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Characterization and function of *homeobox* genes encoding class2 KNOX transcription factors involved in the development of aerial organs in *Prunus persica* (L. Batsch)

Coordinator
Prof. Anna Maria Innocenti

Supervisor
Prof. Maria Beatrice Bitonti

Doctoral student
Dr. Emiliano Condello

Co-supervisor
Dott.re Donato Giannino

Dipartimento di Ecologia
Università della Calabria

Settore disciplinare scientifico: BIO/01

PhD thesis, Doctorate Research Program in Plant Biology
Ecology Department – University of Calabria
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CONTENTS

CHAPTER I

General introduction and scope of the thesis

INDEX OF CHAPTER I

PRUNUS PERSICA (L. Batsch): A MODEL SPECIES FOR FRUIT TREES

In the following paragraphs we selected some information from a vast body of knowledge regarding the biology and cultivation of peach trees. We mainly focused on those topics that may hopefully enhance the comprehension of models, experiments and results dealt with in this thesis.

PEACH ORIGIN AND ECONOMIC CONTEXT

Probably the first fruit crop domesticated in China about 4.000 years ago, the peach cultivars grown today derive largely from ecotypes native to southern China. Peaches were moved to Persia (Iran) along silk trading routes (the epithet *persica* denotes Persia) and then to Europe. Greeks and Romans spread them throughout Europe and England (300-400 BC), subsequently Portuguese introduced them to South America and Spaniards to Florida in the 16^{th}-17^{th} centuries. In Italy, the first peach cultivation appeared at the end of '800 near Ravenna and today Italy is the first European producer and the second in the World (ca. 1,4 - 1,5 M of tons per year), after China, and a major licenser of new varieties after the US (source: FAOSTAT, www.faostat.fao.org). The export flux of fresh products is ca. 400.000t (1/3 of production) and acts as "commercial tow" for other Italian products in the north Europe. Italian peach cultivation covers ca. 100.000 ha mainly located in Emilia-Romagna (about 1/3 of the production), Campania (1/4), Veneto and Lazio. Varieties are cultivated based on their chill requirement (400-900 hrs) for flowering and maturation precocity so that complementarities between northern and southern regions contribute to provide fruits from May to September and consequently limit internal competitions (Fideghelli, 2000).

BIOLOGICAL ASPECTS

Botanical features and growth requirements.

Peach is a small deciduous tree growing to 5–10 m tall, belonging to the family *Rosaceae*, sub-family *Prunoideae*, genus *Prunus*, species *persica*. Peach trees have lanceolate (7–15 cm long and 2–3 cm broad), stipulate leaves, borne on long, slender, relatively unbranched shoots, the flowers are arranged singly, or in groups of two or more, at intervals along the shoots of the previous year's growth. A typical cluster is the triple bud, consisting of two lateral flower buds and a central vegetative one. Flowers open before vegetative resumption are hermaphrodites and have a hollow tube at the base bearing five sepals, five petals, usually concave or spoon-shaped,

pink, and a great number of stamens (until 20-30). The pistil consists of a single carpel with its ovary, style and stigma. The ovules are usually two, but only one is fecundated and becomes mature. The fruit is a drupe having a thin outer skin (epicarp) enclosing the flesh of the peach (mesocarp), the inner layers of the carpel becoming woody to form the stone (endocarp), while the ovule ripens into the kernel or seed.

Mediterranean climate are excellent for peach cultivation, it tolerates wide temperature range (from -18° to subtropical temperature, where the winter is very short). Culture methods are classified in: volume shape, perpendicular and inclined V systems. All the shapes derived from the pruning and the modern fruit cultivation are directed to reduce the volume to shorten the work. Peach water needs vary with the soil property, rainfall, rootstock, cultivar, etc. It was calculated that an hectare of peach orchard consume 2500-4000m^3 of water (that is 250-400mm of rainfall). In addition, supplemental irrigation during the growing season can lead to improvements in yield and tree growth for young trees especially (Layne *et al.,* 1981) and can improve long-term tree survival (Layne and Tan, 1984). Application of fertilizer through a micro-irrigation system (fertirrigation) may enable better timing of fertilizer application and reduce rates applied (Smith *et al.,* 1979; Layne *et al.,* 1996). Peach varieties are classified based on chill and heat requirements. The former is necessary for flower opening in spring (and to complete organ flower development), the latter establishes the meristem destiny (vegetative or reproductive) during the spring-summer period. These requirements are quantified in chill and heat units and expressed as the number of hours to be accumulated below and above established temperatures, respectively. In the Mediterranean areas, peach varieties need 400-900 hours of chill, and the precocity of production can be assessed with respect to the climate of a region (Faust, 1989). An efficient RS-CV combination is crucial for the success of a qualitative production. The lists of peach varieties and rootstocks and the guides for a correct (and recommended) use of both are available on the web and specifically related to each Italian shire (http://www.ivalsa.cnr.it/archivio%20fruit/pesco/cultivar/fr_pesche.htm). Rootstock selection depends on different factors: soil, previous culture, water availability, cultivar, etc. The most widespread rootstocks are : Franco Slavo, franco selections, P.S. series, GF677, Sirio, Hansen, Barrier 1, Plum, M.r.S. 2/5, Penta and Tetra. The choice of rootstock has also been noted to have profound effects on peach tree growth and yield (Marangoni *et al.,* 1985; Layne, 1987).

Vegetative and reproductive organs: a brief synopsis.

The peach shoot apical meristem (SAM) maintains the typical dicotyledonous features: it is made of a surface layer, the *tunica*, which covers the inner tissues that form the *corpus*. The

tunica consists of two layers (L1 and L2), which undergo anticlinal divisions (perpendicularly to the surface) and their daughter cells remain in the same layer as their parent, whereas the corpus (L3) undergoes both anticlinal and periclinal (tangentially to the surface) divisions. All three cell layers participate in stem growth and organ formation (Poethig and Sussex, 1985a, 1985b). In aerial organs the epidermal cell layer is exclusively L1-derived; the L2 comprises cells forming mesodermic tissues and germ cells, while the L3 contributes to both leaf mesophyll and the vasculature (Stewart and Burk, 1970). The SAM also shows cytological zonations, independent of the layered structure: a central zone (CZ) surrounded by a peripheral zone (PZ) and a rib zone (RZ), which are distinguishable by cell features, specific growth characteristics and gene expression patterns. The CZ is located at the distal portion of the apical dome and comprises slowly growing cells, which replenish the PZ and are required for the maintenance of the meristem. The PZ consists of rapid dividing cells in which leaf primordia are initiated. The RZ is sited at the base of the SAM and is formed by strong mitotic activity cells and delivers a contribution to the formation of the central tissues in the plant (Carraro *et al.,* 2006).

Vegetative growth.

Axillary meristems produced by the main axis may grow into lateral shoots while the main axis continues to extend. This mode of branching is called syllepsis (Hallé, Oldeman and Tomlinson, 1978). The variation of syllepsis according to genetic and environmental factors partly explains the considerable plasticity of the shape of trees. The extent of syllepsis determines the spatial arrangement of leaves and the total leaf area of the current year's shoot and thus how the tree grows (Remphrey and Powell, 1985). In peach trees, most shoot elongation occurs during the first 15 weeks after anthesis, whereas branch diameter growth continues until autumn (Grossman and DeJong 1995a).

Floral Buds.

After the vegetative resumption, the bud development and differentiation is regulated by several factors: a) the length of the shoot bearing the buds; b) leaf development and expansion of the shoot bearing the buds; c) the time interval between flower anthesis and the formation of new buds, d) the co-presence of fruits; e) nutritional conditions and hormone balance of the shoot (Grossman and De Jong 1995b). Vegetative growth and flowering are often considered as antagonistic (Krekule, 1979): according to Crabbé (1984), flowers appear on shoots of medium vigour rather than on shoots of low vigor. Moreover, positive relations between shoot length and flower bud formation were observed (Jackson and Sweet, 1972). A detailed report on anatomy of bud development in relation to phonological stages is described by Reinoso *et al.* (2002).

Drupe development and ripening.

Fruit development and ripening involve a series of biochemical and structural changes that make the fruit acceptable for eating. In climacteric fruits, such as peach drupe, these changes are under the control of the plant hormone ethylene (Pech *et al.,* 1994), which control color, aromas, texture and flavor (Tonutti *et al.,* 1991). Stages of development and enzymes involved in fruit ripening are described by Zanchin *et al.* (1994). At present, in Italy is addressing the analysis of several important biochemical pathways (e.g. softening, ripening, aroma development, etc.) based on oligonucleotide microarrays derived from ESTs collection (http://www.itb.cnr.it/estree/).

THE LEAF CURL DISEASE

Generalities.

Peach trees are attacked by several viral, bacterial and fungal pathogens and pests. Nowadays, the major enemies of peach cultivation are the SHARKA virus (*Plum Pox Virus*) and nematodes (www.actahort.org/books/478), which have urged the development of world-wide projects to produce new resistant varieties. However, a relevant parasite is *Taphrina deformans*, a biotrophic fungus that, under the right conditions, can cause severe early defoliation and crop loss on all known peach cultivars. The Taphrina diseases are best known in Europe and North America, but probably occur all over the world. Taphrina causes defoliation leading to small fruit or fruit drop and attacks buds and twigs up to devitalizing the tree (Pscheidt, 1995).

Symptoms.

The most common and striking symptom of leaf curl occurs on the foliage. Infected leaves are severely deformed and often display a variety of colours ranging from light green and yellow to shades of red and purple (accumulation of anthocyanin pigment). The fungus causes the meristematic cells at leaf margins to proliferate quickly and randomly, which results in the leaves becoming variously wrinkled, puckered, and curled. As these infected leaves mature, naked asci containing ascospores of the pathogen are produced on the surface giving them a dusty appearance, after which the leaves turn brown, shrivel, and fall down from the tree. Peach blossoms also become infected. They are distorted, shrivel, and usually fall before the symptoms are well developed. The leaf curl fungus may attack young peach shoots causing the terminal portion to become stunted, swollen, twisted, and pale yellow or green. Such twigs generally produce nothing but tufts of curled leaves at their tips. Many affected twigs will die back. The

tips of plum shoots become greatly enlarged and are often twisted and curled. Many infected fruits drop prematurely whereas those that remain may become crooked at the stem end like a small yellow squash, while others develop reddish to purple, wart-like deformities on the fruit surface

Course of disease.

The development of the fungus within the leaf is a continuous process, but to facilitate the study of host-parasite relationships five stages were defined (Syrop, 1975a and b). The fungus apparently over-winters as ascospores or thick-walled conidia on the tree, perhaps in the bud scales. In the spring, these spores are blown to young tissues, germinate and penetrate the lower surface of developing leaves directly through the cuticle or through stomata of a healthy leaf (Stage 1). From the spongy mesophyll where the fungus established itself, the binucleate mycelium grows between cells and invades the tissues extensively inducing excessive cell enlargement and cell division, which result in the enlargement and distortion of the leaves districts (Stage 2). Later, the myceliar hyphae, which are present only in the red regions of the leaf, grow outward in the area between the mesophyll cells, below the epidermis, and between adjacent epidermal cells below the cuticle (Stage 3) where they cause a distortion of the leaf cell walls by secretion of polysaccharides-degrading enzymes including cellulase. Intercellular and sub-epidermal hyphae can only be distinguished by their relative positions in the infected leaf, while the sub-cuticular hyphae occupy a different location and have a distinctive morphology. The fungal mycelium grow rapidly between the cells of the host tissues, stimulating excessive cell division (hyperplasia) followed by the enlargement (hypertrophy) and differentiation of the leaf cells which result in an absolute cell disorder (Stage 4). Leaf curls show also by an uneven expansion of the leaf thickness. Experimental data suggest that Taphrina deformans, which is known to deliver indolacetic acid and zeatin-like substances in axenic cultures, induce the accumulation of auxin and cytokinins in curled leaves (Perley and Stowe, 1966; Johnston and Trione, 1974; Sziràki *et al.,* 1975; Yamada *et al.,* 1990; Bassi *et al.,* 1984). In the final stage of infection (Stage 5), from the sub-cuticular spaces the asci, which are formed exclusively on the upper leaf surface, grow and enlarge itself and perforate the cuticle by lysis and not by mechanical action which results in a compact, felt-like layer of naked asci. Each ascus usually produces eight, one-celled, oval ascospores, which are released into the air as the mature asci rupture. The ascospores multiply by budding inside or outside the ascus producing blastospores (conidia) during warm, moist weather. The conidia may continue to bud and eventually produce tremendous numbers of thick, weather-resistant walls spores which are capable of surviving hot, dry summers and freezing winters for two years or more. If preventive measures are not taken

before trees break dormancy in early spring, control of peach leaf curl is impossible. Trees are susceptible to infection only during the relatively short time between swelling and opening of the buds. In fact, during cool, rainy weather in early spring, (optimum 15.5° to 21°C although little infection occurs below 7 °C), from bud swell to bud opening, the spores germinate and infect the swelling leaves and flowers within the buds.

MAJOR TARGETS OF MOLECULAR BREEDING AND THE POST-GENOMIC ERA

P. persica is considered one of the best genetically characterized fruit tree species within the *Rosaceae*. Peach has distinct advantages making it suitable as a model species: i) it has a relatively short juvenility phase (2-3 years) compared to most other fruit tree species, such as apple, pear, and citrus, that have juvenile phases ranging from 6-10 years; ii) while some *Prunus* species such as cultivated plums and sour cherries are polyploid, peach is a diploid species with 2n = 16 (Jelenkovic and Harrington, 1972); iii) it has a comparatively small genome size of ca. 300 Mbp (Baird *et al.,* 1994), ca. twice that of *Arabidopsis thaliana*; iv) it is autogamous, hence reduced genetic variability occurs in the progeny; v) it is highly syntenic with others fruit trees (almond, cherry, apricot, plum, apple and pear) whereby the knowledge in molecular field may be easily exported to them.

Recently the grade of sinteny with Arabidopsis was also estimated

Breeding practices in *Prunus* address several traits including seasonality, lowered chilling requirements, fruit quality and novel fruit types, tree habit, plant architecture, adaptability, resistance to biotic and abiotics stresses, etc. Italian targets in peach cultivation to enhance competitiviness are: fruit quality, lowering of production costs, environmental safety. A proper and correct choice of cultivar (CV) and rootstock (RS) combination and environmental safe agro-techiques are fundamental to achieve these objectives. An efficient RS-CV combination solves problems of soil decline, vigour regulation and affects the ripening time and fruit quality. Several projects are focussed in the selection of new rootstocks tolerant or resistant to soil pathogens and pests (www.actahort.org/books/478). As for the European market standards, the fruit quality traits are more important than a wide choice of varieties. Aroma composition and stability at low temperature, high antioxidant content, allergens absence, elimination of wooliness, control of post harvest ripening and long shelf life are among the major traits aimed to enhance peach cultivars. (Fideghelli, 2000)

Two major efforts to develop peach as a model for genomics of *Rosaceae* have been initiated: (1) Structural genomics – the development of complete physical and genetic map of the peach genome and the anchoring of the genetic markers of important *Rosaceae* species maps on

this physical map; (2) Functional genomics – the development of an extensive EST database for fruit, shoot and seed tissues and integration of the tentative unigene set onto the physical and genetic maps of peach (Jung *et al.*, 2004).

Molecular markers are important tools for breeding selection, genotype identification, and studying the organization and evolution of plant genomes. Genome mapping is one of the most important applications of molecular markers. The linkage between molecular markers and genes controlling important horticultural traits (either monogenic or polygenic) makes their introgression via marker-assisted selection (MAS) faster, and may be used as a first step towards saturation of the target region for positional cloning (Tanksley *et al.*,1995). Several classes of DNA-based markers are used for these purposes. Co-dominant highly reproducible and easily transferable markers, such as restriction fragment length polymorphisms (RFLPs) and simple sequence repeats (SSRs), are the best possible choice, since they provide more genetic information than dominant markers and can easily be transferred to other mapping populations. In the last decade, the advent of DNA-based markers has been greatly facilitated the construction of peach genetic maps have been published for peach (Rajapakse *et al.*, 1995 ; Foolad *et al.*, 1995; Viruel *et al.*, 1995; Joobeur *et al.*, 1998 ; Wang *et al.*, 1998). From these, a general *Prunus* genetic map was developed based on the inter-specific cross between the cultivar Texas of almond and Earlygold of peach (TxE) which is considered the reference map for the genus *Prunus* (Joobeur *et al.*, 1998). The current map has 562 markers, covering 519 cM (average density, 0.92 cM per marker). TxE map was originally reported as a saturated linkage map of 246 markers (235 RFLPs and 11 isozymes) in the expected eight linkage group by Joobeur *et al.* (1998). An updated map with an addition of 96 simple sequence repeats (SSRs) has been reported by Aranzana *et al.* (2003) and the current map with 220 additional markers (89 SSRs, five sequence-tagged sites, and 126 RFLPs has been reported by Dirlewanger *et al.* (2004). Other interesting maps were constructed using inter-specific crosses, such as those derived from backcross between peach and *P. ferganensis* (P x F; Dettori *et al.*, 2001) which includes the position of 216 markers disperse on 662cM (Verde *et al.*, 2005). On this map, 10 qualitative traits loci (QTL) involved in qualitative fruit control, resistence to powdery mildrew desease and internode length control, were localised. Comparing the positions of anchor markers of the TxE map with those of 13 maps constructed with other *Prunus* populations, it was shown that the genomes of the diploid species, peach, almond, apricot, cherry, *P. davidiana*, *P. cerasifera*, and *P. ferganensis*, are essentially syntenic and collinear (Dirlewanger *et al.*, 2004). In *Prunus* mapping, the polymorphisms generated by a single nucleotide substitution (Single Nucleotide Polymorphism, SNP) are still poorly exploited. Expressed sequence tag (EST) collections constitute an almost inexhaustible fountain of SNP and, thus,

several programs to localize SNP into EST sequences were developed. SNP markers allow to obtain high-resolution genetic-molecular maps as well as dense physical maps which are the starting point for identification, sequencing and cloning of targets genes. At present, Clemson University (USA, www.genome.clemson.edu/gdr/) is addressing the construction of dense physical map of peach whereas, in Italy, the ESTree Inter-University Centre (www.itb.cnr.it/ESTree) is producing a EST database containing few thousand of EST in addition to the world database of about 18.000 sequences. (Abbot et at., 2002). Microarray chip on.

STUDIES OF GENE FUNCTION IN FRUIT TREES BY GENETIC TRANSFORMATION

Genetic transformation is a fundamental tool to study gene function of trees and to achieve the improvement of tree varieties. Traditional approaches tree breeding are limited by the reproductive cycle, with long juvenile periods, high degree of heterozigosity and auto-incompatibility events. The application of transformation overcomes many drawbacks associated with conventional breeding strategies as demonstrated by the production of transgenic trees with high performance traits in wood and fruit production (Pena *et al.,* 2001; Tzfira *et al.,* 2004). Nowadays, the genes of interest are mostly transferred to trees exploiting disarmed and genetically engineered Agrobacterium strains, which drive foreign DNA into plant cells. Target genes are co-transferred with those required for transformation, including genes that confer resistance to antibiotics necessary for selection of transformed cells. However, given European directives and public concern with genetically modified organisms, the use of antibiotic resistance genes is prohibited into food and several strategies to engineer health and environmental friendly genetic constructs and respective methods of transformation are being developed (Kapuscinski *et al.,* 2003; Halpin C, 2005). However, few of these new methodologies are effective in herbaceous crops, for which a real market production of transgenics exists.

Concerning most tree fruit species, the transformation of market cultivars is quite limited to a few genotypes or to seedlings: so far the papaya tree resistant to the ringspot virus has been the only commercialized transgenic fruit tree (Lius *et al.,* 1997). Technically speaking, transformation of *Rosaceae* trees was applied successfully to: apple, pear, plum, sweet and sour cherry, apricot, almond, and peach (Petri and Burgos, 2005). As for the last three trees, the efficiency of transformation is far from being satisfactory for the regeneration rate of transformed tissues is low, if not absent. Moreover, though seeds show the highest regeneration they are not a convenient material to preserve the elite traits of the parents. In fact, since the seed

derived plants express genetic diversity due to gamete recombination, producing fruit trees by vegetative propagation is highly convenient so that the clone lines maintain the traits of the mother plant.

As for peach trees, transformation events were reported from immature endosperm (Meng and Zhou, 1981), embryo derived callus cultures (Hammerschlag *et al.,* 1985; Bhansali *et al.,* 1990; Scorza *et al.,* 1990), cotyledons (Mante *et al.,* 1989; Pooler and Scorza, 1995), leaf explants (Gentile *et al.,* 2002), but regeneration of plants from transgenic tissues was very modest. Recently, the recovery of transgenic lines was achieved from embryo sections of mature seeds of the only cultivar Miraflores, with a 50% regeneration (Perez-Clemente *et al.,* 2004), which renders this protocol appealing but still not suitable for post-genomic programs.

So far the functional studies of peach genes has not been approached by transformation of peach itself; heterologous hosts - *Drupoideae* trees or Arabidopis - are good candidates, but clear disadvantages are envisaged and predictable as compared to the achievement of transgenic peach trees. Virus induced gene-silencing (VIGS) offer an attractive and quick alternative to down regulate or silence a gene without the need to genetically transform the plant. Recombinant virus carrying plant genes (or portions) in sense and/or antisense orientation is used to infect the plant. When the virus spreads systemically, the endogenous gene transcripts, which are homologous to the insert in the viral vector (VIGS-vector), are degraded by post-trascriptional gene silencing (PTGS) (Baulcombe, 1999). The cloning of tobacco rattle virus (TRV) genome (Ratcliff *et al.,* 2001) into binary *Agrobacterium tumefaciens* plasmids opened the possibility of studying genes expressed in early organ development, because of its ability to reach growing points and to deliver the silencing signal to meristems. TRV is an ssRNA virus with a bipartite genome. The component called RNA1 encodes, among other genes, an RdRP, whereas its genome partner, called RNA2, encodes the coat protein (Angenent *et al.,* 1986; MacFarlane, 1999). The target gene fragment for silencing is inserted into the RNA2 element. Inoculation, either mechanical or via infiltration, requires the presence of both genome components. In the case of agro-infiltration, two different Agrobacterium clones, one carrying the RNA1 genome and another with the RNA2 containing the target gene fragment, are mixed together and co-infiltrated into the leaf tissues (Fig. 1) (English *et al.,* 1997). There are several advantages in using VIGS method: the facility of application with leaf infiltration, non-dependence on the whole coding sequence of the target gene in order to elicit silencing and rapid results within days after inoculation. Consequently, it is feasible to carry out high-throughput VIGS of many genes in a target plant genome. A further advantage of VIGS is its conditional nature: the target mRNA is not silenced until the virus vector infects the plant and can suppress genes that are essential for host cell growth and development. Conversely, most mutations in such genes and/or their

silencing based on a genetic transformation approach would result in lethal or difficult to retrieve phenotypes.

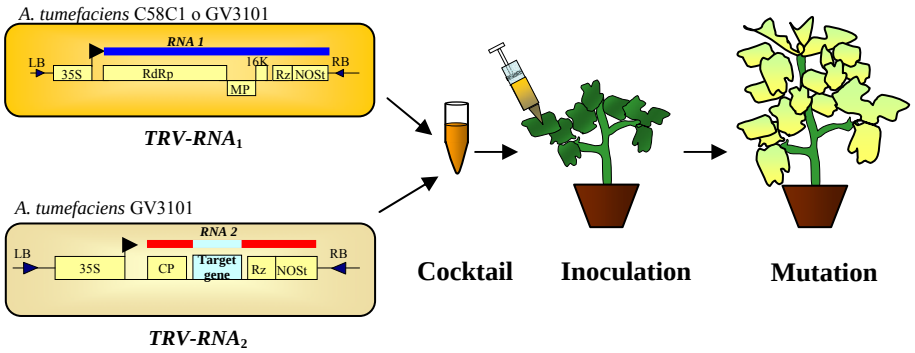

Figure 1 - Schematic representation of the VIGS procedure.
Leaves of young seedling were inoculated with TRV vectors by agro-infiltration. The inoculation mixture consisted of equal amounts of two *Agrobacteria* strains containing *TRV-RNA*1, which harbours the virus polymerases and movement proteins, and *TRV-RNA2*, which carries the coat protein and a fragment of the target gene, respectively. The constructs act synergistically to ignite mechanisms of PTGS in plant and interfere with the target gene. The mutant phenotypes usually appear ca. 21 days later and have a transient appearance (ca. 3 weeks).
LB-RB, left and right T-DNA borders; **35S**, CaMV 35S promoter**; RdRp**, RNA-dependent RNA polymerase; **Mp**, movement protein; **16K**, cystein-rich protein of 16 kDa; **NOSt**, nopaline synthase terminator; **CP**, capside protein; **Rz**, self-cuting ribozime;

HOMEOBOX TRANSCRIPTION FACTORS AND PLANT ORGAN DEVELOPMENT

A short premise.

The topic of this thesis focuses on peach class 2 *Knotted*-like genes (*KNOX*), which encode transcription factors containing a DNA binding homeodomain and belong to a wide gene group characterized by the homeobox sequence. Scientists have devoted much effort producing information on class 1 *KNOX* to unravel their functions in model and crop species. To date the class 2 *KNOX* roles in plant development are still undefined and at the levels of hypotheses. The next paragraphs encompass an overview on plant homeobox genes and mostly on class 1 *KNOX* so as to provide general information and a thesis-supportive background. The data literature ("the state of the art") specifically related to class 2 *KNOX* are reported in the introductions of the chapters II-VI to facilitate the understanding of experiments and results of this thesis.

PLANT HOMEOBOX GENES.

Generalities.

Homeobox genes (HG) encode homeodomain-containing proteins and control a wide range of developmental programs in all eukaryotic lineages (Hayashi and Scott, 1990; Dessain *et al.,* 1992). In animals, they play roles from the earlier step of embryogenesis, such as setting up an anterior-posterior gradient in the egg of *Drosophila melanogaster* (Cooperstock and Lipshitz, 2001), to the latest phases of cellular differentiation, such as the neuron differentiation in Caenorhabditis elegans (Cameron *et al.,* 2002). Many plant HG are involved in the transcriptional regulation of development, particularly in stem cell niches associated with post embryonic growth. The activity of the shoot apical meristem (SAM), which contains the stem cell population from which all aerial organs are ultimately derived, is controlled in part by the activities of HG.

