

AperTO - Archivio Istituzionale Open Access dell'Università di Torino

## Production and fingerprinting of virus-free clones in a reflowering globe artichoke

### This is the author's manuscript

*Original Citation:*

*Availability:*

This version is available <http://hdl.handle.net/2318/76953> since

*Terms of use:*

Open Access

Anyone can freely access the full text of works made available as "Open Access". Works made available under a Creative Commons license can be used according to the terms and conditions of said license. Use of all other works requires consent of the right holder (author or publisher) if not exempted from copyright protection by the applicable law.

(Article begins on next page)



# UNIVERSITÀ DEGLI STUDI DI TORINO

***This is an author version of the contribution published on:***  
*Plant Cell, Tissue and Organ Culture (PCTOC), 100, 32010,*  
*DOI:10.1007/s11240-009-9654-3*

***The definitive version is available at:***  
*<http://link.springer.com/article/10.1007%2Fs11240-009-9654-3#>*

## **Production and fingerprinting of virus-free clones in a reflowering globe artichoke**

Acquadro A.<sup>1</sup>, Papanice M.A.<sup>2</sup>, Lanteri S.<sup>1</sup>, Bottalico G.<sup>2</sup>, Portis E.<sup>1</sup>, Campanale A.<sup>3</sup>, Finetti-Sialer M.M.<sup>2\*</sup>, Mascia T.<sup>2</sup>, Sumerano P.<sup>4</sup> and Gallitelli D.<sup>2</sup>

1) Di.Va.P.R.A. Plant Genetics and Breeding, University of Torino, via L. da Vinci 44, I-10095 Grugliasco (Torino), Italy

2) Dipartimento di Protezione delle Piante e Microbiologia Applicata, Via Amendola, 165/A, 70126 Bari, Italy

3) Istituto di Virologia Vegetale del CNR, Sezione di Bari, Via Amendola, 165/A, 70126 Bari, Italy

4) Consorzio di Difesa e Valorizzazione delle Produzioni Agricole della Provincia di Brindisi, Brindisi, Italy

\*Present address: Istituto di genetica vegetale del CNR, Via Amendola 165/A, 70126, Bari.

### **Corresponding Author:**

#### **Prof. Sergio Lanteri**

DiVaPRA, Plant Genetics and Breeding

University of Torino

via L. da Vinci 44, 10095

Grugliasco (TO)

ITALY

Tel. (+39) 011-6708806 Fax (+39) 011-2368806

e-mail:sergio.lanteri@unito.it

**Abstract**

Most of the 24 viruses which infect globe artichoke are detrimental to the crop's performance and hamper the development of a nursery activity in the respect of current EU legislation. We describe a procedure to sanitize globe artichoke “Brindisino” from *Artichoke Italian latent virus* (AILV) and *Artichoke latent virus* (ArLV), while preserving its valuable early flowering trait. ArLV was successfully eliminated by meristem-tip culture, while AILV was removed when two rounds of meristem-tip culture were spaced out with *in vitro* thermotherapy. *In vivo* thermotherapy, followed by meristem-tip culture, was also successful in producing virus-free material but was less efficient in terms of the number of plants recovered post treatment.

Due to the multi-clonal composition of the populations at present in cultivation, the selected and sanitised clones were fingerprinted by applying microsatellite and AFLP (amplified fragment length polymorphism) markers. One AFLP primer combination produced 28 informative fragments used to evaluate genetic relatedness among the clones in study. Our results demonstrates that AFLP-based molecular fingerprinting enables to verify the true to clone correspondence in nurseries, ensure the effective correspondence between the real and the declared identity of a clone, so that to avoid commercial frauds, and might represents a valuable tool for assessing somaclonal variation occurring during ‘in vitro’ propagation.

**Keywords:** Globe artichoke, sanitization, thermotherapy, plant viruses, genotyping, SSRs, AFLPs

## Introduction

Globe artichoke (*Cynara cardunculus* var. *scolymus* L.) is an allogamous perennial member of the family Compositae (Asteraceae). It is cultivated for its large fleshy immature inflorescences (capitula), that are widely consumed in both fresh and preserved form. Production is mainly concentrated in the Mediterranean area, but it also occurs in the Middle East, North Africa, South America, United States, and China (FAO data 2005: <http://faostat.fao.org/>). Varietal groups in the globe artichoke are defined on the basis of their capitulum appearance and their harvest time. Early flowering types can be induced to produce capitula between autumn and spring, if dormant underground shoots used for propagation are watered during the summer; whereas, late flowering types produce capitula only during spring and early summer (Mauromicale and Ierna 2000). Italy harbours the most abundant *in situ* diversity for the globe artichoke, and is the leading world producer. Within the country, most of the production takes place in Apulia, Sicily and Sardinia, areas in which it represents an important component of regional economic stability and social development. Most commercially cultivated varietal types are derived via vegetative propagation, and these multi-clonal populations have been shown, using DNA markers, to retain a significant component of genetic variation (Lanteri et al. 2001; Portis et al. 2005) mainly due to the limited selection criteria adopted by farmers. An additional source of variation may be via spontaneous mutations, not necessarily detectable at the phenotypic level and propagated over time as they do not underwent any meiotic sieve. Clones within a varietal type share morphological/agronomical traits and may be often individually identified only by applying molecular marker techniques (Lanteri et al. 2004a; Mauro et al. 2009).

Vascular and leaf fungi *Verticillium dahliae* and *Leveillula taurica*, and a spectrum of viruses are among the most damaging pests of the crop. While most pests and fungal pathogens can be chemically controlled, no such control is available for viral diseases. As a result, disease minimization strategies are restricted to preventing virus transmission or eliminating viruses from infected stocks by either heat treatment (thermotherapy) (Mink et al. 1998) or meristem-tip culture (Faccioli and Marani 1998), or a combination of both (Sharma et al. 2008). To date 24 plant viruses, belonging to thirteen genera (Gallitelli et al. 2004; Martelli and Gallitelli 2008) are known to infect globe artichoke. Most are endemic to the Mediterranean Basin, and can be very damaging to the artichoke industry. In Apulia, the globe artichoke “Brindisino” is frequently infected, singly or in combination, by the *Artichoke Italian latent virus* (AILV) and the *Artichoke latent virus* (ArLV). However, the *Tomato spotted wilt virus* (TSWV) and the *Artichoke mottled crinkle virus* (AMCV) are more damaging, as they typically severely affect plant growth, and reduce economic yield by distorting the capitulum. TSWV infected plants also act as reservoirs of inoculum transmitted by thrips on other susceptible crops (Gallitelli et al. 2004). The continued presence of virus-infected plants reflects a lack of active selection for non-infected plants, due to the common farmer practice of mowing the field at the end of the season, and only collecting the following season's propagative material some months later.

