New Phytologist

***New Phytologist* Supporting Information**

Article title: Exploring the metabolic basis of growth/defense tradeoffs in complex environments with *Nicotiana attenuata* plants co-silenced in *NaMYC2a/b* expression

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Article acceptance date: 03 January 2023

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Methods S1 Metabolite extraction and analysis

Table S1 Primers used in this study

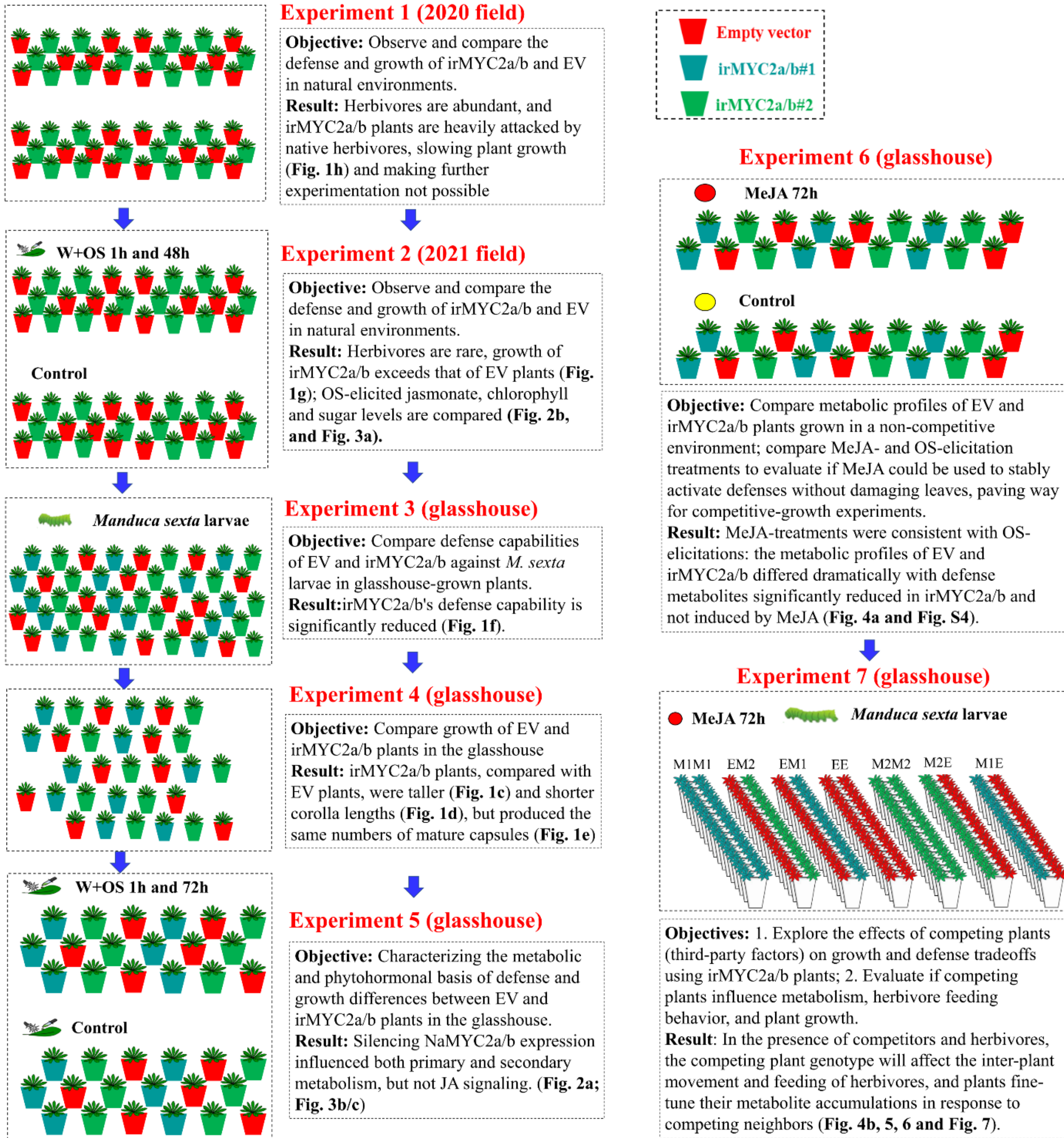


Fig. S1 Experimental design and rationale

Timeline for competitive experiment

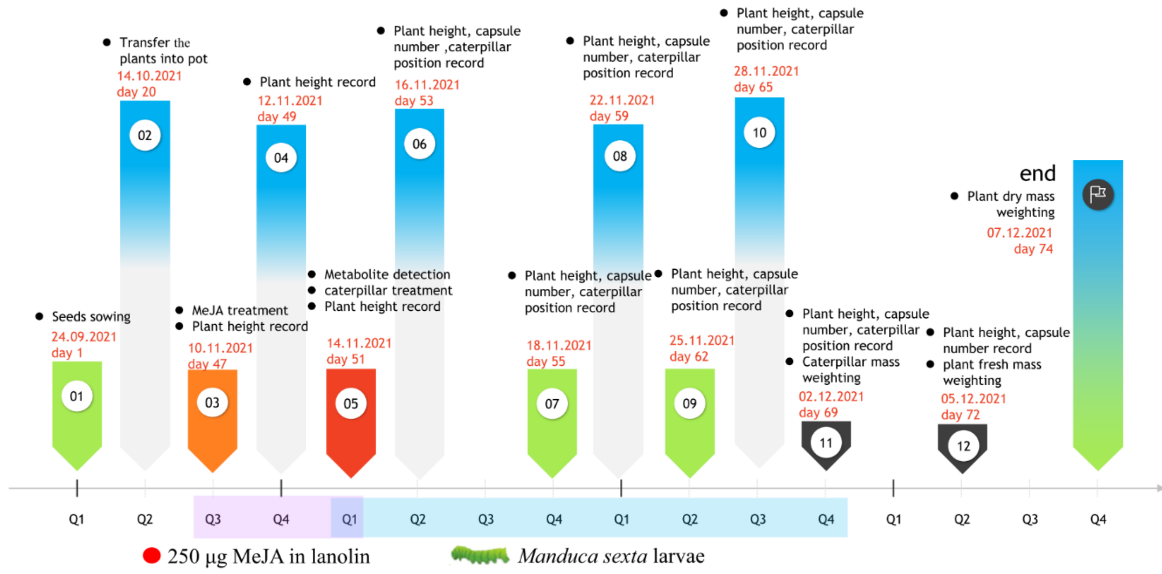


Fig. S2 Timeline of competitive experimental treatments from Fig. 4b, Fig. 5, Fig.6 and Fig.7