The homeobox motif was originally described as a conserved DNA motif of 180 base pairs encoding a protein domain, the homeodomain (HD), of 60 amino acids. Proteins containing the homeodomain typically act as transcriptional regulators by binding to cis-acting elements in the promoters of downstream target genes and facilitating the recruitment of factors such as RNA polymerase II (Reiser *et al.,* 2000). The HD structure has been determined by NMR and X-ray crystallography; it consists of three α-helix arranged in a helix-loop-helix-turn-helix motif, which pack around a hydrophobic core. The third α-helix is involved in recognition of specific sequences of DNA in the promoter and/or enhancer of target genes. It binds the major groove of the double helix of the DNA, whereas the other two helix regulate the juxtapositions of the third

helix and in the maintenance of structure stability (Gehring *et al.,* 1990). Finally, the HD is also able to interact with proteins which act as transcriptional activators or repressors (Chan and Mann, 1996; Mann and Chan, 1996).

Plant homeobox genes and homeotic functions: a question to solve.

The term homeosis describes the alternative developmental path of a certain organ. Pattern formation is one area for comparison between plant and animal development. In Drosophila, the homeobox containing Hox genes (*HOX*) establish segmental identity and hence they were called homeotic genes, referring to their function (Cooperstock and Lipshitz, 2001). Similarly, in plants, the specification of a) the radial pattern of floral organs and b) the organ identity in developing flower requires a set of master regulatory genes (Jack, 2001; Goto *et al.,* 2001). The master regulatory genes identified to date are not members of the homeobox family, but they are mostly transcription factors of the MADS box family (Jack, 2001). The HG and MADS genes are not homologous, both in plants and animals (Gehring *et al.,* 1994; Banerjee *et al.,* 1987; Pellegrini *et al.,* 1995). Hence in plants, homeotic functions have been attributed to *MADS* genes, but not to HG genes (including *KNOX* genes, topic of this thesis) so far. The very few MADS proteins in animals have not been assessed as master regulators of pattern formation (in other words: they are not homeotic genes), for they are involved in muscle differentiation and in wing vein and tracheal development (Taylor *et al.,* 1995; Gunthorpe *et al.,* 1999, Montagne *et al.,* 1996; Riechmann *et al.,* 2000). Thus, it seems that plants and animals independently evolved the master regulatory processes that serve their logically similar mechanisms of spatial pattern formation.

Classification of plant homeobox genes.

Several groups of HG exist in the model plant Arabidopsis thaliana and include: the *WOX*, *BELL*, *HD-ZIP*, and *KNOX*, all of which encode proteins that contain the DNA-binding homeodomain motif (HD) (Lincoln *et al.,* 1994; Reiser *et al.,* 1995; Long *et al.,* 1996; Himmelbach *et al.,* 2002; Haecker *et al.,* 2004). Features of the first four groups are summarised below, followed by an overview on KNOX, the major topic of this thesis.

The Arabidopsis *WUSCHEL* gene (*WUS*) is the founding member of the *WUSCHEL*-related homeobox subfamily (*WOX*), several *WOX* members control various aspects of plant development (Matsumoto and Okada, 2001; Haecker *et al.,* 2004; Nardmann *et al.,* 2004; Park *et al.,* 2005; Wu *et al.,* 2005). WUS contains the HD and three main motifs at the C-terminus: an acidic domain typical of transcriptional activators), a WUS box (TLPLFPMH) with unknown function, and an EAR-like domain (ASLELTLN), for transcriptional repression (Ohta *et al.,*

2001; Hiratsu *et al.,* 2004; Tiwari *et al.,* 2004). *WUS* mutants are unable to maintain a pool of undifferentiated stem cells (Laux *et al.,* 1996). In the SAM, cells expressing WUS define a new domain within the central zone, referred to as the organizing centre (Schoof *et al.,* 2000). *WUS* activity results in signalling to the overlying stem cells, inducing the *CLAVATA 3* gene (*CLV3*), which then signals back to repress *WUS*. The *CLV/WUS* negative feedback loop ensures homeostasis of the SAM by regulating the number of stem cells present in the central zone (Brand *et al.,* 2000; Schoof *et al.,* 2000). The stability of *WUS* expression also involves signalling from leaf primordia and tissue underlying the organizing centre (Stuurman *et al.,* 2002). At the end of flower development, *WUS* terminates stem cell maintenance, and this function involves the direct activation of the *AGAMOUS* (*AG*) together with *LEAFY* genes (*LFY*) (Lenhard *et al.,* 2001; Lohmann *et al.,* 2001). However, the mechanism by which *WUS* represses the differentiation of stem cells is unknown (Heacker *et al.,* 2004).

BELL genes encode atypical homeodomain protein characterized by a three amino acid extension to the loop (TALE) connecting the first and second helices of the homeodomain (Reiser *et al.,* 1995; Bürglin, 1997). TALE-HD proteins constitute a super-class including plant KNOX and BELL proteins. BELL proteins contain two highly conserved domains: SKY and BELL (Bellaoui *et al.,* 2001). Specific BELL proteins were shown to interact or heterodimerize with specific KNOX proteins in both monocots and dicots (Bellaoui *et al.,* 2001; Smith *et al.,* 2002). The cooperative interactions between KN1 and KIP (a maize [Zea mays] BELL protein) were reported to mediate high DNA binding affinity to the KN1 DNA binding motif TGACAG(G/C)T (Smith *et al.,* 2002). Hence, BELL and KNOX homeodomain proteins may work together to regulate diverse developmental processes. The Arabidopsis *Bel1* mutants show a defect in ovule development, primarily in integument morphogenesis and identity (Robinson-Beers *et al.,* 1992; Modrusan *et al.,* 1994; Reiser *et al.,* 1995). As a consequence, female gametophyte development is arrested and mutant plants become female sterile. The expression analysis indicates that *BELL1* is expressed in different organs, such as flowers, seedlings, leaves and roots.

Homeodomain-leucine zipper (HD-Zip) proteins constitute a large plant specific family of transcription factors. The HD-Zip proteins differ from both homeodomain transcription factors of other eukaryotes and from other known homeodomain proteins in plants in that they contain a leucine zipper domain C-terminal to the homeodomain. The HD-Zip proteins are active as protein dimers in the binding of DNA, with the zippers juxtaposing two homeodomains onto the DNA. *HD-Zip* genes (*HD-Zip*) are present in a wide range of plants and act in developmental processes, including vascular tissue and trichome development, and several of them are involved in the mediation of external signals to regulate plant growth. The Arabidopsis *HD-Zip* (47

members) are grouped into four different classes *HD-Zip* I-IV, based on sequence criteria, and their functions investigated (Henriksson E., *et al.,* 2005; Johannesson *et al.,* 2003; Söderman *et al.,* 1996; Wang *et al.,* 2003).

KNOX GENES

Generalities: features, classification and expression patterns.

The *KNOTTED1* gene from maize (*ZmKN1*) was the first plant homeobox gene shown to have role in the SAM (Vollbrecht *et al.,* 1991). *ZmKN1* ectopic expression results in the formation of knot-like meristematic structures (from which the gene name derived) in the vicinity of leaf veins (Smith *et al.,* 1992; Sinha *et al.,* 1993). Loss-of-function studies demonstrated that *ZmKN1* is essential for shoot meristem formation and maintenance (Kerstetter *et al.,* 1997; Vollbrecht *et al.,* 2000). The *KNOTTED1*-like homeobox genes (KNOX) are found in all monocot and dicot species examined to date, and subsets of these genes regulate meristem function in all higher plants. Thus, *KNOX* likely represent ancient and conserved mediators of meristematic potential (Scofield and Murray, 2005).

KNOX proteins together with the BELL contain the atypical homeodomain with three extra amino acids lop extension between helix 1 and 2 (Bertolino *et al.,* 1995) and belong to the TALE superclass. KNOX are closely related to myeloid ecotropic viral integration site (MEIS) proteins of humans owing to a conserved N-terminus region. This domain, called MEINOX after KNOX and MEIS (Burglin, 1997), defines a subclass of the TALE that predates the divergence of fungi and metazoans from plants (see also the paragraph: KNOX proteins).

KNOX genes fall into two classes on the basis of aminoacidic identity within the homeodomain, gene expression patterns and intron position. Class 1 *KNOX* genes refer to *ZmKN1* as founder member in terms of sequence identities, are expressed in the SAM and down regulated in leaf primordia, and do not usually contain an intron nearby the ELK domain. Class 2 *KNOX* genes have distinctive amino acids in the helix 3 of the homeodomain, show widespread expression and harbour an intron upstream the ELK domain; they have yet undefined function in development (Serikawa *et al.,* 1997). The two classes appear to be conserved among both dicots and monocots (Kerstetter *et al.,* 1994; Bharathan *et al.,* 1999; Reiser *et al.,* 2000).

Roles in organ development.

In the chapter IV we investigated the role peach class 2 *KNOX* in the shoot apical meristem and during a few developmental stages of leaf, stem, flower and drupe. Below, the major functions of *KNOX* genes (mostly class1 members) in organ development are summarised.

Shoot apical meristem.

The loss-of-function mutants homozygous for the class 1 *KNOX* gene *STM* lacked characteristic cell divisions that generate the typical tunica-corpus organization, failed to establish a population of self-renewing stem cells and exhibited cotyledons with fused petioles and. *STM* is required for both the initiation and maintenance of SAM, and for defining the boundary between organs to prevent fusion (Vollbrecht *et al.,* 2000). The *stm* and *wus* (*WUSCHEL* gene) mutants are both characterised by the loss of meristematic identity (Laux *et al.,* 1996) but they define distinct meristem alterations: in *stm* meristems, cells are consumed into developing organs, whereas in *wus* meristems disorganised and non-meristematic structures are continually used to re-initiate transient shoot development (Endrizzi *et al.,* 1996; Laux *et al.,* 1996; Mayer *et al.,* 1998). Therefore, *STM* is required to prevent meristematic cells from adopting organ-specific fates, whereas WUS is involved in maintaining stem-cell potential. The ectopic *STM* and *WUS* activities regulate different downstream genes: the former triggers *KNAT1/BP* and *KNAT2* expression (which are class 1 *KNOX*) and prompts cell proliferation that generates leaf peripheral lobes, the latter activates *CLV3* (but not *KNAT1/BP* or *KNAT2*) and mildly enhances leaf cell proliferation (Lenhard *et al.,* 2002). However, the pathways regulated by *WUS* and *STM* appear to converge in the suppression of cell differentiation: the co-expression of both produced a synergistic effect while the increase of *WUS* activity could partly compensate for loss of *STM* function. These data suggest that *WUS* can induce meristem identity in the absence of *STM*, although *STM* is still required to maintain proper meristem cell numbers and organisation. Finally, two main pathways seem to operate for proper meristem organisation and its indefinite function: a) the *KNOX* pathway is required to suppress differentiation throughout the meristem dome, thus allowing stem cell daughters to be amplified before they are incorporated into organs, and b) the *WUS/CLV* pathway regulates stem cell identity/numbers in the CZ (Scofield and Murray, 2006).

KNAT1, KNAT2 and *KNAT6* genes are Arabidopsis class 1 *KNOX* and expressed in the shoot apex. Their single mutations do not produce SAM alteration; hence they are proposed to have redundant functions (Byrne *et al.,* 2000; Byrne *et al.,* 2002; Douglas *et al.,* 2002; Venglat *et al.,* 2002; Dean *et al.,* 2004). These KNOXs are likely to restrict the expression of the *ASYMMETRIC LEAVES1* (*AS1*) and *AS2* genes to organ primordia, and thus to prevent inappropriate leaf development at the shoot apex. In turn, *AS1* and *AS2* negatively regulate *KNOX* expression, excluding them from organ primordia. *AS1* and *AS2* encode transcription factors containing a Myb and a leucine zipper domain, respectively (Byrne *et al.,* 2000; Semiarti *et al.,* 2001; Byrne *et al.,* 2002). Reciprocal negative interactions between KNOX and Myb

factors were also found in maize (Timmermans *et al.,* 1999; Tsiantis *et al.,* 1999a) and snapdragon (Waites *et al.,* 1998).

Leaf development.

Many developmental processes participate in leaf ontogeny such as positioning and initiation of leaf primordia, specification of leaf identity, establishment of dorsiventrality, the control of cell division and expansion and pattern formation. Scientists have been trying to unravel the genetic circuit that underlies each of this process (Micol and Hake, 2003) and in this paragraph mostly class 1 *KNOX* specific roles are outlined (further information is also in the paragraph: regulatory and target genes of *KNOX*).

Shoot meristems produce leaves on their flanks in regular patterns (phyllotaxy) and leaf initiation involves a balance between cell proliferation and commitment to make primordia. Several Arabidopsis genes, such as *CLAVATA1*, *CLAVATA3*, *WUSCHEL*, *KNOTTED1*-like, play key roles in these processes. When expressed in the leaf primordium, however, these 'meristem' genes can profoundly affect leaf shape and size, possibly by regulating hormone gradients and transport (see also the paragraph *KNOX* and phytohormones). Leaves that have just appeared as bumps from the meristem are in plastochron 1 (P_1 leaves), those cells that will become the next leaf are named P_0. The P_0 cells are different from the adjacent sisters for they divide at higher rates, change the growth axis from isodiametric to axial, loose the indeterminate nature, and gain the leaf identity. The boundary that establishes the P_0 cells from the meristem is continually renewed and reinterpreted with every initiating leaf. In many plant species *KNOX* genes are expressed in shoot meristems but not in P_0 cells, consistent with the hypothesis that *KNOX* genes must be repressed to initiate a leaf (Tsiantis and Hay, 2003).

Classical analyses and molecular studies in Arabidopsis, indicate that the SAM produces anadaxial-promoting signal, the perception of which is likely to be mediated through the HD-ZIP proteins PHB PHV, and REV (genes: *Phabulosa*, *PHB*, *Phavoluta*, *PHV,* Revoluta, *REV*). Abaxial cell fate is likely to be the default state in the absence of the SAM adaxial signal, and the transcription factor *YABBY* and *KANADI* genes promote it. *YABBY* genes encode a zinc finger and helix-loop-helix motif and their expression is restricted to abaxial organ domains by the activity of *PHB* and related genes. *KANADI* genes are assumed to act upstream of the *YABBY* genes, but their precise relationships remain to be determined (Kerstetter *et al.,* 2001). In *A. majus* the *PHANTASTICA* gene (*PHAN*) has role in the promotion of organ asymmetry via *PHB*-like genes functions. In Arabidopsis the *PHAN* orthologues are the transcription factors genes *ASIMMETRIC LEAVES* (*AS1* and *AS2*). *AS1* and *AS2* repress *KNAT1* and *KNAT2*, but are negatively regulated by *STM* (see the paragraph on regulatory genes of *KNOX*). Moreover, the

SYMMETRICA (*SYM*) gene may function between the *AS1* and *KNOX* genes. The Arabidopsis *AS2* and *BLADE-LIKE PETIOLE* (*BLP*) genes are involved in the positioning of the central and proximodistal leaf axes and are required in the leaf for repression of *KNAT* genes. *AS2* encodes a nuclear-localized protein with Cys repeats and a Leu-zipper-like sequence and belongs to a novel TF family, named LOB after the expression of genes at lateral organ boundaries (Shuai *et al.,* 2002). Some of the genes involved in axis specification encode epigenetic regulators of gene expression. The *ARGONAUTE1* (*AGO*) is required for RNA-interference mechanisms, whereas mutations in *PICKLE* (*PKL*), which encodes a chromatin remodeling factor, enhance a dorsoventral defect in *crc* mutants.

Several experiments indicated that one of the *STM* roles is to exclude the *GA-oxidase5* gene expression supporting the hypothesis that *KNOX* genes negatively regulate gibberellic acid (GA) in the SAM, thus keeping cells from expanding and differentiating. GA acts antagonistically to *KNOX* genes in simple (Arabidopsis) and compound (tomato) leaves and the differential regulation of *KNOX* genes is likely involved in the generation of dissected leaf morphology (Hareven *et al.,* 1996; Janssen *et al.,* 1998).Mutants of the auxin transport (PAT) indicate that the determination of leaf shape and vascular patterning are interdependent processes. One role of auxin transport in meristems is to downregulate *KNOX* genes so that leaf initiation can occur. The auxin localization patterns in wild type leaves appear complementary to sites of ectopic meristem formation in *35S:KNAT1* leaves: ectopic shoots occur at the indentations of leaf margins, whereas auxin levels are highest at the lobes of the margins. Hence, auxin may modulate the downstream effects of *KNOX* genes on cell expansion and/or division patterns.

Most species with dissected leaves express *KNOX* genes, whereas those that have simple leaves do not. Species with a simple leaf expressing *KNOX* genes are exceptions, but they were shown to actually be dissected at leaf initiation. *KNOX* expression occurs along the leaf margins of young leaves, leading to the elaboration of the lobes and leaflets. The pea and Medicago spp. that have no longer a dissected leaf are due to mutations in *LEAFY* orthologs. *LEAFY* is an Arabidopsis gene that regulates the initiation of flowers from the inflorescence meristem. Dissected (wild type) leaves do not express *KNOX* genes, but instead express *LEAFY*. Hence, it appears that the phylogenetic clade that includes pea and *Medicago* gained dissected leaves, not by expressing *KNOX* genes, but expressing genes normally restricted to the floral meristems.

Reproductive meristems and flower organs.

The SAM and the floral meristems (FM) contain a stem cell reservoir at the apex that provides cells for the organogenesis occurring at the flanks. Both meristem types share regulatory genes

and mechanisms for the development and maintenance. In Arabidopsis, the class 1 *KNOXs* show distinct expression patterns during reproductive growth, though their transcripts are always excluded from founder cells and from the incipient floral meristems.

The Arabidopsis *STM* is expressed throughout the apical region of the FM, but not in the basal domain that corresponds to the cryptic bract (Long *et al.,* 1996; Long and Barton, 2000). Later, *STM* expression is restricted to the centre of the developing flower (carpel) as the outer floral organs are initiated in concentric whorls. *STM* activity persists in the placentae of the two central carpels and is excluded from initiating ovules just as in leaf and floral meristem inception (Long and Barton, 2000). In the mild *stm-2* mutant, the reproductive SAM terminates in flowers that lack a central gynaecium (Clark *et al.,* 1996; Endrizzi *et al.,* 1996). Hence, *STM* is also required for reproductive SAM maintenance and likely for carpel development (Scofield and Murray, 2006).

The *KNAT2* and *STM* expression pattern are similar during flower development, suggesting that they have overlapping and/or redundant roles (Pautot *et al.,* 2001). Interestingly, *KNAT2* overexpression produces ectopic carpels and carpelloid organs and causes the conversion of ovules to carpels (Pautot *et al.,* 2001), although *KNAT2* is not required for carpel development in wild type flowers (Byrne *et al.,* 2002).

KNAT1/BP has a distinct role from those of *STM* and *KNAT2*. The transcript is not detected in the reproductive SAM, but localises to the cortical tissues of the stem, floral pedicels and to the marginal tissues and style of the gynaecium (Lincoln *et al.,* 1994). In *knat1/bp* loss-of-function mutants (*KNAT1* is also called *BREVIPEDICELLUS*, *BP*) flowers point downward and show reduced radial growth. These abnormalities are caused by reduced cell division and impaired differentiation. The bp mutation in a Landsberg erecta (Ler) background enhances the aberrations of inflorescence architecture, suggesting that *KNAT1/BP* and the *ERECTA* functions co-participate and/or overlap in the floral internode and pedicel growth (Douglas *et al.,* 2002; Douglas and Riggs, 2005). The effects of bp indicate that its function is critical for normal pedicel and inflorescence development, but not in carpel development for *KNAT1*/*BP* over-expression does not cause any ectopic carpel formation (Lincoln *et al.,* 1994; Chuck *et al.,* 1996; Pautot *et al.,* 2001).

In the end, Arabidopsis *KNOX* genes appear to be functionally redundant and are exploited to confer an undifferentiated identity upon specific populations of cells, followed by augmentation with additional factors to generate vegetative (SAM) or reproductive (carpel) stem cell niches.

Secondary growth in woody plants.

There is a body of evidence sustaining that the SAM and the vascular cambium share major genetic regulatory mechanisms. (Scofield and Murray, 2006). In Arabidopsis, *KNAT1/BP* and *STM* promote stem cell fate in the cambium during secondary growth, beyond their established roles in SAM fate (Ko and Han, 2004). The *knat1/bp* mutants exhibit: short internodes and pedicels, siliques pointing downward (Douglas *et al.,* 2002, Venglat *et al.,* 2002); precocious lignification and lignin content increased, accompanied by raised activity of major genes involved in cell wall and lignin biosynthesis. Conversely, plants over-expressing *KNAT1/BP* showed a decrease in cell wall lignification. Furthermore, the protein KNAT1/BP directly binds to the promoters of genes encoding lignin biosynthetic enzymes. Taken together, these data strongly suggest that *KNAT1*/*BP* plays a role in down regulating genes involved in lignification (Mele *et al.,* 2003).

KNOX genes participate in xylogenesis of hybrid aspen. In fact, a poplar *KNAT1*/*BP* ortholog was expressed across the cambium and expansion zones of secondary xylem and then repressed in sectors of maturation (Hertzberg *et al.,* 2001). In *Populus tremula*, the *STM* ortholog mRNA localised to the cambium and was proposed to regulate other *KNOX* genes in maintaining the identity of cambium cells. The *STM*-like gene *ARK1* of *P. trichocarpa* x *P. alba* was also active in the cambium and its over-expression in poplar lead to phenotypes with a low lignin content and altered xylem by acting on genes involved in the stem secondary growth (Groover, 2005). These data suggest that the down-regulation during fibre differentiation is necessary for the induction of secondary cell-wall synthesis, similarly to the function of *KNOX* genes in the apical meristem when lateral organs are initiated (Hertzberg *et al*., 2001).

Regulatory and target genes of *KNOX* genes.

Class 1 *KNOX* genes are down regulated in leaf founder cells within the meristem that marks a change in cell fate from meristem to leaf (Chuck *et al.,* 1996). The MYB-TF *PHANTASTICA* (*PHAN*) of snapdragon, *ROUGH SHEATH2* (*RS2*) of maize and *ASYMETRIC LEAVES1* (*AS1*) of Arabidopsis are repressors of *KNOX* genes and expressed in patterns complementing those of *KNOX* genes (Waites and Hudson, 1995; Schneeberger *et al.,* 1998; Tsiantis *et al.,* 1999a).

In Arabidopsis, single or double recessive *as1* and *as2* mutants result in leaf morphological abnormalities accompanied by the ectopic expression of *KNAT1*/*BP*, *KNAT2* and *KNAT6*, but not of *STM* (Byrne *et al.,* 2000; Byrne *et al.,* 2002), suggesting that *STM* suppresses *AS1* and *AS2*, which down regulate the other *KNOX* genes (Byrne *et al.,* 2000). Moreover, *AS1* and *AS2* mutually interact to form complexes and may regulate the same downstream targets

(Lin *et al.,* 2003). Although the Arabidopsis *phan* mutants differ from *as1* (lateral organs are abaxialized and loss of radial symmetry occurs), they display endogenous *KNOX* ectopic expression in leaves. PHAN protein was proposed to delimit the borders of *KNOX* expression (Waites and Hudson, 1995; Waites *et al.,* 1998). The Arabidopsis *YAB3* and *FIL* (*YABBY* family) induce abaxial fate in the leaves cells (Siegfried *et al.,* 1999) and the double *yab3 fil* mutants forming ectopic meristems on the lateral organs trigger *STM*, *BP* and *KNAT2* mis-expression (Kumaran *et al.,* 2002). *AS1* and *PHAN* are alleged to repress homeobox genes in plants with a mechanism similar to those of animal Polycomb-group genes. These modify chromatin structure and define the "cellular memory" of the differentiated state. In support of this, transgenic tobacco plants expressing a Drosophila polycomb chromodomain show alterations in leaf shape that resemble *as1*, *rs2* and *phan* mutant phenotypes (Ingram *et al.,* 1999). The Arabidopsis *PICKLE* and *SERRATE* also play a role in *KNOX* regulation by epigenetic mechanisms. *PICKLE* encodes a chromatin re-modelling factor and forms a complex with histone deacetylases (Ogas *et al.,* 1999). *SERRATE* encodes a zinc finger protein (Luo *et al.,* 1999) and acts in a microRNA gene-silencing pathway to a) regulate expression of the *HD*-Zip III *PHABULOSA* (PHB) and b) limit the competence of shoot tissue to respond to *KNOX* expression (Grigg *et al.,* 2005).

Recently, RNA silencing mechanisms were also found to regulate Arabidopsis *KNOX* genes: both the *RNA-DEPENDENT RNA POLYMERASE6* (*RDR6*) and *ARGONAUTE1* (*AGO1*) play a role in the process. *RDR6* is a key component for plant post-transcriptional gene silencing (PTGS), and likely to regulate leaf patterning. *rdr6-as1* and *rdr6-as2* double mutants exhibited more severe defects in the leaf adaxial-abaxial polarity and vascular development, more intensely lobed leaves and ectopic expression of *BP* than the *as1* and *as2* single mutants. Higher levels of microRNA165/166 also occurred and may lead to mRNA degradation of III *HD-ZIP* genes. Taken together, these data suggest that *AS1-AS2* pathway and the *RDR6*-associated epigenetic pathway are both required for repression of *BP* (and *MIR165*/*166*) in normal leaf development (Li *et al.,* 2005). The Arabidopsis *AGO1*-like genes are also crucial in PTGS and microRNA and trans-acting siRNA pathways (Baumberger *et al.,* 2005). *AGO1* mutations lead to defective miRNA functions, affecting the expression of several target genes (Kidner and Martienssen, 2005) and inducing pleiotropic morphological defects in plant architecture. The *AGO1-37* protein interacts genetically with *AS1* and *AS2* in repressing class 1 *KNOX* in developing leaves and in promoting the juvenile leaf fate and specifying both leaf and petal adaxial and abaxial identities (Yang *et al.,* 2006).

Relationships between *KNOX* genes and phyto-hormones.

Proper SAM function requires the maintenance of a delicate balance between the production of lateral organs from its flanks and indeterminate growth at its centre. In definition of this balance, the cooperation between phytohormones and *KNOTTED1*-like transcription factors has primary role.

As for cytokinins, transgenic lines of distinct plant species (eg.: tobacco, maize, lettuce) overexpressing class 1 *KNOX* exhibited a higher cytokinin content than the wild types, and leaf senescence was delayed (Kusaba *et al.,* 1998a; Ori *et al.,* 1999; Frugis *et al.,* 2001). These data suggest a role of KNOX in regulating cytokinin biosynthesis. Conversely, transgenics plants over-expressing the prokaryotic cytokinin biosynthetic enzyme isopentenyltransferase (*ipt*) produce phenotypes resembling those of *KNOX* overexpression (Sinha *et al.,* 1993) and induce ectopic accumulation of *BP* and *STM* transcripts (Rupp *et al.,* 1999). These data suggest that cytokinin may act upstream *KNOX* genes. In summary, a mutual interaction with feedback-loop mechanisms is proposed to occur between cytokinin and *KNOX* transcription (Hay *et al.,* 2004).

