < 0.01$); $n = 5$ biological replicates consisting of pools of 10 seedlings. Error bars are SEM. (*B*) Differences from midparent level of SA at 15 DAS correlate with deviations in mature rosette size from the midparent value (at ~45 DAS) for the reciprocal hybrid combinations. (*C*) Differences in SA levels between parents at 15 DAS correlates strongly with the level of better-parent heterosis for mature rosette size (at ~45 DAS).

higher absorption efficiency through the leaves than SA] on soil grown plants (commencing at 14 DAS and assessed at 25 DAS) or through the addition of SA to the agar growth medium (from germination to assessment at 15 DAS). In both the SA- and BTH-treated plants, the *PR1* SA-responsive target gene was up-regulated (Fig. S6*B*), SA-responsive cell expansion genes were down-regulated (Fig. S6*B*), and rosette growth was reduced in both parents and hybrids (Fig. 7 *A* and *B*). Both assays yielded similar patterns of altered growth levels. The reduction in rosette size was most apparent in the C24/Ler hybrid (suppressed SA network), whereas the Col/Ler hybrid (unaltered SA network) was the least sensitive of the lines (Fig. 7 *C* and *D*). The elevated SA levels caused a greater reduction in the growth of the C24/Ler hybrid compared with its parents (Fig. 7*B*), leading to a reduction in the level of heterosis under high SA conditions (Fig. 7*E*). Col/Ler showed a proportionally lower level of reduced growth compared with its parents (Fig. 7*D*), resulting in a higher level of heterosis under elevated SA conditions (Fig. 7*F*). These results agree with a decreased SA level and transcriptional response contributing to the greater growth of hybrids involving C24, and support the suggestion that the SA pathway is not involved in the heterotic growth of the Col/Ler hybrid.

We introduced the *Pseudomonas NahG* gene, which catabolizes salicylic acid to catechol (38), into the parental lines with the expectation that by mimicking the reduced SA state of the hybrids, we could phenocopy the increased vegetative biomass of the hybrids. *PR1* was down-regulated in the *NahG* transgenic plants indicating reduced SA levels (Fig. S7*A*). Among the parental lines, growth enhancement in response to *NahG* activity correlated with the basal level of SA in each parent, with C24 having the largest response and Col the least. By 35 DAS, C24-*NahG* plants had rosettes 23% larger with 43% increase in fresh weight over wild-type C24 (Fig. 8 *A*–*C*) and were phenotypically more comparable to the nontransgenic C24 hybrids than to the nontransgenic parental lines. C24-*NahG* plants produced more seed than wild-type C24 (Fig. S7*B*). The growth enhancement was established early in the life cycle with the C24-*NahG* seedling larger than wild-type C24 at 18 d (Fig. 8*B*), the larger leaves a result of increased cell size (Fig. 8*D*), as seen in the C24 hybrids. Genes regulating cell expansion that responded to SA application and are differentially expressed in the C24 hybrids show different levels of expression in C24-*NahG* compared with wild-type C24. *TCH4* (*TOUCH 4*/*XTH22*), a promoter of cell expansion is higher in C24-*NahG*, whereas *EXT3* (*EXTENSIN 3*), a repressor of cell expansion, is lower in C24-*NahG* compared with wild-type C24 (Fig. 8*E*).

Ler-*NahG* plants showed smaller increases in vegetative growth over wild-type counterparts, with 16% increase in rosette size and 33% in aerial biomass by 35 DAS (Fig. 8 *A*–*C*). Under our conditions, there was no effect of the *NahG* transgene on the vegetative growth of Col (Fig. 8 *A*–*C*), but there was an increase in seed yield (Fig. S7*B*).

The *NahG* gene was introduced into C24/Col (decreased SA response hybrid) and Col/Ler (unaltered SA response hybrid) hybrids. *NahG* activity was found to promote substantial increases in vegetative biomass and size in the C24/Col hybrid (Fig. 8*F*), and a significant, but more modest, increase in vegetative growth of the non-C24 (Col/Ler) hybrid (Fig. 8*F*).

