result from disturbs in the cell wall moiety but that remains to be verified. Based on the analysis of the EST libraries we verified that the plant response is very complex and involves different defense mechanisms, which also seems to be interconnected. This study provided a better understanding of the genetic components related to the resistance mechanism of *C. reticulata* against Xf. The genes identified in this work could be valuable molecular targets for developing CVC resistant varieties of sweet orange. Support: Fapesp: 04/14576-2

[114]

Development of SNP Markers for Citrus Genotype Analysis

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Citus EST sequences obtained from our lab and downloaded from Harvest database were analyzed using multiple sequence alignment software. Putative SNPs (single nucleotide polymorphisms) were obtained and some of them having base change in restriction sites were chosen to test the possible use of restriction enzymes and single strand conformation analysis in the identification of SNPs. Primers were specifically designed according to the DNA sequences of the corresponding EST, which would amplify around 200 bp long DNA fragment containing the restriction site in the middle. Most of the PCR yielded the expected products. PCR products were digested with corresponding restriction enzymes and subjected to agarose gel electrophoresis (PCR-RFLP). The results showed that the banding patterns of citrus samples could be classified into three categories: a single band without cleavage product, two cleaved bands without the original intact band, three bands with both cleaved and un-cleaved products. These were the perfect patterns as expected. The PCR products were also analyzed for their single strand conformation polymorphisms (PCR-SSCP). The PCR products were denatured, quickly re-natured and then separated by PAGE to reveal the SSCP. Around half of the PCR products showed polymorphisms in their single strand conformations, and the genotypes revealed by these polymorphic bands were in good agreement with those obtained by PCR-RFLP. It suggested that both PCR-SSCP and PCR-RFLP can be used in the analysis of citrus genotypes. PCR-RFLP was preferable when the SNP is located in a restriction site while SSCP can be used when the SNP is not located in a restriction site.

[115]

Differential Gene Response of Citrus to CiLV

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The leprosis virus (CiLV), which induces local lesions in its plant hosts and does not invade them systemically, is currently considered the most important viral agent affecting citrus in Brazil due to the severe economic losses it can causes. The false spider mite, *Brevipalpus phoenicis*, is the only vector to Citrus species in Brazil. Sweet oranges (*Citrus sinensis*) are considered the most susceptible species to CiLV and, even though there are differences in susceptibility among them, none of the commercial varieties are resistant to the disease. It has been accepted that some mandarins (*C. reticulata*) and the Murcott tangor (*C. sinensis* x *C. reticulata*) exhibit higher levels of resistance. The main objective of this work was to identify differential expression of some genes related with the defense in the Pêra sweet orange and Murcott tangor plants, previously identified through suppression subtractive hybridization (SSH) and ESTs. RNAs were extracted from leaves collected in different periods after the mite vector inoculation. Quantitative real-time PCR (RT-qPCR) was used for gene expression analyses. The preliminary results indicate that is a larger expression of DNAJ 24 hours after the CiLV inoculation in the Murcott tangor.

[116]

Haploid Plant of *Citrus Clementina* Hort. ex Tan. `Clemenules´ Selected to Establish the First Reference whole Citrus Genome Sequence

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In the last years, structural genomic programs have created a new interest in obtaining haploid plants. The use of totally homozygous lines or monoploid lines presents a great advantage for the accomplishment of sequencing projects. It is particularly true for the commercial Citrus species characterised by a high heterozygosity, which makes difficult the assembly of large genomic sequences. The International Citrus Genomics Consortium, decided in 2007, to establish the first whole citrus genome sequence from a homozygous citrus plant. The long juvenile period, and the self-incompatibility of many genotypes, practically make impossible to obtain totally homozygous citrus plants

by traditional methods. Thus, it was decided to select a haploid plant of clementine due to the earlier implementation of very important molecular resources in clementine and the existence of different haploid plants in France, Italy and Spain. We present here the project of gynogenesis *in situ*, induced by irradiated pollen, used at IVIA to recover haploid clementine lines. Twenty five plantlets were obtained after embryo rescue. Flow cytometry, chromosome counts, and SSR marker analysis allowed the identification of 10 different haploid plants, one aneuploid plant and for the first time a doubled haploid of clementine 'Clemenules'. One of the haploid lines, recovered directly from the embryo without callus stage, displays a good vigorous growth and has spontaneously produced a dihaploid line. These two lines have been extensively characterised at molecular, histological and morphological level. This haploid genotype has been selected by the International Citrus Genomics Consortium to establish the reference sequence of the nuclear genome of citrus.

