Regulation of the expression of ethylene biosynthesis genes *in Hevea brasiliensis* shoots

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IRRDB Workshop Biotec 2009
on Hevea Genome & Transcriptome

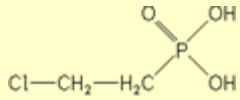
3-5 June 2009 - Montpellier, France

Natural Rubber Production: Tapping & Ethephon Stimulation



Ethephon

(2-chloroethyl-phosphonic acid)

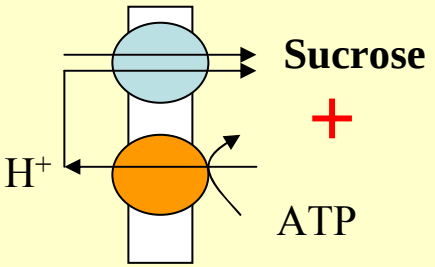


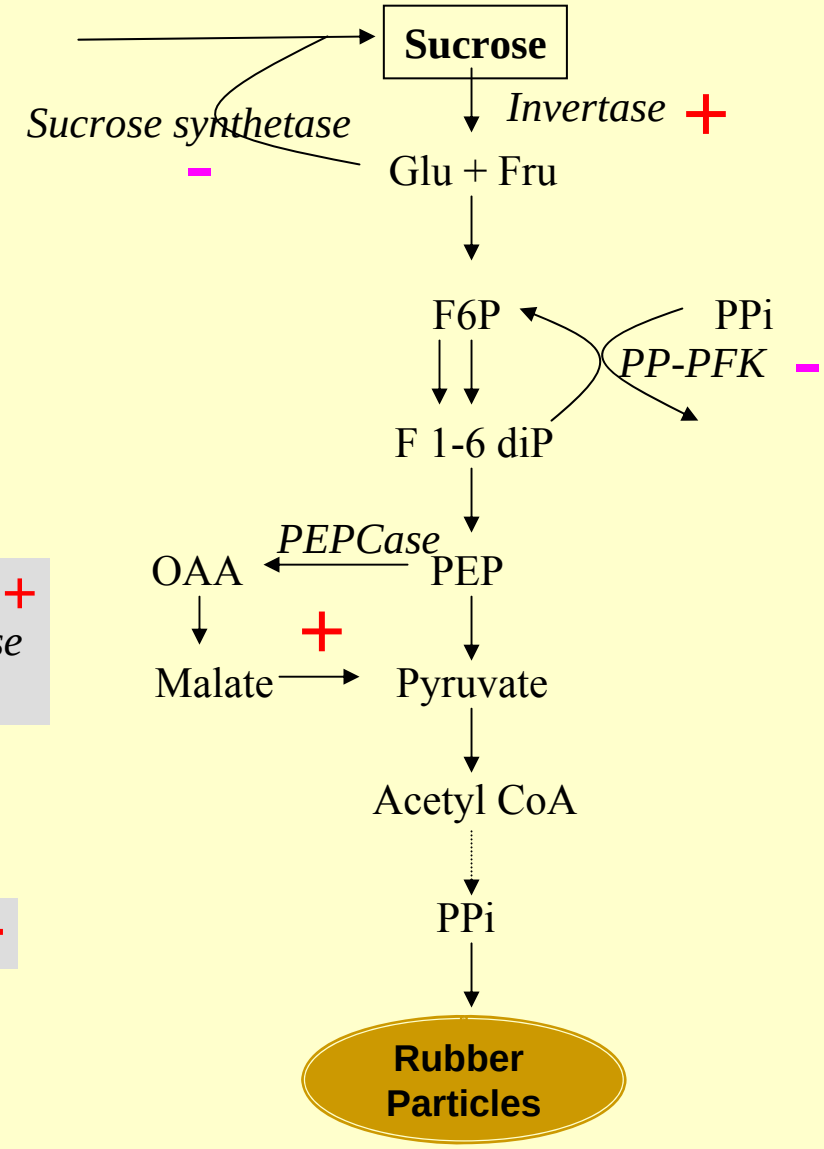
In-situ latex regeneration → **Latex low** → **Production**

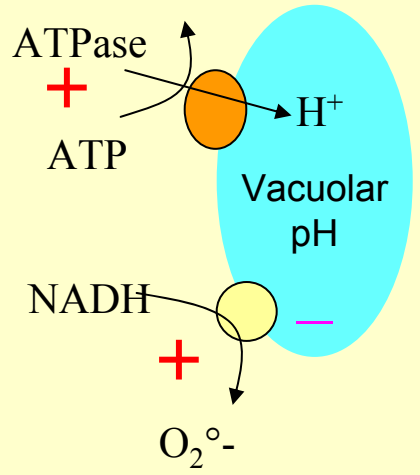
Tapping Panel Dryness: dry cut and brown bast

Stimulation of the laticifer metabolism by ethylene

(from J. d'Auzac)







