



“FLORAL BIOLOGY AND S-INCOMPATIBILITY IN FRUIT SPECIES”

| INTERNATIONAL WORKSHOP |

San Michele all'Adige (Trento) and Bologna – Italy –
22-25 June 2011

FONDAZIONE EDMUND MACH



ISTITUTO AGRARIO
DI SAN MICHELE ALL'ADIGE



ALMA MATER STUDIORUM
UNIVERSITÀ DI BOLOGNA

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The PhD Programme in Genomics and Molecular Physiology of Fruits (GMPF) of Fondazione E. Mach and University of Bologna, with the patronage of ISHS (International Society for Horticultural Science), have planned a first Workshop on “floral biology and s-incompatibility in fruit species”, within the activities connected to the Summer Schools, for the students attending the Doctorate courses, including those located at the single Universities which are supporting the programme.

The First Session of the Workshop will deal with the molecular basis of phase transition and flower development. The second session, during the afternoon of the first day and the morning of the second day, covers the update achievements on S-locus alleles and relative determinants in pome, stone and other fruit tree species. Both fields have evident connections with phylogenesis and evolution of fertility.

The Workshop represents an important forum for discussion and exchange of ideas between younger generations of researchers and more experienced and established scientists with the perspective to set an official Working Group, belonging to ISHS. We hope to offer matter of cooperation to the scientists who started to collaborate during the Pear Symposium at Peniche (Portugal) in 2007.

We hope the Workshop will be fruitful and you will enjoy your staying in S. Michele all'Adige and Bologna.

The Conveeners
Francesco Salamini and Silvano Sansavini

Genomics and Molecular Physiology of Fruits PhD Programme

Genomics and Molecular Physiology of Fruits (GMPF) is an international PhD Programme in Fruit Plants Genomics and Molecular Physiology that represents 17 research institutions from 11 different countries.

The programme was launched in 2009 and it is based at Fondazione Edmund Mach, IASMA Research and Innovation Centre in San Michele all'Adige (TN), Italy.

GMPF offers once a year PhD scholarships for high level international research projects and collaborations in fruit biology through a network involving prestigious institutions.

The PhD fellowships are advertised internationally and are awarded following stringent selection procedures. Successful candidates carry out research projects involving at least two of the participating institutions and will have access to state-of-the art facilities in the participating institutions. The outstanding quality of the research organizations involved in the PhD programme will ensure access to top level research formation and training to GMPF fellows.

Students will also have the opportunity to receive specialized summer courses aimed at methodological and technological updating.

More information available at www.gmpf.eu

Overall Programme

June 22, 2011 – Arrival and Get Together

Arrival at Bologna airport (or directly at San Michele all'Adige) and transfer to San Michele all'Adige at two different times, minibus or car (journey takes 3 hrs)

14.00 – Welcome Desk open at San Michele all'Adige

19.00 – Guest meeting and refreshment at San Michele. Welcome by Prof. F. Salamini (President of Fondazione Edmund Mach, FEM), dr. R. Viola (Director of FEM Research and Innovation Centre) and Prof. S. Sansavini (DCA, University of Bologna)

20.30 – Opening lecture: Dr. R. Velasco (Head of the Genomics and Biology of Fruit Crops Department of FEM Research and Innovation Centre). "*Fruit Tree Genomics at FEM-IASMA*"

June 23, 2011 - Workshop

Morning:

09.30 – 11.35 - Session 1 (Coordinator A. Ramina, University of Padua)
"*Floral biology: transition phase and floral development*"

11.55 – 12.30 – Poster viewing

Afternoon:

14.00 – 17.30 - Session 2 (Coordinator S. Sansavini, University of Bologna)
"*S-incompatibility: Molecular mechanism and gene determinants (first part)*"

June 24, 2011 - Workshop

Morning:

09.00 – 11.10 – Session 2 (Coordinator F. Salamini, Fondazione Edmund Mach)
"*S-incompatibility: structural genomic of S-locus*"

11.30 - 12:30 – Business Meeting and proposal for the constitution of a new ISHS group dedicated to S-incompatibility

Afternoon: visit FEM Station and GMPF Facilities and field plots

Late Afternoon: FEM Historical cellar & wine tasting

Evening: Farewell Dinner

June 25, 2011 – Transfer to Bologna and Departure

7.30 – Bus transfer San Michele all'Adige – Bologna University (3 hrs)

10.30 – Visit Experimental Farm of University of Bologna: Fruit plots, Cadriano (apples, pears, apricot, cherry)

12.30 - Country lunch at Cadriano Experimental Farm

Afternoon: optional guided visit of Bologna downtown

Transfer to airport

Scientific Programme

Session 1 - Phase transition and flower development (Coordinator A. Ramina)

June 23 (9:30 – 11:35)

- 9:30 F. Fornara and L. Colombo, Dept. of Biology, University of Milano, Italy
Control of flowering time by day length in Arabidopsis.
- 9:55 H. Flachowsky and V. Hanke, Pillnitz Dresden Institute for Genetic and Breeding, Germany
Genetic control of flower development in apple and the use of transgenic early flowering apple plants for breeding
- 10:20 P. K. Boss, CSIRO, Glen Osmond, Australia
Dwarfing and floral induction in Vitis vinifera.
- 10:45 G. Barcaccia, A. Botton, G. Galla, L. Baldoni, G. Perrotta, A. Ramina, University of Padova and Verona; CNR-IGV, Perugia, Italy
Comparative genomics for identifying flower organ identity genes in peach and olive
- 11:10 M. Flaishman – Dept. of Horticulture, ARO, The Volcani Center, Bet Dagan, Israel
Deciduous fruit trees under global warming: flower development and fruit set

Session 2 – S-incompatibility (Coordinator S. Sansavini, University of Bologna)

June 23 (14.00 – 17.30)

- 14:00 L. Dondini, S. Sansavini – DCA, University of Bologna, Italy
Introduction. Gametophytic incompatibility in pome and stone fruits: genes controlling S-locus
- 14:25 M. Goldway – MIGAL, Galilee Technological Center Tel-Hai Academic College, Israel
The self-incompatibility fertilization system in Rosaceae, agricultural and genetic aspects
- 14:50 J. Sanzol – Unidad de Fruticultura, CITA-Aragón, Zaragoza, Spain
A pistil S mutated S-allele conferring self-fertility in European pear
- 15:15 H. Sassa – Graduate School of Horticulture, Chiba University, Japan
Structural features of the S locus of apple
- 15:40 P. De Franceschi, L. Dondini – DCA, University of Bologna, Italy
Structural and functional conservation of S-specificities among Pyrinae species.
- 16:05 R. Tao – Kyoto University, Japan
S-locus mutation and self-compatibility on stone fruits
- 16:30 A. Hegedus – Corvinus University of Budapest, Hungary
S-locus genotyping on stone fruits
- 16:55 R. Socias I Company - CITA, Zaragoza, Spain
The double expression of Sf in almond

June 24 (9.15 – 11.10) - (Coordinator F. Salamini, Fondazione Edmund Mach)

- 9:00 M. Caruso, G. Distefano, S. La Malfa, F. R. Tadeo, M. Talon, A. Gentile – DI-SPA, University of Catania, Italy; IVIA - Valencia, Spain
New insights into the molecular basis of self-incompatibility in Citrus

- 9:25 S. Collani, F. Alagna, C. Colao, G. Galla, A. Ramina, L. Baldoni, G. Perrotta, R. Muleo, G. Barcaccia – University of Padova, Verona, Viterbo; CNR of Perugia, Italy
Self-incompatibility in olive: a new hypothesis on the S-locus genes controlling pollen-pistil interaction
- 9:50 D. Serafini Fracassini– Dipartimento di Biologia, Università di Bologna, Italy
Post-translational modification by transglutaminase of proteins involved in incompatibility.
- 10:15 G. Cai – Dipartimento Scienze Ambientali, Università di Siena, Italy
Pollen cytoskeleton, transglutaminases and self-incompatibility
- 10:40 M.W. Davey – Catholic University of Leuven, Heverlee, Belgium
Genetic engineering of the Self-Incompatibility mechanism in Elstar apple leads to distinct levels of self-fertility resulting from both S3- and S5-RNase gene silencing

