Project Nº: **015453**

Project acronym: **CIBEWU**

CITRUS BREEDING FOR EFFICIENT WATER AND NUTRIENT USE

Instrument : Specific targeted research or innovation project
Thematic Priority: **INCO-DEV-2**

PUBLISHABLE FINAL ACTIVITY REPORT

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Project coordinator organization name: **CIRAD** **(International Center for Research in Agronomy for Development)**

CIBEWU: Citrus Breeding for Efficient Water and nutrient Use;
an Euro-Mediterranean initiative to face the Mediterranean citrus rootstock challenge
http://inco-cibewu.cirad.fr

1. General context and objectives

Mediterranean citriculture faces up an increasing combination of biotic (Citrus tristeza virus (CTV) /phytophthora) and abiotic stresses (salinity/drought/alkalinity). Most of these constraints should be individually managed by adapted rootstock choice. However, spread of CTV all over the Mediterannean Basin will soon prohibit the current use of the traditional sour orange rootstocks, that provides adequate tolerance to salinity and alkalinity. Therefore, there is an urgent need to select new citrus rootstocks. All the required traits are present in the citrus germplasm with a very notable complementarity of *Poncirus trifoliata* for tolerance to biotic stress and some *Citrus* species for adaptation to abiotic stresses. However, the complexity of citrus biology and genetic makes it difficult to combine them through traditional breeding. The project proposes to enhance the efficiency of citrus germplasm utilization for rootstock breeding with two main breeding strategies: sexual recombination and somatic hybridization. Knowledge and methodologies will be developed by combining physiological, genomic, genetic and biotechnological approaches. The project focuses on tolerance to salinity and tolerance to iron deficiency associated with alkaline soils. Mains technical objectives of the project were the following: (i) local germplasm conservation and evaluation and implementation of regional citrus rootstock germplasm network, (ii) identification of physiological indicator of tolerance and development of standardized protocols for physiological evaluation (iii) search of candidate genes and study of their physical distribution on the genome, (iv) comparative genetic mapping between *Citrus* and *Poncirus* and analysis of recombination at intergeneric level, (v) development and optimisation of new breeding methods including Assisted Marker Selection and somatic hybridization, (vi) implementation of a regional network for new rootstock agronomical evaluation. Training and methodology transfer to the end users were also important components of the project.

2. Consortium

The consortium associates 6 organisms of five countries of the Mediterranean Basin

Partic. Role*	Partic. no.	Participant name	Participant short name	Country
CO	1	Centre International de Recherche Agronomique pour le Développement	CIRAD	France
CR	2	Instituto Valenciano de Investigationes Agrarias	IVIA	Spain
CR	3	Institut National de la Recherche Agronomique	INRA	Morocco
CR	4	Institut National de la Recherche Agronomique en Tunisie	INRAT	Tunisia
CR	5	Institut National Agronomique de Tunisie	INAT	Tunisia
CR	6	Cukurova University	CU	Turkey

*CO = Coordinator CR = Contrator

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3. Work performed, result achieved at the end of the Project

Activities have been conduced in the framework of 6 thematic work packages:

3.1. WP1: Citrus rootstock germplasm Network

WP leader: Samia Lotfy (INRA Morocco); samilotfy@yahoo.fr

The global objective of WP1 was to improve the management of citrus rootstock germplasm at the regional level. This will contribute to the identification of new genitors for abiotic stress tolerance. It will also result in an immediate improvement of the quality of planting material by an assessment of genetic conformity of mother trees. Specific technical and scientific objectives were as following:

- enrichment and assessment of genetic conformity of collections,
- physiological evaluation of local germplasm (tolerance to salinity/water stress and iron chlorosis),
- implementation of computerized database system for the network management.

3.1.1. Enrichment and assessment of genetic conformity of collections

Genetic characterization of local collections

CIRAD, IVIA, INRAM, INRAT, Cukurova University

CIRAD proposed standardized protocols for genetic characterization of rootstocks collections. Flow cytometry was used by all partners to analyze the ploidy level of the rootstock genotypes in collection. Ten SSR markers from genomic libraries and EST databases were systematically used to verify the genetic conformity of the trees in collection. Rootstock collections of Spain (IVIA), Tunisia (INRAT), Turkey (Cukurova University) and France (CIRAD) have been characterized. Molecular characterization of the INRA Morocco collection will be completed early 2009. Some zygotic plants, polyploid variants as well as traceability errors have been identified among mother trees of collections. Theses of types genotypes have been eliminated to ensure conform propagation of rootstocks.

Prospection and genetic characterization of local germplasm in Tunisia

INRAT, INAT, CIRAD

A prospection was organised in Tunisia in order to identify the different rootstocks used in the diverse pedoclimatic Tunisian regions. INRAT and INAT collaborated in making a survey of the citrus farms. The overall aim of this work was to collect and to analyse the genetic diversity of the rootstocks used in the different regions. This was completed by a study of the agro-morphological and physiological variability of this germplasm related to abiotic stress (mainly salinity). A total of 205 accessions was collected. The “Bigaradier” (Sour Orange) is by far the most widely used rootstock in Tunisia. However, the survey showed that other citrus (lime, lemon and orange) are being used non-grafted and sometimes are used for rootstocks for other citrus trees in the oasis of southern Tunisia and were selected by farmers as adapted to the particular conditions of the oasis. From each of the accessions, leaves, and fruits when possible were collected. The trees were observed at different phenological stages according to the dates of the collecting trips. So they were marked and other visits will be done in order to carry out the flower observation and to complete the fruit collection. Twenty pairs of SSR primers were selected according to previous studies and were used to genotype the 205 accessions. The 20 marker set displayed good quality patterns and generated 120 alleles in total. The data analysis was carried out using DARwin software and results in 33 unique genotypes. The diversity structure was consistent with taxonomic recent data.