Wounding

Chitinase

Hevein +

HRGP

SOD

cytosolic pH +

Glu + NH_4 +

↓ *Glutamine synthetase*

Gln

Calmoduline +

Phosphorylation

Nucleotides adenines +

ATP/ADP -

Latex biosynthesis +

Senescence

NADH oxydase +

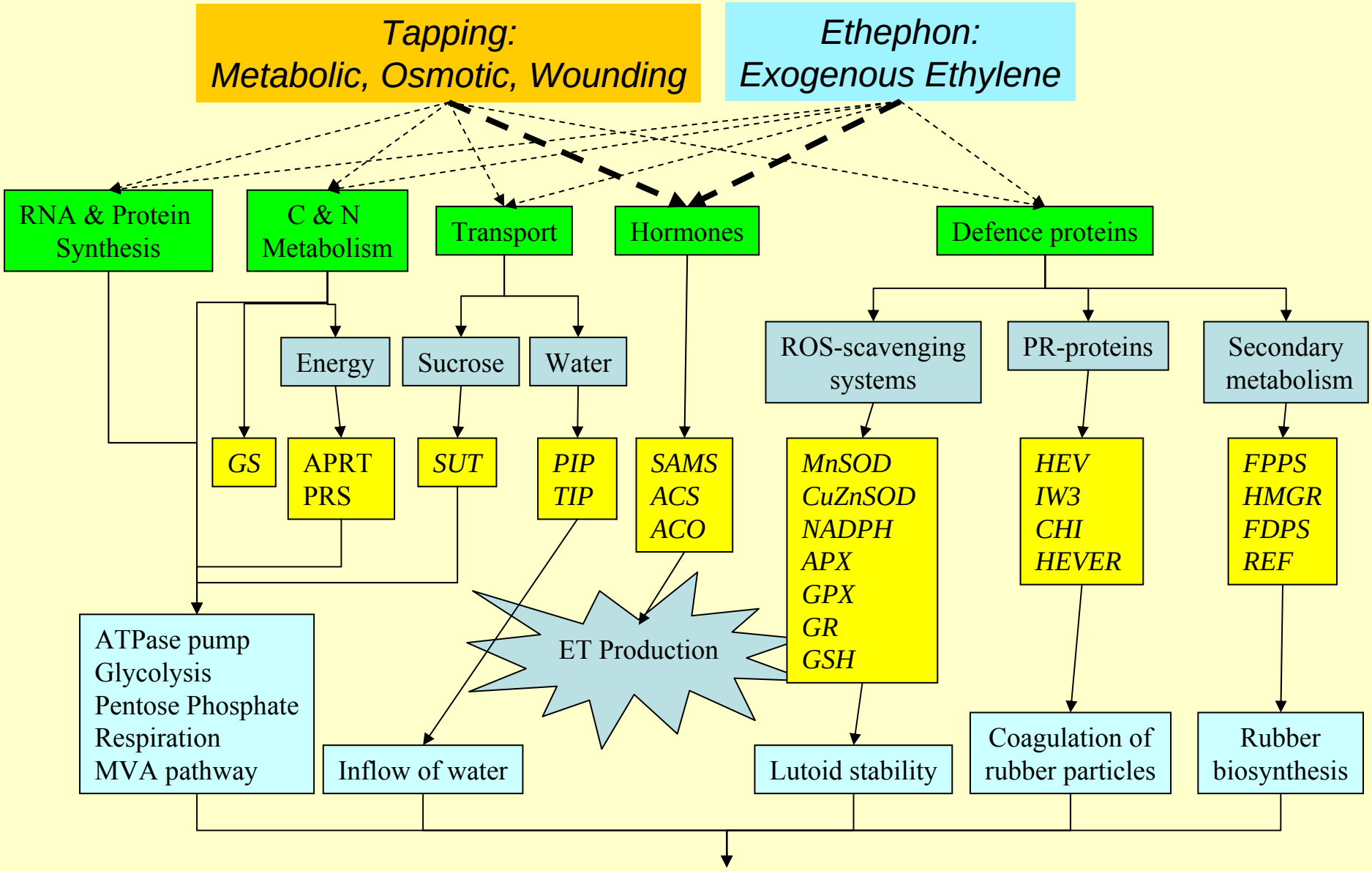
Peroxydase

Catalase -

SOD +

Thiols

Wounding & ethylene regulation of the gene expression in laticifers

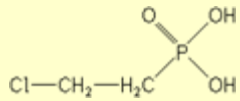


Latex production vs TPD

Exogenous & endogenous ethylene production in bark tissue

Ethephon

(2-chloroethyl-phosphonic acid)



In-situ latex regeneration → **Latex low** → **Production**

Siwey: Endogenous ethylene production in bark tissue after tapping and after ethephon treatment

Audley (1975): ethephon could not induce ethylene production in 2-year-old stem

Sivakumaran (1984): Endogenous ethylene concentration in bark were higher in wound-susceptible clones than wound-resistant clones

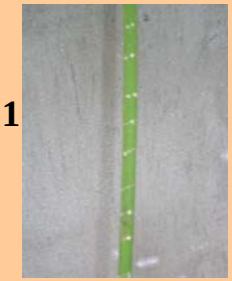
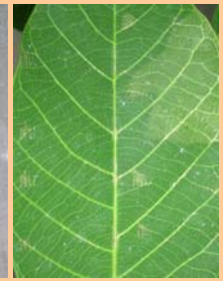
	Ethylene (nl/g/h)			
Tapping	Apparent	Exogenous	Endogenous	Endog./Exogenous
1	334	124	210	169 %
2	66	31	35	111 %
3	118	62	56	91 %
4	94	64	31	49 %

Releasing ethylene of bark tissues after ethephon treatment

A model to study the regulation of the gene expression by ethylene and wounding.

Application and symptoms observed after ethylene and wounding treatments in 3-month-old shoots

	Ethephon 2.5 %	Ethylene 1ppm
Day 0		
Day 2		
Day 4		
Day 7		

	Wounding	
Day 0		
Day 1		

Isolating & characterization of *ACS* and *ACO* genes involved in ethylene biosynthesis in *Hevea brasiliensis*

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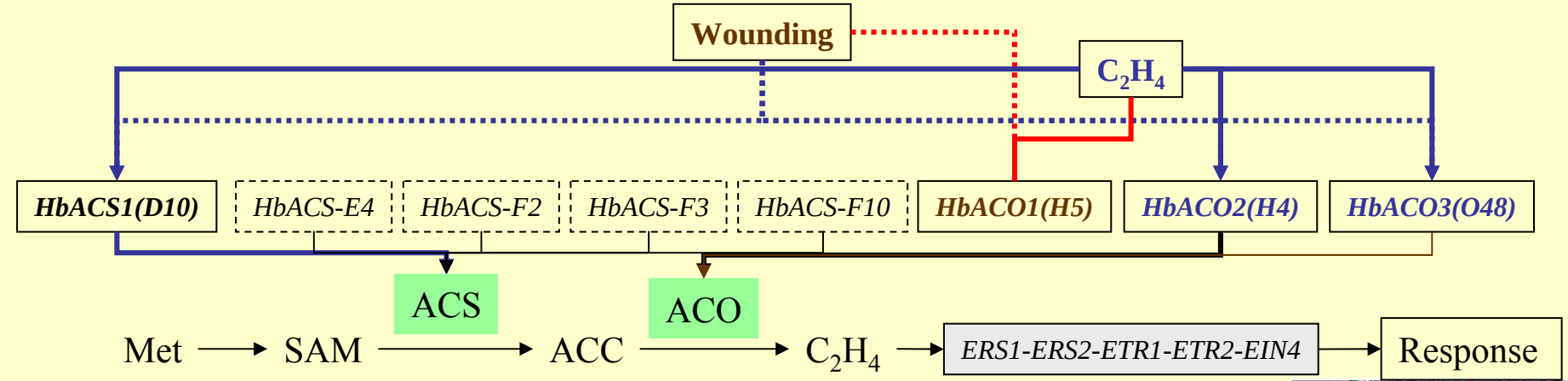
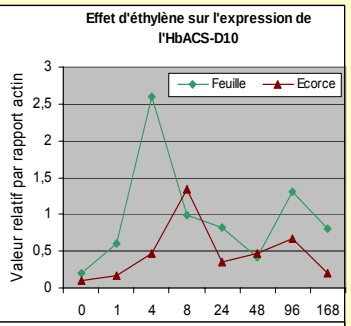
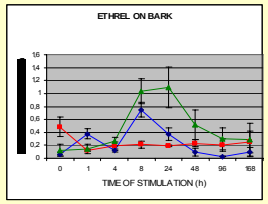
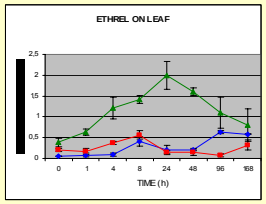
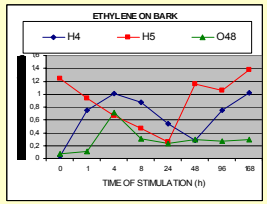
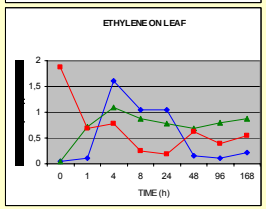