ABSTRACTS

CONTROL OF FLOWERING TIME BY DAY LENGTH IN *ARABIDOPSIS*

Fabio Fornara

Universita' degli Studi di Milano, Dipartimento di Biologia, Milano, Italy

Flowering is a crucial event for most plants species and maximizing its success requires careful timing. Synchronizing flowering with the most favorable season depends on integrating several environmental inputs with an endogenous molecular network. Among the environmental signals driving phase change, day length plays a major role. In the model plant species *Arabidopsis thaliana* flowering is promoted by long days typical of spring or early summer, but delayed during short winter days. This response is mediated by the photoperiod pathway that comprises at its core the *GIGANTEA* (*GI*)-*CONSTANS* (*CO*), and *FLOWERING LOCUS T* (*FT*) genes. Mutations in either gene delay flowering under long day conditions, but have little or no effect when plants are grown under short day conditions. Recently, it has been demonstrated that this gene network is active in phloem companion cells that function as sensors for photoperiod changes. To identify novel regulators of the pathway, we over-expressed a library of transcription factors in phloem companion cells, using the *SUCROSE TRANSPORTER 2* (*SUC2*) promoter. We identified a group of related genes belonging to the DOF family, called *CDFs* (*CYCLING DOF FACTORS*). Genetic and molecular analyses suggest that *CDFs* act as potent floral repressors that act upstream of *CO*. Affecting the abundance of *CDFs* in the plant results in altered cycling of *CO* transcription, which in turn leads to defects in timing of the floral transition. Increasing day length induces *CO* expression by triggering degradation of *CDFs*, and this process is mediated by *GI* protein. Mutations in *GI* impair degradation of *CDFs*, allowing accumulation of high amounts of repressor proteins, and maintaining the plant in a flowering incompetent state. By mutant and expression analyses we demonstrated that the activity of *GI* is dependent on three related F-box proteins that mediate degradation of *CDFs*. This regulatory network represents the interface between light signals and production of florigenic compounds, such as *FT*.

GENETIC CONTROL OF FLOWER DEVELOPMENT IN APPLE AND THE USE OF TRANSGENIC EARLY FLOWERING APPLE PLANTS FOR BREEDING

H. Flachowsky and M.-V. Hanke

Julius Kühn-Institut, Federal Research Centre for Cultivated Plants, Institute for Breeding
Research on Horticultural and Fruit Crops, Dresden, Germany

In recent years much effort has been made to understand the genetic regulation of reproductive processes in annual/biennial and perennial plants. A number of genes were identified which are triggers of the transition from the juvenile to the adult stage, floral induction, flower initiation, flower organ development as well as bud and seed dormancy. Whereas the picture in *Arabidopsis thaliana* is next to complete much less is known in perennial trees like apple *Malus × domestica* Borkh.. Results of recent studies suggest a number of similarities to cues and pathways known in annual model plants, but individual pathways seem to be different. During the last decade different flowering gene homologs have been isolated from apple and functionally characterized. Their mRNA expression level was measured to study its correlation to biological processes like floral initiation and flower organ development. A number of selected genes were ectopically expressed in *Arabidopsis*. Only a few out of them have really been functionally tested in transgenic apple plants. Most studies were focused on genes involved in floral induction. Genes, that are able to break the juvenile stage of apple, are of particular interest to shorten long-lasting generation cycles, which is the major drawback in fruit breeding programs. Using the *BpMADS4* gene, a *FRUITFULL*-like gene of silver birch, we produced plants with a juvenile phase of only a few months. These transgenic plants were used for crosses to introduce genes for resistance to plant pathogens from apple wild relatives to the cultivated apple. Transgenic seedlings were selected which flowered within a few months. These seedlings were evaluated on the presence of the resistance genes using molecular markers and challenging assays. Selected seedlings were then used for pseudo-backcrosses with high quality apple cultivars to refuse the linkage drag coming from the wild apples. Using the system described here one crossbred generation per year is feasible.

DWARFING AND FLORAL INDUCTION IN *VITIS VINIFERA*

Paul K Boss

CSIRO Plant Industry, Glen Osmond, Australia

As grapevine flowering has a major impact on the yield of vines there has been much interesting in understanding the biology behind this important developmental process. The study of flowering in grapevine provides some unique challenges due to the unusual shoot architecture of the plant and the extension of the process over two growing seasons. However, with the realisation that tendrils were modified inflorescences, researchers began to reveal methods of converting one to the other. These studies with plant growth regulators or their inhibitors, had suggested a link between gibberellins and fruitfulness in grapes. Ultimate proof for this link came from the discovery of a dwarfed mutant grapevine that is insensitive to gibberellins. Understanding the various stimuli that promote fruitfulness in grapevines can help modify yield in the vineyard, but also gives us insight into the possible evolutionary strategy employed by this species to ensure reproduction.

COMPARATIVE GENOMICS FOR IDENTIFYING FLOWER ORGAN IDENTITY GENES IN PEACH AND OLIVE

G. Barcaccia¹, A. Botton¹, G. Galla¹, L. Baldoni², R. Muleo⁴, G. Perrotta³, A. Ramina¹

1) Dept. of Environmental Agronomy and Crop Science, University of Padova, Italy 2) CNR – Institute of Plant Genetics, Italy 3) ENEA, TRISAlIA Research Center, Italy 4) Dept. of Crop Production, University of Tuscia, Italy

Peach and olive produce fruits with closely similar features, in both cases being drupes. Despite this similarity, the flowering induction pathways of these two species are quite different. In the case of peach, the transition phase occurs in the productive season preceding bloom. Flower bud differentiation occurs during the summer and stops when the endodormancy is established. At this stage, sepals, petals and stamens are completely differentiated, while the pistil is at a primordium stage with the appearance of the carpellar leaf from which the ovary will develop. The most advanced stage of differentiation is observed in anthers, where the pollen mother cells can be recognised. The differentiation is resumed at the end of endodormancy and the first event is the microsporogenesis. Thereafter, flower differentiation and spores development proceed up to the completion of the ovule and embryo sac development occurring at bloom. As far as olive is concerned, phase transition occurs in the spring of the bloom season. In this species, flower bud differentiation is controlled by temperature (vernalization) and light, presumably by long days. Taking into account these different behaviours, it might be hypothesized that different master regulators are involved in controlling the upstream processes of the flowering pathways, while the function of genes controlling ovary development may be conserved in agreement with the same fruit type.

In order to shed light on the outlined aspects, specific bioinformatic analyses were carried out in peach and olive, leading to the *ab initio* identification of genes responsible for phase transition and flower differentiation ('ABCDE' genes). Since in peach most of the putative MADS-box genes have been already identified and the expression patterns studied, these data were used to validate our bioinformatic approach, which was carried out on the recent public release of the genome. A double experimental approach was set up, starting from available genes whose function was already characterized not only in model plants but also in crop species. HMM patterns were set up, starting from multiple sequence alignments of proteins involved in phase transition and flower differentiation, and used to query the genome for putative orthologs. Concurrently, a BLAST search was carried out using the same sequences. Results of both approaches were cross-checked and a list of candidate genes generated and used to further validate peach genes previously characterized. The same pipeline was used to search for olive candidate genes. Since the genome sequence of this species is not available, a 454 collection recently generated from flower buds at different developmental stages was used as a target. In order to do this, nucleotide sequences were translated in all possible frames, so that the same approach adopted for peach could be carried out.

In peach, the list of candidates was implemented with further members, such as four AP2- and two AP3-like genes, with a putative 'A' and 'B' function, respectively. In olive, a higher number of candidates was identified compared to peach, probably due both to the larger size and the polyploid origin of its genome, as well as to the presence of different alleles of the same gene (being most loci heterozygous in olive and homozygous in peach). Detailed phylogenetic analyses were performed at the amino acid level, pointing out homogeneous clusters in which candidates of the same class group together with proteins already characterized in model species (*i.e.* *Arabidopsis* and *Antirrhinum*). Expression analyses of candidates are currently in progress in order

to assess the organ specificity and the timing of expression. The role of these genes in determining discrepancies and similarities in terms of phase transition pathway and fruit type, respectively, will be critically discussed.