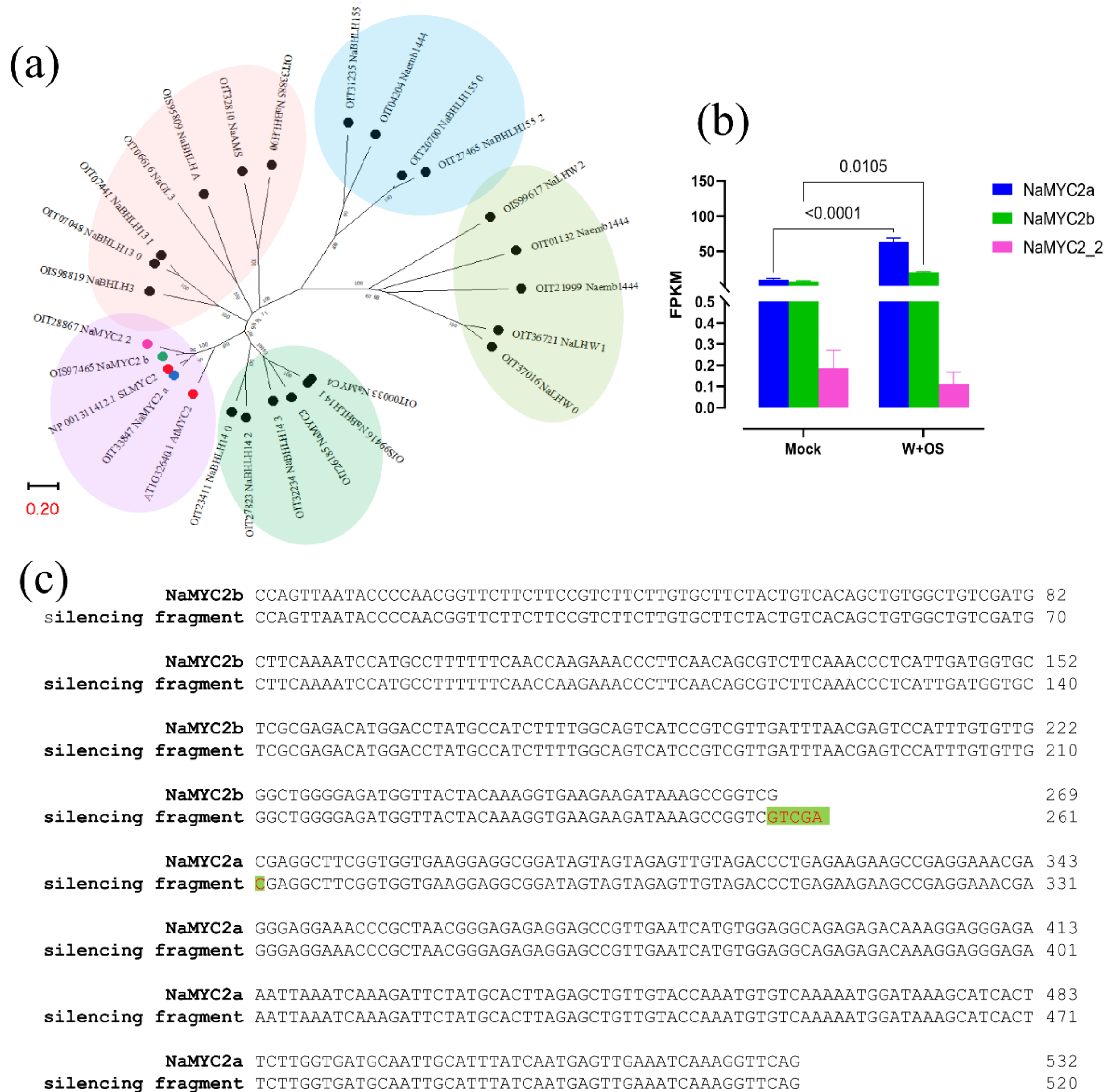


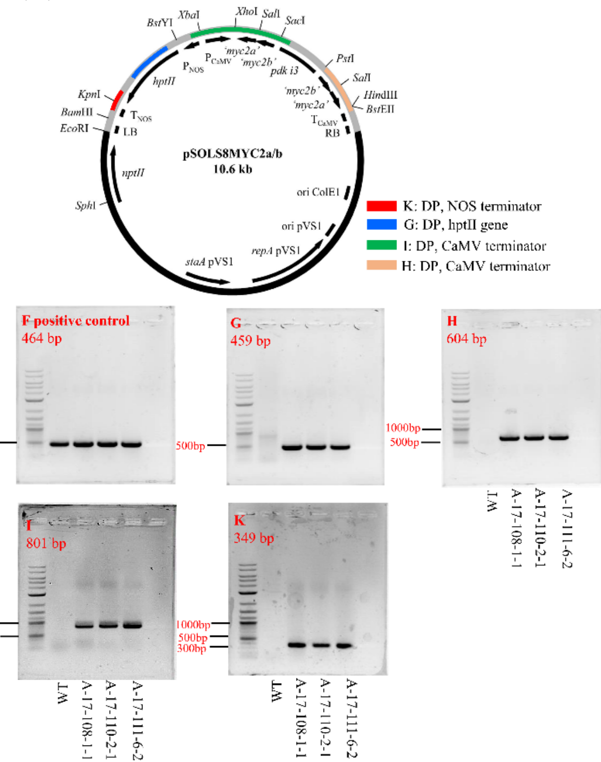
Fig. S3 irMYC2a/b line construction. **(a)** Phylogeny of bHLH-MYC-like family in *N. attenuata*. The consensus tree generated was tested by bootstrapping (1000 times); **(b)** transcript levels of MYC2-likes after W+OS elicitation in EV *N. attenuata* leaves;**(c)**: Sequence fragments of *NaMYC2a* and *NaMYC2b* for RNAi silencing (SalI sites were marked in green).

