Progress towards a high-throughput RNA extraction protocol And a few words about pyrosequencing

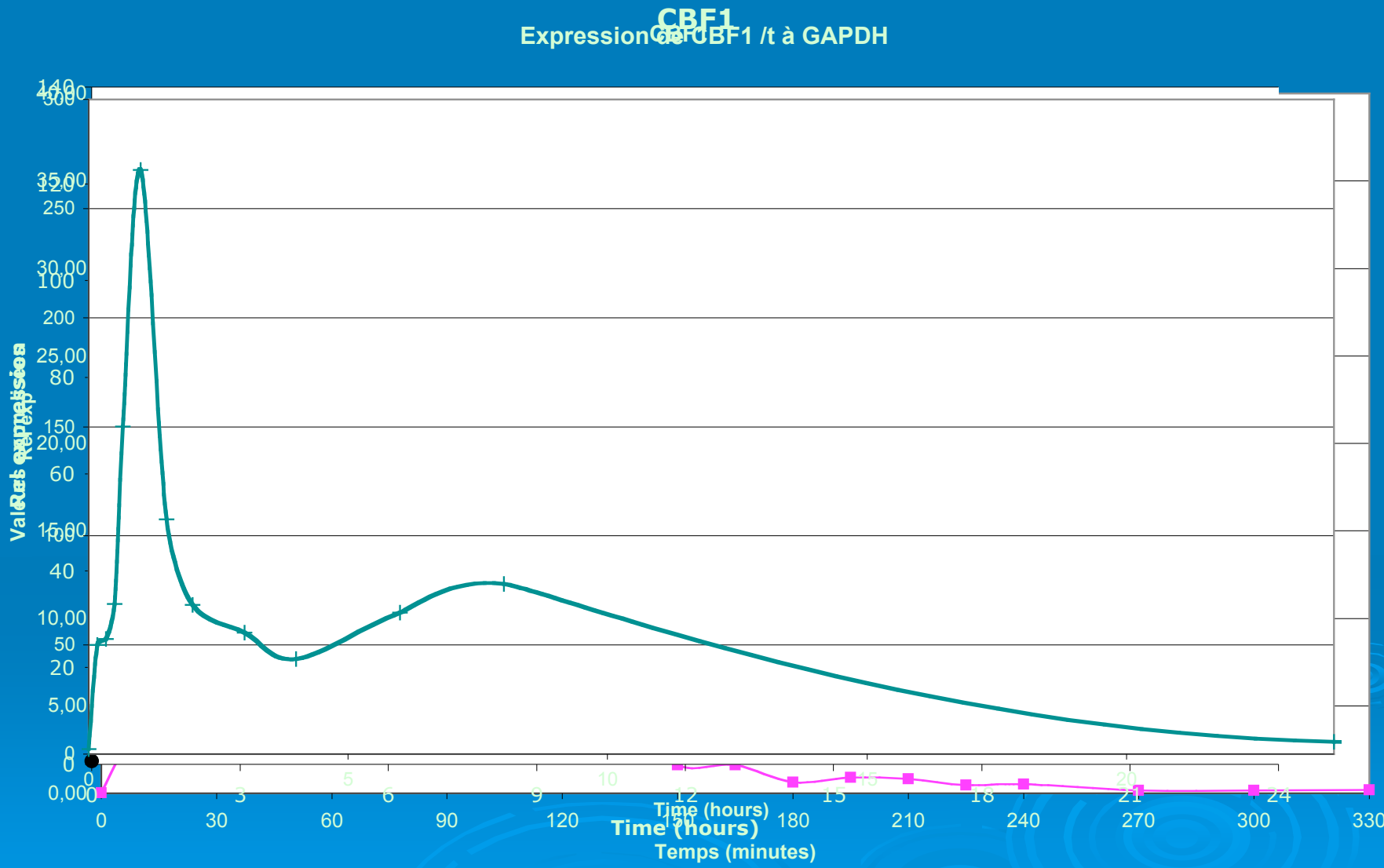
Heather McKhann
EPGV

The problem…

Need for reliable method for the preparation of large numbers of RNA samples for subsequent cDNA synthesis for studies of gene expression: Q-PCR, pyrosequencing