With regard to gibberellins, *KNOX* are likely to down tune the gibberellin (GA) content (Kusaba *et al.,* 1998b; Rupp *et al.,* 1999; Hay *et al.,* 2002). The over-expression of tobacco *NTH15* (a *STM* ortholog) altered tobacco leaf morphology, and suppressed the transcription of giberellic acid 20-oxidase gene (*GA20ox*), which lead a decrease in bioactive GA levels. In wild type plants, *NTH15* mRNA occurs in SAM corpus cells, whereas *Ntc12* transcript occurs in leaf primordia and rib meristem but not in the corpus, indicating that the expression patterns of these two genes is complementary. *NTH15* directly represses *Ntc12* expression in the corpus by binding to a specific sequence in the first intron of Ntc12 so as to maintain the indeterminate state of meristematic cells (Sakamoto *et al.,* 2001). In Arabidopsis *stm* mutants, the *AtGA20ox1* was expressed at high levels in the normal domain of *STM* expression, supporting the idea that *STM* excludes GA biosynthesis from the SAM and that a reduced GA regime is favourable for meristematic activity. In addition, constitutive GA signalling enhanced weak *stm* phenotypes, resulting in shoot-less plants that fail to form a meristem (Hay *et al.,* 2002; Hay *et al.,* 2004). Overall, the repression of GA activity by *KNOX* is a key component of meristem function: low GA levels may allow random cell division patterns in the SAM corpus, while high GA levels induce an ordered arrangement of cell divisions that favour determinate growth in the leaf (Sakamoto *et al.,* 2001). Moreover, it was also speculated that the transfer of the KNOX/GA regulatory module from the meristem to the leaf may have contributed to the generation of the diverse leaf morphologies observed in higher plants (Hay *et al.,* 2004).

Concerning auxin, it accumulates in leaf founder cells at the flanks of the SAM where *KNOX* genes are down regulated, suggesting antagonism between the two (Reinhardt *et al.,*

2000). This is indirectly demonstrated by the occurrence of reduced apical dominance in plants over-expressing *KNOX* genes, which implies a decrease of auxin concentration at the shoot apex. The phenotypes of Arabidopsis pinformed1 mutant (with incorrect auxin transport into leaves) and maize seedlings treated with inhibitors of polar auxin transport (PAT) were similar to class 1 *KNOX* over-expressing mutants (Hay *et al.,* 2004). The maize PAT inhibitors treated shoots failed to initiate new leaves associated to the failure of *KNOX* down-regulation in the SAM. PAT inhibitors-cultured shoots formed abnormal leaf with ectopic expression *KNOX* genes. Subsequent transfer of PAT inhibitors-cultured apices to PAT inhibitors-free media resulted in the resumption of leaf initiation from the SAM and the restoration of normal expression patterns of *KNOX* (Scanlon, 2003).The wild-type maize seedlings grown on PAT inhibitors phenocopy the rs2 mutants, which accumulate inappropriately at least three *KNOX* genes in leaves and display decreased polar auxin transport in the shoot (Tsiantis *et al.,* 1999b). Taken together, these data suggest that the proper leaf initiation is correlated with auxin transport acting on *KNOX* down-regulation.

Responses to biotic and abiotic factors.

Biotic factors.

Micro-organisms (MO) and plant tissues establish relationships that lead to symbiotic or pathogenic interactions and plants have evolved recognition systems to discriminate "friends from foes". The timely recognition of a pathogen infection and the rapid activation of the plant defence response is a fundamental mechanism of resistance. Pathogen-induced signal transduction pathways converge into plant nucleus, where defence-related genes are activated (Rushton and Somssich, 1998). Pathogen-induced low molecular weight molecules, including salicylic acid, jasmonic acid, ethylene and reactive oxygen species, act as signals to trigger a variety of plant pathways (Reymond and Farmer, 1998), which result in: hypersensitive response (HR), enhancement of preformed barriers and de novo synthesis of pathogenesis-related (PR) proteins encoded by the defense-related genes (Somssich and Hahlbrock, 1998). The HR is a whole process of defence response including: recognition of pathogens, host cell death, accumulation of antimicrobial compounds and inhibition of pathogen growth. The HR cell death is a type of programmed cell death (PCD) and in the resistance gene-activated PCD system, a single gene in the host (the *R* gene) confers resistance only to those pathogen isolates containing a cognate avirulence gene (*Avr*) (Flor, 1971).This 'gene-for-gene' type resistance is sustained by an elicitor-receptor model: the plant R protein recognizes directly or indirectly the pathogen-derived Avr product.). In absence of complementarities, due to the loss/mutation of either plant

R or pathogen Avr, the plant becomes sick (compatible reaction). Rapid host cell death would explain resistance to different classes of pathogens, for it may both deplete nutrient supply to biotrophs and arrest ingress of necrotrophs via release of toxic molecules. Transcriptional regulation of defence gene expression is mediated by changes in the activity of DNA-binding transcription factors (TF). TF contribute to the regulation of the plant defence response, including the up-regulation of the PR-genes, through recognition of specific DNA sequences in the promoter region (Rushton and Somssich, 1998). Several TF are reported to participate in defence response: Myb, ethylene-responsive-element binding factors (ERF), basic-domain leucine-zipper (bZIP), WRKY, homeodomain proteins and zinc fingers proteins. The Arabidopsis *Atmyb30* is induced by an avirulent strain of *Xanthomonas campestris* pv. *campestris* and related to the PCD in lesion mimic mutants (Daniel *et al.,* 1999). Tomato and tobacco ERF genes were induced after bacterial, virus and fungal infections (Zhou *et al.,* 1997; Horvath *et al.,* 1998; Durrant *et al.,* 2000). A of bZIP regulates the expression of *PR-1* (Lebel *et al.,* 1998) and *Glutathione S-transferase 6* genes (Chen and Singh, 1999). Plant WRKY DNA-binding proteins recognize various W box elements present in the promoters of many defence related genes (Rushton and Somssich, 1998) and are up-regulated in response to pathogen infection (Eulgem *et al.,* 2000; Yu *et al.,* 2001; Shinozaki and Yamaguchi-Shinozaki, 2000).

Root-knot nematodes and rhizobia establish pathogenic and symbiotic interactions with plant roots which are characterized by the de-novo induction of host structures, termed giant cells and nodules, respectively. In *Medicago truncatula*, *PHAN* and *KNOX*, required for the meristem establishment, were expressed in lateral root initials upon nematode infection, in nematode-induced giant cells and in rhizobia induced nodules. (Koltai et al, 2001). These results reveal common elements of host responses to endosymbionts and parasites and suggest that overlapping regulatory pathways lead to giant cells and nodules. MO are known to alter plant hormone balance by direct production of phyto-hormone like substances or by inducing the transcription of plant hormone endogenous genes (Maor and Shirasu, 2005). In this scenario, the role of *KNOX* in the regulation plant response mechanisms becomes relevant in those MO-plant interactions (pathogenic, resistant or symbiotic processes) that involve the reactivation, direct or indirect, of cell cycle-correlated genes and the alteration of host hormonal levels.

Abiotic factors.

Developmental plasticity in response to environmental and physiological conditions is a unique feature of plant development and is one of the most important current targets of studies of mechanisms that control plant development. Although the regulation of *KNOX* genes by abiotic factors is still largely unknown, some works highlight how the different light conditions may play a role in the modulation *KNOX* expression. Serikawa and colleagues (1997) showed that

Arabidopsis *KNAT3* and *KNAT4* were light-regulated as they show reduced expression in etiolated seedlings. GUS fusions assays with *KNAT3* promoter revealed that its expression is induced in cotyledons, upper hypocotyl and roots by continuous white light whereas it was reduced in those same tissues by continuous red light (Serikawa *et al.*, 1997). Interestingly, continuous far-red light was able to induce expression stronger than that in red light-grown plants in cotyledons, upper hypocotyl but not in roots. The data indicate that *KNAT3* promoter responds differently to red and far-red lights and that *KNAT3* transcription was regulated by more than one light perception pathways. Red light is primarily sensed through phytochrome B and far-red light through phytochrome A (Parks and Quail, 1993; Quail *et al*., 1995). *KNAT3* expression analyses in phytochrome mutant backgrounds (*phyA* or *phyB*) revealed role for phytochrome in regulation of light-mediated *KNAT3* expression (Serikawa *et al.*, 1997)

Protein-protein interactions and movement in the plant cell.

Domain features.

KNOX contain the conserved MEINOX and ELK domains reside upstream the homeodomain (HD) (Sakamoto *et al.,* 1999; Nagasaki *et al.,* 2001), which is involved in the DNA binding. TALE proteins contain the highly conserved WFXN within the third helix of HD, in which the X position is critical for DNA-binding specificity (Treisman *et al.,* 1989). The HD of KNOX contain the WFIN motif, likewise to HD of vertebrate MEIS (Myeloid ecotropic viral integration site) homeodomains proteins, that recognizes the TGACAGG/CT motif. Both STM and KNAT1 specifically associate to the TGACAGG/CT motif in-vitro (Hake *et al.,* 2004). The ELK domain (named after the first three amino acid that form it: E, glutammate; L, leucin; K, lysine) creates an amphipathic helical region, which includes two nuclear localization signals (Meisel and Lam, 1996). The ELK is alleged to be necessary for protein-protein interaction. In the N-terminal half, the MEINOX includes the conserved KNOX1 and KNOX2 sub-domains, separated by a spacer (Burglin TR, 1997) (Fig. 2). The KNOX1 contributes to generate phenotype alteration in KNOX over-expressing Arabidopsis, and is proposed to have a repressive effect on target gene transcription. The KNOX2 is also essential for the generation of abnormal phenotypes in KNOX-transgenics and necessary for dimer formation and trans-activation (Sakamoto *et al.,* 1999). The MEINOX is similar to human MEIS proteins (MEINOX = MEIS+KNOX), which dimerises with the PBX proteins of the TALE superclass (Burglin *et al.,* 1998). KNOX also contain a region of amino acids enriched in proline (P), glutamic acid (E), serine (S) and threonine (T), called PEST sequences (Vollbrecht *et al.,* 1991) which are proposed to signal and

promote protein degradation via ubiquitin-mediated proteolysis (Rechsteiner and Rogers 1996, Rogers *et al.,* 1986).

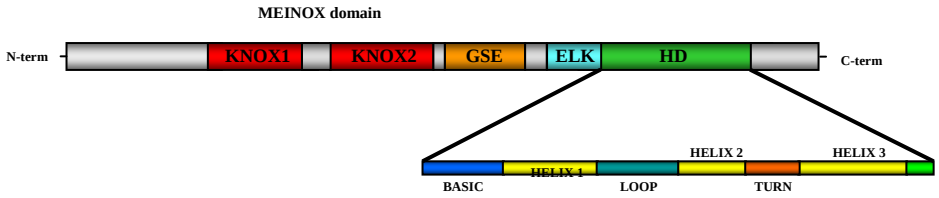

Figure 2 – Schematic representation of a typical KNOX protein.
The MEINOX domain featured the N' terminus half of KNOX proteins and it is formed by two conserved sub-domains (**KNOX1** and **KNOX2**) which are critical for protein-protein interactions. The predicted amphipathic helical region of the **ELK** domain includes two nuclear localization signals and could also act as a protein-protein interaction domain. The homeodomain (**HD**) is composed of three α helices arranged in a helix-loop-helix-turn-helix motif. The third α-helix binds the major groove of the double helix of the DNA of gene targets, whereas the other two helices regulate the juxtapositions of the third helix and in the maintenance of structure stability.

Partners of interaction.

The whole MEINOX mediates the interactions (and selective-specificity) with BELL (Muller *et al.,* 2001; Smith *et al.,* 2002; Chen *et al.,* 2003). It recognises a conserved bipartite domain in the N-terminus of BELL, called MEINOX interacting domain (MID), resulting in the formation of KNOX-BELL heterodimer transcriptional unit (Hake *et al.,* 2004). These complexes occur in both monocots and dicots (Bellaoui *et al.,* 2001; Muller *et al.,* 2001), are essential for normal KNOX function in the SAM (Smith and Hake, 2003). KNOX-BELL relationships are selective: each KNOX protein binds to only a subset of BELL proteins. In Arabidopsis, PENNYWISE (PNY) and POUND-FOOLISH (PNF) are expressed in the SAM and encode BELL proteins, which are essential for specifying floral primordia and establishing early internode patterning events during inflorescence development (Smith *et al.,* 2004). PNY associates with both STM and KNAT1, whereas PNF has the strongest interaction with STM. PNY poorly interacts also with KNAT2 (Smith and Hake, 2003) and in no way with KNAT4 (Byrne *et al.,* 2003) and KNAT3 (Bellaoui *et al.,* 2001). PNY-STM, PNY-BP and PNF-STM regulate early internode patterning events (Byrne *et al.,* 2003; Roeder *et al.,* 2003; Smith and Hake, 2003). PNY-STM and PNF-STM control floral specification and maintain boundaries between the inflorescence meristem and initiating floral primordia. (Smith and Hake, 2003). Finally, KNOX-KNOX associations are also documented: contrary to KNOX-BELL interactions, KNOX homo- and

heterodimers require both the homeodomain and MEINOX (Muller *et al.,* 2001; Nagasaki *et al.,* 2001; Hake *et al.,* 2004). The significance of these interactions is still unknown.

Cell trafficking.

The intercellular movement of regulatory proteins comprise phloem proteins as well as several transcription factors (Balachandran *et al.,* 1997) and is a necessary mechanism for cell-to-cell communication during plant development (Zambryski and Crawford, 2000; Jackson D, 2002; Haywood *et al.,* 2002).

KNAT1 and STM traffic in the Arabidopsis SAM, and the maize KN1 moves from epidermal (L1) cells to underlying cells in the inflorescence SAM, suggesting that hypothetical signal(s) for trafficking are conserved. The SAM trafficking of fluorescently labelled KNOX (GFP-KNOX) has a short range, in fact a steep gradient of GFP fluorescence spans 2-3 cell layers. However, GFP-KN1 can also traffic over 3-5 cell layers in the leaf. Therefore, the KNOX movement in the meristem appears to be restricted and used for short range signalling. KN1 moves unidirectionally from leaf mesophyll to epidermis but not from epidermis to mesophyll. Recently, the set up of novel assays (Kim *et al.,* 2005) allowed to establish that only the HD regions of closely related class 1 KNOX (Arabidopsis STM, KNAT1, or tomato LeT6) contain movement signals.

Finally, the HD of KN1 can directly interact with its mRNA and then acting as a mediator for selective cell-to-cell mRNA trafficking. The proposed interaction between KN1 and its mRNA could be mediated at the sequence level through binding of the HD to specific sequences in the KN1 mRNA (Kim *et al.,* 2005).

SCOPE OF THE THESIS.

The architecture of aerial organs and the vegetative habit of fruit trees are important traits for productivity and quality. Since the determinism and variability of these traits are highly likely to be under *KNOX* control, the knowledge on the *KNOX* features and functions allows their use: a) as markers for assisted breeding and b) in biotechnological approaches to manipulate genetically traits of agronomical interest. Contrary to class 1 *KNOX* genes, class 2 members have been poorly investigated in both plant models and crop species. Interestingly, class 2 *KNOX* genes, but not class 1 members, are detected in peach EST banks during several stages of drupe development, suggesting they may specifically play a role. Peach is a model species for the *Drupoideae* and the knowledge acquired at the molecular level is consequently exported to almond, apricot, plum and fruit and timber cherry.

In this scenario, the major aims of this thesis can be outlined as follows:

- Cloning and characterization of class 2 *KNOX* genes active in the development of aerial organs of peach tree.
- Comprehension of the roles of the peach *KNOPE3*, a putative Arabidopsis *KNAT3* ortholog, in stem, leaf, flower and fruit development and in leaf response during the course of the curl disease.
- Positioning of class 2 *KNOPE3* and *KNOPE4* on the *Prunus* genetic map and production of specific polymorphic markers effective in marker-assisted selection of fruit tree breeding.

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CHAPTER II

Genomic features, organisation and mapping of *KNOPE 3* and *KNOPE 4*

(With: Giulio Testone, Ignazio Verde, Domenico Mariotti, Maria Beatrice Bitonti and Donato Giannino)

Summary

The genes *KNOPE3* and *KNOPE4* were sequenced, including the intron-exon regions, and major nucleotide signals and features identified. Peach class 2 *KNOX* genes constituted a family of 3-4 estimated members and *KNOPE3* was highly likely to be a single copy gene in the cultivar Chiripa. The intron positions (but not the intron sizes and the identities) of both genes were conserved with respect to those of Arabidopis *KNAT3* and *KNAT4*. Interestingly, the intron II of *KNOPE4* harbored a stretch putatively recognized by class 1 *KNOX* genes. Cleavage amplified polymorphism (CAP) markers were produced specifically for each of the two genes and the study of CAP segregation in a *Texas* x *Earlygold* F_2 population indicated that *KNOPE3* mapped on linkage group 1 at 71 cM and *KNOPE4* on linkage group 7 within 62 66 cM of the *Prunus* reference map.

INTRODUCTION

The *KNOX* genes fall into two classes, distinguished by sequence similarities, intron position, and expression patterns (Kerstetter *et al.*, 1994; Reiser *et al.*, 2000; Bharathan *et al.*, 1999). Class 1 and 2 *KNOX* genes were found in angiosperms, gymnosperms (Sundas-Larsson *et al.*, 1998), ferns and bryophytes (Champagne and Ashton, 2001) and in green algae (Serikawa and Mandoli, 1999; Hake *et al.*, 2004). They are thought to have a monophyletic origin and class 1 and 2 duplication probably occurred ca. 400 million years ago, before divergence of the non-vascular plants (*Bryophytes* and hornworts) from the *Tracheophyta* and after the land plant lineage formation. Duplication and divergence have resulted in the formation of multiple class 1 and 2 *KNOX* genes in flowering plants (Bharathan *et al.*, 1999, Reiser *et al.*, 2000).

KNOX make relatively small gene families, varying from 8 to 13 members in Arabidopsis and maize, which have the best characterized *KNOX* (Kerstetter et al., 1994). As for non-coding regions, promoters have been characterized for several class 1 *KNOX*, such as *STM* and *BP* (Hake *et al.*, 2004), and class 2 *KNAT3*, *KNAT4* and *KNAT5* (Serikawa *et al*., 1997; Truernit *et al*., 2006). In addition, one intron was strictly conserved within the homeobox domain of all *KNOX* members, falling near the N-terminal end of the second helix. Moreover, a small intron constantly features in the region immediately upstream the ELK of class 2 *KNOX* genes, allowing further distinction of class 1 from class 2 members (Kerstetter, 1994). Introns of class 1 *KNOX* were demonstrated to contain sequences necessary for the correct regulation of transcription (Muller et al., 1995; Hake et al., 2004). Some rice class 2 *KNOX* members intronic regions were shown to be alternatively transcribed in distinct tissues (Tamaoki *et al*., 1995). Finally, *KNOX* genes of maize, rice, and Arabidopsis (for these species check in the GenBank database), tomato (Hareven *et al.*, 1996), pea (Hofer *et al.*, 2001), and conifers (Guillet-Claude *et al*., 2004) have been located onto physical and genetic maps.

RESULTS

Features of class 2 *KNOPE3* and *KNOPE4* genes.

KNOPE3 (DQ786755) and *KNOPE4* (EF107110) full-length cDNAs were 1895 and 1342 nt long, the former encompassed an open reading frame (ORF) of 1347 nt, the latter an ORF of 1254 nt. Very short stretches in the 5'UTR of both genes were rescued and no typical regulatory signals were found (e.g.: the Kozak or Shine-Dalgarno sequences). The *KNOPE3* 3'UTR included a canonical poly-adenylation signal (AATAAA, 1835-1840), a U-rich stretch

(TTTTCT, 1805-1809) followed by a cleavage site (CA, 1817-1818) and terminal U-rich site (1862-1866), which are usually located upstream the poly A tail of many plants (Graber *et al*., 1999). Interestingly, a K-Box occurred (TGTGAT, 1430-1435), which regulates RNA mediated gene down-regulation in Drosophila (Lai *et al*., 1998) (Fig. 1). Conversely, no standard 3'UTR signals appeared in *KNOPE4*, and the AAGTAA (1279-1284) is proposed as possible functional poly-A signal (Graber et al., 1999) (Fig. 2). By performing PCR on genomic DNA with primer couples designed along the *KNOPE3* and *KNOPE4* cDNAs, introns (Fig. 1 and 2, respectively) were identified within the ORFs of both genes. Both *KNOPE3* and *KNOPE4* harboured 5 introns, which conserved the same positions as those of Arabidopsis *KNAT3* (Fig. 3). The intron I and II fell in the KNOX2 sub-domains (QFM-THY and LTG-VSP), the intron III in the ELK domain, the IV in the homeodomain and the V was upstream the stop codon. The I-V introns of *KNOPE3* differed in sizes and sequence identities as compared to the respective I-V introns of *KNOPE4*. They were all rich in AT content and followed canonical cleavage consensuses. The nucleotide identity of each intron ranged around the 10% (only introns III were 27% identical). Interestingly, a DNA motif (TGACAGGT, pos: 1344 on**)** recognized by class 1 KNOX proteins was found in intron II of *KNOPE4* (Smith et al., 2002). Finally, *KNOPE3* and *KNOPE4* cDNAs were 54% identical, whereas their genomic sequences were just 24 % identical.

KNOPE3

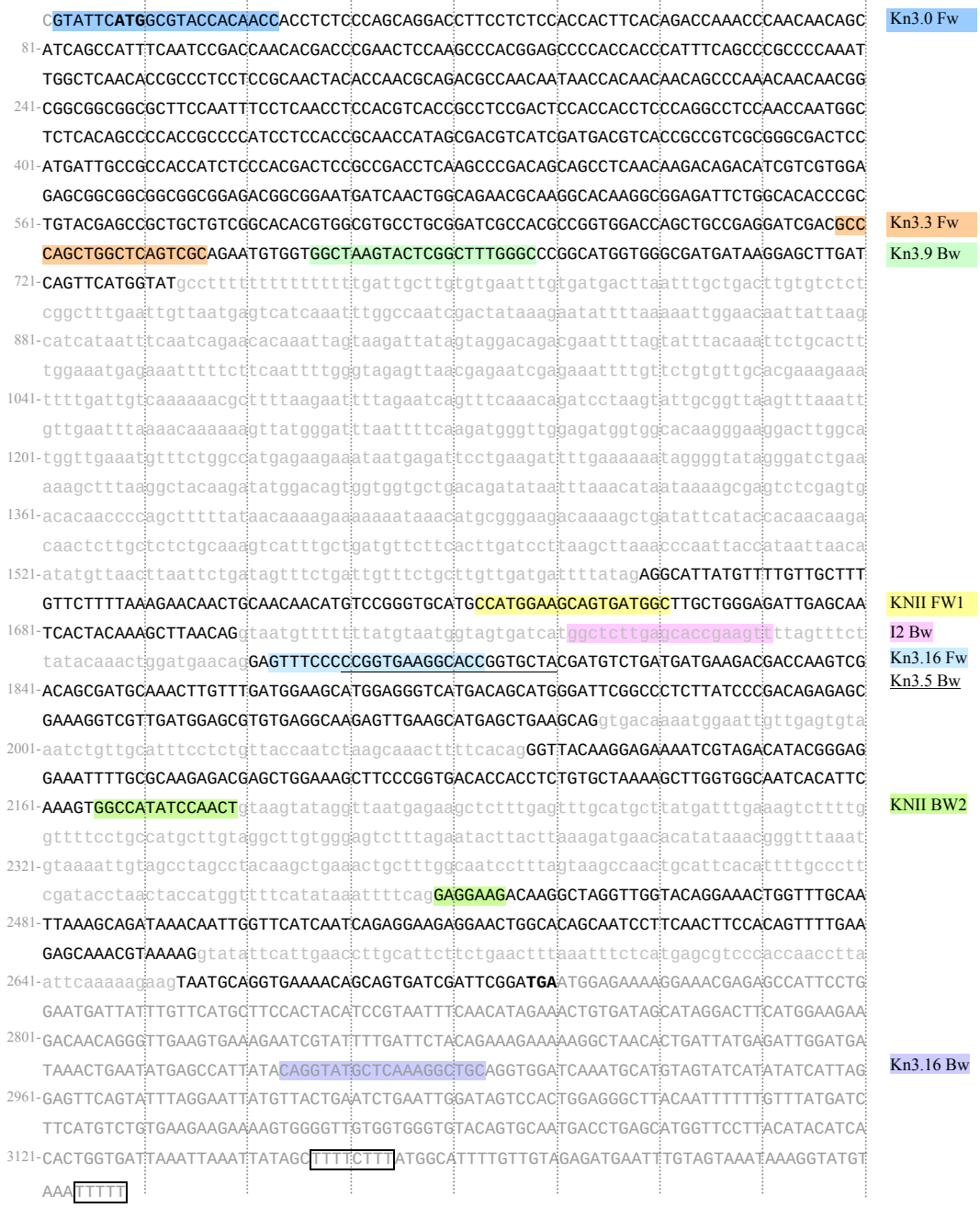

Figure 1. The nucleotide sequences of the *KNOPE3* gene. Nucleotide sequence of *KNOPE3* genomic DNA is shown. Numbers at left side show nucleotide positions. The primer name, used for PCR to amplify the gene, and the corresponding sequence are coloured. The small letters indicate intron sequence; grey capital letters are 5' and 3' UTR. Canonical poly-adenylation signals are boxed.

KNOPE4

Figure 2. **The nucleotide sequences of the *KNOPE4* gene.** Nucleotide sequence of *KNOPE4* genomic DNA is shown. Numbers at left side show nucleotide positions. The primer name, used for PCR to amplify the gene, and the corresponding sequence are coloured. The small letters indicate intron sequence; grey capital letters are 5' and 3' UTR. DNA motif recognized by class 1 KNOX proteins is boxed. The alternative spliced sequence is double underlined.

