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An Asymmetric PCR-ELISA Typing assay for *Citrus tristeza virus* (CTV)

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Abstract. Typing of CTV based on coat protein (CP) gene has been approached in diverse ways. The basis for the assay focused on in this paper was presented some years ago and has been subject to several improvements since its appearance. Although the results obtained with this assay have been presented in diverse scientific meetings, theses and technical seminars, so far no paper has explained the rationale of the method or detailed the protocol. This is presented here as well as the discussion of recent developments regarding the set of probes, hybridisation procedure and software for interpreting the results.

Keywords. Citrus – Hybridization – PCR ELISA – Tristeza – Typing.

Un essai de typage par PCR-ELISA asymétrique pour le virus de la tristeza des agrumes

Résumé. *Le typage du CTV, utilisant le gène de la protéine capsidique, a été réalisé en suivant différentes procédures. Les principes fondamentaux de l'essai, décrits dans ce travail, ont déjà été présentés il y a quelques années mais, dans le temps, on a apporté des améliorations importantes. Bien que les résultats obtenus dans cet essai aient été illustrés à l'occasion de diverses conférences scientifiques, de nombreux séminaires techniques et dans des mémoires de thèse, aucun travail n'a expliqué jusqu'à présents la logique de cette méthode ou proposé un protocole détaillé. C'est là donc l'objet de cette présentation où, en plus, on va parcourir les avancées récentes concernant les sondes, la méthode d' hybridation et le logiciel pour l'interprétation des résultats.*

Mots-clés. *Agrumes – Hybridization – PCR ELISA – Tristeza – Typage.*

I – Introduction

Several studies support the existence of a not yet fully clarified relationship between CP gene sequence and CTV symptoms, and different approaches have been made to develop a typing system based on the CP gene (Permar *et al*., 1990; Gillings *et al*., 1993; Pappu *et al*., 1993; Niblet *et al*., 2000; Zemzami *et al*., 2002; Halbert *et al*., 2004). Zemzami *et al*. (2002) further developed the set of short (16-20) discriminating probes presented by Niblett *et al*., (2000) for targeting the seven phylogenetic groups which are now soundly recognized (see an accompanying paper in this volume). In the same work the format of the discriminating hybridisation step was presented as an asymmetric PCR-ELISA. The rationale of this procedure is the use of a PCR reaction in which the amount of one primer is about one order of magnitude higher than the other. This originates a large amount of single-stranded molecules that in the following steps are hybridised to a set of strain discriminating probes immobilized in the wells of an ELISA plate. During the PCR reaction, the DNA molecules are labelled by Digoxigenin (Dig) which is included in the deoxy-nucleotide mixture as Dig-dUTP. The hybridised products are quantified through an ELISA assay using commercial alkaline-phosphatase conjugated anti-Dig antibodies. This information is used through a software analysis to estimate the composition of the samples in terms of the seven phylogenetic groups.

This paper presents and discusses the latest developments regarding the set of probes, hybridisation procedure and software for interpreting the results. The software and the probes sequence is available upon request to the author, gnolasco@ualg.pt. A more complete paper is now in press (Nolasco *et al*., 2008).

II – Material and methods

1. cDNA synthesis

cDNA synthesis is done using random primers. Typically this is done mixing 5µl total RNA with 1µl random primers (0.5 µg/µl, random p(dN)6, Roche, refª 11034731001), denaturing for 5 min at 95 ºC and quickly transfer to ice. The reverse transcription is done for 1h at 37 ºC using SuperScriptTM III Reverse Transcriptase (Invitrogen, refª 18080-044) following the manufacturer instructions.

2. Dig Labelling by Asymmetric PCR

One microlitre of cDNA is used as template in the asymmetric PCR reaction. This is done with the primers CTV43 (forward) 5'-ATGTTGTTGCNGCNGAGTC-3 and CTV42 (reverse) 5'-CTCAAATTGCGRTTCTGTCT-3 which amplify a fragment starting at position 59 and finishing at position 473 in the CP gene. Primer CTV 43 is used at of 200 nM (final concentration) and primer CTV 42 at 20 nM. The deoxy-nucleotide and Dig labelling mixture contains (final concentration) 80 µM of each of dATP, dGTP, dCTP, 76 µM dTTP and 2 µM of Dig-11-dUTP (Roche Applied Science ref. 11093088910). MgCl2 concentration is 2.5 mM and typically one unit of Taq polymerase is used in a 50 µl reaction. Thermocycling is done after an initial denaturation of 2 min at 92 ºC and consists of 50 cycles with the following steps: 92ºC for 30 s, 52ºC for 30 s, 72ºC for 45 s. A final extension period of 5 min at 72º C completes the process.

