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Abstract

Tomato yellow leaf curl virus (TYLCV) and its related viruses are prone to recombination. It was reported that random homologous recombination between 20% diverging TYLCV related species is rarely deleterious and may be associated with a fitness advantage. Indeed, TYLCV-IS76, a recombinant between the 20% divergent TYLCV and tomato yellow leaf curl Sardinia virus, exhibited a higher fitness than that of parental viruses. As this typical fitness advantage was observed with TYLCV-IS76 representatives of different pedigrees, it was thought that it is induced by beneficial intra-genomic interactions rather than by specific mutations. This hypothesis was further supported with TYLCV-IS141, a TYLCV recombinant with a short TYLCSV inherited fragment of around 141 nts, slightly longer than that of TYLCV-IS76. Indeed, the typical fitness advantage was detected irrespective of the position of the recombination breakpoint (loci 76 or 141) and the sequences of the TYLCV and TYLCSV inherited fragments.

Keywords

Plant resistance, geminivirus, emergence, positive selection, convergent evolution

Introduction

Recombination is an important source of variability for some viruses and particularly those belonging to the genus *Begomovirus*, family *Geminiviridae* (Lefeuvre and Moriones, 2015). Tomato yellow leaf curl virus (TYLCV), a begomovirus infecting tomato worldwide, is particularly prone to recombination (Davino et al., 2012; Garcia-Andres et al., 2007; Martin et al., 2011; Urbino et al., 2013). Moreover, it was reported that random homologous recombination between 20% diverging TYLCV related species is rarely deleterious (Vuillaume et al., 2011). Genetic variability is a vector of adaptation to new environments and the contribution of recombination was demonstrated with TYLCV-IS76, a recombinant between TYLCV and tomato yellow leaf curl Sardinia virus (TYLCSV). TYLCV-IS76 virtually replaced its parental viruses in Southern Morocco (Souss region) (Belabess et al., 2015), and the replacement coincided with the shift from susceptible tomato cultivars to resistant cultivars bearing the Ty-1 resistant gene. The fitness of TYLCV-IS76 in Ty-1 resistant plants was shown experimentally to be higher than that of representatives of parental viruses (Belabess et al., 2016), which was fully consistent with an adaptation scenario to the new Ty-1 resistant environment. Typically, the competitiveness of TYLCV-IS76 is associated with a drastic negative impact on TYLCV accumulation, the most competitive parental virus in Ty-1 resistant plants. This negative effect was induced by a wild type TYLCV-IS76 clone, as well as with a TYLCV-IS76 clone generated artificially from a non-Moroccan TYLCV parent, but was not induced by a TYLCSV parental-type clone (Belabess et al., 2016). Hence,

it was thought that the typical competitiveness feature of TYLCV-IS76 was mediated by a beneficial modification of the intra-genome interaction network rather than by a direct effect of specific mutations inherited from one of the parent. As stated previously by Martin et al. (Martin et al., 2011), this network might include inter-amino-acid contacts that determine and maintain proper protein folding, Watson-Crick base pairing within functionally meaningful secondary structures that form in single stranded DNA (ssDNA) and RNA molecules, and DNA and amino acid sequence motifs that mediate protein-protein, protein-DNA and protein-RNA interactions.

It was previously shown that homologous recombination breakpoints of TYLCV recombinants generated in plants co-infected with clones of TYLCV and Tomato leaf curl Comoros virus (ToLCKMV, formerly named Tomato leaf curl Mayotte virus), are not randomly distributed (Martin et al., 2011). Using a permutation-based genetic association test, these authors showed that the over-all patterns of recombination are strongly influenced by negative selection against individual recombinants displaying disrupted intra-genomic interactions. Interestingly, the results obtained with TYLCV-IS76, suggest that intra-genomic interactions induced by interspecies recombination are not necessarily deleterious or neutral but may be beneficial. It is noteworthy that the inferences deduced from the TYLCV/ToLCKMV recombinant library are expected to be relevant for TYLCV/TYLCSV recombinants because the genomic divergence between both pairs of viruses is similar, about 20%. The objective of the present study was to further support that the beneficial effect of the recombination in TYLCV-IS76 is due to improved intra-genome interactions rather than by specific mutations inherited from a particular parent.

TYLCV-IS76 was considered as an unusual TYLCV/TYLCSV recombinant because, unlike the previously reported TYLCV/TYLCSV recombinants, its TYLCSV-derived region is extremely short, only 76 nts on the virion sense gene side starting from the origin of

replication (OR). Interestingly, other TYLCV/TYLCSV recombinants with a similar recombination profile were suspected to be positively selected in Ty-1 resistant plants (Belabess et al., 2018; Panno et al., 2018). They belong to a recombinant type named hereafter TYLCV-IS141 according to the length of the short TYLCSV-derived region, around 141 nts, extending from the OR to the initiation codon of the V2 gene. Representatives of this recombinant were detected for the first time in 6 out of 10 Ty-1 resistant plants sampled in Sicily between 2012 and 2015 (Belabess et al., 2015; Belabess et al., 2018). The hypothesis of a positive selection of TYLCV-IS141 recombinants in Ty-1 resistant plants was suggested by field and laboratory results. Whereas TYLCV-IS141 recombinants were detected in co-infection with parental viruses in susceptible plants, in Ty-1 resistant plants, they were detected alone (Panno et al., 2018). Moreover, TYLCV-IS141 recombinants were found to be generated in Ty-1 resistant plants co-inoculated with TYLCV and TYLCSV clones, and their accumulation in parallel lineages is consistent with positive selection (Belabess et al., 2018).

TYLCV-IS141 was considered as an excellent model virus to further support the positive effect of modified intra-genomic interactions on fitness. Here we show that positive selection of recombinants with short TYLCSV-derived regions was detected in Ty-1 resistant environment, irrespective of the position of the recombination breakpoint (loci 76 or 141) and the sequences of the TYLCV and TYLCSV inherited fragments. Altogether, the results support that the fitness advantage of IS76 and IS141 type recombinants is induced by beneficial intra-genome interactions rather than by specific mutations inherited from a particular parental genome.

Materials and Methods

Plant material

The resistant cultivar “Pristyla" carrying the Ty-1 resistance allele in a heterozygote state (Ty-1/ty-1) and a nearly isogenic susceptible cultivar (Gautier Semences, France), were previously described (Belabess et al., 2016). Seven-day-old seedlings were transplanted into individual pots. All plants were grown in containment growth chambers under 14h light at 26±2°C, and 10h dark at 24±2°C, and were watered with 15:10:30 NPK fertilizer + oligoelements.