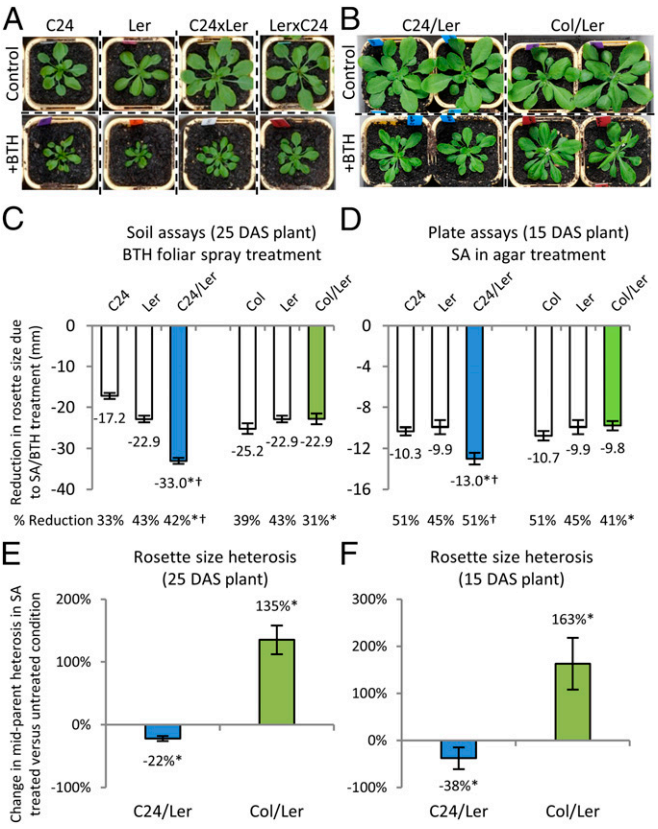

Fig. 7. Effects of increasing SA concentrations and signaling on vegetative heterosis. (*A*) Example of the greater reduction in rosette size of C24/Ler compared with parental lines. (*B*) An example showing Col/Ler is less sensitive to BTH/SA treatment than C24/Ler. (*C* and *D*) Absolute reductions in rosette size caused by BTH or SA treatments. Percentages below bars represent the relative decrease; note that hybrids are larger than parents under control conditions when interpreting percentage decrease. Asterisk indicates different from MPV (Student's *t* test $P < 0.01$). †, C24/Ler different from Col/Ler (Student's *t* test, $P < 0.01$). (*E* and *F*) Changes in the levels of heterosis in response to salicylic acid (or BTH) treatment; standardized to untreated control conditions. Asterisk indicates different from untreated control (Student's *t* test $P < 0.01$). For both BTH and SA assays, n = parental lines (~40), hybrid lines (~50). Error bars are SEM.