DECIDUOUS FRUIT TREES IN A WARMING WORLD: FLOWERING, FRUIT SET AND DEVELOPMENT

Moshe A. Flaishman

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Bet Dagan, Israel

Current and future global warming is expected to change climate in the classic regions of deciduous fruit tree cultivation. Thus, a broader and deeper understanding of the relevant physiological processes that regulate tree physiology and fruit production under warm climate conditions is imperative for sustainable fruit growing in world regions that currently still enjoy temperate climate but expected to suffer climatic changes. Being a significant research and practical center of horticulture in a warm-climate region, Israel does considerable efforts in developing new cultivars and practices of fruit production suitable for cultivation under warm conditions, and could thus serve as a good example of the development of special horticultural strategies for warm climates.

In deciduous fruit trees, flower differentiation depends on the conditions prevailing during the previous summer and autumn, during winter dormancy and also on spring temperatures. Exposure of trees to high temperatures or water deficits during flower differentiation alters their development, thus inducing the occurrence of flower malformations such as double pistils, resulting in the reduction of the number of flowers and formation of various abnormal fruits. High temperatures during mid dormancy are correlated with flower bud drop, for instance, spring flower bud drop prior to swelling is best known among apricots and is genotype dependent. With the anticipated global warming, high-latitude areas will experience the largest relative changes in climate worldwide, with the greatest effect on winter temperature. Growing fruit tree species with a large chilling requirement, in milder winters such as Israel result in inadequate chilling and hence delayed and erratic bud burst in the spring. Fruit breeders in Israel and elsewhere have developed and are developing commercial fruit tree cultivars and horticultural treatments suited to a wide range of mild winter conditions.

In this paper morphological, histological and biochemical studies of the genetic regulation of flower and fruit formation, will serve to monitor reproductive development in cultivars with limited adaptation to warm regions. This study will illustrate new approaches to overcome physiological problems in flower and fruit development of deciduous fruit trees in temperate regions.

GAMETOPHYTIC INCOMPATIBILITY IN POME AND STONE FRUITS: GENES CONTROLLING S-LOCUS

Luca Dondini, Paolo De Franceschi, Silviero Sansavini

Dipartimento di Colture Arboree, Bologna University, Italy

Angiosperms are the most prevalent and evolutionarily advanced group of plants. A critical step for the flowering plant success was the wide promotion of cross-fertilization as reproductive strategy. Evolution favoured the development of genetic barriers to prevent self-fertilization or fertilization among closely related individuals within several families. Gametophytic Self-Incompatibility (GSI) system represents one of these strategies and it occurs in Rosaceae species (i.e. pome and stone fruits). Among these species, self-incompatibility is determined by haplotypes of a single self-recognition locus (named Sterility locus or S-locus) and as “self” and “non-self” is indicated the genetic identity or not at the S locus. Within Rosaceae the pistil specificity is controlled by an S locus-encoded ribonuclease (S-RNase) and the pollen determinant, according to update researches, is a pollen-expressed protein containing an F-box domain (S-Fbox). A protein degradation model was proposed to explain S haplotype-specific rejection of pollen tubes by S-RNase: an S-Fbox allelic variant specifically recognizes its non-self S-RNases and mediates their degradation by the ubiquitin–26S-proteasome system. The genotyping of the S-alleles (S-genotyping) is the most powerful support for breeding programs aimed to define the inter-fertility groups among varieties belonging to species in which self-incompatibility is predominant. Moreover this molecular approach is the base for the identification of mutated alleles that could explain the occasional self-fertility and/or self-fruitfulness that have been reported in certain genotypes.

In spite of the availability of a large literature on this topic, several aspects of the GSI in the Rosaceae have to be more in depth analysed. It is still intriguing to note that GSI evolved differently in genera belonging to Prunoideae (i.e. *Prunus*) in respect to the Pyrinae ones (i.e. *Malus* and *Pyrus*). In *Prunus* a single F-box gene determines the pollen S while in apple, European and Japanese pears multiple S-locus F-box genes (named S-locus F-Box Brothers or S-FBBs) were recently identified as candidates for this specificity: all these genes exhibit S-haplotype-specific polymorphisms, pollen-specific expression and linkage to the S-RNase. This divergent structure inside the S-locus led several Authors to discuss more aspects of the pistil S and pollen S interaction. In *Prunus* mutants in which insertions or deletions in pollen determinant genes resulting in the breakdown of SI have been described. This observation lead to think that *Prunus* pollen S determinant inhibit mechanisms that are able to inactivate the cytotoxic effects of the S-RNase. In the Pyrinae non functional mutants of pollen S linked to self-compatibility have never been reported as well as a detoxification of the SRNase without the pollen S contrarily to *Prunus*. In this subtribe it has to be clarified the role of multiple SFBB genes and how multiple S-Fbox proteins may recognize a wider group of non-self S-RNases. In *Petunia* it was demonstrated that three types of divergent pollen S are able to recognize each a subset of non-self S-RNases and this model was named ‘collaborative’ (Kubo et al 2010). May it also fit with the Pyrinae model system? All these aspects of GSI will be analysed in depth by the invited speakers of this Workshop as well as the existence and the relative role of other genes that do not belong to the S Locus but that are involved in GSI in pome and stone fruits (i.e. transglutaminase) and the possibility to overcome Self-incompatibility by genetic engineering approach.

THE SELF-INCOMPATIBILITY FERTILIZATION SYSTEM IN ROSACEAE, AGRICULTURAL AND GENETIC ASPECTS

Martin Goldway, Raffi Stern, Annat Zisovich, Amir Raz, Gal Sapir, Doron Schnieder, Reut Niska

The molecular genetic lab. in agriculture, MIGAL-Galilee technological center, Kiryat Shmona, Israel

The Rosaceae family carries the gametophytic self-incompatibility fertilization system (GSI). The GSI is controlled by a single multi-allelic locus (S-locus). The S-locus contains two haplotype-specific genes: the S-RNase gene, which is expressed in the pistil, and an F-Box gene named SFB (S-haplotype-specific F-Box), which is expressed in the pollen tube. Thus, cultivars of the Rosaceae depend on cross-pollination and therefore commercial orchards contain at least two cultivars that flower synchronically. The cultivar couples may be fully compatible, i.e., they differ in both of their S-loci, or semi-compatible, i.e., they share one of the two S-loci. In 1999 we published a paper showing that semi-compatibility between apple cultivars (Topred and Jonathan) led to fruitset and yield reduction in comparison to fully compatible cultivars (Topred and Golden delicious). We proposed that the reduction was due to rejection of half of the pollen by the semi-compatible cultivars. This phenomenon was also found in our following work in pears, plums and apricots. In pears, we quantified the contribution of each seed to be one to two mm. of the fruit's diameter.

Thus, in the Mediterranean basin and other regions with sub-optimal conditions for growth and pollination, full compatibility is beneficial for ensuring high quality fruit with satisfactory yields.

Usually S-RNase alleles serve as the genetic marker of the S-haplotype yet, SFB genes are also S-haplotype specific and are a source for markers. The markers are identified by PCR, RFLP or DNA chromatography (Denaturing High-Performance Liquid Chromatography - dHPLC). Due to the importance of distinguishing the compatibility between cultivar, S-genotypes are determined intensively. For the benefit of the pear community, we and laboratories from Italy, Japan, Spain and Portugal determined 133 pear cultivars – most of the world cultivated varieties.

Nonetheless, there are cases in which a suitable fully compatible pollinator is lacking, therefore seedlings of the wild Syrian pear (*Pyrus syriaca*) were S-genotyped and examined for their fertilization abilities exhibiting high potency as pollinators for the Spadona cultivar.

