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Supplemental Data

Rapid Synthesis of Auxin via a New Tryptophan-Dependent Pathway Is Required for Shade Avoidance in Plants

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Supplemental Experimental Procedures

Detailed Growth Conditions

For the greenhouse experiment described in Figure 1, seedlings were grown for 4 weeks on a greenhouse bench without supplemental lighting (peak photosynthetically active radiation at midday was 1200 μmol m^{-2} s^{-1}). Seedlings were then aligned in front of banks of incandescent lamps covered with either opaque screens (-FR treatment) or FR filters (+FR treatment). Plants were irradiated with FR from 10:00 to 19:00 every day. The R:FR ratios, measured with a Skye SKR 100/SKR 110 radiometer pointed to the light sources (Skye Instruments), were 0.24 and 0.68 for +FR and -FR treatments, respectively (Izaguirre et al., 2006). The drop in R:FR caused by FR supplementation was equivalent to the effect of neighbor proximity in a canopy of leaf-area index = 0.5. For responses to picloram (Sigma), seedlings were grown on ½ MS supplemented with varying amounts of picloram for 3 days under Wc. The plates were then either left in Wc or transferred to simulated shade for 3 days before hypocotyl measurements were made. For microarray experiments, seedlings were grown under simulated white light condition (R: 13 $\mu E \cdot m^{-2} \cdot s^{-1}$; B: 1.23 $\mu E \cdot m^{-2} \cdot s^{-1}$; (R:FR ratio of 1.1)) for 7 days and were then

treated with simulated white light or simulated shade for 1 hour. Whole seedlings were collected. For 5-MT sensitivity tests, seedlings were grown for 9 days on ½ MS supplemented with 20 μM of 5-MT (Sigma) in Wc.

Protein Sequence Alignment

Protein sequence alignment was carried out using clustalW program (http://www.ebi.ac.uk/Tools/clustalw2/index.html) and visualized as box shade alignment (http://www.ch.embnet.org/software/BOX_form.html).

Constructs

For complementation experiments, genomic *TAA1* DNA, including 2 Kb of upstream sequence and 800 bp of downstream sequence, was PCR-amplified from Col-0 genomic DNA and cloned into the pJHA212K vector using EcoRI and PstI sites (Yoo et al., 2005). For expression pattern analysis of *TAA1*, the *uidA* gene was first cloned into pJHA212K using SalI/BamHI; the 2 Kb promoter of *TAA1* was PCR-amplified and cloned in pJHA212K-GUS using KpnI/SacI; finally the genomic DNA of *TAA1* and the 800bp DNA downstream region of *TAA1* was PCR-amplified and inserted using the SalI site. For TAA1 localization test, *TAA1* cDNA was first amplified from a cDNA library from Col-0 and was then cloned into a modified pPZP212 vector with YFP (Chen et al., 2005). For complementation tests with the mutant form of *TAA1*, cDNA of *TAA1* was cloned into a modified pPZP212 vector with 3X Flag tag (Wang et al., 2005). This clone was then used as a template for DpnI-directed mutagenesis to generate the indicated mutation using QuikChange® Site-Directed Mutagenesis Kit from Stratagene.

Quantification of free IAA and IAA biosynthesis rate

Col-0 and *sav3-2* seedlings were grown under Wc for 7 days after germination. They were then treated with or without simulated shade for one hour and the aerial parts of seedlings were pooled, weighed and frozen in liquid nitrogen for quantification of free IAA content. Four

replicates were analyzed for samples without shade treatment and three replicates were analyzed for samples with shade treatment. The frozen samples (15 mg of plant tissue (fresh weight)) was homogenized in 0.5 ml 50 mM Na-phosphate buffer pH 7.0 containing 0.02% diethyldithiocarbamic acid (antioxidant) and 500 pg $^{13}C_6$-IAA internal standard, using the Retsch vibration mill (Retsch GmbH & Co. KG) and a 3 mm tungsten carbide bead at a frequency of 30 Hz for 2 min. The pH was adjusted to 2.7 and the sample was then purified by solid phase extraction on a 500 mg Isolute C_8-EC column (International Sorbent Technology) conditioned with 2 ml methanol and 2 ml 1% acetic acid. The column was washed with 2 ml 10% methanol in 1% acetic acid, eluted with 2 ml 70% methanol in 1% acetic acid and the sample was evaporated to dryness. The sample was dissolved in 0.2 ml 2-propanol and 1 ml dichloromethane, and IAA was methylated by adding 5 µl 2 M trimethylsilyl-diazomethane in hexane (Aldrich). The sample was then left at room temperature for 30 min. 5 µl of 2 M acetic acid in hexane was added to destroy excess diazomethane and the sample was evaporated to dryness. The sample was then trimethyl-silylated and analyzed by gas chromatography-selected reaction monitoring–mass spectrometry as described (Edlund et al., 1995).