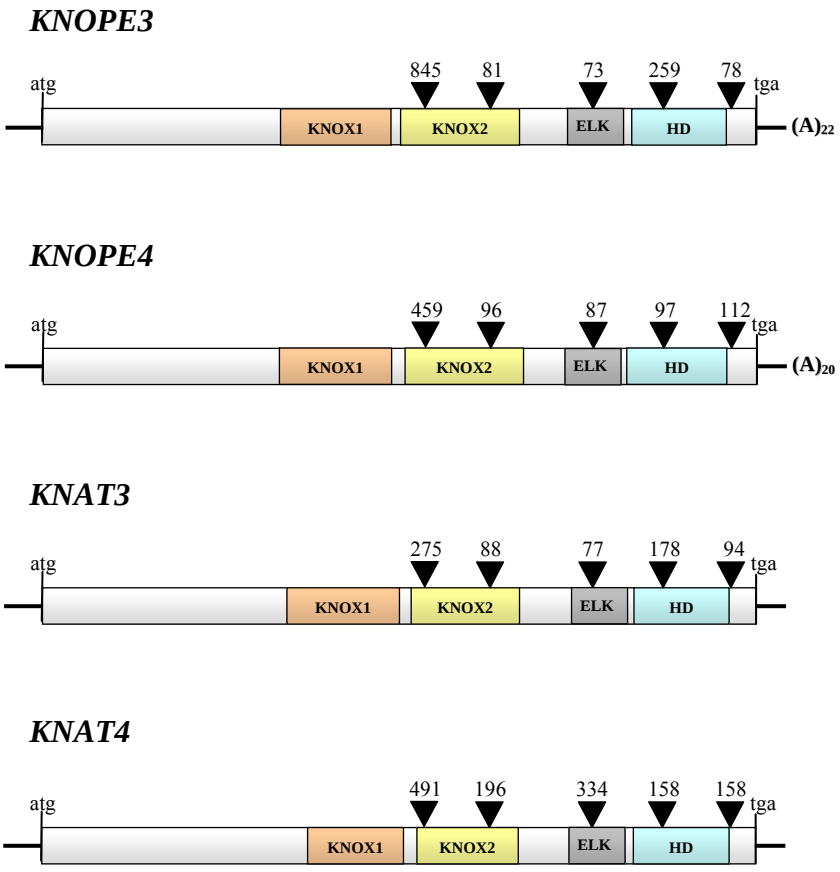

Figure 3. Intron position of class II *KNOPEs*. Introns position of *KNOPE3* and *KNOPE4* matched exactly with those of other class II genes, such as *KNAT3* and *KNAT4* from *A. thaliana*. Black triangle and number indicates intron position and length, respectively.

Class 2 *KNOX* belong to a small member family in Chiripa.

Southern blot analysis was first carried out with a *KNOPE3* cDNA probe (Fig. 4A) 76% identical to *KNOPE4* and spanning a highly conserved region of plant class 2 *KNOX*. The signal pattern suggested the occurrence of two or three class 2 *KNOX* members in the cultivar Chiripa (Fig. 4B, left panel). Subsequently, the DNA was hybridised at high stringency with a *KNOPE3* genomic probe (Fig. 4A), encompassing introns II-V (identity with *KNOPE4* ca. 40%) and one Hind III cleavage site. A single band was observed (Fig. 4B, right panel) when genomic DNA was digested with EcoRI, EcoRV and XbaI, whereas two signals occurred with Hind III and the ca. 450 nt band was consistent with the size predicted by restriction analysis (Fig. 4A). This pattern indicated that *KNOPE3* was in single copy.

***KNOPE 3* and *KNOPE4* position on *Prunus* reference map.**

KNOPE3 and *KNOPE4* were mapped to specific linkage groups (LG) by scoring the segregation pattern of cleavage amplified polymorphism (CAP) markers in the TxE population (Joobeur et al 1998). Primer pairs were designed on the sequences of *Chiripa KNOPE3* and *KNOPE4*, encompassing introns so as to maximize the chances of polymorphism occurrence (Fig. 7A and 8A). Two *KNOPE3* primers (see the material and methods) yielded a PCR product of ca.900 nt, using T, E and F_1 genomic DNA, which were cloned and sequenced. HincII only cut in the Earlygold fragment, generating a polymorphic signal patterning (Fig. 7B). Similarly, a 950 nt stretch of *KNOPE4* was amplified and a CAP for Earlygold was achieved by HinfI digestion (Fig. 8B). Fig. 5 and 6 summarises all the nucleotide differences for potential markers within the amplified regions of *KNOPE3* and *KNOPE4*. Overall, 19 nucleotide differences and 5 IN/DEL were scored in *KNOPE3* fragments of parental lines, and the HincII site fell into Earlygold was generated by C insertion. As for *KNOPE4*, 11 polymorphic events and 2 IN/DEL occurred , and the HinfI site fell into Earlygold was generated by T/A substitution. Data were processed by MAPMAKER ESP 3.0, which indicated that *KNOPE3* was placed on LG1, at 71 cM, co-segregating with the AC18 and AC23 markers of the *Prunus* reference map (Fig.7C). Moreover, *KNOPE4* was sited on LG 7 at 64 cM between CC132 and FG24 markers (Fig. 8C).

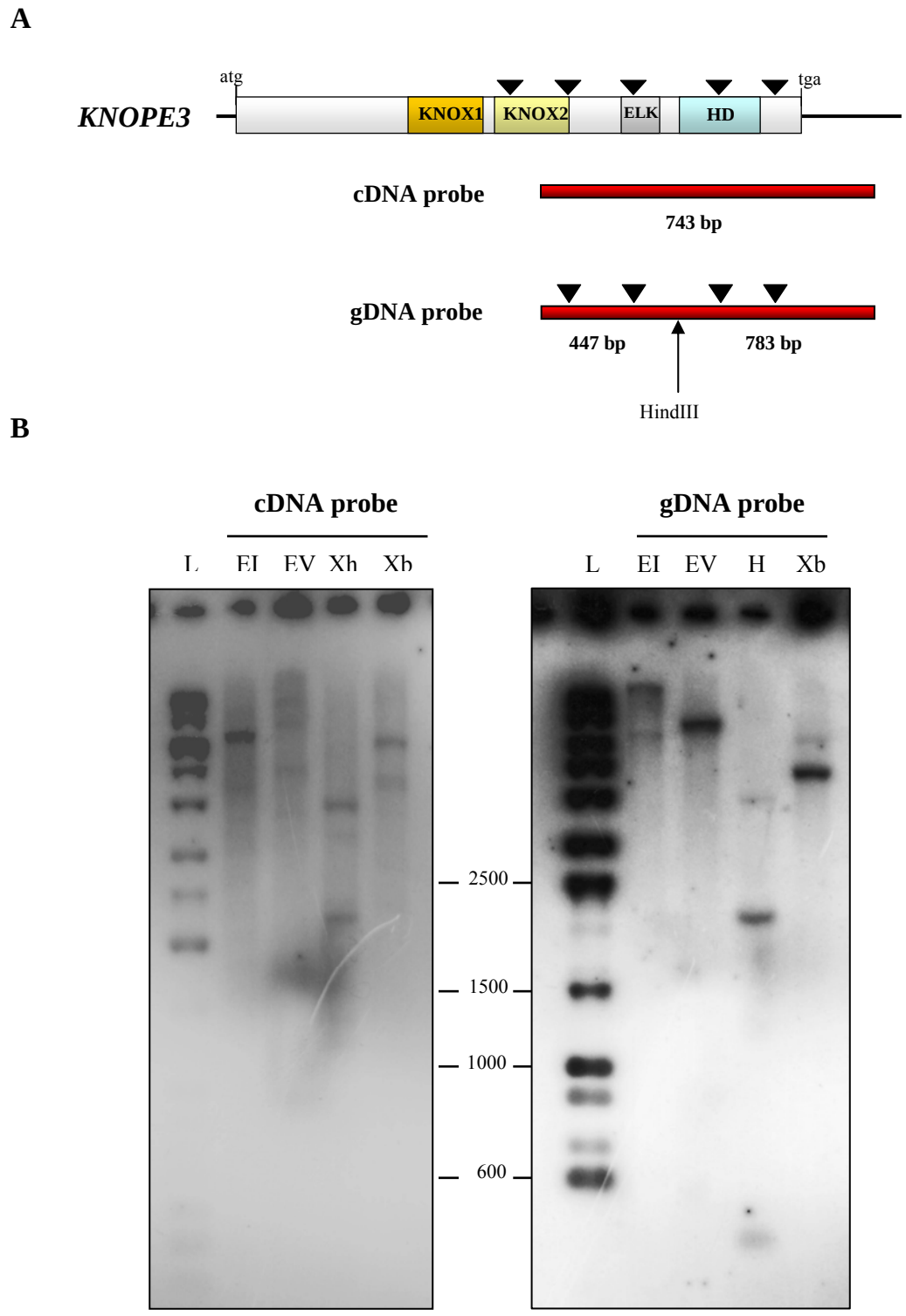

Figure 4. Southern analisys. (A) Organization of the gene encoding *KNOPE3* and probes used in Southern analisys. Black triangle indicates intron position. **(B)** Left: Southern blot was performed using a cDNA probe and suggests that class 2 *KNOX* belong to a small family. Right: using a genomic probe including *KNOPE3* introns, the hybridisation profile suggests a single copy gene in Chiripa genome.
L, ladder **EI**, EcoRI; **EV**, EcoRV; **Xh**, XhoI; **Xb**, XbaI, **H**, HindIII

KNOPE3

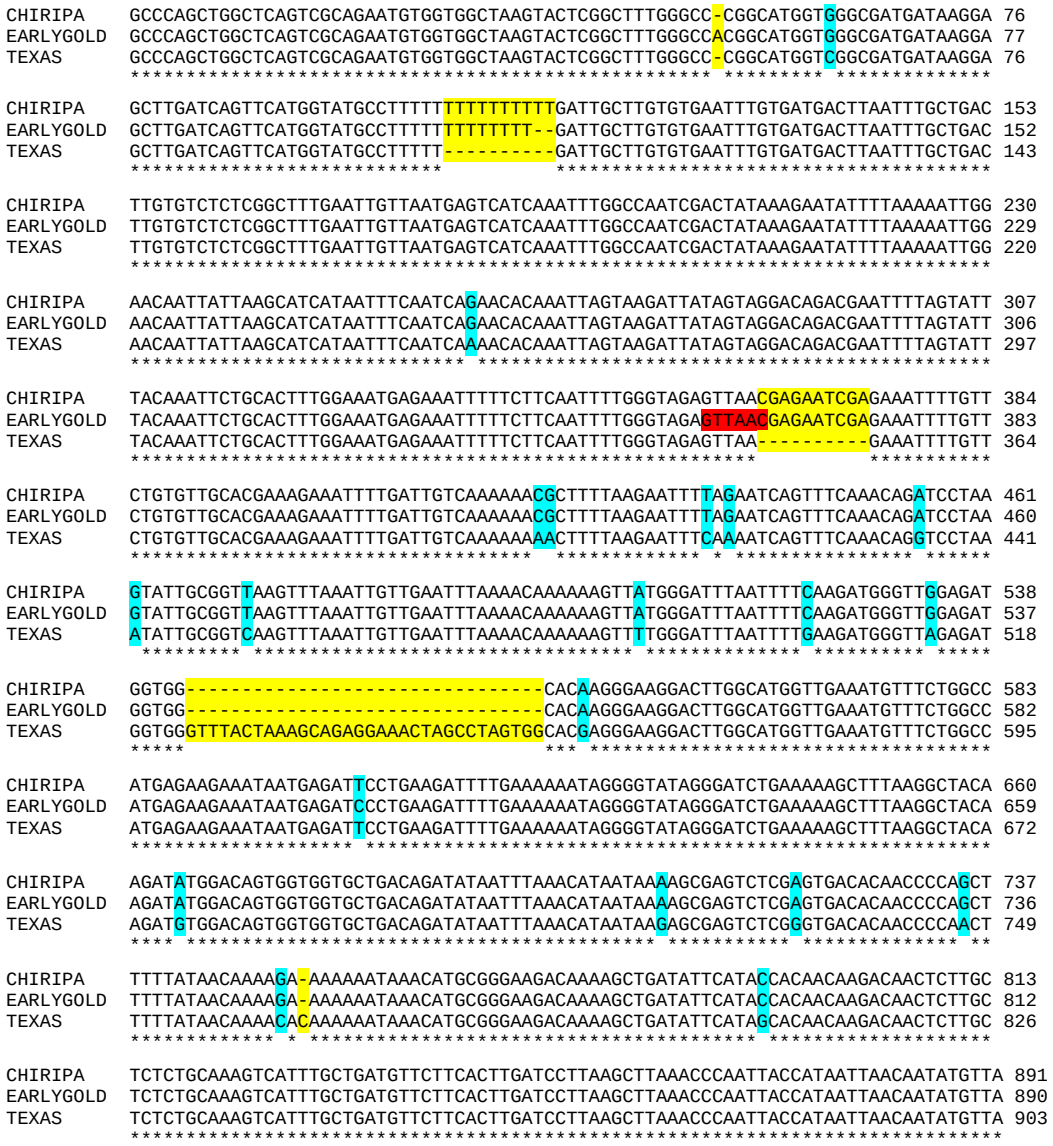

Figure 5. *KNOPE3* alignment among Chiripa (*Prunus persica*), Earlygold (*Prunus persica*) and Texas (*Prunus dulcis*) cultivars. In blue and yellow are SNPs and IN/DEL, respectively, nucleotides polymorphism. In red is the site digested by HincII.