Construction of recombinant TYLCV-IS76” by site-directed mutagenesis

TYLCV-IS76 is a TYLCV/TYLCSV recombinant that was cloned from a naturally infected tomato plant collected in the field (Belabess et al., 2015). TYLCV-IS76’ has the same recombination pattern as TYLCV-IS76 but its TYLCV region is 100% identical to the homologous region of TYLCV-IL[RE:STG4:04] (GenBank accession number AM409201), the parental TYLCV clone used experimentally (Belabess et al., 2016). TYLCV-IS76” has the same recombination pattern as TYLCV-IS76’ but its TYLCSV region was replaced here with the homologous region of TYLCSV-ES[MA:Aga5a:12] (GenBank accession number LN846598), the parental TYLCSV clone used experimentally (Belabess et al., 2016). The TYLCSV region of TYLCV-IS76 and TYLCV-IS76’ differ from that of TYLCV-IS76” at positions 32 and 44. TYLCV-IS76” was constructed with the QuikChange Lightning Site-Directed Mutagenesis Kit (Agilent Technologies, USA) according to the manufacturer’s instructions. Primers were designed using the web-based QuikChange Primer Design Program available online at www.agilent.com/genomics/qcpd (Table S1). The amplification product was digested with 2 µL of DpnI at 37°C for 5 h, and introduced into XL10-Gold competent cells. Plasmid DNA was purified with the Wizard sv plus minipreps DNA purification system (Promega, USA), and sequenced with plasmid specific primers (M13-40 Forward and SP6) and insert-specific primers (Beckman Coulter Genomics).

Agroinfectious TYLCV clones

The agro-infectious clones for TYLCV-IL[RE:STG4:04] (GenBank accession number AM409201) and TYLCV-IS76' were previously reported (Belabess et al., 2016). Additional agroinfectious clones were prepared here for each of the following TYLCV/TYLCSV recombinants: (i) TYLCV-IS76"; (ii) TYLCV-IS141[Fr:Lab; 17] (TYLCV-IS141-Lab) (GenBank MG489967), previously cloned in an *E. coli* vector from the Pristyla tomato plant R16, coinfected with parental TYLCV-IL[RE:STG4:04] and TYLCSV-ES[MA:Aga5a:12] (Belabess et al., 2018); and (iii) TYLCV-IS141[IT:Sic1; 13] (TYLCV-IS141-Sic) cloned and sequenced here from a resistant tomato plant sampled in December 2013 in Sicily (Belabess et al., 2015) (GenBank MG489968),. The cloning was as described for TYLCV-IS141-Lab (Belabess et al., 2018). To prepare agroinfectious clones, the cloned genomes were inserted as a tandem repeat into the binary vector pCAMBIA2300. The recombinant dimer construction was isolated from *E. coli* and introduced into *Agrobacterium tumefaciens* (strain C58) via electroporation. Agroinfectious constructions of TYLCV-Il, TYLCV-IS76' and TYLCV-IS76" are cloned in PCambia 0380 and those of TYLCV-IS76, TYLCV-IS141-lab and TYLCV-IS141-Sic are cloned in PCambia 2300.

Agro-inoculation, and randomized experimental design

Tomato plants were agroinoculated 14 days after sowing with various combinations of agroinfectious clones in two different experiments as described in Table 1. Agrobacterial suspensions were prepared for single and mixed infections as described in (Belabess et al., 2016). Plants used as negative controls were agroinfiltrated with bacteria of the C58 MP90 strain of *A. tumefaciens* containing an empty pCAMBIA2300 plasmid. Plants were arranged

in a complete randomized block design. TYLCV-IS141-Lab and TYLCV-IS141-Sic were tested in Experiments 1 and 2 respectively, in single infection and in competition with TYLCV-IL (Table 1). At 30 dpi, the height of the plants was measured between the petioles of the cotyledons and that of the youngest leaf. In Experiment 1, plants were analyzed at 10, 30, 60 and 120 dpi, except the plants coinfected with TYLCV-IL and TYLCV-IS76' which were analyzed at 30 dpi only. It should be noted that after the 30 dpi sampling, only 10 randomly chosen plants were retained per treatment for the last samplings. In Experiment 2, plants were analyzed at 30 and 60 dpi, and only 6 randomly chosen plants were retained to be sampled at 120 dpi.

Total DNA extraction

At each collection date, five leaf discs were collected from each plant, from the youngest leaf for which five leaflets were visible, as described in (Belabess et al., 2016). Total DNA from each sample was extracted according to the protocol of (Dellaporta et al., 1983) with the previously reported modifications (Belabess et al., 2016), and stored at –20°C until use.

Real-time PCR quantification of each virus

The within-plant accumulation of TYLCV-IL, TYLCSV-ES and TYLCV-IS76' clones was estimated with real-time PCR (qPCR) as described in (Belabess et al., 2016). The forward primer used for TYLCV-IS76" was modified from that of TYLCV-IS76 to take in account the difference of nucleotide at position 32. Primer pairs were designed on both sides of locus 141 for specific detection of IS141-Lab and IS141-Sic recombinants (Table S1). The specificity of each primer pair was tested with viral DNA of non-targeted viral clones.

The viral DNA content of each plant sample was quantified in duplicate using the LightCycler 480 SYBR Green I qPCR mix (Master, Roche, Germany), and standardized with an estimation of the plant DNA content by a qPCR quantification of the nuclear-encoded large subunit ribosomal RNA gene (*Solanum lycopersicum* L. 25S ribosomal RNA gene) as described in (Belabess et al., 2016). DNA accumulations were expressed either with fluorescence values or with the number of viral DNA targets present in each sample. The copy numbers of the target were interpolated from the appropriate standard curve obtained for each virus with 10 fold serial dilutions of recombinant plasmid extracts ranging between 10^1 and 10^8 copies.

Statistical analysis

All statistical analyses were performed using R Studio software, version 3.0.3.(R_Development_Core_Team, 2010). Viral DNA accumulations were compared between or within plants of Experiment 1 and experiment 2. Because data did not always fill assumptions of normal distribution of experimental errors and equal variances between treatments for ANOVA tests, a non-parametric test was used (Kruskal-Wallis test). Non-parametric comparisons for each pair were performed with Wilcoxon method.

Phylogenetic analysis

Viral DNA sequences were aligned using the dynamic alignment method of DNAMAN (version 5.0; Lynnon BioSoft, Quebec, Canada) with default parameters. Phylogenetic trees were constructed using the maximum likelihood method (ML) implemented in PhML (Guindon et al., 2010). The best nucleotide substitution model that fitted the data was determined using likelihood-based criteria (AIC) with SMS (Smart Model Selection) (Lefort

et al., 2017). The branch support was calculated with the fast likelihood-based method (aLRT) implemented in PhyML(Anisimova and Gascuel, 2006).