(a) **Hygromycin screening results of T1, T2, and T3 plants**

line(T1)	Resistant seedlings	Hygromycin sensitive seedlings	not germinated	
A-17-108	35	19	6	58%
A-17-109	39	18	3	65%
A-17-110	43	16	1	72%
A-17-111	46	11	3	77%
A-17-112	40	20	0	67%
A-17-113	50	9	1	83%
A-17-170	0	60	0	0%
A-17-180	45	14	1	75%
A-18-008	41	19	0	68%
A-18-009	43	17	0	72%
A-18-010	0	60	0	0%
WT + hyg	30	0	0	100%
WT - hyg	0	30	0	0%

line(T2)	Resistant seedlings	Hygromycin sensitive seedlings	not germinated	
A-17-108-1	**57**	**0**	**3**	**95%**
A-17-108-3	53	0	7	88%
A-17-110-2	**57**	**0**	**3**	**95%**
A-17-110-10	57	0	3	95%
A-17-111-6	**56**	**0**	**4**	**93%**
A-17-111-8	52	0	8	87%
A-17-112-4	57	0	3	95%
A-17-112-5	55	0	5	92%
WT + hyg	0	30	0	0%
WT - hyg	30	0	0	100%

line(T3)	Resistant seedlings	Hygromycin sensitive seedlings	not germinated	
A-17-108-1-1	**60**	**0**	**0**	**100%**
A-17-108-1-2	60	0	0	100%
A-17-110-2-1	**60**	**0**	**0**	**100%**
A-17-110-2-2	60	0	0	100%
A-17-111-6-1	60	0	0	100%
A-17-111-6-2	**60**	**0**	**0**	**100%**
A-17-112-4-1	59	0	1	98%
A-17-112-4-2	60	0	0	100%
WT + hyg	0	60	0	0%
WT - hyg	60	0	0	100%



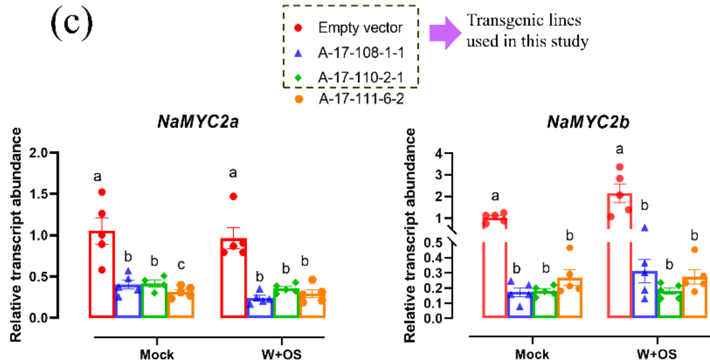


Fig. S4 irMYC2a/b line construction. **(a)** Phylogeny of bHLH-MYC-like family in *N. attenuata*. The consensus tree generated was tested by bootstrapping (1000 times); **(b)** transcript levels of MYC2-likes after W+OS elicitation in EV *N. attenuata* leaves*;* **c)**: Sequence fragments of *NaMYC2a* and *NaMYC2b* for RNAi silencing (SalI sites were marked in green).