3. ELISA plate preparation

The hybridisation probes are biotinylated and immobilized in the wells via a previous streptavidin coating. Microlon (Greiner) 600 microplates with round or flat bottom wells are used. All volumes are 100 µl per well. The microplate wells coated with 10 µg/ml streptavidin in 50 mM sodium carbonate buffer (pH 9.6) overnight at 4ºC or at 37ºC for 1.5 h. The microplates are then washed three times with PBS-Tween as in standard ELISA procedures. The biotinylated probes are added, 20 pmol per well, in hybridisation buffer (0.75M NaCl, 0.425 M NaH2PO4, 0.005 M Na EDTA, pH 7.4, containing 0.1 % n-lauroylsarcosine) and incubated at 37ºC for 30 min. The probes are arranged in rows as shown in Table 1. The plate is washed as before and used immediately for the following steps or prepared in advance and kept empty at 4ºC or at room temperature up to three months.

Table 1. Layout of the first 8 columns of the ELISA plate. Each probe, indicated on the left side, is arranged on one row. Each column corresponds to one sample whose PCR product are arranged in the 8 corresponding wells. In this case the samples are a set of CP gene variants representative of each of the seven phylogenetic groups which are indicated on the top. The numbers inside the border are the initial rates of hydrolysis estimated through fitting of the Michaelis-Menten equation.

	Gp 1	Gp 2	Gp 3a	Gp3b	Gp 4	Gp 5	Gp M
	#1.1tt	#13.3	#bb6	#28c	#134a	#010.8	#20.2
B1	0.643	0.006	0.006	0.007	0.006	0.010	0.006
B2	0.005	0.488	0.006	0.007	0.003	0.011	0.007
B3a	0.031	0.010	0.219	0.022	0.048	0.006	0.094
B10	0.009	0.005	0.071	0.299	0.002	0.002	0.002
III	0.005	0.009	0.031	0.278	0.018	0.002	0.002
B4	0.007	0.006	0.007	0.003	0.443	0.004	0.005
B5	0.005	0.003	0.004	0.008	0.013	0.552	0.003
B8	0.000	0.031	0.007	0.006	0.013	0.055	1.426

4. Hybridisation

For each sample, 45 μl of the Dig labelled PCR products are added to 855 μl of hybridisation buffer and arranged, 100 μl per well, in one column of the plate. The plate is sealed with tape and incubated for 1h30 at 45ºC. The plate is then washed three times for 3 min. with pre-warmed (45 ºC) low molarity tris washing buffer (Tris-HCl 10mM, pH8, 15 mM NaCl).

5. Colorimetric development and absorbance reading

Anti-DIG-F(ab')$_2$ alkaline phosphatase conjugate (Roche Applied Science 11093274910), 0.075 units per well in 100 μl of PBS-Tween containing 2 % PVP-40, 0.2 % BSA, are added and incubated for 30 min at 37 ºC. The wells are washed 5 times with PBS-Tween and 1 mg/ml of p-nitrophenyl phosphate in substrate buffer (9.7 % diethanolamine, pH 9.8) are added, 100 μl per well. The plate reader should be ready before adding the substrate. This should be preferably added with a 8-multichannel pipette by columns to avoid differences in the reaction times delivery, with 3 min intervals. If available, incubation is allowed at a certain temperature among wells of each sample. The absorbance is read at 405 nm starting immediately after substrate (e.g. 37ºC).

6. Analysis of the results

This is done in two steps using a specially developed software. In a first step the raw data is imported and an array with the initial rates of the substrate hydrolysis is calculated. In a second step this array is compared to the array of the standards stored in the software and its composition in terms of the presence of variants of each of the seven phylogenetic groups is determined.

II – Results and discussion

1. Experimental protocol

The nucleic acid to be typed may be CTV RNA directly extracted from infected plants when the objective is to analyse an unknown isolate, or colonies of transformed bacteria harbouring the CP gene when the objective is to make a previous screening before choosing clones to be sequenced. Previously CP gene amplified products may also be used.

In comparison with the previous versions of the protocol, it should be noticed that the hybridisation and washings are now performed at 45ºC (instead of 37 ºC) and at a lower molarity. These changes were found advantageous for a better control of the stringency of the procedure. However, it should be taken in consideration that accidental variations in the concentration of NaCl may result in erroneous results due to a change in the ability of the probes to hybridise to mismatched targets.

For easiness of operation to follow the hydrolysis of the substrate, longer periods e.g. 5 or 10 minutes may eventually be more convenient in some equipment. In these cases it may be useful to lower the substrate concentration or incubation temperature to slow down the reaction rate. The reaction should be followed until the faster reacting wells reach an absorbance of about 2.8 OD.

The raw data is used in a first step to estimate the initial rate of hydrolysis on each well. The software supplied with some equipment brands may provide a way to do these calculations, frequently through a linear regression for the first data points. Alternatively an application in Visual Basic is available upon request (MonoMadjust). This is based on the Michaelis-Menten kinetic model of a single substrate reaction and produces a more accurate non-linear curve fitting using all data points. An example of the output of this step is presented in Table 1. When running the software, care should be taken regarding the absorbance values that can be used. Depending on

the age of the instrument lamp or other factors, the highest absorbance values (e.g. higher than 2.8) are biased and no longer follow the single-substrate model. This can be noticed by a sharp bending of the curve before the plateau. These values should not be considered to curve fitting, an option which can be selected in the software.