Conversely, some Rosaceae cultivars are self compatible (SC) due to mutations. For example, in apricot, of the genus *Prunus*, the Sc-SFB allele is mutated and in loquat, of the genus *Pyrus*, the S6 locus carries a mutation that confers SC. The SC mutations not only provide markers for breeding SC cultivars, but also contribute to our understanding of the GSI system. Examples will be discussed.

A PISTIL S MUTATED S-ALLELE CONFERRING SELF-FERTILITY IN EUROPEAN PEAR

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Unidad de Fruticultura
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European pear exhibits a gametophytic self-incompatibility system controlled by the polymorphic S-locus. I have investigated self-fertility in two European pear cultivars, 'Abugo' and 'Ceremeño', using fruit/seed setting and pollen tube growth examination. S-genotyping through S-PCR and sequencing identified a new S-RNase allele in the two cultivars, with identical deduced amino acid sequence as S_{21} , but differing at the nucleotide level. The analysis of progenies and test-pollinations suggested that the new allele is a pistil-mutated variant of S_{21} conferring self-compatibility (S_{21}^*). Moreover, the cultivars 'Abugo' and 'Ceremeño' could be S-genotyped as $S_{10}S_{21}^*$ and $S_{21}^*S_{25}$ respectively, being S_{10} , S_{21} , S_{25} functional S-alleles. Reciprocal crosses between cultivars bearing S_{21} and S_{21}^* indicated that both alleles exhibit the same pollen function; however, only the cultivars bearing S_{21}^* had impaired pistil-S function as they failed to reject either S_{21} or S_{21}^* pollen. Finally, RT-PCR analysis showed absence of S_{21}^* -RNase gene expression in styles of 'Abugo' and 'Ceremeño', suggesting a possible origin for S_{21}^* pistil-dysfunction.

STRUCTURAL FEATURES OF THE S LOCUS OF APPLE

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Apple (*Malus × domestica*) exhibits gametophytic self-incompatibility (GSI) in which at least two specificity genes, pistil *S* and pollen *S* genes, located at the complex *S* locus region control self/non-self discrimination between pistil and pollen. The pistil *S* gene encodes a highly polymorphic extracellular ribonuclease, *S*-RNase, that is considered to function as a cytotoxin and arrest growth of self-pollen tube. Recent studies on Solanaceae, *Antirrhinum* and *Prunus* suggested that pollen *S* genes encode F-box proteins (SLF/SFB), raising a possibility that SLF/SFB ubiquitinates non-self *S*-RNases for degradation to allow compatible pollen tubes to cope with cytotoxic effect of the non-self *S*-RNases.

We found that apple *S* locus contains more than ten F-box genes that are related with each other and specifically expressed in pollen, and named them *SFBB*. Genetic analysis and FISH (fluorescence in situ hybridization) experiments revealed that all the *SFBB* genes are linked to the *S*-RNase gene, and are located at the heterochromatic region of a chromosome. The heterochromatic localization of the *S* locus would contribute to repress recombination at the region to maintain the pair of pistil *S* and pollen *S* alleles, the *S* haplotype, for the GSI function. These findings would be consistent with the idea that multiple *SFBB* genes at the *S* locus region are involved in pollen *S* specificity in apple.

STRUCTURAL AND FUNCTIONAL CONSERVATION OF S-SPECIFICITIES AMONG PYRINAE SPECIES

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Gametophytic self-incompatibility (GSI) is the main mechanism that controls fertilization in many rosaceous species, including those belonging to the subtribe Pyrinae (formerly the Maloideae). In natural conditions, S-specificities are subject to frequency-dependent balancing selection; the genetic imprint left by this kind of selection is visible on the S-RNase gene in terms of high sequence diversity, evidence of positive selection, and shared ancestral polymorphisms: thus, some alleles have been maintained almost unaltered during evolution in different but related species, as is the case of *Malus* and *Pyrus* species.

We have questioned whether the same expected features can be extended to whole S haplotypes, thus including not only the female S determinant (the S-RNase) but also its male counterpart, most likely provided by S-locus F-box Brothers (SFBB) genes - even though direct functional evidence is still needed. On the one side, coevolution between female and male S genes has been postulated several times, given that the generation of any new S-specificity requires a coordinated change on both sides in order to maintain the full S-haplotypes functionality. But on the other side, recent models for S-RNase-based GSI suggest a key difference between the control of female and male S functions: while the former entirely depends on the single S-RNase gene, the latter might likely be provided by multiple SFBB genes acting in a collaborative way. Even though this hypothesis makes it necessary to re-discuss the mode of coevolution between the female and male S genes, it might provide a suitable explanation for the complex phylogenetic profiles of SFBB genes, which are only in part in agreement with that of the S-RNase.

Phylogenetic and segregation analyses of S-locus genes, together with the increasing amount of genomic information available for apple and pear, provide a precious tool for understanding both the molecular mechanism of S-RNase-based GSI, and the complex evolutionary pattern of S-haplotypes.

S LOCUS MUTATION AND SELF-COMPATIBILITY IN STONE FRUITS

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Most *Prunus* fruit tree species exhibit a homomorphic monofactorial gametophytic self-incompatibility (GSI) system, in which self-/nonself-recognition is controlled by a single multiallelic locus, called the S locus. A self-incompatibility (SI) reaction occurs when the same "S allele" specificity is expressed in both the pollen and pistil. During the last two decades, much progress has been made in our understanding of the molecular basis of the gametophytic self-incompatibility system in *Prunus*. The pistil and pollen S specificity determinants of *Prunus* have been shown to be the S-ribonuclease (S-RNase) and the S haplotype-specific F-box protein (SFB), respectively. Identification of the pistil S and the pollen S determinants led to the development of PCR-based S genotyping and marker-assisted selection for self-compatible (SC) individuals. Molecular and genetic analyses of *Prunus* SC S haplotypes and tetraploid sour cherry (*P. cerasus*) reveal the possible existence of a distinct recognition mechanism in the S-RNase-based GSI system of *Prunus*. Although the function of SFB has yet to be clarified, it is now clear that the dysfunction of either the pistil or pollen S determinant leads to SC in *Prunus*. Thus suppression of either *S-RNase* or *SFB* expression could be used to produce self-compatible cultivars in *Prunus*. However, this technique may not be applicable to apples and pears because the recognition mechanisms in the S-RNase based GSI in these fruit species seem to be similar to those of Solanaceae and Plantaginaceae, where dysfunction of the pollen S determinant results in self and cross incompatible pollen production. We are still far away from a full understanding of the GSI mechanism in *Prunus*; yet, further studies will gradually open up the unknown features of GSI in *Prunus* leading to the identification or development of SC selections and SC breeding techniques.

S-LOCUS GENOTYPING ON STONE FRUITS

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S-allele diversity among cultivated self-incompatible (SI) fruit trees is of great interest from either a practical or theoretical point of view. Our long-term study is being carried out to S-genotype stone fruit (mainly apricot, sweet cherry, almond and plum) cultivars and landraces. Fruit set, pollen tube growth analyses, stylar ribonuclease detection and activity assays, PCR amplification, cloning and DNA sequencing and bioinformatics are used in the experiments.

Complete S-genotype was determined for 20 Eastern European *Prunus dulcis* cultivars. Based on DNA sequences and fruit set analysis two novel cross-incompatibility groups (CIGs) were proposed: group XXI ($S_{11}S_{31H}$) and XXII ($S_{36}S_{37}$). Five new alleles (S_{31H} , S_{36} - S_{39}) were identified. Since S_{31H} is characterized by similar intron sizes as S_{39} , an allele-specific primer was designed to selectively detect the S_{31H} -RNase allele. Increasing diversity multiplies the chance of the occurrence of different S-alleles with matching intron sizes; therefore, consensus PCR discrimination may not have enough resolution power.