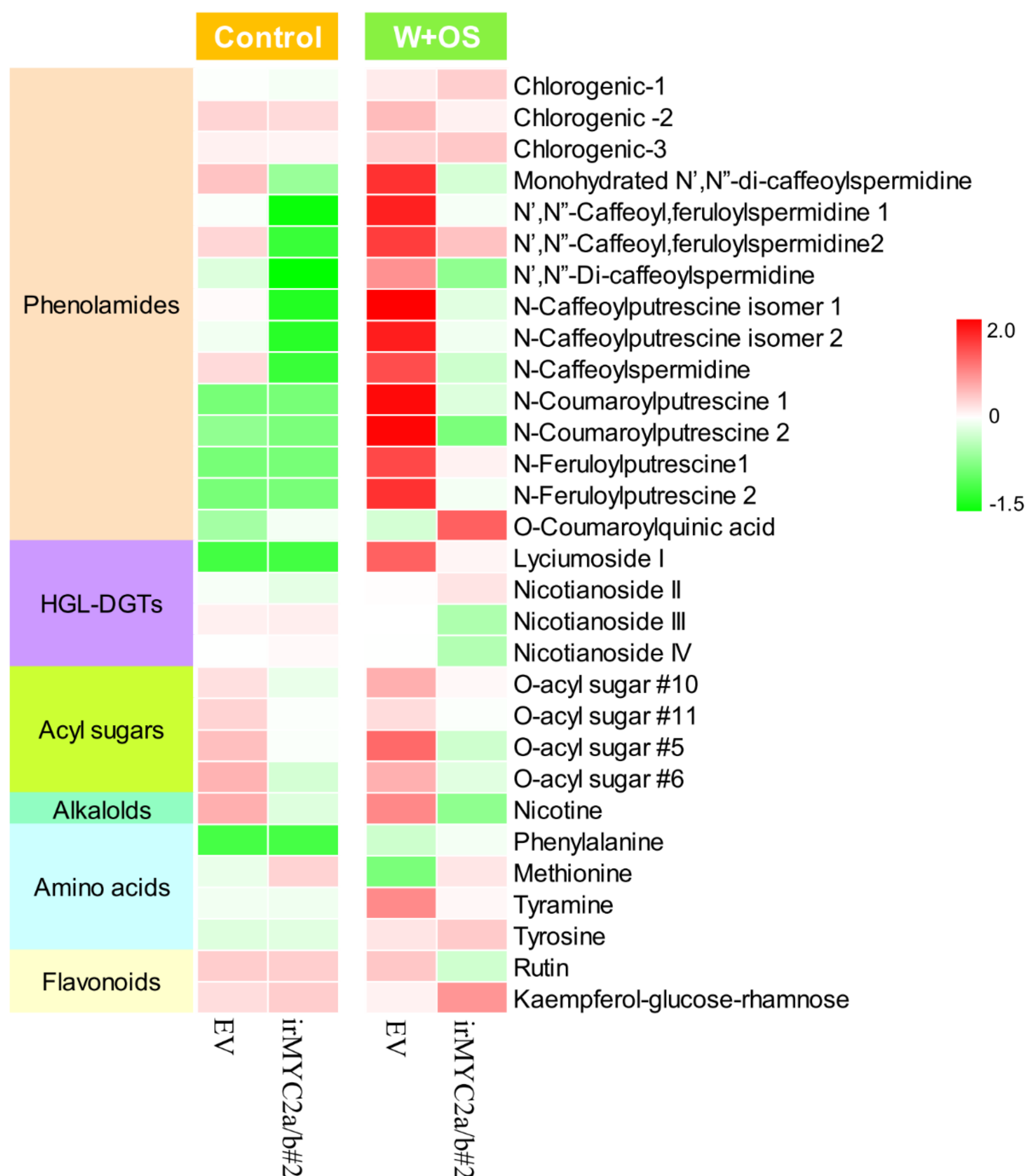


Fig. S5 Effects of W+OS elicitation on metabolites in the field (2021). The data were normalized by log transformation (base 10) and Pareto scaling (mean-centered and divided by the square root of the standard deviation of each variable).