The provision of virus-free planting material should allow a substantial increase in productivity (Gallitelli and Barba 2003), and current EU legislation (Directives 93/61/CEE and 93/62/CEE) requires that nursery production of propagation material must be based on virus-free and true-to-type certified stocks. Seed propagation has been regarded as an attractive means of obtaining virus-free plants, but most of the artichoke-

infecting viruses are both seed- and pollen-transmitted. AILV and ArLV have been detected in both seed coats and fully expanded cotyledons (Bottalico et al. 2002). Thus, these viruses must be able to overcome the meristem exclusion mechanism that controls their systemic spread, probably via RNA silencing (Wang et al. 1997; Brigneti et al. 1998; Martin-Hernandez and Baulcombe, 2008). Some encouraging, but not fully conclusive results for curing infected plants have been obtained by either *in vivo* or *in vitro* thermotherapy. Exclusion of viruses using *in vitro* culture of shoot tips or young capitulum receptacles (Marras et al. 1982; Pecaut et al. 1985) is less effective than meristem-tip culture (Peña-Iglesias and Ayuso-Gonzales 1974; 1982; Harbaoui et al. 1982). However, the latter technique is not suitable in practice as it is associated with loss in earliness (an economically important trait) in reflowering globe artichokes “Brindisino”, “Violetto di Provenza”, and “Catanese”. Pecaut and Martin (1992; 1993) reported that meristem-tip cultures of cultivars of the early Mediterranean group produce either variants distinguished by lateness and deeply divided leaves (denoted as “pastel” variants) or plants with globular heads (denoted as “bull” variants) or plants showing both traits (denoted as “pastel-bull” variants) (Pochard et al. 1969). The frequency of occurrence of such variants increases with the number of passages, and can reach 100% (Pecaut and Martin, 1992; 1993).

In this study, a protocol for curing ArLV and AILV infections in the early flowering globe artichoke ‘Brindisino’ is described. This protocol consists of the use of either meristem-tip culture followed by *in vitro* thermotherapy, or *in vivo* thermotherapy followed by meristem-tip culture.

## Materials and Methods

### *Plant material and virus detection*

Cuttings with three to four developed leaves were harvested from each of 90 one-year-old symptomless plants of globe artichoke “Brindisino” grown in commercial fields of the Brindisi Province (Apulia, Southern Italy). All cuttings were screened by dot-blot hybridization (Gallitelli and Saldarelli, 1996) for presence of AILV, ArLV, AMCV, *Bean yellow mosaic virus* (BYMV), *Broad bean wilt virus* (BBWV), *Cucumber mosaic virus* (CMV), *Pelargonium zonate spot virus* (PZSV), *Potato virus X* (PVX), *Tobacco mosaic virus* (TMV), TSWV, and *Turnip mosaic virus* (TuMV). For this, ~100 mg of young leaf tissue was squashed in a small plastic bag in the presence of 600 µl of 50 mM NaOH and 2.5 mM EDTA, and incubated for 5 min at 25°C. A 5 µl slurry aliquot was spotted onto positively charged nylon membrane (Roche Diagnostics GMBH, Mannheim, Germany), and membranes were then subjected to nucleic acid hybridization using digoxigenin (DIG)-labelled riboprobes transcribed from cloned sequences, and analyzed as described by Gallitelli and Saldarelli (1996). Infected plants were transplanted into 18 cm diameter pots, and maintained in a greenhouse at 22-24 °C prior to sanitation.

### *Virus elimination*

Two strategies were adopted to obtain virus-free plants, both based on meristem-tip culture and thermotherapy. These are outlined in Fig. 1.

**Meristem-tip culture** - The vegetative apex (1-2 cm length) of selected cuttings was excised and sterilized by dipping in 20% v/w sodium hypochlorite (about 1.2 g l<sup>-1</sup> active chlorine), 2.6 mM citric acid, 3 mM ascorbic acid, followed by three rinses in 3 mM citric acid and 2.6 mM ascorbic acid to prevent oxidation. Citric acid and ascorbic acid solutions were sterilized by filtration through 0.45 µm membranes. Meristem-tips, 0.3-0.8 mm in length, with one or two leaf primordia were removed aseptically from each vegetative apex, and incubated for one month at 24°C with a 16 h photoperiod at 40.5 µmol m<sup>-2</sup>s<sup>-1</sup> photosynthetic photon flux (PPF) on a solid medium (pH adjusted to 5.6-5.8 with 1M KOH) consisting of 3 mM CaCl<sub>2</sub>, 1.5 mM MgSO<sub>4</sub>, 19 mM KNO<sub>3</sub>, 20 mM NH<sub>4</sub>NO<sub>3</sub>, 1.2 mM KH<sub>2</sub>PO<sub>4</sub> containing Nitsh and Nitsh (1969) micro-nutrients along with 25 mg l<sup>-1</sup> FeSO<sub>4</sub>, 25 mg l<sup>-1</sup> Na<sub>2</sub>EDTA, 400 µg l<sup>-1</sup> Thiamine HCl, 100 mg l<sup>-1</sup> Myo-Inositol, 1 mg l<sup>-1</sup> Dymethylaminopurine, 250 µg l<sup>-1</sup> gyberellic acid, 1 mg l<sup>-1</sup> indolacetic acid and 3% (w/v) sucrose (Morone Fortunato et al., 1989). Medium was sterilised by autoclaving at 120°C for 20 min. Tips were excised from growing explants and were transferred to a 0.7% plant agar (Duchefa-Biochemie, Haarlem, The Netherlands) medium containing macro- (Murashige and Skoog 1962) and micro- (Nitsh and Nitsh 1969) nutrients, B5 vitamins (Lloyd and McCown 1980), along with 500 mg l<sup>-1</sup> FeSO<sub>4</sub> 7H<sub>2</sub>O, 550 mg l<sup>-1</sup> Na<sub>2</sub>EDTA, 1 mg l<sup>-1</sup> kinetin, 0.5 mg l<sup>-1</sup> alpha-naphthaleneacetic acid (NAA) and 2% (w/v) sucrose for one month, and grown under the same conditions as described above. After three passages, shoot regenerants were rooted onto a sterile solid medium (pH adjusted to 5.6-5.8 with 1M KOH) consisting of 0.7% plant agar (Duchefa-Biochemie, Haarlem, The Netherlands) along with 3 mM CaCl<sub>2</sub>, 1.5 mM MgSO<sub>4</sub>, 19 mM KNO<sub>3</sub>, 20 mM NH<sub>4</sub>NO<sub>3</sub>, 1.2 mM KH<sub>2</sub>PO<sub>4</sub>, macro- and micro-nutrients and vitamins (Murashige and Skoog 1962), 27.8 mg l<sup>-1</sup> FeSO<sub>4</sub>·7H<sub>2</sub>O, 37 mg l<sup>-1</sup> Na<sub>2</sub>EDTA, 10 mg l<sup>-1</sup> indolacetic acid and 0.2 % (w/v) sucrose (Morone Fortunato et al., 1989). After 45 days, plantlets were transplanted into pre-compressed peat disks (Jiffy pots) soaked in water, and plantlets were acclimatized by gradually removing the plastic film placed over the Jiffy pots. After an additional 20 day-period, plants were transplanted to 10 cm diameter pots containing

sterile potting mixture consisting of Brill type 3 special (Brill, Maubec, France), and grown in an insect-free greenhouse.