Eleven cultivars of *P. salicina* were genotyped. Our results clarified and harmonized two different allele nomenclatures. The S_5 -allele-specific primer can be used as a reliable marker for self-compatibility (SC) in Japanese plum. One CIG has been established (CIG VII, S_5S_6) and others were extended. A table was assembled including 49 cultivars assigned to I–VII CIGs. These data were used later as the basis of worldwide classification of Japanese plum cultivars and was completed by other studies.

More than 120 *P. armeniaca* cultivars have been S-genotyped and 13 novel S-alleles were identified and characterized from Eastern European and Asian accessions. Many Turkish cultivars were classified into new CIGs, III–XIV, which was surprising as apricot has been known traditionally as a mainly SC species in Europe. Among Turkish apricots only seven SC cultivars have been determined. The fact that all five S-alleles detected in the Hungarian germplasm were also present in Turkish apricots, furnished molecular evidence supporting the long-suspected historical connection between Hungarian and Turkish apricots. The connection between these germplasm seems to be relatively recent. Our results confirmed that the Turkish germplasm had contributed considerably to the formation of several precious Hungarian apricot cultivars.

SC in apricot is due to a loss-of-function mutation within the pollen *SFB* gene of the S_c -haplotype. A new co-dominant marker was designed based on PCR of *SFB*-alleles to allow high throughput identification of self-compatible apricot cultivars and selections. *SFB*₈ was clarified to be the first known progenitor allele of a naturally occurring self-compatibility allele in *Prunus*, and consequently S_c can be considered as S_8 . Results suggest that the mutation rendering the S_c -haplotype non-functional might have occurred somewhere east from Central Turkey. Allele frequency data and single nucleotide polymorphisms in the *S-RNase* gene were used to reconstruct the putative dissemination routes of self-compatibility in apricot and clarify its crop evolutionary consequences.

Thirty Turkish *P. avium* cultivars and 17 selections were S-genotyped. New alleles have been identified and characterized. All 17 selections are perspective sources of resistance to fruit cracking. Wild cherry alleles were abundant in the S-locus of such

selections pointing to a good opportunity of their mutual compatibility with most of the commercial cherry cultivars.

Our results supplied information on S-allele diversity from regions between the main cultivation centres and the centres of origin of stone fruits. The increased number of the S-alleles present in tree fruit cultivars native to the regions from Eastern Europe to Central Asia holds considerable implications in relation with the S-genotyping methods, cultivation and breeding strategies as well as crop evolution.

THE DOUBLE EXPRESSION OF S_i IN ALMOND

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The search for sources of almond self-compatibility other than the cultivars from the Italian region of Puglia was undertaken in order to avoid the problems derived from inbreeding depression in some offspring. The S_i allele was identified by specific primers in several genotypes which later on were shown to be self-incompatible. Sequencing of this allele from both SC and SI genotypes showed their full genetic identity, not only in the coding region of both the S_i -RNase and the SFB_n , but also in the 5' regulatory sequence of the S_i -RNase. In addition, two RNase bands were obtained in the SI genotypes with the S_i allele as opposed to the production of a single RNase band in the SC genotypes. Thus, the inactive S_i (S_{in}) does not produce RNase and is linked to SC, whereas the active S_i (S_{ia}) is distinguished by producing RNase, as the other SI alleles, and is linked to SI. The genetic identity of both alleles is further confirmed by the recognition of the S_{in} allele by the S_{ia} -RNase. Consequently, the presence of the S_i gene is not the exclusive source of self-compatibility in almond and the reason for the different expression of the S_i is independent of the complete genetic identity in the whole chromosome region bordering the S locus. Thus, other factors outside the S locus must be involved in the expression of almond self-compatibility.

NEW INSIGHTS INTO THE MOLECULAR BASIS OF SELF-INCOMPATIBILITY IN CITRUS

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Compared to what is known in other fruit tree species, self-incompatibility in citrus is still poorly understood. Although in recent years several efforts have been made to study citrus reproductive biology, little is known about the molecular mechanisms regulating reproduction and the key genes involved in self-incompatibility.

Here we report the identification of possible candidate genes regulating pollen-pistil interaction and self-incompatibility in clementine (*Citrus clementina* Hort. ex Tan.). These genes have been identified comparing transcriptomes of laser-microdissected stylar canal cells isolated from two clementine genotypes differing for self-incompatibility response: 'Comune', self-incompatible; and 'Monreal', a natural self-compatible mutation of 'Comune'. These genotypes were previously characterized by histological assays, which demonstrated that the mutation leading to self-compatibility in 'Monreal' affected the style functions regulating pollen rejection.

Transcriptome profiling was performed using Affymetrix Citrus Genechip representing up to 33,000 citrus transcripts. This analysis allowed the identification of 10 genes overexpressed in 'Comune' stylar canals and 6 genes overexpressed in 'Monreal' ones. Most of them are not functionally annotated in citrus or other plant species. Interestingly, 3 of the ten overexpressed genes in Comune clustered in a range of about 10 kb in the clementine genome. The results of microarray hybridizations were validated using real time PCR. Moreover, a time course analysis was performed to investigate the expression patterns of the candidate genes in virgin and self-pollinated styles with stigmas of both genotypes during pollen tube growth. Further characterization is needed to reveal the specific role of the differentially expressed genes in the interaction between pollen tubes and stylar canal cells. The understanding of self-incompatibility mechanism in clementine, which is related to seedlessness, is of outstanding interest for breeding.

SELF-INCOMPATIBILITY IN OLIVE: A NEW HYPOTHESIS ON THE S-LOCUS GENES CONTROLLING POLLEN-PISTIL INTERACTION

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Olea europaea L. is one of the oldest agricultural tree crop species and, in spite of the great economical and cultural impact, a few studies have been carried out on its reproductive barriers. The aim of this research was to elucidate the self-incompatibility system in olive from cyto-histological and bio-molecular standpoints. Self-incompatibility is one of the most effective systems adopted by flowering plants to prevent inbreeding, maintaining so diversity within the species. Olive is actually classified as a gametophytic self-incompatible plant because of distinctive morphological traits, as wet-type pistil and bi-nucleated pollen. According to this background, experiments were carried out with the aim of cloning the genes involved in the gametophytic self-incompatibility (GSI) but, despite several thorough attempts, any homolog of the S-locus ribonuclease, known to be primarily involved in GSI pathway, was identified. At the same time, preliminary cyto-histological analyses highlighted the behaviour of pollen tubes in stigmatic surface and transmitting tissues of pistils that was in disagreement with a GSI system. Overall findings led us to hypothesize that a sporophytic self-incompatibility (SSI) mechanism would occur in olive. Detailed cytological analyses of 34,000 pollen grains performed using pistils of self-compatible and self-incompatible cultivars under self-pollination and open-pollination conditions were crucial to shed light on the pollen-pistil interactions at the microscopic level. As common trait a low germination rate for pollen grains in all olive cultivars was observed. The most important finding is that no pollen tubes were found into the stigma and the style transmitting tissue of cultivar Leccino, while several pollen tubes penetrated into the stigma surface and only one was able to grow through the style in cultivar Frantoio. Based on this cytological evidence, the main genes known to play a crucial role in the SSI pathway, as SLG (S-locus glycoprotein), SRK (S-locus receptor kinase) and SCR (S-locus cysteine rich protein), were sought using degenerate and non-degenerate primers designed on consensus sequences obtained by multiple alignments of sequences belonging to SSI-related species (e.g. *Brassica* spp.). The use of both cDNA and genomic DNA as templates allowed us to clone the full-length of SLG-like and SRK-like genes and their intron sequences. Several sequences were initially recovered for olive by RACE experiments: protein domain analysis and multiple alignments were then useful to select one single *OeSLG* (accession no. HM070826) and one single *OeSRK* (accession no. HM070828). The former included the Beta-lectin, the S-locus glycoprotein and PAN-AP plant domains whereas the latter included also the protein kinase catalytic (PKc-like) domain and the DUF3403 (functionally uncharacterized) domain. Homologous sequences sharing SLG and/or SRK-related domains were also found in olive flower-specific transcriptome databases. Quantitative Real-Time PCR assays, replicated using different subdomain-specific primer combinations, showed an antagonist gene expression pattern in flowers of cultivars Leccino and Frantoio for the putative female determinants. In particular, the *OeSRK* proved to be strongly expressed in Leccino pistils at 3-4 days after pollination,

whereas in the Frantoio the expression was high at the time of pollination and very low 1-2 days after. Recently, a candidate for the male determinant was isolated from olive flower-specific transcriptome databases through the definition of the SCR-like pattern according to SP11 of *Brassica* spp. (gi|6572991.1.1) and the selection of most similar ORFs in the resulting NJ tree. A strong anther-specific expression of the OeSCR-like gene at the time of pollen dispersal was documented by replicated Real-Time PCR experiments. On the whole, a new hypothesis for the genetic system controlling the self-incompatibility reaction can be postulated for olive.