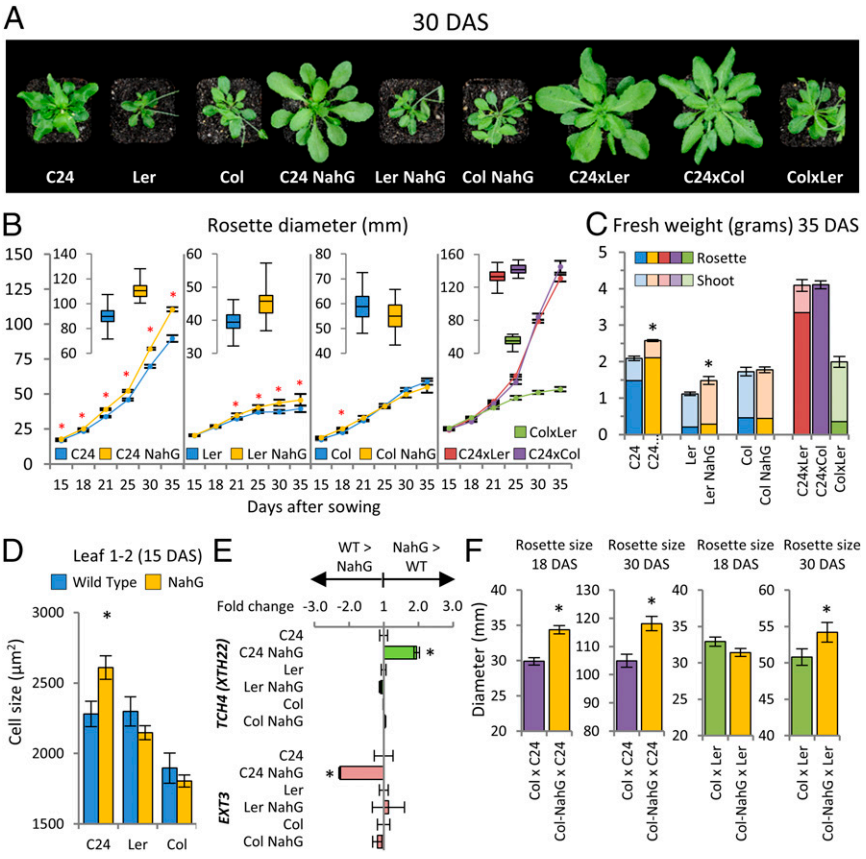

Fig. 8. Increases in plant vigor caused by the expression of the SA-catabolizing enzyme *NahG*. (*A*) Representative wild-type parental and hybrid lines and *NahG* transgenic parental lines at 30 d after sowing. (*B*) Rosette size of wild-type and *NahG* transgenic plants at different time points in development; $n = 12–17$ plants. Box and whisker plot inset shows spread of rosette sizes at 35 d. There was no difference in the flowering times between wild-type and *NahG* parental lines (Dataset S1, Table S5). (*C*) Aerial fresh weight at 35 d after sowing. Error bars represent SEM of the total weight comprised of both vegetative and any reproductive structures; $n = 12–17$ plants. (*D*) Palisade mesophyll cell size of the larger of the first two leaves at 15 d after sowing; $n = 10–14$. (*E*) qRT-PCR mRNA expression levels of SA-regulated genes in 15 DAS seedlings that promote cell expansion (*XTH22*; green) and repress cell expansion (*EXT3*; red). *XTH4* and *XTH33* levels were no different between C24 wild-type and C24 *NahG*. (*F*) Rosette size of wild-type and *NahG* hybrid lines at 18 and 30 d after sowing. All error bars represent SEM. Asterisks represent significant difference between *NahG* transgenic and wild-type line (Student's *t* test, $P < 0.05$).

Although we cannot exclude potential effects of the catechol by-product of NahG-mediated SA degradation, the phenotypic effects seen in the *NahG* transgenic plants of increased rosette biomass through greater cell expansion and increased seed yields are known to be directly associated with reduced SA levels in *Arabidopsis* (39–42). NahG-generated catechol may not accumulate to high levels (43), which in any case would cause growth inhibition (44) and not the growth enhancement observed.

Discussion

To understand the transcriptional changes responsible for heterosis, we examined a series of *Arabidopsis* F1 hybrids that show differences in their levels of hybrid vigor. Hybridization of the parental accessions resulted in substantial transcriptional reprogramming with 6–8% of genes showing altered expression levels in the hybrid. Almost half of these genes were common to at least two of the hybrids we studied. An analysis that discounted redundancies among the biological process GO terms showed a preponderance of defense- and stress-related processes associated with the differentially expressed genes in all three hybrids. Though changes to abiotic stress response genes were common to all three of the hybrids, the changes in defense response gene expression were more prominent in hybrids having C24 as a parent.

Our analysis of genes with altered activities in the hybrids suggests an improved tolerance to abiotic stresses and a suppression of genes associated with basal defense levels. Changes in stress response gene expression may be linked to the increased growth performance of *Arabidopsis*, crop, and wild species hybrids under a range of environmental conditions (10, 45). A lower level of defense-related metabolism could be significant in generating the greater growth of the hybrids, given the negative tradeoffs between defense robustness and plant growth and yield (7, 8).