Philippe MILLASSEAU and Sylvie LEVEQUE

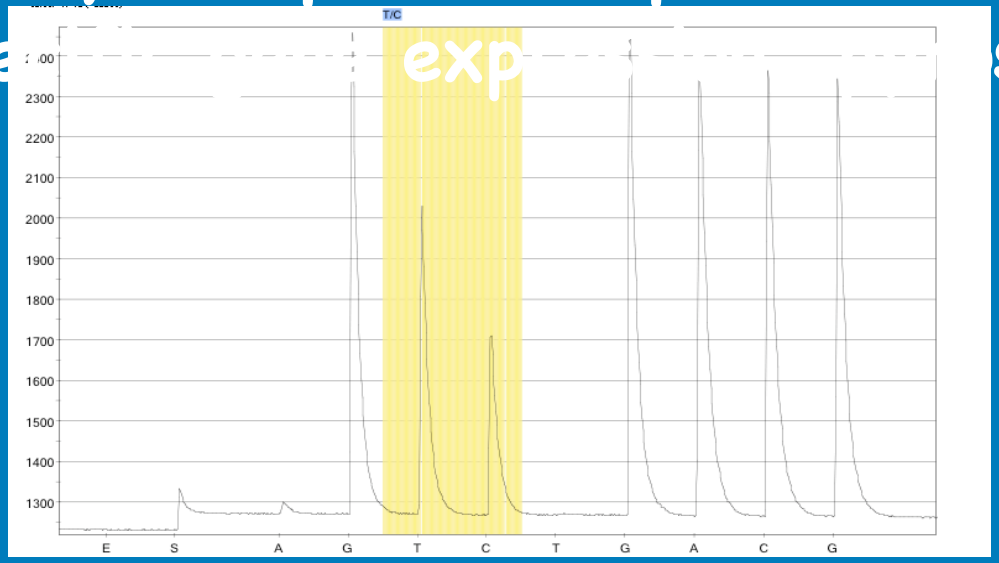
Kinetics of gene expression



E P G V
Etude du Polymorphisme des Génomes Végétaux

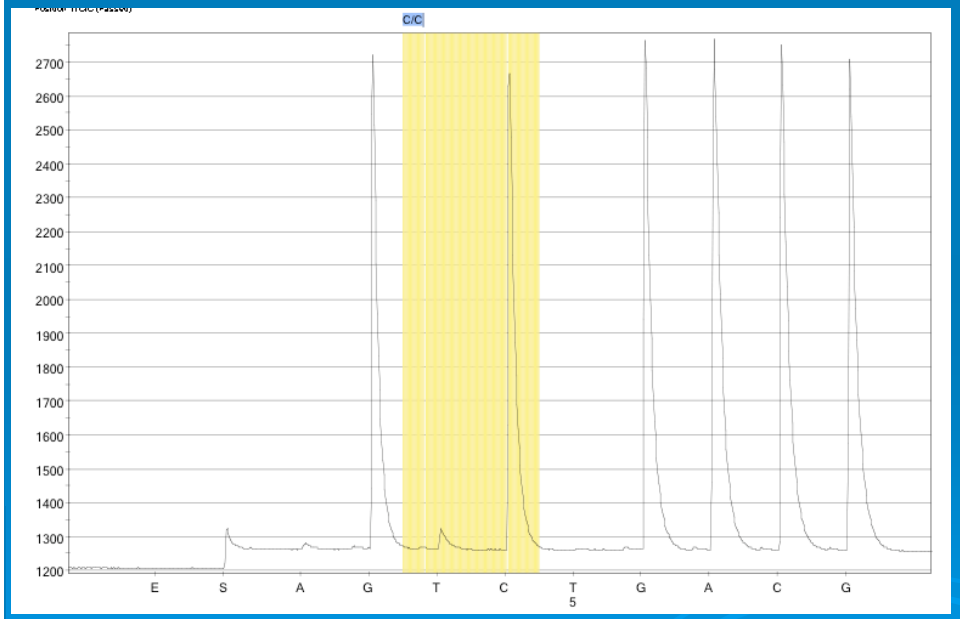
Hybride parent 1 x parent 2

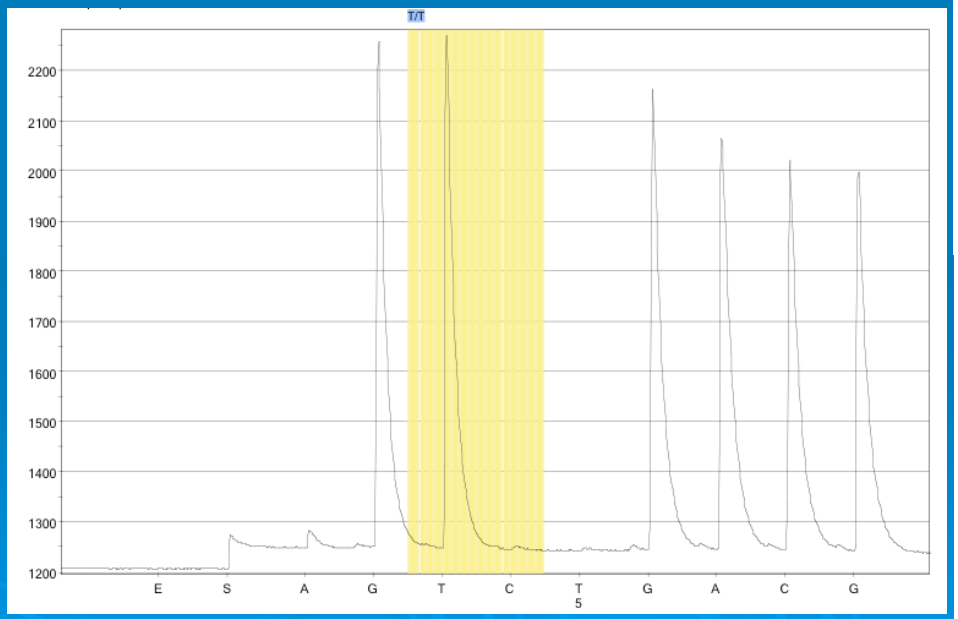
Allele-spe exp sequencing



Parent 1

Parent 2





INRA

E P G V
Etude du Polymorphisme des Génomes Végétaux

CNG
CENTRE NATIONAL DE GENOTYPAGE

cea

BEFORE…

Collection of leaves into **Liquid nitrogen** 

→ **Storage at -80°C**

↓

Grinding of leaves in liquid nitrogen

In mortar

←

Extraction of mRNA **from 70 mg of leaves**

←

cDNA

↑

Retrotranscription RT **from 80 ng of mRNA**

Vf (cDNA) = 160 µL

THE TESTS

1. Machines for grinding
2. Extraction of total vs. mRNA
3. Different quantities of plant tissue
4. Different tissues
5. Extraction in lysis buffer
6. Temperature

1. Machines for grinding

Paint shaker
Quiagen grinder for 2 x 5 tubes
Qiagen grinder for 2 x 24 tubes
« Electric tooth brush grinder »

2. Extraction methods: Total vs. mRNA

Total RNA - TRIzol

mRNA - Dyna Beads

Jost R, Berkowitz O, Masle J.,
Biotechniques. 2007 Aug;43(2):206-11.