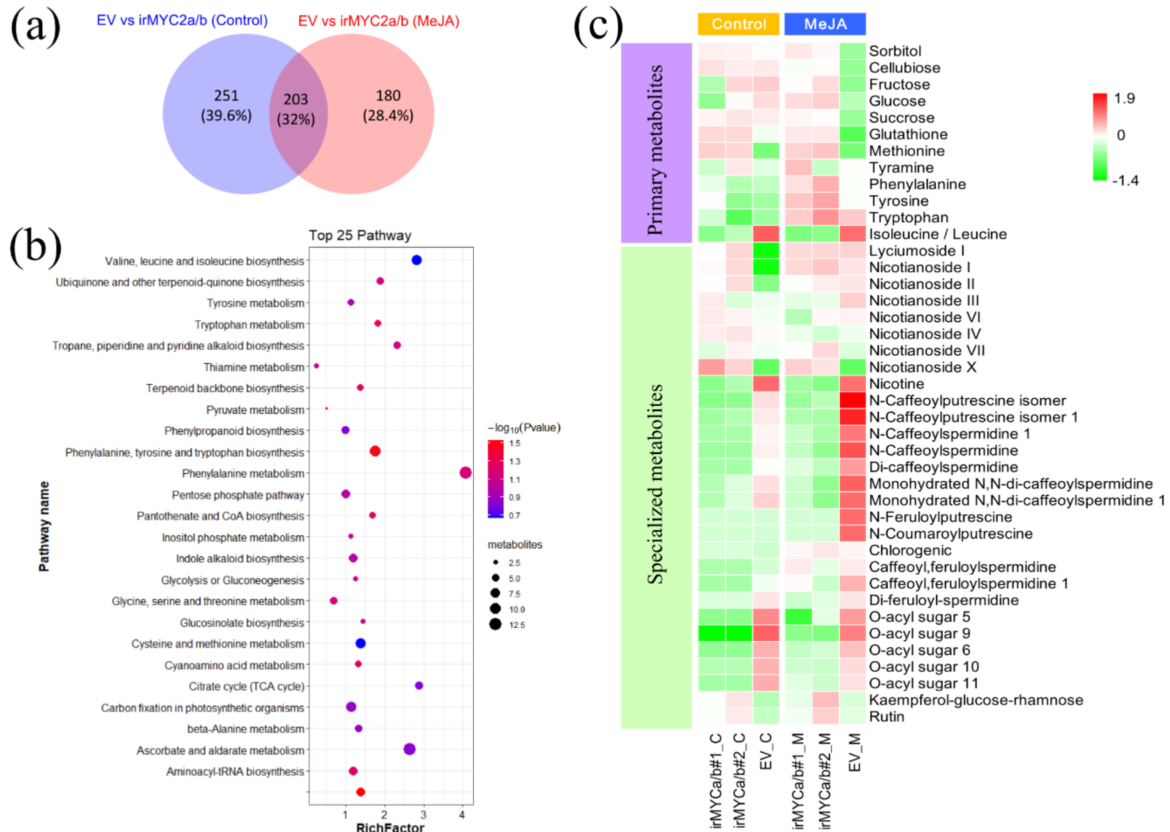


Fig. S6 Metabolomic analysis in a non-competitive environment. **(a)** Venn diagram of differential MS features between EV and irMYC2a/b (the differential MS features were obtained using partial least squares discriminant analysis (PLS-DA) with the variable importance in projection (VIP-value) > 1). **(b)** Metabolic pathways with significant differences between EV and irMYC2a/b; **(c)** Metabolite heatmap of EV and irMYC2a/b. The data were normalized by log-transformation (base 10) and Pareto scaling (mean-centered and divided by the square root of the standard deviation of each variable).