Among the defense and stress-related differentially expressed genes of the hybrids were those encoding enzymes in the biosynthetic pathways of SA and auxin together with downstream genes regulated by these two hormones. These altered gene activities are of potential importance in generating the heterotic phenotype given that SA and auxin are key regulators of plant defense responses and are also major controllers of plant structure and growth (37, 42, 46). We found that the reprogramming of the IAA pathway (occurring in all except the C24/Ws hybrid) and the SA pathway (occurring in hybrids having C24 as a parent) was correlated with increased leaf cell number and leaf cell expansion, respectively. This relationship is consistent with the known functions of these two hormones, with auxin controlling leaf size through cell proliferation (46) and lower SA concentrations increasing leaf size through greater cell expansion (41, 42). Heterosis in leaf cell size and leaf cell number occurs throughout the life history of the *Arabidopsis* hybrid, being identifiable in early development and contributing to a greater photosynthetic capacity of the hybrids relative to the parents (3, 47). These morphological and anatomical characteristics of the hybrid may be an important prerequisite for hybrid vigor, with the differences in the levels of vegetative heterosis between hybrids related to the different exploitation of the SA and IAA pathways.

The reduced SA-related gene activities in the C24 hybrids were found to be associated with a decrease in SA concentration. The decrease in SA concentration between hybrids was found to correlate with mature rosette size. Similarly, SA levels are negatively correlated with plant biomass in C24/Col recombinant inbred lines (48), providing support for the concept that a reduction in SA concentration and associated changes in gene expression levels in the F1 contribute to increased vegetative plant biomass. Hybrids with C24 as a parent presented a special

situation among the hybrids in this study, because the C24 accession has substantially higher concentration of SA than the other parental accessions, yet its growth is not reduced relative to the other parental accessions. We assume that C24 must have genes that promote high SA concentrations and others that confer a tolerance to high SA levels in terms of growth. Hybridization with Ler, Col, or Ws represses the high SA-producing alleles of C24, allowing increased activity of the C24 SA-tolerant growth-related alleles in C24 hybrids. We were able to mimic the effect by introducing the *NahG* gene into C24, resulting in hybrid-like increases in biomass and seed yields. Although we only observed these SA-related changes in relation to C24, substantial natural variation for SA response occurs across *Arabidopsis* accessions (49–51), and it is likely that other hybrid combinations will show decreased SA production/response to produce heterotic hybrids. One example is the Est-1 accession, which has elevated levels of SA, shows greater growth when constitutively expressing *NahG*, and produces highly heterotic hybrids when in combination with a low SA accession such as Col or Ler (52, 53).

Greater leaf growth of hybrids in other species is mainly due to increased cell numbers rather than cell expansion (10, 54). Leaf heterosis in maize occurs through increased cell numbers linked with changes in auxin response (55, 56). Altered auxin responses are also observed in rice hybrids (57). Although changes in auxin response are common, the molecular triggers appear to differ between hybrid systems. Micro-RNAs and other small RNAs are linked with changes to the IAA network in hybrid rice (57), whereas in maize hybrids, allele variants of the auxin-regulated transcription factor *ZmARGOS1* are contributors (55). In the *Arabidopsis* hybrids, the increased auxin response mainly involves changes at the level of auxin biosynthesis involving repression of genes involved in producing the defense compounds, indole glucosinolates, and camalexin. Changes in flavonoid biosynthesis also contribute because flavonoids are negative regulators of polar auxin transport (27, 28). Even moderate decreases in flavonoid biosynthesis gene expression improve plant growth (27). We found differentially expressed genes in the hybrids included those reported to be important for regulating cold, drought, and macronutrient stress responses that could be related to the changes in auxin and flavonoid biosynthesis. Cross-talk between networks could explain changes in stress response genes in the hybrids despite the absence of an abiotic stimulus. The changes to the IAA pathway may explain the residual heterosis in the SA-treated C24 hybrid and why C24-*NahG* cannot fully mimic the C24 hybrid phenotype. The increased cell proliferation promoted by an increased auxin response provides an obvious growth-promoting complement to the increased cell expansion stimulated by the decrease in SA, and may explain why the larger hybrids are those with changes to both hormone pathways.

