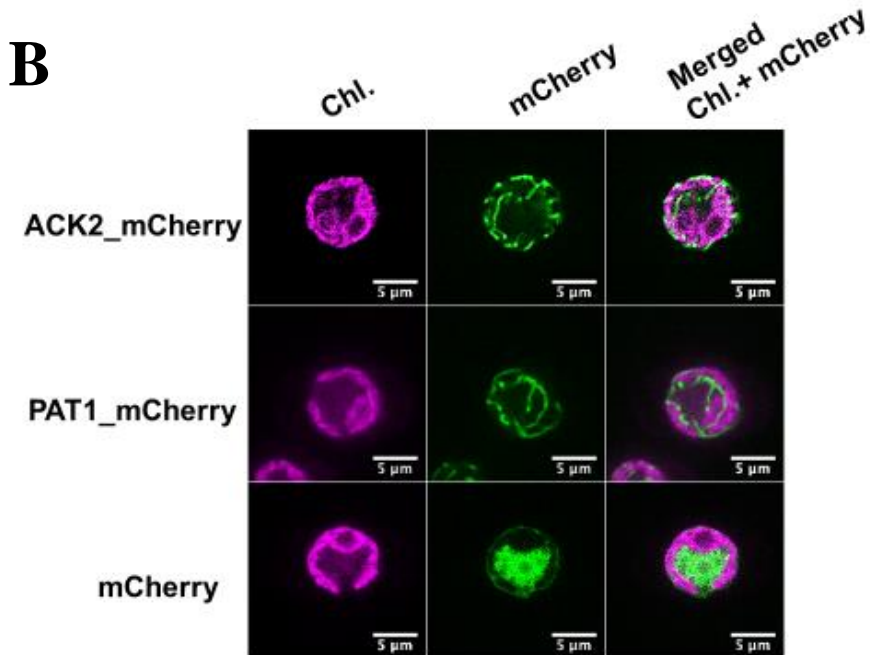


### Supplemental Figure 1. Relative Fluorescence Intensity of *Chlamydomonas* Cells Expressing Fluorescent Fusion Proteins.

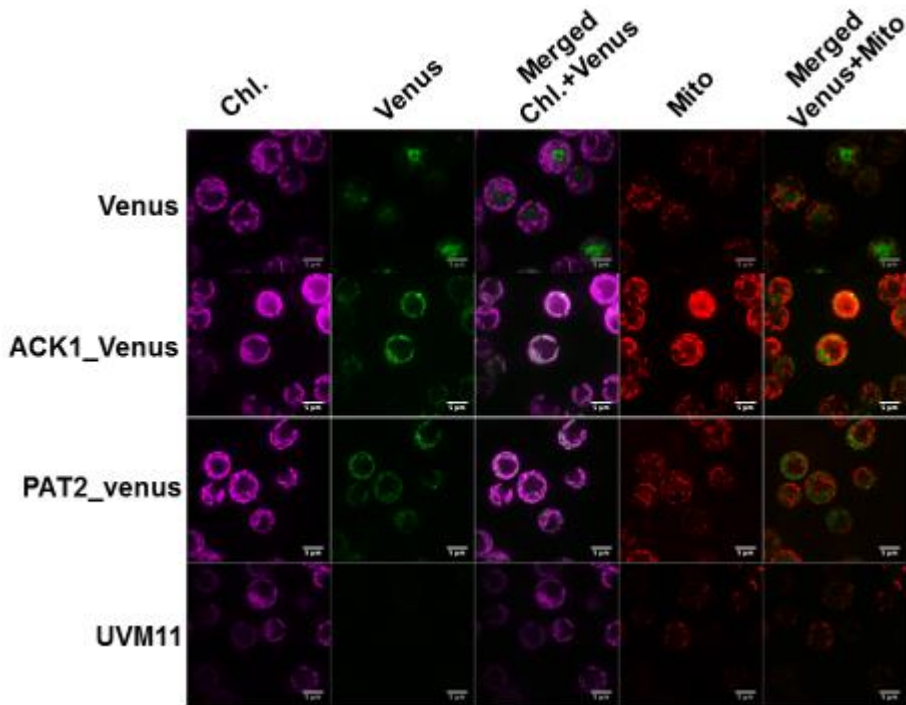
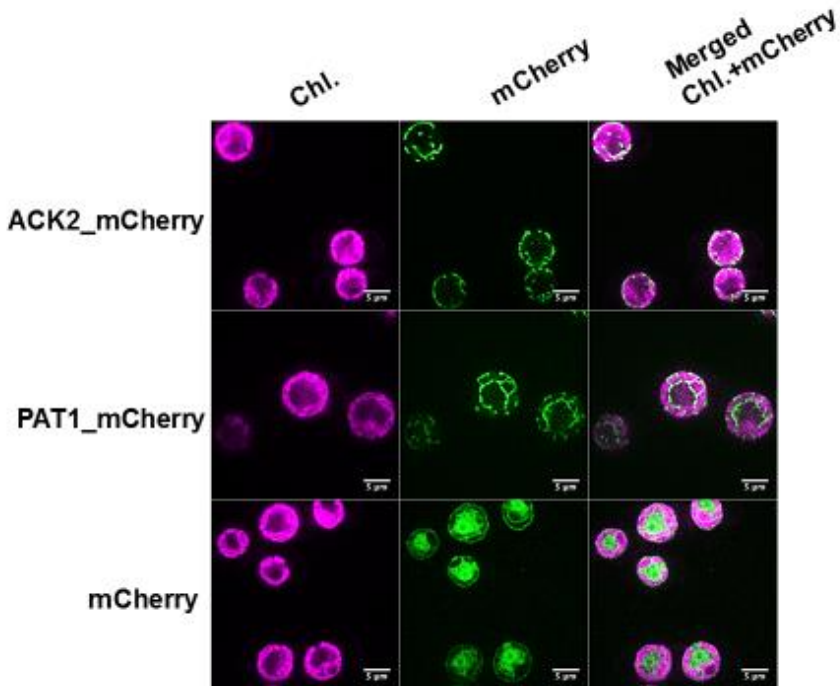
Fluorescence of the Venus (**A-D**: ACK1, ACK2, PAT1, and PAT2) and mCherry (**E-F**: ACK2 and PAT1) fusion proteins from *Chlamydomonas* transformants (from 1-12), visualized using a TECAN microtiter-plate reader. The UVM11 strain was used as a negative control while pLM004\_Venus and pLM004\_mCherry were used as positive controls (indicated with '+' on the x-axis). The y-axis represents the relative fluorescence compared to the UVM11 strain, and the x-axis represents individual transformants. Fluorescence from the cells was monitored at 3 different cell densities; the results for the different densities were similar.

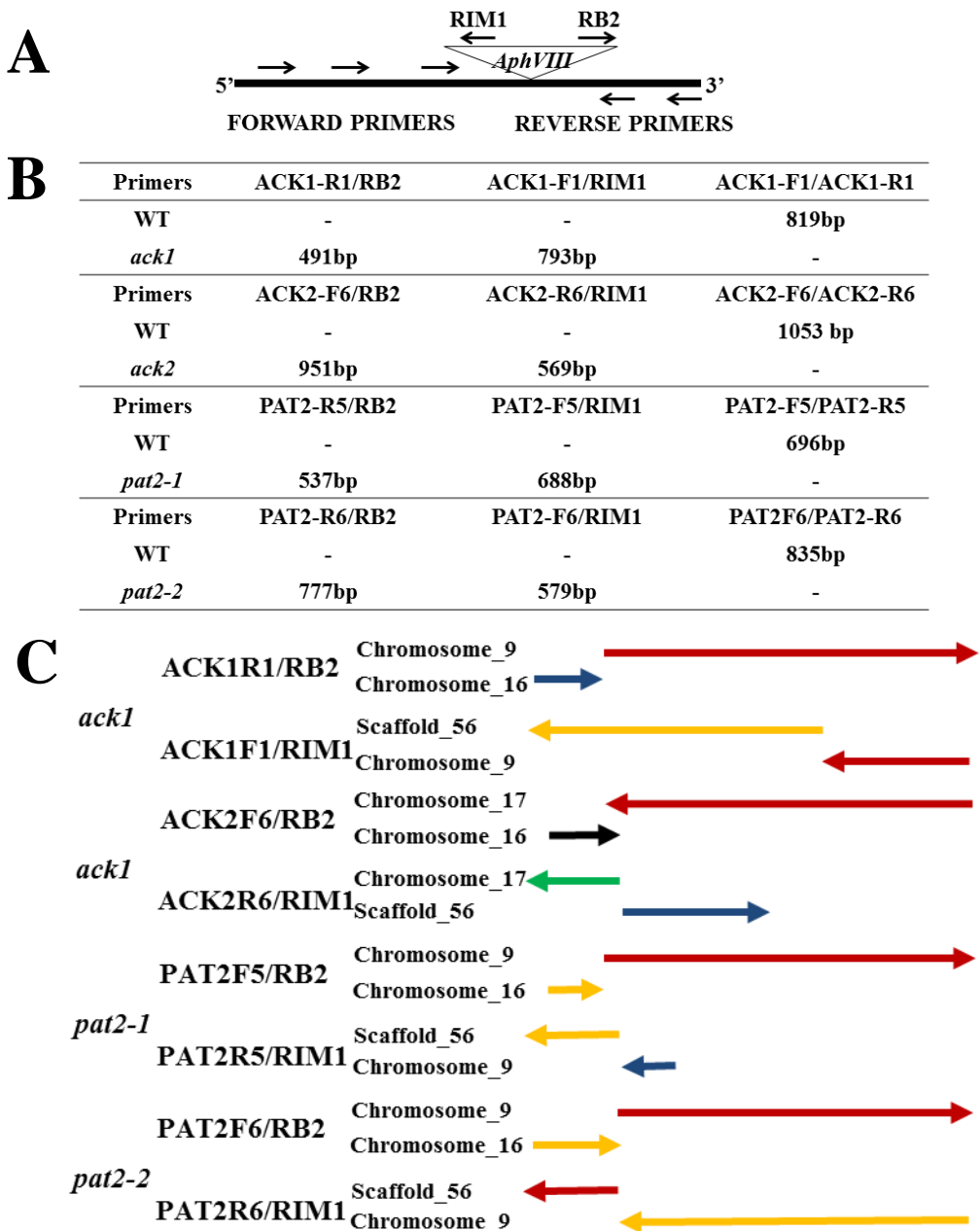


### Supplemental Figure 2. Localization of ACK2 and PAT1 Using mCherry Fusion Proteins.

**(A).** Schematic of the pLM004\_mCherry with the *ACK2* or *PAT1* gene fusions. The black arrows represent the promoters, including the tandem *HSP70* and *RBCS2* promoter driving expression of the *AphVIII* gene, and the *PSAD* promoter driving expression of the genes of interest. The *ACK2* and *PAT1* cDNAs are highlighted in yellow; the *mCherry* gene is in red. The black box in front of the *mCherry* gene represents a small linker region and the black box after the *mCherry* gene represents the *Cyc6* terminator.

**(B).** Localization of *ACK2* and *PAT1* proteins. For all panels, column 1 shows the auto-fluorescence of chlorophyll (Chl.), column 2 the images of the mCherry fluorescence signal (mCherry), and column 3 the merged images of column 1 and column 2. Scale bars are 5  $\mu$ m.

**A****B****Supplemental Figure 3. Localization of ACK 1/2 and PAT 1/2 in Multiple Cells.****(A).** Localization of ACK1 and PAT2 Venus fusion proteins. Scale bars are 5 μm.**(B).** Localization of ACK2 and PAT1 mCherry fusion proteins. Scale bars are 5 μm. The different columns are the same as in **Figure 3B**.



### Supplemental Figure 4. Genetic Analyses of *ack* and *pat* Single and Double Mutants.

(A). Schematic showing the positions of the RB2 and RIM1 primers at the borders of the *AphVIII* insertion with representations of primers within the *PAT/ACK* target gene.

(B). Amplicon sizes from WT and the different *PAT* and *ACK* mutants by PCR.