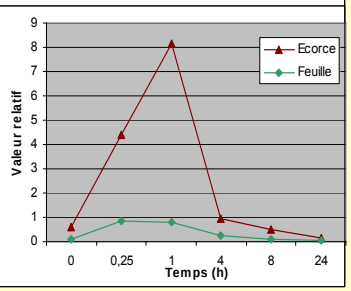


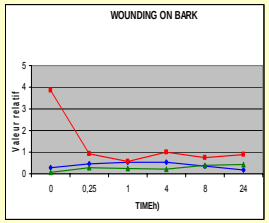


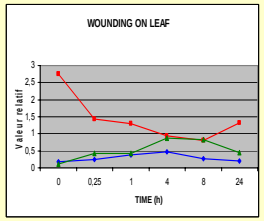


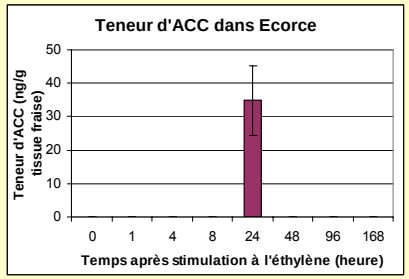


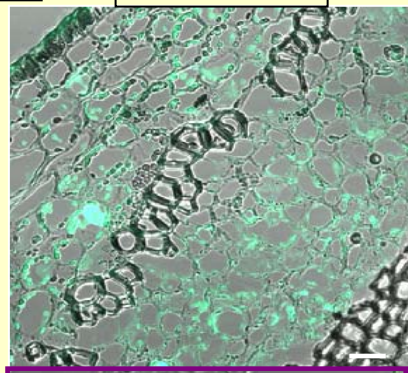


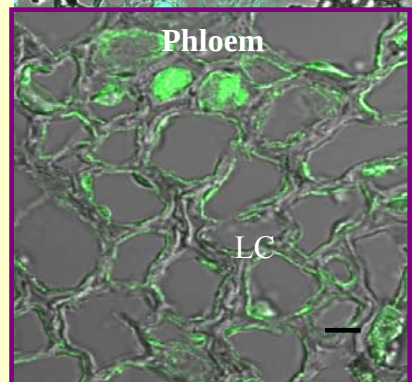






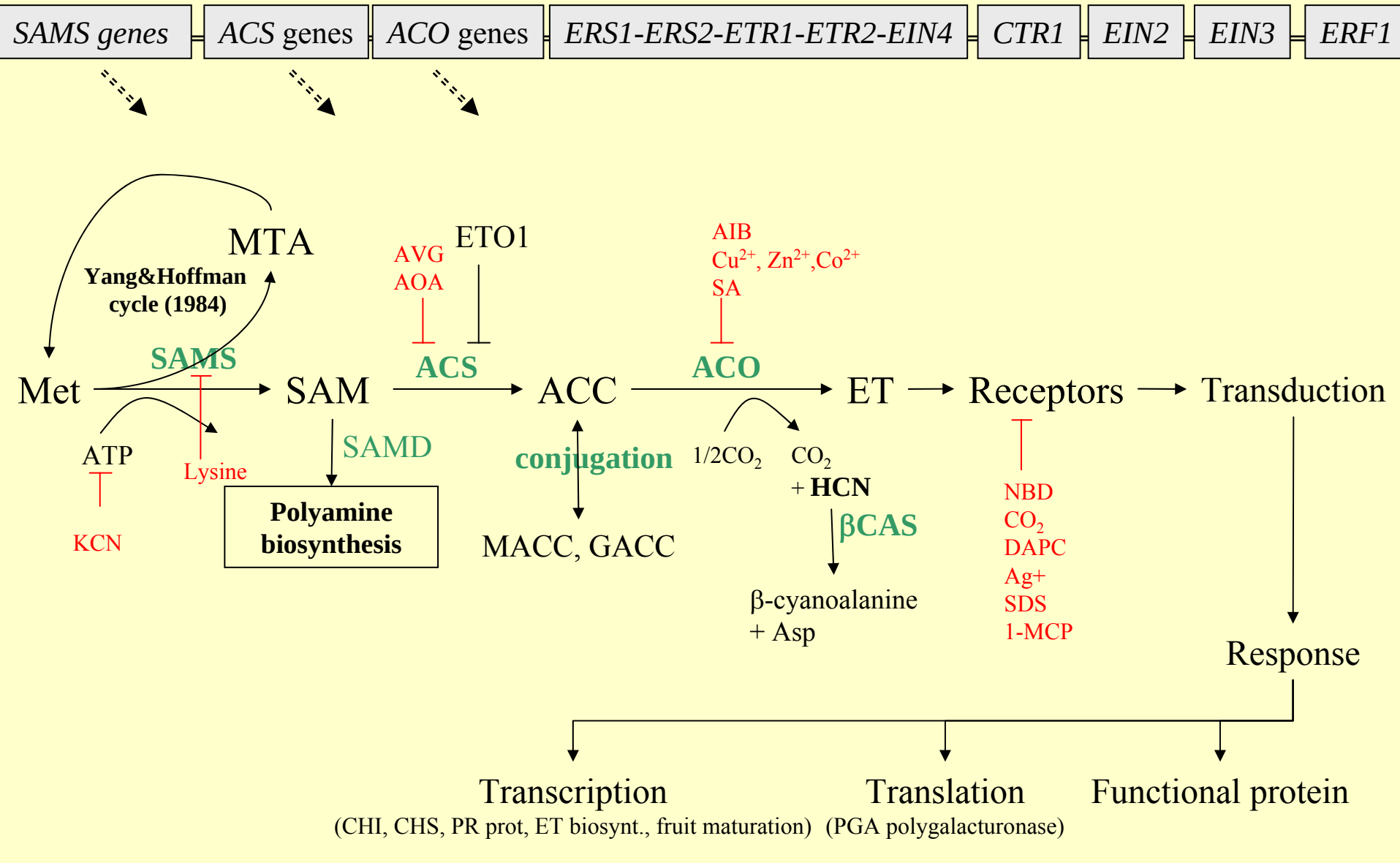






ACS amino-transferases (cofacteur : pyroxydal phosphate)
ACO super famille Fe $^{2+}$/Ascorbate oxydase.

Effect of wounding & ethylene on the expression of genes involved in ethylene biosynthesis and related pathways in 3 clones with contrasting metabolism



Step for the analysis of the gene expression using real-time RT-PCR Light Cycler 480 (Roche)

cDNA, EST, reference genes (Ubi, Actin, etc.)

Design of primers (CDS – 3'UTR)

Order of primers

Putative specific primers for a gene

Real-time PCR

Validation of primers
-Amplification curve
- Melting peak

Amplicon

Reamplification by PCR

Amplicon

Cloning & sequencing & Blast

Gene validation

Test with range of dilution 10^{-2} to 10^{-7}