3. Quantity of plant material
Micro-extraction: 1 leaf, 1 root

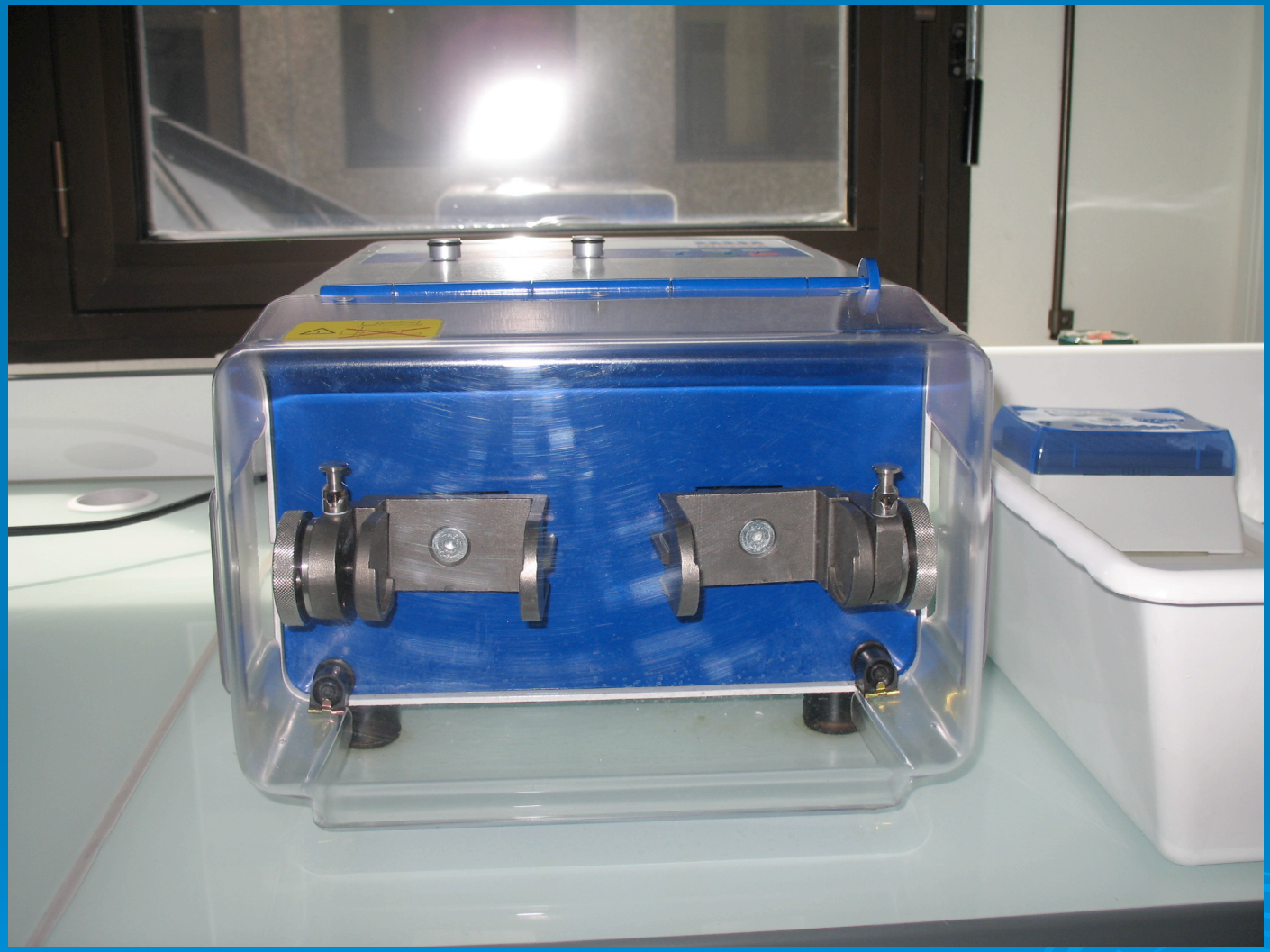
4. Oligo dT vs. random priming

Results

Toothbrush: grinding not homogenous

Paint shaker: Problem of support for tubes

Quantity of RNA (total or mRNA) is not proportional to the amount of original material: grinding is not complete (all methods)