(C). Junction analyses of the four single mutants by PCR amplification and sequencing. The amplicons described in (B), generated with RB2 and primers for *PAT* or *ACK* sequences represent chimeric products containing the *Cyc6* sequence of the 3' end of the insertion cassette and a region of the *PAT* or *ACK* gene contiguous to the 3' end of the cassette. The amplicons generated with RIM1 and primers for *PAT* or *ACK* represent chimeric products containing the *PSAD* promoter at the 5' end of the insertion cassette and a region of the *PAT* or *ACK* gene contiguous to the 5' end of the cassette. Different colors of arrows represent the lengths of the alignment (the matches are exact). Black: less than 40 nucleotides. Blue: 40-49 nucleotides. Green: 50-79 nucleotides. Orange: 80-199 nucleotides. Red: more than 200 nucleotides.

**Supplemental Figure 5. Genomic and CDS Sequences of *ack1*, *ack2*, *pat2-1* and *pat2-2* Mutants.**

Sequences from the genes are in uppercase while the sequences of the *PSAD* promoter, *AphVIII* gene, and *Cyc6* terminator are in lowercase. Blue nucleotides represent UTR, red nucleotides exons, and black nucleotides introns. The forward and reverse primers for the PCR are highlighted in green, while the RB2 and RIM1 primers are highlighted in pink and yellow, respectively. Newly introduced nucleotides are shown in bold.

***ack1* GENOMIC SEQUENCE**

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### **ack1 CDS SEQUENCE**

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#### **ack2 GENOMIC SEQUENCE**

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### ***ack2* CDS SEQUENCE**

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### ***pat2-1* GENOMIC SEQUENCE**

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### **pat2-1 CDS SEQUENCE**

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### **pat2-2 GENOMIC SEQUENCE**

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CTGCGCTGCACGCACGGACTTGAGGAAAGCAAGTACCGTTTGGTCACTCCGTTGCTCTACAATATTC  
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**GGCAACAACACGTACAAAGCGGTGCAGCAGTCCACGGTGGCCATCGCCATGGCCCCGTGATGCA**  
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 GCGCTTGCCTGCGCGGTGACCGGGCGGTGCTGAGGGGACGACGTGGTAATAGCACATGGCAAT  
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GAGGCATCAGGGCTGGGGGGAGCGAGCTGCCTTGAGGGAAGGGCGTGGATGTTGTTGGGAAAGG  
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### **pat2-2 CDS SEQUENCE**

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CGGGCGTGCAGGACGCCGAGGCCGCGCGGATGATCGCAACGGCCAGCACAGCGAGCTGCTGGA  
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CCCCACCACCTCCTGGCCGAGGCGGCCAAGCTGGGCCTGGACCTGTCCGGCTGCAACATCCACA  
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GGTGGCCGACATCATCAACACCATCTGCGTACCTCCATCCAGGCCTCCCGCATGTCATCCGCCCG  
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**Supplemental Figure 6. Alignments of WT and Truncated ACK and PAT Proteins.**

(A). Alignment of amino acid sequences of WT ACK1 and the truncated protein of the *ack1* mutant. (B). Alignment of amino acid sequences of WT ACK2 and the truncated protein of the *ack2* mutant. (C). Alignment of amino acid sequences of WT PAT2 and the truncated protein of the *pat2-1* mutant. Identical or highly conserved amino acids in all positions are given in red letters (high consensus region), a low consensus amino acid position is given in blue letters, while black letters indicate an overall lack of strong consensus. Alignments were performed using <http://multalin.toulouse.inra.fr/multalin/>.

(A). Alignment of amino acid sequences of WT ACK1 and the truncated protein of the *ack1* mutant.

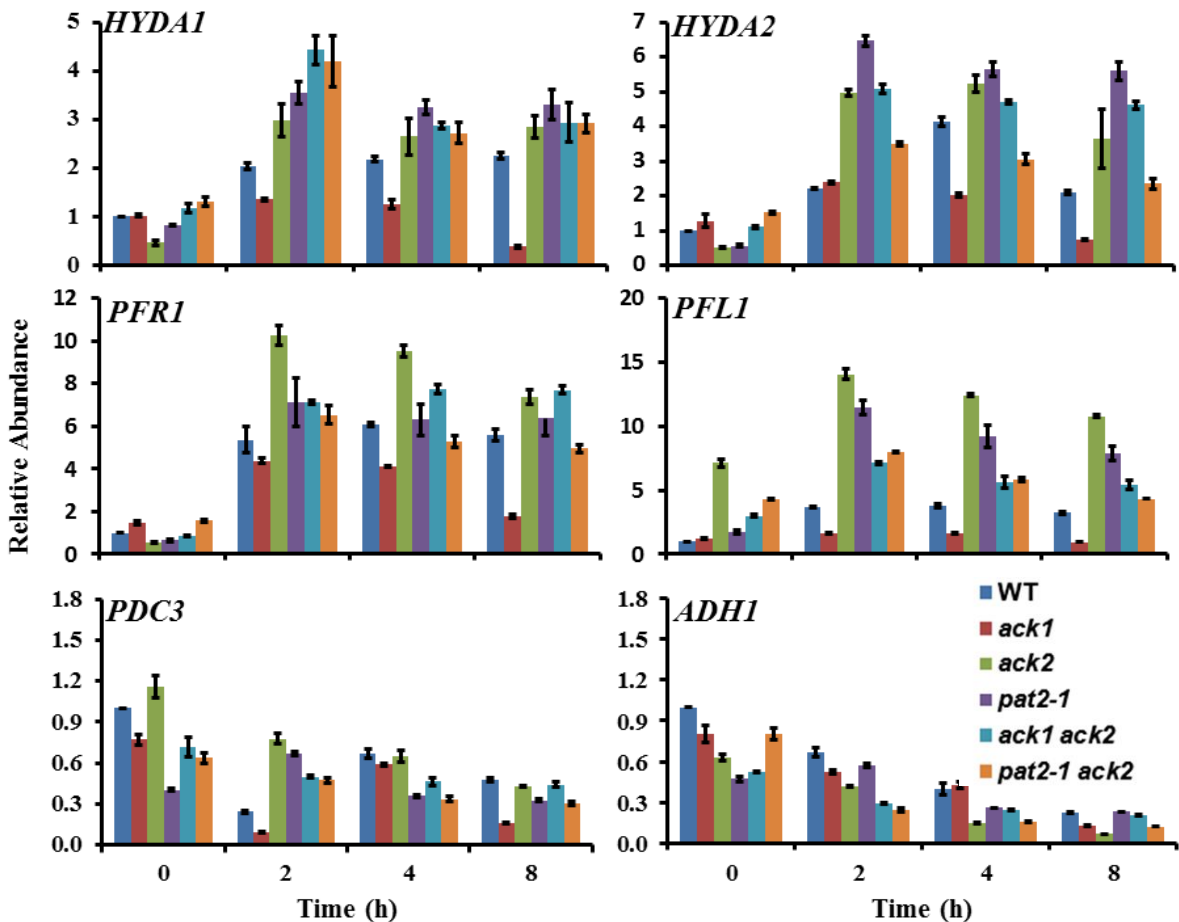
	1	10	20	30	40	50	60
	-----+-----+-----+-----+-----+-----+-----+-----						
ACK1	MLAGKYPVGSAYPPRNTAPSGKGSKYL VYNAGSSSIKFKIFTAETLLPGAAGLFDRI						
ack1	MLAGKYPVGSAYPPRNTAPSGKGSKYL VYNAGSSSIKFKIFTAETLLPGAAGLFDRI						
Consensus	MLAGKYPVGSAYPPRNTAPSGKGSKYL VYNAGSSSIKFKIFTAETLLPGAAGLFDRI						
	61	70	80	90	100	110	120
	-----+-----+-----+-----+-----+-----+-----+-----						
ACK1	ANCYLKASVPDDGGKPKKFEKKIPAKDHLTAMQDIMSFI						
ack1	ANCYLKASVPDDGGKPKKFEKKIPIVTRALVNTSPPRSPSHGRPRSPFALS						
Consensus	ANCYLKASVPDDGGKPKKFEKKIPakdralar#dimpridengrprfafalc.....						
	121	130	140	150	160	170	180
	-----+-----+-----+-----+-----+-----+-----+-----						
ACK1	GLDMSQPKLLTPDYIRCIQDASHFAPLHNPPHLQGIYASQKVF						
ack1	GLDMSQPKLLTPDYIRCIQDASHFAPLHNPPHLQGIYASQKVF						
Consensus	.....						
	181	190	200	210	220	230	240
	-----+-----+-----+-----+-----+-----+-----+-----						
ACK1	MPPESSYYVGLPYDMEYKHKIRRYGFHGTSHKYLVDQAAAMLGK						
ack1	MPPESSYYVGLPYDMEYKHKIRRYGFHGTSHKYLVDQAAAMLGK						
Consensus	.....						
	241	250	260	270	280	290	300
	-----+-----+-----+-----+-----+-----+-----+-----						
ACK1	VAAYRGGQCVDTSMGHTPLEGLLMGTRCGDIDPAVYLHIQNLGL						
ack1	VAAYRGGQCVDTSMGHTPLEGLLMGTRCGDIDPAVYLHIQNLGL						
Consensus	.....						
	301	310	320	330	340	350	360
	-----+-----+-----+-----+-----+-----+-----+-----						
ACK1	LGLTGSNDLRTVIEGAGKGEPRSQLGLDMFYRRYRKYIGAYNAHLDGQY						
ack1	LGLTGSNDLRTVIEGAGKGEPRSQLGLDMFYRRYRKYIGAYNAHLDGQY						
Consensus	.....						
	361	370	380	390	400	410	420
	-----+-----+-----+-----+-----+-----+-----+-----						
ACK1	NSALYRGLICDRMQALGIAYDKDANAAAYGGKQADISGPGARIRALVIPTDEEL						
ack1	NSALYRGLICDRMQALGIAYDKDANAAAYGGKQADISGPGARIRALVIPTDEEL						
Consensus	.....						
	421	431					
	-----+-----						
ACK1	LQVYRGAALAA						
ack1	LQVYRGAALAA						
Consensus	.....						