For IAA biosynthesis measurements, Col-0 and *sav3-2* seedlings were grown under Wc for 7 days after germination. Seedlings were pretreated with ½ MS containing 30% ^2H_2O for 0.5 hours. They were then treated with Wc or shade for 2 hours and the aerial parts of 10 seedlings were collected and frozen in liquid nitrogen for each sample. Each treatment contains four replicates. Samples were homogenized, extracted and purified as described above. IAA synthesis rates were measured by GC-SRM-MS as described in Ljung et al.2005 (Ljung et al., 2005). For calculation of the relative synthesis rate of IAA, enrichment is expressed as the ratio of deuterium-labeled IAA (*m/z* 203+204+205) to unlabelled IAA (*m/z* 202), after correction for natural isotope distribution to *m/z* 203, 204 and 205. Four replicates were analyzed for all samples.

Supplemental Figure Legends

Figure S1. Description of the Mutant Screen and Molecular Characterization of the *sav3* Alleles

(A) Schematic diagram of the mutant screen.

(B) Hypocotyl length distribution of wild-type seedlings grown under Wc or simulated shade. Pictures of representative seedlings are also shown.

(C) DNA agarose gel picture of RT-PCR products of *TAA1* (nucleotides 501 -1145 of the coding region, *TAA1*-C), full-length *TAA1* (*TAA1*-F) and ubiquitin control (*UBQ*) (left panel). In the right panel, relative expression levels of *TAA1* transcript were shown. Total RNAs were extracted from 5-day old seedlings grown under Wc. The expression levels of *TAA1* were measured using quantitative RT-PCR (qRT-PCR) in triplicates and were normalized against a reference gene (AT2G39960). Error bars represent standard error of means (SEM).

Figure S2. Phenotypes of *sav3* Mutants

(A) *sav3* partially suppresses the phenotype of *phyb9*, a null mutant of *phyB*. Hypocotyl elongation in response to shade was quantitatively measured.

(B) Canopy height of *sav3-1* grown in the greenhouse with or without supplementary FR light.

(C) Hypocotyl elongation in response to high temperature. Seedlings were grown under Wc at 22°C for 3 days. They were then left at 22°C or transferred to 29°C and allowed to grow for 4 more days.

Mean values of at least 12 seedlings are shown and error bars represent SEM.

Figure S3. Expression Analysis of TAA1-dependent and Independent Early Response Genes

(A) Expression patterns of *TAA1*-independent early response genes. Expression values of *PAR1 ATHB2, HFR1* and several other *TAA1*-independent shade-induced genes were obtained from our microarray data. For each gene, median expression value was set to one.

(B) Expression levels of *TAA1*-dependent early response genes, *IAA19 and IAA29*, under Wc or shade, treated with or without 1μM IAA. 5-day-old, Wc-grown seedlings were soaked in control (1/2 MS) or 1/2 MS plus 1μM IAA solution and were then treated with simulated Wc or shade for 1hour. Expression levels were quantified using qRT-PCR in triplicates and normalized by the expression level of the reference gene. Error bars represent SEM.

Figure S4. Expression Analysis of *TAA1* and Rescue with *iaaM*

(A) Quantification of *TAA1* mRNA levels. 5-day old WT seedlings were treated with Wc or shade for 2 hours and relative expression levels of *TAA1* were measured using qRT-PCR and were normalized to the reference gene.

(B) Hypocotyl phenotypes of transgenic lines expressing the bacterial *iaaM* gene under the control of the *TAA1* promoter in *sav3-2* background. 5-day old T_2 seedlings were treated with Wc or shade for 4 days. Transgenic lines (*sav3-2*) containing *TAA1* cDNA driven by *TAA1* promoter and *iaaM* gene driven by the 35S promoter were included as controls. Mean values of at least 12 seedlings are shown.