KNOPE4

```
Chiripa    AGTGATGGCTTGCTGGGAGCTCGATCAATCTCTACAAAGCTTAACAGGTCATTCCTATTTCTTTTCATATTTCTACATGT 80
Earlygold  AGTGATGGCTTGCTGGGAGCTCGATCAATCTCTACAAAGCTTAACAGGTCATTCCTATTTCTTTTCATATTTCTACATGT 80
Texas      AGTGATGGCTTGCTGGGAGCTCGATCAATCTCTACAAAGCTTAACAGGTCATTTCTATTTCTTTTCATATTTCTACATGT 80
           ************************************************** ************************

Chiripa    ACAGCAGATAAGTGGCAAGTTTACGAATGGTTTATAACTGAACAGCTTGAGATGCATTGACAGGTGTGTCAACGGGTGAA 160
Earlygold  ACAGCAGATAAGTGGCAAGTTTACGAATGGTTTATAACTGAACAGCTTGAGATGCATTGACAGGTGTGTCAACGGGTGAA 160
Texas      ACAGCAGATAAGTGGCAAGTTTACGAATGGTTTATAACTGAACAGCTTGAGATGCATTGACAGGTGTGTCAACGGGTGAA 160
           ********************************************************************************

Chiripa    GGCACCGGTGCTACAATGTCCGACGACGACGACCAGGTCGACAGTGACATCAACTCGTATGATGGAAGCCTGGACGGGCC 240
Earlygold  GGCACCGGTGCTACAATGTCCGACGACGACGACCAGGTCGACAGTGACATCAACTCGTATGATGGAAGCCTGGACGGGCC 240
Texas      GGCACCGGTGCTACAATGTCCGACGACGACCACCAGGTCGACAGTGACATCAACTCGTATGATGGAAGCCTGGACGGGCC 240
           ***************************** ************************************************

Chiripa    TGACACCATGGGATTCGGTCCTCTCGTTCCGACTGAGAGTGAGAGGTCCTTAATGGAGCGTGTAAGGCAAGAATTGAAGC 320
Earlygold  TGACACCATGGGATTCGGTCCTCTCGTTCCGACTGAGAGTGAGAGGTCCTTAATGGAGCGTGTAAGGCAAGAATTGAAGC 320
Texas      TGACACCATGGGTTTCGGTCCTCTTGTTCCGACTGAGAGTGAGAGGTCCTTGATGGAGCGTGTAAGGCAAGAATTGAAGC 320
           ************ *********** ************************* ***************************

Chiripa    ATGAACTGAAACAGGTATATGAACTATAGGACTTGCAAAGCATGTATAATATATTTGCTAGATGGGATATAGAAATGTAG 400
Earlygold  ATGAACTGAAACAGGTATATGAACTATAGGACTTGCAAAGCATGTATAATATATTTGCTAGATGGGATATAGAAATGTAG 400
Texas      ATGAACTGAAACAGGTATATGAACTATAAGACTTGCAAAGCATGTATAATATATTTGCTAGATGGGATATAGAAATGTAG 400
           ************************* ************************************************

Chiripa    CTAATAACATCTGCTTCTCAGGGTTACAAGGAAAAGATTGTAGACATTAGGGAGGAAATTCTACGCAAGAGAAGAGCAGG 480
Earlygold  CTAATAACATCTGCTTCTCAGGGTTACAAGGAAAAGATTGTAGACATTAGGGAGGAAATTCTACGCAAGAGAAGAGCAGG 480
Texas      CTAATAACATCTGCTTCTCAGGGTTACAAGGAAAAGATTGTAGACATTAGGGAGGAAATTCTACGCAAGAGAAGAGCAGG 480
           ********************************************************************************

Chiripa    TAAACTGCCAGGTGACACCACTTCTGTCCTAAAATCTTGGTGGCAATCACATTCTAAGTGGCCTTACCCTACGGTGAGTT 560
Earlygold  TAAACTGCCAGGTGACACCACTTCTGTCCTAAAATCTTGGTGGCAATCACATTCTAAGTGGCCTTACCCTACGGTGAGTT 560
Texas      TAAACTGCCAGGTGACACCACTTCTGTCCTAAAATCTTGGTGGCAATCGCATTCCAAGTGGCCTTACCCTACGGTGAATT 560
           ********************************************** ***** ********************** **

Chiripa    TCAACTTTGATATAATCCCCTTGTAGCTTTCTGTTTAGCAAATTAATTCTTTTTAAAATGGCTATTAAAACCGTCGTTCG 640
Earlygold  TCAACTTTGATATAATCCCCTTGTAGCTTTCTGTTTAGCAAATTAATTCTTTTTAAAATGGCTATTAAAACCGTCGTTCG 640
Texas      TCAACTTTGATATAATCCCCTTGTAGCTTTCTGTTTAGCAAATTAATTCTTTTTAAAATGGCTATTAAAACCGTCGTTCG 640
           ********************************************************************************

Chiripa    CTTGGAATAGGAGGAAGACAAAGCCAGGTTGGTGCAGGAAACAGGCTTGCAATTGAAACAGATCAATAACTGGTTCATAA 720
Earlygold  CTTGGAATAGGAGGAAGACAAAGCCAGGTTGGTGCAGGAAACAGGCTTGCAATTGAAACAGATCAATAACTGGTTCATAA 720
Texas      CTTGGAATAGGAGGAAGACAAAGCCAGGTTGGTGCAGGAAACAGGCTTGCAATTGAAACAGATCAATAACTGGTTCATAA 720
           ********************************************************************************

Chiripa    ATCAAAGAAAAAGGAATTGGCACAGTAACATTTCCTCAACTTCTGTTTTGAAGGGCAAACGCAAGAGGTAGGATTGTATT 800
Earlygold  ATCAAAGAAAAAGGAATTGGCACAGTAACATTTCCTCAACTTCTGTTTTGAAGAGCAAACGCAAGAGGTAGGATTGTATT 800
Texas      ATCAAAGAAAAAGGAATTGGCACAGTAACATTTCCTCAACTTCTGTTTTGAAGAGCAAACGCAAGAGGTAGGATTGTATT 800
           ************************************************** ************************

Chiripa    TAACATATATGGTCGTTTTGTTCACATAAGTTTGTTTTCAAGAACTTTATATAAATATTTTTTTTTTT-CATTTGTAATT 879
Earlygold  TAACATATATGGTCGTTTTGTTCACATAAGTTTGTTTTCAAGAACTTTATATAAATATTTTTTTTTTT-CATTTGTAATT 879
Texas      AAACATATATGGTCGTTTTGTTCACATAAGTTTGTTTTCATGAACTTTATATAAATATATTTTTTTTTACATTTGTAATT 880
            ************************************ ***************** ********* ***********

Chiripa    TGGGAAAAAAAGTAATGCAGGTGACATCAGCAGCCAACGATTGAAGTAACTGTGTGTGACACTTGACAAGTCCACAACTT 959
Earlygold  TGGGAAAAAAAGTAATGCAGGTGACATCAGCAGCCAACGATTGAAGTAACTGTGTGTGACACTTGACAAGTCCACAACTT 959
Texas      -GGGAAAAAAAGTAATGCAGGTGACATCAGCAGCCAACGATTGAAGTAACTGTGTGTGACACTTGACAAGTCCACAACTT 959
            ********************************************************************************

Chiripa    ATGG 963
Earlygold  ATGG 963
Texas      ATGG 963
           ****
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Figure 6. *KNOPE4* alignment in Chiripa (*Prunus persica*), Earlygold (*Prunus persica*) and Texas (*Prunus dulcis*) cultivars. In blue and yellow are SNPs and IN/DEL, respectively, nucleotides polymorphism. In red is the site digested by HinfI.

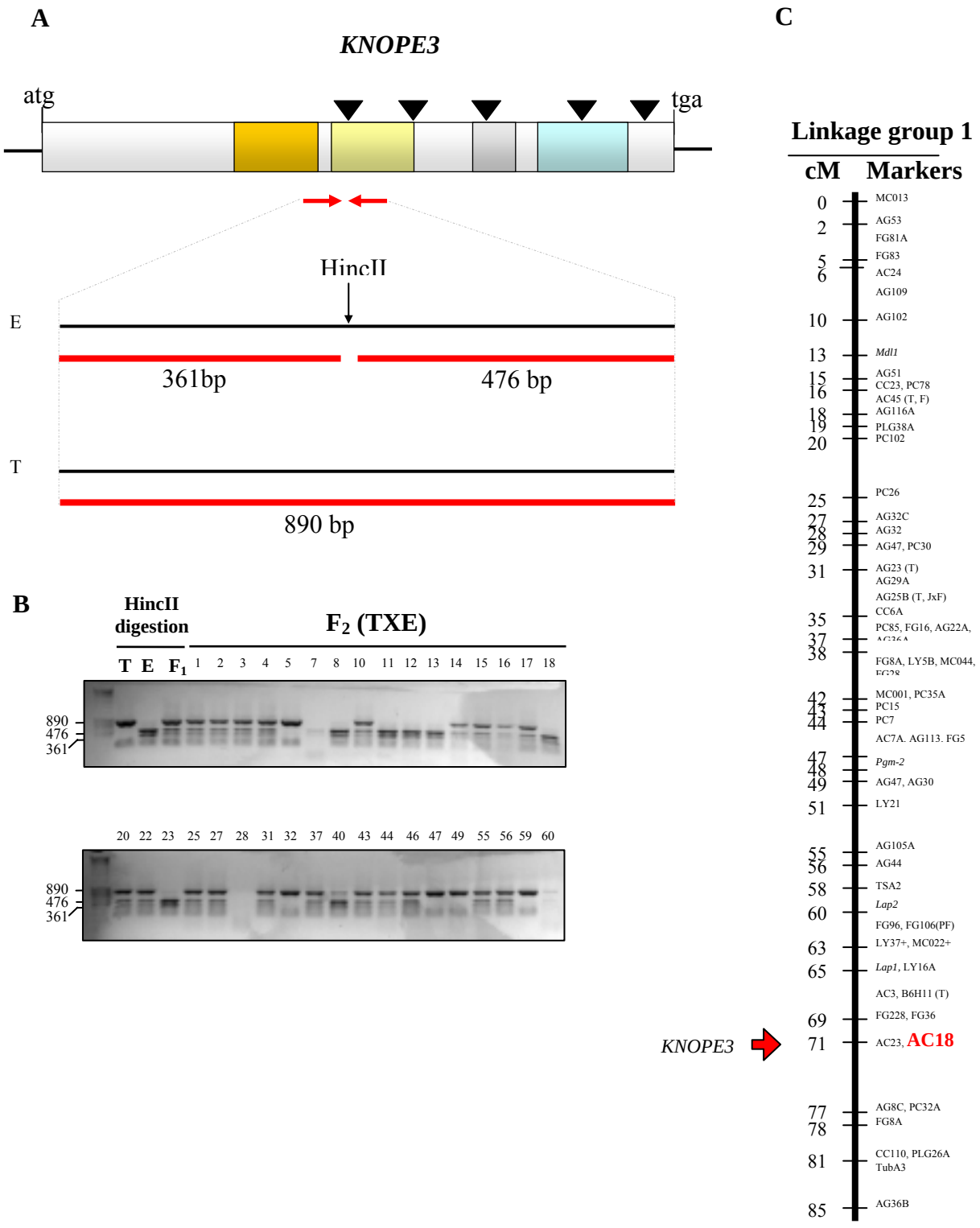

Figure 7. *KNOPE3* map position. *KNOPE3* were mapped to specific linkage groups (LG) by scoring the segregation pattern of CAP markers in the TexasXEarlygold F_2 population. **(A)** Primer pairs (red arrows), designed on the sequences of *Chiripa KNOPE3*, yielded a PCR product of ca. 890 nt (black line), using Texas (T) and Earlygold (E) genomic DNA. HincII only cut in the Earlygold fragment (red line). Black triangle indicates intron position; **(B)** Parental, F_1 and F_2 genomic DNA was first amplified and subsequently digested with HincII which produced the pattern as in figure. **(C)** Data were analysed by using MAP MAKER 3.0 and *KNOPE3* was sited on linkage group 1 at 71 cm (red arrow), segregated with AC18 marker of the *Prunus* reference map.

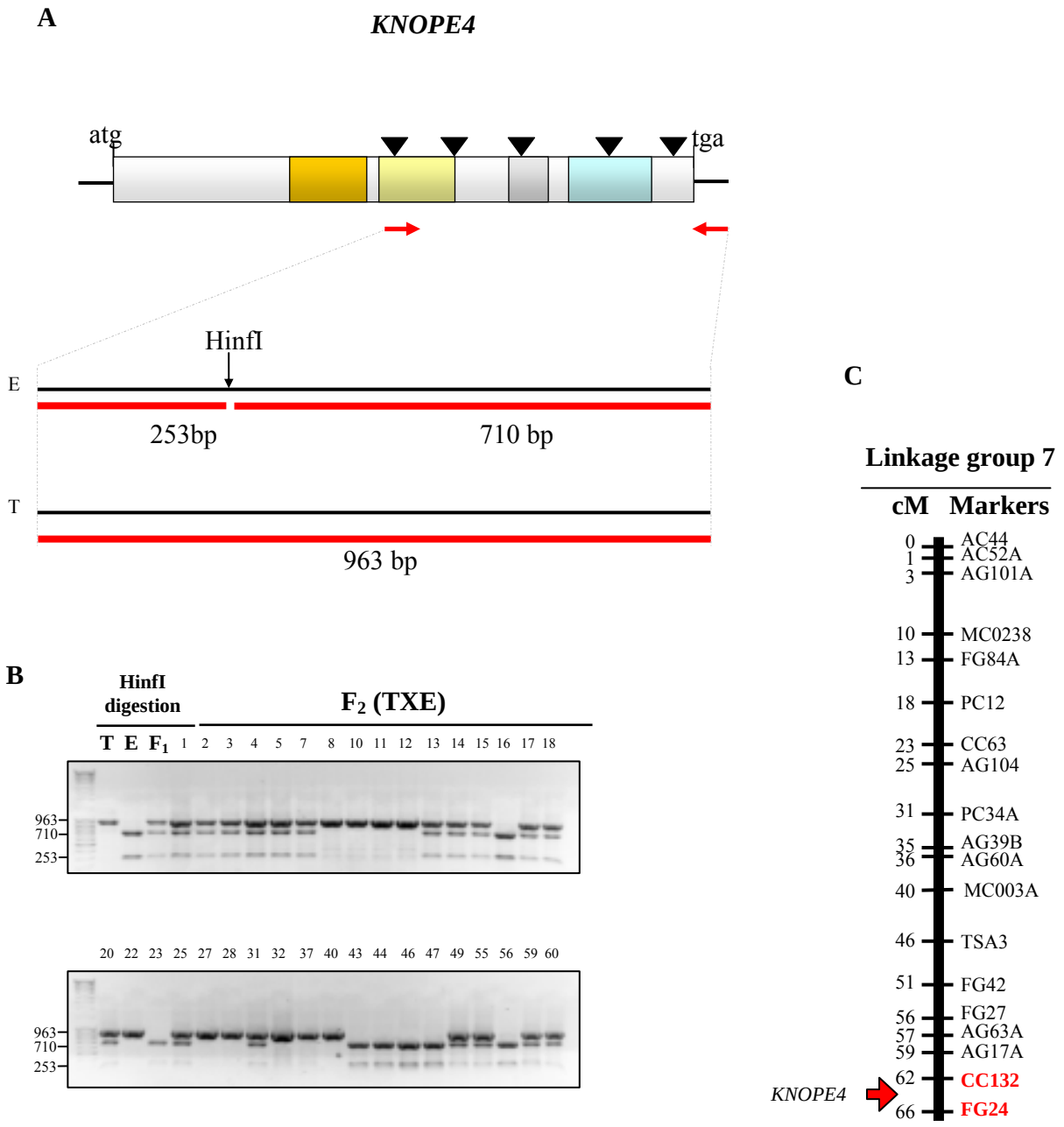

Figure 8. *KNOPE4* map position. *KNOPE4* were mapped to specific linkage groups (LG) by scoring the segregation pattern of CAP markers in the TexasXEarlygold F_2 population. **(A)** Primer pairs (red arrows), designed on the sequences of *Chiripa KNOPE4,* yielded a PCR product of ca. 960 nt (black line), using Texas (T) and Earlygold (E) genomic DNA. HinfI only cuts in the Earlygold fragment (red line). Black triangle indicates intron position; **(B)** Parental, F_1 and F_2 genomic DNA was first amplified and subsequently digested with HinfI which produced the pattern as in figure. **(C)** Data were analysed by using MAP MAKER 3.0 and *KNOPE4* was sited on linkage group 7 between 62 and 66 cM, segregated with CC132 and FG24 markers of the *Prunus* reference map.

DISCUSSION

In our experiments for gene search, two distinct class 2 *KNOPEs* members were fully sequenced. Southern analyses suggested that a few more members may occur in the *Chiripa* genome, though *KNOPE3* was highly likely to be a single copy (per haploid genome). Four class 1 *KNOPE* members were assessed in the same cultivar (Testone et al., unpublished, personal communication from the IBBA-CNR) and overall peach KNOX are estimated around eigth components. In Arabidopsis also eight *KNOX* members were precisely counted, four for each class, since the genome was fully sequenced (TAIR, www.arabidopsis.org/). The kind *Malus* (n=17) is genetically related to *Prunus* (n=8) and class 1 and 2 members of apple were putatively computed over ten (Watillon *et al.*, 1997).

KNOPE3 and *KNOPE4* were organized in five exons and four introns similarly to the Arabidopsis putative orthologs, and both harboured an intron inside the ELK domain, which further assigned them to class 1 (Reiser *et al.,* 2000). The *KNOPE3* and *KNOPE4* genes shared a low identity (27%) at the genomic level, which increased to 54% for the transcribed sequence, implying that they are not tight paralogues.

The intron V of *KNOPE4* was found to participate in alternative splicing events. In fact, in the *KNOPE4* transcript, eight nucleotides were alternatively edited in leaves. The spliced stretch implied a frame shift that resulted in an eleven amino acid shorter deduced protein. Tissue specific alternative message editing has been observed for class 1 and 2 *KNOX* in different plant species (Lincoln *et al*., 1994; Ito *et al*., 2002; Di Giacomo et al., unpublished, personal communication from the IBBA-CNR) and the *KNOPE4* intron V coding regions may be responsible for regulating stability and/or trafficking of the encoded protein.

Interestingly, the *KNOPE4* intron II encompassed a stretch (TGACAGGT), which was demonstrated to bind class 1 KNOX proteins (Smith *et al*., 2002). In fact, both Arabidopsis *STM* and *KNAT1*/*BP* contain the WFIN in the homeodomain, which specifically interacts with the TGACAGG(C)T motif (Hake *et al.*, 2004). In animal TALE proteins, the third amino acid of WFIN is replaceable and such variation determines the DNA-binding specificity (Treisman *et al.*, 1989; Chang *et al*., 1997). Moreover, the HD of maize KN1 protein was shown to bind its own mRNA for directional transport in the cell (Kim *et al*., 2005). Considering these aspects, we are tempted to speculate that *KNOPE4* unprocessed mRNA might be recognised by peach class 1 *KNOX* (or even by itself) in processes of transcriptional regulation or in mechanisms of message trafficking.

KNOPE3 and *KNOPE4* were positioned on LG1 (71 cM) and LG7 (60-64 cM) of the reference *Prunus* TxE genetic map, respectively. The markers in the neighbourhood of both

genes were RFLP and did not result associated to any EST or QTL (www.mainlab.clemson.edu), and we could not retrieve data useful to address functions for the two *KNOPE*s. The information on *KNOX* genes from several plant species revealed that they are sparse on diverse chromosomes and/or LG and no indication of extensive clusters occurred, which are observed instead in the animal *HOM*-C or *Hox* members (Krumlauf, 1994). Considering that *KNOPE3* and *4* mapped on two distinct LG, it is likely that genome spreading of *KNOPEs* also occurs in peach.

Finally, two CAP markers were produced and validated to distinguish the two genes in almond and peach. Moreover, a refined analysis to score for polymorphic sequences of *KNOPE3* and *KNOPE4* provided tools to study the variability between the Earlygold and Chiripa cultivars.

MATERIALS AND METHODS

Plant materials and growth conditions.

In a orchard eighteen adult plants were cultivated and they derived from a seed lot of the open pollinator 16 *Prunus persica* cultivar Chiripa (OP 16). These plants have been confined and left to pollinate freely (peach autogamy is estimated 95%), seeds were collected from each mother plant and lots were kept separate. Seeds were washed with tap water, laid on filter paper, dried in the hood overnight, then treated with Ziram (1%) and copper oxychloride (4 g/l of pure Cu), dried for 2 h, and finally stored in paper bags at 4-6°C for at least four moths (vernalization). Sowing was carried out using peat/mould and sieved soil (4:2) in baby rooms at 25°C 16/8h of light/dark and a light intensity of 100 μmol $m^{-2}s^{-1}$ of photosynthetically active radiation (PAR). Seedlings were acclimated and grown in the green house at 22°C under natural photoperiod and light intensity. In destructive analyses, tissues were immediately frozen in liquid nitrogen after sampling and stored at –80°C.

Isolation and sequence analysis of cDNA and genomic clones.

As for *KNOPE3*, RT-PCR experiments were performed using oligo-dT cDNA, which was reverse transcribed from RNA of fully expanded leaves. Primers KNIIFW1 (5'-GCAATGGAAGCTGTGATGGC-3') and KNIIBW2 (5'-CTTCCTCAGTAGGGTAAGGCC -3') were designed based on conserved AMEAVMA (in the MEINOX) and DEETPYPW (in the homeodomain), respectively. The 390 nucleotide (nt) KNIIFW1 – KNIIBW2 fragment was cloned and sequenced. The full length *KNOPE3* cDNA was rescued by PCR and 3' RACE methodologies, starting from fully expanded leaves RNA and according to the manufacturer's instructions (Life Technologies). 5' region of cDNA was isolated by primers based on sequence identities with *M. domestica*: KN3.0FW (5'-GTATTCATGGCGTACCACAACC-3') and KN3.5BW (5'-CGGGTGAAGGCACCGGTGCTA-3'), which lead to the cloning of 944 nt fragments. The 3' RACE primers were: an oligo-dT anchor primer provided by the kit for the cDNA synthesis and KNIIFW1 primer. The intron search within the *KNOPE3* was approached by PCR on genomic DNA (gDNA) using the combinations: KN3.3FW (5'-GCCCAGCTGGCTCAGTCGC-3') / KNIIBW2 and KNIIFW1 / KN16BW (5'-CAGGTATGCTCAAAGGCTGC-3'). The sequences of products amplified from cDNA and gDNA were

aligned and compared by ClustalW (http://www.ebi.ac.uk.clustalw), optimised by visual inspection (PILEUP program), and intron size and locations established.

As for *KNOPE4*, the 3' RACE with oligo-dT anchor primer and KNIIFW1 primer lead to a second transcript, named *KNOPE4*. The full length *KNOPE4* cDNA was rescued by 5' RACE methodologies, starting from fully expanded leaves RNA. Three backward primers were used for cDNA synthesis and amplifications combined with forward adapter primers provided by the kit : KN3.5BW, KN4.24BW (5'-GGAAGAAGACGAACTGGAGTG-3') and KN4.28BW (5'-GCAAAGACCAAAGTAATGGCG-3'): PCR experiments lead to the cloning of 413, 314 and 184 nt fragments, respectively and reaction conditions were the same as described above. Intron identification was achieved by gDNA PCR experiments using KN4.12FW (5'-TGTGATCGCTTGTTGGGAG-3') / KN3.19BW (5'-CACTTGACAAGTCCACAACTTATGG-3') and KN4.13FW (5'-AAAGCGGACTTAGTGGGC-3')/ KN3.5BW.

The final PCR conditions for both genes were: 500 ng of genomic DNA or 200 ng of cDNA, 1 mM of each primer, 0.5 mM dNTPs, Taq DNA polymerase (TaqQUIA, Quiagen) 2.5 U, 1/10 of 10X Taq Buffer (Quiagen), 2.5 mM $MgCl_2$, in a final volume of 50 µl. Cycling conditions included an initial cycle at 95°C for 5 min followed by 35 cycles at 95°C for 40s, either 55°C (in cDNA based and RACE-PCR experiments) or 60°C (with genomic DNA) for 30-60 s and 72°C for 30-90s, final extension at 72°C for 5 min. All PCR fragments were cloned into pGEM-T easy vector system (PROMEGA). The GenBank accession numbers of *KNOPE3* and *KNOPE4* complete cDNAs are DQ786755 and EF107110.

Southern blot analysis.

The technique was performed as described in Giannino *et al*. (2000). Filters were hybridized at 60°C, washed twice (2X and 1X SSC/0.1%SDS) at 60°C for 10 min and exposed to Biomax films (Kodak) for 4-12 h at -80°C. *KNOPE3* probes included the region between the primers KN3.16FW (5'- GTTTCCCCCGGTGAAGGCACC -3') / KN3.16BW (5'-CAGGTATGCTCAAAGGCTGC-3') and were 743 and 1230 nt long in cDNA and genomic sequences, respectively. Hind III cuts at 293 nt of *KNOPE3* cDNA probe and does not cut in introns.

Mapping.

A F_2 mapping population of 56 individuals derived from an almond [*Prunus dulcis* (Mill) D. A. Webb; syn. *P. amygdalus* Batsch] 'Texas' X peach 'Earlygold' (TxE) interspecific cross (Joobeur *et al.* 1998) was used for mapping the candidate genes. Trees of the mapping population are maintained in the field at CRA – Istituto Sperimentale per la Frutticoltura, Ciampino, Rome and DNA was provided by Dr. Ignazio Verde. *KNOPE3* primers: Kn3.3Fw and I2Bw (5'-GGCTCTTGAGCACCGAAGTT-3'); *KNOPE4* primers: KN4.12FW and KN4.19BW (see above). The PCR conditions were: 30 ng of genomic DNA, 0,4 µM of each primer, 0.5 mM dNTPs, 1.25 U of Taq DNA polymerase (Quiagen), 1/10 of 10X Taq Buffer (Quiagen), 2.5 mM $MgCl_2$, in a final volume of 25 µl. Cycling conditions included an initial cycle at 95°C for 5 min followed by 35 cycles at 95°C for 30s, 62°C for 60 s and 72°C for 90s with a final extension at 72°C for 7 min. PCR products were digested with 5 enzyme units (Hinc II for *KNOPE3* and HinfII for *KNOPE4* fragments) in a final volume of 30 µl and 15 µl was electrophoresed onto 1% agarose gel. The data obtained from polymorphism segregation pattern were included in the TxE map (Joobeur et al 1998) using MAPMAKER EXP 3.0b (Lander *et al.*, 1987; Lincoln *et al.,* 1992). The mapping was done following the procedures described in Joobeur *et al.* (1998) . Initially, the "group" command was used to assign the *loci* to linkage groups (LOD threshold > 5 and recombination fraction < 20). Loci were then placed within the respective TxE linkage group using the "try" and "ripple" commands. After mapping, the "error

detection" command was used and possible errors were re-examined. Kosambi's (Kosambi, 1944) mapping function was used to convert recombination fractions into centimorgan map distances.

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CHAPTER III

Features of the deduced product of KNOPE3 and KNOPE4

(With: Giulio Testone, Domenico Mariotti, Maria Beatrice Bitonti and Donato Giannino)

Summary

Identity and phylogenetic analyses of KNOPE3 and KNOPE4 deduced proteins confirmed that both proteins could be assigned to the class 2 of KNOX. Interestingly, the two proteins contained a C-terminus putative nuclear localization signals, which is highly conserved in eukaryotes. KNOPE3 and KNOPE 4 are two distinct proteins for they were found to be remarkably different in the N terminus region. KNOPE4 N terminus showed very low identity degree to all the other class 2 proteins, representing a novel putative KNOX transcription factor. KNOPE4 was also likely to be synthesised in two isoforms from the same gene due to alternative splicing events of the intron V. The genetic phylogram indicated that KNOPE3 was closest to apple KNAP3, whereas KNOPE4 was next to tomato LET12, further suggesting the divergences between the two proteins.

INTRODUCTION

The functions of conserved domains in the KNOX proteins have been analysed by expression of chimeric/truncated coding sequences *in planta* and assessing the ability of each of the resultant proteins to bind cis-acting elements in DNA, form dimers and modulate target gene expression in yeast (Serikawa *et al*., 1997; Sakamoto *et al*., 1999; Nagasaki *et al*., 2001).

The DNA binding of individual homeodomain proteins is generally weak. Protein–protein interaction appeared to be necessary to confer high affinity binding of HD proteins to their cognate target sequence, and ternary homeodomain protein complexes were found to play important roles in the combinatorial control of gene expression (Gehring, 1990). This feature makes it possible for HD proteins to be involved in a series of developmental processes with different protein partners.

Every member of the KNOX family has at least one interaction partner amongst the BELL proteins and vice versa, supporting the notion that BELL-KNOX heterodimerization plays a general role in TALE protein function. In addition, several homo- and heterodimerizations within each TALE family have been detected in Arabidopsis. More specifically KNAT3 interacts with KNAT4 and the whole group BEL1 to 9; KNAT4 binds to KNAT3, KNAT1 and BEL6; KNAT5 recognizes itself and the 1 to 10 BEL proteins (Hackbusch *et al.,* 2005). The dense TALE network indicates potential functional redundancy amongst TALE proteins, which might account for the fact that null mutations in TALE genes frequently do not exhibit obvious aberrant phenotypes.

RESULTS

Features of the deduced products KNOPE 3 and KNOPE4.

KNOPE3 and KNOPE4 consisted of 448 and 417 amino acids and weighed 50,05 and 47,18 kD, respectively (http://www.expasy.ch/cgi-bin/protparam). Both proteins contained the typical domains of KNOX factors (Fig. 1). KNOPE3 shared the highest identity with KNAP3 (91%) of *Malus domestica* and KNAT3 (68%) of *Arabidopsis thaliana*. KNOPE4 also shared the highest identity with KNAP3 (56%) and KNAT3 (64%). The overall identity between KNOPE3 and KNOPE4 was 56%, which raised to 86% by comparing the C terminus regions from QNA (pos. 172-174) and ESA onwards (pos. 153-155), respectively, up to 98% by aligning their homeodomains (Tab. 1 lists the HD homology and identity with respect to class 2 and 1 KNOX of other species; Fig. 2 shows amino acids allignement). The remaining upstream N terminus of

KNOPE3 was 81% identical to that of KNAP3, whereas KNOPE4 N-terminus shared very low identity (6-12%) with those of other proteins. Moreover, extensive analyses indicated that KNOPE4 N-terminus had no significant homology with any other protein deposited in available databases so far. Interestingly, putative nuclear localization signals (NLS) were predicted in pos. 416-436 of KNOPE3 and in pos. 396-417 KNOPE4 (http://cubic.bioc.columbia.edu/predictNLS/; Fig. 1). Moreover, KNOPE4 was putatively synthesized in two isoforms from the same gene since eight nucleotides of intron V were found to be alternatively spliced. Both edited and non edited cDNAs occurred in leaves: the edited variant produced a protein with eleven aminoacids less than the non edited form (for details see Fig. 1 and Chapter II). The deduced short isoform contain the NLS, whereas the long isoform did not.

Finally, predictive analyses by web on-line programmes (see the material and methods) identified a N-O-glycosilation sites at pos. 326 (T) and 307 (T) for KNOPE3 and KNOPE4, respectively. Phosphorylation sites were also predicted at pos. 428 (T) of KNOPE3 and 359 (S) of KNOPE4 (Fig. 1).

***KNOPE3* and *KNOPE4* fall into class 2 KNOX and are divergent.**

A phylogram (Fig. 3) was constructed by clustering class 2 KNOX proteins from dicot and monocot species available in several databases. KNOPE3 and KNOPE4 were confirmed to belong to the highly supported monophyletic group of class 2 proteins. KNOPE3 was closest to apple KNAP3, while KNOPE4 was closest to LeT12 (bootstrap values 98 and 61, respectively), implying that a good grade of divergence occurred between the two KNOPEs.

KNOPE3

```
    MAYHNHLSQQDLPLHHFTDQTQQQHQPFQSDQHDPNSKPTEPHHPFQPAP
51- NWLNTALLRNYTNADANNNHNNSPNNNGGGGASNFLNLHVTASDSTTSQA
    SNQWLSQPHRPILHRNHSDVIDDVTAIAGDSMIAATISHDSADLKPDSSL
151- NKTDIVVESGGGGGDGGMINWQNARHKAEILAHPLYEPLLSAHVACLRIA
    TPVDQLPRIDAQLAQSQNVVAKYSALGHGMVGDDKELDQFMRHYVLLLCS
251- FKEQLQQHVRVHAMEAVMACWEIEQSLQSLTGVSPGEGTGATMSDDEDDQ
    VDSDANLFDGSMEGHDSMGFGPLIPTESERSLMERVRQELKHELKQGYKE
351- KIVDIREEILRKRRAGKLPGDTTSVLKAWWQSHSKWPYPTEEDKARLVQE
    TGLQLKQINNWFINQRKRNWHSNPSTSTVLKSKRKRSNAGENSSDRFG
```

KNOPE4

```
    MNFHQHHFLSNSEHHNFFFPLSLSFSQLQKLLFSEPRFLVCTETKSSVLF
51- LQFNCKDQSNGVSSSSSSPAAANPSRNGVPLLRLGPPPLSGAPTWLNNAA
    FRQQNSFLHDARNDDVVISPSGKSSNCSGRNRREISGYDGEEEEEDELEC
151- ESARFKADLVGHPLYEQLVSAHVSCLRIATPVDQLPRIDEQLVQSQRVVD
    KYSALRANGDVQVMDEKELDLFMTNYVLLLCSFKEQLQQHVRVHAMEAVI
251- ACWELDQSLQSLTGVSTGEGTGATMSDDDDQVDSDINSYDGSLDGPDTMG
    FGPLVPTESERSLMERVRQELKHELKQGYKEKIVDIREEILRKRRAGKLP
351- GDTTSVLKSWWQSHSKWPYPTEEDKARLVQETGLQLKQINNWFINQRKRN
    WHSNISSTSVLKGKRKR
```

KNOPE4 alternative product