Results

The intra-plant frequency of TYLCV-IS141 recombinants increased after their generation

The generation and accumulation of TYLCV-IS141 recombinants observed previously in three Ty-1 resistant tomato plants (R5, R6, R16) co-inoculated in parallel with TYLCV-IL and TYLCSV-ES parental viruses, suggests that these recombinants were positively selected (Belabess et al., 2018). To further support the selection hypothesis, we tested if the intra-plant accumulation of recombinants bearing a recombination breakpoint around position 141 increased over time. To do this, DNA extracts from formerly collected leaf samples (Belabess et al., 2018) were used to monitor DNA accumulation of such recombinants and that of parental viruses between 1 and 12 months post-inoculation (mpi), using qPCR (Fig. 1). TYLCV-IS141 recombinants were below the detection level at 1 mpi. Their detection was possible from 2 mpi in plants R6 and R16 and from 5 mpi in plant R5. Finally, at 12 mpi, following the accumulation of TYLCV-IS141 recombinants and the parallel decrease of the parental genomes, the frequency of the recombinants was about 10^3 to 10^4 time higher than those of the parental viruses, which altogether support the positive selection of TYLCV-IS141 recombinants.

TYLCV-IS141 exhibits a high selective advantage over the parental TYLCV in Ty-1 resistant plants

To further confirm the suspected selective advantage of TYLCV-IS141 in Ty-1 resistant plants (Belabess et al., 2018; Panno et al., 2018), we estimated the intra-plant DNA accumulation of a TYLCV-IS141 clone isolated from plant R16 (TYLCV-IS141-Lab) and compared it to that of TYLCV-IL[RE:STG4:04]), the parent from which it inherited most of its genome (95%) (Belabess et al., 2018), and which is the most competitive parent in Ty-1 resistant plants (Belabess et al., 2016; García-Andrés et al., 2009). DNA accumulation was used as a proxy for fitness and was monitored over time in single and competition infection in both Ty-1 resistant plants and nearly isogenic susceptible plants. Typical symptoms of leaf curling and yellowing were observed at 18 dpi in all infected plants of the susceptible cultivar whereas resistant plants were symptomless. The height of the infected plants of the susceptible cultivar were approximately half that of mock inoculated ones (Tukey's test, P= 0.05), but no difference was detected with the resistant plants (Suppl Fig.1). Plants were sampled at 10, 30, 60 and 120 dpi for the quantification of viral DNA accumulations. Fig. 2 shows DNA accumulations at 30 and 120 dpi whereas the full data set is presented in Suppl. Fig. 2. In Ty-1 resistant plants, the DNA accumulation of TYLCV-IS141-Lab was significantly higher than that of TYLCV at 30, 60 and 120 dpi, irrespective of the infection status (single or mixed); in the 10 dpi samples of Ty-1 resistant plants, viral accumulations were similar (Fig. 2 and Suppl Fig. 2). In susceptible plants, the DNA accumulation of TYLCV-IS141-Lab is significantly higher than that of TYLCV at 60 and 120 dpi, in mixed infection and at 120 dpi in single infection. At 10, 30 and 60 dpi in single infection, its accumulation was similar to TYLCV or slightly lower (Fig.2 and Suppl. Fig. 2). These results confirm that TYLCV-IS141-Lab has a fitness advantage in Ty-1 resistant plants and that the fitness cost in susceptible plants, if any, is low.

The competitiveness of representatives of TYLCV-IS141 and TYLCV-IS76 are similar

The recombination profile of TYLCV-IS141 type recombinants is very similar to that of TYLCV-IS76, the invasive recombinant reported from Morocco. A high selective advantage was reported for TYLCV-IS76 (Belabess et al., 2016), and the typical feature of this advantage was its tremendous negative impact on the accumulation of TYLCV in Ty-1 resistant tomato plants. Thus, to further characterize the competitiveness of TYLCV-IS141-Lab, we compared it with that of a representative of TYLCV-IS76. To compare only the effect of recombination and not that of additional mutations inherited from different parental viruses, we engineered TYLCV-IS76", which like TYLCV-IS141-Lab, is composed of TYLCV and TYLCSV genome fragments exhibiting 100% identity with, respectively TYLCV (GenBank N° AM409201), and TYLCSV (LN846598), the clones used previously in competition tests (Belabess et al., 2016).

As TYLCV-IS76" was not tested before, we included the previously reported TYLCV-IS76' as a positive control of the typical fitness phenotype of TYLCV-IS76 type recombinants (Belabess et al., 2016). Both representatives of TYLCV-IS76 exhibited 100% infection success (Table 1) and similar accumulation profiles in the susceptible and Ty-1 resistant cultivars at 30 dpi (Fig. 2). In spite of 2 mutations in their TYLCSV-derived region, the deleterious effect on TYLCV-IL DNA accumulation in the resistant cultivar, typical of the wild type TYLCV-IS76, was detected for both recombinants (Fig. 2). Thus, TYLCV-IS76" can be considered as a valid control of the typical fitness of TYLCV-IS76 recombinants.

The isogenic recombinants TYLCV-IS141-Lab and TYLCV-IS76" were compared in Ty-1 resistant plants, in single and mixed infections with the parental TYLCV. Interestingly, from 30 dpi, both recombinants exhibited a significant negative impact on TYLCV accumulation in

Ty-1 resistant plants (Fig. 2, suppl Fig. 2). The accumulation gap detected between recombinants and TYLCV increased over time and reached an accumulation ratio of at least 10^3 at 120 dpi, in the same range as that previously reported with TYLCV-IS76 and TYLCV-IS76' (Belabess et al., 2016) which altogether indicate that TYLCV-IS141-Lab exhibit the typical fitness feature of TYLCV-IS76 recombinants. Taken together, these results show that the typical competitiveness phenotype of TYLCV-IS76 recombinants is not impaired when the locus 76 recombination breakpoint is shifted to locus 141.

The competitiveness of TYLCV-IS141-Lab and TYLCV-IS141-Sic are similar

As the recombinant region of TYLCV-IS141-Lab inherited from TYLCV is 100% identical to the parental TYLCV, the fitness advantage exhibited by TYLCV-IS141-Lab (Experiment 1, Table 1) was obviously induced by the short region inherited from the TYLCSV parent. Moreover, it should be noted that the TYLCSV parental clone did not exhibit any negative impact on TYLCV accumulation (Belabess et al., 2016), whereas TYLCV-IS141-Lab, which is 100% identical to TYLCSV in the cognate region, has a negative impact on TYLCV accumulation. Thus, as the TYLCSV derived region induced its negative impact on TYLCV accumulation when it is carried by TYLCV-IS141-Lab and not by TYLCSV, it is inferred that the fitness advantage of TYLCV-IS141-Lab is determined by interactions between TYLCSV and TYLCV genomic regions rather than independently by mutations inherited from one parent. To further support this hypothesis, we tested if TYLCV-IS141-Sic, a recombinant that differ from TYLCV-IS141-Lab by 59 mutations in the TYLCV region and by 21 mutations in the TYLCSV region, exhibit too the typical fitness advantage in Ty-1 resistant plants characterized by the negative impact on TYLCV accumulation.