Error bars represent SEM.

Figure S5. Biochemical Characterization of TAA1

(A) Optimal temperature and pH of TAA1. The borate buffer assay was employed to assay the production of IPA. 100 μl of reaction buffer containing 0.5M borate buffer (pH8.5 or as indicated), 10 μM of PLP, 1mM pyruvate and 1 μg of TAA1 was incubated at 55 ºC or indicated temperature for 5 minutes. The production of IPA was monitored by absorption at 330nm.

(B) Aminotransferase activity of TAA1 towards various L- amino acids. 100 μl of reaction mixtures containing 50 mM K_2HPO_4/KH_2PO_4 (pH 8.5), 10mM α-ketoglutarate, various amino acids (5mM) and 3μg of TAA1 were incubated at 37℃ for 20 min. The reaction was stopped by heat inactivation (95℃ for 5 min). Production of L-glutamate was measured using a kit from

Boehringer Mannheim/ R-Biophram, which is based on an L-glutamic acid colorimetric method. The amount of L-glutamate was expressed as absorption at 492nm. The line marks the production of L-glutamate by TAA1 using L-Trp as a substrate. Using this line as a cutoff, TAA1 can use at least 6 out of the 19 amino acids (Phe, Tyr, Trp, Leu, Ala and Met) (Glu is not assayed due to the limit of the assay). Mean values of 3 measurements are shown. Error bars represent SEM.

(C) Km and Vmax of TAA1 to L-Phe and L-Tyr. Initial velocities of TAA1 in buffers with various concentrations of Phe or Tyr (0.125-4 mM) were measured using the borate buffer assay as described above except that 20 mM sodium pyruvate was utilized. Production of β-phenylpyruvate or p-hydroxyphenylpyruvate was measured by absorption at 310 nm. Km and Vmax were determined as described in the Materials and Methods.

Figure S6. Analysis of *Yucca* Pathway

(A) A subset of *Yucca* genes are induced by shade. *Yucca* genes are the rate-limiting enzymes for the tryptamine pathway of IAA biosynthesis (Zhao et al., 2001). Relative expression levels of *Yucca* gene family members from the microarray experiment are shown.

(B) Hypocotyl phenotypes of y*ucca* mutants in shade. 5-day old seedlings were treated with Wc or shade (R:FR = 0.7) for 4 days before measurement. Mean values of at least 12 seedlings are presented. Error bars represent SEM.

Supplemental References

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Edlund, A., Eklof, S., Sundberg, B., Moritz, T., and Sandberg, G. (1995). A Microscale Technique for Gas Chromatography-Mass Spectrometry Measurements of Picogram Amounts of Indole-3-Acetic Acid in Plant Tissues. Plant Physiol *108*, 1043-1047.

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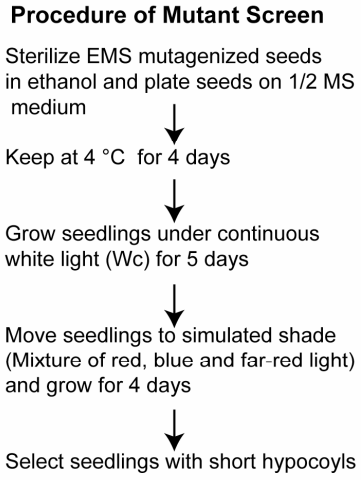