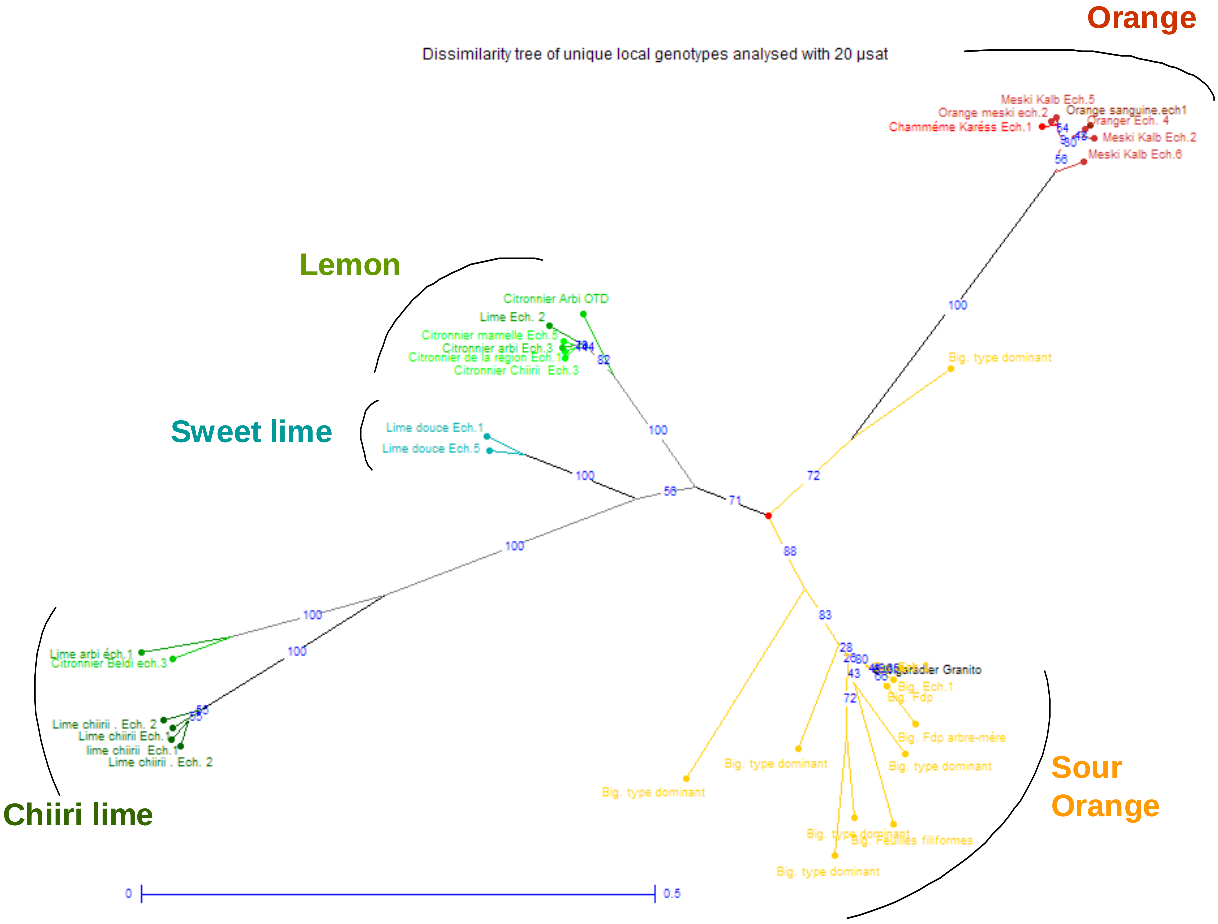

Figure 1. Dissimilarity tree of unique local Tunisian genotypes analysed with 20 microsatellite markers

3.1.2. Physiological characterization of local collections

INRAM, INAT, Cukurova University

Physiological evaluation of rootstock collections was done for salt and water stress an iron chlorosis deficiency by INRAM while INAT focused on salt stress tolerance and Cukurova university focused on iron cholrosis. The results, completed by agronomical evaluation, will allow short term recommendations to the citrus grower and for long term the best accession will be used as progenitor in breeding schemes. Illustrations of results are given below.

Tolerance of trifoliate hybrid citrus rootstocks under salt stress conditions

INRAM

Citrus in Morocco are mainly planted on sour orange rootstock which should be abandoned due to its susceptibility to Tristeza disease. Salinity also becomes a serious problem in citrus growing areas of morocco. The choice of tolerant rootstock is the most practical means to overcome the problem. *Poncirus trifoliata* and several of its trifoliate hybrids (citrange,, citrumello, citrandarin) are known to be resistant to CTV. The purpose of the present work was to evaluate nine trifoliate citrus rootstocks, for their behavior against salt stress. Seedlings of ten citrus rootstocks were evaluated for their leaf chloride accumulation under salt conditions. This study was carried out in greenhouse conditions at INRA Experimental station EL Menzeh, Morocco. 3-month-old plants were grown, in 5 liter containers of sand and peat moss in proportion of, (2/3.1/3.v/v), and irrigated four times per week and fertilized with complete nutritional solution. Na^+Cl^- at 0 (control), 2 and 5 g/l was added to the watering solution for 2 additional months. All rootstocks showed specific symptoms of toxicity when irrigated with Na^+Cl^- solution. The severity of symptoms increased with time and salt concentration and varied between rootstocks. Diverse effects of salt stress on stem, leaves and roots fresh and dry weights were also observed according to the rootstock genotype. Rootstock leaf analysis revealed that accumulation of chloride, varied in relation with the rootstocks genotype and salt concentration. Among the 9 trifoliate hybrids tested Citrange C35, Cleopatra mandarin X Carrizo citrange 30057, Cleopatra Mandarin X Carrizo citrange 30576 have been identified, in these experimental conditions as the less sensitive to salt stress among trifoliate hybrids.

Tolerance to Iron chhlorosis

Cukurova university

The intensity of iron chlorosis can be quantified by total-Fe, active-Fe, leaf chlorophyll meter or by visiual ratings of leaf chlorosis. The use of visiual ratings and readings of a portable chlorophyll meter are the most efficient approaches to define iron chlorosis in citrus. The seeds of the citrus genotypes of Cukurova University local germplasm were sown on peat medium. When the plants were about 8 months old, pH of the nutrient solution was insreased to 7.8 by adding $CaCO_3$ 0.5 gL^{-1} + 35mM $NaHCO_3$ to the nutrient solution for the irrigation of Fe-deficiency stress plants. Since the pH of Fe-stress plants were increased, the composition of the nutrient solution was changed for new condition. Local trifoliata, 08A 30.15 Rubidoux trifoliate and Benecke trifoliate exhibited severe chlorosis and was more chlorotic than the other genotypes. Cleopatra mandarins among mandarin and its hybrids were more tolerant than Sunki and Calamondins. Macrophylla was the best in lemon and lemon hybrids group. Volkameriana and Rangpur were almost the same. Gou Tou was hyghly tolerant to iron deficiency. Alanya Dilimli sweet orange is a variety selected in south of Turkey. It is known to be very resistant to high pH conditions. The results confirm that Alanya Dilimli is very tolerant to high pH. According to the results, Macrophylla, Antalya Cleopatra mandarin, Tuzcu 31-31 sour orange, Gou Tou sour orange and Alanya Dilimli sweet orange were more tolerant to iron chlorosis than the others in citrus rootstock collection of Çukurova University. In addition we have tested the somatic hybrid frm CIRAD FLHORAG1 that was introduced for agronomical purpose. It presented a very high tolerance as the best génotypes of the collection. .

3.1.3. PHENOTATE computerized database system for the citrus germplasm network management

Centro de Investigacion Principe Felipe (CIPF), Cukurova University, IVIA, CIRAD

For this task the CIBEWU consortium contracted a collaboration with the CIPF to develop the database system of CIBEWU from the PHENOTATE software (http://bioinfo.cipf.es/phenotate/). The main objective was to implement a database system capable to store citrus rootstock collection data. The novel system had to be made capable to collect data consisted of descriptive ”passport” data, molecular data, physiological descriptors of tolerance to abiotic stresses, data on tolerance to diseases, soils characteristics, tree data (height of the tree, volume of canopy…), production data (precocity, yield…), and fruit quality data (sugar and acid content, juice content, colour…) in a predefined and controlled manner. Therefore all information had to be described on the base of a list of 87 descriptors mostly adopted from the IPGRI list of descriptors. Based on a generic data model (PhenoModel) for the definition of phenotypic information, the Phenotate application was adopted to acquire specific functionalities required within the CIBEWU project. The Phenotate Client is a Java application which can be locally installed through Java Web Start Technology over the Internet and permits future updates in an automated manner. The application consists of a work-desktop and contains several areas. The *Traits/Descriptor Area* contains all the traits available for a given project. The *Collection Panel* is used to create and browse the data of a given collection. The *Annotation Table* permits to view and edit annotations of one or more elements of a collection. Finally the *Statistics Panel* is used to view generated charts. The developed system provide now a generic solution for the storage of phenotype data suitable to exchange and analyse contained information in an intuitive manner. The developed software provides the desired solution to the specific needs of the CIBEWU project. Through a client software, project members are enabled to upload, share, query, and retrieve phenotypic information. The specially developed module to address common statistical questions allow the members to quickly inquire the dataset while combining/filtering different data characteristics/parameters. Further improvements of the client software will enhance these features due to the foreseen close and user-feedback driven development.