*In vitro and in vivo thermotherapy* - *In vitro* thermotherapy was carried out by exposing 3-4 cm long explants from the first passage for a period of 15 days at 38°C with 16 h photoperiod and 67,5  $\mu\text{mol m}^{-2}\text{s}^{-1}$  PPF. Meristem-tips were prepared and incubated as described above. For *in vivo* thermotherapy, cuttings were acclimatized at 28-30 °C for one month prior to their exposure at 38 °C for up to 150 days. Within the first two weeks of this treatment, all leaves senesced, and only plants producing new growth was used for collecting meristem-tips.

#### *DNA extraction and genotyping*

Genomic DNA was extracted from young leaves as described by Lanteri et al. (2001). This provided template for subsequent microsatellite or simple sequence repeat (SSR) and amplified fragment length polymorphism (AFLP) analyses. Eight microsatellite loci were assayed, including CDAT-01, CLIB-02, CMAL-21, CMAL108, CELMS-13, CELMS-14, CELMS-20, and CELMS-38 (Acquadro et al. 2003; 2005a,b; 2009) using the protocol described by Acquadro et al. (2003). Briefly, each 20  $\mu\text{l}$  PCR contained 10 pmol of both forward and reverse primers in the presence of 12.5 ng genomic DNA, 1.5 mM  $\text{MgCl}_2$ , 0.2 mM dNTP and 1U *Taq* polymerase (Promega, Madison, Wisconsin, USA) in the buffer supplied by the manufacturer. A touchdown protocol was used consisting of an incubation at 94 °C for 5 min, followed by 11 cycles of 94 °C for 30 sec, 60 °C for 30 sec, decreased by 0.5 °C per cycle and 72 °C for 60 sec, and then 24 cycles of 94 °C for 30 sec, 55 °C for 30 sec, and 72 °C for 60 sec.

The AFLP analysis was conducted as described by Vos et al. (1995), but as modified by Lanteri et al. (2004a). Briefly, 5  $\mu\text{l}$  of  $\sim 100$  ng  $\mu\text{l}^{-1}$  DNA was digested with *EcoRI* and *MseI*, and ligated to corresponding adapters. Digested ligated DNA fragments were pre-amplified with primers whose sequences were complementary to adapter sequences, with an additional selective nucleotide at the 3' end (*EcoRI*+A and *MseI*+C). Subsequently, selective amplifications were conducted using primers carrying three selective nucleotides. As a pilot, six AFLP primer combinations (PCs) were tested against three clones, and the PC E+ACA/M+CAA was finally selected for the analysis of the entire clone set. Two independent AFLP reactions were produced from each plant obtained from meristem-tip culture, and only clear and reproducible amplified polymorphic fragments were scored.

Both SSR and AFLP amplicons were electrophoresed on 6.5% polyacrylamide gels, using a LI-COR Gene Reader 4200 apparatus, as described by Jackson and Matthews (2000).

#### *Analysis of DNA fingerprint data*

None of the SSR loci were polymorphic, so the microsatellite data were not subjected to statistical analysis. Each AFLP fragment was assumed to represent a single bi-allelic locus, and was scored as 1 when present and 0 when absent. A probability of identity (PI) was calculated following Paetkau et al. (1995). The binary 1/0 matrix was imported into NTSYS-pc software (Rohlf 1993), and used to calculate genetic similarities between clones based on Jaccard's Similarity Index (JSI) (Jaccard 1908), using the SIMQUAL (Similarity of Qualitative Data) routine. These similarity coefficients were used to construct a UPGMA dendrogram. A co-phenetic matrix was produced



using the hierarchical cluster system, by means of the CPH routine, and correlated with the original distance matrices, in order to test for association between clusters and the JSI matrix. The clustering ability of each polymorphic fragment was tested to determine the minimum number of fragments needed to uniquely fingerprint all clones.

## Results

### *In vitro* sanitation

Of the 90 symptomless plants, 58 were found to be infected with AILV and 32 with both AILV and ArLV. No other viruses were detected. Twenty AILV-infected and 20 AILV- and ArLV-infected cuttings were used as sources of explants. After the first passage, all explants were assayed for the presence of AILV and ArLV, and while no ArLV was detectable, AILV was still present in these tissues. Four explants from each meristem were then subjected to *in vitro* thermotherapy. A mean of three of the four explants survived, and produced rosettes consisting of six or seven new shoots (Fig. 2a). At least six shoots per explant survived thermotherapy and all were subjected to a second cycle of meristem-tip culture. In all, 34 acclimatized plantlets, traceable to seven different maternal plants, were obtained. These seven clones were designated as 8.2A, 3A, 10.2A, 5.2B, 9.2B, 7B1 and 10.2B, respectively. Over two years, all plants obtained from *in vitro* thermotherapy were assayed on a monthly basis for the presence of AILV and ArLV, and all were found to be virus-negative.

### *In vivo* sanitation

Only three of 20 acclimatized plants that were subjected to thermotherapy survived the treatment. Within one month, these developed five or six lateral shoots per plant (Fig. 2b), and these were virus tested 60 days, 90 days, and 150 days following thermotherapy. At 60 days, neither virus was detected, but at 90 days, AILV, but not ArLV, was found to have been re-established in all the shoots. At 150 days, AILV was still present, so a further cycle of meristem-tip culture was carried out. Seven meristem-tips survived culture and sub-culture, at which point all material was free of both ArLV and AILV. Two clones (1/2/4 and 2/7/1) were successfully acclimatized and transferred to an insect-free greenhouse.