Gene calibrator
(cloned gene into plasmid)

Plant samples
(3 independent samples per treatment)

Extraction

Total RNA

Reverse-transcription

cDNA

Pool of cDNAs

real-time PCR with range of cDNA dilutions (1/5-1/1600)

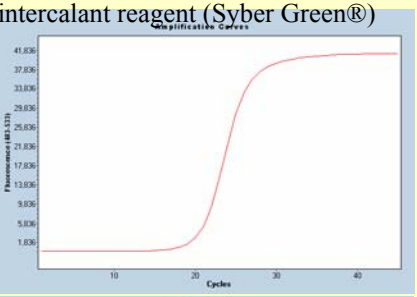
Standard curves
(PCR efficiency)

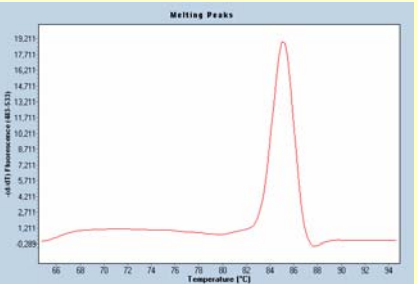
Real-time RT-PCR

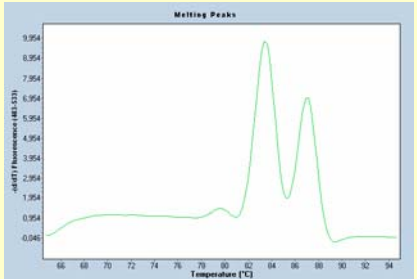
Relative quantification of the expression of each studied gene by qPCR

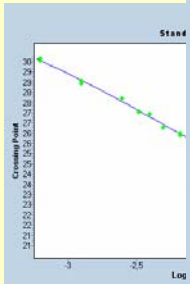
$$\text{Ratio} = \frac{\text{Efficiency}^{-\Delta\Delta(\text{Cp gene} - \text{Cp actin})}}{\text{Effciency}^{-\Delta\Delta(\text{Cp calibrator gene} - \text{Cp calibrator actin})}}$$

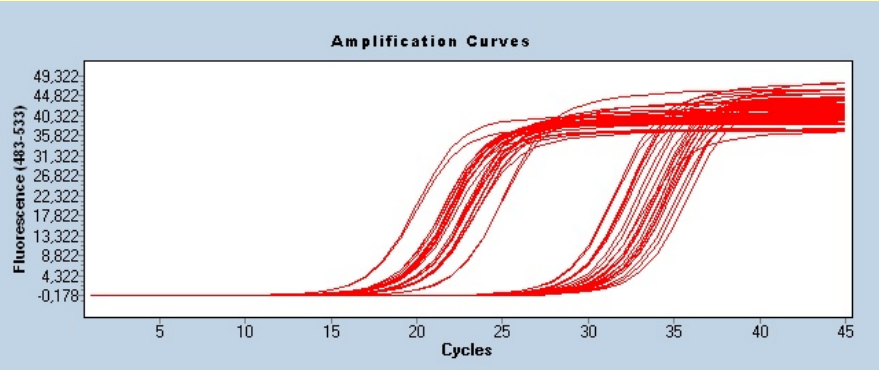
Quantity of amplicon using fluorescent intercalant reagent (Syber Green®)







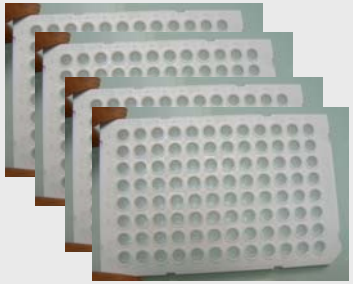




High-throughput gene expression analysis using 384-well plate on real-time PCR Light Cycler 480 (Roche)