3.2. WP2: Physiology and molecular physiology: ‘Identification of physiological and molecular indicators for abiotic stress tolerance’

WP leader: Dr Mehdi Ben Mimoun; arbo_inat@yahoo.com

Objective

The objective of this work package was to develop knowledge on the variability of physiological behaviour and gene expression of citrus when submitted to abiotic stresses and to identify improved physiological and molecular indicator for evaluation of tolerance to salt stress and Iron chlorosis. The analyses were conduced in a reference collection including about 29 genotypes mainly from Citrus genus but also representatives of relative genera. The Citrus genotype has been chosen to cover a wide range of global behaviour against abiotic stresses.

3.2.1. Physiological diversity and indicators for salt stress tolerance

INAT

The studies concerned non grafted plants. Detailed protocol of the experiments should be encountered in the deliverable D3 (Protocols for physiological studies of answer to abiotic stresses, 2006). Two treatments were installed a control without salt and a treatment with 70 mM NaCl (7, 7 dS.m^{-1}). The experiment was done the first year (2007) for 120 days and 147 days for the second year (2008). The experimental design was a 10 × 2 factorial of ten rootstocks × two salt treatments (0 mM and 70 mM NaCl) with five replicate plants in each treatment. For the evaluation of the salt tolerance foliar toxicity symptoms, growth measurements, dry weight, leaf area, number of leaves, mineral analysis, SPAD measurement, Total chlorophyll content and chlorophyll fluorescence, were used. The experiment had identified six physiological indicators for salt tolerance correlated with global behaviour of the genotypes in seedling plants one and two years old. Physiological indicators differ according to their efficiency along the salt treatment experiment and present advantages and inconveniences presented in table 1.

Table 1. Advantage and inconvenient of some physiological indicators of salt tolerance.

Physiological indicators	Days of treatment	Advantage	Inconvenient
Toxicity symptoms	143	Easy to observe	Late
Number of leaves	105	Easy to observe	Late
Total dry biomass	60	Rapid Easy to observe	Destructive Not enough selective
Chlorophyll content	60	Rapid Can be used in field or greenhouses	Destructive Not enough selective
SPAD	143	Easy to observe Non destructive	Late Over estimate chlorophyll content
Fv/Fm	143	Easy to observe Non destructive	Late Not enough selective

According to all results (table2), a classification of the genotypes of the reference collection after 143 days of salt treatment could be done as following:

- Very sensitive: *Kumquat marumi*, *M. Fuzhu* and *P. Flying dragon*.
- Sensitive: *Bintangor Sarawak, C. Volkameriana, Mexican Lime, M. Changsha,C. amblicarpa* and *M. Willowleaf.*
- Acceptable: *C. Macrophylla, Lime Cheiri, S.O. Gou tou* and *Citrumello 4475*.
- Tolerant: *S.O. Maroc, S.O. Tunisie, M. Cleopatra* and *M. Cleopatra Antalya, M. Sunki, M. Shekwasha, M. Cleoatra. x P. trifoliata, Rough lemon, P. Pomeroy* and *Pomelo Duncan.*
- Most tolerant: *S.O. Tuzcu 31-31, Lime rangpur, Lime Hammamet* and *Citrange Carrizo*

Table 2. Answer to physiological indicators of salt treatment for citrus genotypes

Genotypes	Visual Injury	Dead plant	Dry biomass	Chlorophyll content	SPAD	Fv/Fm	Salt Sensitivity *
	143 days	143 days	60 days	60 days	143 days	143 days	
Bintangor Sarawak	+	+			+	+	4
Citron Eureka	+		+	+		+	4
Rough lemon red	+				+		2
Citrus Macrophylla	+		+	+			3
Citrus volkameriana	+		+	+	+		4
Kumquat Marumi	+	+	+	+	+	+	6
Pomelo Duncan	+			+			2
Poncirus Pomeroy	+			+			2
Ponc.Flying-dragon	+	+	+	+	+	+	6
Poncirus Rubidoux	+		+				2
Lime cheiri	+				+	+	3
Lime Hammamet	+						1
Lime Mexicaine	+		+	+	+		4
Lime rangpur				+			1
M. Changsha	+	+			+	+	4
M. Cleopatra Tuzcu	+			+			2
M. Cléopatre			+	+			2
M. Fuzhu	+	+	+	+	+	+	6
M. Nasnaran	+	+	+	+		+	5
M. Shekwasha			+		+		2
M. Sunki	+			+			2
M. Willowleaf	+		+	+	+		4
S.O. Gou tou	+			+	+		3
S.O. Tunisia	+						2
S.O. Maroc				+	+		2
S.O. Tuzcu 31-31				+			1
Citrange carrizo	+						1
Citrumelo 4475	+			+	+		3
M. Cleopatre x P. trifoliata swingle	+		+				2

*6: Very sensitive 4-5: Sensitive 3: Acceptable 2: Tolerant 1: Most tolerant

3.2.2. Physiological diversity and indicators for Iron Chlorosis tolerance

Cukurova University

The studies concerned non grafted plants. Detailed protocol of the experiments should be encountered in the deliverable D3 (Protocols for physiological studies of answer to abiotic stresses, 2006). Two treatments were installed a control without iron deficiency and a treatment where the pH were increased by adding CaCO3 0.5 gL-1 + 35mM NaHCO3. The plants were maintained under stress conditions along 4 months. For the evaluation of the iron tolerance, SPAD chlorophyll readings on young leaves, total Fe concentration in young leaves, active Fe concentration in young leaves, shoot dry weight and root dry weight were used. As result, the intensity of iron chlorosis can be quantified by total-Fe, active-Fe, leaf chlorophyll meter or by visual ratings of leaf chlorosis. The most tolerant rootstocks in the collection reference were Tuzcu 31-31 sour orange and Gou Tou sour orange, and the most sensitive genotypes were Rubidoux trifoliata, Flying Dragon and Poncirus trifoliata (as used sensitive reference to iron chlorosis).

Table 3. Classification of references collection genotypes according to tolerance of iron chlorosis

Genotypes	Tolerance to iron chlorosis*
Antalya Cleopatra mandarin	5
C-35 citrange SRA 731	2
Carrizo Citrange SRA 796	4
Citrumelo 4475 SRA 732	2
Citrus volkameriana	3
Cleopatra mandarin SRA 948	5
Cleopatra X P. Trifoliata "Swingle"	2
Duncan Pumelo	2
Flying Dragon	2
Fuzhu mandarin SRA 599	2
Gou Tou sour orange	5
Marumi kamkat	2
Meksika Lime	2
Poncirus Pomeroy	1
Red Rangpur Lime	3
Rubidoux	2
SRA 4 Eureka	2
SRA 413 Changsa	4
SRA 779 Alemow	2
SRA 952 Maroc Bigarade	5
Sarawak Bintangor SRA 683	4
Shekwasha mandarin SRA 847	4
Sunki mandarin	4
Tuzcu 31-31 sour orange	5
Tuzcu 891 sour orange	4
Local trifoliata	1
Nasnaran mandarin SRA 896	4
Rough lemon red SRA 778	4

*1: very sensitive 2: sensitive 3: acceptable 4: tolerant 5: very tolerant

3.2.3 Analysis of candidate genes expression in the reference collection

Salt stress experiment
INAT, IVIA (genomic center)

The analysis of candidate genes expression has been done during the third year of the project. Five candidate genes were chosen to perform the experiment for respectively salt and iron tolerance by P5 and P6 in collaboration with P2. For the salt experiment, ten two-years-old seedlings for each rootstock were grown in plastic pot in sand. As sensitive genotypes *Fuzhu Mandarin*, *Kumquat Marumi*, *Lime mexicaine, Citron eureka, Poncirus flying dragon* and *Poncirus Pomeroy* were selected. The tolerant genotypes were *Sour Orange Tuzcu 31-31, Sour Orange INRAT, Antalya Cleopatra mandarin* and *Rough lemon.* Seedlings were progressively adapted to salt during one week to reach the concentration of 90 mM NaCl. Then two harvesting time: 24 hours and 1 week were done. For each rootstock and harvesting time a control treated with 0 mM NaCl with 2 biological repetitions and the treatment 90 mM NaCl with 3 biological repetitions were used. Total RNA was isolated from frozen root tissue for each sample. Genes expression was analysed by Real Time-PCR. The result showed that in salt stress experiment, ACCO2 expressions in roots were efficient to discriminate tolerant / most sensitive genotypes. SLAH1 was repressed in all the sensitive rootstocks in response to salt stress but not exclusively because in Cleopatra a tolerant genotype showed the same profile. However, more information is needed for using these two genes as molecular indicators to evaluate salt stress.