### Effect of sanitation on earliness

A set of 30 plants derived from clones 8.2A, 7B1, 5.2B and 9.2B and 1/2/4 were planted in a commercial artichoke field at the end of August 2005. Plants derived from clones 8.2A and 7B1 flowered by the end of November 2005; while, those derived from the three clones flowered one month later, at the same time as most wild-type plants flowered in the field. When visually inspected, treated plants showed larger plant size and brighter green foliage than non-treated plants (Fig. 2c), and produced leaves and capitula that were 1.5-2 folds more abundant than all other wild-type plants of the globe artichoke “Brindisino” type.

### Genotyping

All SSR primer pairs generated amplicons, consisting of either one or two alleles, as expected. In all, 13 alleles were detected across the nine clones, but no polymorphism was detected (Fig. 3). The six AFLP PCs applied in the pilot study (three genotypes) generated 382 bands of which 91 were polymorphic. The PC E+ACA/M+CAA was chosen since originated the highest number of polymorphisms and produced 28 polymorphic bands (ranging in size from 150 to 750bp) when applied to whole set of the 9 sanitized plants, (Fig. 4). Three (2/7/1, 3A and 1/2/4) of the nine clones showed clone-specific fragment(s), potentially convertible to sequence tagged site assays, if there was a need for a simple means of identifying these clones. The PIs ranged from 0.605 to 0.702 for each locus and we estimated that the combined probability of finding two identical genotypes was  $1.75 \times 10^{-5}$ .

#### *Genetic similarity and cluster analysis*

Based on the UPGMA dendrogram of AFLP analysis, the co-phenetic correlation coefficient (r-value) of 0.937 between the data matrix and the co-phenetic matrix for AFLP data showed that the clusters were well supported by the similarity matrices (Fig. 5). Each clone had a unique AFLP fingerprint. Clones 5.2B and 10.2A were the most similar to one another (JSI of 0.789), while 8.2A and 3A were the least similar to the cluster containing all the other clones. The minimum number of fragments required to uniquely fingerprint all clones was four, and the AFLP profiles relating to these fragments are reported in Table I.

## Discussion

In establishing an *in vitro* shoot proliferation protocol for globe artichoke, it is important that the highest frequency of shoot organogenesis is obtained from a single meristem-tip. This is usually achieved by multiple passages, which, in the case of “Brindisino” and other reflowering globe artichokes (Pecaut and Martin 1992; 1993 and references therein), carries a risk of compromising important agronomic traits, such as early flowering and capitulum shape. Despite the risk or recovery of undesirable traits, meristem-tip culture does not appear to guarantee virus elimination, as some viruses persist through generations of sub-culturing - as observed in this (with AILV), and as reported previously by Faccioli and Marani (1998). Thus, micropropagation using meristem-tip explants is not an ideal system for obtaining virus-free globe artichoke plants.

Thermotherapy has been exploited for virus elimination in a number of woody plants (Mink et al. 1998), but cannot be applied to heat-sensitive plants and heat-tolerant viruses. Although little information has been reported on the effects of high temperature on the virus life cycle, it has been assumed that it delays virus movement through the plant, and hence inhibits colonization of the meristem-tip (Mink et al. 1998). Other studies have indicated that thermotherapy inhibits viral replication while the virus is being degraded, thus resulting in its elimination from shoot-tips (Cooper and Walkey, 1978; Kassanis, 1957). Recently, Wang et al. (2008) have demonstrated that *Raspberry bushy dwarf virus* (RBDV) can be eradicated from raspberry plants by using a combination of meristem-tip culture, thermotherapy, and cryotherapy. As RBDV RNA is highly degraded at high temperatures, it has been suggested that RNA degradation could be due to RNA silencing that is enhanced at elevated temperatures (Szittyá et al. 2003; Qu et al. 2005; Chellappan et al. 2005). Moreover, RNA silencing could have been also implicated in the exclusion of the virus from meristems (Foster et al. 2002; Mochizuki and Ohki, 2004; Qu et al. 2005; Schwach et al. 2005). However, silencing is not confirmed as small interfering RNAs specific for RBDV have not been detected, and despite the fact that the virus is apparently not present in meristem-tips, thermotherapy followed by meristem-tip culture has not resulted in the recovery of any RBDV-free regenerants (Wang et al. 2008).

In raspberry, thermotherapy induced enlargement of cells of the apical dome and of both cells and vacuoles of the youngest leaf primordia so that the latter accumulated more water that was lethal when shoots were exposed to cryotherapy. Thus, results of Wang et al. (2008) suggested a model in which thermotherapy reduced the proportion of apical meristem invaded by the RBDV by degrading its RNA and reducing the number of virus-infected cells that survived the subsequent cryotherapy. These combined effects resulted in a situation in which shoot tips (> 10%) that survived and were able to regenerate could escape RBDV infection.

In this study, we have shown that virus-free plants of globe artichoke “Brindisino” can be obtained by a combination of meristem-tip culture and thermotherapy. ArLV infection was eradicated by meristem-tip culture, while AILV by a combination of meristem-tip culture and *in vitro* or *in vivo* thermotherapy. Comparing data of this study with those of Wang et al. (2008), Jovel et al. (2007), and Siddiqui et al. (2008), it can be assumed that RNA silencing as well as other mechanisms may have been involved in the eradication of the two viruses from globe artichoke.

ArLV belongs to the genus *Potyvirus*, family *Potyviridae* and probably codes for the HC-Pro protein known to suppresses local but not systemic RNA silencing (reviewed by Li and Ding, 2006). Thus, silencing could account for ArLV exclusion from apical meristem. On the other hand, Kobayashi and Zambryski (2007) have demonstrated that, in *Arabidopsis thaliana*, the silencing signal moves more extensively in the hypocotyl

and root compared with tissues of tips of cotyledons, and that a putative boundary for silencing spread may exist just below the shoot apical meristem blocking its movement upward from the root and hypocotyl. While these data may explain why ArLV is found in globe artichoke cotyledons (Bottalico et al. 2002), they do not provide any explanation why ArLV is not able to enter apical meristem as the silencing signal may not enter these cells. Since both viral movement and silencing signal follow the route of plasmodesmata and correlate with the degree of their aperture (Kobayashi and Zambryski, 2007), it is likely that ArLV exclusion from the apical meristem is a matter of restriction rather than the result of degradation by silencing. However, this is different with AILV as this virus belongs to genus *Nepovirus*, family *Comoviridae*, and unlike potyviruses no silencing suppressors have been identified among the genes coded by this group of viruses. After an initial phase of symptom appearance, plants infected by nepoviruses usually undergo a phase of recovery in which symptoms disappear.