The infectivity of TYLCV-IS141-Sic was similar to that of TYLCV-IL in the susceptible and the resistant cultivars with 100% infection success of agroinoculated plants (Experiment 2, Table 1). The same typical symptoms of leaf curling and yellowing were observed at 18 dpi on all infected susceptible plants whereas infected *Ty-1* resistant plants exhibited no symptoms. Infected susceptible plants were significantly smaller than mock-inoculated plants whereas the height of resistant plants were all similar Suppl Fig 3.

The fitness of TYLCV-IS141-Sic and TYLCV were compared in single and mixed infections with the same procedure as that with TYLCV-IS141-Lab. Plants were sampled at 30, 60 and 120 dpi for the quantification of viral DNA accumulation. Fig. 3 shows DNA accumulations at 30 and 120 dpi whereas the full data set is presented in Suppl. Fig. 4. In the case of mixed infections in Ty-1 resistant plants, the DNA accumulation of TYLCV is significantly lower than that in single infection at 30 and 120 dpi (Fig. 3); at 120 dpi, the accumulation ratio was of 700. On the contrary, the accumulation of TYLCV-IS141-Sic is not negatively affected by the competition. The negative impact on TYLCV DNA accumulation is similar to the one induced by TYLCV-IS141-Lab or TYLCV-IS76" (see above). The fitness advantage of TYLCV-IS141-Sic in Ty-1 resistant plants is not associated with fitness cost in susceptible plants (Fig. 3). In spite of the contrasting origins of TYLCV-IS141-Sic and TYLCV-IS141-Lab - natural versus artificial - and different filiations, they both exhibit the typical competitiveness trait of TYLCV-IS76 recombinants, i.e. the tremendous negative impact on TYLCV accumulation. Taken together, these results support that the high competitiveness of TYLCV-IS141 is determined mainly by the modification of intra-genomic interactions induced by the recombination rather than by the direct effect of mutations inherited from a particular parental genome.

Evidence of independent generation and emergence of TYLCV-IS141-type recombinants

TYLCV-IS141 recombinants can be generated and positively selected in Ty-1 resistant tomato plants co-inoculated with TYLCV and TYLCSV [Fig. 1 and (Belabess et al., 2018)]. Moreover, the selective advantage of TYLCV-IS141 in Ty-1 plants was not associated to a unique genotype but was detected with TYLCV-IS141 recombinants exhibiting distinct filiations (Figs 2 and 3). Taken together, these results suggest that TYLCV-IS141 recombinants may be generated and emerge in any location where TYLCV, TYLCSV and Ty-1 resistant tomato plants are present. This prediction was tested by comparing the sequence of the complete DNA genomes of three TYLCV-IS141 clones isolated from different regions of Italy. One clone isolated in Sicily in 2013 (Belabess et al., 2015) was sequenced in this study (GenBank MG489968). Two other TYLCV-IS141 sequences were available in GenBank from clones isolated in 2016, one from Sicily (MF405078) (Panno et al., 2018) and one from Sardinia (MH817479) (Granier et al., 2019). The sequence of their TYLCSV moiety were compared with the homologous sequence of parental-type TYLCSV genomes available in GenBank (Fig. 4A) and similarly for the TYLCV moiety (Fig. 4B). The TYLCSV moiety of the two TYLCV-IS141 from Sicily belong each to a highly supported clade (≥ 95% bootstrap) which do not include the TYLCSV moiety of the TYLCV-IS141 from Sardinia. Although the genetic diversity of representatives of the Mediterranean invasive TYLCV is much lower than that of representatives of the Mediterranean native TYLCSV , the TYLCV moiety of the Sicilian TYLCV-IS141 clones belong to a 100% supported clade to which the TYLCV moiety of TYLCV-IS141 from Sardinia does not belong (Fig. 4B). These results are consistent with independent generations and emergences of three wild type TYLCV-IS141 recombinants.

Discussion

Tomato yellow leaf curl disease (TYLCD) is a major constraint for tomato production and particularly in Mediterranean countries. The most efficient and widespread management method is to use tomato cultivars with the resistance gene *Ty-1*. Previous results showed that Ty-1 resistant cultivars have positively selected the recombinant TYLCV-IS76 and suggested that its selective advantage is associated to new intra-genomic interactions induced by the interspecies recombination, rather than to specific mutations (Belabess et al., 2016). The discovery of TYLCV-IS141 in field conditions (Belabess et al., 2015; Granier et al., 2019; Panno et al., 2018) as well as its positive selection in experimental conditions (Belabess et al., 2018) represented an excellent opportunity to further explore the determinism of the selective advantage of TYLCV-IS76.

Firstly, based on intra-plant DNA accumulations of TYLCV-IS141 recombinants monitored in controlled conditions (Figs1-3), we confirmed previous results that suggested that such recombinants exhibit a selective advantage over representatives of parental viruses in Ty-1 resistant plants (Belabess et al., 2018; Panno et al., 2018). Most importantly, like TYLCV-IS76, TYLCV-IS141 recombinants exhibit a strong negative impact on the accumulation of the parental TYLCV. These results suggest that the negative impact on TYLCV is a typical feature of these recombinants. It was not detected with a TYLCV clone from Spain although this clone was isolated from a symptomatic Ty-1 resistant tomato plant in the field and was shown to accumulate at higher level than TYLCV-IS76 in single infection of Ty-1 resistant tomato plants (Torre et al., 2018). The detection of three genetically distinct field representatives of TYLCV-IS141 in Ty-1 resistant plants reveals that these recombinants are generated easily in natural conditions, are frequent enough to be detectable, and altogether confirm their positive selection in Ty-1 resistant plants.

Secondly, the results show that the selective advantage of TYLCV IS141 and TYLCV-IS76 recombinants is associated neither to a unique position of the non-OR recombination

breakpoint (locus 76 and locus 141) nor to a specific sequence of parental viruses. Indeed, together with previous results (Belabess et al., 2016) we show that the typical competitiveness feature was invariably detected with TYLCV-IS76 representatives differing by up to 29 mutations, with TYLCV-IS141 representatives differing by up to 80 mutations, and TYLCV-IS76 and TYLCV-IS141 representatives differing by up to 90 mutations.

In a previous report, a TYLCV-IS141 type recombinant was identified in susceptible tomato plants that were co-infected with TYLCV and ToLCKMV, a begomovirus that exhibit 82% overall nucleotide identity with TYLCV(Urbino et al., 2013). Interestingly, this recombinant, named R10, was the most frequently detected TYLCV/ToLCKMV recombinant and most importantly was detected in plants coinfected in parallel, which is consistent with positive selection. These complementary results show that the selective advantage of TYLCV-IS141 type recombinants is not a specifically associated to the TYLCSV parent and that positive selection of TYLCV-IS141 type recombinants can occur in the absence of a Ty-1 resistance gene.