**(B).** Alignment of amino acid sequences of WT ACK2 and the truncated ACK2 protein of the *ack2* mutant.

	1	10	20	30	40	50	60
	-----+-----+-----+-----+-----+-----+-----						
ACK2	MQRVLYLNAGSSSLKFKLFTAAGTSLGEAGLGGQFDRIGDVANCTLKASQPGAAPGMKPR						
<i>ack2</i>	MQRVLYLNAGSSSLKFKLFTAAGTSLGEAGLGGQFDRIGDVANCTLKASQPGAAPGMKPR						
Consensus	MQRVLYLNAGSSSLKFKLFTAAGTSLGEAGLGGQFDRIGDVANCTLKASQPGAAPGMKPR						
	61	70	80	90	100	110	120
	-----+-----+-----+-----+-----+-----+-----						
ACK2	KMELKVPAQDHVGAMKGILDFIKENVSATFASEVVAVGHRIVHGLDLSQPVLLTPSSTAT						
<i>ack2</i>	KMELKVPAQDHVGAMKGILDFIKENVSATFASEVVAVGHRIVHGLDLSQPVLLTPSSTAT						
Consensus	KMELKVPAQDHVGAMKGILDFIKENVSATFASEVVAVGHRIVHGLDLSQPVLLTPSSTAT						
	121	130	140	150	160	170	180
	-----+-----+-----+-----+-----+-----+-----						
ACK2	IKQAAYLAPLHNPPGLQGI EAATEVFSGYPQVAVFDATAFHQTMPPHAYMYGLPYELYEKH						
<i>ack2</i>	IKQAAYLAPLHNPPGLQGI EAATEVFSGYPQVAVFDATAFHQTMPPHAYMYGLPYELYEKH						
Consensus	IKQAAYLAPLHNPPGLQGI EAATEVFSGYPQVAVFDATAFHQTMPPHAYMYGLPYELYEKH						
	181	190	200	210	220	230	240
	-----+-----+-----+-----+-----+-----+-----						
ACK2	KIRRYGFHGTSHKYLVEQAAMLGKPYSDTNVITCHLNGSSVAAYRGGQCYDTSMGLTP						
<i>ack2</i>	KIRRYGFHGTSHKYLVEQAAMLGKPYSDTNVITCHLNGSSVAAYRGGQCYDTSMGLTP						
Consensus	KIRRYGFHGTSHKYLVEQAAMLGKPYSDTNVITCHLNGSSVAAYRGGQCYDTSMGLTP						
	241	250	260	270	280	290	300
	-----+-----+-----+-----+-----+-----+-----						
ACK2	LEGLLMGTRCGMDPAVYLHIQNCGLNVKETDTLLNKKSGLLGLTGSNDLRAVIEGAGK						
<i>ack2</i>	LEGLLMGTRCGMDPAVYLHIQNCGLNVKETDTLLNKKSGLLGLTGSNDLRAVIEGAGK						
Consensus	LEGLLMGTRCGMDPAVYLHIQNCGLNVKETDTLLNKKSGLLGLTGSNDLRAVIEGAGK						
	301	310	320	330	340	350	360
	-----+-----+-----+-----+-----+-----+-----						
ACK2	GELRSQLGLDMFTYRKYIGAYTAALDGKYDAVYVFSAGIGENSPFIRKLI AEPLHARGN						
<i>ack2</i>	GELRSQLGLDMFTYRKYIGAYTAALDGKYDAVYVFSAGIGENSPFIRKLI AEPLHARKV						
Consensus	GELRSQLGLDMFTYRKYIGAYTAALDGKYDAVYVFSAGIGENSPFIRKLI AEPLHARGN						
	361	370	380	390	400	410	414
	-----+-----+-----+-----+-----+-----+-----						
ACK2	QLDHAANDTAVGRAGVISTPGSRVQLLYIPTDEELSI AEQTLQVAKGALGAAAA						
<i>ack2</i>	AGSL						
Consensus	agdh.....						

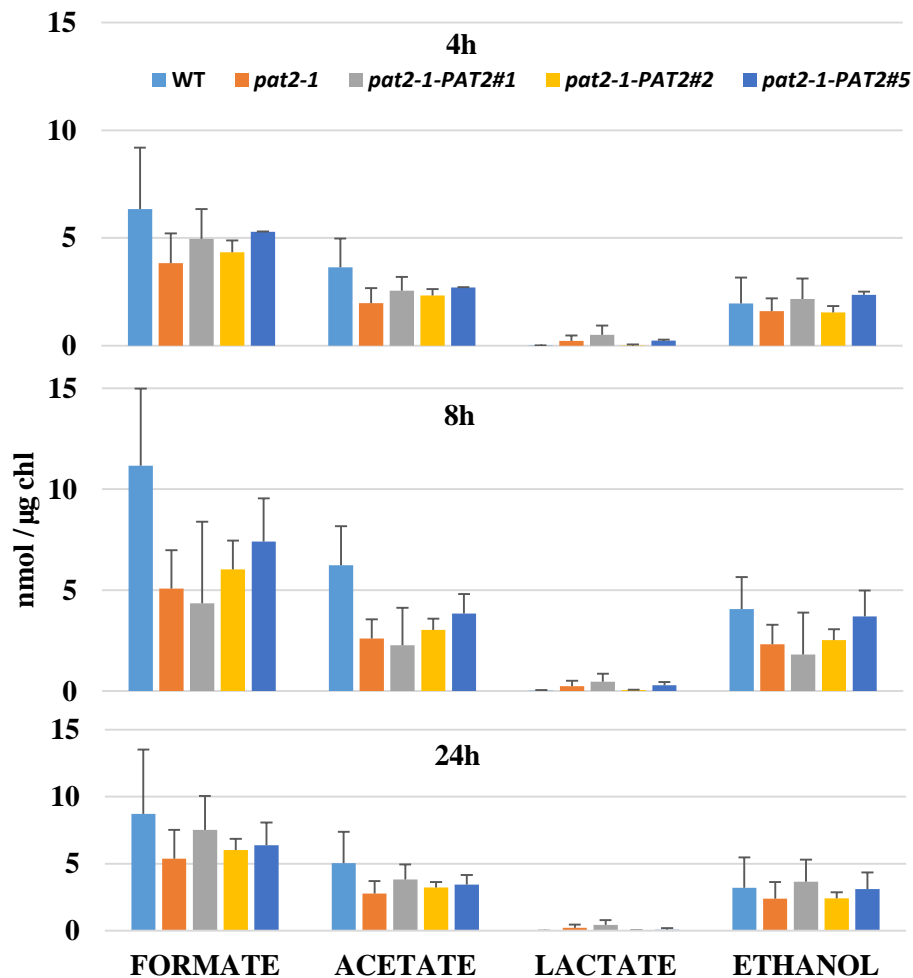
(C). Alignment of amino acid sequences of WT PAT2 and truncated PAT2 protein of the *pat2-1* mutant.

	1	10	20	30	40	50	60	70	80	
PAT2	-----+-----+-----+-----+-----+-----+-----+-----+-----+-----									
pat2-1	-----+-----+-----+-----+-----+-----+-----+-----+-----+-----									
Consensus	-----+-----+-----+-----+-----+-----+-----+-----+-----+-----									
	81	90	100	110	120	130	140	150	160	
PAT2	-----+-----+-----+-----+-----+-----+-----+-----+-----+-----									
pat2-1	-----+-----+-----+-----+-----+-----+-----+-----+-----+-----									
Consensus	-----+-----+-----+-----+-----+-----+-----+-----+-----+-----									
	161	170	180	190	200	210	220	230	240	
PAT2	-----+-----+-----+-----+-----+-----+-----+-----+-----+-----									
pat2-1	-----+-----+-----+-----+-----+-----+-----+-----+-----+-----									
Consensus	-----+-----+-----+-----+-----+-----+-----+-----+-----+-----									
	241	250	260	270	280	290	300	310	320	
PAT2	-----+-----+-----+-----+-----+-----+-----+-----+-----+-----									
pat2-1	-----+-----+-----+-----+-----+-----+-----+-----+-----+-----									
Consensus	-----+-----+-----+-----+-----+-----+-----+-----+-----+-----									
	321	330	340	350	360	370	380	390	400	
PAT2	-----+-----+-----+-----+-----+-----+-----+-----+-----+-----									
pat2-1	-----+-----+-----+-----+-----+-----+-----+-----+-----+-----									
Consensus	-----+-----+-----+-----+-----+-----+-----+-----+-----+-----									
	401	410	420	430	440	450	460	470	480	
PAT2	-----+-----+-----+-----+-----+-----+-----+-----+-----+-----									
pat2-1	-----+-----+-----+-----+-----+-----+-----+-----+-----+-----									
Consensus	-----+-----+-----+-----+-----+-----+-----+-----+-----+-----									
	481	490	500	510	520	530	540	550	560	
PAT2	-----+-----+-----+-----+-----+-----+-----+-----+-----+-----									
pat2-1	-----+-----+-----+-----+-----+-----+-----+-----+-----+-----									
Consensus	-----+-----+-----+-----+-----+-----+-----+-----+-----+-----									
	561	570	580	590	600	610	620	630	640	
PAT2	-----+-----+-----+-----+-----+-----+-----+-----+-----+-----									
pat2-1	-----+-----+-----+-----+-----+-----+-----+-----+-----+-----									
Consensus	-----+-----+-----+-----+-----+-----+-----+-----+-----+-----									
	641	650	660	670	680	690	700	710	720	
PAT2	-----+-----+-----+-----+-----+-----+-----+-----+-----+-----									
pat2-1	-----+-----+-----+-----+-----+-----+-----+-----+-----+-----									
Consensus	-----+-----+-----+-----+-----+-----+-----+-----+-----+-----									
	721	730	740	750	760	770	780	790	92	
PAT2	-----+-----+-----+-----+-----+-----+-----+-----+-----+-----									
pat2-1	-----+-----+-----+-----+-----+-----+-----+-----+-----+-----									
Consensus	-----+-----+-----+-----+-----+-----+-----+-----+-----+-----									



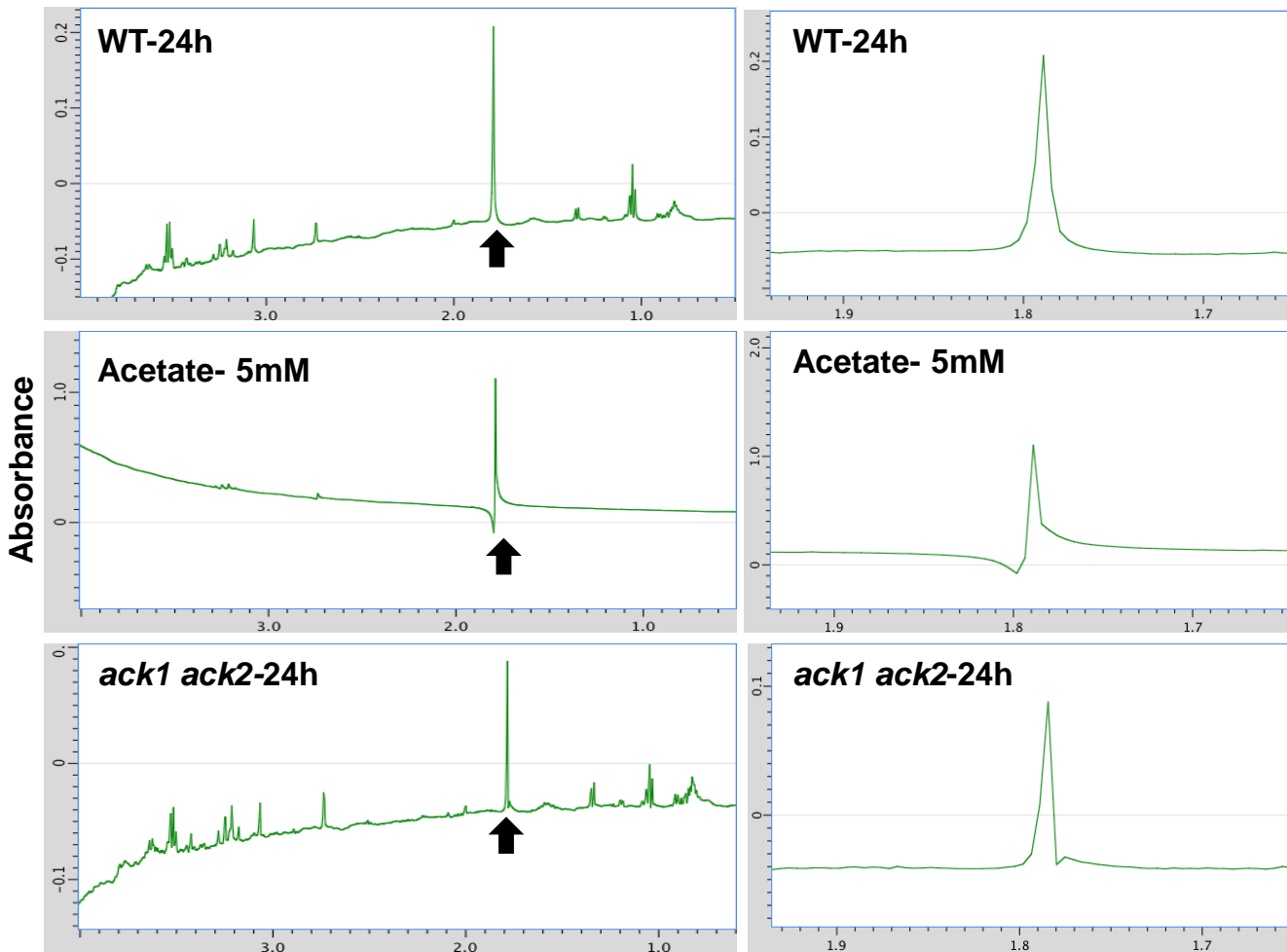
### Supplemental Figure 7. Changes in Abundance of Transcripts Encoding Fermentative Enzymes in *pat* and *ack* Mutants.