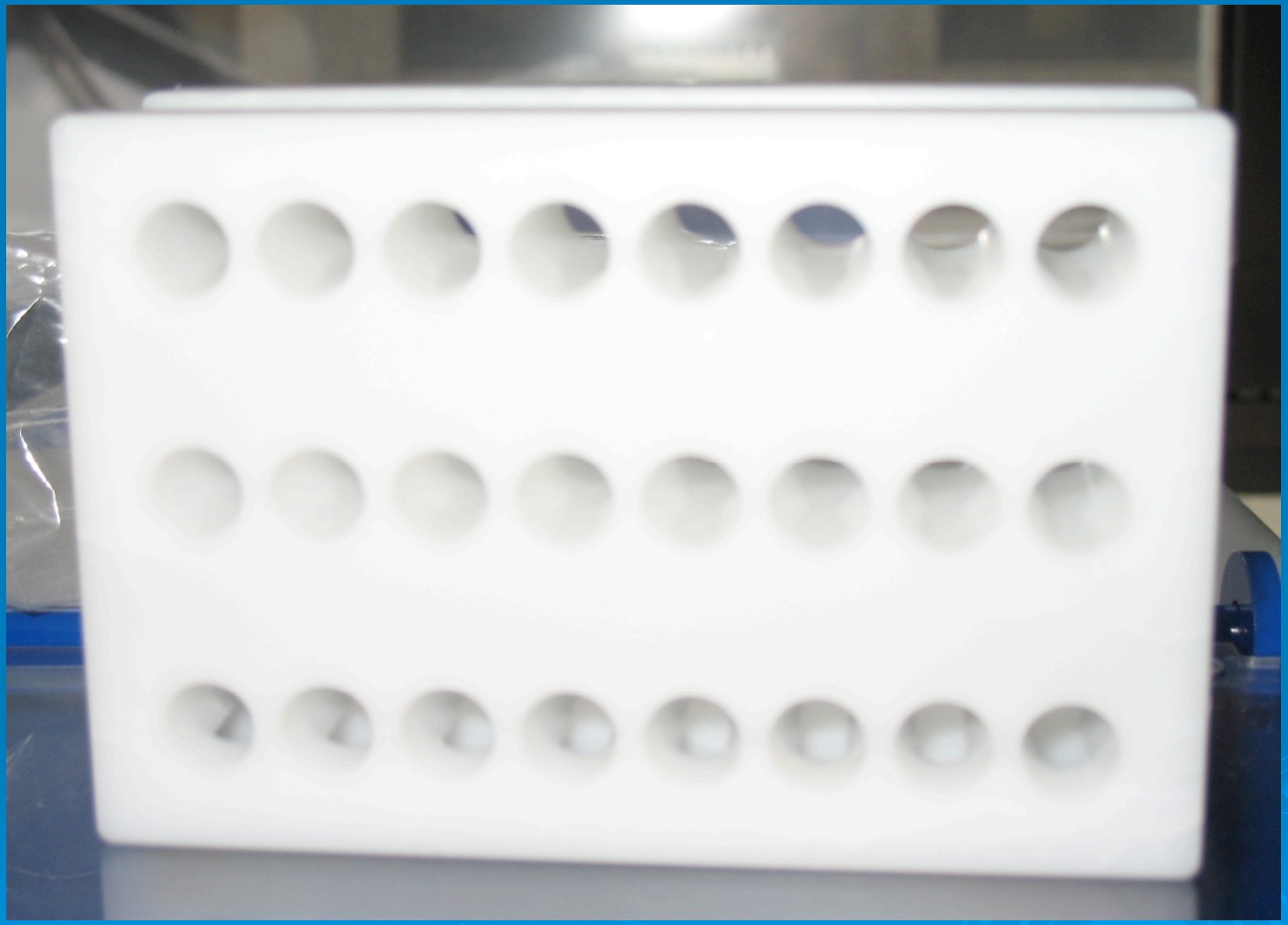
INRA

E P G V

Etude du Polymorphisme des Génomes Végétaux

CNG
CENTRE NATIONAL DE GENOTYPAGE

cea



INRA

E P G V
Etude du Polymorphisme des Génomes Végétaux

CNG
CENTRE NATIONAL DE GENOTYPAGE

cea

Results (2)

Yields based on Ct values slightly higher using TRIzol, but less adapted to high throughput

Ct values lower when oligo dT primers are used, as compared to random primers

Based on these results it was decided to grind with Qiagen (2 x 24), 2 x 1.5 min, 30 Htz, 1.5 or 2 ml tubes, containing 2 inox beads of 3 mm diameter