Altered defense and stress response gene activities have also been observed in *Arabidopsis* allotetraploids (58), hybrids in crop species including rice, maize, and wheat (59–62), and even in natural populations of hybrids (63, 64). This seemingly general outcome of hybridization suggests that our findings of heterosis in *Arabidopsis* hybrids centering on shifts in the tradeoff relationship between growth processes and defense response could apply to hybrids in other species. In our hybrids, the factors responsible for the lower basal level of defense response gene expression did not compromise the inducible immune response of the hybrids to at least *Pseudomonas syringae* infection; but this may not always be the case because changes in immunity have been reported for hybrids across a number of species, some showing improvements, and others being more susceptible to attack (64, 65). *Arabidopsis* hybrids between the Col and Sei accessions show hyperresistance to *Pseudomonas* and elevated levels of defense response gene expression and SA concentrations (66). Consistent with our findings, the Col/Sei hybrid does not show heterosis for rosette biomass.

Elevated SA response, changes in auxin concentrations, increased defense gene expression, and reduced growth parameters all occur in a less frequent hybridization outcome termed hybrid weakness (45, 52, 67, 68), and at its extreme, hybrid incompatibility (69), providing additional support to the competitive interaction of defense pathways and growth regulation determining levels of heterosis. Hybrid vigor and hybrid weakness may represent opposite outcomes of changes to SA- and IAA-regulated pathways that are determined by interactions between defense and stress response allele and epiallele combinations provided through the cross to produce the hybrids.

In conclusion, our work suggests that a suppression of defense response gene activities are important for generating the hybrid vigor phenotype. Our results with the SA pathway showed that it is possible to change processes identified as altered in hybrids to generate improved parental lines and perhaps to produce more vigorous hybrids.

Materials and Methods

All F1 hybrid seed were generated through hand pollination as described in ref. 3. All plants in the same experiment were grown under the same conditions in randomized blocks with position rotated daily. Appropriate parental lines were grown alongside F1 hybrids. For transcriptomes, total RNA was isolated from aerial tissues of 15-d-old seedlings, replicates consisted of a pools of 5–15 seedlings harvested at time ZT8 $\pm$ 1.5 (Dataset S1, Table S6). Libraries were sequenced on the Illumina platform. Sequenced reads were mapped to the TAIR10 reference genome using BioKanga (sourceforge.net/projects/biokanga/). Raw and processed reads were deposited in the Gene Expression Omnibus database (accession no. GSE64475). Midparent expression levels were generated for each biological replicate and various pairwise comparison performed using DESEQ (70). Our main transcriptome dataset was derived from a simultaneous sowing of two biological replicates for each of the three parental lines and their six reciprocal hybrid combinations (replicates A and B; Dataset S1, Table S6). To be confident that the transcriptional changes identified in the F1 are an effect of hybridization (thus potentially important in generating the hybrid phenotype) and not an environmental influence over a single sowing, data from another 3–5 replicates of C24, Ler, and their reciprocal F1 hybrids from three additional independent sowings were also examined (replicates C–G; Dataset S1, Table S6). Correlations of individual gene expression levels showed good consistency between the independent C24, Ler, F1 C24/Ler sowings (Fig. S8). GO analysis was performed using singular enrichment analysis in agriGO (71). REViGO (9) was used to generate GO term redundancy clusters. Rosette diameters were measured from images using ImageJ (rsb.info.nih.gov/ij/). Size of palisade mesophyll cells were determined from cleared samples using DIC (differential interference contrast) optics. The midparent values were generated through averaging across ranked parental values. *Pseudomonas* infections were performed as described in ref. 72 using 24 leaves per genotype across eight 10-wk-old plants grown under short-day conditions. LC-MS/MS determination of SA, JA, and ABA *in planta* concentrations was performed as described in ref. 73 with minor modifications (*SI Materials and Methods*). Further details, including methods for identifying hormone-regulated gene networks in the hybrids, SA/BTH application assays, SA reduction through *NahG*, and bioinformatics analysis, can be found in *SI Materials and Methods*.

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