Iron Chlorosis experiment
Cukurova University, IVIA (genomic center)

For the iron tolerance, Citrumelo, *Cleopatra mandarin, Sunki mandarin and Gou Tou sour orange* uniform one years old seedlings were transplanted to vermiculite-sand mixture (1:1 ratio). After 2 weeks , 100uM FeEDTA applied to control plants with pH 5,5 and the chlorosis treatment was obtained through watering seedlings with a nutrition solution containing 10 uM FeEDTA and raising pH to7,5. Root samples were harvested at 1 day and 5 day after imposing iron deficiency and alkanity. Root samples were sent to IVIA in June 2008. Total RNA was isolated from frozen root tissue for each sample. Genes expression was analysed by Real Time-PCR. The results showed that IRT1, Fe-S and FR04 genes seem to be useful to differentiate susceptible and tolerant genotypes. However, more information is needed for using them as indicator genes to evaluate iron chlorosis.

3.3. Genomic: ‘Candidate genes, microarrays generation and physical mapping’

WP leader: Manolo Talon (IVIA); mtalon@ivia.es

Objectives

This work package played a central role in the project. It is the place where genomic developments related to candidate genes were established and the Center of Genomics of IVIA was is the main contributor to this work package. The specific objectives were:

- To identify candidate genes involved in the response of citrus to abiotic stresses (salinity, iron chlorosis) and in the tolerance mechanisms,

- To develop new tools for molecular physiology studies and particularly for the analysis of the expression of the candidate genes in various genotypes and environmental conditions,
- To establish a first draft of physical map of citrus and to allocate candidate genes in the genome.

3.3.1. Microarrays for gene expression studies

IVIA (genomic center), CIRAD and external partners

Part of the initial work related to gene expression analyses in the searching for candidate genes was performed with a 7K citrus cDNA microarray. However, in the frame of the CIBEWU proposal and a productive collaboration between the Spanish Citrus Genomic Consortium, INRA, CIRAD and Genoscope a second generation citrus cDNA microarray was developed. This tool, released in 2007, contains 20.000 citrus unigenes and constitutes a powerful tool for gene expression determinations and therefore one of the mayor contributions of this workpage. The 20.000K citrus cDNA microarray considerably speeded up the rate and accuracy of gene expression analyses allowing identification of putative candidate genes in a magnitude superior to what was initially planned.

3.3.2. Identification of candidate gene for abiotic stress tolerance

IVIA (genomic center), INAT, Cukurova University

Most of the salt stress gene expression analysis was performed with tolerant Cleopatra and sensitive Carrizo Citrange rootstocks. Analyses of enriched functional categories of differentially expressed genes showed that the tolerant rootstock induced wider stress responses in gene expression while repressing central metabolic processes such as photosynthesis and carbon utilization. These features were in agreement with phenotypic changes in the patterns of photosynthesis, transpiration, and stomatal conductance and support the concept that regulation of transpiration and its associated metabolic adjustments configure an adaptive response to salinity that reduces Cl^- accumulation in the tolerant genotype. These analyses rendered numerous candidate candidates, such as a number of uncharacterized membrane transporter genes that were differentially regulated in the tolerant and the sensitive genotypes. For instance, there were a high number of differentially induced genes from the POT family that contains genes encoding active transporters that mediate H^+-energized symport of anions into plant cells (CNRT1-A, B and C). Citrus genes homologous to antiporters (SOS1), co-transporters (CCC), chloride channels (ICLn, CLC-1,2,3, CLC-d, g), ABC transporters, receptor kinases, mechanosensitive ion channels, and ethylene signalling genes were considered also to be putative candidate genes for chloride homeostasis and/or salt responses. The expression of several of these selected candidate genes has been analyzed in various genotypes of a rootstock reference collection to associate and confirm expression responses with salt tolerance. As above, most of the iron deficiency expression analysis was performed with selected tolerant Sour orange and sensitive Poncirus trifoliate rootstocks. Analyses of enriched functional categories of differentially expressed genes showed that the tolerant rootstock induced faster responses in gene expression related to the categories of “transition metal ion transport” and “metal ion transmembrane transport activity” than the Poncirus genotype. These analyses rendered several candidate genes, such as the iron transport Fe-Sulfur-assembly protein LSCA1, that were differentially regulated in the tolerant and the sensitive genotypes. Other relevant candidate genes related to the homeostasis, metabolism and/or transport of iron were: IRT1 that probably encodes an iron transporter protein, various putative root chelate reductases such as FRO1, 2,3, 4 and 5, and other additional shoot chelate reductases, i.e. FRO6 and 7. Cytoplasmic aconitase and ATP-Citrate liase B2 genes were also significantly differentially expressed en both genotypes involving citrate, an iron chelating molecule in the response to iron deficiency. Other biochemical pathways including genes with altered expression under this condition were associated with sulphate metabolism and alcohol catabolism. The expression of several of these selected candidate genes has been analyzed in various genotypes of a rootstock reference collection to associate and confirm expression responses with salt tolerance.

Additional work has revealed that during abscission induced by abiotic stresses an activation program dominated by the expression of genes associated with protein synthesis, protein fate, cell type differentiation, development and transcription. In addition, over-representation of particular members of different transcription factor families suggests important roles for these genes in the differentiation of the cell separation layer inside the LAZ. Thus, this work expands the list of putative candidate genes for abiotic stress.

3.3.3. Physical mapping of Clementine

IVIA (genomic center), CIRAD

CIBEWU also contributed to the generation of 3 BAC libraries to help the completion of the physical mapping of Clementine. A total of 57000 clones were picked with an average size of 1118-125 kb, representing 18x coverage of the Clementine genome. BAC-end sequencing was carried out in 2006 and sequence analysis for repetitive elements, ESTs, SNP and SSR performed during the following years. BES sequencing produced 25 Mb of genomic sequence that allowed identification of the repetitive fraction (12.5% of the genome) and estimation of gene content (35,000 genes) of this species. Collections of 3,814 SSRs and 5,893 putative SNPs were also identified. To generate the physical mapping of citrus, 20.000 clones digested with several enzymes rendered a set of restriction fragments that after labeling with different fluorescent dyes, were sized on a capillary DNA analyzer. The resulting fingerprints were edited with a fingerprint-editing computer program and contigs were assembled with the FPC computer program to obtain approximately 1,200 contigs. Comparison among contigs obtained by fingerprint analysis and physical maps which were obtained by walking of BAC clones with both molecular markers and BAC end sequences showed consistent assembly. Further evaluations, assignment of molecular markers for the contigs, and gap filling are improving the final resolution. Thus, a full draft is expected to be freely delivered.

More than 60 putative candidate genes for iron deficiency and salinity responses according to the microarray analyses have been located in the BAC ends sequences. From this listing, for instance, the following ones posses associated ESTs and are very likely regulated by iron chlorosis, such as NRAMP2, and salinity, CCC1, NHX2, ICln, LKR-SDH and ABC transporter, among others.