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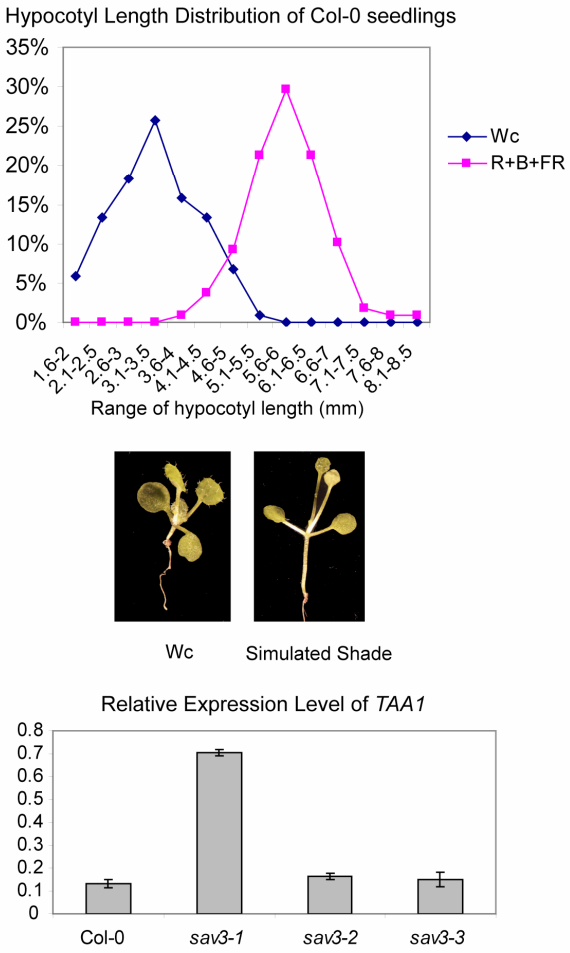

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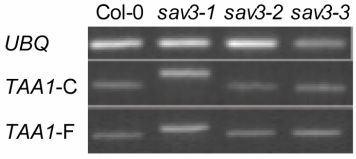

Figure S1

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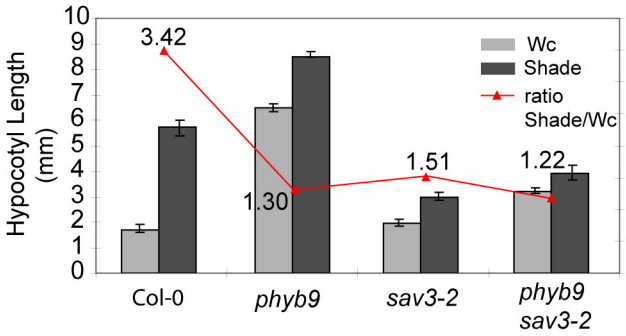

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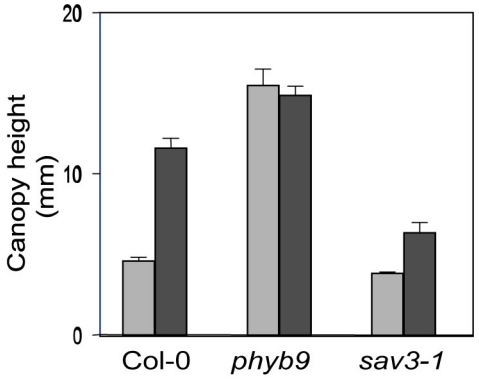

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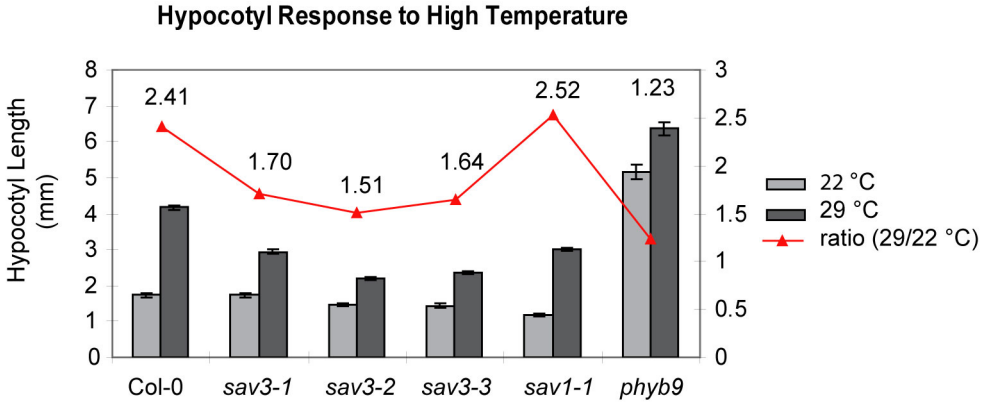