Similarly, we can propose a model in which AILV could partially or totally escape silencing by a recovery-like mechanism that allows it to enter apical meristem thus yielding regenerants that are still infected. This hypothesis seems supported by the notion that AILV-infected tobacco plants undergo recovery but recovered tissue still contain highly infectious virus (Mascia et al., 2009). However, if explants of the first subculture are exposed to thermotherapy, AILV may not survive there either because RNA silencing is enhanced at high temperature or because meristematic cells receive big damage from the thermotherapy or by both so that virus-free regenerants can be obtained by the next cycle of meristem-tip culture. The surprising observation that AILV was undetectable in the shoots after 60 days of thermotherapy but reappeared within 30 days, is similar to the proposed model in this study. By extending thermotherapy for periods of up to five months, virus-free plants were obtained. The number of viable regenerants obtained in this study after five months of thermotherapy was about 10%, and similar to findings of Wang et al. (2008).

To date, the presence of AILV in globe artichoke has been reported only in Italy and Greece, at least five other viruses belonging to the same family, namely *Artichoke vein banding* (AVBV), *Artichoke yellow ringspot* (AYRSV), BBWV, *Artichoke Aegean ringspot* (AARSV) and *Tomato black ring* (TBRV) (Martelli and Gallitelli 2008) share a number of biological and physico-chemical properties with AILV. These can infect globe artichoke worldwide with analogous sanitation problems.

In this study, out of 60 plants obtained, only nine virus-free plants, of which seven were obtained from *in vitro* (8.2A, 10.2A, 5.2B, 9.2B, 7B1, 10.2B) and two (1/2/4 and 2/7/1) from *in vivo* virus-free plants. The *in vitro* and *in vivo* thermotherapy treatments (outlined in Fig. 1) were successful in maintaining early flowering habit and capitulum shape typical of the globe artichoke “Brindisino” type. Although both methods can deliver virus-free plants, both have disadvantageous features. The *in vitro* approach is economical in terms of time and space, since the heat treatment needs to be applied to only a few explants, which can be accommodated in a small facility; however, two passages of meristem-tip culture are required, with associated risks of recovery of off-types. The *in vivo* protocol requires only one round of meristem-tip culture, but is more demanding of heated and lit space, since rooted cuttings have to be maintained for long periods at high temperatures; furthermore, it is less efficient in terms of the number of viable plants recovered post treatment. The sanitised plants obtained here represent a starting point for the provision of productive and virus-free propagation material to farmers.

When regenerant plants were genotyped using molecular markers, SSR and AFLP markers, interesting findings were observed. Although the most informative eight microsatellites (out of 93; see Acquadro et al. 2003, 2005a, 2005b, 2009) were selected, as previously observed (Portis et al. 2005, Mauro et al. 2009), only

monomorphic patterns were generated from individuals within a given globe artichoke varietal type. On the other hand, AFLP analysis revealed polymorphism. Previously, it has been reported that AFLP assays many loci simultaneously, therefore, AFLPs are particularly suitable for the detection of intra-varietal polymorphism (Lanteri et al. 2004b). Indeed, for the nine clones investigated in this study, one AFLP primer combination generated 28 informative fragments, which made possible to obtain a unique fingerprinting for each, thus enabling assessment of clonal fidelity of material propagated in nurseries. AFLP markers have been widely used to assess genetic stability of plants regenerated through tissue culture (Polanco and Ruiz 2002; Vendrame et al. 1999; Chuang et al. 2009). Although out of the aim of this study, the present results confirm that AFLP markers are highly reproducible and allow the identification of a great number of polymorphisms among genotypes, confirming their suitability for evaluating the genetic fidelity of globe artichoke plantlets originated through meristem-tip culture.

## Figure legends

Fig. 1. A flow chart of the production of virus-free plants of early varietal types of globe artichoke using meristem-tip culture accompanied by either *in vivo* or *in vitro* thermotherapy. For *in vitro* thermotherapy, explants of the first passage are exposed to thermotherapy, and then used to obtain new meristem-tips that are grown on establishment medium, allowed to proliferate by not more than three passages, rooted, and acclimatized. For *in vivo* thermotherapy, after a period of acclimatization, off-shoots are exposed to thermotherapy, and then used to obtain meristem-tips that are transferred to an establishment medium, proliferated by not more than three passages, rooted, and acclimatized.

Fig. 2. Panel a: rosette generated from an explant that survived *in vitro* thermotherapy. Panel b: Leaves of plants exposed to *in vivo* thermotherapy dried down within two weeks and new growth was used for meristem-tip culture. Panel d: a demonstration field set up by the Regional phytosanitary extension service (Apulia) with virus-free plants of globe artichoke "Brindisino" (on the left) and standard material (on the right) routinely used by farmers. Picture took Nov. 2008.

Fig. 3. SSR profiles of nine sanitized globe artichoke clones.

Fig. 4. AFLP profiles of nine sanitized globe artichoke clones, generated by primer combination E+ACA/M+CAA. Each clone is represented by two independent reactions. Arrows indicate polymorphic fragments, black boxes indicate clone-specific fragments.

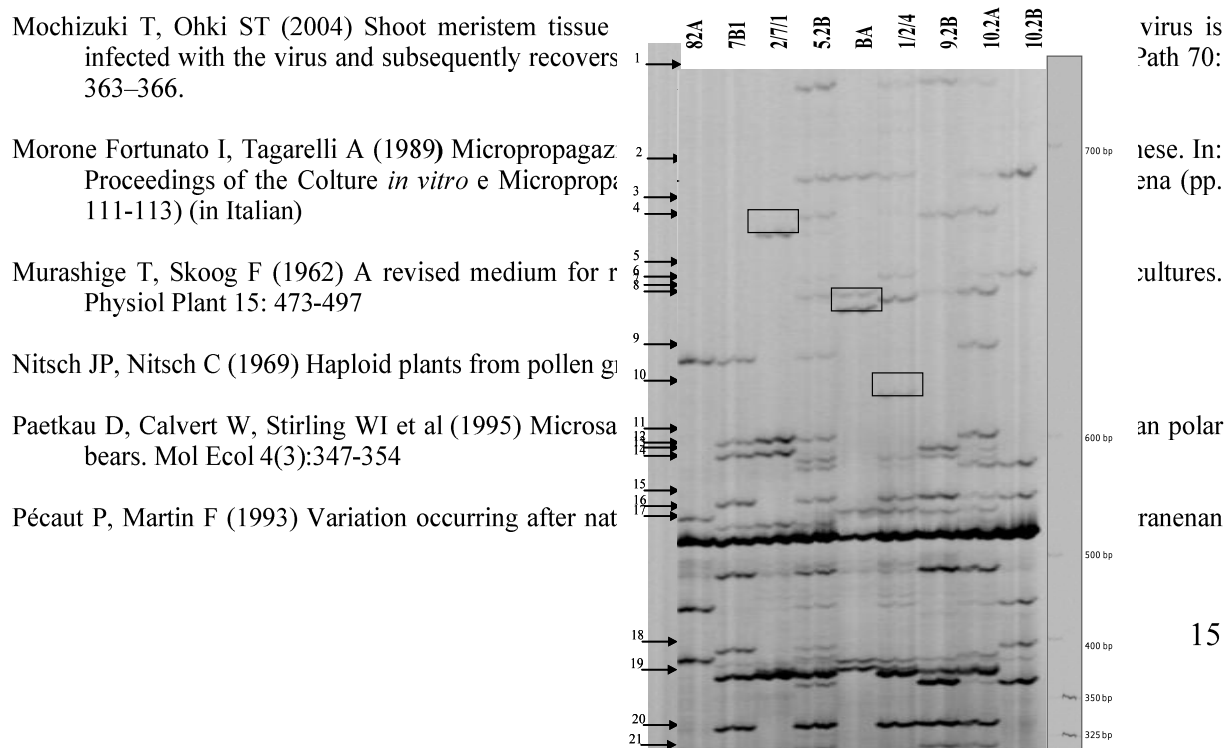
Fig. 5. UPGMA dendrogram based on AFLP data (primer combination E+ACA/M+CAA).