POST-TRANSLATIONAL MODIFICATION BY TRANSGLUTAMINASE OF PROTEINS INVOLVED IN INCOMPATIBILITY

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Gametophytic self-incompatibility (SI) is dependent from mechanisms blocking pollen growth at the upper third of the style. In *Rosaceae*, the stylar S locus encodes for glycoproteins having ribonuclease (S-RNases) activity, even if also other genes still to be identified, are involved. Two main models based on S-locus are proposed: the system S-Rnase, identified in *Rosaceae*, *Campanulaceae*, *Solanaceae*, *Scrophulariaceae* and that of *Papaver rhoeas* in which cytoskeleton modifications play a central role.

To identify one of the pollen putative determinants we focused our attention on an enzyme, the transglutaminases (TGase), able to post-translationally cross-link proteins directly or by conjugating polyamines (PAs). PAs in bound form greatly increased in SI either in *Rosaceae* (Del Duca et al 2010, *Amino Acids* 38, 659-667) or *Citrus*, with a maximum in concomitant with the pollen tube arrest. The SI caused the TGase activity increase, whereas the compatible pollination caused its decrease. Also the glutamyl-PAs, products of the enzyme activity, reached a maximum concomitant with the block of the tube growth. High molecular mass cross-linked products were observed at the tube tip by confocal microscopy. Aggregates of tubulin and punctuate aggregates of actin were observed in SI, suggesting a role of cytoskeleton also in *Rosaceae* (Di Sandro et al 2007, *Acta Hort.* 800, 423-426), as verified in vivo and in vitro (Del Duca et al 2009, *Biochem. J.*, 418, 651-664). The over expression of TGase could inhibit tube growth in SI by causing an abnormal functioning of the cytoskeleton.

Other data suggest a role of an extracellular TGase as modulator of cell wall building and strengthening (Iorio et al 2008, *Plant Biosystems* 142, 1-6; Di Sandro et al 2010, *Biochem. J.* 429, 261-271): it catalyzed the cross-linking of polyamines into proteins, both released by the pollen tube itself. A similar distribution of TGase was observed on tube surface of pollen germinating inside the style, consistent with a role for TGase in the interaction between the pollen tube and the style in the extracellular matrix (ECM) during fertilization.

An ongoing approach is based on the fact that TGase, in animal systems, can act as a protein disulfur isomerase (PDI) which play a role in the ECM assembly. PDI also re-naturates the RNase by reconstituting the disulfur bridges. S-RNases, the stylar determinants in incompatibility, have been very well characterised in pear¹. Thus a molecular approach to clone pear S-RNases and TGase has been performed¹, based on the high homology between the pear and apple TGase sequences, confirmed by the publication of *Malus domestica* cv Golden Delicious genome.

Another mediator of the SI response is the enzyme phospholipase (PL) involved in the cell signalling and cytoskeleton reorganization during pollen tube growth; in particular, the stimulatory effect of TGase on the PLA activity has been identified, suggesting a synergic role of both enzymes in SI response.

POLLEN CYTOSKELETON, TRANSGLUTAMINASES AND SELF-INCOMPATIBILITY

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The cytoskeleton of pollen tubes is a network of single and polymer-forming proteins that are involved in many aspects of pollen germination and growth, from the asymmetrical distribution of membrane-bounded organelles to the deposition of cell wall material. It is known that actin filaments (AFs) and possibly microtubules (MTs) are regulated by a number of associated proteins, which control both the polymerization state and the bundling activity (Ren & Xiang 2007). Although the specific function of some associated proteins is recognized, the involvement of post-translational modifications of proteins in the regulation of the cytoskeleton dynamics is still unknown. In the self-incompatibility response, changes to both AFs and MTs is likely to be triggered by specific proteins, resulting in either the depolymerization of cytoskeleton filaments or the formation of aberrant structures that seriously interfere with the cytoskeleton functions (Bosch et al. 2008, *Mol. Plant* 1, 879-887).

Transglutaminases (TGases) are proteins widespread in all plant organs and cell compartments and catalyze the post-translational conjugation of polyamines to different targets, including proteins of the photosynthetic complex, of the cytoskeleton and of the cell wall. In apple pollen, an extracellular form of TGase is likely to promote the apical growth of pollen tubes (Di Sandro et al. 2010, *Biochem. J.* 429, 261-271). During self-incompatibility, the activity of TGase is usually enhanced leading to the formation of high molecular mass cross-linked products, including aggregates of tubulin and actin. Coupled with altered levels of polyamine (PA) content, these evidences suggest an involvement of TGase in the incompatibility response (Del Duca et al. 2010, *Amino Acids* 38, 659-667).

Recently, we showed that purified pollen TGase might catalyze the post-translational modification of purified actin and tubulin, leading to the formation of amorphous aggregates of actin and tubulin; as consequence, both the affinity and the activity of motor proteins (kinesin and myosin) decrease (Del Duca et al. 2009, *Biochem. J.* 418, 651-664). If these processes take place *in vivo* as well, the general organization of the cytoskeleton and the organelle transport would be compromised. These results suggest that the inhibition of tube growth in the incompatible response might be also mediated by an abnormal functioning of the cytoskeleton caused by the conjugation of PAs catalyzed by cytoplasmic TGase.

In apple pollen, TGase was found in the form of more or less consistent aggregates on the pollen tube surface; this pattern was observed in pollen tubes germinated both *in vitro* and in the style, suggesting a role for extracellular TGase in the interaction between pollen tubes and the extracellular matrix during fertilization (Di Sandro et al. 2010). We have investigated the distribution of TGase in pear pollen tubes and we found that TGase is likely to be secreted by a mechanism involving both membrane dynamics and AFs. Since AFs are consistently perturbed during the self-incompatibility response, it is likely that the distribution and activity of extracellular TGase is also affected, leading to the blocking of pollen tube growth.

GENETIC ENGINEERING OF THE SELF-INCOMPATIBILITY MECHANISM IN ELSTAR APPLE LEADS TO DISTINCT LEVELS OF SELF-FERTILITY RESULTING FROM BOTH S_3 - AND S_5 -RNASE GENE SILENCING

Rozemarijn S.G. DREESEN, Johan KEULEMANS, Mark W. DAVEY*

Like most Rosaceae fruit crops, apple (*Malus x domestica*), has a gametophytically-determined self-incompatibility (GSI) system. This GSI prevents in-breeding and so promotes outcrossing and genetic diversification within the species. GSI is governed by a single polymorphic gene locus, called the S-locus, which contains 2 tightly-linked genes (S-genes) that are expressed either in the pistil or in pollen. The pistil S-gene was identified as a T2-type ribonuclease protein or S-RNase, while more recently the GSI pollen factor was found to be an F-box-type protein, named *SLF/SFB*. These 2 proteins determine the genetic relatedness between pollen and pistil in such a way that fertilization only occurs when at least one of the pollen S-alleles is different from the maternal S-alleles expressed in the pistil. Recognition of the germinating pollen tube as "self" results in it being targeted for destruction by the action of the S-RNases, which enter the pollen cytoplasm and degrade RNA.