```
351- GDTTSVLKSWWQSHSKWPYPTEEDKARLVQETGLQLKQINNWFINQRKRNW
    HSNISSTSVLKSKRKSNAGDISSQRLK
```

Figure 1. Amino acid sequences and secondary structure predictions for *KNOPE3* and *KNOPE4*. The amino acid sequences of *KNOPE3* and *KNOPE4* are shown with the predicted secondary structure derived by class 2 KNOX proteins alignement in other plants. The KNOX1 (orange), KNOX2 (yellow), ELK domain (grey), and Homeodomain (blue) are coloured. Bold and coloured letters are protein signals describe in the legend.

Protein sequences	% identity	% similarity	% identity in HD
Knope3 (peach; DQ786755)	100	100	100
Knope4 (peach; EF107110)	52	60	98
Knat3 (Arabidopsis, P48000)	69	75	100
Knat4 (Arabidopsis, P48001)	63	68	98
Knat5 (Arabidopsis, P48002)	55	66	90
Knap3 (apple; O04136	87	90	100
Osh45 (rice; BAA08553)	58	64	96
Let12 (tomato; O22300)	60	68	100
Nth23 (tabacco, BAA25921)	42	51	85
Stm (Arabidopsis, Q38874)	22	35	55
Knat1 (Arabidopsis, P46639)	26	41	53
Knap1 (apple; O04134)	25	39	53
Knap2 (apple; O04135)	25	38	55
Kn1 (maize; P24345)	27	38	55

Table 1. Comparison of the deduced amino acidic sequence of *KNOPE3* with *KNOPE4* and members of the class 1 and class 2 *KNOX* gene in plants. Sequence similarities were calculated with the GCG program GAP. The name of each protein is listed in column 1, with its accession number and the species from which the corresponding gene was isolated, shown in parenthesis.

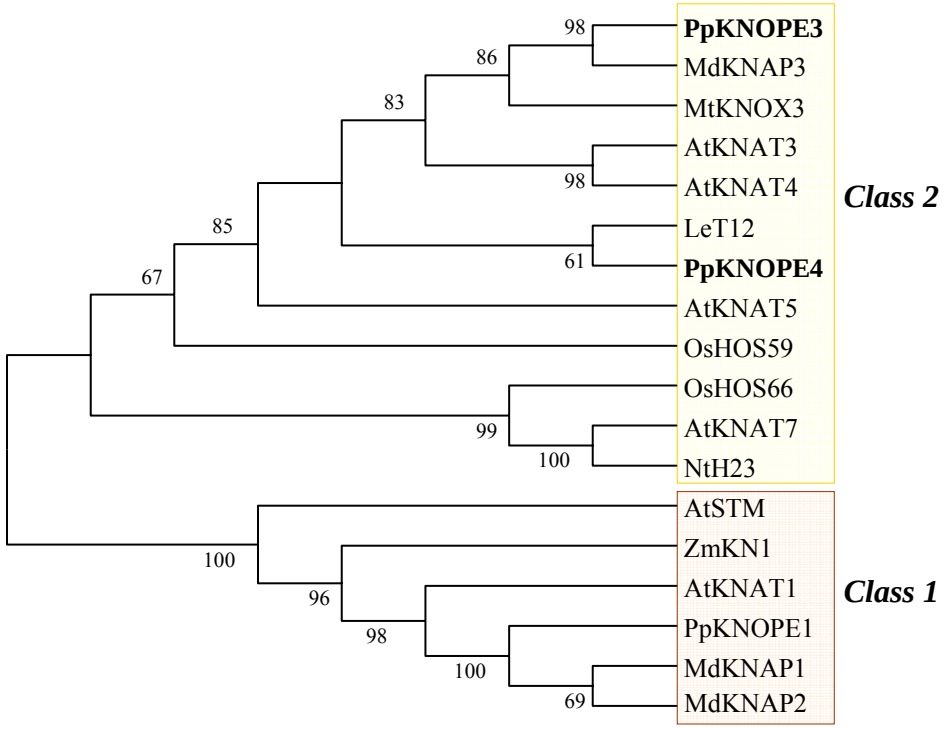

Figure 3. Phylogenetic tree. Phylogenetic relationships of 18 homeodomain proteins based on analyses of amino acid sequences. Numbers along branches denote bootstraps (1000 replicates), which assign proteins to a clade; the genetic distances are measured by horizontal bars (MEGA2 program and clustering analysis by the minimum evolution criterion). KNOPE3 and KNOPE4 fell into class II monophyletic group.

DISCUSSION

The deduced proteins KNOPE3 and 4 respect the typical KNOX structure: the N-terminal MEINOX (split into KNOX1 and KNOX2), the ELK and the HD domains. The HD of KNOPE3 and 4 shared the 55% and 52% identities, respectively, with that of maize KN1 (Tab 1.), which is a widely accepted criterion to assign KNOX to class 2 and distinguish them from those of class 1. Moreover the HD of both proteins included the three HSN residues which are peculiar of class 2 KNOX (Kerstetter *et al.*, 1994; Lincoln *et al.*, 1994). The ELK domain was proposed to have NLS function in KNOX (Reiser *et al.*, 2000), however a new NLS was scored at the C terminus of the KNOPE3 and the KNOPE4 short putative isoform. Some class 2 KNOX (eg.: KNAT5, Hos 59 and 66) also maintained the same NLS sequence as that of KNOPEs, others included similar but not an identical stretch. Therefore, it will be useful to perform assays of protein movement and localisation in the cell. To this aim GFP fusion constructs containing the full and 413-436-truncated KNOPE3 proteins have been prepared and are ready to be bombarded into onion leaf. The N terminus of KNOPE3 had significant identity with apple KNAP3, while KNAP4 encompassed a peculiar region with scarce identity to all the other class 2 KNOX. The phylogram further suggested that the divergence between KNOPE1 and 2 and we hypothesise that this reflects distinct functions for KNOPE3 and 4.

MATERIALS AND METHODS

Alignments and phylogenetics.

The alignment of *KNOPE3* and *KNOPE4* deduced protein with other *KNOX* genes was carried out by ClustalW (http://www.ebi.ac.uk.clustalw) and optimised by visual inspection (PILEUP program). Phylogenetic trees were constructed by MegaBlast2 using bootstrap values performed on 1,000 replicates and the 50% value was accepted as an indicative of a well-supported branch. The sequences of class 1 KNOX were used to create an out-group. Comparison of the deduced amino acid sequence of *KNOPE3* and *KNOPE4* with members of the class 1 and class 2 *knox* gene families in plants were calculated with the GCG program GAP. PutativegGlycosilation sites were established (http://www.cbs.dtu.dk/services/NetOGlyc/) according to Julenius *et al.* (2005), whereas the NetPhosK method without ESS filtering (http://www.cbs.dtu.dk/services/NetPhosK/) was used to predict phosphorylation sites (Blom *et al*., 2004).

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CHAPTER IV

Patterns of *KNOPE* 3 expression and transcript localisation in aerial organs

(With: Giulio Testone, Domenico Mariotti, Maria Beatrice Bitonti and Donato Giannino)

Summary

The *KNOPE3* and *KNOPE4* transcripts were detected in several organ tissues including vegetative and floral buds, leaves, stems, flowers, drupe and roots. The transcript localization was carried out for *KNOPE3* during stem, leaf and fruit development. The *KNOPE3* message was not detected in the apical meristem, but occurred in the cortex of subtending stems. Proceeding downwards the stem, the *KNOPE3* mRNA was confined to the cells of the phloem and was no longer revealed in the cortex. The expression of *KNOPE3* and *4* was differentially regulated during stem maturation. RT-PCR stem analysis moving top-down on a shoot indicated that the *KNOPE3* message decreased accordingly, whereas the *KNOPE4* transcript was unvaried. During leaf development, the *KNOPE3* message localization pattern changed: at very early stages it featured in the bundles, subsequently it spread in the mesophyll and localized to phloem cells. The gene was down regulated in the epidermis cells and the bundle sheaths at all leaf stages. In pre anthesis buds, the *KNOPE3* transcript marked the bundles of all flower elements, was signaled intensely in the cortex of receptacles, in sepals and anther endothecia. Finally, drupes were sectioned 3 and 16 weeks after full bloom and the *KNOPE3* mRNA just localized to the vascular bundles. The varied activity patterns support the idea that *KNOPE3* plays several and different roles based on timing and places of expression. A constant trait of *KNOPE3* was the association of its transcript to the phloem (and companion) cells, suggesting it may play a role in the correct development and function of vessels. Secondly, the message absence in the SAM and in the cambia suggests that it may not participate in determining the meristem fate of cells, contrary to class 1 *KNOX*. Third, we propose that it is involved in organ development, but after the cell differentiation has occurred.

INTRODUCTION

The class 2 *KNOX* members have widespread and overlapping expression patterns within a given plant species. This variety suggests that class 2 *KNOX* play several and distinct roles, depending on when and where each member is active. Arabidopsis *KNAT3* is the best investigated class 2 member (Serikawa *et al.,* 1997), for which three main expression patterns were distinguished: (1) in leaves, buds and pedicels at early development; (2) at and near the junction between two organs at specific times, e.g.: the hypocotyl-root boundary of young seedlings, the anther-filament border of mature flowers, and the junction of ovule-funiculus and siliques-peduncle in elongating fruit; and (3) in adult tissues such as the style of siliques, the petioles of mature leaves, and the root. *KNAT3* transcripts have never been observed in the meristems of the root, shoot or inflorescence (Serikawa et al 1997). As for the tomato *LeT12*, the expression was detected in the whorls of developing buds, but decreased in the distal region of developing sepals. In older buds, *LeT12* transcript localised to tapetal tissue, the stylar transmitting tract and developing ovules and the placental-ovular junction (Janssen *et al*., 1998). Tobacco *NTH23* transcript occurred in most organs, and GUS fusion studies revealed activity in the basal region of blades of leaf primordia and in young leaves around shoot apices. (Sentoku *et al*., 1998). Maize class 2 *KNOX* expression was also organ-widespread, but tissue specific preferences were also detected: *KNOX1* in roots, *KNOX2* in leaves, *KNOX6* and *KNOX7* in meristematic tissues (Kerstetter *et al*., 1994). The rice *OSH45* transcript featured in most tissues, though highly abundant in roots and leaf blades, moderate in shoot meristems, stems, leaf sheathes and etiolated leaf blades and low in flowers (Tamaoki *et al*., 1995). The apple *KNAP3* (the only class 2 KNOX characterised in fruit trees so far) showed strong expression in sepals, petals and fully expanded leaves (Watillon *et al*., 1997).

It has been difficult to propose single and/or specific functions for class 2 *KNOX* genes, considering that diverse expression patterns occurs in many distinct tissues at different times (Serikawa *et al.,* 1997). Moreover, the over -expression of class 2 *KNOX* have not produced clear mutant phenotypes, whereas putative knock out *KNAT3* and *KNAT4* mutants have only been described in symposia (Chandler and Wolfgang, 2004). The class 2 *KNOX* activity in young leaves suggests a role in the early growth, concurrent with differentiation events, such as the expansion or thickening of the leaf blade. The possibility of time and space dependent diverse roles correlates with the observation that HD-containing proteins often work as homo - or heterodimers with other transcription factors, and these interactions can result in very different specificities in terms of targets and their regulation (Goutte and Johnson, 1994; Kues *et al*., 1994; Wilson *et al.,* 1995). Alternatively, the products of the target genes may be multifunctional

and play different roles depending on the type of cells in which they are transcribed (Serikawa *et al*., 1997).

In this chapter, we first monitored the transcript abundance of *KNOPE3* and *KNOPE4* in various tissues, and subsequently the localization profiles of *KNOPE3* mRNA so as to preliminary attribute a role in stem, leaves, flower buds and drupes.

RESULTS

KNOPE3 and *KNOPE4* are transcribed in most tissues.

KNOPE4 cDNA harbours a NcoI site in the KNOX2-HD region, which is absent in the respective *KNOPE3* stretch. RT-PCR technology was first performed using a pair of primers strictly conserved in the two genes (Fig. 1B) which yielded two overlapping products (namely 391 and 387nt for *KNOPE3* and *KNOPE4*, respectively.), which were digested with NcoI. The consequent *KNOPE4* bands sized 226 and 161nt, which were distinguished electrophoretically from that of *KNOPE3*. The transcript of both genes were signaled in apical tips, leaf pedicels, herbaceous stems, fully expanded leaves, roots and vegetative buds at the vegetative resumption. The mRNA of both genes was lowly abundant in stems and roots (Fig. 1B).

RT-PCR with *KNOPE3* specific primers (Fig. 1C)was used to monitor gene expression in a new set of tissues, further including swollen floral buds (sampled in March), sepals, petals, and gynoecium of open flowers. The transcript occurred in all these tissues, highest abundant in leaf pedicels and pre-shooting vegetative buds, lowly abundant in herbaceous stems and petals, moderately abundant in apical tips, fully expanded leaves, floral buds, sepals, gynoecium and roots.

KNOPE3 transcript levels and localization vary in stem development.

The *KNOPE3* transcript localisation was investigated by in situ hybridisation in shoot apical meristem, stem sections at 0.2 mm, 2 and 5 cm below the apex of an elongating shoot (April) borne on a one year old branch. The transcript was absent in the apical dome and was detected from the 4th leaflet on (Fig. 2B, see also Fig 6 set). The mRNA signal spread evenly in the stem cortex (Fig. 2C), but was absent in the pith and procambium, and marked the phloem region (Fig. 2D) within the bundles of leaf petioles (leaf traces). At 2 cm (Fig. 3), the message localised specifically to the phloematic region of vascular bundles (Fig. 3B-C-D), whereas at 5 cm the mRNA maintained a strong signal within the leaf traces and vascular cylinder of the stem (Fig. 4B-D). More specifically, in leaf traces it marked groups of cells sub-adjacent to the phloem cap

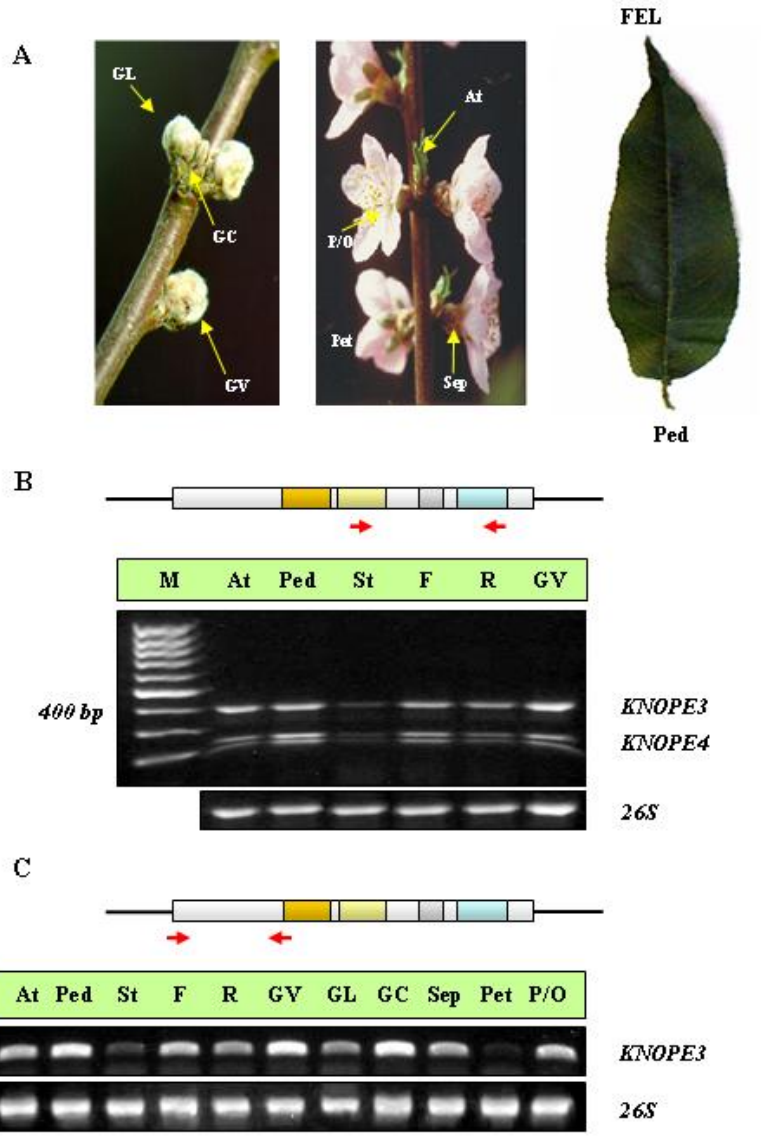


Figure 1. Tissue specific expression. (A) Organs and tissues monitored for *KNOPE3* and *KNOPE4* expression. **(B)** Message abundance of *KNOPE3* and *KNOPE4*. RT-PCR was performed using two primers conserved perfectly (in red, see chapter 2) in both genes. PCR products were NcoI restricted to distinguish the two transcripts. NcoI cuts in *KNOPE4* but not in *KNOPE3*. Both genes were transcribed in all tissues tested and the highest abundance was in petioles and vegetative buds. 26S shows equal aboundance of transcript. **(C)** Message abundance of *KNOPE3*. RT-PCR was performed using highly specific primers for *KNOPE3* (in red, see chapter 2). The lowest abundance of the message was signalled in herbaceous stems and petals. 26S shows equal aboundance of transcript.
At, apical tips; **Ped**, pedicel; **St**, herbaceous stem; **FEL**, fully expanded leaf; **R**, root; **GV**, vegetative bud; **GL**, lateral bud; **GC**, central bud; **Sep**, sepal; **Pet**, petal; **P/O**, pistil e ovary.

and sets of cells siding on the cambium (Fig. 4C), likely to be new phloem bundles. Similarly, in stem vascular bundles the message signal occurred in phloematic cells but not in the cambium (Fig. 4E). Interestingly, no transcript featured in xylem cells and phloem cap schlerenchima. (Controls: Fig. 4F)

Shoots derived from secondary branches of mature plants and generated at the vegetative resumption of year 2006 were used as target model. RNA was extracted from a pool of the 2nd, 6th, 13th and 18th internodes of 19-internode stems and named as stage 1,2,3,4, respectively (Fig. 5A). Histological sections of 1-4 stages were stained with phloroglucinol and the wood ring was found to increase accordingly, marking the process of stem maturation (Fig. 5A). Five month old shoots were sampled (August 2006) and RT-PCR was performed using gene specific primers. The *KNOPE3* expression was down regulated from the apical to the basal internodes, so that its abundance diminished to coincide with the lignin layer increase (Fig. 5B), whereas *KNOPE4* maintained a constant level of expression along the axis. Peach key genes involved in the lignification pathway were used to mark the woodiness of the stem sectors (Fig. 5B). They were up regulated in areas with higher content of lignin in agreement with the behaviour reported for ortholog genes in poplar (CL1,4-coumarate:CoA ligase1; CCR, cinnamoyl-CoA reductase. Hertzberg et al. 2001).