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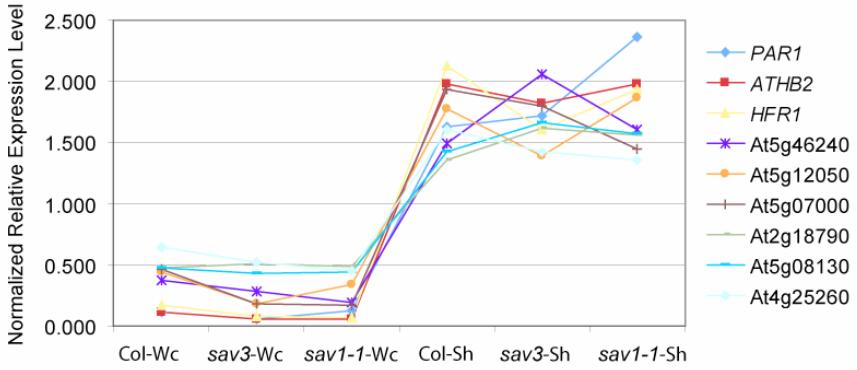
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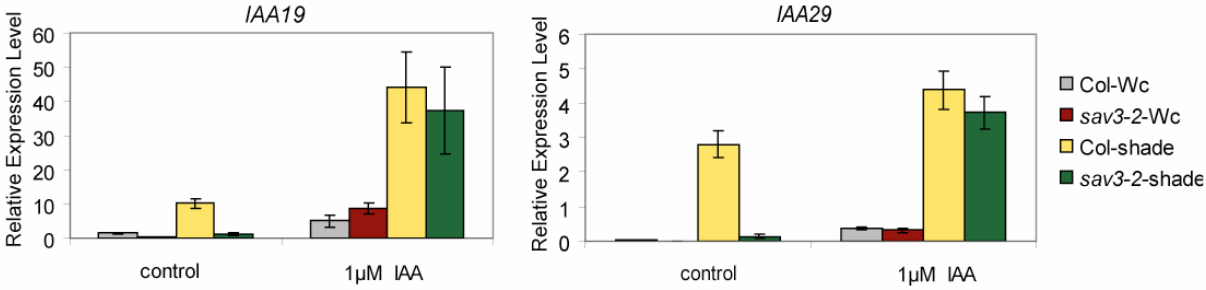
2.000
1.500
1.000
0.500
0.000
Col-Wc
sav3-Wc
sav1-1-Wc
Col-Sh
sav3-Sh
sav1-1-Sh
PAR1
ATHB2
HFR1
At5g46240
At5g12050
At5g07000
At2g18790
At5g08130
At4g25260

B


IAA19
Relative Expression Level
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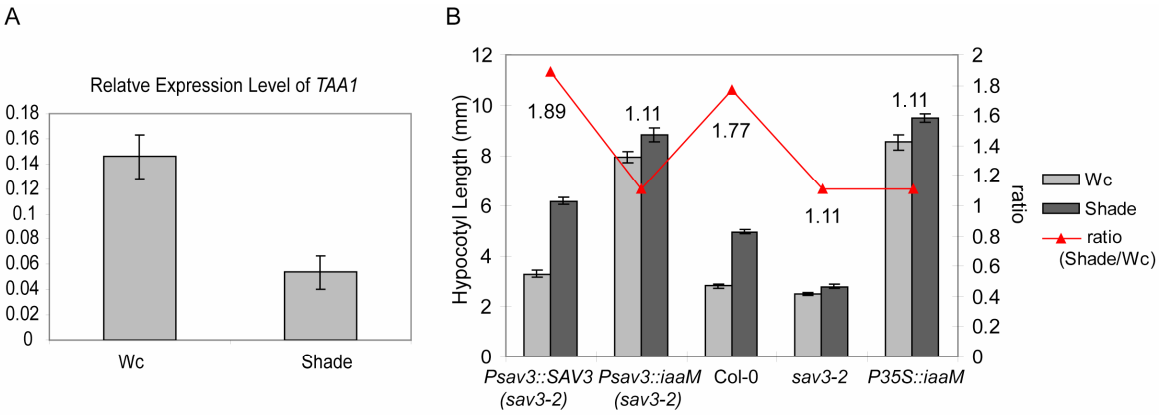
50
40
30
20
10
0
control
1μM IAA
IAA29
Relative Expression Level
6
5
4
3
2
1
0
control
1μM IAA
Col-Wc
sav3-2-Wc
Col-shade
sav3-2-shade