To evaluate the impact of the self-compatibility trait in apple, the highly self-incompatible cultivar 'Elstar' (S_3S_5) was transformed with T-DNA constructs containing either the full-length or the 3'-terminus S_5 -allele in either *sense* or *anti-sense* orientations. The resultant transgenic lines were then screened for their ability to set fruit following cross- and self-pollination under controlled greenhouse conditions. Transgenic S-silenced lines were identified that displayed significant fruit set in the absence of any active pollination vector.

A detailed molecular and physiological analysis of both full-length and 3'-terminus S_5 -RNase *anti-sense* lines, identified 12 lines with an altered SI-behaviour relative to wild-type 'Elstar'. In all lines there was a complete silencing of the S_5 -allele but a variable degrees of S_3 -allele silencing, depending on the transgenic line. Interestingly, Southern blot analysis indicated that the presence of an altered SI-phenotype is exclusively linked to the presence of inverted repeat structures around the right border (IR-RB) of the T-DNA, suggesting post-transcriptional gene silencing (PTGS) of the S_5 -gene (and S_3 gene) by an RNA interference type reaction. The degree of self-compatibility observed also varied between lines and both complete and intermediate levels were found. These phenotypes were found to be related to the level of S-protein expression in the pistil. Intermediate self-fertile phenotypes were found to be a consequence of a slower growth of self-pollen tubes and were only observed in a subset of lines generated using the 3' terminus of the S_5 -cDNA in *anti-sense* orientation. For an as yet unknown reason, the GSI-reproductive barrier in these lines is still partially active. There is a strong interest in the introduction of a self-fertile character into apple as this could potentially reduce the dependency of fruit set on weather conditions and insect vectors during flowering, and ensure higher and more consistent production yields. Therefore S-RNase-silenced apple trees with a range of self-fertile phenotypes are a potentially powerful tool to learn more about the biological implications of removal of the GSI reproductive barrier and the possible impact of this technology on current production problems.

POSTERS

TRANSCRIPT LEVELS OF GENES INVOLVED IN FLORAL TRANSITION IN APPLE TREES AND ITS RELATION WITH BIENNIAL BEARING

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Perennial plants potentially have a long juvenile phase. When mature, flowering is recurrent but can be strongly inhibited by concurrent fruiting, leading to alternate bearing. In fruit trees and particularly in apple, this generates major agronomic issues. Therefore, development of novel cultivars with intrinsic regular bearing is highly desirable.

A previous study, using QTL detection and candidate gene mapping, indicated that previously recognized flowering genes may not be responsible for alternate bearing, as they did not co-locate with QTLs, whereas several hormone-related genes were located within the QTL intervals. The aim of this study was to investigate through an expression study the role of the positional candidate genes in the regulation of flowering. Shoot apical meristems were collected from bourse shoots during floral induction and during fruit development on a set of cultivars carrying heavy crop ("on" trees) versus trees carrying light crop ("off" trees). Transcript levels of eight genes were studied, corresponding to marker genes for flowering transition, key flowering genes and hormone-related genes.

RNA profiling confirmed that floral induction takes place within 90 days after full bloom and suggested that the presence of fruit influenced the expression of key flowering genes and genes involved in hormone degradation. Since fruit are known to produce high concentrations of hormones, these results raise questions concerning hormone synthesis in fruit, their possible transport to the meristem and regulatory mechanisms triggered within the meristem. Based on these results, we propose a putative regulatory network scheme for the control of flowering in apple.

GENETIC BASIS OF FRULET ABSCISSION IN APPLE

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Apple (*Malus x domestica*) is one of the most important fruit crops in Europe and worldwide. The high number of flowers (and consequently of fruitlets after fertilization) can diminish the size and the quality of the fruit production. Furthermore, a broad fruit set in apple trees cause a reduction in the number of buds the subsequent year, therefore producing reduced fruit yield. Thus, thinning, or clearance of fruitlet load, is necessary. Reduction of fruitlet number (thinning) is carried out chemically and in some cases manually or mechanically to help the physiological drop of fruitlets in apple. This procedure at the pre-harvest production process is expensive both ecologically (chemicals) and economically (manual or mechanical). The molecular basis controlling the abscission of fruitlets remains unknown. To better understand the genetic traits that lead to the abscission, a population of Golden delicious apple trees has been treated with benzyladenine (BA) as a thinning agent. The collection of central (king flowers) and lateral fruitlets at different stages of development pre- and post-treatment and the comparison of these samples with samples of non-treated trees will permit us to identify genes responding to the treatment, therefore involved in the process of abscission. A transcriptomic analysis will be performed comparing different tissues (embryo, cortex and mesoderm) of apple seeds before and after the treatment to identify putative genes expressed in the embryo that could be involved in the signaling and/or initiation of the abscission process.

In *Arabidopsis*, *SHATTERPROOF* genes control a network of genes leading to the formation of the valve margin in the fruit. The valve margin is a specialized structure that thanks to the formation of different layers of cells with or without lignification leads to the opening of the mature silique permitting the dispersal of the seeds. On the other hand, *FRUITFUL* gene is expressed in the valves of the *Arabidopsis* fruit repressing the expression of the *SHP* genes and the downstream network that they control to form the valve margin. The process of lignification or not lignification of the different layers of cells of the *Arabidopsis* siliques is the mechanism that allows the dehiscence. A similar mechanism could permit the abscission of the apple fruitlets after the thinning. In the case of the *SHP* genes, four orthologues have been identified in the recently sequenced genome of the apple variety Golden delicious, whereas a single *FUL* orthologue is present. The duplication of the *SHP* genes in apple could be in the basis of a specialization of one or more of them in controlling the lignification in the abscission zone of the apple fruit. By studying the expression patterns of the apple orthologues of *SHP* and *FUL* we would like to understand the network of genes controlling abscission in apple.

Methodologies: Laser microdissection, *in situ* hybridization, RNA-seq, Real Time PCR

Achieved results: The project has recently started and we are in the process of collecting the samples after the treatment with BA in the experimental population of apple trees. RNA-seq will be performed in dissected embryos from central and lateral fruitlets of treated and non-treated trees.

ISOLATION OF A POLLEN-EXPRESSED ACTIN HOMOLOGOUS PROTEIN AS A POSSIBLE INTERACTER WITH S-RNASE IN *PRUNUS AVIUM*

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Many species in the Rosaceae, Solanaceae and Plantaginaceae exhibit the S-RNase-based self-incompatibility (SI) system. In this system, the pistil and pollen specificities are conferred by S-RNase and S locus F-box protein (SLF/SFB). In addition to these determinants, other SI general factors or modifiers are shown to be required for the SI reaction. Although such factors have been cloned and characterized in Solanaceae and Plantaginaceae, there is no information at the molecular level in the Rosaceae. To isolate SI general factors of in *Prunus*, yeast two hybrid (Y2H) screening was conducted against sweet cherry pollen cDNA library using the N-terminal (NT) and the C-terminal (CT) regions of *Prunus avium* (*Pav*) S⁶-RNase as the bait. Thirty one genes including an actin homolog (*PavAct1*) were isolated by the Y2H screening. Since it was reported that some of the T2/S-type RNase family proteins could bind to actin, the interaction between actin and S-RNase was further examined by Y2H and pull-down assays. Full-length (FL) *PavAct1* showed no interaction with the NT, CT and FL of S³- and S⁶-RNases in yeast. Although pull-down assay showed no interaction between GST-tagged recombinant *PavAct1* and non-reduced S⁴- and S⁶-RNases prepared from the style extracts, interaction was detected between the recombinant *PavAct1* and reduced S⁴- and S⁶-RNases. Furthermore, EDC-cross linking assay and filamentous actin (F-Act) co-sedimentation assay using the rabbit actin showed that reduced S⁴- and S⁶-RNases interacted with both the globular actin (G-Act) and F-Act. These result collectively suggested that S-RNase that might be fully or partially reduced in the cytosolic environment in pollen tube could bind actin to disrupt the actin dynamics. Further studies are required to elucidate whether the cytotoxic target of S-RNase in the SI reaction in *Prunus* was the pollen RNA alone and/or actin.