***KNOPE3* is developmentally regulated in leaf.**

Transversal sections were performed on a shoot at the vegetative burst (late March, Fig. 6A), and the *KNOPE* message was monitored in the surrounding leaves (Fig. 6B). The signal was detected from the 4th leaf onwards with a differential pattern (Fig. 6C). From the 4th to the 6th leaf the mRNA stain was visible in the vascular bundles (Fig. 6D), whereas afterwards the signal spread in the mesophyll parenchyma, though absent in the outermost layer of mesophyll and the epidermis and still featured in the phloem rather than xylem of the central vein (Fig. 6F).

In fully expanded leaves (main rib length 5 cm, Fig. 7A), borne on an adult plant and sited 8 cm below the shoot apex, the *KNOPE3* transcript was detected in mesophyll and vascular bundles, but it was absent in adaxial epidermis(Fig. 7B-C). In particular it appeared to be more abundant in spongy cells than in the palisade layer (Fig. 7D).

Finally, in petiole, both the vasculature structure and mRNA localization pattern reminded the main vein of the leaf (Fig. 7F). In the bundle sheath (collenchymatic tissue), surrounding the main (Fig. 7C) and secondary (Fig. 7E) veins of the leaf and the petiole vasculature (Fig. 7F), signal was not detectable.

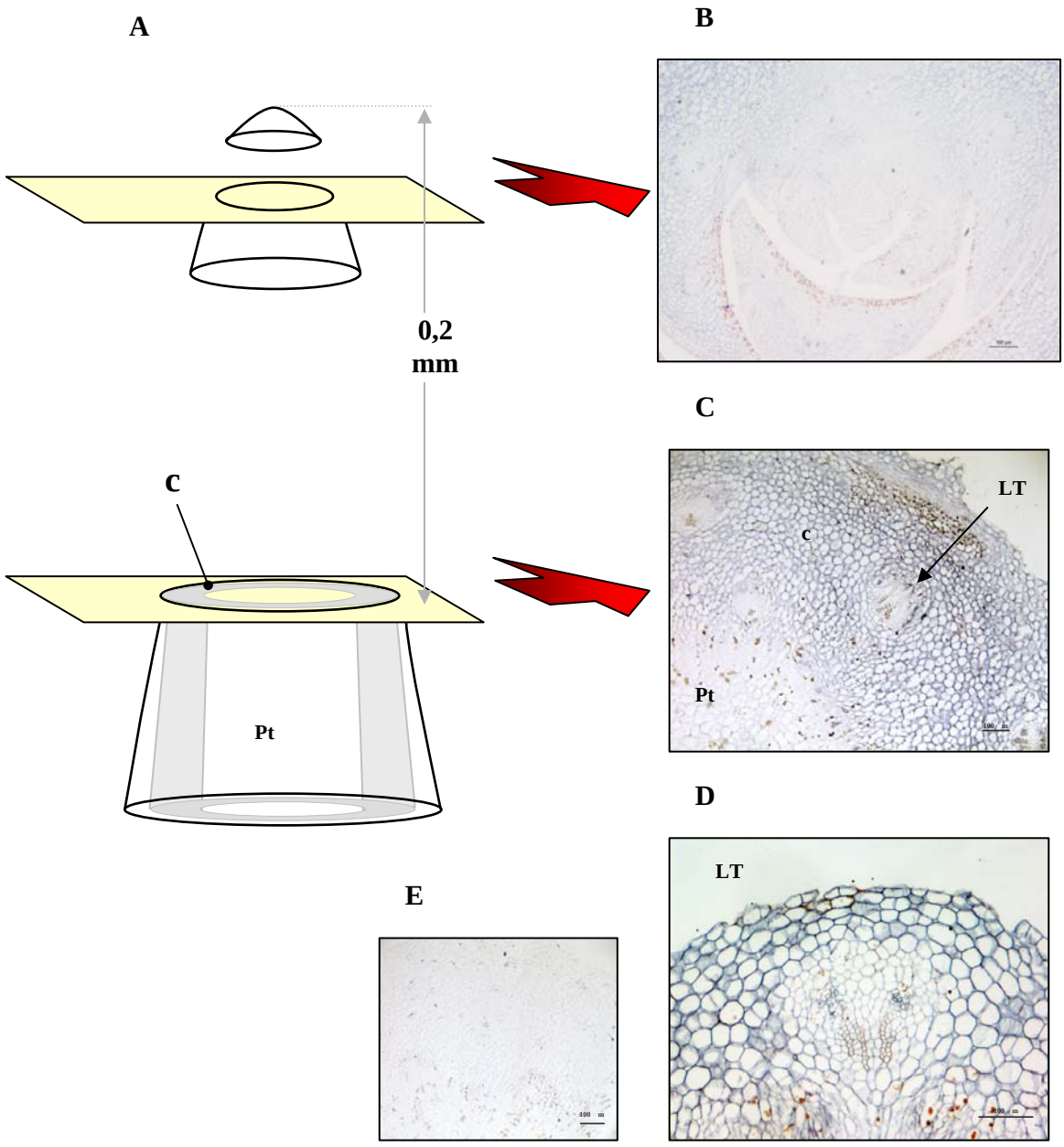


Figure 2. *KNOPE3* transcript localization in stem. (A) Schematic representation of sections position through elongating shoot (April), the blue area showed the transcript localizaton. **(B)** In shoot apical dome the signal was absent, whereas it was present in surrounding leaflets. **(C)** Transversal section at 0,2 mm below the shoot apical meristem. The signal (blue) was evenly diffused in the cortex (c) and absent in the pith (Pt). **(D)** A slightly stronger signal was observed in phloem region (P) within the bundles of leaf petioles (leaf traces, LT). **(E)** Control, no signal was observed when using a *KNOPE3* probe in sense orientation.

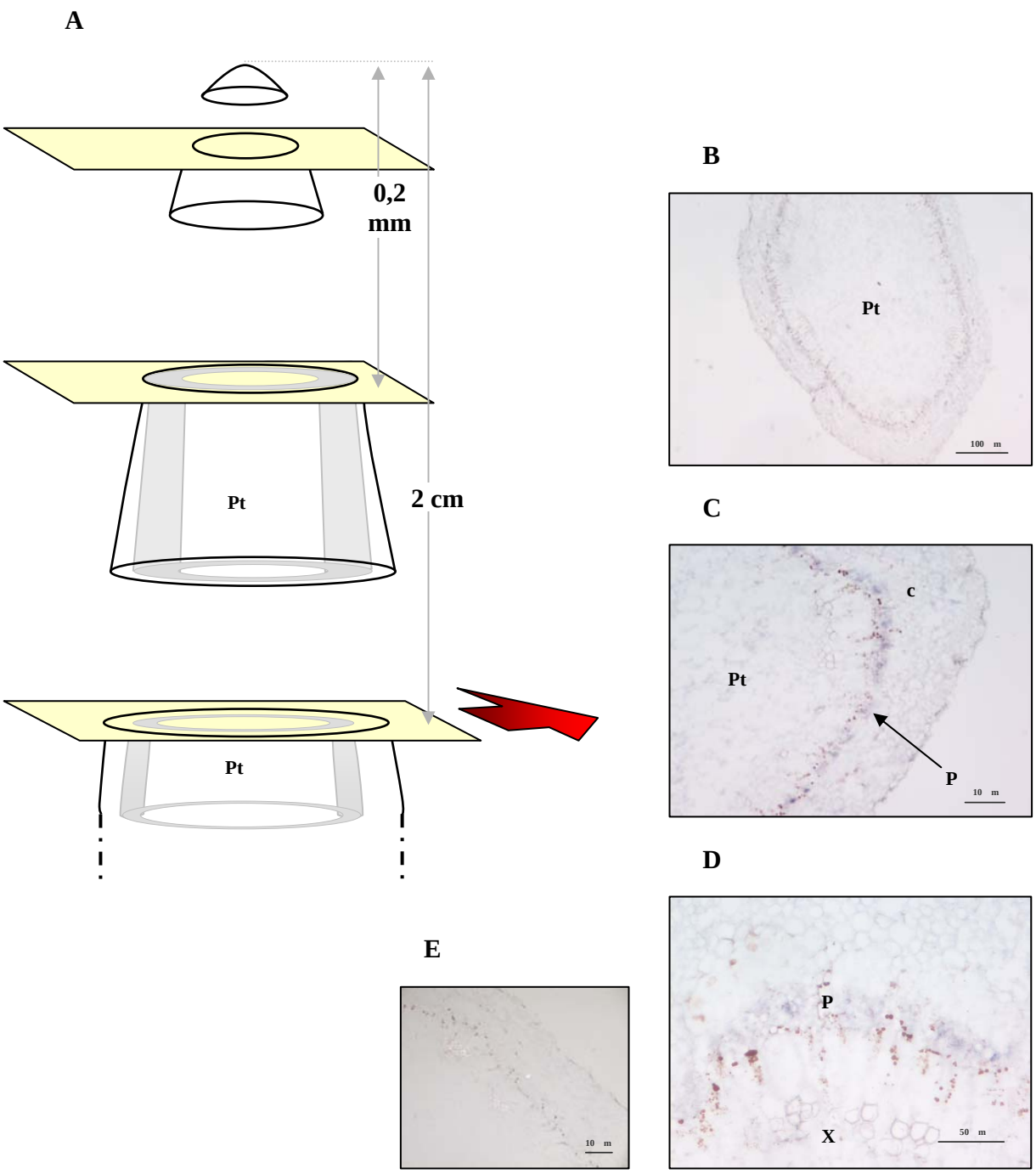


Figure 3. *KNOPE3* transcript localization in stem. (A) Schematic representation of sections position through elongating shoot (April), the blue area showed the transcript localization. **(B)** Middle sections of internodes sited ca. 2 cm below the apex. The signal marked the vascular cylinder. **(C)** and **(D)** The magnification showed that the message signaled the phloem area (P) of the vascular bundles rather than xylem cells (X). **(E)** Control, no signal was observed when using a *KNOPE3* probe in sense orientation.

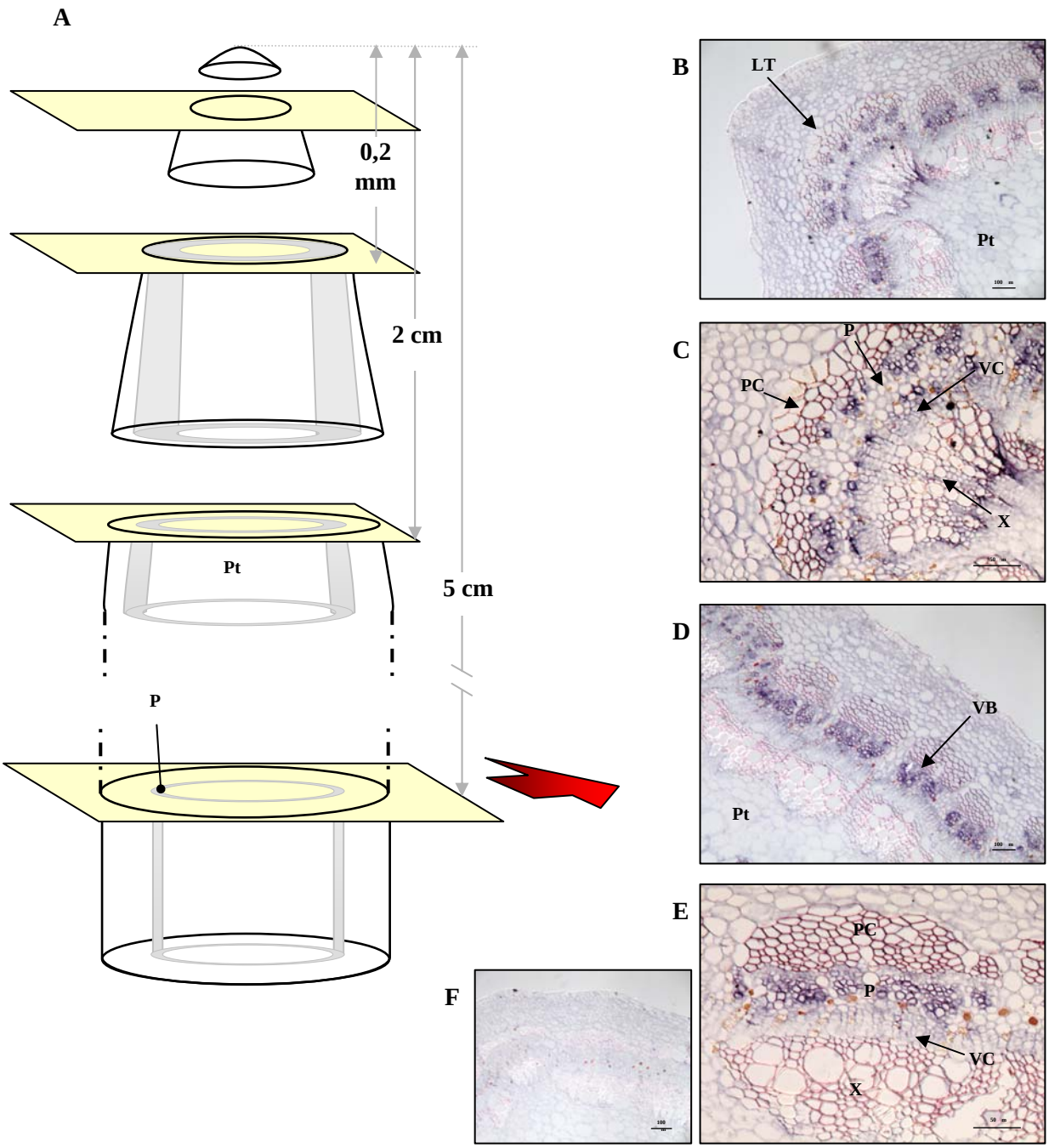
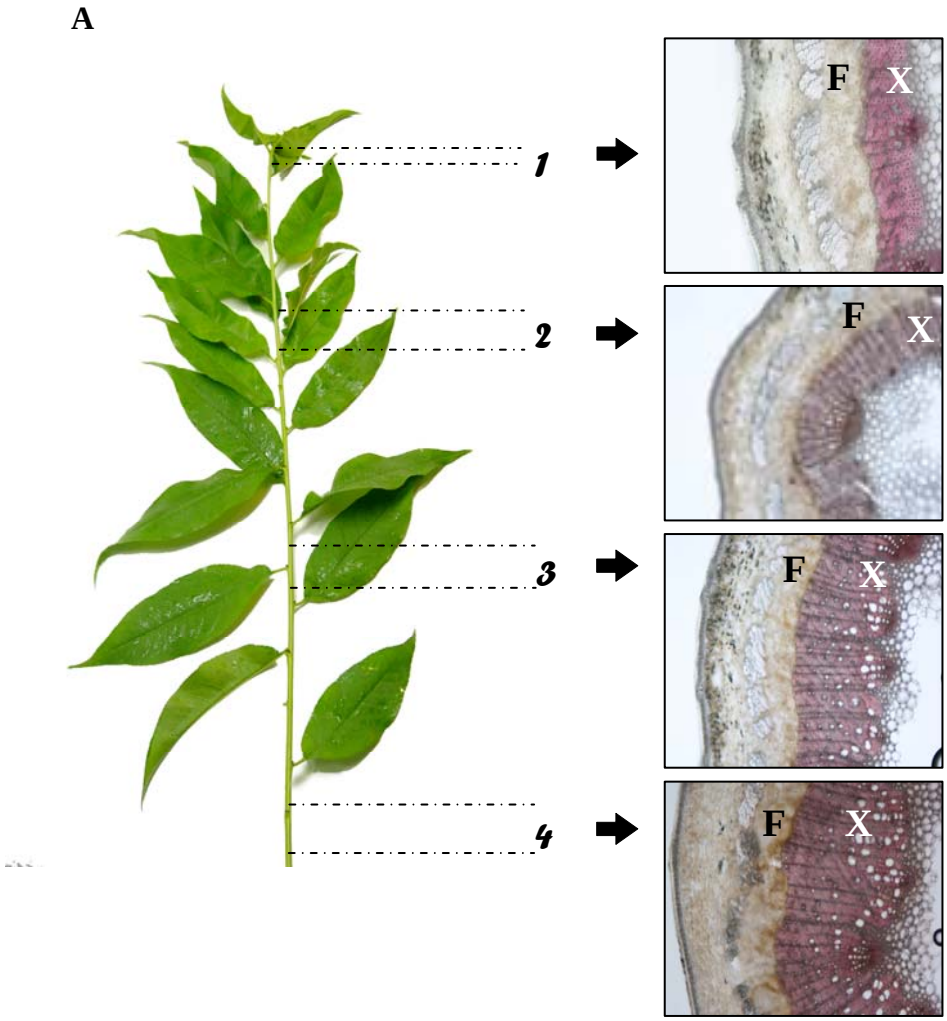


Figure 4. *KNOPE3* transcript localization in stem. (A) Schematic representation of sections position through elongating shoot (April), the blue area showed the transcript localizaton. **(B)** Middle sections at ca. 5 cm below the apex. The transcript featured bundles areas of leaf traces. **(C)** Leaf traces magnification. The signal marked groups of cells sub-adjacent to the phloem cap and sets of cells siding on the cambium. **(D)** Vascular cylinder showed an intense stain. **(E)** Magnification of vascular bundle. The signal occurred in groups of phloematic cells. **(F)** Control, no signal was observed when using a *KNOPE3* probe in sense orientation.



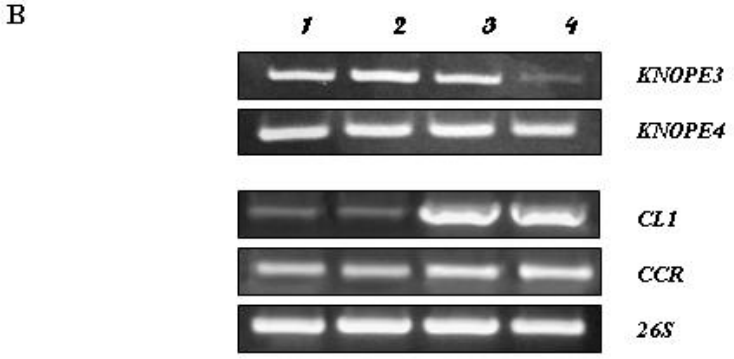


Figure 5. ***KNOPE3*** **and** ***KNOPE4*** **during stem maturation. (A)** Five month old shoot (August) was sectioned at different levels and lignin depostion (stage 1-4) was marked by phloroglucinol-HCl staining (red colour). **(B)** RT-PCR analisys. *KNOPE3* transcript level dropped at stage 4 where the highest content of lignin occurs. On the contrary, the message of *KNOPE4* maintained a constant pattern along the axis. Key genes involved in the lignification pathway were used to mark the woodiness of the examined stem sectors. They were up regulated in areas with higher content of lignin in agreement with the behaviour reported for ortholog genes in poplar (CL1,4 coumarate:CoA ligase1; CCR, cinnamoyl-CoA reductase. Hertzberg et al. 2001).

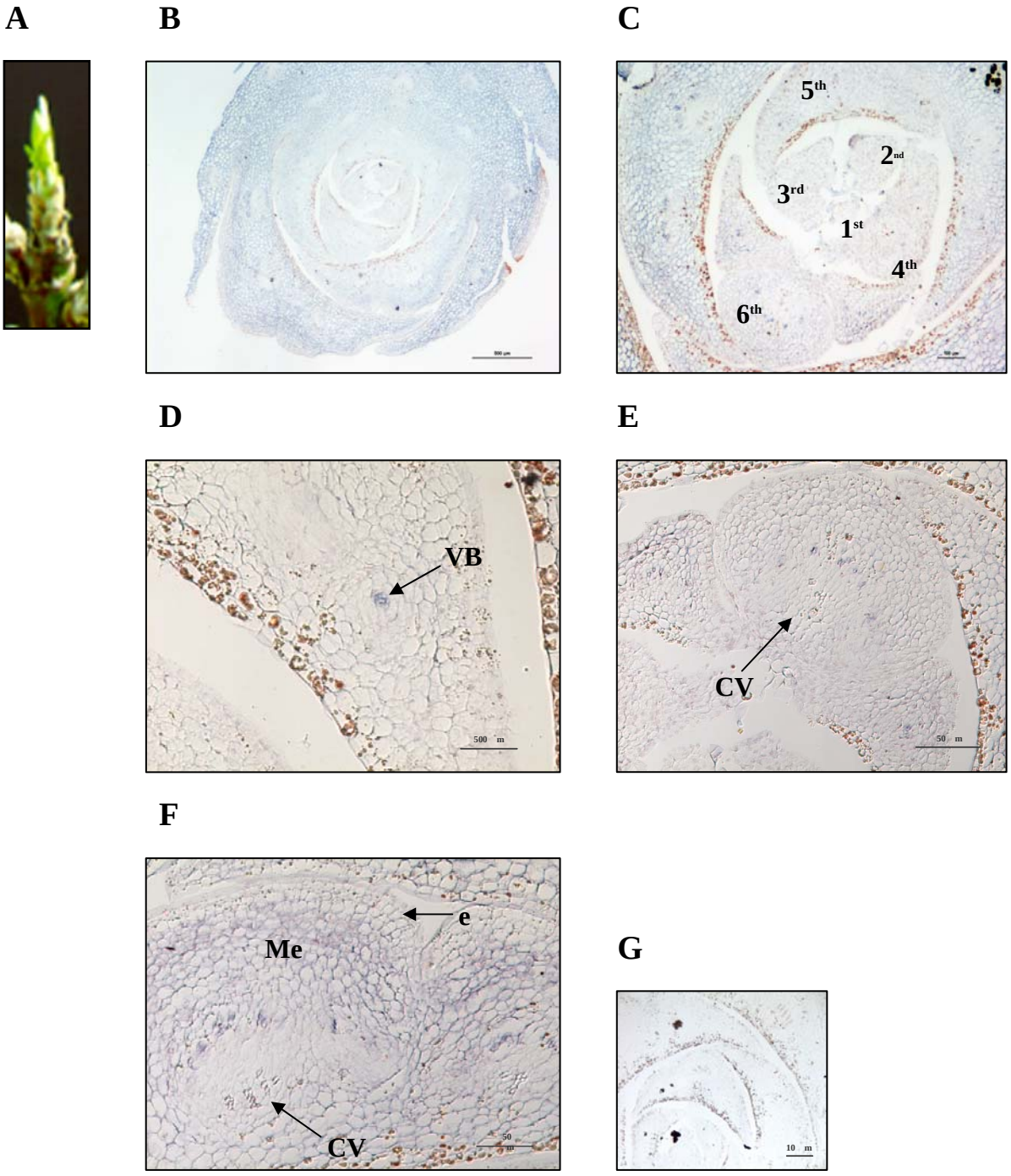


Figure 6. *KNOPE3* transcript localization during leaf development. (A) Leaves surrounding apex at the vegetative burst (late March) was monitored for *KNOPE3* transcript localization. **(B)** The signal appeared to increase from the leaf primordia to the leaflet surrounding the apex. **(C)** Number indicate peach phyllotaxis (120°). **(D)** A slight signal compared in the vascular bundle (VB) of the 4th leaf. **(E)** In the 6th leaf, central vein (CV) showed the phloem localization of the transcript. **(F)** From the 7th-8th leaf, mRNA signal spread in the mesophyll (Me), though absent in the outermost layer of mesophyll and the epidermis (e). **(G)** Control, no signal was observed when using a *KNOPE3* probe in sense orientation.

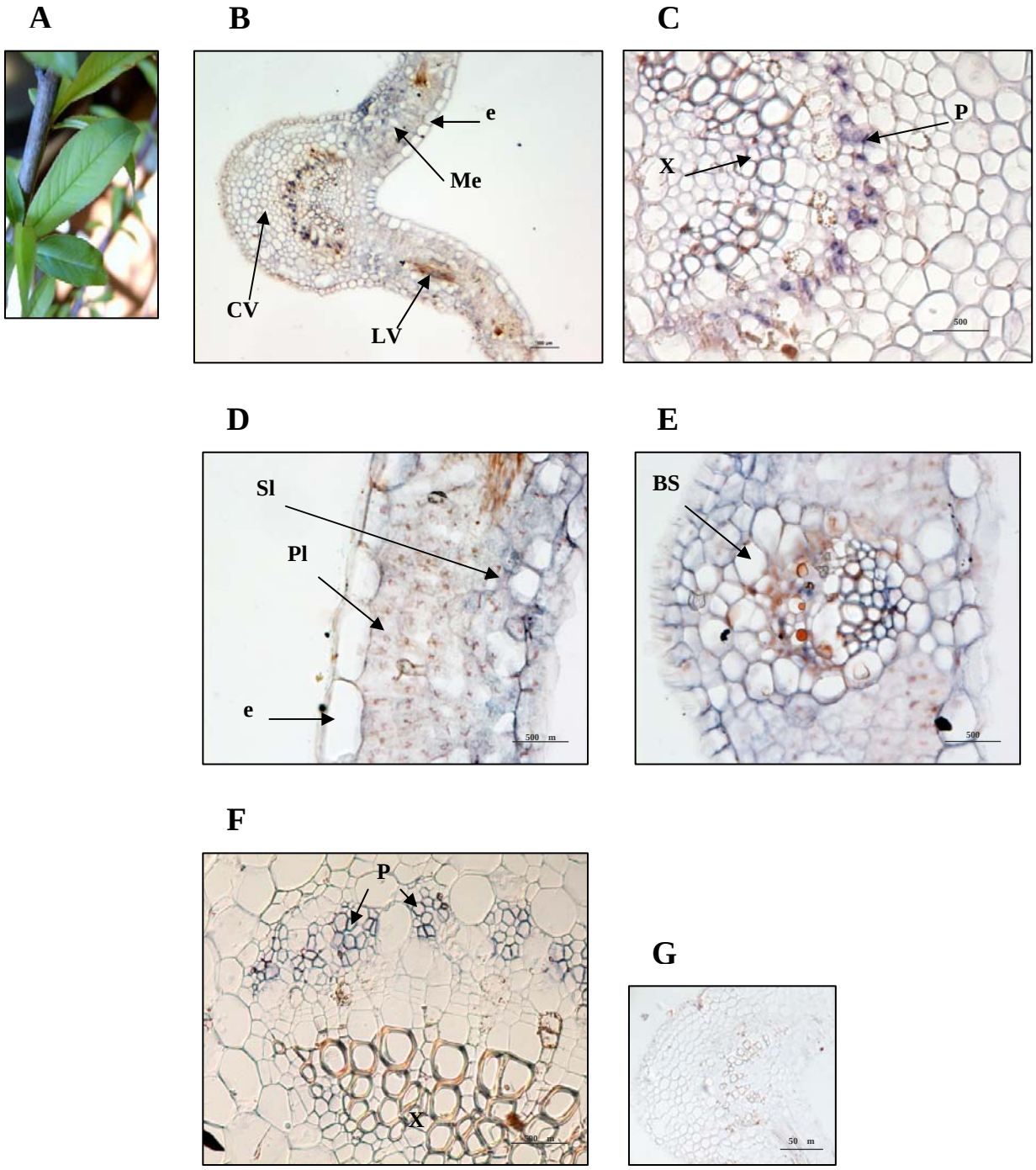


Figure 7. *KNOPE3* transcript localization during leaf development. (A) Fully expanded leaf (main rib length 5 cm) was monitored for *KNOPE3* transcript localization. **(B)** *KNOPE3* transcript was detected in central vein (CV), lateral vein (LV) and diffused in mesophyll (Me), but it was absent in adaxial epidermis (e). **(C)** The magnification of the central vein showed a phloematic localization. **(D)** In particular the signal appeared to be more abundant in spongy cells (Sl) than in the palisade layer (Pl). **(E)** In the bundle sheath (BS) signal was not detectable. **(F)** Transversal section of petiole reminded the main vein of the leaf, both in the vasculature structure and in mRNA localization pattern. **(G)** Control, no signal was observed when using a *KNOPE3* probe in sense orientation.

***KNOPE3* localization in floral bud.**

Transcript localization of *KN0PE3* was investigated during the course of floral bud before antesys (February, Fig. 8A). The message occurred in the outer cells of the stigma orifice, in the vascular bundles of the style (Fig. 9B) and in the inner layers of the ovary (Fig. 9A). It also appeared in the upper part of the filament (the connettive), between the lobes of the anthers, localized in the vascular bundle (Fig. 9D). Around the pollen sacs (Fig. 9C), the signal was in the endothecia rather than in epidermis (Fig. 9E).

The perianth (pt, petals and sp, sepals) vascular system was completely marked (Fig. 8D) and the sepals mesophyll too (Fig. 8E). A considerable signal was evident in the cortical region of the receptacle and in the net vasculature (Fig. 8C). Here it could be observed the distinctive scheme of the stele, which it alternated traces and gaps bundle of the floral organs (Fig. 8B).

***KNOPE3* transcript marks the vascular bundles of drupe.**

In fruits at very early development of canonical stage I (3mm of diameter, Fig. 10A), an intense stain indicated that the transcript was mainly localized in the vascular bundles of the mesocarp (Fig. 10B-C) but was absent in the developing seed (Fig. 10B). A faint labeling was observed in funiculus (Fig. 10D).

In fruit at later development of canonical stage I of development (5cm of diameter, Fig. 11A), the signal was detected in the vasculature of endocarp (Fig. 11B-C) and mesocarp (Fig. 11D-E). No signal was observed in the inner portion of the pulp (Fig. 11A, compact tissue).

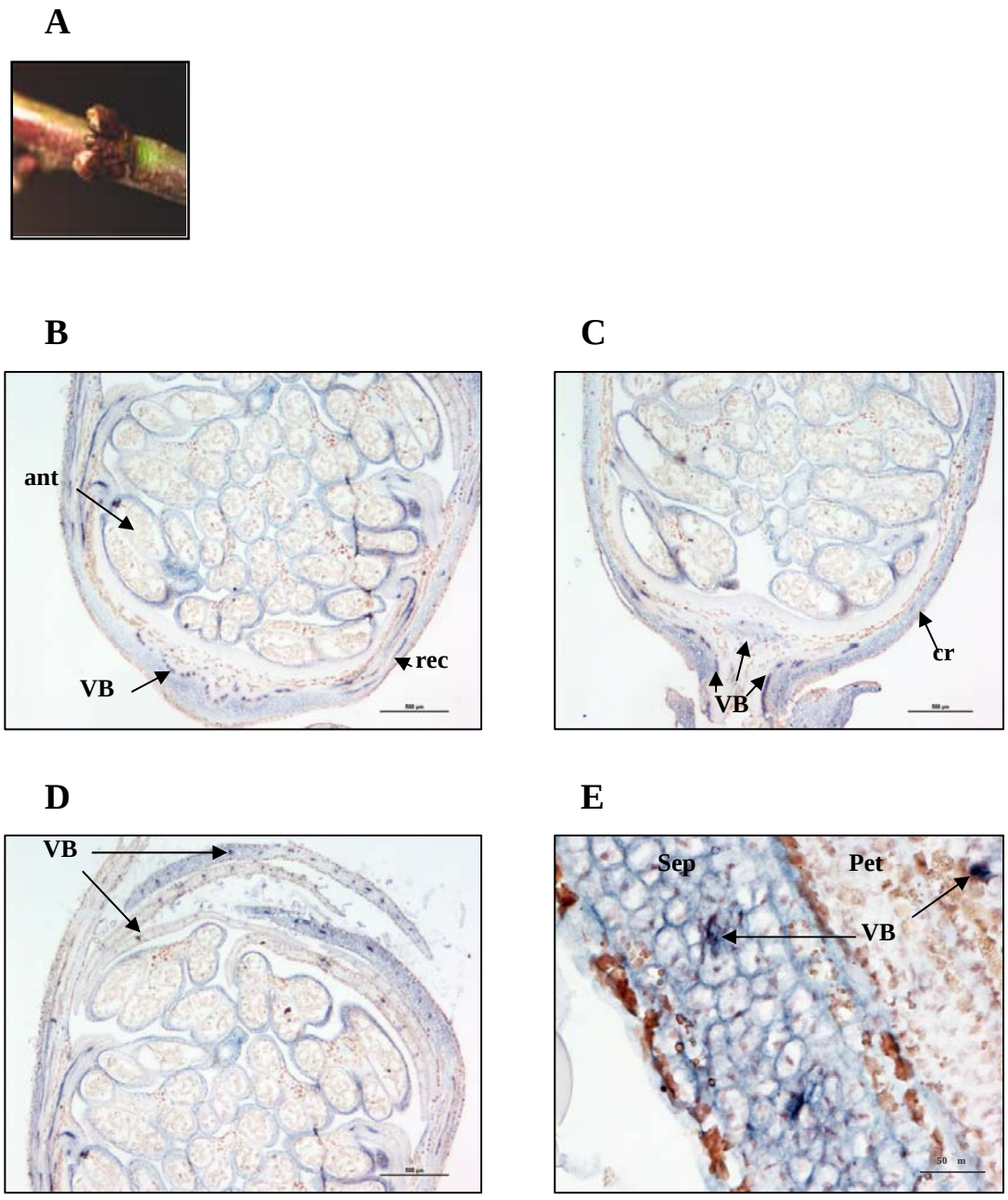


Figure 8. *KNOPE3* transcript localization in floral bud. (A) Floral bud before antesys (February) was monitored for *KNOPE3* transcript localization. **(B)** The signal was diffused in the receptacle (rec) and anthers (ant) outermost layer, moreover it marked strongly the net vasculature (VB). **(C)** Middle longitudinal section showed a signal distribution in the cortical region (cr) of the receptacle. **(D)** The transcript labelled the vasculature net of perianth. **(E)** Magnification of sepal (Sep) and petal (Pet) section. *KNOPE3* stained the sepals mesophyll rather than petal one.

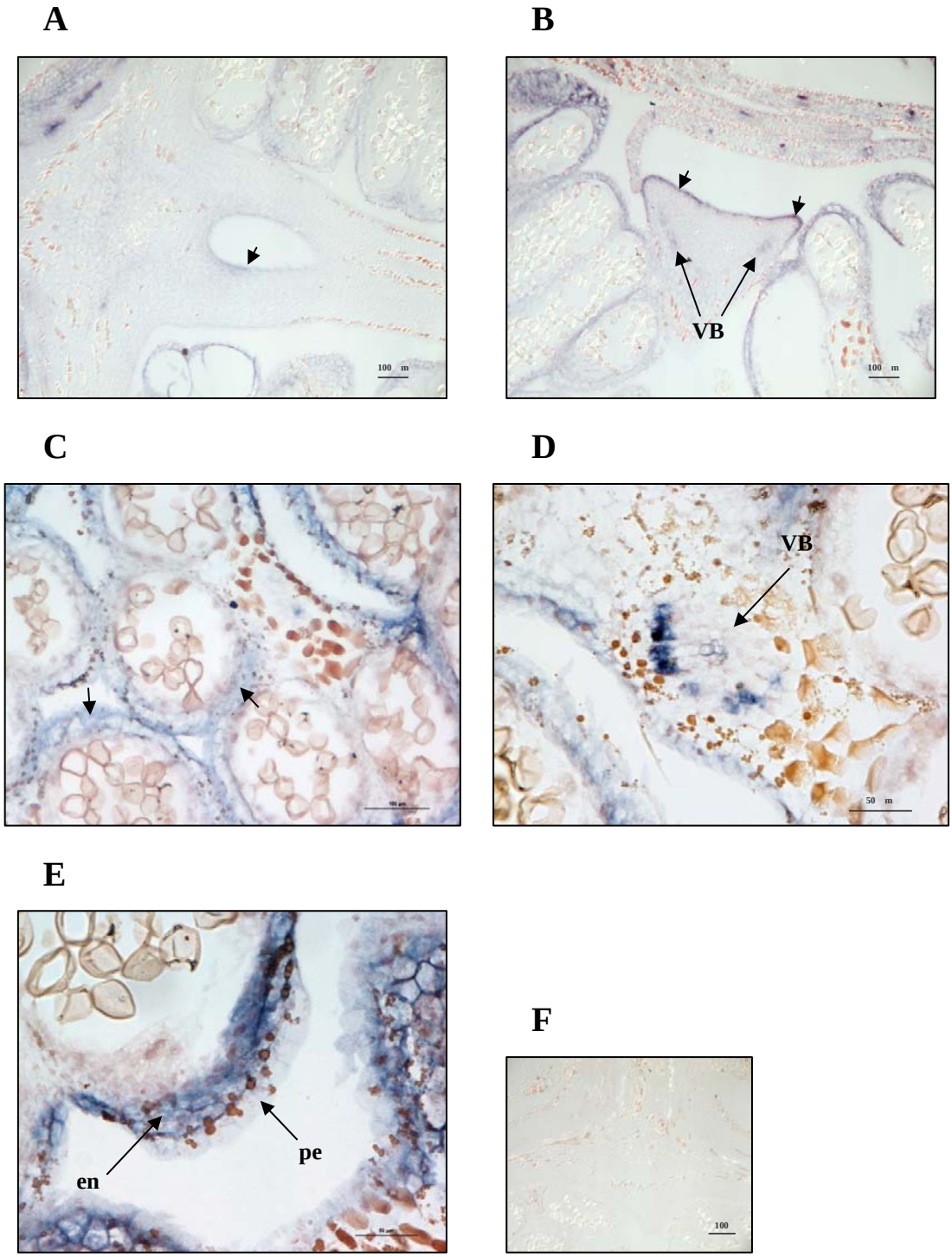


Figure 9. *KNOPE3* transcript localization in floral bud. (A) A slight signal was observed in the inner layer of the ovary. **(B)** *KNOPE3* transcript marked the stile vascular bundle (VB) and the peripheral cells of the stigma orifice. **(C)** A continuous signal stained the anthers perimeter. **(D)** Vascular bundle of filament showed a strong labeling. **(E)** Magnification of anther sidewall showed a signal accumulation in endothecium (en) rather than perithecium (pe). **(F)** Control, no signal was observed when using a *KNOPE3* probe in sense orientation.

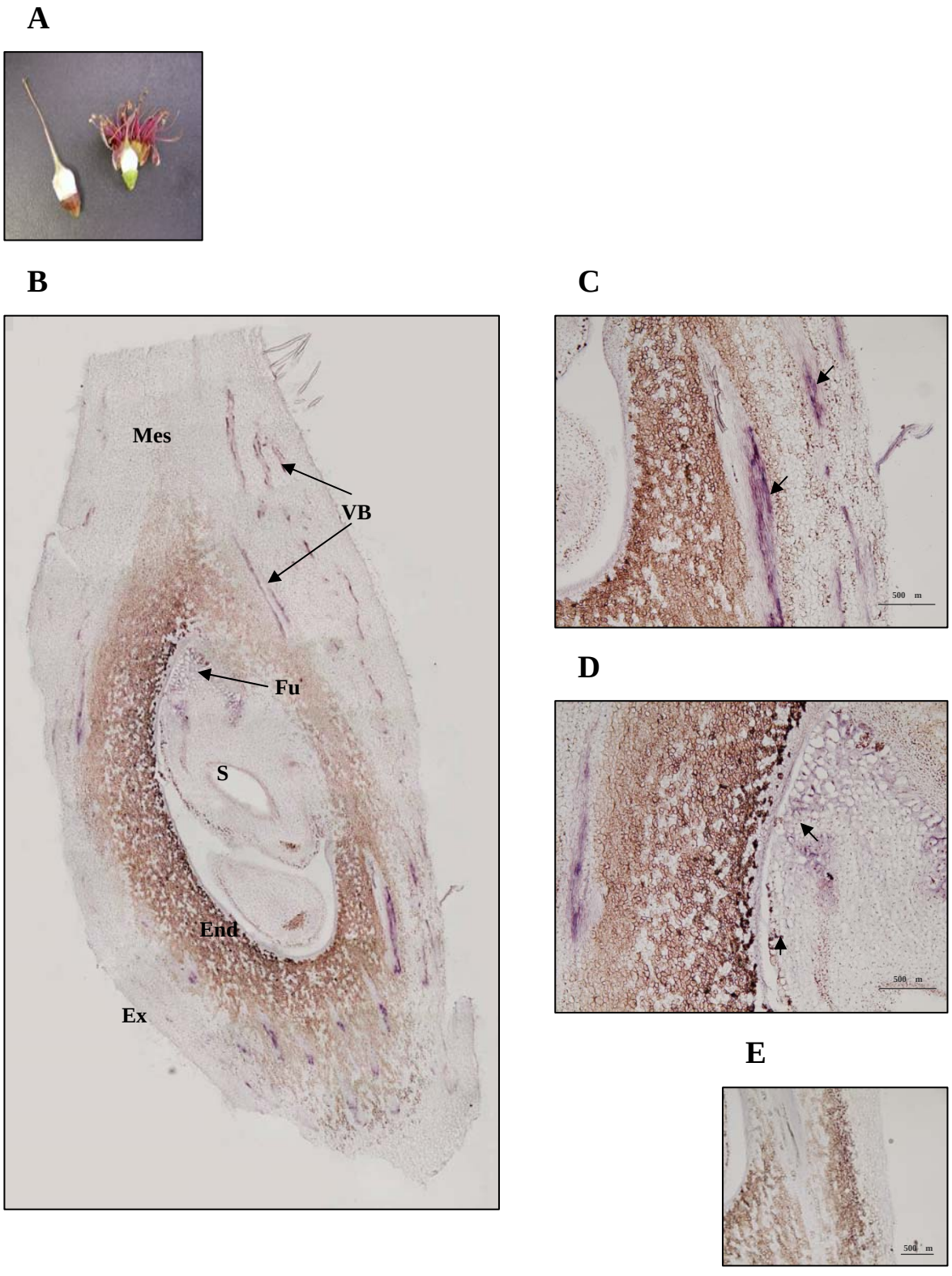


Figure 10. *KNOPE3* transcript localization in fruit. (A) Fruits at early development stage (stage I, 3 mm of diameter) was monitored for *KNOPE3* transcript localization. **(B)** Intense stain indicated the transcript mainly localized in the vascular bundles (VB), moreover a slight signal was detected in funiculus tissue (Fu). **(C)** Magnification of mesocarp vasculer bundle. **(D)** Magnification of funiculus bending. **(E)** Control, no signal was observed when using a *KNOPE3* probe in sense orientation.

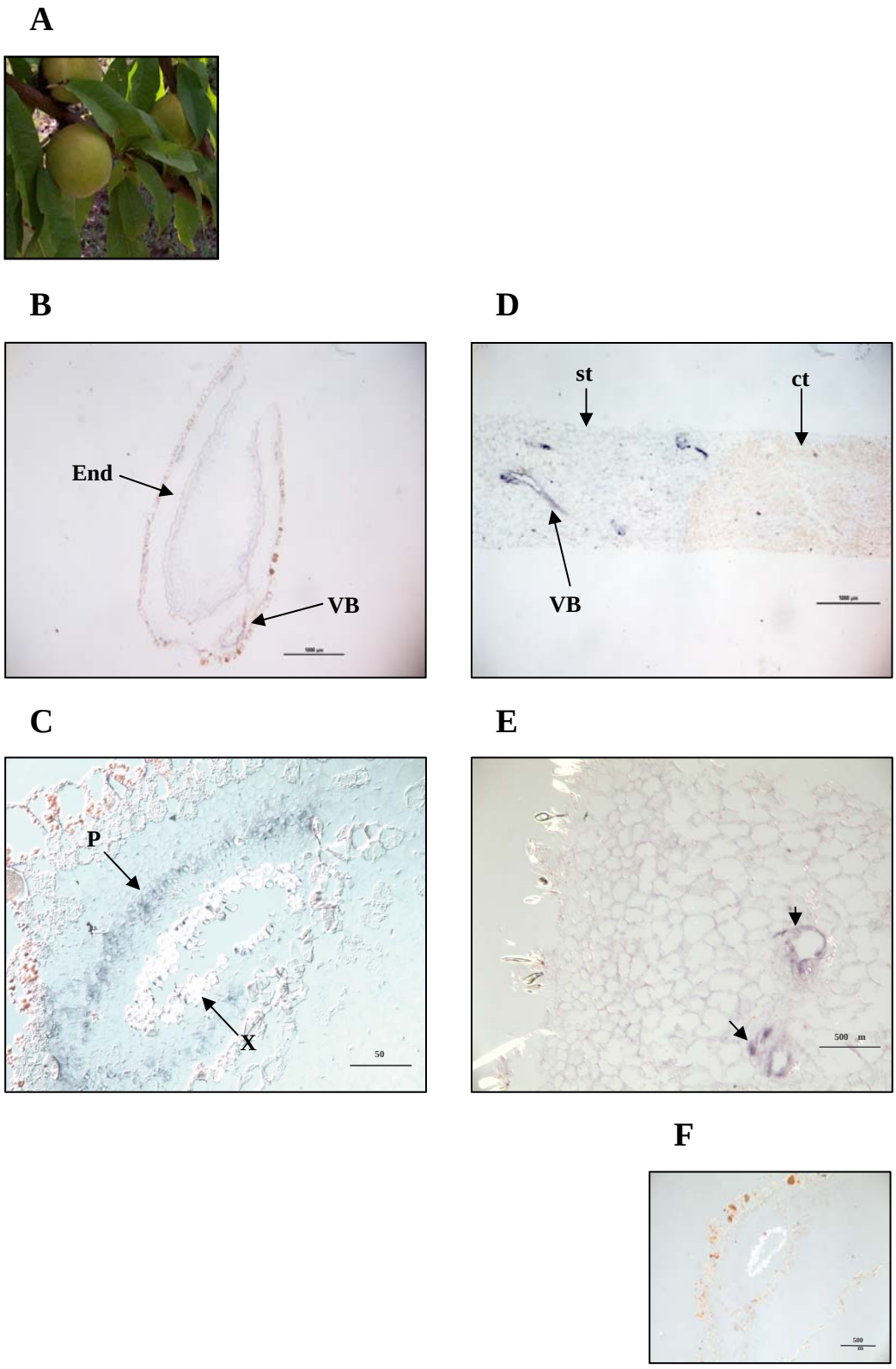


Figure 11. *KNOPE3* transcript localization in fruit. (A) Fruits at late development stage (stage IV, 5 cm of diameter) was investigated for *KNOPE3* mRNA localization. **(B)** Transversal section of the seed showed labelling in vascular bundle (VB) of endocarp wall (End). **(C)** Magnification of endocarp vascular bundle. An intense stain was detectable in phloem (P) rather than xylem (X). **(D)** Longitudinal section of mesocarp tissue. The transcript marked vascular bundle of the outer part of mesocarp (slack tissue, st); in the inner part, vascular bundle appeared absent (compact tissue, ct). **(E)** Magnification of the outer mesocarp. **(F)** Control, no signal was observed when using a *KNOPE3* probe in sense orientation.

DISCUSSION

The *KNOPE3* and *KNOPE4* transcripts appeared to be ubiquitous, though their abundances varied depending on tissues examined. These outcomes are in agreement with patterns reported for several plant species (Reiser *et al*., 2000). However, literature data concerning transcript localization have been very poor so far and are basically related to the herbaceous species Arabidopsis and tomato (Serikawa *et al.*, 1997; Jansen *et al*., 1998). Our experiments were mainly focused on monitoring the *KNOPE3* behavior during the development of organs (of agronomical interest) in a fruit tree species.

The transcription analysis of *KNOPE3* was monitored in stems along the axis of five month old shoots, which contained an increasing degree of lignification from the apex to the basis. In brief, from distal to basal internodes: the *KNOPE3* message decreased gradually, the *KNOPE4* mRNA was maintained abundant. The *KNOPE3* transcript occurred in the cortex of stem immediately underneath the apex, proceeding downwards it was confined to the phloem region and no longer in the cortex and at further distances just restricted to phloem associated cells. The gene was found to be constantly repressed in the cambia, pith, xylem, and phloem cap. Therefore, it is not surprising that the message decrease was associated to stem maturation (see also the scheme of *KNOPE3* transcript localization pattern in Fig 4A), however, the gene must play a role in distinct developmental phases of phloem cells. As for *KNOPE4* behavior, a set of experiments are needed to formulate any hypothesis, which were not addressed in this work.

There is a body of evidence that class 1 *KNOX* are involved in lignification (including those of peach, Giulio Testone, personal communication): an inverse relationship between these genes' expression and lignin deposition was observed (Mele *et al.*, 2003; Groover *et al.,* 2006). Arabidopsis *KNAT1* was also demonstrated to interact with the promoters of lignin synthesis key genes (Mele *et al.*, 2003). The class 1 *KNOX* expression patterns in stem development/maturation are consistent with the hypothesis that the SAM and the vascular cambia are regulated by overlapping genetic programs. Again, if we observe that *KNOPE3* territory falls out of the cambium, this gene must have a distinct role from class 1 genes, most likely occurring after the action of these latter. This hypothesis is further supported by the *KNOPE3* down regulation in apical meristem and the transcriptional onset from the 4th leaf, in which the message labeling marked the vascular bundles. This behavior is exactly opposite to that of class 1 KNOX, which have to be repressed to prompt the leaf initiation (Hake *et al.*, 2004). In older leaves (7th onwards) the message featured in the phloem cells and in the mesophyll, but not the epidermis. In expanded but still developing leaves, the mRNA stain just featured in the phloem vessels and companion cells and spread evenly in the mesophyll cells. An identical pattern was observed for

Arabidopsis *KNAT3* expression in the SAM and in the first lateral leaf primordia (Serikawa *et al.*, 1997), though the analysis was not extended to expanded leaves. Concerning these latter in peach, *KNOPE3* maintains the activity in the mesophyll and the phloem cells and is repressed in the epidermis, bundle sheaths and collenchyma cells that support the vascular system. The *KNOPE3* message occurrence in the developing leaf and its localization in the palisade may lead to think that the gene is also associated to photosynthetically active cells, or at least to those equipped with chloroplasts. Hence, it may be regulated by the light, and in support of this the Arabidopsis *KNAT3* promoter (a *KNOPE3* ortholog) was demonstrated to respond differently to red and far-red light (Serikawa *et al.*, 1997).

The *KNOPE3* mRNA localization profile in flower mainly confirmed the gene action in the vascular bundles of all the elements (eg.: stamen threads, ovary, pistils, sepals, petals, receptacle, pedicel etc.) Similar patterns were reported for the *Let12* transcript in tomato flowers (Jansen *et al.*, 1998). Again, the diffuse signal staining in the cortex of the receptacle and in the sepals led to think that a relationship between the gene transcription and "green tissues" may exist and be mediated by the light. It is intriguing that the mRNA marked intensely the anther endothecia, suggesting a specific role worth being investigated.

The *KNOPE3* expression has been observed in all the drupe developmental stages as the gene sequence is retrievable from fruit specific EST databanks (http://www.itb.cnr.it/estree/). In situ hybridizations at S1 and S4 stages showed that the message mainly localized to vascular bundles, including those that innervate the mesocarp and feed the seed through the funiculus. *KNOPE3* participates to fruit development from the very early to late stages, and its role appear to be tightly related to the vasculature. The proper function of the latter is fundamental for fruit development and quality and *KNOPE3* may be involved in affecting the fruit tracts.