Perspective

The work developed in this workpage provides a set of new and valuable putative genes related to tolerance to salinity, iron deficiency and associated damaging responses such as abscission and arrest of growth among others. The efficiency of this approach is based on the use of a 20K cDNA microarray developed in the frame of CIBEWU in collaboration with other groups. This genomic tool, that is available for research, has considerably speeded up the rate and accuracy of gene expression analyses allowing identification of many putative candidate genes in a simple experiment. The amount of information generated with the microarray has allowed publication of a high number of manuscripts not only for the teams involved in CIBEWU but also for many other citrus research teams. In the frame of this proposal, confirmation of the relationship between candidate genes and tolerance to the several abiotic stresses has been shown in a number of cases as initially planned, but the number of putative candidate genes and the information generated overcame the best optimistic initial plans. Thus, the association between gene and stress tolerance still remains to be verified for a majority of putative genes which sequence and annotation from now on are freely available for the citrus research community. This workpage also provides numerous genomic resources such as BAC clones, BES, SSR and SNPs markers and 25 Mb of genomic sequence. More than 7000 genes have been located into the BAC ends and among them more than 250 are according to the microarray analyses, related to salinity and iron deficiency. Since only a minor portion of them have been fully analyzed, many more are expected to be identified in this listing. Thus, several candidate genes unequivocally located in specific BACs, showing differentially expression in tolerant and sensitive rootstocks have been found. These are equally available for the citrus community for breeding, improvement or other developments. Finally, further fingerprinting work on the BAC clones has rendered a set of restriction fragment patterns that after evaluation, assignment of molecular markers for contigs, and gap filling for improvement of final resolution is rendering a first draft of the citrus physical mapping, a powerful tool for citrus breeders and researches.

3.4. WP4: Genetic: ‘Allelic diversity of candidate genes, genetic mapping and recombination between Citrus and Poncirus’

WP Leader: Patrick Ollitrault (CIRAD); ollitrault@cirad.fr

Context and global objective : Strong complementarities exist between certain species of Citrus and *Poncirus trifoliata* for the tolerances to diseases and abiotic stresses. These two genera are sexually compatible and their hybrids are fertile. Thus the implementation of introgression schemes between the two genera appears pertinent to solve the Mediterranean rootstock challenge. The optimisation of such breeding strategy is the final target of this workpackage. Marker assisted selection should improve greatly such strategies. It depends of the availability of numerous markers in heterozygosity in the parental line of the segregant populations, the development of knowledge about diversity of candidate genes and the analysis of association between phenotypical and molecular polymorphism. Moreover, the analysis of synteny among *Citrus* and *Poncirus* species would allow to exploit results obtained from one species to the others.

3.4.1. Comparative genetic mapping

Comparative mapping between Citrus species and Poncirus

IVIA, Center of Plant Protection and biotechnology -Laboratory of Genetic-

100 new ESTs SSR markers have been developed to increase the shared heterozygotic markers between the progenitors of segregating populations. Two relatively large populations (over 150 progenies each) used for citrus rootstock breeding programs were genotyped. One was obtained from the cross *C. reshni* x *P. trifoliata* ten years ago and the other was derived

through self-pollination from this cross *C. reshni* x *P. trifoliate* (RxP). This F_2 population derived from RxP could be genotyped only for 44 loci. The genotyping data of the RxP family for 83 maker loci was used to construct linkage maps of the two parental species, *Poncirus trifoliata,* donor of disease resistance and *Citrus reshni*, donor of salt tolerance. Another segregating population derived from the cross between *P. trifoliata* and *C. aurantium* (the well adapted species to the Mediterranean semi-arid conditions, Sour orange rootstock) was also used to enrich the previously reported linkage maps of these species with codominant markers for comparative purposes among *Citrus* species and *Poncirus*. Since the group had previously reported the linkage maps of *C. volkameriana* (Ruiz and Asins 2003), and those of the scion cultivars Chandler, a *C. grandis* hybrid, and Fortune, a mandarin hybrid, it was possible to compare the linkage maps of these species (*C. aurantium, C. volkameriana, C. grandis, Fortune mandarin)* to those obtained for *C. reshni*, *P. trifoliata* and its hybrid *C. reshni* x *P. trifoliata* by using a set of common markers. Within the *C. aurantium* x *Poncirus trifoliata* progeny, five salt tolerance candidate genes have been genotyped and mapped.

Implementation of the reference genetic map of Citrus clementina

CIRAD, IVIA (Center of Genomic, Center of Plant Protection and biotechnology- IVIA/CIRAD lab), INRAM, Cukurova University

Considering the ongoing project of citrus whole genome sequencing in the framework of the International Genomic Citrus Consortium (ICGC) that is based on whole genome shotgun approach of an haploid Clementine, it was decided by the steering committee of the CIBEWU project to focus more than previously planned in the establishment of a saturated Clementine genetic map with SSR markers to give a strong impact of the CIBEWU results at international level. The first draft of the whole genome sequence will be available end of 2009 and will allow a direct physical relative positioning of the candidate genes and the markers used for genetic mapping. In order to extend the number of mappeable locus and directly join genetic and physical map, 194 new polymorphic SSR markers were developed by CIRAD and IVIA from BACend sequences. A population of 250 Chandler pummelo x Clementine hybrids (*C. maxima* x *C. clementina*) was established in Corsica and 186 hybrids were genotyped during the CIBEWU project. This population has been selected by the ICGC for implementing the saturated reference genetic map of Clementine and INRA from France, IFAS (Florida) and UCR (California) contributed to the project. 220 polymorphic markers have been analysed. Taking advantage of the important allelic differentiation between Clementina and Chandler, 3 linkage maps have been developed using Join Map 4 (LOD 4.0; $\theta = 0.25$; Kosambi's mapping function; table 2): for Clementina (203 loci) and Chandler (108 loci) as well as a consensus map (220 loci). Total size of Clementina and consensus maps are around 1000 cM while the one of Chandler is 745 cM. The short term objective is to analyse at list 400 SSR markers early 2009. Moreover, the saturation of the Clementine genetic map with SNPs identified in Clementine BES is palned before end 2009. The genetic map will be anchored to the diploid physical map developped by IVIA by SSR, Indel and SNP BacEnds markers and will help the whole haploid clementine genome assembly. All genotyping data will be stored in the online TropGene database (http://tropgenedb.cirad.fr/).

3.4.2. Intergeneric recombination between Citrus and Poncirus

CIRAD, IVIA (Center of Plant Protection and biotechnology -Laboratory of Genetic-)

One of the important limitations to establish intergeneric breeding schemes between *Citrus* and *Poncirus* is the biology of reproduction. Indeed *Poncirus* and most of the potential *Citrus* genitors for rootstock breeding are apomictic. Monoembryonic cultivars are generally assumed to produce sexual progenies. In case of F1 hybrid between Cleopatra mandarin and *Poncirus trifoliata*, CIRAD results showed that the selection of monoembryonic seeds is not sufficient to ensure the zygotic origin of the embryo. Their results also highlighted the potential bias associated with the M13 tailing methodology for SSR analysis, in case of unequal allelic competition during PCR as it may occur with interspecific or intergeneric hybrids. For intergeneric progenies studies we now use standard PCR protocols associated with silver staining for PAGE or by using forward primers directly marked with dye for capillary sequencer genotyping. The analysis of intergeneric genetic segregation and recombination from a backcross progeny between Clementine and (Clementine x Poncirus) done by CIRAD in collaboration with INRA France revealed that recombination was not too much affected by the differentiation between homeolog chromosomes. However, very important segregation distortion were observed that globally affected Clementine and Poncirus alleles in a similar range. However, it appeared that in some linkage groups, Poncirus alleles were preferentially under-selected while the reverse situation was observed in other parts of the genetic maps. CIRAD and IVIA are currently analysing F2 populations obtained by selfing F1 hybrids between Cleopatra Mandarin and Poncirus. Indeed these F2 population will be a strong tool do decipher the molecular bases of the intergeneric phenotypic differentiation (particularly for abiotic stress tolerances).