RT-qPCR was used to determine transcript levels from the indicated fermentative genes in WT and single and double mutant strains under dark anoxic conditions. There were no significant differences between the values based on *t*-tests. Errors bars represent SD.



**Supplemental Figure 8. Accumulation of External Metabolites in the Complemented Strains of *pat2-1*.**

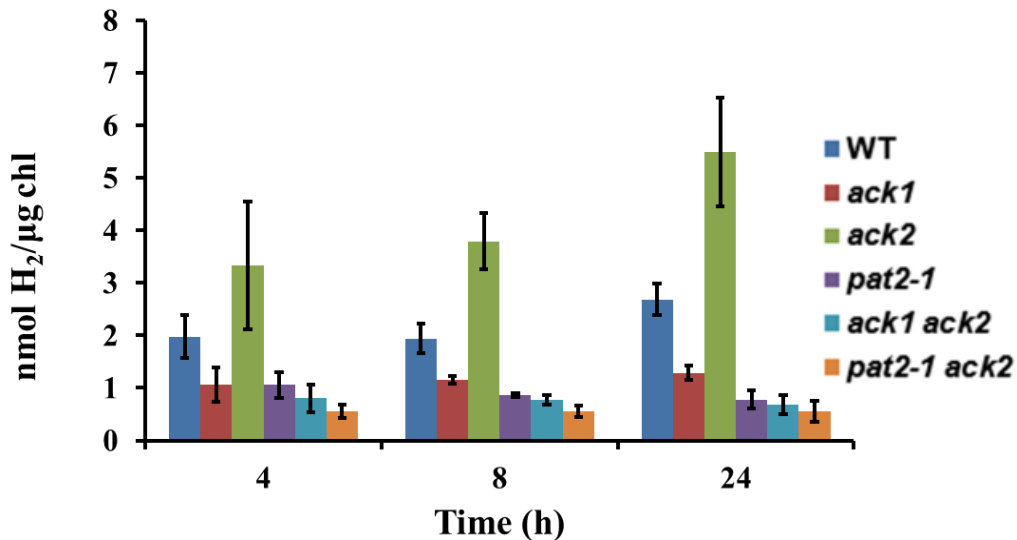
HPLC quantitation of extracellular metabolites secreted by the indicated mutants after 4, 8 or 24 h of dark anoxic acclimation. Errors bars represent SEM.



**Supplemental Figure 9. Nuclear Magnetic Resonance (NMR) to Confirm the Acetate Production in *ack1 ack2* Double Mutants.**

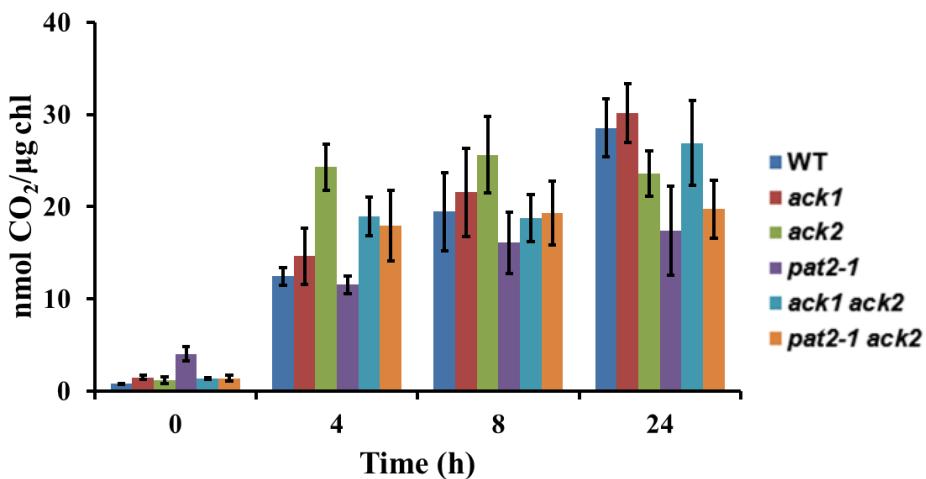
NMR confirmation of acetate secreted by the WT and *ack1 ack2* mutant strains after 24 h of dark anoxic acclimation. 5 mM acetate was applied as positive controls. Arrows represent the acetate absorbance peak. The right pics are the close-up pic of the acetate absorbance peaks.





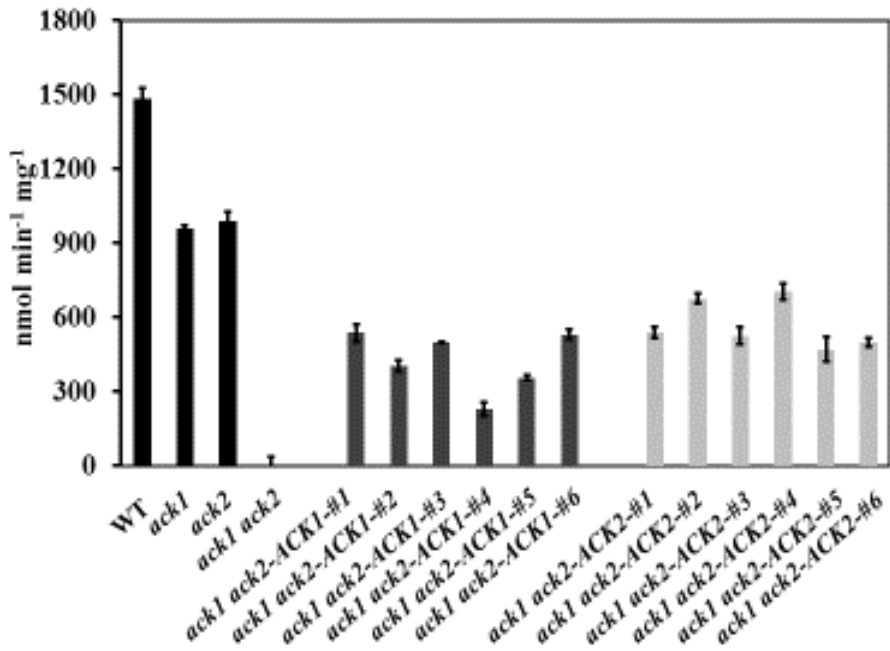
### Supplemental Figure 10. Fermentative H<sub>2</sub> Production by Various Mutants.

Fermentative H<sub>2</sub> production at the indicated times of dark anoxic acclimation determined using gas chromatography from cultures of the indicated mutants. There were no significant differences between the values based on *t*-tests. Errors bars represent SD.



### Supplemental Figure 11. Fermentative CO<sub>2</sub> Production by Various Mutants.

Fermentative CO<sub>2</sub> production at indicated times of dark anoxic acclimation determined using gas chromatography from cultures of the indicated mutants. There were no significant differences between the values based on *t*-tests. Errors bars represent SD.



**Supplemental Figure 12. *In Vitro* Acetate Kinase Activity in Mutant and Complemented Strains.**

Acetate kinase activity was measured in the *ack1*, *ack2* and *ack1 ack2* mutants. The *ack1 ack2-ACK1-#1* to #6 lines are the *ack1 ack2* double mutants transformed with *ACK1*, while *ack1 ack2-ACK2-#1* to #6 are *ack1 ack2* double mutants transformed with *ACK2*. Error bars represent SD.

A

1 10 20 30 40 50 60 70 80 90 100 110 120 130  
 AC51-194063  
 AC52-391501  
 AC53-196311  
 AC54-511767  
 Consensus  
 MGLAARACCRARVPLSLEGRARVRLTQSVRYLGVATAVRVLVYIADVTVEEGYSGEHLGLAQLAARLAPASGAGRQPRGCLPRAAGSCGPPYPERAGSSALFRQALFKSKAPVYVHAFGRPPRFVSARGGATRA

131 140 150 160 170 180 190 200 210 220 230 240 250 260  
 DGGVYVALLSGSSTFSLGATRASLPVLRHARRARPARAGCSAREADLCLDARASGARTFARVPLGFLGGAGAGVYVSGSGGAARAGVYVPLGLLIARVAVSAGDEPARAGDGGGGGVEVARVRA

261 270 280 290 300 310 320 330 340 350 360 370 380 390  
 AC51-194063  
 AC52-391501  
 AC53-196311  
 AC54-511767  
 Consensus  
 GAARVADSSAKRSRHPITGGSVYVPPSPQLSPPPPVPVVSAPQARAPPPTPTATPVNHLSTQARLSASQDHSKSTSYVTSGSGRGRISLAAARREARQITLEPSSPSTRSLRALHLQTVQSRITAVN

391 400 410 420 430 440 450 460 470 480 490 500 510 520  
 DDFAREGDEALHNKGRHS-PLCGNINQENVPVHSVAREINNAHYLDLRIETAGDRDRVATVGGNDGEEVRLYSELXSKRYCRANV-KSLGKRVGRSDVYHNSLVLPELPAHPLACRIGRIGV

521 530 540 550 560 570 580 590 600 610 620 630 640 650  
 AC51-194063  
 AC52-391501  
 AC53-196311  
 AC54-511767  
 Consensus  
 IYVFGFARLSEARLISGSRVYVIT-THRPKPKTGLKATVNDLCTEKNHNTVYVNLVLEL-PLIAREVE-GSKHGRQVYVTAER-PPHARPELKYIYSGTQPKK-

651 660 670 680 690 700 710 720 730 740 750 760 770 780  
 SVVFGFARLSEARLISGSRVYVIT-THRPKPKTGLKATVNDLCTEKNHNTVYVNLVLEL-PLIAREVE-GSKHGRQVYVTAER-PPHARPELKYIYSGTQPKK-

781 790 800 810 820 830 840 850 860 870 880 890 900 910  
 AC51-194063  
 AC52-391501  
 AC53-196311  
 AC54-511767  
 Consensus  
 AHWYVVDGGRCPDTHAQITTEGHHITPLPGATPL-KPESRITLFFGVEVPLDEK-GNEL-GQPCGVLCKKPKPSRHRVTVGDRHARE-QTYFSAKYGYFVIGDGRDRDHYVHITGDDVYDLV

911 920 930 940 950 960 970 980 990 1000 1010 1020 1030 1040  
 AC51-194063  
 AC52-391501  
 AC53-196311  
 AC54-511767  
 Consensus  
 SGRHIGTEVESHLVEHPSGERRVYV-GVENPIKQSTYVYVITLVEGPDARAKELISYVYKTLGEPFAPPDVIHAPLKTARSKLHRRVLRKLRASGKEDL-GDTSILNDPSSVQVVLVEGK

1041 1050 1060 1070 1080 1090 1100 1110 1120 1130 1140 1150 1160 1170  
 AC51-194063  
 AC52-391501  
 AC53-196311  
 AC54-511767  
 Consensus  
 LGVDVGGARLVLYLQGLPPAPARALLTVYRDTGALDKLKSGLVHARLRASGRADMDLRAGVGVYVLSVSHVSRHGRGSSSTTASRALLSTTGMLNFRAPVYAGGSGGGGSLGRARSSAH

1171 1180 1190 1200 1210 1220 1230 1240 1250 1260 1270 1280 1290 1300  
 AC51-194063  
 AC52-391501  
 AC53-196311  
 AC54-511767  
 Consensus  
 HAPPPAPQALSPHGPGICGAGGSSGGGGGGGGVHGPELSSAPARDELLQLQARAAASASGGGGGGGSSCHSVSIVSNAVGAARVAVSAGAGRQLQPGGGVGGSSGGRLSALPSSSSAVYNTGATT

1301 1310 1320 1330 1340 1350 1360 1370 1380 1390 1400 1409  
 AC51-194063  
 AC52-391501  
 AC53-196311  
 AC54-511767  
 Consensus  
 GTGTATVAGRGGSTCGSSHGVGGGGGGGGGCGAEGPTATALLTVMDLEETYESGHRARQALVDSLITTTLAGNTSGRHALHGGPGPAGGGGGVDDGTANG