It is noteworthy that the typical competitiveness feature was observed with experimentally generated recombinants, i.e., TYLCV-IS141-Lab isolated from a plant co-inoculated with TYLCV and TYLCSV or with TYLCV-IS76' and TYLCV-IS76" generated in vitro. As their negative impact on TYLCV accumulation was detected as early as 30dpi and even 10dpi for TYLCV-IS76", it is thought that compensating mutations are not required to express the typical competitiveness feature.

Together with our previous results, it can be inferred that the typical competitiveness feature of TYLCV-IS76 and TYLCV-IS141 recombinants in Ty-1 resistant tomato plants is mainly determined by intra-genomic interactions modified by the recombination event. It should be reminded that the parental TYLCSV from which the TYLCSV fragment of TYLCV-IS141-

Lab and TYLCV-IS76" was derived, did not exhibit the typical competitiveness feature of the recombinants (Belabess et al., 2016), which confirm that the TYLCSV inherited sequences are not determining by themselves, the typical competitiveness feature of the recombinants. Further studies are needed, to identify which TYLCV and TYLCSV elements, nucleotides or peptides, are involved in the beneficial intra-genome interactions created by the recombination events. A set of recombinants with different recombination profiles should be tested, and their design may take advantage of potential intra-genomic interactions deduced from a set of TYLCV recombinants generated experimentally (Martin et al., 2011).

The fact that TYLCV-IS141 has the same competitiveness feature as TYLCV-IS76 in experimental conditions does not necessarily mean that it may have the tremendous invading potential of TYLCV-IS76 (Belabess et al., 2015). Indeed, in natural conditions, fitness does not depend solely on intra-plant accumulation, the feature used as a proxy for fitness in our study. However, the preliminary field results on TYLCV-IS141 recombinants are consistent with its positive selection because they were obviously frequent enough to be detected in the collected Ty-1 resistant plant samples.Unlike the previous TYLCV/TYLCSV recombinants detected in Italy, always detected in co-infection with parental viruses (Davino et al., 2012; Davino et al., 2009), TYLCV-IS141 was detected without them in some Ty-1 resistant tomato plants sampled in Sicily (Belabess et al., 2015; Panno et al., 2018) and without TYLCV, the most competitive parental virus, in tomato samples collected in Sardinia (Granier et al., 2019). Moreover, it is noteworthy that TYLCV-IS141 does apparently emerge more easily than TYLCV-IS76. Indeed, according to sequence comparisons (Belabess et al., 2015), and experimental results (Belabess et al., 2018), TYLCV-IS76 is thought to have emerged once only, whereas TYLCV-IS141 has most likely emerged several times. It is noteworthy that convergent evolution of TYLCV/TYLCSV recombinants has been reported previously (Davino et al., 2009; Fiallo-Olive et al., 2019). The invasion potential of TYLCV-IS141

recombinants need to be confirmed with field data that are presently very limited. Indeed only about 50 samples in total were sampled from three locations (Belabess et al., 2015; Granier et al., 2019; Panno et al., 2018).

To summarize, the pending issues are the identification of the intra-genomic interactions that determine the selective advantage of TYLCV-IS76 and TYLCV-IS141 recombinants, and the determination of the invading potential of TYLCV-IS141 through a large field survey.

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Figure legends

Fig.4. Maximum likelihood phylogenetic tree showing the relationships between the DNA sequences of three TYLCV-IS141 recombinants and representatives of parental viruses, i.e.

TYLCSV and TYLCV. (A) The sequence of the TYLCSV moiety of the recombinants (about 130 nts depending of the viral clones) were compared with the sequence of the homologous region of parental-type TYLCSV genomes available in Genbank. The best substitution model that fitted the TYLCSV moiety sequences was HKY85 +G. The tree was rooted using a TYLCSV from Spain (GenBank accession number KC953604). (B) Similar to A, with the TYLCV moiety of the recombinants (2634 nts). The best substitution model that fitted the TYLCV moiety sequences was TN93 +G+I. The tree was rooted using a TYLCV-IL from Iran (GenBank accession number GU076444). Numbers associated with nodes represent branch support.