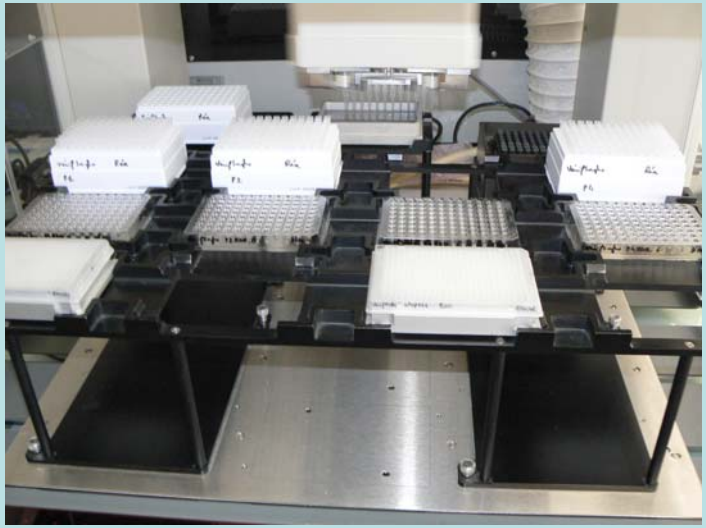
1. Manual preparation of 96-well plates

Primers, water & SyberGreen

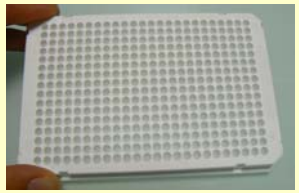


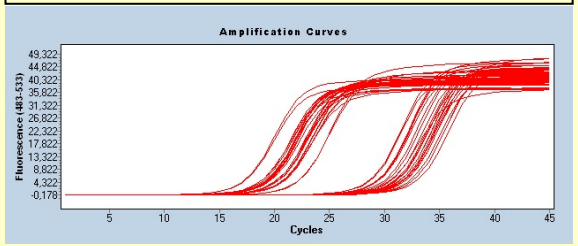
DNA templates

2. Preparation of 384-well plates using Robot Beckman Coulter



3. Real-time PCR analysis using 384-well plate on PCR Light Cycler 480 (Roche)



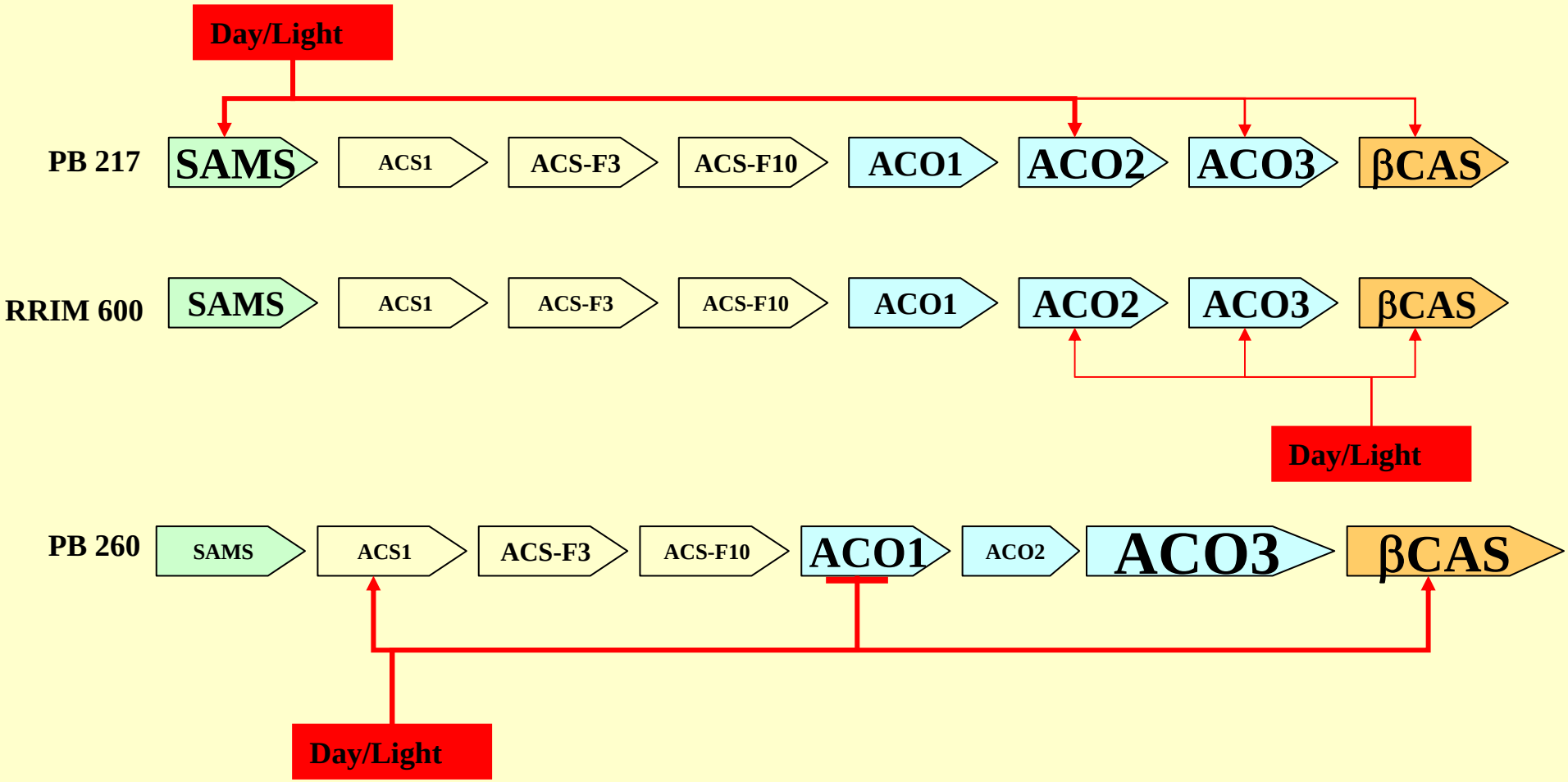


Comparison of the expression of genes involved in ethylene biosynthesis during daytime in three clones with constrasting metabolism.

Value must be multiplied by 10^{-4}. (*) a-b Fisher test (LSD). All values with the same letter are not significantly different (P <0.05). Each value is the mean of 3 replications.