Table 1 reports the initial hydrolysis rates obtained for a set of CP gene variants representative of each of the seven phylogenetic groups. These rates of reaction were obtained using preparations with equal starting concentrations of each variant. This is the standard array of rates of hydrolysis that will be used by a second Visual Basic application (CalcType, available upon request) to search for the presence of variants of each group when analysing an unknown sample. This standard array is obtained only once and can be used for the subsequent experiments as it is permanently stored in the software.

When an unknown sample is being analysed the software will compare its array of reaction patterns with the standard array. Let the following array represent the ELISA rates of reaction of the CP gene of each group with the set of probes, i.e, the data presented in **Table 1** (for simplification only a 3 x 3 array is here represented),

	Probe 1	Probe 2	Probe 3	…..
Gp 1	a_{11}	a_{12}	a_{13}	….
Gp 2	a_{21}	a_{22}	a_{23}	…..
Gp 3	a_{31}	a_{32}	a_{33}	….
…..	…	…	…	….

Suppose that the analysis of an unknown sample resulted in the following array representing the ELISA rates of reaction with the set of probes:

Probe 1	Probe 2	Probe 3	…..
P_1	P_2	P_3	….

Assuming that the ELISA rates of reaction are proportional to the amount of DNA present on the sample, if the unknown sample is composed by a certain amount (X1) of DNA from Gp 1, a certain amount (X_2) of DNA from Gp 2, etc…, then it is possible to write the following system of linear equations:

$$P_1 = X_1 . a_{11} + X_2 . a_{21} + X_3 . a_{31} + ….$$
$$P_2 = X_1 . a_{12} + X_2 . a_{22} + X_3 . a_{32} + ….$$
$$P_3 = X_1 . a_{13} + X_2 . a_{23} + X_3 . a_{33} + ….$$
$$.... = …. ... + … …..+ …. …..+ …$$

Solving the system in order to the array X1, X2 X3, ….. will give the amount of DNA present in the sample from each of the groups. In practice, due to experimental errors, etc.., the system may originate negative solutions for the amount of DNA of each group. Although this has no real meaning, it is used by the software as a first approach. In following successive iterations, the software CalcTypeVel will find the closest solution in which all the Xi are greater than or equal to zero. This software integrates in a Visual Basic module running on Microsoft Office Excel with the Solver add-inn which must be installed. The absence of a perfect linearity may lead to an erroneous over interpretation of quantitative results by the user. Thus, although the results internally managed by the software are quantitative the output is qualitative, in terms of presence or absence of sequence variants of each group.

The software also provides an estimate of the error in the solution. A large error means that there was not any combination of the standards that could be used to explain the unknown sample. This

should be taken as an indication of bad quality experimental procedures (e.g. a not homogeneous concentration of probes, variations in the amount of PCR product added to each well, etc..) or an indication that the unknown sample might be composed by sequence variants that react in an unknown way to the panel of probes, i.e, hypothetically, the unknown sample belongs to a new group not previously characterized. If this situation happens, this sample may be added to the panel of standards for future assays. Eventually the design of a new probe may be necessary.

2. Comparison with other hybridisation assays

A limitation of the previous membrane-based assays (Niblett *et al*., 2000) is that the result of the hybridisation of the target to each probe of the panel is analysed on an all-or-none basis. As such, each probe had to be designed in such a way that it only hybridises to its homologous group. This originated a limited discriminating ability as it is not possible to find in the CP gene regions of the desired length which have enough nucleotide differences to fully differentiate among the seven groups. Some discrepancies or difficulties in interpreting the results have been reported when using this approach (Herron *et al*., 2005; Halbert *et al*., 2004). In contrast, in this assay, each probe can react with variants of more than one group. This allows much more flexibility in the design of probes, allowing a better discrimination.

3. Typical results

Assays run with cloned CP genes in diverse proportions showed that in artificial mixtures of two phylogenetic groups, the presence of one group could be detected when it represented only 10 % of the sample. More than one hundred samples have been characterized in parallel through this assay and by cloning and sequencing the following SSCP choice of the variants. In all the cases, the phylogenetic groups detected by sequencing were also detected by PCR ELISA Typing assay. In some samples in which the number of sequenced clones was small, e.g. 3 or 4, it was possible to detect by this typing assay the presence of additional phylogenetic groups, probably represented in lower amount. Mixtures of up to five groups could be detected in nature, although this situation was not common. The ability to detect mixtures of five groups was verified through the use of artificial mixtures.

4. Relationship with symptoms

Although it is tempting to establish a direct relationship with symptoms, some care should be made when doing such extrapolations. Also the degree of confidence in these relationships depends on the group. Considering isolates composed by just one phylogenetic group, group M, group 2 corresponds to mild isolates which do not originate stem pitting on sweet orange nor the quick decline of plants grafted on sour orange. Group 3a corresponds to severe isolates which originate diverse degrees of decline and also stem pitting on branches of grapefruit, sweet orange and some mandarins when grafted on tolerant rootstocks. Group 1 corresponds to isolates which originate decline and quick decline of trees grafted on sour orange. Groups 3b and 5 are usually not mild but more variable in the ability to induce stem-pitting or decline. Extrapolation of these relationships for isolates harbouring mixtures of these groups is not clear at all.

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