[117]

Albinism in *Citrus* and *Poncirus*: Genetic Aspect

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Variegated shoots with a white over green periclinal chimera structure occurred in satsuma mandarin trees with a frequency of 10 shoots / 1 ha / 20 years. The variegated shoots were also observed in many kinds of *Citrus* plants such as citron, pummelo, calamondin, lemon, sweet orange and sour orange as well as trifoliate orange. Some of these variegated citrus plants are valuable as ornamental plants. Albino seedlings were steadily produced from polyembryonic seeds of these variegated plants. However, one albino seedling often appeared in a polyembryonic seed of self-pollinated Hanayu (*Citrus hanaju* Hort. ex Shirai), suggesting albino seedling formation was not due to fungi infection. When monoembryonic seeds derived from self-pollination of Hanayu were choused for segregation analysis, the S_1 showed both green and albino seedlings in a ratio of 3:1. When Hanayu was backcrossed with Hanayu F_1 hybrids, the BC_1 families showed both green and albino seedlings in ratios of 3:1 and 1: 0. Thus, this genotype is caused by homozygosity for a recessive Mendelian gene *al*. Second, to find the *al* gene in *Citrus* cultivars, Hanayu and monoembryonic Hanayu F_1 hybrids with a heterozygous genotype (al^+al) were crossed with several cultivars. In any crosses, however, albino seedlings were not generated. Third, Hanayu and monoembryonic Hanayu F_1 hybrids with the heterozygous genotype (al^+al) were also crossed with variegated sour orange and variegated Buda's Hand. In the two crosses, however, albino seedlings were not generated. Interestingly, some zygotic seedlings derived from self-pollination of variegated sour orange were normal green. In self-pollinated trifoliate orange Flying Dragon (*Poncirus trifoliata* Raf. var. *monstrosa*), the S_1 showed both green and yellow seedlings in a ratio of 3:1. The yellow seedlings developed to some extent but showed low vigor. This genotype is caused by homozygosity for a recessive Mendelian gene *yel*. However, interaction of the two genes *al* and *yel* was obscure.

[118]

Flower Formation and Phase Development Mechanism in Citrus plant:Progress Report

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Citrus plant usually has a long juvenile phase which brings a great obstacle to fruit early production and breeding of new varieties. To understand the mechanism in flower formation and phase development in citrus plant, continuous effort has been made in cytological observation and related genes characterization. Progress has been acquired in following aspects. 1) Investigation of the process of flower formation in various citrus plants by paraffin section showed that in Citrus reticulata and C. sinensis, morphological development of floral bud is initiated, but in Poncirus trifoliata, the deciduous citrus plant, the floral bud is formed in early summer, when the spring flush is in self-cutting. The former need low temperature to differentiate the flower bud, and the latter need low temperature to pass the dormancy of the flower bud. 2) A cDNA library of suppression subtractive hybridization was constructed to screen genes involved in phase change and flower development. 125 and 149 non-redundant expressed sequence tags (ESTs) were identified. These cDNAs covered a broad repertoire of flowering development related genes, such as FT, FLC, LFY, etc. Real-time PCR and in situ hybridization was conducted to identify the spatial and/or temporal patterns of suspected flower related genes. 3) Promoter region 2148 bp of Pc. LFY was isolated by chromosome walking. GUS expression driven by Pc. LFY promoter with deletion of different region was then introduced into Arabidopsis thaliana. GUS staining was first detected in 7-day-old seedlings, strongly staining in first two true leaves and relatively weaker staining in the hypocotyls, but no staining in cotyledon and radicles. In terms of floral organ, GUS staining was found in floral bud, sepal and peripheral petal, but not in stamen, pistil and siliques. 4) FLC homologue