3.4.3. Candidate gene allelic diversity

CIRAD, IVIA, INRAM, INRAT, Cikurova University

Allelic diversity of 12 candidate genes for salt stress tolerance and 3 candidate genes for iron chlorosis tolerance was analysed by direct sequencing of PCR products in a set of genotypes representative of the *Citrus* and *Poncirus* basic taxa. Interspecific SNPs were identified with a high frequency (over 20 per 1000 bp) and permitted to distinguish the haplotypes of the different species. Interspecific Indel polymorphism was also encountered for 10 genes. Direct sequencing of 30 genotypes representative of citrus rootstock diversity has been done for 6 candidate genes for salt stress tolerance. Phylogenetic origin of the alleles of secondary species and hybrid genotype has been infered from the SNPs polymorphism. Primers have been designed in conserved regions of the genes flanking polymorphic ones to allow further routine analysis of large population and assisted marker selection by SSCP (SNPs differentiation) or PAGE (Indel differenciation). The identified SNPs will also be included in a new generation of SNPs for high throughoutput genotyping. These new tools open the way to efficient association genetic studies as well as QTLs analysis and validation of genes by co-positioning of QTLs and genes.

3.4.4. Search of markers linked to genes for salt tolerances

CIRAD, IVIA

The usual method to locate loci implied in the control of genetic variance of quantitative traits (QTLs) requires segregating population of plants with each one genotyped with molecular markers. However, plants from such segregating populations can also be grouped according to phenotypic expression of a trait and tested for differences in allele frequency between the population bulks: bulk segregant analysis (BSA). As QTLs analysis, BSA is based on linkage disequilibrium. A polymorphic molecular marker closely-linked to a major QTL regulating a particular trait will mainly co-segregate with that QTL, i.e. segregate according to the phenotype. Thus, if plants are grouped according to expression of the trait and extreme groups tested with that polymorphic marker, one should expect to differentiate susceptible and tolerant bulk by the presence/absence of fragments closely linked with strong QTLs. CIRAD and IVIA (center of Plant protection and Biotechnology-IVIA/CIRAD laboratory) have searched markers of salt stress tolerance by BSA. One susceptible and one tolerant bulks were established from an IVIA population of 76 F1 hybrids between Cleopatra mandarin and Poncirus Rubidoux. The 2 bulks and the progenitors were characterized for 86 SSR markers. Seven markers differentiated the 2 bulks. We can supose that these markers are linked with major genes for salt tolerance. To have a more complete coverage of the genome, the study is pursued with AFLP markers and will be extended to bulks of others families to validate the already identified markers. The laboratory of genetic from the IVIA Plant Protection and Biotechnology center is conducing a QTLs analysis in *C. reshni* x *P. trifoliata* progeny of 150 F1 hybrids. Genotyping has been done with 83 maker loci (mostly SSR). Phenotyping for salt tolerance has been initiated and will continue during 2009.

3.5. WP5: Biotechnology and breeding strategy 'Improvement of somatic hybridization and ploidy manipulation strategies for rootstock breeding'

WP leader: Raphael Morillon (CIRAD); morillon@cirad.fr

Global objective: the objective of this workpackage was the improvement of somatic hybridization and ploidy manipulation strategies for rootstock breeding. This workpackage associated biotechnological activities such as somatic embryogenesis, somatic hybridization but also characterization of autoteraploids and alloteraploid at the physiological and the molecular level.

3.5.1. Induction of embryogenic callus lines

Establishment of embryonic nucellar callus lines is a prerequisite for an efficient citrus somatic hybridization program. Since leaf protoplasts do not have the capacity for plant regeneration, at least one of the parental protoplasts must be obtained from embryonic lines. Such lines are generally obtained by ovule culture of polyembryonic genotypes. Plants regenerated from such callus display little or no somaclonal variation even after protoplast isolation. Conversely, induction of embryogenic callus lines from ovules of monoembryonic cultivars is a difficult task. Capacity for embryogenic callus induction is very variable according to the genotype. It should be relatively easy in the mandarin group while its much more difficult for other genotypes like Sour Orange or Poncirus and its hybrids (Citrange, Citrumello, Citrandarin) while they are very important genetic resources for rootstock breeding. The objective in the framework of the CIBEWU project was to test different procedure for embryogenic callus induction and to establish new callus lines from the different rootstock collection of the CIBEWU partners.

Very significant advances have been obtained with the constitution of cellular resources of very important germplasm. Indeed embryogenic callus lines of considered recalcitrant genotypes such as sour orange and trifoliate hybrids have been obtained respectively by IVIA-INRAT and INRAM. A total of 43 new callus lines have been induced by CIRAD, IVIA, INRAM, INRAT and Cukurova University (figure 2) Procedure for efficient induction has been shared between the partners.

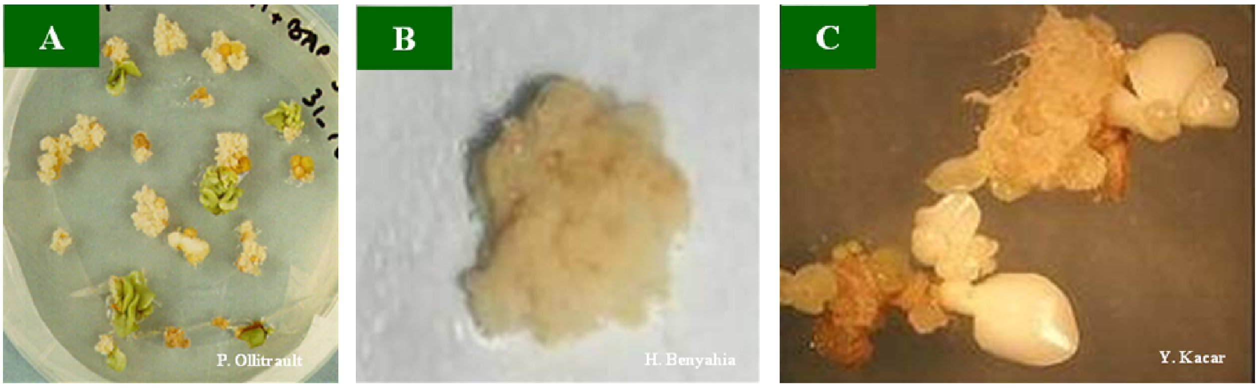

Figure 2: Embryogenic callus induction from ovule (A), embryo (B) and style (C)

3.5.2. Creation of new somatic hybrids

Complementary genitors should be found in citrus germplasm to combine the desired traits particularly between *Poncirus* or trifoliate hybrids (citrange, citrumello) for resistances to diseases and *Citrus* species for abiotic tolerances. Somatic hybridization allows to cumulate in an allotetraploid hybrid all dominant genes for tolerance to biotic and abiotic factors of the two parents, irrespective to their heterozygozity level. The biodiversity available is also very large because somatic hybrids can be obtained between sexual incompatible species and genera. Moreover tetraploid level *per se* seems to improve tolerances to salt and hydric stress. This technique has been largely exploited in Florida and promising results have been obtained at the agronomical level and for some tolerances to pathogens such as Tristeza and Phytophthor. Cybridization (the association of the nucleus of one species with cytoplasmic organites –chloroplast or mitonchodria- of an other species) is an other application of protoplast fusion. Such cybrids can be obtained by chance in classical somatic hybridization procedure or tageted by asymetric hybridisation. In the framework of CIBEWU project, the interest of such cybrids is that they are a unique material for the analysis of the impact of cytoplasm genomes and nucleo-cytoplasmic interaction in phenotypic variation. It should be particularly interesting to test the hypothesis that a part the inter-varietal variability of ROS (Reactive Oxygen Species) accumulation level under stress should be associated with polymorphism of cytoplasmic genomes.