## References

- Acquadro A, Portis E, Lanteri S (2003) Isolation of microsatellite loci in artichoke (*Cynara cardunculus* L. var. *scolymus* L.). *Mol Ecol Notes* 3:37-39
- Acquadro A, Portis E, Lee D et al (2005a) Development and characterization of microsatellite markers in *Cynara cardunculus* L. *Genome* 48:217-225
- Acquadro A, Portis E, Albertini E et al (2005b) M-AFLP based protocol for microsatellite loci isolation in *Cynara cardunculus* L. (*Asteraceae*). *Mol Ecol Notes* 5:272-274
- Acquadro A, Lanteri S, Scaglione D et al (2009) Genetic mapping, diversity assessment and annotation of genomic microsatellites isolated from globe artichoke. *Theor Appl Genet* 118 (8): 1573-1587
- Bottalico G, Padula M, Campanale A et al (2002) Seed transmission of Artichoke Italian latent virus and Artichoke latent virus in globe artichoke. *J Plant Pathol* 84 (3):167-168
- Brigneti G, Voinnet O, Li WX, Ji LH, Ding SW, Baulcombe DC (1998) Viral pathogenicity determinants are suppressors of transgene silencing in *Nicotiana benthamiana*. *EMBO J.* 17:6739-6746
- Chellappan P, Vanitharani R, Ogbe F, Fauquet CM (2005) Effect of temperature on geminivirus-induced RNA silencing in plants. *Plant Physiol* 138: 1828-1841
- Chuang SJ, Chen CL, Chen JJ, Chou WYY, Sung JM (2009) Detection of somaclonal variation in micro-propagated *Echinacea purpurea* using AFLP marker. *Sci Hortic* 120(1): 121-126.
- Cooper VC, Walkey DGA (1978) Thermal inactivation of cherry leaf roll virus in tissue cultures of *Nicotiana rustica* raised from seeds and meristem tips. *Ann. Appl. Biol.* 88: 273-278.
- Faccioli G, Marani F (1998) Virus elimination by meristem tip culture and tip micrografting. In: Hadidi A, Khetarpal RK, Koganezawa H, (eds) *Control of Plant Virus Diseases*, APS Press, St. Paul, MN, pp 346-380
- Foster TM, Lough TJ, Emerson S, Lee R., Bowman JL, Forster RLS, Lucas WJ (2002) A surveillance system regulates selective entry of RNA into the shoot apex. *Plant Cell* 14: 1497-1508.
- Gallitelli D, Barba M (2003) Normativa vivaistica in Italia. In: *Atti Giornate Nazionali di studi sul Carciofo, Vivaismo e strategie di sviluppo del Carciofo*, Samassi (Ca): 47-51
- Gallitelli D, Rana GL, Vovlas C et al (2004) Viruses of globe artichoke: an overview. *J Plant Pathol* 86(4): 267-281
- Gallitelli D, Saldarelli P (1996) Molecular identification of phytopathogenic viruses. In: JP Clapp (ed) *Methods in Molecular Biology* 50, *Species Diagnostics Protocols: PCR and other Nucleic Acid Methods*. Humana Press, New York, pp 57-79
- Harbaoui Y, Smaijn G, Welvaert et al (1982) Assainissement viral de l'artichaut (*Cynara scolymus*) par la culture in vitro d'apex meristematiques. *Phytopat medit* 21: 15-19
- Jaccard P (1908) Nouvelles recherches sur la distribution florale. *Bull Soc Vaud Sci Nat* 44: 223-270
- Jackson JA, Matthews D (2000) Modified inter-simple sequence repeat PCR protocol for use in conjunction with the Li-Cor gene ImagIR(2) DNA analyzer. *BioTechniques* 28: 914-917
- Jovel J, Walker M, Sanfaçon H (2007) Recovery of *Nicotiana benthamiana* plants from a necrotic response induced by a Nepovirus is associated with RNA silencing but not with reduced virus titer. *J Virol* 81:12285-12297
- Kassanis B (1957) Effects of changing temperature on plant virus diseases. *Adv Virus Res* 4:221-241