Clone	Daytime	SAMS	ACS1	ACSF10	ACO1	ACO2	ACO3	CAS
PB 217	8h00	1953 ± 110^{b}	0.6 ± 0.5^{bc}	20 ± 8^{a}	35 ± 26^{bc}	817 ± 533^{b}	216 ± 113^{b}	819 ± 220^{bc}
	12h00	2997 ± 409^{a}	0.5 ± 0.3^{c}	19 ± 5^{a}	60 ± 54^{bc}	1031 ± 529^{b}	$257 \pm 1\ 10^{b}$	1339 ± 343^{b}
	16h00	3499 ± 1547^{a}	3.9 ± 4.9^{bc}	21 ± 5^{a}	41 ± 13^{bc}	4874 ± 3673^{a}	514 ± 247^{b}	1062 ± 118^{bc}
RRIM 600	8h00	413 ± 31^{c}	0.1 ± 0^{c}	1.4 ± 0.7^{b}	6 ± 3^{c}	55 ± 18^{b}	40 ± 21^{b}	214 ± 41^{c}
	12h00	509 ± 116^{c}	0.2 ± 0.2^{c}	1.9 ± 1.9^{b}	9 ± 9^{c}	118 ± 66^{b}	62 ± 58^{b}	293 ± 119^{c}
	16h00	496 ± 61^{c}	0.1 ± 0^{bc}	2.0 ± 0.7^{b}	4 ± 1^{c}	101 ± 19^{b}	107 ± 102^{b}	579 ± 268^{bc}
PB260	8h00	16 ± 7^{c}	4.1 ± 2.5^{bc}	12 ± 7^{ab}	507 ± 419^{a}	4 ± 3^{b}	273270 ± 128410^{a}	1531 ± 317^{b}
	12h00	16 ± 2^{c}	5.4 ± 1.1^{b}	24 ± 19^{a}	282 ± 162^{ab}	7 ± 9^{b}	354130 ± 188870^{a}	3803 ± 1266^{a}
	16h00	13 ± 4^{c}	14.7 ± 6.8^{a}	14 ± 11^{ab}	186 ± 80^{bc}	8 ± 2^{b}	314300 ± 260920^{a}	3794 ± 570^{a}

Day/light regulation of the accumulation of transcripts for genes encoding enzymes related to ethylene biosynthesis & cyanide detoxification in 3 clones with contrasting metabolism

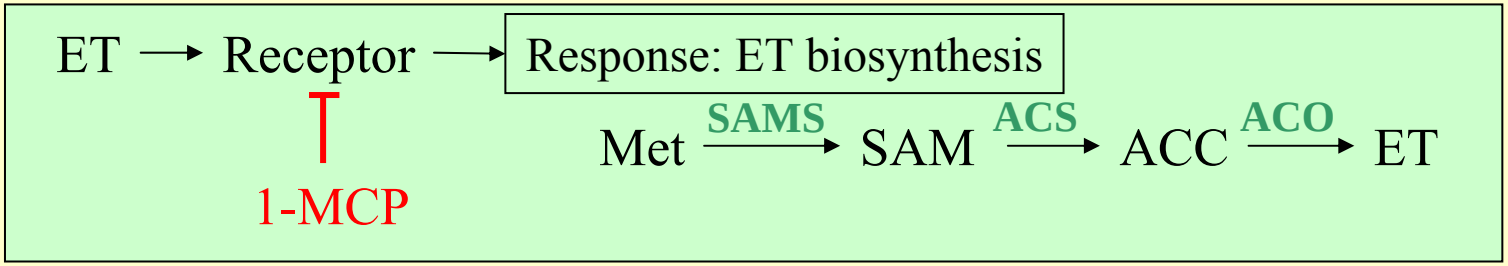


→ **The effect of a treatment must be related to the expression of a control sampled at the same daytime**

Up-regulated genes (p-Value<0.25) | Non-regulated genes | Down-regulated genes

Treatment		Clone	SAMS	ACS1	ACSF3	ACSF10	ACO1	ACO2	ACO3	CAS	UBI
Wounding	**15 min**	PB217	1,6	41,4	4,4	9,6	0,6	2,4	0,8	1,0	0,3
		RRIM 600	1,1	30,5	0,6	11,3	0,5	1,5	0,4	0,8	1,2
		PB 260	1,8	42,8	0,1	9,8	0,2	2,0	0,5	0,3	1,9
MCP/Wounding	**15 min**	PB217	1,2	4,3	0,2	5,9	0,3	0,4	0,0	0,8	0,6
		RRIM 600	1,0	8,5	3,4	1,3	0,1	1,2	0,2	1,2	0,8
		PB 260	1,2	52,1	19,6	9,2	1,2	2,5	0,8	1,4	1,7
Wounding	**4h**	PB217	1,4	7,0	65,5	3,3	0,7	7,1	1,5	1,1	1,1
		RRIM 600	4,2	3,0	4,0	2,8	0,5	3,0	1,4	0,9	0,8
		PB 260	1,3	5,6	4,9	0,5	0,2	4,8	1,4	1,5	0,6
MCP/Wounding	**4h**	PB217	0,8	2,3	15,5	0,8	1,6	7,5	53,2	1,6	1,1
		RRIM 600	0,7	1,0	1,4	0,7	1,5	1,2	0,8	1,1	0,9
		PB 260	1,7	3,7	5,2	1,3	2,1	2,6	1,5	1,3	1,7
Ethylene	**4h**	PB217	1,0	1,0	7,0	0,5	0,4	0,9	0,4	0,6	0,5
		RRIM 600	0,9	1,7	17,5	2,1	1,9	1,3	0,9	1,0	0,8
		PB 260	1,7	1,8		0,5	1,3	1,6	1,7	2,8	1,4
MCP/Ethylene	**4h**	PB217	0,6	1,6	0,4	1,1	1,6	1,7	0,4	0,9	0,9
		RRIM 600	1,3	0,6	1,3	1,2	0,9	0,8	1,0	1,1	1,0
		PB 260	1,3	5,9		1,7	1,4	1,1	1,7	1,4	0,8
Ethylene	**24h**	PB217	1,1	1,1	3,1	1,4	0,3	1,4	0,4	1,5	1,7
		RRIM 600	1,0	5,1	0,4	1,2	0,5	1,6	2,0	1,7	1,5
		PB 260	0,7	1,2		0,5	0,9	0,9	0,8	0,9	0,5
MCP/Ethylene	**24h**	PB217	0,9	5,5	0,7	0,2	0,4	1,1	0,2	0,2	1,8
		RRIM 600	1,3	0,8	33,1	1,5	1,2	0,7	0,8	0,9	1,4
		PB 260	1,4	2,2		1,9	0,5	1,6	1,1	0,5	1,3

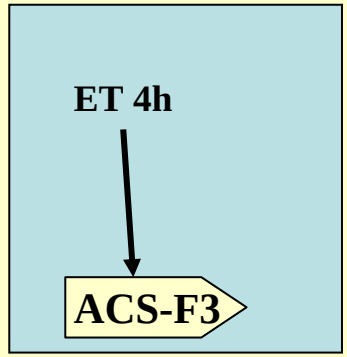
Ratio of up- or down-regulation calculated with the level of gene expression in treated & control tissues sampled at the same daytime