Somatic hybridizations have been realized at CIRAD and IVIA with special emphasis in intergeneric (*Citrus* + *Poncirus*) combinations. Plants have been regenerated from 6 intergeneric and 1 interspecific combinations (figure 3). Ploidy analysis by flow cytometry, SSR markers (nuclear and chloroplastic) and targeted PCR analysis (mitochondria) have allowed to infer the genomic constitution of these hybrids (table 3). Organite inheritance was always monoparental. Mitochondrial genome arised from the callus parent while segregation was observed for the chloroplasts. Interesting new allo-tetraploid somatic hybrids have been obtained for 4 intergeneric and 1 interspecific combinations. Diploid cybrids with trifoliate hybrid nucleus and *C. deliciosa* or *C. macrophylla* mitochondria have also been identified for 3 intergeneric combinations.

Methodology as well as the training required for the production of somatic hybrids by fusion of protoplast was transferred to INRAM, INRAT and Cukurova University. Protoplast fusion experiments start in the laboratories of thess research organism and vegetal material is under regeneration process.

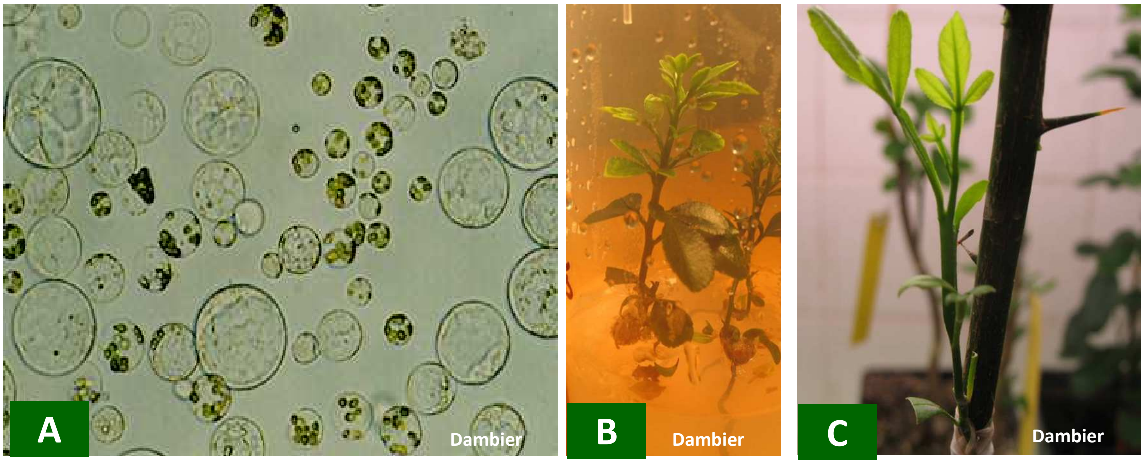

Figure 3: plant regeneration from protoplast fusion
A: mix of leaf and callus protoplasts; B: 4x hybrid Shamouti Orange + *P. trifoliata*
C: 2x cybrid Chios mandarin + 4475 citrumelo

3.5.3. Physiological behaviour of polyploid citrus rootstocks

This work was mainly conduced by CIRAD withfruitfull collaboration with IVIA for genomic tools and tetraploid germplasm.

Investigation of autotetraploids and allotetraploid seedlings when subjected to moderate salt stress

CIRAD investigated diploids of Willow leaf mandarin *(Citrus deliciosa)* and trifoliate orange (*Poncirus trifoliata L.)* as well as their respective autotetraploids and their allotetraploid intergeneric hybrid (FLHORAG1: *Citrus deliciosa* + *Poncirus trifoliata L.)* Leaf anatomy and plant growth of tetraploids were compared to diploids. FLHORAG1 inherited trifoliate leaf shape from its trifoliate orange parent. All the tetraploid plants were greener when compared to their respective diploids. As well leaf thickness of tetraploids was greater. Investigation of stomatal size revealed that tetraploids presented much bigger stomata than diploids but at a lower density. Plants were then subjected to a moderate salt stress for eight weeks. At the end of the experiment, trifoliate orange tetraploids, Willow leaf mandarin and FLHORAG1 were clearly more tolerant to salt stress than the chlorotic trifoliate orange. Trifoliate orange and FLHORAG1 were clearly sodium excluders whereas Willow leaf mandarin genotypes accumulated sodium. Trifoliate orange and FLHORAG1presented much higher leaf chloride concentrations than Willow leaf mandarin genotypes. Using cDNA-AFLP methodology, we isolated genes up or down regulated depending of the ploidy status and the growth conditions. Several transcript derived fragments (TDFs) showing qualitative expression alterations were identified. Diversity analysis of has shown that autetraploids were very close related to their respective diploids. FLHORAG1 inherited genes expression profiles from both its parents but with a dominance of its Willow leaf mandarin parent. Purification, sequencing and analysis of the TDFs revealed several genes involved in the biosynthesis of osmoticum or involved in the regulation of transcription.

Physiological indicators and gene expression of autotetraploid and allotetraploid seedlings when subjected to sever salt stress.

CIRAD investigated the same diploid, autotetraploid and alloteraploid seedlings when subjected to high salinity constraint. Plants were watered each day in order to apply a constant water potential, from 50 mM to 400 mM NaCl. Several physiological parameters such as maximum quantum yield of PSII and the leaf and root chloride and sodium content were monitored. Interestingly, in these salt stress conditions, tetraploid plants were more sensitive than their respective diploid. Indeed, chloride accumulation in leaves was greater in tetraploid when compared to diploid. Gene expression of several candidate genes involved in signal transduction, sodium and chloride transport, osmotic adjustment and regulation of the stomata opening, detoxification processes were investigated by qRT-PCR. In high salt stress conditions, a good correlation was observed between greater sensitivity to the stress and an earlier induction of those genes. Taking together, these results suggest that in high salt stress condition with no water limitation, Willow leaf mandarin genotypes did not limit their chloride and sodium absorption from the soil, and transferred ions directly from roots to leaves. We may suppose that the specific manner to impose the salt stress associated to the specific anatomy of tetraploid seedlings such the greater cell size and the greater leaf thickness were the main criteria leading to higher ions accumulation in leaves of tetraploid seedlings. In those conditions, because of their specific anatomy, autotetraploid were disadvantaged when compared to their respective diploids.

Physiological indicators of water deficit tolerance of autotetraploid seedlings and the same rootstocks grafted with Valencia orange, when subjected to water deficit.

CIRAD investigated the physiological responses to water deficit and genomic expression of autotetraploid Rangpur lime seedlings when compared to its diploid parent. The same rootstock grafted with Valencia delta orange was also studied. Plants were submitted to a progressive water deficit for eight weeks. At the end of the experiment, autotetraploid Rangpure lime, seedlings and rootstocks grafted with Valencia delta, were clearly more tolerant to water shortage than the respective diploid seedlings and rootstocks (Figure 4).

Figure 4: Diploid and autotetraploid Rangpur Lime rootstocks grafted with Valencia delta orange after withdrawing irrigation for 11 days.