Grinding in extraction buffer

Test grinding in buffer at ambient temperature

Results: Results are satisfactory

Ct values higher than -20°C but still exploitable

Test grinding small quantities of tissue

1 leaf, 1 root

Results: necessary to use smaller tubes (0.5 ml) with 3 beads (3mm)
Concentrations difficult to measure but Ct values demonstrate presence of mRNA (need to optimize dilutions)

Test of CBF expression

	Valeurs de Ct							
	GAPDH		CBF1		CBF2		CBF3	
	T0	T1h	T0	T1h	T0	T1h	T0	T1h
1 feuille_T° ambiante	27,88	23,46	35,23	23,75		23,81	32,83	26,19
1 feuille_T° ambiante	25,59	23,15	32,96	23,73	34,55	22,90	34,21	24,96
1 feuille_-20°C	20,91	6,83	24,57	6,04	25,21	8,21	27,75	20,77
1 feuille_-20°C	20,96	6,72	24,67	6,10	24,64	7,50	27,84	
1 racine_T° ambiante	22,55	22,71	30,20	29,93	29,42	28,08	31,02	28,79
1 racine_T° ambiante	22,65	22,33	30,74	29,76	29,69	28,18	31,04	28,65
1 racine_-20°C	20,04	20,17	26,29	25,92	25,76	25,81	29,88	26,67
1 racine_-20°C	20,05	20,04	26,34	26,45	26,04	25,79	30,05	26,15

Valeurs de Ct							
GAPDH		CBF1		CBF2		CBF3	
T0	T1h	T0	T1h	T0	T1h	T0	T1h
15,30	16,10	30,10	18,80	25,90	17,10	25,30	16,00

Results- CBF expression

	Expression relative					
	CBF1		CBF2		CBF3	
	0	T1h	0	T1h	0	T1h
1 feuille_T° ambiante	0,61	81,69		78,58	3,24	15,05
1 feuille_T° ambiante	0,60	66,99	0,20	118,77	0,25	28,63
1 feuille_-20°C						
1 feuille_-20°C						
1 racine_T° ambiante	0,50	0,67	0,86	2,43	0,28	1,48
1 racine_T° ambiante	0,37	0,58	0,76	1,72	0,30	1,25
1 racine_-20°C	1,31	1,85	1,89	2,00	0,11	1,10
1 racine_-20°C	1,28	1,18	1,57	1,85	0,10	1,44

Avant…

Prélèvements des feuilles à ≠ temps

azote liquide

→ **Stockage à -80°C**

↓

Broyage des feuilles sous azote liquide

en mortier

←

Extraction des ARNm **sur 70 mg de feuilles**

←

ADNc

↑

Rétro Transcription RT **sur 80 ng d'ARNm**

Vf (ADNc) = 160 µL

Plan de manips

Prélèvements des feuilles à ≠ temps

À T° ambiante

→ **Stockage à -80°C**

↓

Broyage des feuilles sous azote liquide

en mortier

←

Extraction des ARNm **sur 70 mg de feuilles**

←

ADNc

↑

Rétro Transcription RT **sur 80 ng d'ARNm**

Plan de manips

Prélèvements des feuilles à ≠ temps

À T° ambiante

Stockage à T° ambiante en tampon de lyse

Broyage des feuilles sous azote liquide

en mortier

Extraction des ARNm **sur 70 mg de feuilles**

ADNc

Rétro Transcription RT **sur 80 ng d'ARNm**

Plan de manips

Prélèvements des feuilles à ≠ temps

À T° ambiante

Stockage à T° ambiante en tampon de lyse

Broyage du matériel à T° ambiante – 48 éch / 3 min

Extraction des ARNm **sur 70 mg de feuilles**

ADNc

Rétro Transcription RT **sur 80 ng d'ARNm**

Plan de manips

Prélèvements des feuilles à ≠ temps

À T° ambiante

Stockage à T° ambiante en tampon de lyse

Broyage du matériel à T° ambiante – 48 éch / 3 min

Extraction des ARNm **sur 1 feuille / 1 tige / 1 racine**

Rétro Transcription RT **sur 80 ng d'ARNm**

ADNc

Après… !!!!!!!!!!!!!!!!!!!!!!!!