Figure S3


A
Relatve Expression Level of TAA1
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0.16
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
Wc
Shade
B
Hypocotyl Length (mm)
12
10
8
6
4
2
0
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
ratio
1.89
1.11
1.77
1.11
1.11
Wc
Shade
ratio
(Shade/Wc)
Psav3::SAV3 (sav3-2)
Psav3::iaaM (sav3-2)
Col-0
sav3-2
P35S::iaaM

Figure S4

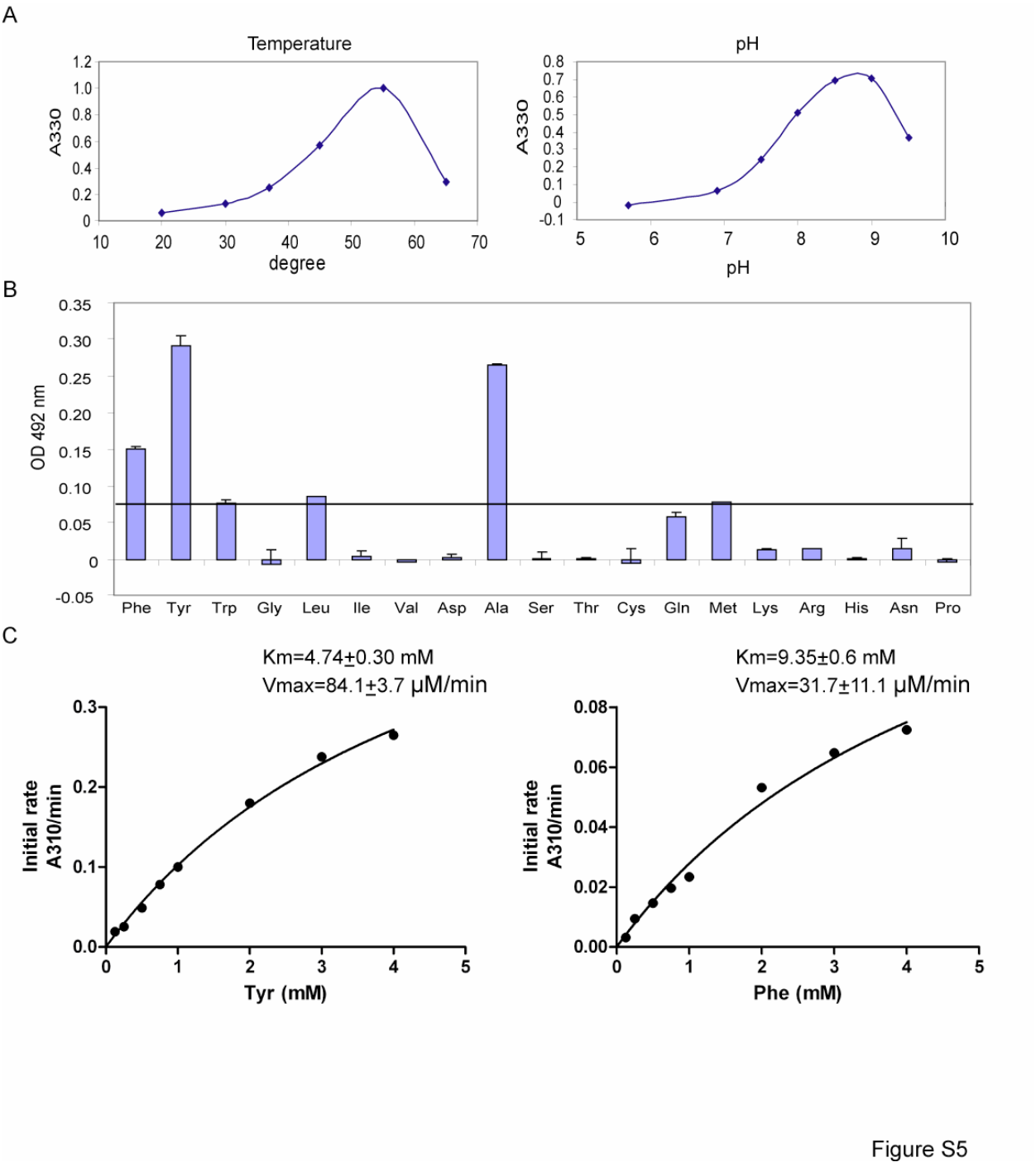
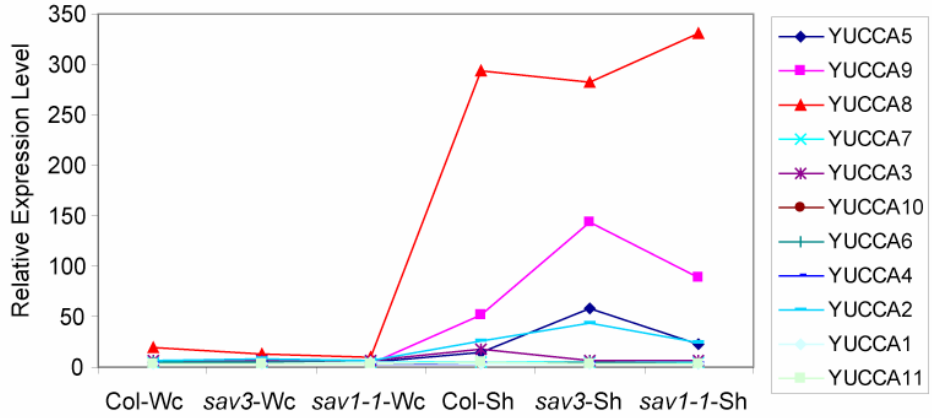