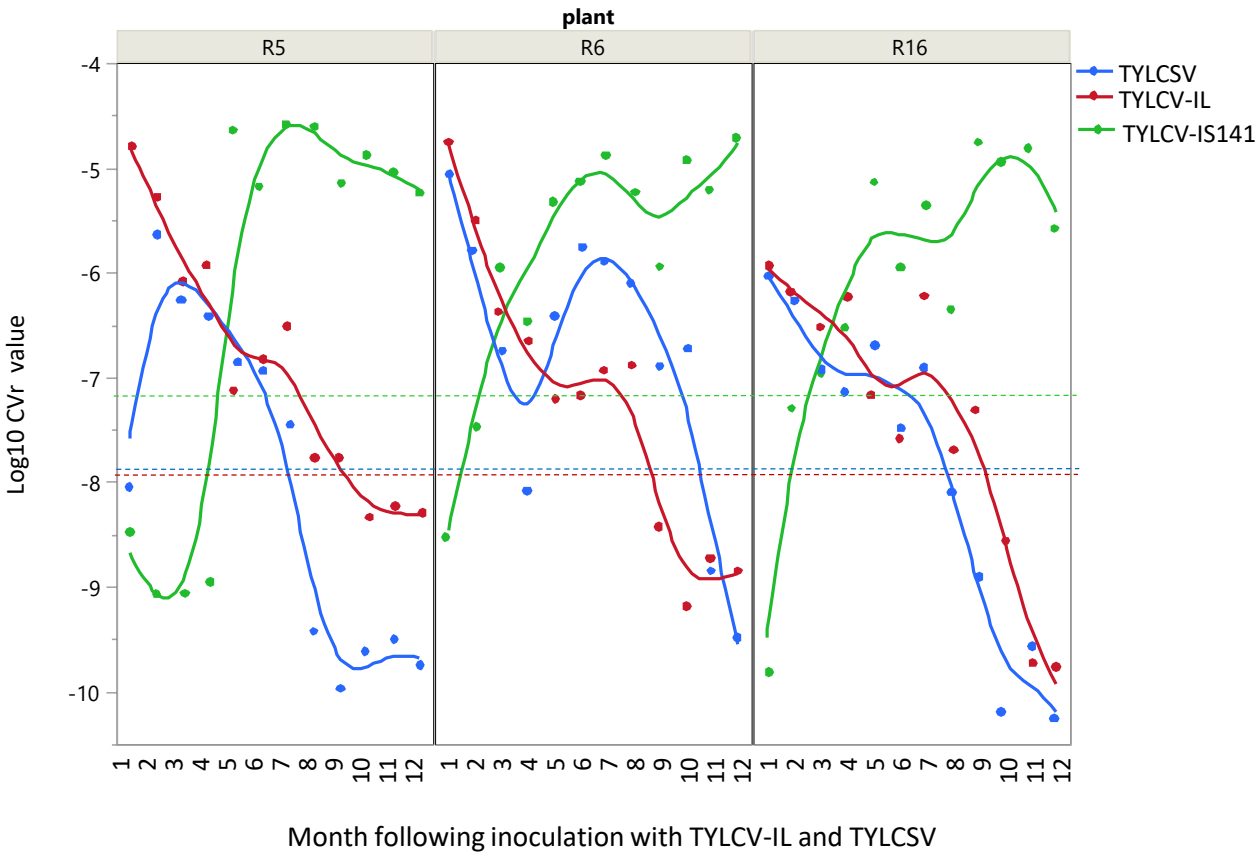


Fig 1. Dynamics of DNA accumulation of TYLCV-IL, TYLCSV and TYLCV-IS141 recombinant types in three tomato plants of the Ty-1 resistant cv. Pristyla. Plants were monitored during 12 months (1 to 12) following their coinoculation with TYLCV-IL and TYLCSV. Viral DNA was quantified by real-time PCR from leaf samples collected each month from each plant. The logarithm of the Calibrated Value of fluorescence (logCVr) reflects viral DNA accumulation. Curves were obtained by smoothing method with lambda =0,013.

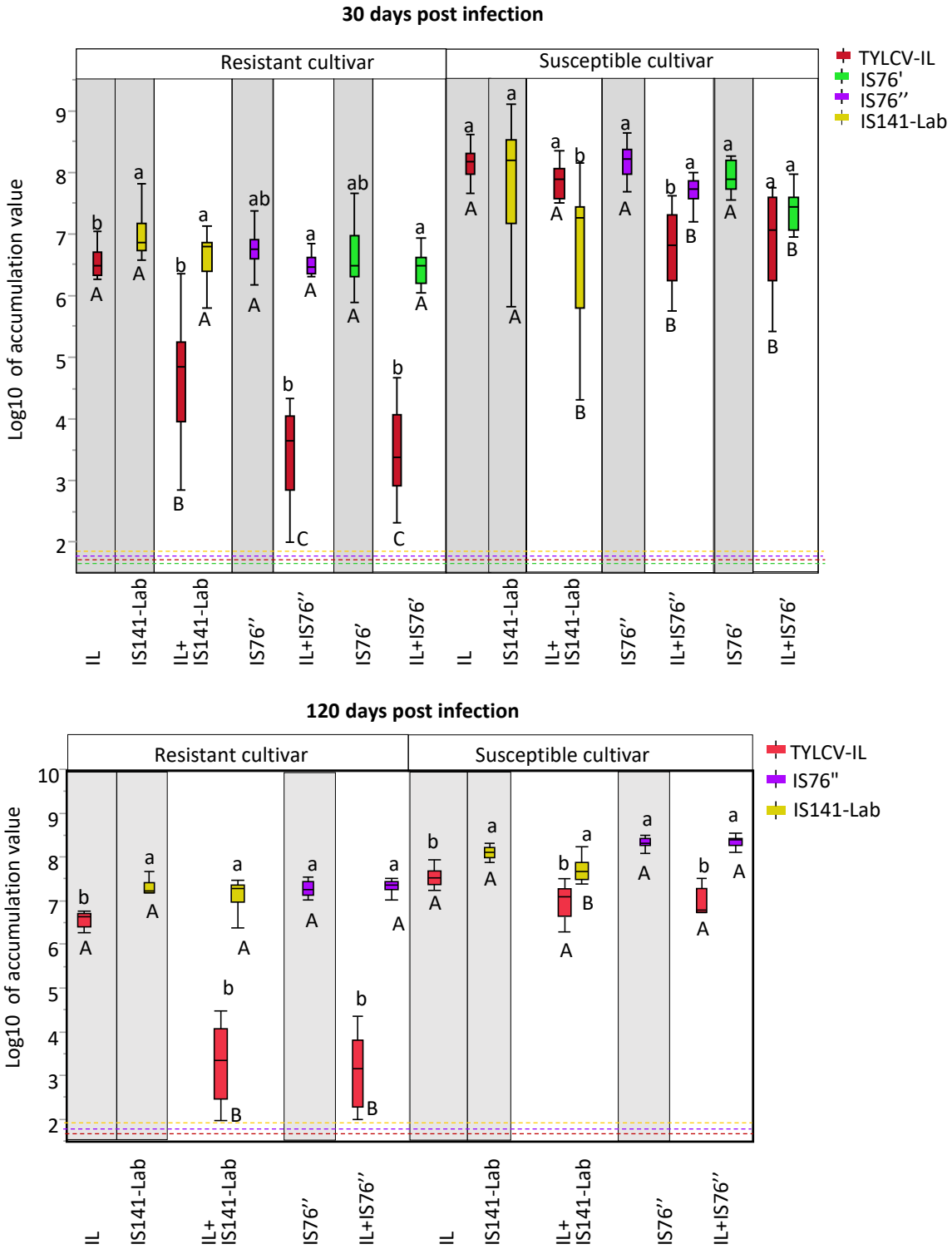


Fig.2. DNA accumulations of TYLCV-IL (IL), TYLCV-IS76' (IS76'), TYLCV-IS76" (IS76") and TYLCV-IS141-Lab (IS141-Lab) clones in agroinfected tomato plants of the Ty-1-resistant cv. Pristyla and a nearly isogenic susceptible cultivar. Plants were infected with one or two viral clones as indicated at the bottom of the figure. Boxplot background are shaded for single infection treatments. Viral DNA was quantified with real-time PCR from leaf samples collected from 11 to 15 plants at 30 days post infection (dpi), and from 8 plants at 120 dpi. Accumulation are reported as logarithm 10 of number of viral copies. Within the boxes, the horizontal line indicates the median value (50% quantile), the box itself delimits the 25 and 75% quantiles, and lines represent the normal range of the values. The red, yellow, green and purple dotted lines represent the mean values obtained with mock-inoculated plants tested with TYLCV-IL, TYLCV-IS76' TYLCV-IS76" and TYLCV-IS141-Lab primers, respectively. Box plots with different letters indicate significant differences. The statistical analyses were according to Tukey's test with the 30 dpi samples (P=0.05) and according to Wilcoxon test with the 120 dpi samples (P=0.05). Capital letters correspond to comparisons of accumulations of the same virus between treatments in each cultivar and at each date, whereas small letters correspond to comparisons in each cultivar and at each date of different viruses either co-infected in the same plant or single-infected.