ALPHA- AND BETA-TUBULIN ISOFORMS ACCUMULATE DIFFERENTLY DURING BUD DEVELOPMENT IN *VITIS VINIFERA* L.

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Microtubules (MTs) are central components of the plant cell cytoskeleton and play important roles during plant growth and morphogenesis, such as in the synthesis and deposition of cell wall components, in the process of cell division, in organelle positioning and, more generally, in plant cell shaping. Most of MT functions depend on the cooperation between tubulin and MT-associated proteins (MAPs), which regulate the nucleation of MTs, their association with the plasma membrane, the number and orientation of MTs and the formation of MT bundles (Hammond et al. 2008, *Curr Opin Cell Biol* 20:71-76). In addition to MAPs, the regulation of MT functions is also achieved through the expression of distinct tubulin isoforms. Multiple isoforms of α - and β -tubulin accumulate in higher plant cells and may originate either by transcription of different genes or by post-translational modifications, such as tyrosination, acetylation, glutamylation, glycylation and transamination (Wloga and Gaertig 2010, *J Cell Sci* 123:3447-3455). Different tubulin isoforms might affect the binding of MTs to different associated proteins thus generating MTs with different organizations and functions. It is known that tubulin isoforms are differentially expressed in vegetative and reproductive structures according to the developmental program of plants (Yoshikawa et al. 2003, *Plant Cell Physiol* 44:1202-1207). In grapevine (*Vitis vinifera* L.), vegetative and reproductive structures appear on the same stem, a fact that makes this plant species an excellent model to study the accumulation of tubulin isoforms during the development of floral organs. To approach this problem, we used a proteomic analysis to screen the protein content of developing buds. Proteins were extracted from different grapevine samples (buds, leaves, flowers and tendrils) using an optimized extraction protocol; proteins were then separated by two-dimensional electrophoresis (2-DE) and analysed by immunoblot with anti-tubulin antibodies directed against the α - and β -subunits. Both subunits have a similar pI around 4.8-5 and group in separate clusters. Eight α -tubulins and seven β -tubulins were identified after immunoblots on 2-DE gels. More acidic α -tubulins were detected in buds, while more basic α -tubulins were prevalently found in tendrils and flowers. Similarly, more acidic β -tubulins were used in the initial stages (buds) while a basic β -tubulin was essentially used in leaves and two central β -tubulins are characteristically used in tendrils and flowers (Parrotta et al. 2010, *Planta* 231:277-298). As a parallel approach to the proteomic study, we have also analyzed the genome of grapevine to identify putative tubulin genes, taking as reference the α - and β -tubulin genes of *Arabidopsis thaliana*. By comparing the *Arabidopsis* tubulin genes to the 12X grapevine genome, we identified about 120 gene alignments. Once the presence of genes was confirmed, their genomic sequence was analyzed by online protein databases in order to verify that alignments might correspond to annotated tubulins. After a series of controls, we putatively identified 7 α -tubulin and 9 β -tubulin genes, a number that corresponds comparatively to the number of spots identified by 2-DE.

Our current attempt is to obtain the sequence of protein spots in order to understand if tubulins used during the transition from buds to leaves/flowers/tendrils are either distinct isoforms or post-translational modifications of a few expressed genes.

THE STUDY OF SELF-(IN)COMPATIBILITY IN CEBAS-CSIC ALMOND BREEDING PROGRAMME

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Most cultivated almonds [*Prunus dulcis* (Miller) D.A. Webb] exhibit self-incompatibility (SI) of the gametophytic type. Because of this reproductive barrier, cross-fertilization is required to obtain a yield. Some almond cultivars with a self-compatible phenotype have also been found mainly in Apulia (Italy). Due to the great advantage of self-compatibility (SC) to ensure self-fertilization even in the absence of pollinator insects, some of these cultivars have been used as parents in the CEBAS-CSIC almond breeding programme to introduce this character in the progenies. Initially SC was detected by recording fruit set after bagging flower buds and also by fluorescence microscopic observation of pollen tube growth through the pistil following hand-self-pollination. Later, the identification of the pistil-S product and the finding that in almond the S_r -RNase (self-fertility-RNase) does not have RNase activity, allowed the identification of SC by isoelectric focusing of stylar proteins and staining for activity. Different crossing strategies ensuring 100% of self-compatible descendants were conducted in the 90's and early 2000's. In this way, homozygous self-compatible almonds were experimentally obtained for the first time. These strategies have been proved to increase the efficiency of the programme by avoiding the laborious task of evaluating self-compatibility in the progenies. Over the last few years there has been an intensive research on the molecular genetics of SI in a wide range of *Prunus* species, generating more useful and efficient primers, which allow an accurate identification of the S genotype using PCR methods. Using two sets of consensus primers in plant material from CEBAS-CSIC almond germplasm collection, 30 almond S-RNases were characterised for the first and second intron. Later the cloning and sequencing of these S-RNases confirmed the existence of new alleles, allowed the resolution of synonyms and the correction of S-genotypes, and consequently to update the previously proposed cross-incompatibility groups in almond. In addition, amino acid sequence comparison revealed the occurrence of intragenic recombination and indicated the significance of the region between RC4 and C5 in defining specificity. Recently, when evaluating the suitability of local almond selections as pollinators of the premium quality almonds 'Marcona' and 'Desmayo Largueta', a selection with the S_r haplotype but with a self-compatible phenotype was identified. These results indicate that in almond SC is not due to a mutation in the S-RNase amino acid sequence, and the absence of ribonuclease activity of S_r -RNase could be explained by the action of other factor(s) involved in the functioning of the SI system. To identify proteins other than S-RNase and SFB that may be involved in almond SI, a comparative analysis of the quantitative differential expression of proteins in pollen and pistil tissue following compatible and incompatible crosses has been performed using 2D-DIGE and HPLC-MS/MS techniques. Identification by HPLC-MS/MS and searches with SEQUEST against NCBI database revealed the existence of 48 candidate proteins, 21 of which were up-regulated in the incompatible interactions. Further research to identify proteins and genes participating in the functioning of SI in almond is in progress.

SYRIAN PEAR (*PYRUS SYRIACA*) AS A POLLINATOR FOR EUROPEAN PEAR (*PYRUS COMMUNIS*) CULTIVARS

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In Israel four European pear cultivars are grown: 'Spadona' is the main cultivar and 'Coscia', 'Gentile' and 'Spadochina' are its pollinators. However, molecular S-genotyping revealed that 'Spadona' is semi-compatible with its three pollinators. This explains, at least in part, the relatively low pear yield of in Israel. The Syrian pear (*Pyrus syriaca*) grows wild in Israel and blooms intensively, overlapping the blooming of the cultivated European pears. Cross-fertilization between Syrian pear and 'Spadona' was shown to be efficient suggesting that Syrian pear might be a potent pollinator for 'Spadona'. Twenty six Syrian pear seedlings, from different sites in north-east Israel were S-genotyped from which, twenty four different S-RNase alleles were cloned; ten of them are new, whereas the other fourteen had been identified previously in other *Pyrus* species. Eleven of the Syrian pear seedlings are fully compatible (according to their S-genotype) with the four European pear cultivars. In addition, seedlings of two *P. betulifolia* and one from *P. korshinskii* wild pear species were also S-genotyped: From these seedlings four S-RNases were cloned, two are new, one had been cloned previously and one, from *P. betulifolia* 1, was found to be differs in only one amino acid from the *P. syriaca* S7 allele cloned in this work and therefore was named *P. betulifolia* S7a.

Computer modeling indicates that the RHV conformation of *PsyS7* and *PbeS7a* is effected by the different amino acid. To prove that *PsyS7* and *PbeS7a* are functionally different, pollination experiments were carried out: *P. betulifolia* '1 flowers (carrying S7a) were hand pollinated with the pollen of Syrian pears Sassa 2 and 3 (carrying S7). No fruits were found on selfed *P. betulifolia* indicating that it is not self compatible. Seeds from fruit of the cross pollination are currently S-genotyped, If *PsyS7* will be identified in the seeds it will be concluded that the two alleles are functionally different.

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