Prélèvements des feuilles à ≠ temps

À T° ambiante

Stockage à T° ambiante en tampon de lyse

Broyage du matériel à T° ambiante – 48 éch / 3 min

Extraction des ARNm **sur 1 feuille / 1 tige / 1 racine**

Rétro Transcription RT **sur 5 à 20 ng d'ARNm**

ADNc

Vf (ADNc) = 80 µL

Perspectives

Extraction in 96 well plates- 48 samples

Quantification of RNA

Stability of samples over time?

A few words on pyrosequencing

Pilot project: Projet innovant
(DGAP 2008):
Developpement de methodes pour la quantification allélique et application

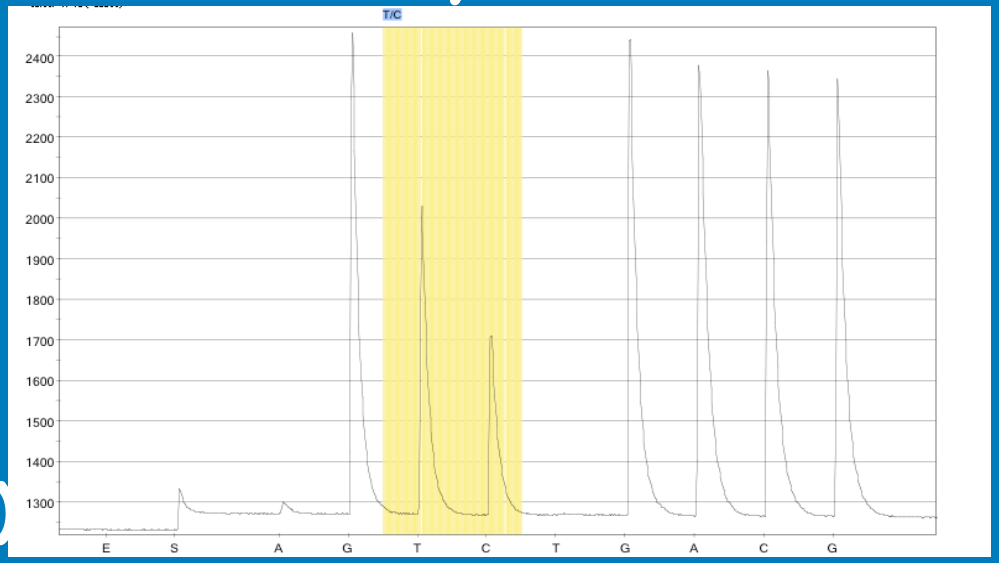
Fabrice Foucher
Phillipe Barre
Heather McKhann

1. Arabidopsis *CBF* genes

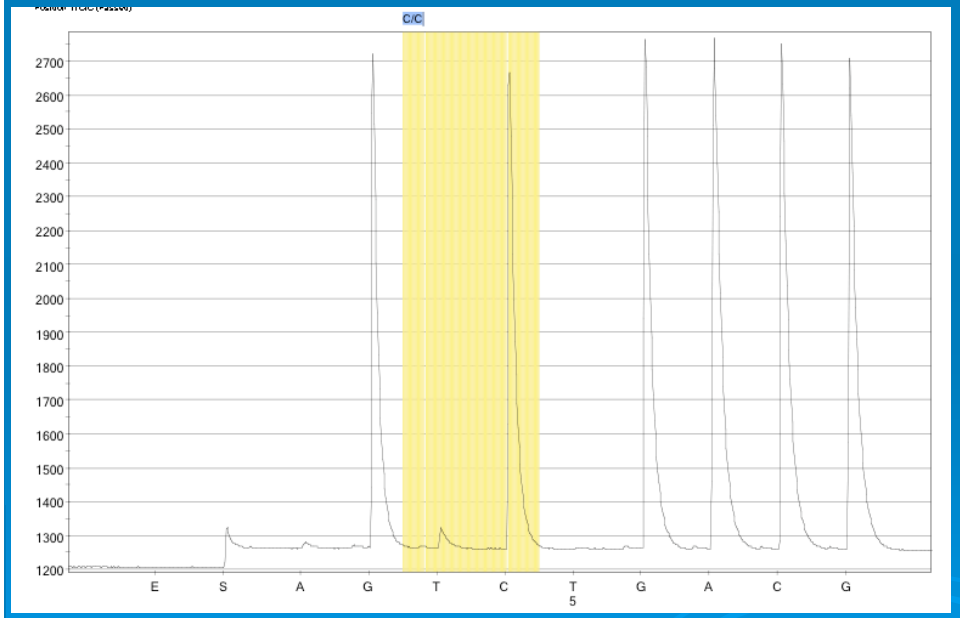
Objective: look at allele-specific Expression in an F1 hybrid between 2 accessions with contrasted freezing tolerance