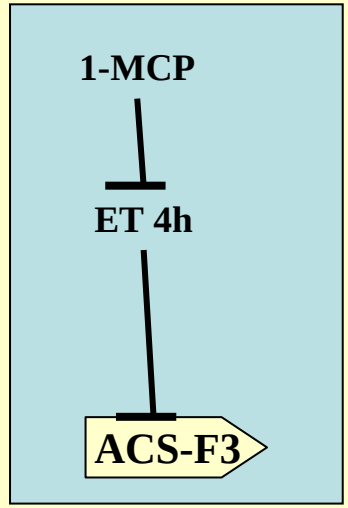


Regulation of the expression of gene *ACS-F3* in clone RRIM 600 upon ethylene treatment. Three biological replicates

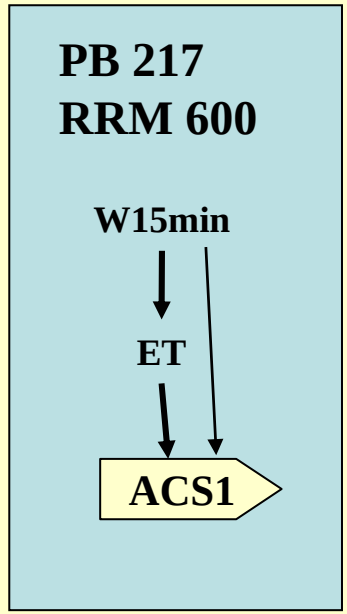
Pretreatment	Treatment		Expression ACSF3
None	**Control (8h00-12h00)**	Mean	0.00261
None	**Ethylene 4h (8h00-12h00)**	Mean	0.04560
		Ratio	17,5
		p-value	0,21
1-MCP 16h	**Control (8h00-12h00)**	Mean	0.01040
1-MCP 16h	**Ethylene 4h (8h00-12h00)**	Mean	0.01335
		Ratio	1,3
		p-value	0,69

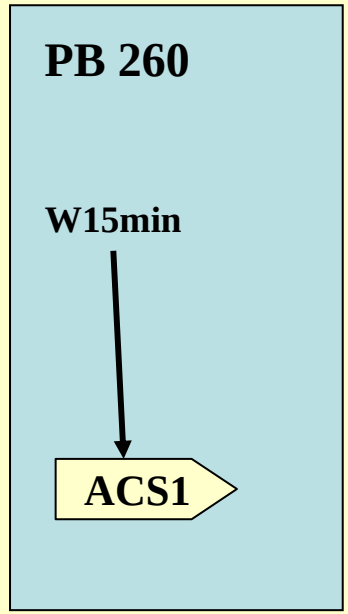
				ACSF3
Ethylene	4h	RRIM 600	ratio	17,5
			p-value	0,21
MCP/Ethylene	4h	RRIM 600	ratio	1,3
			p-value	0,69



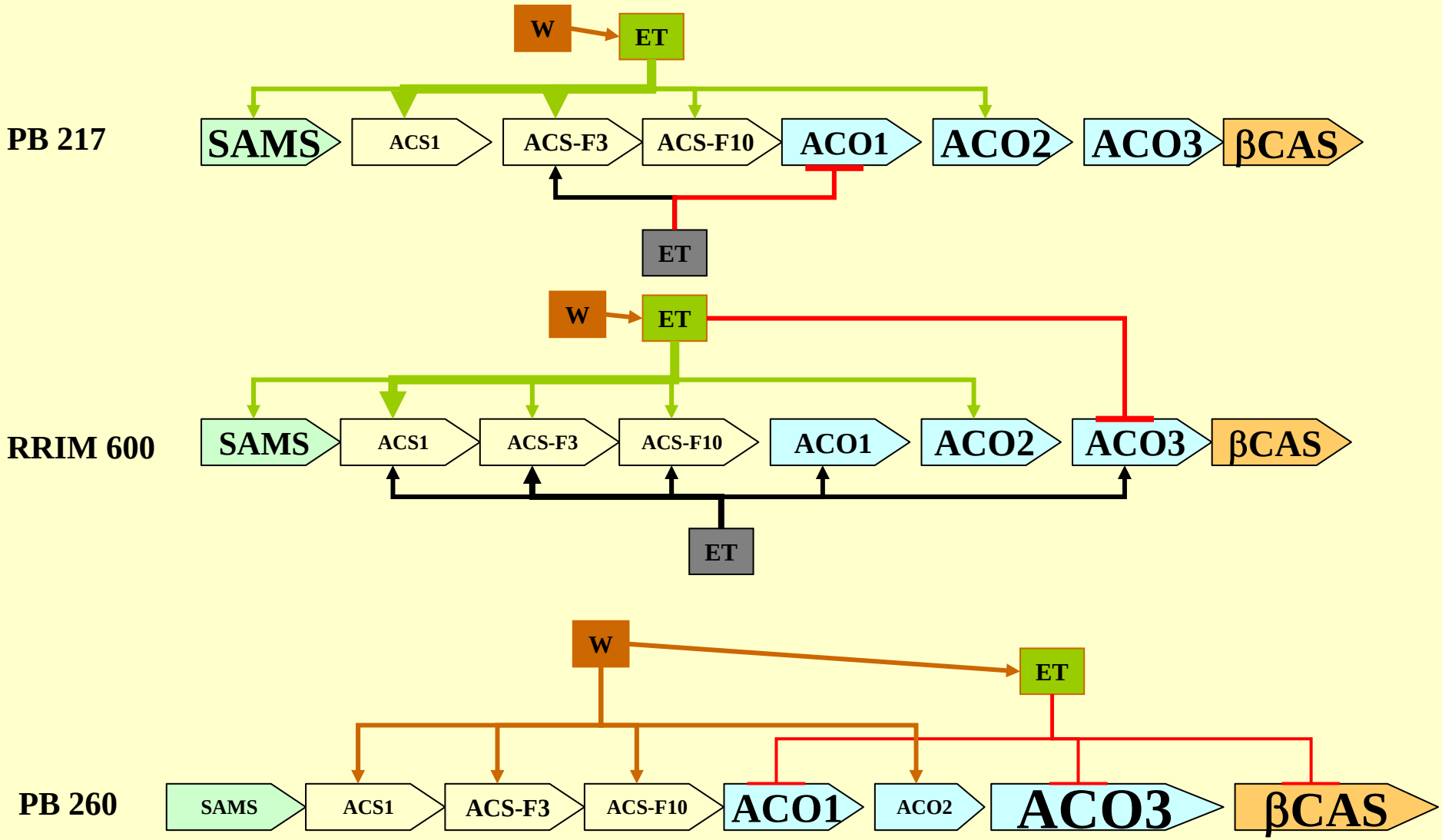


Treatment		Clone		ACS1
Wounding	15 min	PB217	ratio	41,4
			p-value	0,10
		RRIM 600	ratio	30,5
			p-value	0,24
		PB 260	ratio	42,8
			p-value	0,21
MCP/Wounding	15 min	PB217	ratio	4,3
			p-value	0,18
		RRIM 600	ratio	8,5
			p-value	0,13
		PB 260	ratio	52,1
			p-value	0,21