Methods S1 Metabolite extraction and analysis

Briefly, 100 mg of frozen ground leaf tissue was extracted with 1 mL precooled (−20 °C) 80% methanol (vol/vol) containing internal standards (For each sample, 10 ng D_4-ABA, 10 ng D_6-JA, 10 ng D_6-JA-Ile, 10 ng D_6-SA, 600ng testosterone). Sample mixtures were homogenized in a ball mill (Genogrinder 2000; SPEX CertiPrep) for 60s at a rate of 1100 strokes min^{-1} then centrifuged at 4°C and 15,000 ×g for 30 min. Supernatants were transferred into 1.5-mL microcentrifuge tubes and recentrifuged for 30 min. 600 µL of supernatant were transferred to a 2-mL vial for secondary metabolite detection and a 100 µL aliquot of the supernatant was used for phytohormone quantification. For the primary metabolite quantification, 2 µL of the remaining extract was added to 98 µL aqueous solution containing a mix of ^{13}C,^{15}N-labeled amino acids standards (949 nM $^{13}C_3$,$^{15}N_1$-Ala; 186 nM $^{13}C_6$,$^{15}N_4$-Arg; 1500 nM $^{13}C_4$,$^{15}N_2$-AsxAsn; 1209 nM $^{13}C_4$,$^{15}N_1$-AsxAsp; 648 nM $^{13}C_5$,$^{15}N_2$-GlxGln; 516 nM $^{13}C_5$,$^{15}N_1$-GlxGlu; 1465 nM $^{13}C_2$,$^{15}N_1$-Gly; 41 nM $^{13}C_6$,$^{15}N_3$-His; 196 nM $^{13}C_6$,$^{15}N_1$-Ile; 522 nM $^{13}C_6$,$^{15}N_1$-Leu; 216 nM $^{13}C_6$,$^{15}N_2$-Lys; 8.1 nM $^{13}C_5$,$^{15}N_1$-Met; 255 nM $^{13}C_9$,$^{15}N_1$-Phe; 240 nM $^{13}C_5$,$^{15}N_1$-Pro; 410 nM $^{13}C_3$,$^{15}N_1$-Ser; 404 nM $^{13}C_4$,$^{15}N_1$-Thr; 191 nM $^{13}C_9$,$^{15}N_1$-Tyr; 210 nM $^{13}C_5$,$^{15}N_1$-Val) to quantify amino acid content, another 2 µL of the extract was diluted 500 times in 500 pg mL^{-1} sorbitol solution to quantify sugar content.

For photosynthetic pigment measurements, 100 mg of frozen ground leaf tissue were extracted with 1 mL methanol. Sample mixtures were homogenized in a ball mill (Genogrinder 2000; SPEX CertiPrep) for 60s at a rate of 1100 strokes min^{-1} then twice centrifuged at 4°C and 15,000 ×g for 30 min each time. Then 600 µL supernatant was transferred to a sample vial and injected directly into an HPLC system.

Analysis of amino acids and sugars was performed on a Bruker Elite EvoQ Triple quadrupole-MS equipped with a HESI (heated electrospray ionization) ion source. Samples were analyzed in MRM mode with the parameters previously published (Schäfer *et al.*, 2016).

For chromatographic separation of secondary metabolites, 1 µL of each sample was separated with an Acclaim™C18 column (150 ×2.1 mm, 2.2 µm, ThermoFisher Scientific) equipped with a UPLC SecurityGuard™ ULTRA cartridge (Phenomenex, Catalogue#: AJO-8782). The mobile phase was composed of eluent A (de-ionized water with 0.05% formic acid and 0.1% acetonitrile) and eluent B (0.05% formic acid in acetonitrile) with a flow rate of 0.400 $mL\cdot min^{-1}$. Gradient elution was as follows: 0 to 0.5 min, isocratic elution with 90% A; 0.5 to 23.5 min, 90 to 10% A; 23.5 to 25 min, and isocratic elution with 10% A. MS detection of specialized metabolites was performed on a microTOF-Q II MS system (Bruker Daltonics, Bremen, Germany) equipped with an electrospray ionization (ESI) source. MS ion collection was carried out in positive ion mode. Nitrogen was used as the collision gas; the capillary voltage was 4500 V, and the capillary exit was 140 V. The dry gas flow rate and temperature were 10 $L\cdot min^{-1}$ and 200°C, respectively. Full scan mode was used for mass spectral acquisition at a repetition rate of 5 Hz.