B

ALDH1-102889  
 ALDH2-130434  
 ALDH3-135619  
 ALDH8-412458  
 ALDH5-283125  
 ALDH6-390966  
 ALDH7-406746  
 ALDH4-150809  
 Consensus  
 MSLRAALGHARR-VAHEGSLVGV

1 10 20 30 40 50 60 70 80 90 100 110 120 130  
 HNLNGALAPHLNHSYCSAGIGASREQPPSAGCGATGATATATSPSSSGRPAPPTASVYPTSSVYPTASFLPTASVYPTASFLAGAAARGLVRAITRQLALAAITSAALPAPSPRGLPQPLPQSLGG

131 140 150 160 170 180 190 200 210 220 230 240 250 260  
 Consensus  
 MSLRAALGHARR-VAHEGSLVGV

261 270 280 290 300 310 320 330 340 350 360 370 380 390  
 ALDH1-102889  
 ALDH2-130434  
 ALDH3-135619  
 ALDH8-412458  
 ALDH5-283125  
 ALDH6-390966  
 ALDH7-406746  
 ALDH4-150809  
 Consensus  
 AKPSKL-IT-EVVRSDIITDYATEGRYVLEGGILLNSDFSPGNARMLCLVSPKGLVGLVITLPPFVYVNLAVSKLAPALHAGNAVILKPTT---GQAVAGIHWVQCFHARGLPQALSVATGRGE

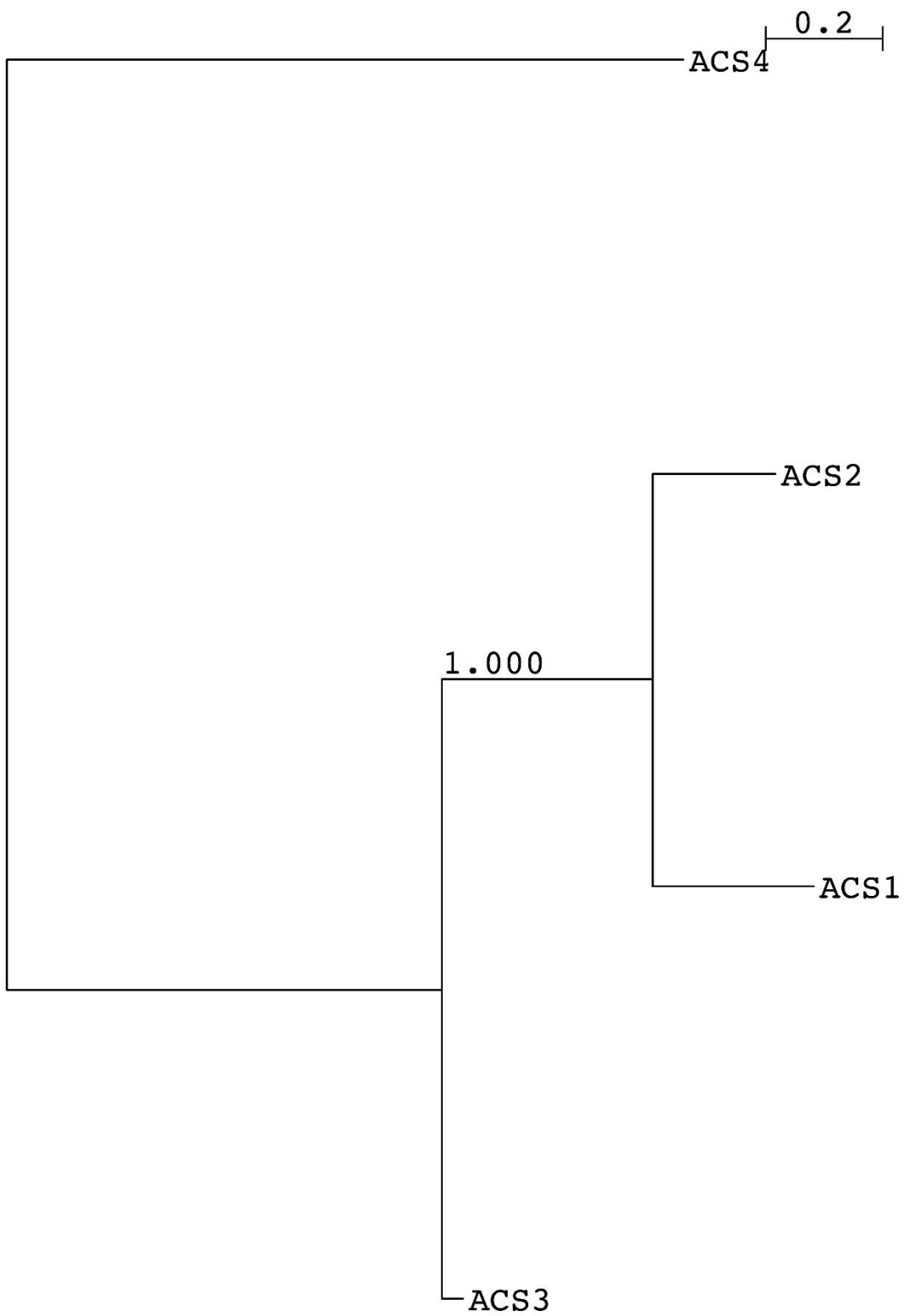
391 400 410 420 430 440 450 460 470 480 490 500 510 520  
 ALDH1-102889  
 ALDH2-130434  
 ALDH3-135619  
 ALDH8-412458  
 ALDH5-283125  
 ALDH6-390966  
 ALDH7-406746  
 ALDH4-150809  
 Consensus  
 LGDFLITHPDVCISFVGGDTG---TSIARKAGRV---PLNELGKGVKIVCED---ADLDAANKIHKGGFVSGQRCTAVKLVVRA---PVADR

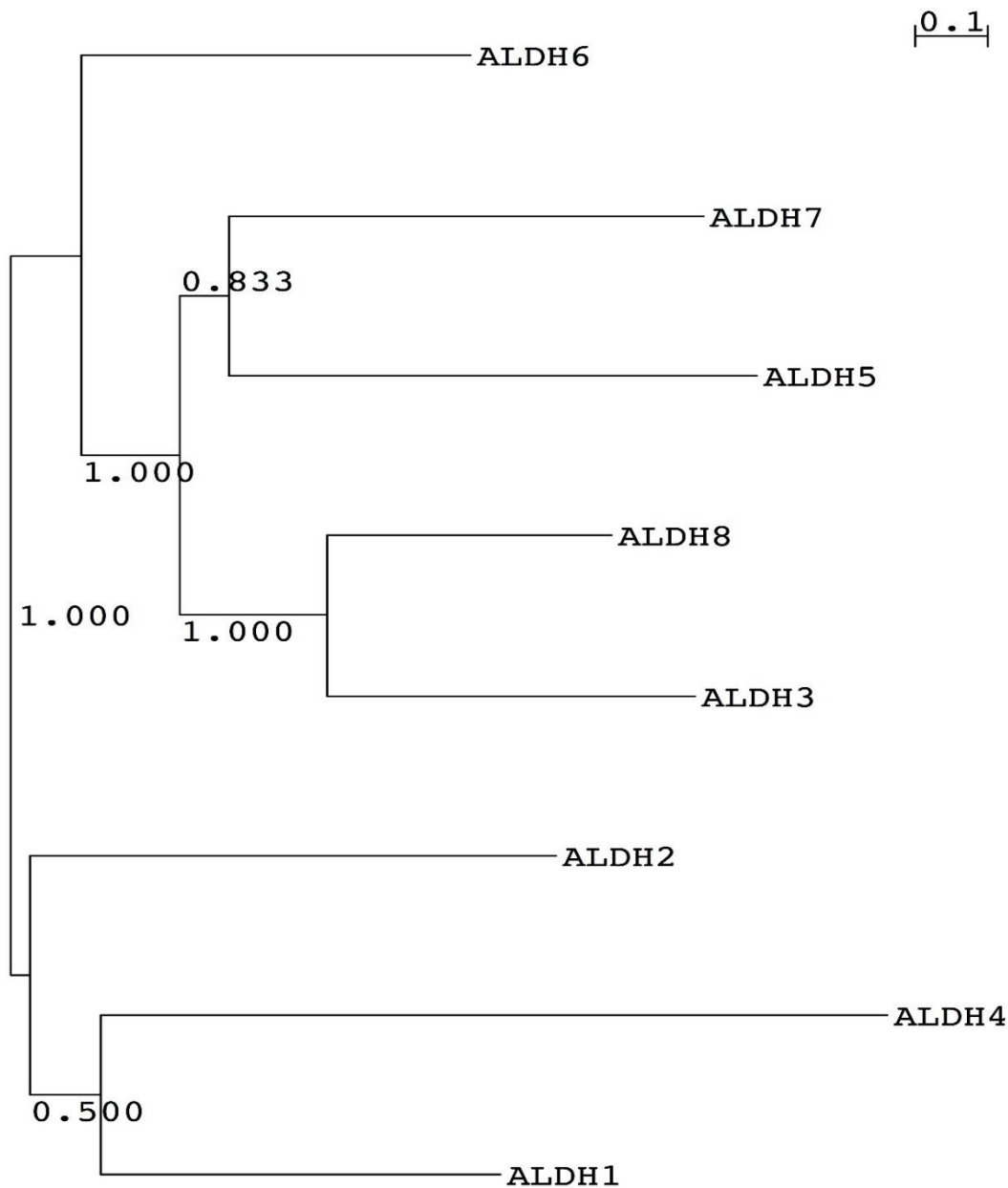
521 530 540 550 560 570 580 590 600 610 620 630 640 650  
 ALDH1-102889  
 ALDH2-130434  
 ALDH3-135619  
 ALDH8-412458  
 ALDH5-283125  
 ALDH6-390966  
 ALDH7-406746  
 ALDH4-150809  
 Consensus  
 LVAARVAGVAKLSVGR-EDDCDITPV-VSESSANFTEGLANDAKKAGATFVTEGAR---REG---NLITPVLIDHTVADLAEPEFGVPLVHRRVSSYVAEREH---CNKSKY

651 660 670 680 690 700 710 720 730 740 750 760 770 780  
 ALDH1-102889  
 ALDH2-130434  
 ALDH3-135619  
 ALDH8-412458  
 ALDH5-283125  
 ALDH6-390966  
 ALDH7-406746  
 ALDH4-150809  
 Consensus  
 GLQGVCFTRDINAIKISDAHEGTQVQVNSAPRAG-DHFFPQGRDGGTSGQGRINSANIKIKSTVNLDKESYTLG

781 790 800  
 ALDH1-102889  
 ALDH2-130434  
 ALDH3-135619  
 ALDH8-412458  
 ALDH5-283125  
 ALDH6-390966  
 ALDH7-406746  
 ALDH4-150809  
 Consensus  
 GHTFTLTPSAGHAGLNEQVNDVAGVGN-VPVPLPFFSFTGARGSLDGHVYRAGVYVYQITVYKHPKEDIRGTSNPATLSSSSGSKS---SGCKPRLPLGRDVRGS