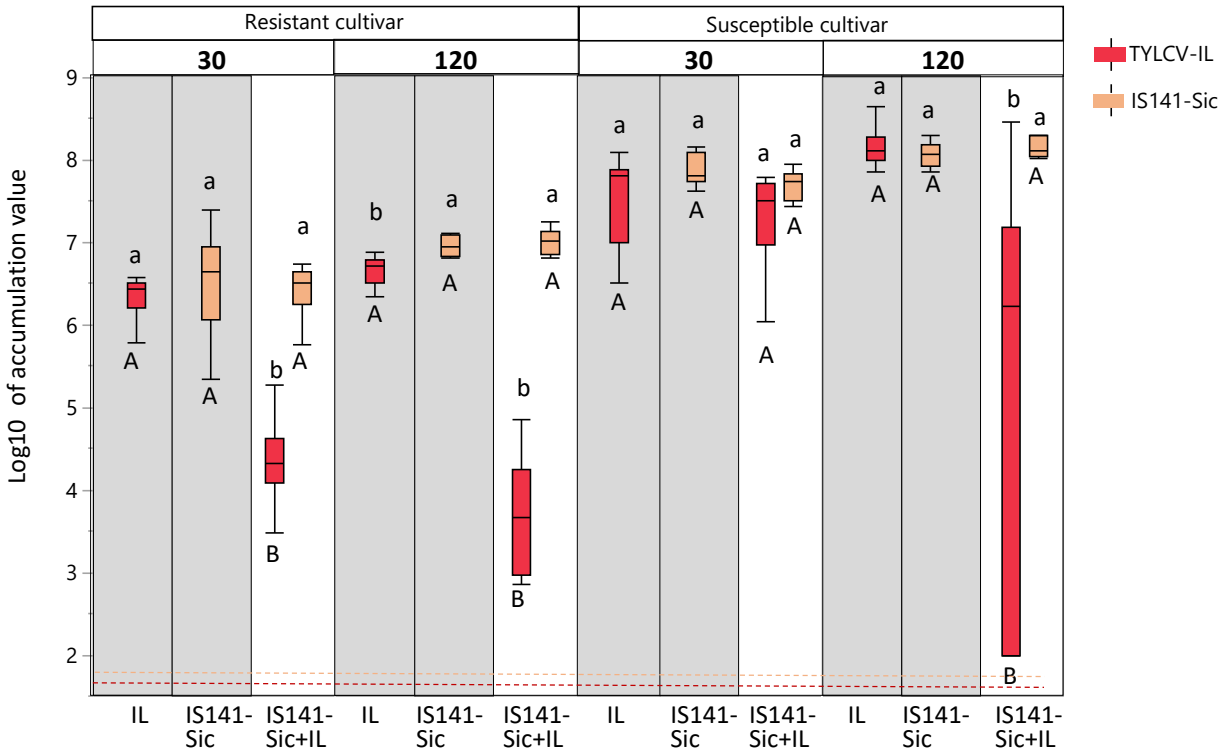
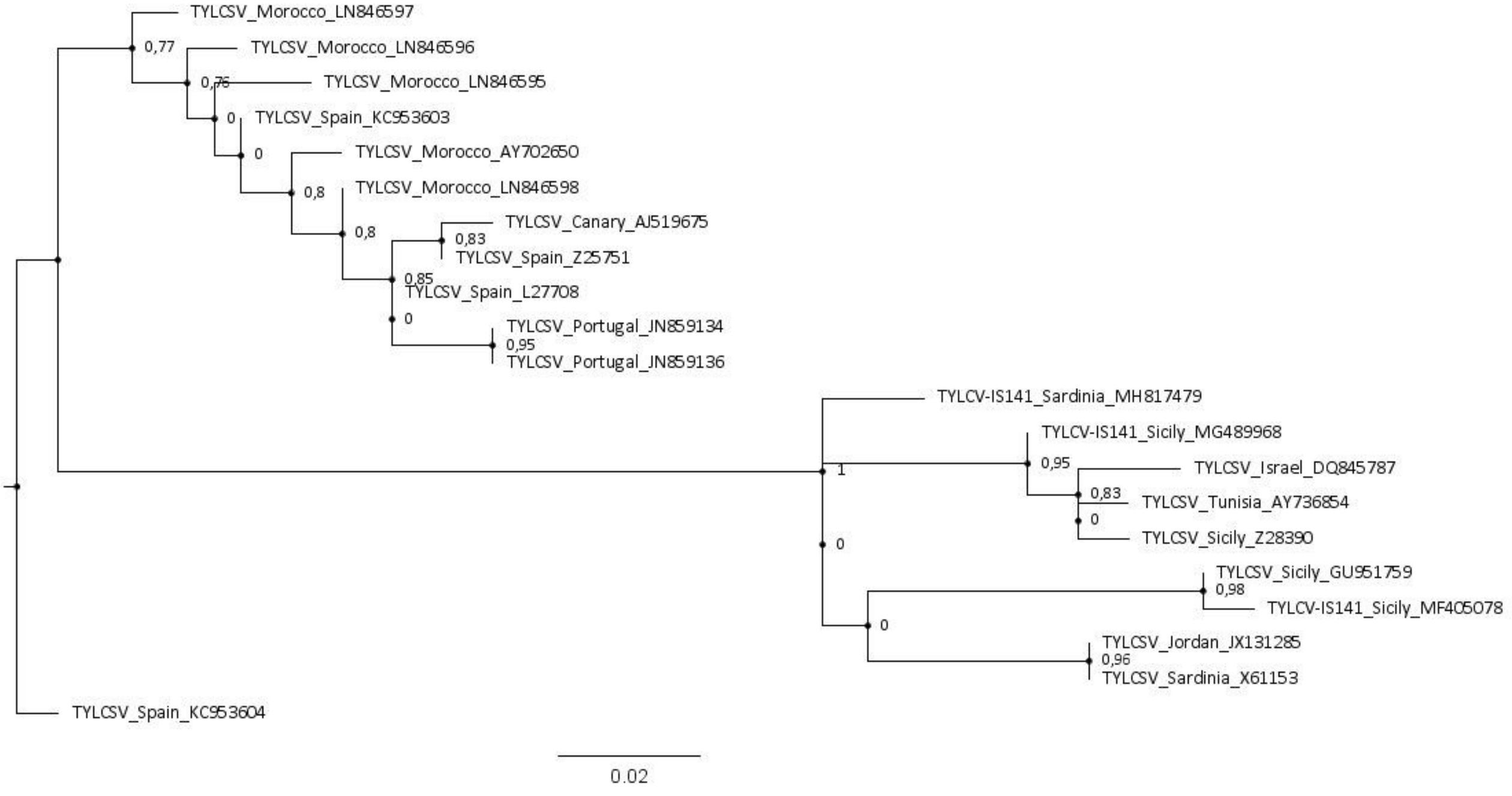


Fig.3. DNA accumulations of TYLCV-IL (IL) and TYLCV-IS141-Sic (IS141-Sic) clones in agroinfected tomato plants of the Ty-1-resistant cv. Pristyla and a nearly isogenic susceptible cultivar at 30 and 120 days post infection. The infection status of plants is indicated at the bottom of the figure. Boxplot background are shaded for single infection treatments. Viral DNA was quantified with real-time PCR from leaf samples collected from 8 or 9 plants per treatment (Table 1). Report of viral DNA accumulations, box plots, positive thresholds, and statistics including the capital and small letters as in Fig.2.