Analysis of photosynthetic pigments was performed using an Agilent 1260 Infinity II HPLC system equipped with a DAD detector. Chromatographic peak separation was conducted on an Acclaim™C30 column (250×4.6 mm, 5 µm, ThermoFisher Scientific). The mobile phase was composed of eluent A (methanol: ethyl acetate [1:1 v/v]), eluent B (acetonitrile), and eluent C (200 mM acetic acid in de-ionized water). Elution gradient was 0 to 3.6 min, isocratic elution with 14.5% A, 85% B, 0.5% C; 3.6 to 27 min, 14.5% to 34.5% A, 85% to 65% B, 0.5% C; 27 to 45 min isocratic elution with 34.5% A, 65% B, 0.5% C. The injection volume was set at 20 µL

and the flow rate was 1 mL·min^{-1}. Chromatographic peaks were identified by comparing retention times and UV spectra with those of standards in the 440 to 475 nm detection range.

Table S1 Primers used in this study

Name	Sequence 5' to 3'	Purpose
NaMYC2a-F	CCAAGGATTCGGGCTTCAGA	qPCR
NaMYC2a-R	GATCGGGCTCAGCGTGAATA	qPCR
NaMYC2b-F	AAGGCATCACTGCTTGGAGA	qPCR
NaMYC2b-R	TCTTGATTTGGTGGAGGACCAG	qPCR
NaIF5a-F	GTCGGACGAAGAACACCATT	qPCR
NaIF5a-R	CACATCACAGTTGTGGGAGG	qPCR
NaMYC2a-A-F	GCGGCGCTGCAGCCAGTTAATACCCCAACGGTTCTTC	amplification, cloning
NaMYC2a-A-R	GCGGCGGTCGACGACCGGCTTTATCTTCTTCACCTTTG	amplification, ligation
NaMYC2b-A-F	GCGGCGGTCGACGAGGCTTCGGTGGTGAAGGAGGCG	amplification, ligation
NaMYC2b-A-R	GCGGCGAAGCTTCTGAACCTTTGATTTCAACTCATTG	amplification, cloning
NaMYC2a-C-F	GCGGCGGAGCTCCAGTTAATACCCCAACGGTTCTTC	cloning
NaMYC2b-C-R	GCGGCGCTCGAGCTGAACCTTTGATTTCAACTCATTG	cloning
ECI3-21	CTTTGCCGTGAATTGCCTGAG	DP, positive control
ECI4-23	GATACTGCTATGCGAAACCTGTC	DP, positive control
HYG10-21	GAGTTTAGCGAGAGCCTGACC	DP, hptII gene
HYG11-21	GAAGAAGATGTTGGCGACCTC	DP, hptII gene
TER1-22	CTTATCTGGGAACTACTCACAC	DP, CaMV terminator
INT5-22	TTGGATTGATTACAGTTGGGAC	DP, CaMV terminator
INT6-22	TCTTCTTCGTCTTACACATCAC	DP, CaMV promoter
PROM1-20	GACGCACAATCCCACTATCC	DP, CaMV promoter
HYG12-21	GATGGCTGTGTAGAAGTACTC	DP, NOS terminator
NOT5-23	CCCGATCTAGTAACATAGATGAC	DP, NOS terminator

DP, diagnostic PCR to confirm complete T-DNA integration.

Reference

Schäfer M, Brütting C, Baldwin IT, Kallenbach M. 2016. High-throughput quantification of more than 100 primary- and secondary-metabolites, and phytohormones by a single solid-phase extraction based sample preparation with analysis by UHPLC–HESI–MS/MS. *Plant Methods* **12**(1): 30.