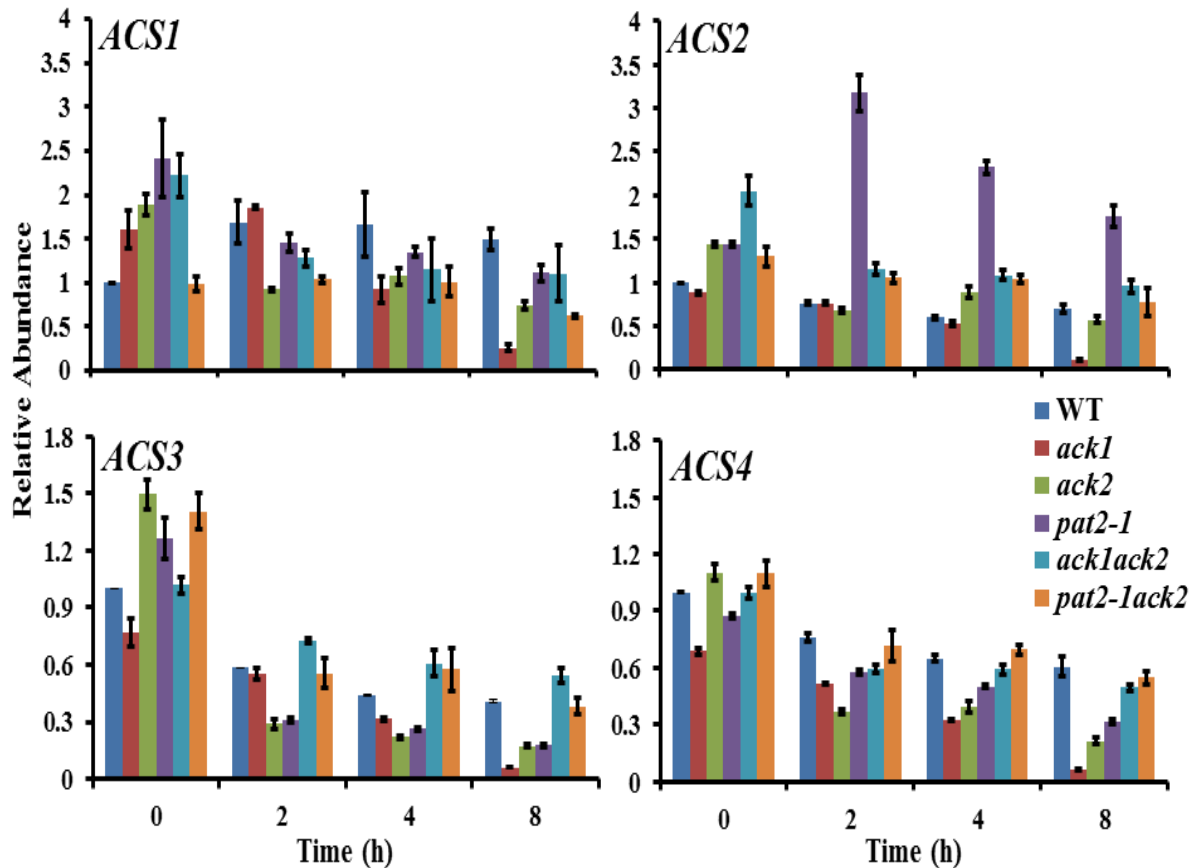
C



**D**

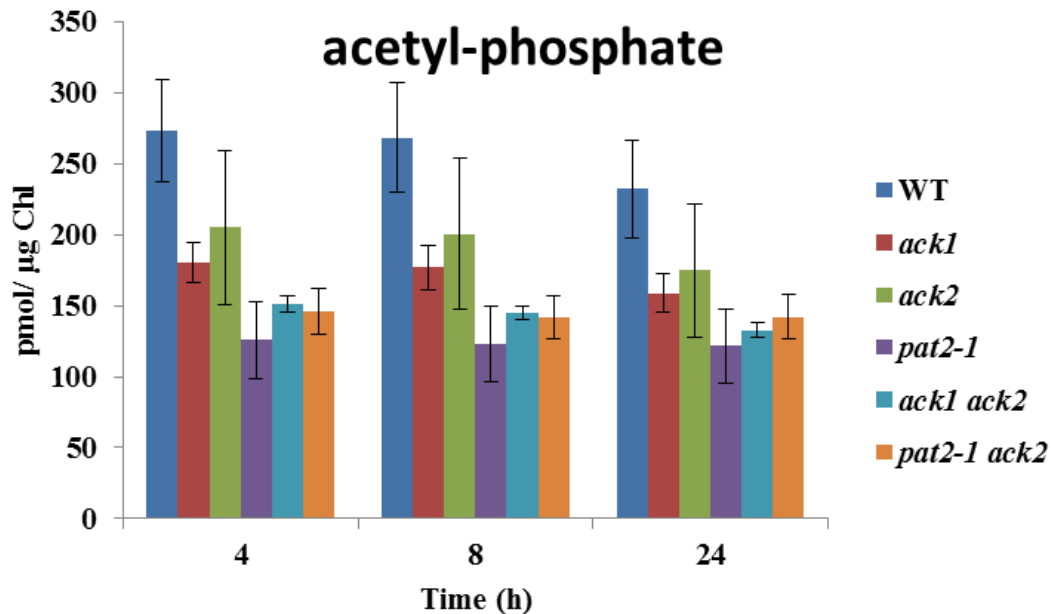
### Supplemental Figure 13. Alignments and Phylogenetic Analyses of Chlamydomonas ACS and ALDH Proteins.

Alignments of 4 ACS (A) and 8 ALDH (B) protein homologs along with phylogenetic analysis (C and D for ACS and ALDH, respectively). Protein IDs are given on the right. Alignments was performed using the online software <http://multalin.toulouse.inra.fr/multalin/> (Corpet, 1988). Identical or highly conserved amino acids in all positions are given in red letters (high consensus region), a low consensus amino acid position is given in blue letters, while black letters indicate an overall lack of strong consensus. The settings for the alignment were: Symbol comparison table: blosum62; Gap weight: 12; Gap length weight: 2; Consensus levels: high=90% low=50%. MrBAYES (via the Cipres Portal) was used to generate the trees (<https://www.phylo.org/portal2/>) (Miller et al., 2010). Newicktops (via the Mobyly Portal) was used to draw the trees <http://mobyly.pasteur.fr/>) (Bertrand et al., 2009). The numbers associated with the phylogenies (C and D) are boot strap values.



**Supplemental Figure 14. Changes in Levels of Putative Acetyl-CoA Synthetase Transcripts.**

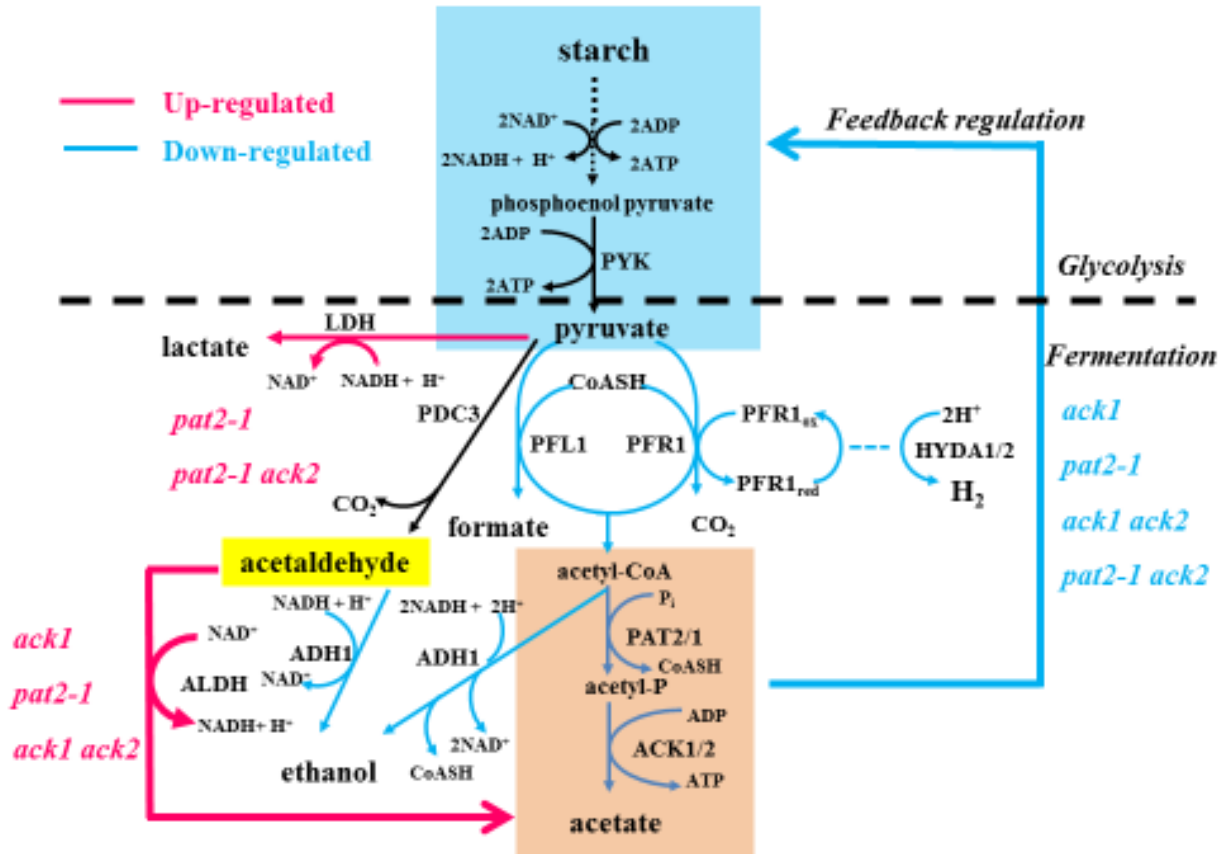
RT-qPCR was used to assess transcript levels of 4 ACS genes in the indicated mutants. Error bars represent SD.



**Supplemental Figure 15. Accumulation of Intracellular Acetyl-Phosphate.**

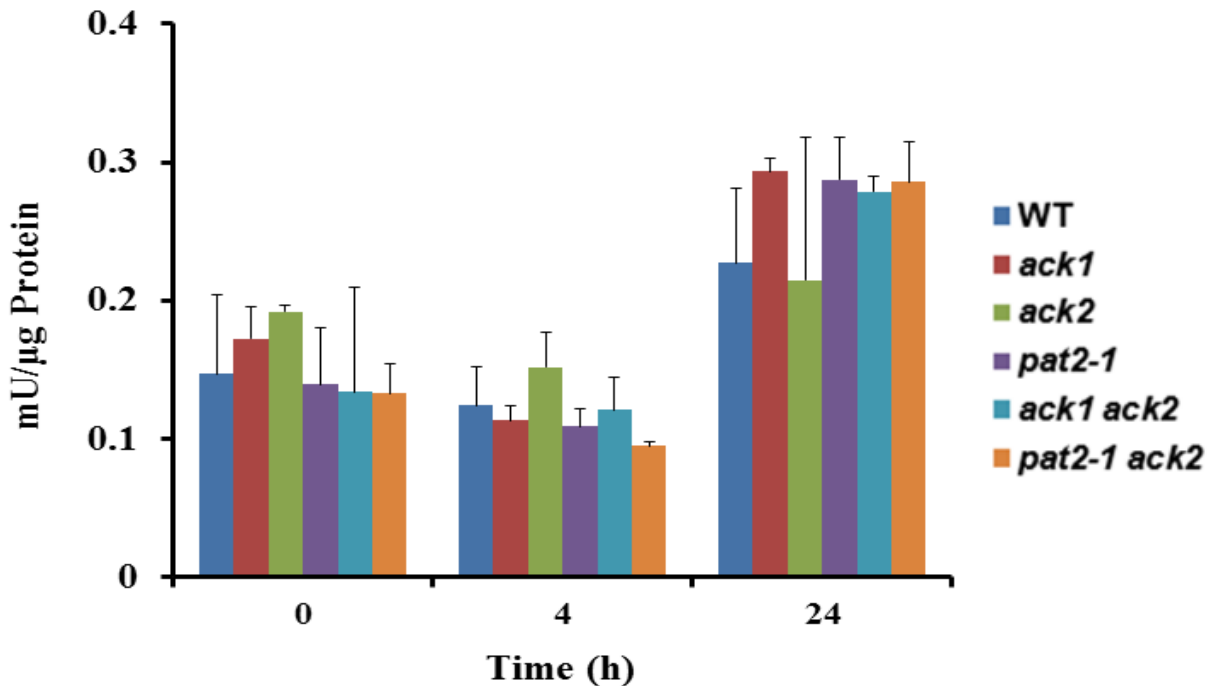
Acetyl-phosphate was measured in the indicated mutants after 4, 8 or 24 h of dark anoxic acclimation. Errors bars represent SD.