TYLCSV_Morocco_LN846597
0,77
TYLCSV_Morocco_LN846596
0,76
TYLCSV_Morocco_LN846595
0
TYLCSV_Spain_KC953603
0
TYLCSV_Morocco_AY702650
0,8
TYLCSV_Morocco_LN846598
0,8
TYLCSV_Canary_AJ519675
0,83
TYLCSV_Spain_Z25751
0,85
TYLCSV_Spain_L27708
0
TYLCSV_Portugal_JN859134
0,95
TYLCSV_Portugal_JN859136
TYLCV-IS141_Sardinia_MH817479
TYLCV-IS141_Sicily_MG489968
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TYLCSV_Israel_DQ845787
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TYLCSV_Sicily_Z28390
0
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0,98
TYLCV-IS141_Sicily_MF405078
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TYLCSV_Sardinia_X61153
TYLCSV_Spain_KC953604
0.02

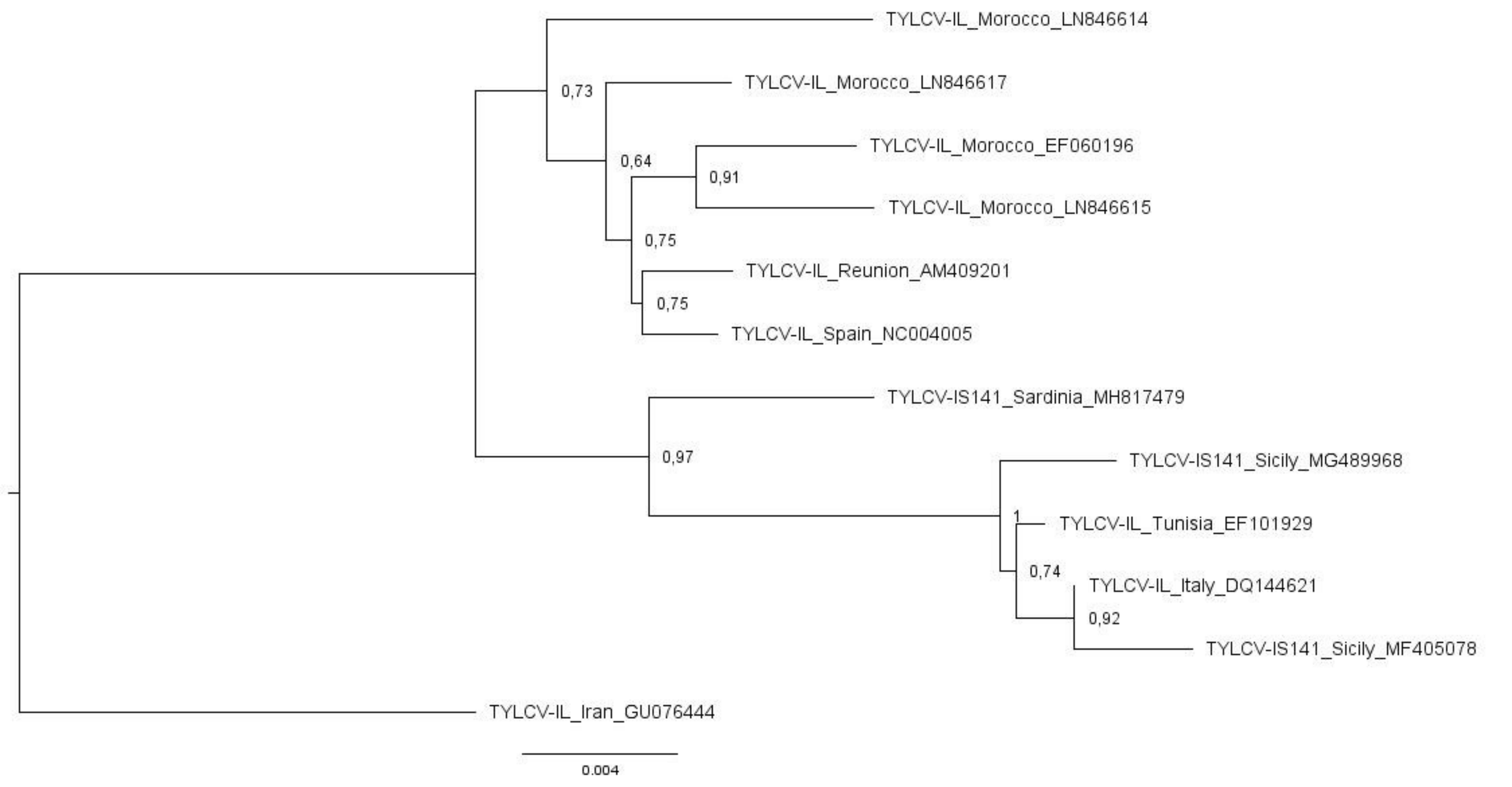
TYLCV-IL_Morocco_LN846614
TYLCV-IL_Morocco_LN846617
0,73
TYLCV-IL_Morocco_EF060196
0,64
0,91
TYLCV-IL_Morocco_LN846615
0,75
TYLCV-IL_Reunion_AM409201
0,75
TYLCV-IL_Spain_NC004005
TYLCV-IS141_Sardinia_MH817479
0,97
TYLCV-IS141_Sicily_MG489968
1
TYLCV-IL_Tunisia_EF101929
0,74
TYLCV-IL_Italy_DQ144621
0,92
TYLCV-IS141_Sicily_MF405078
TYLCV-IL_Iran_GU076444
0.004

Table 1: Origin of leaf samples from which the viral DNA content was quantified by real time PCR. Samples were collected from tomato plants of the Ty-1-resistant cv. Pristyla and from a susceptible nearly isogenic cultivar. Resistant cultivars were heterozygous for the *Ty-1*-resistant allele (Ty-1/ty-1). Plants were agroinfected in single or mixed infection with TYLCV-IL (IL), TYLCV-IS76' (IS76'), TYLCV-IS76'' (IS76''), TYLCV-IS141-Lab (IS141-Lab) or TYLCV-IS141-Sic (IS141-Sic). Mock plants were agroinoculated with an empty vector.

Experiment	Viral inoculum	Number of infected plants/ Number of inoculated plants	
		Susceptible	*Resistant*
1	IL	15/15	15/15
	IS76'	15/15	15/15
	IS76''	15/15	15/15
	IS141-Lab	14/15	15/15
	IL+IS76'	15/15	11/15
	IL+IS76''	15/15	14/15
	IL+IS141-Lab	15/15	15/15
	Mock	5	5
2	IL	8/8	8/8
	IS141-Sic	9/9	9/9
	IL+IS141-Sic	9/9	9/9
	Mock	3	3