A
Temperature
A330
degree
pH
A330
pH
B
OD 492 nm
Phe Tyr Trp Gly Leu Ile Val Asp Ala Ser Thr Cys Gln Met Lys Arg His Asn Pro
C
Km=4.74±0.30 mM
Vmax=84.1±3.7 μM/min
Initial rate A310/min
Tyr (mM)
Km=9.35±0.6 mM
Vmax=31.7±11.1 μM/min
Initial rate A310/min
Phe (mM)

Figure S5

A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200
150
100
50
0
Col-Wc
sav3-Wc
sav1-1-Wc
Col-Sh
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YUCCA5
YUCCA9
YUCCA8
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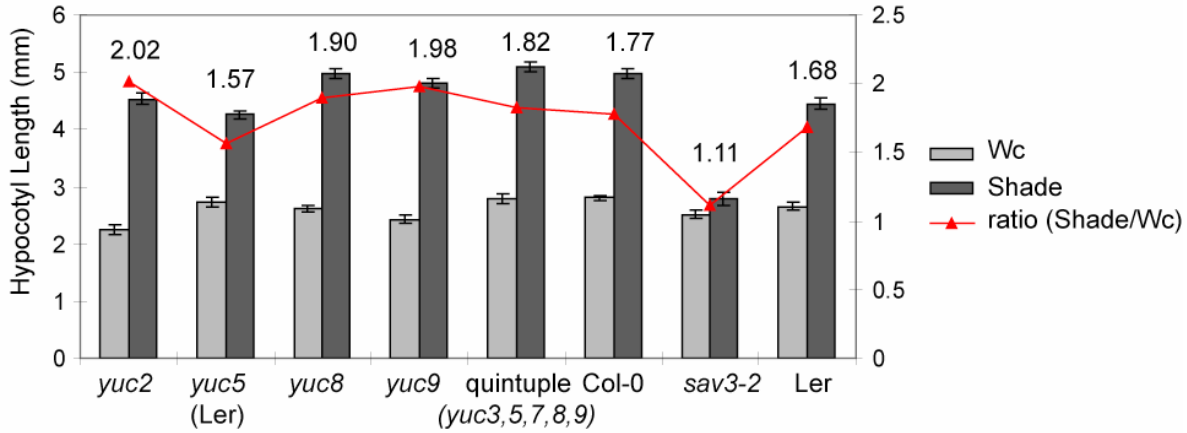
YUCCA3
YUCCA10
YUCCA6
YUCCA4
YUCCA2
YUCCA1
YUCCA11

B


Hypocotyl Length (mm)
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5
4
3
2
1
0
2.5
2
1.5
1
0.5
0
2.02
1.57
1.90
1.98
1.82
1.77
1.11
1.68
yuc2
yuc5 (Ler)
yuc8
yuc9
quintuple (yuc3,5,7,8,9)
Col-0
sav3-2
Ler
Wc
Shade
ratio (Shade/Wc)

Figure S6