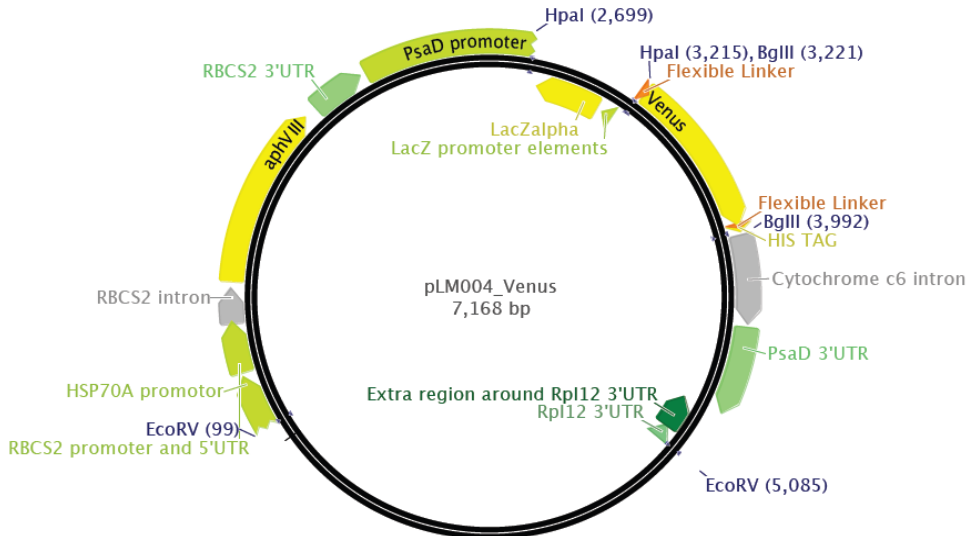
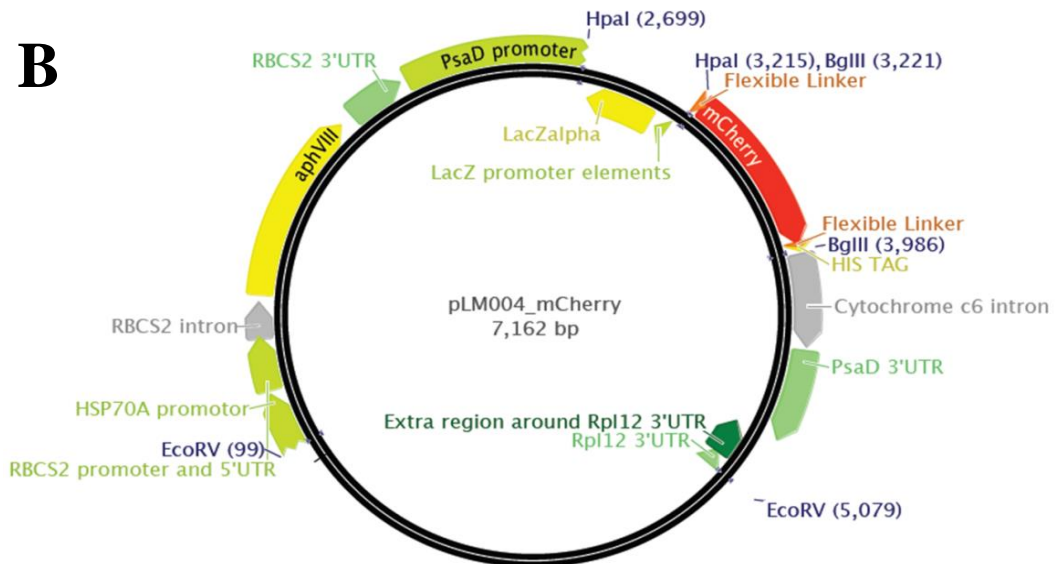
**Supplemental Figure 16. Proposed Anoxic Metabolisms in Single and Double Mutants.**

The light blue background highlights the glycolytic breakdown of starch, and the orange-brown background represents the acetate-producing pathways by the chloroplast and mitochondrial PAT-ACK enzymes. The blue arrows represent reactions and pathways that appear to be suppressed in a subpopulation of mutants (as indicated), while the red arrows represent pathways that appear to be more active in a subpopulation of mutants (as indicated). The black dashed line separates glycolysis from the fermentative pathways of metabolism. The blue dashed line represents omitted electron transfer components involved in hydrogenase reduction (e.g. ferredoxin). The black dashed arrow represents the omitted pathways from starch to phosphoenol pyruvate. Acetaldehyde is highlighted (yellow) to emphasize that the ALDH pathway may be active in some of the mutant strains.



**Supplemental Figure 17. *In Vitro* Aldehyde Dehydrogenase Activity.**

Aldehyde dehydrogenase activity in WT and the indicated mutants after acclimation to dark, anoxic conditions. Errors bars represent SD. One unit (U) is the amount of enzyme that will generate 1.0 mol of NADH per min at pH 8 at room temperature.

**A****B****Supplemental Figure 18. Maps of Plasmids used for Protein Subcellular Localization.**

Schematic representations of the pLM004\_Venus (A) and pLM004\_mCherry (B) plasmids, which were used to generate fluorescent fusion proteins for subcellular localization of the ACK and PAT enzymes. Additional information provided in the Methods.

**Supplemental Table 1. Intracellular Pyruvate Levels.**

Intracellular concentrations of pyruvate during a transition from photoautotrophic growth to fermentative metabolism. Values are presented as fg pyruvate cell<sup>-1</sup>. Values shown are the range of biological duplicates. Two replicate samples of each biological duplicate were analyzed. Single values represent the technical average of a single biological replicate.

<b>Time (h)</b>	<b>WT</b>	<b><i>ack1</i></b>	<b><i>ack2</i></b>	<b><i>ack1ack2</i></b>	<b><i>pat2-1</i></b>	<b><i>pat2-1ack2</i></b>
<b>4</b>	1.3 – 1.4	0.9 – 2.5	1.7 – 1.8	2.0 – 3.8	2.1 – 4.2	2.8
<b>8</b>	1.1 – 1.3	0.2 – 0.5	0.6 – 1.8	2.3 – 5.1	1.9	0.7 – 0.9
<b>24</b>	0.3 – 0.6	0.6 – 0.6	0.6 – 0.6	0.4 – 0.9	0.9 – 1.1	0.4 – 0.7

**Supplemental Table 2. Primers Used in this Study.**

**(A).** Primers used for the genetic screen to identify the mutants, to amplify the genes for complementation, and for genotyping progenies of the genetic crosses.

**(B).** Primers for generating the constructs used for subcellular localization.

**(C).** Primers used for qRT-PCR.

Gene	Use of Primers	Orientation	Sequence	T <sub>m</sub>
<b>A. Primers used for the genetic screen to identify the mutants, to amplify the genes for complementation, and for genotyping progenies of the genetic crosses</b>				
ACK1-F1	Mutant Screen	forward	5'- ATACCTAGAATGCTGGCTGGC -3'	60.6 °C
ACK1-R1	Mutant Screen	reverse	5'- GCATGTCACAGCGAGGAGT -3'	60.0 °C
ACK1-F2	Mutant Screen	forward	5'- ACTCCTCGCTGTGACATGC -3'	60.0 °C
ACK1-R2	Mutant Screen	reverse	5'- AAGCAGGAGGAAGGAGAAGG -3'	60.0 °C
ACK1-F3	Mutant Screen	forward	5'- CCTTCTCCTTCTCCTGCTT -3'	60.0 °C
ACK1-R3	Mutant Screen	reverse	5'- CACCACCCACCTGACTTCTT -3'	60.0 °C
ACK1-F4	Mutant Screen	forward	5'- AAGAAGTCAGGTGGGTGGTG -3'	60.0 °C
ACK1-R4	Mutant Screen	reverse	5'- GCAGCTGCCGTAGATCTGTA -3'	59.2 °C
ACK1-F5	Mutant Screen	forward	5'- TACAGATCTACGGCAGCTGC -3'	59.2 °C
ACK1-R5	Mutant Screen	reverse	5'- TGTAGGTCCCTTCTCCATCG -3'	60.0 °C
ACK2-F1	Mutant Screen	forward	5'- GTATCGCGAGAAACGATGC -3'	59.4 °C
ACK2-R1	Mutant Screen	reverse	5'- CAACAGGTGGAGGTGTGATG -3'	60.0 °C
ACK2-F2	Mutant Screen	forward	5'- CATCACACCTCCACCTGTTG -3'	60.0 °C
ACK2-R2	Mutant Screen	reverse	5'- CAAGCAGATGAGGACAAGCA -3'	60.1 °C
ACK2-F3	Mutant Screen	forward	5'- TGCTTGTCTCATCTGCTTG -3'	60.1 °C
ACK2-R3	Mutant Screen	reverse	5'- TACAATGCCTTGCACGAGAG -3'	60.0 °C
ACK2-F4	Mutant Screen	forward	5'- CTCTCGTGCAAGGCATTGTA -3'	60.0 °C
ACK2-R4	Mutant Screen	reverse	5'- CTGATGACCGGGCAGAGTAT -3'	60.0 °C
ACK2-F5	Mutant Screen	forward	5'- ATACTCTGCCGGTCATCAG -3'	60.0 °C
ACK2-R5	Mutant Screen	reverse	5'- GAGGGAACCCATCACAACCTG -3'	60.4 °C
ACK2-F6	Mutant Screen	forward	5'- CAGTTGTGATGGGTTCCTC -3'	60.4 °C
ACK2-R6	Mutant Screen	reverse	5'- CACCTGCAGGTCTGCTC -3'	60.6 °C
PAT2-F1	Mutant Screen	forward	5'- ATCGAATACTTTCGGCTCGA -3'	59.8 °C
PAT2-R1	Mutant Screen	reverse	5'- ACCGTATGGAACGCAGGA -3'	60.1 °C
PAT2-F2	Mutant Screen	forward	5'- TCCTGCGTTCCATACGGT -3'	60.1 °C
PAT2-R2	Mutant Screen	reverse	5'- TCACCGCACGGTTGTAGTAG -3'	59.8 °C
PAT2-F3	Mutant Screen	forward	5'- CTACTACAACCGTGCGGTGA -3'	59.8 °C
PAT2-R3	Mutant Screen	reverse	5'- TCTCTACGGGGGCTAAAAGC -3'	60.7 °C
PAT2-F4	Mutant Screen	forward	5'- GCTTTTAGCCCCGTAGAGA -3'	60.7 °C
PAT2-R4	Mutant Screen	reverse	5'- TTTCCCCTCTACAGCTTCCA -3'	59.8 °C
PAT2-F5	Mutant Screen	forward	5'- TGGAAGCTGTAGAGGGGAAA -3'	59.8 °C
PAT2-R5	Mutant Screen	reverse	5'- GTGCTCCGCTTCGTCTACTC -3'	60.2 °C
PAT2-F6	Mutant Screen	forward	5'- GAGTAGACGAAGCGGAGCAC -3'	60.2 °C