In those stress conditions, water loss was greater for diploid Rangpur lime seedlings and rootstocks when compared to diploid. As well we observed that in control condition (ie before the beginning of the stress), leaf stomatal conductance of autetraploid seedlings and rootstocks was much lower than for the respective diploid seedlings and rootstocks. This lower stomatal conductance could explain the lower growth rates observed for autetraploid seedlings and rootstocks. As well, in control condition, higher rated of ABA contents were also measured in leaves of autetraploid seedlings and in leaves of Valencia delta grafted on autotetraploid Rangpur lime rootstocks. Taking together, these results suggest that the greater tolerance of autetraploid seedlings is mostly due to a greater regulation of the stomatal conductance and the by a limitation of gas exchanges. When a water stress occurs, autotetraploid Rangpur lime seedlings and rootstocks may be more adapted, the limitation of water loss being lower for those plant when compared to the respective diploid lime seedlings and rootstocks. Gene expression analyzing using microarrays is currently performed.

Conclusions

This work on the influence of ploidy on physiological indicators for salt stress tolerance suggested that tetraploid seedlings are more to tolerant than diploid when confronted to a moderate salt stress. In sever salt stress condition (watering at field capacity) tetraploid seedlings presented an opposite comportment, tetraploid seedlings being more affected by the high salt stress concentrations than diploid. In our hands, the better comportment of tetraploid seedlings was dependent of water limitation when the salt stress occurred. Indeed, experiment in water deficit conditions shown that tetraploid Rangpur line seedlings and Rangpure line rootstocks grafted with Valencia delta orange were more tolerant than the respective diploid seedlings and rootstocks grafted with Valencia delta orange. Monitoring of the ABA leaf content suggested that autotetraploid rootstocks synthesised this hormone at a higher endogenous level than diploid. We may then assume that the better tolerance of tetraploid rootstocks was correlated with their better efficiency to regulate their water exchanges at the stomatal level. Gene expression profiling using microarrays on autotetraploid Rangpur lime when compared with diploid seedlings was performed. As well, gene expression profiling on autotetraploid Rangpur lime rootstocks grafted with Valencia delta compared to diploid Rangpur lime rootstocks grafted with Valencia delta was also initiated. Real time PCR experiments should allow confirming the differences of genome expression at the origin of the phonotypical differentiation of autotetraploid seedlings and rootstocks when confronted to salt stress and water deficit conditions.

3.6. WP6: ‘Agronomical Citrus rootstock evaluation network’

WP leader: Pr. Onder Tuzcu, Cukurova University; otuzcu@ttmail.com

At the starting point of the project, there was no existing regional network for rootstock evaluation. IVIA and CIRAD have developed some promising hybrid rootstocks and Cukurova University has selected elite rootstocks from local germplasm.

All transfer of new rootstocks was done by seeds to avoid pathological problems and under Material Transfer Agreement. CIRAD sent to all the concerned partners seeds of the somatic hybrid FLHORAG1 (C. *reticulata + P. trifoliata)* at the end of 2006, seeds of the FAO- SRA (*Poncirus trifoliata x Sunki mandarin hybrid*) early 2007. Also, seeds of FA 5 (Forner Alcaide - 5) and FA 517 (Forner Alcaide - 517) which are two promising intergeneric hybrid rootstock from IVIA were provided and shared with INRAM, INAT and Cukurova University. Cukurova University has sent the seeds of elite rootstocks (Tuzcu Cleopatra mandarin, Tuzcu 891 sour orange) and also Carrizo citrange, Swingle citrumelo 4475, Volkameriana from local germplasm to INRAM and INAT. Partner in charge of field agronomical evaluation are INRAM, INAT and Cukurova University. The

Varieties grafted on the rootstocks in respect of institutes and priorities of the local citrus industry are : (i) Lane Late navel orange and Clemenules Clementine for Turkey, Lane Late navel orange, Clemenules Clementine and Sidi Aissa Clementine for Morocco and Lane Late navel orange, Clemenules Clementine and Maltaise demi sanguine for Tunisia.

All partners agreed common trial design (random split blocks) and common methodologies for agronomical evaluation based on IPGRI and UPOV descriptors. These common procedure allowed the CIPF to developp on the bases of PHENOTATE software a specific database computerized system for the networking management of the datas.

Plants have been prepared and will be field planted during 2009. All partners are engaged to share all evaluation information thrue the specific PHENOTATE database developed. This first multilocal evaluation will give very important information for further recommandations to the Mediterranean citrus industry.

4. Perspectives for concrete application for the Mediterranean Citrus industry

In the short term, the control of genetic conformity of rootstock collection will give a guaranty on mother trees of rootstock amplification schemes. It will ensure homogeneity of the planting material and conformity of agronomical behavior. In medium term the network for agronomical evaluation of new rootstock will allow sharing pre-existing rootstock innovation of the partners. Multi-local data will give an accurate estimation of new rootstock global behavior and of their adaptation to specific context of the producing countries. For the long term, a new generation of rootstock has been created by somatic hybridization with the specific goals of the Mediterranean Basin. Moreover, CIBEWU project has given basic knowledge on the physiological, genetic and molecular determinant of tolerances to abiotic stresses. This knowledge and molecular tools for early selection will support greatly the management of these tolerance traits both in further somatic hybridization and sexual breeding schemes. Particularly, the greater tolerance to salt/water stresses of tetraploid rootstock open new perspectives with innovative strategies to shorten the release of new rootstocks for the specific constraint of the Mediterranean Basin.

5. Plan for using and disseminating the knowledge

Breeding is an integrative science. Efficient programs require an effective mastering of biotechnological tools (molecular markers, in vitro technique…) as well as geneticist, physiologist, pathologist, etc. For this reason the partners of the five countries developing their own breeding programs participate in most workpackages. Some activities, particularly for the initial development of genomic and molecular tools, need facilities not available in MPC or ACC. After preliminary step of development in EU labs, simplified molecular tools have been transferred to MPC and Turkey laboratories to be used routinely during and after the project. In the same way, efficient methods of somatic embryogenesis and somatic hybridization have been successfully transferred to the same partners. Efficient methodological transfer was an important aspect of the plan for using and disseminating knowledge.

Four main types of deliverables have been obtained from the different task during the project:

- Knowledge has already and will be published in international journals and communicated in congress. 12 publications in international revus have been pubished during 2007 and 2008 and 14 are planed for 2009. 40 communications have been done in several congresses. 9 were done in PAG -San Diego- 2006, 2007 2008 and 2009 congress. A large presentation of the results of CIBEWU has been done during the 11th International Citrus congress, held in China end of 2008, with a total of 17 oral or poster communication done by all members of the CIBEWU project.

- Standardized protocols and methodologies to be used by the partners of the consortium. Standardized protocol for genetic characterization of collection, standardized protocols for physiological evaluation against stress and standardized design for new rootstock multilocal evaluation and protocols for somatic embryogenesis and protoplast fusion have been released (see deliverables).

- New biological resources and tools to be used by the partners of the consortium under specific agreements. New embryogenic callus lines have been obtained and are currently used to develop new rootstocks by somatic hybridization.
  - New somatic hybrids have been created abd will enter in multilocal evaluation after an amplification step.
  - SSR markers, have been developed from BACend sequences of Clementine and released to all member of the consortium. They will be published during 2009.
  - SSCP and Indel markers have been developed from candidate genes for salt stress and iron chlorosis tolerances. They have been shared with the members of the consortium and they will be published during 2009.

- Softwares for network management of genetic resources and new rootstock trials have be release by the Centro de Investigaciones Principe Felipe on the basis of PHENOTATE software end of 2008 for the partners of the consortium

Citrus growers and nurserymen are the final target of the CIBEWU project and need to be informed about this EU initiative. CIBEWU has been presented to the CLAM (Comité de liaison des agrumes Méditerranéens) during its 2006 annual meeting. It was also presented at the the congress of the International Society of Citrus Nursery men in Egypt in 2005. Main results of the project will be diffused by technical notes in the FRUITROP revue published by CIRAD and targeting professional world. For larger public information, a WEB page has been developed: http://inco-cibewu.cirad.fr.

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