Crosses Rub-1 (R) x Cvi-0 (S)
Genes: *CBF1, CBF3*

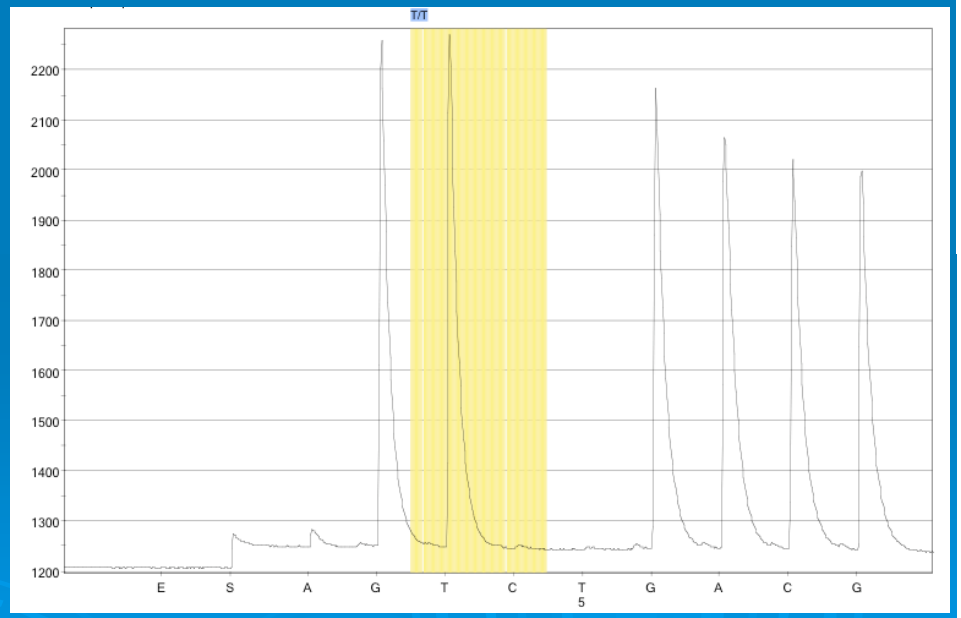
F1 Hybride



Rub-1 (R)



Cvi-0 (S)



INRA

E P G V
Etude du Polymorphisme des Génomes Végétaux

CNG
CENTRE NATIONAL DE GENOTYPAGE

cea

2. Rose genes involved in GA signalling (heterozyous diploid)

Rosa wichurana
Rosa chinensis 'Old Blush'
Rosa *hybrida* 'Felicité Perpetue'
its vegetative mutant 'Little White Pet'

Genes: *GID1, KSN, RGA, SPY*

Questions: Are the 2 alleles regulated differently at the transcriptional level during flowering process and in response to exogenous application of GA?

3. Quantification of allelic frequency in populations of ryegrass

Objective: determine if pyrosequencing is accurate enough to estimate allele frequencies in pooled genotypes.

2 genotypes of ryegrass, P50 et P173
Genes: *Cry2, PhyA*

For expression studies:

Need genomic DNA
Mixes of well quantified parental DNAs
1:9, 2:8, etc. (picogreen)
Q-PCR data

For genomic DNA:

Need mixes of well quantified DNA

Normalisation/statistical analysis

Thank you for your attention

Thanks to:

Jörg Tost
Sylvie Lévêque
Philippe Millasseau
Aurélie Chauveau
Florence Busato
DGAP