PAT2-R6	Mutant Screen	reverse	5'- CTATGCCACCACAGCATGTC -3'	60.1 °C
PAT2-F7	Mutant Screen	forward	5'- GACATGCTGTGGTGGCATAG -3'	60.1 °C
PAT2-R7	Mutant Screen	reverse	5'- CGTCACACACACAGGTGACA -3'	60.3 °C
PAT2-F8	Mutant Screen	forward	5'- TGTCACCTGTGTGTGTGACG -3'	60.3 °C
PAT2-R8	Mutant Screen	reverse	5'- GAGAGCGTGTGTGCATTTGT -3'	60.0 °C
PAT2-F9	Mutant Screen	forward	5'- ACAAATGCACACACGCTCTC -3'	60.0 °C
PAT2-R9	Mutant Screen	reverse	5'- AGCCCAAACAAGGGGTGT -3'	60.4 °C
PAT2-F10	Mutant Screen	forward	5'- ACACCCCTTGTTTGGGCT -3'	60.4 °C
PAT2-R10	Mutant Screen	reverse	5'- CCACAACCACGCAAGACA -3'	60.3 °C
PAT2-F11	Mutant Screen	forward	5'- TGTCTTGCGTGGTTGTGG -3'	60.3 °C
PAT2-R11	Mutant Screen	reverse	5'- GACAGTGACATTGCCGTTTG -3'	60.2 °C
PAT2-F12	Mutant Screen	forward	5'- CAAACGGCAATGTCACGTGTC -3'	60.2 °C
PAT2-R12	Mutant Screen	reverse	5'- CGCCCTACACAACCAAGAG -3'	59.3 °C
RB1	Mutant Screen	forward	5'- ATGGGGCGGTATCGGAGGAAAAG-3'	60.0 °C
RB2	Mutant Screen	forward	5'- TACCGGCTGTTGGACGAGTTCTTCTG -3'	60.0 °C
RIM1	Mutant Screen	reverse	5'- GCTGGCAGGAGTACGGGTTG -3'	58.0 °C
MID-UP	Mating type	forward	5'- ATGGCCTGTTTCTTAGC -3'	61.6 °C
MID-LOW	Mating type	reverse	5'- CTACATGTGTTTCTTGACG-3'	52.0 °C
FUS-UP	Mating type	forward	5'- ATGCCTATCTTTCTCATTCT-3'	52.0 °C
FUS-LOW	Mating type	reverse	5'- GCAAATAACACGTCTGGAAG-3'	52.0 °C
Paro-up	Southern Blot	forward	5'- ATGGGGCGGTATCGGAGGAAAAG -3'	60.0 °C
Paro-dw	Southern Blot	reverse	5'- TACCGGCTGTTGGACGAGTTCTTCTG -3'	60.0 °C
ACK1-COM-F1	Complementation	forward	5'- GAATTCCAAAGACGCAAACATATACG-3'	56.2 °C
ACK1-COM-R1	Complementation	reverse	5'- TAGGATCCAATTATCCGAGCTACACACT-3'	56.8 °C
ACK1-COM-F2	Complementation	forward	5'- GCACTAGCCACAAGTACCTGG-3'	60.0 °C
ACK2-COM-F1	Complementation	forward	5'- GAATTCCCGTAGGGGGTATTGATATACAA G-3'	59.9 °C
ACK2-COM-R1	Complementation	reverse	5'- TAGGATCCCCCTGTCACATTGACTCACG-3'	60.2 °C
PAT2-COM-F1	Complementation	forward	5'- GAATTCATGTCTCTGAACAGTAGCACTAT G-3'	58.5 °C
PAT2-COM-R1	Complementation	reverse	5'- GGATCCGGTGGGCATTGACAGACAA-3'	59.1 °C
PAT2-COM-F2	Complementation	forward	5'- CGACTACTACAACCGTGCGA-3'	59.9 °C
PAT2-COM-F3	Complementation	forward	5'- ATCACGCTGCTGGGAGAC-3'	59.9 °C

**B. Primers for generating the constructs used for subcellular localization**

ACK1-Loc-F	Localization	forward	5'- GTTAACATGCTGGCTGGCAAAGTGCCTGTC GGT -3'	60.0 °C
ACK1-Loc-R	Localization	reverse	5'- GGTAAACAGCGGCCAGCGCCGCGCC -3'	60.0 °C
ACK2-Loc-F	Localization	forward	5'- GGTAAACATGAAGCCGCGCAAATGGGAGC -3'	60.0 °C
ACK2-Loc-R	Localization	reverse	5'- GGTAACTGCCGCTGCGGCTCCCAA -3'	60.0 °C
PAT1-Loc-F	Localization	forward	5'- GGTAAACATGGCCTTCGCGAGCAGCAGCAT -3'	60.0 °C
PAT1-Loc-R	Localization	reverse	5'- GGTAAACCTTGGGCGCTGCTGCCGC -3'	60.0 °C

PAT2-Loc-F	Localization	forward	5'-GGTAAACATGTCTCTGAACAGTAGCACTAT GT -3'	60.0 °C
PAT2-Loc-R	Localization	reverse	5'-GGTAAACGAACGGCCGCGACGGCAGC -3'	60.0 °C
<b>C. Primers used for qRT-PCR</b>				
PFL1	qRT-PCR	forward	5'- ATGTACGCGAACACCCATGAA-3'	54.6 °C
PFL1	qRT-PCR	reverse	5'- GTCACCTGGGCGTACTTGAT-3'	57.1 °C
PFR1	qRT-PCR	forward	5'- CGCGCAGGGCACAATCACAC-3'	62 °C
PFR1	qRT-PCR	reverse	5'- ATCAGACGGCGCCACAAACACA-3'	61.9 °C
ACK1-B	qRT-PCR	forward	5'- CAACTGCGTGTGAAGGCTA-3'	57.4 °C
ACK1-B	qRT-PCR	reverse	5'- AAGGTGCTCGACACGTTCTC-3'	55.2 °C
ACK1-A	qRT-PCR	forward	5'- GCCGTACGACATGTACGAGA-3'	60.0 °C
ACK1-A	qRT-PCR	reverse	5'- AGGTGATGACGTTGGTCTCC-3'	60.0 °C
ACK2-B	qRT-PCR	forward	5'- GCAGCAGCTCGCTTAAGTTT-3'	56.2 °C
ACK2-B	qRT-PCR	reverse	5'- GCACTTTGAGCTCCCATTTG-3'	54.6 °C
PAT1	qRT-PCR	forward	5'- ATCAACTTCTTCGGCACCAT-3'	55.2 °C
PAT1	qRT-PCR	reverse	5'- ATGGAGGACACGAGGCTAGA -3'	54.8 °C
PAT2-B	qRT-PCR	forward	5'- ACCCTCTTCTCTCGGACAT-3'	57.4 °C
PAT2-B	qRT-PCR	reverse	5'- GTCGCCCTTGAGGTTAAACA-3'	55.2 °C
PAT2-A	qRT-PCR	forward	5'- GTTCATGCACACGCTCAAGT -3'	60.0 °C
PAT2-A	qRT-PCR	reverse	5'- CTCCCAGCAGCGTGATCT -3'	60.0 °C
HYDA1	qRT-PCR	forward	5'- CGGGAACGTGGGTAGCATTTAGG-3'	59.8 °C
HYDA1	qRT-PCR	reverse	5'- CGCCAAGGGTCCACATCAGAGT-3'	61.5 °C
HYDA2	qRT-PCR	forward	5'- GCGGGCAAGTTCTTCAATCT-3'	61.6 °C
HYDA2	qRT-PCR	reverse	5'- AGGCCGAATGACTCAGCAAT-3'	62.0 °C
PDC3	qRT-PCR	forward	5'- TACTCCACTGCCGGCTACTC-3'	58.6 °C
PDC3	qRT-PCR	reverse	5'- AGAGCCATGCGCTTGTAGAT-3'	61.6 °C
LDH	qRT-PCR	forward	5'- AGTTAGCCAAGGCGGGTGTG-3'	55.5 °C
LDH	qRT-PCR	reverse	5'- TGTTGAGCGCGAAGATGAGC-3'	61.6 °C
ADH1	qRT-PCR	forward	5'- GCACCTGCTGAACATCAAGA-3'	55.6 °C
ADH1	qRT-PCR	reverse	5'- AGGATGTGGGTGACCTTGTC-3'	56.9 °C
ACS1	qRT-PCR	forward	5'- TCCAACATCAACGTGGAGAA -3'	60.1 °C
ACS1	qRT-PCR	reverse	5'- GTCATTGCCCTCCCAAATA-3'	59.8 °C
ACS2	qRT-PCR	forward	5'- GTGCTGGTGTACGAGAACGA -3'	59.9 °C
ACS2	qRT-PCR	reverse	5'- CGAGGTGTACAGCAGGAACA -3'	59.9 °C
ACS3	qRT-PCR	forward	5'- ACTCATCTGGGAGGGCAAC -3'	60.1 °C
ACS3	qRT-PCR	reverse	5'- AGGTAGATGGTCACGGCATC -3'	60.0 °C
ACS4	qRT-PCR	forward	5'- GACGAGAAGGGCAACGAGT-3'	60.4 °C
ACS4	qRT-PCR	reverse	5'- GAAGTAGTAGCCGGGGAAGG-3'	60.1 °C
ALDH1	qRT-PCR	forward	5'- CGCAGACCTGATCGACTACA -3'	60.0 °C
ALDH1	qRT-PCR	reverse	5'- GTTGACCGGGTAGTTGAAGG -3'	59.5 °C
ALDH2	qRT-PCR	forward	5'- GGTTCATCGTTTCTTCGCTTC -3'	59.8 °C

ALDH2	qRT-PCR	reverse	5'- AACTCGGATTTGGTGGTCAG -3'	60.0 °C
ALDH3	qRT-PCR	forward	5'- CAGCTGGAGACACTGGACAA -3'	60.0 °C
ALDH3	qRT-PCR	reverse	5'- AAGGTGTAGGCCAGGTAGGG -3'	60.0 °C
ALDH4	qRT-PCR	forward	5'- CTACCGTCCTTGTTGCACCT -3'	60.1 °C
ALDH4	qRT-PCR	reverse	5'- TCTCATCCACCTGTGTGTGC -3'	60.8 °C
ALDH5	qRT-PCR	forward	5'- ATCTGGGAGGAGGAGGTGTT -3'	59.6 °C
ALDH5	qRT-PCR	reverse	5'- ATGTAGGTGGTGGCGAAGTC -3'	60.0 °C
ALDH6	qRT-PCR	forward	5'- GACAAAGGCACCTTCGATGT -3'	60.1 °C
ALDH6	qRT-PCR	reverse	5'- TGATGCTGCAGAATGAGGTC -3'	60.0 °C
ALDH7	qRT-PCR	forward	5'- CGAAGGTGCTGAAAGCCTAC -3'	60.0 °C
ALDH7	qRT-PCR	reverse	5'- GCCCTAGGCCAGTCGTA ACT -3'	60.7 °C
ALDH8	qRT-PCR	forward	5'- CCTACCGCGCTAAGTACCTG -3'	60.0 °C
ALDH8	qRT-PCR	reverse	5'- GACCCGCGTAGTAGTCAAG -3'	60.0 °C



**Supplemental Table 3. Constructs Used in this Study**

Use	Construct	Plasmid name	Insert or PCR product	Primers	Plasmid backbone	Cloning method
Localization	Venus	pLM004_Venus	-	-	pUC19	-
	Venus:ACK1	pLM004ACK1_Venus	ACK1CDS	ACK1-Loc-F/ ACK1-Loc-R1	pUC19	HpaI/HpaI
	Venus:ACK2	pLM004ACK2_Venus	ACK2CDS	ACK2-Loc-F/ ACK2-Loc-R1	pUC19	HpaI/HpaI
	Venus:PAT1	pLM004PAT1_Venus	PAT1CDS	PAT1-Loc-F/ PAT1-Loc-R1	pUC19	HpaI/HpaI
	Venus:PAT2	pLM004PAT2_Venus	PAT2CDS	PAT2-Loc-F/ PAT2-Loc-R1	pUC19	HpaI/HpaI
	mCherry	pLM004_mCherry	-	-	pUC19	-
	mCherry:ACK2	pLM004ACK2_mCherry	ACK2CDS	ACK2-Loc-F/ ACK2-Loc-R1	pUC19	HpaI/HpaI
	mCherry:PAT1	pLM004PAT1_mCherry	PAT1CDS	PAT1-Loc-F/ PAT1-Loc-R1	pUC19	HpaI/HpaI
Complementation	PSAD:ACK1	pJM43Ble_ACK1	ACK1 cDNA	ACK1-COM-F1/ ACK1-COM-R1	pSP124	EcoRI/BamHI
	PSAD:ACK2	pJM43Ble_ACK2	ACK2 cDNA	ACK2-COM-F1/ ACK2-COM-R1	pSP124	EcoRI/BamHI
	PSAD:PAT2	pJM43Ble_ACK2	PAT1 cDNA	PAT2-COM-F1/ PAT2-COM-R1	pSP124	EcoRI/BamHI