- Kobayashi K, Zambryski P (2007) RNA silencing and its cell-to-cell spread during *Arabidopsis* embryogenesis. *Plant J* 50:597–604.
- Lanteri S, Di Leo I, Ledda L et al (2001) RAPD variation within and among populations of globe artichoke (*Cynara scolymus* L.), cv ‘Spinoso sardo’. *Plant Breeding* 120: 243-247
- Lanteri S, Saba E, Cadinu M et al (2004a) Amplified fragment length polymorphism for genetic diversity assessment in globe artichoke. *Theor Appl Genet* 108: 1534-1544
- Lanteri S, Acquadro A, Saba E et al (2004b) Molecular fingerprinting and evaluation of genetic distances among selected clones of globe artichoke (*Cynara cardunculus* L. var. *scolymus* L.) ‘Spinoso sardo’. *J Hort Sci Biotech* 79: 863-870
- Li F, Ding SW (2006) Virus counterdefense: diverse strategies for evading the RNA-silencing immunity. *Ann Rev Microbiol* 60: 503-531
- Lloyd GB, McCown BM (1980) Commercially feasible micropropagation of montian laurel, *Kalmia latifolia*, by the use of shoot tip culture. In: *Proceedings International Plant Propagation Society* 30: 412-427
- Marras, F, Foddai A, Fiori M (1982) Possibilità di risanamento del carciofo da infezioni virali mediante coltura *in vitro* di apici meristematici. In: *Proceedings of Giornate Fitopatologiche*, Sorrento (pp 151-158) (in Italian)
- Martelli GP, Gallitelli D (2008) Viruses of *Cynara*. In: Rao GP, Khurana SMP, Lanardon S (eds) *Characterization, Diagnosis & management of Plant Viruses*, Vol 1: Industrial Crops. Studium Press Texas, USA, pp 445-479
- Martin-Hernandez AM, Baulcombe DC (2008) Tobacco rattle virus 16 kilodalton protein encodes a suppressor of RNA silencing that allows transient viral entry in meristems. *J Virol* 82: 4064-4071
- Mascia T., Prigigallo I., Gallitelli D. 2009 Artichoke italian latent virus and RNA silencing: implications in sanitation schemes of nepovirus infected plants. *Proc. XV Annual Congress of the Italian Phytopathological Society Locorotondo (BA), Italy, 28 Ottobre -1 novembre 2009*; p.64
- Mauromicale G, Ierna A (2000) Panorama varietale e miglioramento genetico del carciofo, *Inf agrario* 26:39-45
- Mauro R, Portis E, Acquadro A et al (2009) Genetic diversity of globe artichoke landraces from Sicilian small-holdings: implications for evolution and domestication of the species. *Cons Genetics* 10:431-440
- Mink GI, Wample R, Howell WE (1998) Heat treatment of perennial plants to eliminate phytoplasmas, viruses and viroids while maintaining plant survival. In: Hadidi A, Khetarpal RK, Koganezawa H, Hadi (eds) *Control of Plant Virus Diseases*. APS Press, St. P:

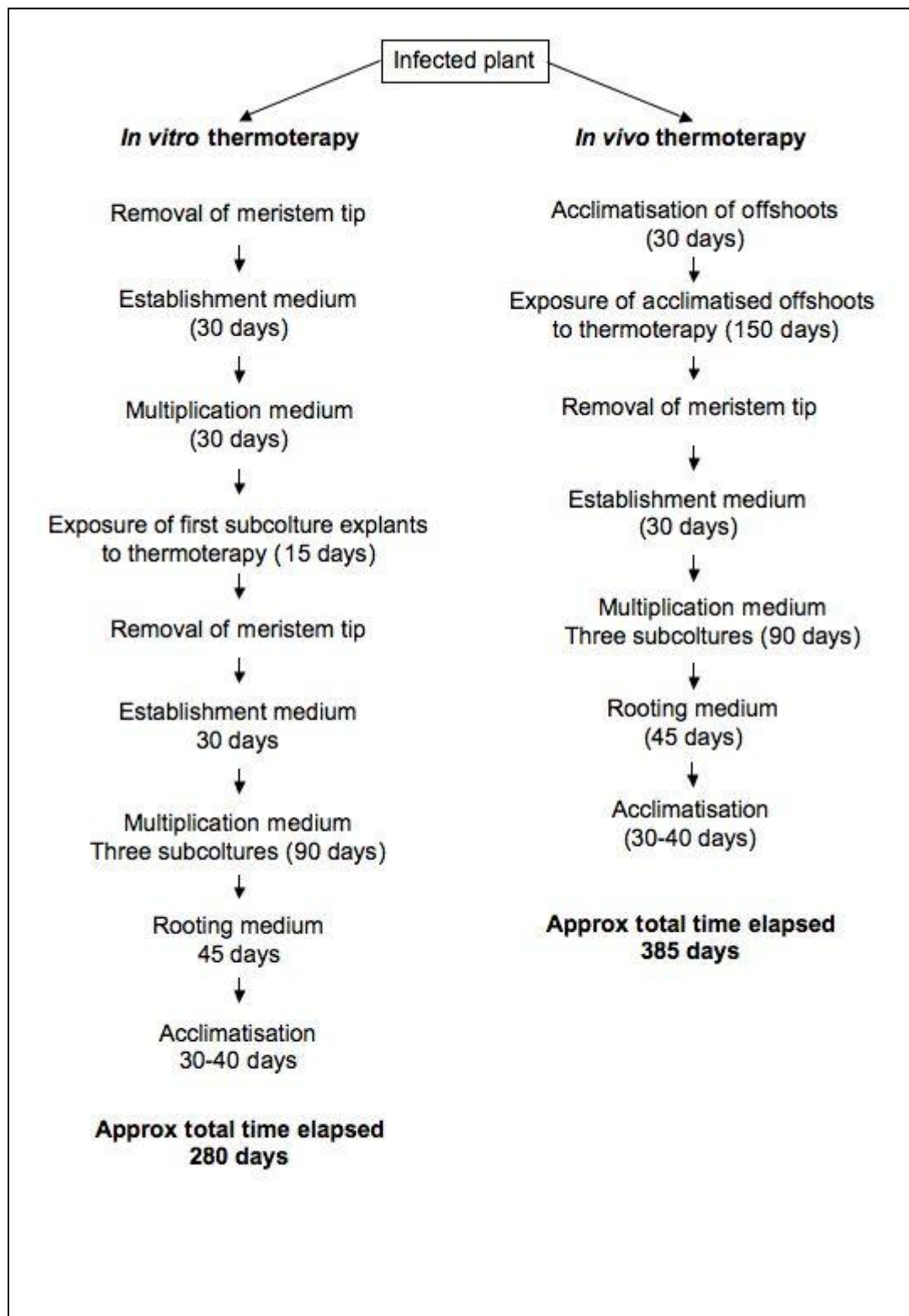


- cultivars of globe artichoke (*Cynara scolymus* L). *Agronomie* 13 : 909-919
- Pécaut P, Martin F (1992) Non-conformity of *in vitro* propagated plants of early Mediterranean varieties of globe artichoke (*Cynara scolymus* L.). *Acta Hort* 300: 363-366
- Pécaut PJ, Corre J, Lot H et al (1985) Interet des plantes sains d'artichaut régénérées par la culture *in vitro*. *PHM-Rev. Hort*: 256
- Peña-Iglesias A, Ayuso-Gonzales P (1974) The elimination of viruses from globe artichoke (*Cynara scolymus* L.) by etiolated meristem tip culture. In: *Proceedings 19th International Horticulture Congress, Warsaw (63)* (in English)
- Peña-Iglesias A, Ayuso-Gonzales P (1982) The elimination of some globe artichoke viruses by shoot apex culture and *in vitro* micropropagation of the plant. In: *Proceedings of the 4th Conference of the ISHS Vegetable Virus Working Group, Wellesbourne (47)* (in English)
- Pochard E, Foury C, Chambonnet D (1969) Il miglioramento genetico del carciofo. In: *Atti del 1° Congresso internazionale di studi sul carciofo, Bari 1967 (117-143)* (in Italian).
- Polanco C, Ruiz ML (2002) AFLP analysis of somaclonal variation in *Arabidopsis thaliana* regenerated plants, *Plant Sci* 162: 817-824
- Portis E, Mauromicale G, Barchi L et al (2005) Population structure and genetic variation in autochthonous globe artichoke germplasm from Sicily Island. *Plant Sci* 168: 1591-1598
- Qu F, Ye XH, Hou GC, Sato S, Clemente TE, Morris TJ (2005) RDR6 has a broad-spectrum but temperature-dependent antiviral defense role in *Nicotiana benthamiana*. *J Virol* 79: 15209–15217.
- Rohlf FJ (1993) *NTSYS-PC Numerical taxonomy and multivariate analysis system, version 1.80*. Owners manual. Exter Software, New York
- Sharma S, Singh B, Rani G et al (2008) *In vitro* production of Indian Citrus RingSpot Virus (ICRSV) free Kinnow plants employing thermotherapy coupled with shoot tip grafting. *Plant Cell Tiss Org Cult* 92 (1): 85-92
- Schwach F, Vaistij FE, Jones L, Baulcombe DC (2005) An RNA-dependent RNA polymerase prevents meristem invasion by potato virus X and is required for the activity but not the production of a systemic silencing signal. *Plant Physiol* 138: 1842–1852
- Siddiqui SA, Sarmiento C, Kiisma M, Koivumäki S, Lemmetty A, Truve E, Lehto K (2008) Effects of viral silencing suppressors on tobacco ringspot virus infection on two *Nicotiana* species. *J Gen Virol* 89: 1502-1508.
- Szittya G, Silhavy D, Molnar A, Havelda Z, Lovas A, Lakatos L, Banfalvi Z, Burgyan J (2003) Low temperature inhibits RNA silencing-mediated defence by the control of siRNA generation. *EMBO J* 22: 633–640.
- Vendrame WA, Kochert G and Wetzstein HY (1999) AFLP analysis of variation in pecan somatic embryos. *Plant Cell Rep* 18: 853-857.
- Vos P, Hogers R, Bleeker M et al (1995) AFLP: A new technique for DNA fingerprinting. *Nucleic Acids Res* 23: 4407-4414
- Wang D, MacFarlane SA, Maule AJ. (1997) Viral determinants of pea early browning virus seed transmission in pea. *Virology* 234:112–117.
- Wang Q, Cuellar WJ, Rajamäki ML, Hirata Y, Valkonen JPT (2008) Combined thermotherapy and cryotherapy for efficient virus eradication: relation of virus distribution, subcellular changes, cell survival and viral RNA degradation in shoot tips. *Mol Plant Path.* 9: 237-250

**Table 1.**

Fingerprinting of the 9 clones of globe artichoke “Brindisino” in study on the basis of the presence (1) and absence (0) of the 4 AFLP fragments identified

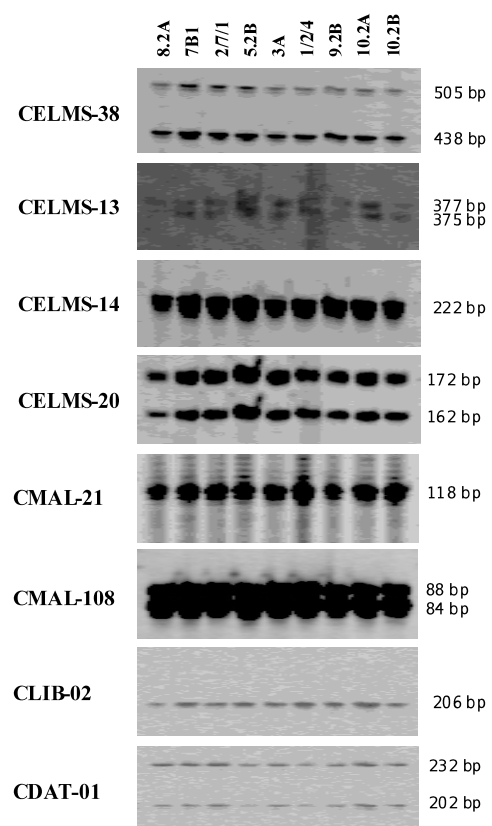
<b>AFLP bands</b>	<b>5.2B</b>	<b>10.2A</b>	<b>9.2B</b>	<b>1/2/4</b>	<b>7B1</b>	<b>2/7/1</b>	<b>10.2B</b>	<b>82A</b>	<b>3A</b>
<b>15</b>	1	1	1	1	1	0	1	0	0
<b>16</b>	0	1	1	1	0	0	0	1	1
<b>26</b>	1	1	0	1	0	1	0	1	0
<b>28</b>	1	0	1	1	1	0	0	0	0



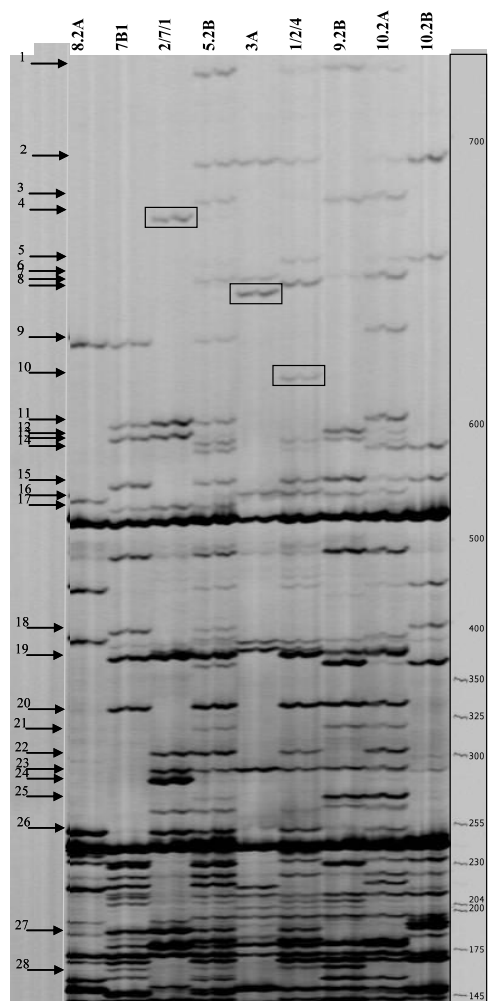
**Figure 2**



**Figure 3**



**Figure 4**



**Figure 5**