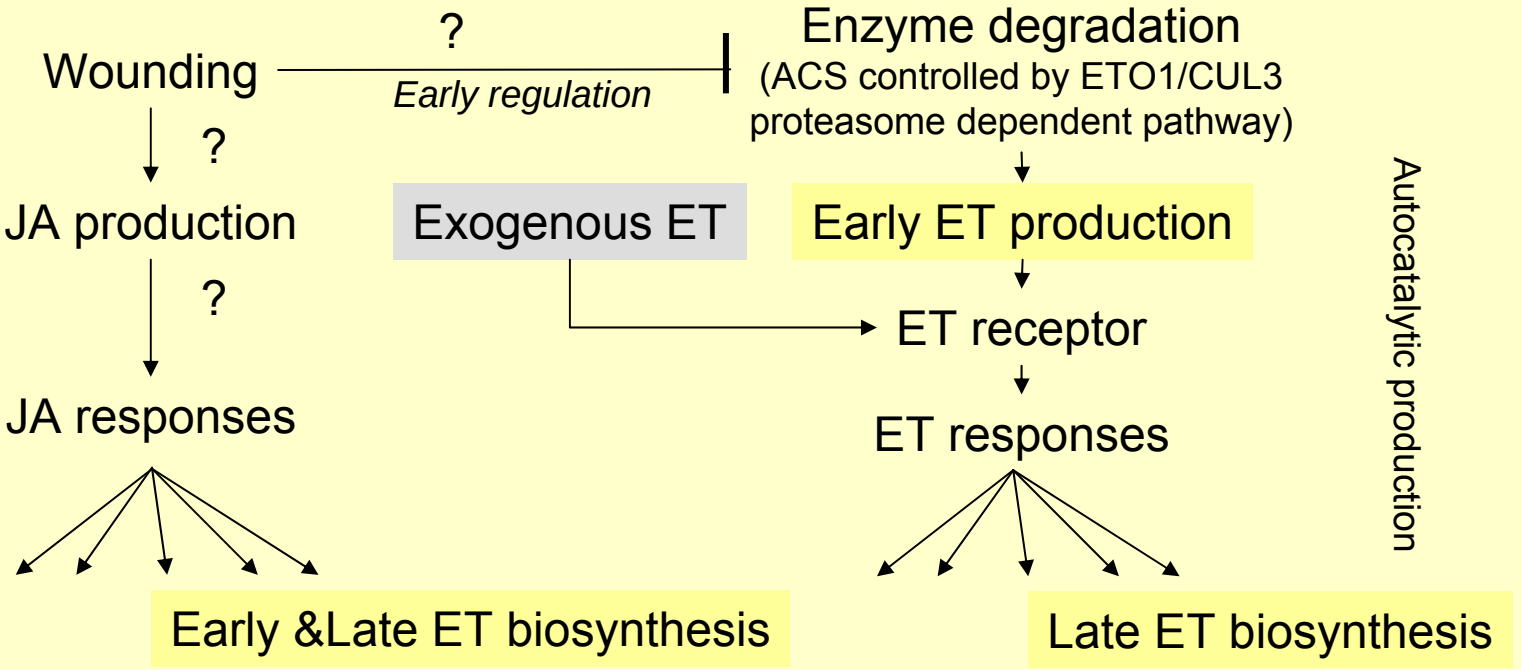


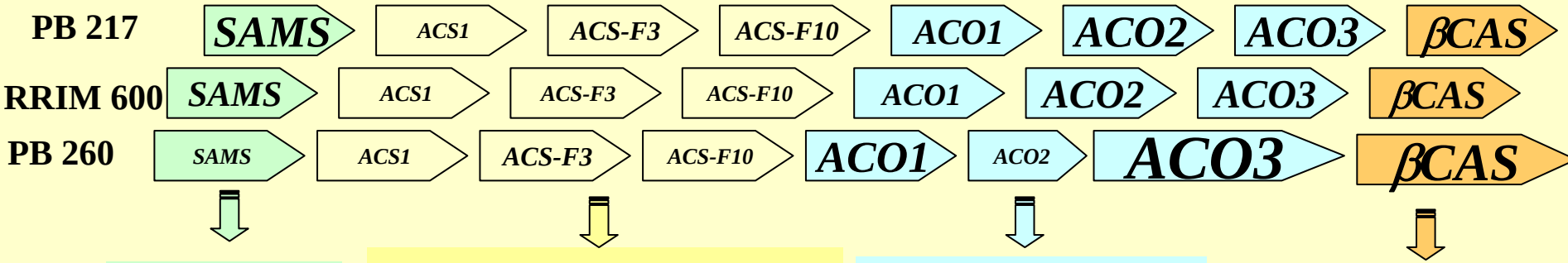


Early and late wounding & ethylene effects on the accumulation of transcripts for genes encoding enzymes related to ethylene biosynthesis & cyanide detoxification



Regulation of the ethylene biosynthesis





SAMS
limiting factor
in PB 260 ?

ACS
very low & transient
all studied clones
highly regulated by wounding

ACO
clonal variation
source of variability
for genetic studies

βCAS
not a limiting factor
for CN detoxification

Acknowledgements

Plant Development and Genetic Improvement Research Unit
CIRAD www.cirad.fr

BURST Group – Biology of Responses to STresses in tropical perennial species
Leclercq, Montoro, Oliver, Rio

GS Group – Gene & Selection – Xavier SABAU

Funding from the *Institut Français du Caoutchouc*