Overall, our results present novel and detailed information on *KNOPE3* transcript localization during organ development of peach trees. Taken together these data suggest that the class 2 genes may be involved in later events, while the class 1 members have roles in meristem development. Although *KNOPE3* may be involved in a general maturation process of several tissues (Serikawa *et al.*, 1997), in peach it was always associated to phloem and companion cells and hence high likely to play a specific role.

MATERIALS AND METHODS

RT-PCR analysis.

Tissue specific expression.

Total RNA was extracted from tissue plants as reported in Giannino *et al.* (2000), DNAse-treated (RQ1, Promega), quantified by both spectrophotometer assays and by comparing with standard scales in ethidium-bromide stained gels. DNA-free RNA (3 μg) was reverse transcribed at 55°C into a single strand cDNA by olido-$(dT)_{20}$ by Superscript III-RT and following the manufacturer's instructions (INVITROGEN). *KNOPE3* and *KNOPE4* transcripts (391 nt and 387 nt, respectively) were detected by KNIIFW1 and KNIIBW2 primers. *KNOPE4* was distinguished by the presence of a single nucleotide polymorphism which create a NcoI restriction site. *KNOPE3* transcript (5' region) was also detected with KN3.0 FW and KN3.9 BW (5'-GGCTAAGTACTCGGCTTTGGGC-3') primers. Peach *26S* rRNA transcript was isolated by 26SFw (5'-AGCATTGCGATGGTCCCTGCGG-3') and 26SBw (5'-GCCCCGTCGATTCAGCCAAACTCC-3') and used to verify the same cDNA amount in tested tissues.

Expression during stem maturation

Total RNA was extracted from stem tissue as described above. As for *KNOPE3*, transcript (391 nt) was detected by KN3.0 FW/KN3.9 BW, whereas for *KNOPE4* (590 nt) by KN4.14FW (5'-CTCTCTCTCTTTCTCTCAGC-3') / KN4.21BW (5'-CCAAGTCATGGATGAGAAAG-3').

Peach EST sequences PP_LEa0033M22f and PP_LEa0024P21f (EST database: http://www.itb.cnr.it/estree), highly homolog to 4-coumarate:CoA ligase 1 (*CL1*) and Cinnamoyl-CoA reductase (*CCR*) of Arabidopsis, respectively, which are involved in lignification process, was used as xylem deposition markers. *CL1* transcript (515 nt) abundance was detect by CL1Fw (5'-ACAAATCAAGTATCGTCTGACCCTG-3') / CL1Bw (5'-GTGTTGCTATCAAGTCCTTCCGC-3') whereas *CCR* transcript (554nt) by CCRFw (5'-CTTTGGCTGAGGACGCTGC-3') / CCRBw (5'-CACTAAACCAAGAACACCAACCG-3').

Histochemical stains.

Fifty micrometers of fresh tissue, secondary branch (five months old) of mature plants, were sectioned by vibratome in presence of 0.4 M Mannitol and 50 mM HEPES. For lignin visualization, the tissues were stained with phloroglucinol-HCl [1% (wt/vol) phloroglucinol in 6N HCl] for 5 min and then observed under a light microscope.

In situ hybridization.

Excised tissues were fixed, dehydrated, embedded in paraffin, cut into 8 μM sections and hybridized (53°C) to a digoxygenin-labelled antisense RNA probe as described by Cañas *et al.* (1994). The fragment spanning *KNOPE3* cDNA stretch (9–405) was linearised by SpeI and NcoI. Digoxigenin-labelled RNA anti-sense and sense probes were *in vitro* synthesized by T7 and SP6 polymerases, respectively (Giannino *et al.*, 2000).

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CHAPTER V

KNOPE 3 and hormones relationships: gene response to cytokinin and auxin treatment and to pathogens causing hormone alteration

(With: Giulio Testone, Adriana Chiappetta, Domenico Mariotti, Maria Beatrice Bitonti and Donato Giannino)

Summary

The *KNOPE3* transcription was found to be affected negatively in leaves treated with auxin and cytokinins. The gene message disappeared in a time lapse comprised between 0.5 and 2 hours post 6-BAP treatment and was restored within 4 hours, whereas the gene expression was progressively down regulated in 4 hour time lapse following the IAA treatment. The leaf distortions caused by the parasite *Taphrina deformans* are known to be associated to cytokinin and auxin misbalances. The *KNOPE3* transcript localization was monitored in healthy and infected curly leaves, in the former the message localized to the mesophyll and phloem cells, whereas in the latter the mRNA stain was just confined to a few phloematic cells, indicating the *KNOPE3* turn off in the mesophyll. Immunolocalization experiments showed that zeatin marked the vascular bundles of uninfected leaves, whereas it was scattered evenly in curled sectors of attacked leaves and intensely signaled in those tissues that undertook the uncoordinated cell division. The comparison between *KNOPE3* mRNA and zeatin localizations suggest that cytokinin accumulation and/or misbalance may mediate the *KNOPE3* down regulation.

INTRODUCTION

Class 2 KNOX and hormones.

Several relationships between class 1 *KNOX* genes and phyto-hormones have been assessed and are under intensive survey (a topic overview is in chapter 1). Briefly, in the meristem, class 1 *KNOX* activity repress gibberellin synthesis (Hay *et al.*, 2004), the auxin down regulates class 1 *KNOX* expression, and a mutual regulation occurs between cytokinins and class 1 *KNOX* (Hay *et al.*, 2004). As far as class 2 *KNOX* and hormones are concerned, data have been remarkably scarce. To date, Arabidopsis *KNAT3* was reported to be down-regulated in roots grown on cytokinin containing media, whereas *KNAT4* transcription was not affected (Truernit *et al.*, 2006). Regarding auxin, this hormone was demonstrated to have no effect on *KNAT3* and *KNAT5* expression in root (Truernit *et al.*, 2006).

We were not able to retrieve any data about class 2 *KNOX* and hormone relationships in aerial organs from any of the crop species reported in literature. In this chapter, *KNOPE3* transcription was monitored in auxin and cytokinin treated leaves to ascertain whether these hormones could affect the gene activity.

KNOX *roles in plant-microbe interactions.*

Transcription factors have been demonstrated to contribute to the regulation of plant responses (see chapter 1) in the interaction with micro-organisms (MO), including the control of defence systems against pathogens (Rushton and Somssich, 1998). So far, class 1 *KNOX* genes have been reported to be up-regulated in *Medicago truncatula* roots interacting with root-knot nematodes and rhizobia. The authors proposed the occurrence of common pathways of host responses to parasites and endosymbionts, leading to giant cells and nodules, respectively (Koltai *et al*, 2001).

MO may directly produce phyto-hormone like substances or induce the transcription of plant hormone genes (Maor and Shirasu, 2005). In this scenario, *KNOX* genes may play distinct roles in those MO-plant interactions (pathogenic, defense or symbiotic processes) that involve the reactivation, direct or indirect, of cell cycle-correlated genes and the alteration of host hormonal levels.

The ascomycete *Taphrina deformans* (see chapter I) causes the leaf curl disease of several *Prunus* spp., including peach. Moreover, this fungus is able to produce cytokinin and auxin-like substances (Perley and Stowe, 1966; Johnston and Trione, 1974; Sziràki *et al.*, 1975; Bassi *et al.*, 1984; Yamada *et al.*, 1990) and probably alters the balance of hormones produced by the plant itself. Due to the exiguousness of data concerning class 2 *KNOX* roles in plant-MO interactions, even in model species, we monitored the *KNOPE3* transcript and the zeatin

localization pattern during the course of leaf curl disease to assess whether any relationship existed between the gene and the hormone activities.

RESULTS

Leaf *KNOPE3* transcription is down regulated by cytokinin and auxin treatment.

Fully expanded leaves were immerged in BAP or IAA containing solutions (see material and methods) and the *KNOPE3* transcription was monitored in a time lapse of 30 minutes, 2 and 4 hours (Fig. 1). In leaves immerged in hormone-free solution, the *KNOPE3* expression was unvaried after 30 min, but lowered within 4 hr (Fig. 1, panel 1). This pattern may be due to the occurrence of a response to stress effects (e.g.: anoxia and/or wounding). In IAA-treated leaves, the message decreased at 2 and 4 hr more intensely than in leaves immerged in IAA-free solution (Fig 1., panel 3). In BAP-treated leaves, the transcript abundance fell at 0.5 and 2 hr and was partially restored within 4 hr below the level of untreated samples (Fig.1, panel 5). These patterns indicated that auxin and cytokinins negatively affect *KNOPE3* transcription with distinct action timing.

KNOPE3 transcript diminishes in leaf affected by the curl disease and the message decrease is associated to cells accumulating cytokinins.

To monitor *KNOPE3* mRNA localization in leaves challenged by *Taphrina deformans*, we referred to: a) the model of Syrop (1975a-b), which defined the developmental stages of the biotrophic ascomycete in almond leaf and related them to cyto-histological modifications of the host, and b) biochemical and histological data specifically regarding diseased peach leaves (Bassi *et al.*, 1984). (More details are reported in chapter I from page 6).

In situ hybridisation experiments were performed on: a) uninfected healthy leaves (Fig. 2A) and b) curly sectors of attacked leaves (Fig. 2D). In these latter the epidermis and the palisade layer were no longer distinguishable one from the other (compare Fig. 2B and Fig. 2E), and the fungus asci featured at different maturation stages (not in figures), corresponding to stages 4 and 5 according to Syrop (1975a-b) In healthy leaves, the transcript signal was intense in the vascular bundles and diffuse in the palisade and spongy layers (Fig. 2B). In distorted sectors of infected leaves, the message stain disappeared from the mesophyll cells and marked faintly few phloem and phloem-associated cells (Fig. 2E). The cellular disorder causing curliness

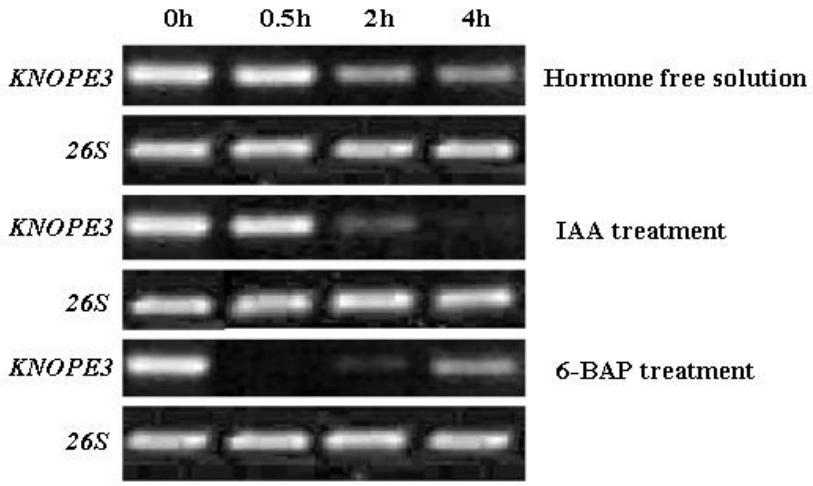

Figure 1. *KNOPE3* expression analysis in leaf treated with cytokinin and auxin.
1st panel. Fully expanded leaves soaked into hormon free solution were used as controls.
3rd panel. Leaves were soaked into a solution containing 6-Benzyl Amino Purine (6-BAP) sampled after 0.5, 2 and 4 hours.
5th panel. Leaves were soaked into a solution containing Indol Acetic Acid (IAA) sampled after 0.5, 2 and 4 hours.
RNA was isolated from a pool of treated leaves and RT-PCR was performed to reveal message of *KNOPE3*. 26S message was used to check for an effective reverse transcription and that an equal cDNA synthesis from distinct tissues occurred.

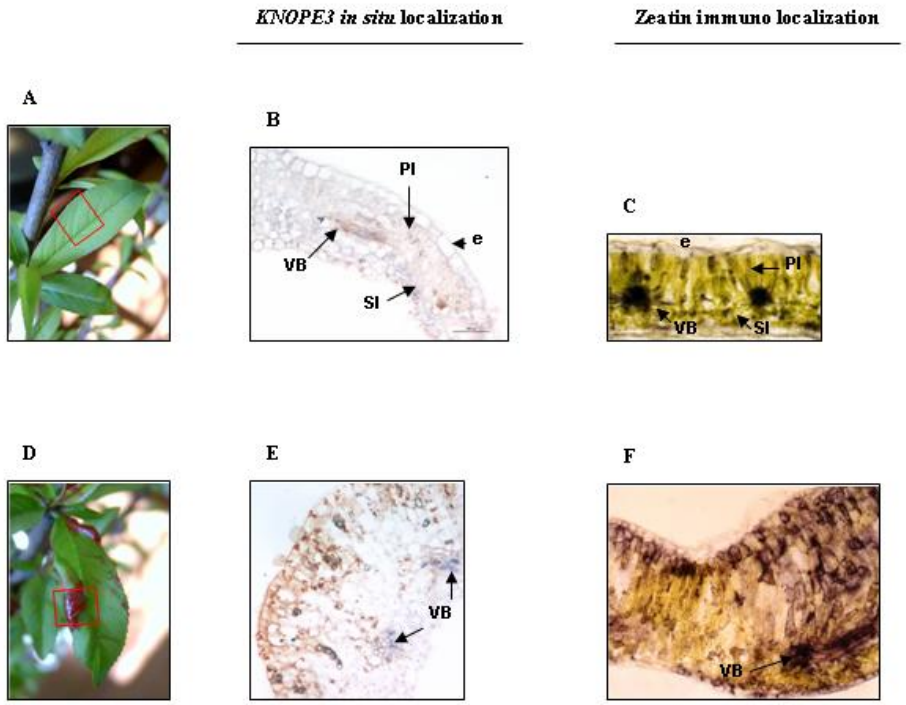

Figure 2. *KNOPE3* is down regulated in leaf affected by the curl disease. (A) Healthy leaf monitored for transcript localization. The sector analyzed is boxed in red. **(B)** Leaf lamina showed tissue differentiation in palisade (Pl) and spongy layer (Sl). *KNOPE3* labelling diffused in mesophyll and it marked strongly the vascular bundles (VB). No signal was detected in epidermis (e). **(C)** Zeatin signal strongly marked the vascular bundles (VB). **(D)** Infected curl and reddish leaf. The sector analyzed is boxed in red. **(E)** Leaf lamina exhibited outgrowth on ad axial side and the palisade and spongy differentiation was not recognizable. The message abundance decreased significantly in mesophyll cells, but persisted in vascular bundles (VB). **(F)**. Zeatin labelling spread in the upper layers of the mesophyll.

suggested the occurrence of hormone misbalances, hence, zeatine (Z) specific iits transcript mis-location is accompanied by zeatin accumulation.

RT-PCR analysis evidenced that *KNOPE1* transcript sensibly increased in curly-swollen sectors of leaves challenged by the pathogenic fungus *T. deformans* with respect to healthy leaves (data not shown). In attacked leaves, *KNOPE1* message featured in palisade cells in green to red areas and it was spread abundantly in all those sectors affected by cell disorders. On the contrary, *KNOPE1* was expressed at very low level in vases of healthy leaves. Moreovimmuno-localisation was performed on both healthy and infected leaves. In the former, Z localised to the vascular bundles as a large spotted and intense signal (Fig. 2C), which likely encompassed phloem cells, companion cells and parenchimatous cells associated to the phloem. In distorted sectors of attacked leaves, the Z signal spread chaotically in the areas of disordered cell division (Fig. 2F). In control experiments, tissues were hybridized with a sense probe for the *in situs* and with just the secondary antibody for immunolocalization. In both cases, no signal above background was detected (data not shown).

DISCUSSION

The peach *KNOPE3* response to hormones was tested in fully expanded leaves treated with 6-BAP and auxin independently. The transcription was repressed within 30 minutes and lowly restored within four hours after 6-BAP treatment. This suggests that the gene perceived sensibly and rapidly the exogenous cytokinin supply, though the hormone effect appeared to be strong in a short time lapse since a certain degree of transcriptional restore occurred. Cytokinin negative effects on Arabidopsis *KNAT3* expression were observed in roots (Truernit *et al.*, 2006). Moreover, in *Medicago truncatula*, most of class 2 *KNOX* genes were down regulated in leaves after cytokinin treatments (Di Giacomo et al., unpublished, personal communication, IBBA-CNR). Moreover, in Arabidopsis plants over-expressing the peach class 1 *KNOPE1*, which putatively accumulate cytokinins, preliminary RT-PCR analyses indicated that *KNAT3* was down-regulated (Testone et al., unpublished, IBBA-CNR laboratory). Taken together, these findings are in agreement with our results that assess *KNOPE3* down regulation by cytokinin. As for auxin treated leaves, the *KNOPE3* mRNA gradually decreased in the four hour time interval. At the moment, the available literature reports that *KNAT3* and *4* expressions in roots are not affected by auxin treatments. Our results suggest that *KNOPE3* may act with tissue specific

response to auxin signals, though further investigations are necessary (e.g. *KNOPE3* auxin response in other organs).

The *KNOPE3* down regulation and transcript disappearance from the mesophyll was observed in leaves attacked by the parasite *T. deformans* as compared to healthy leaves. As a consequence of a collaboration between the IBBA-CNR and the Botanics Laboratory of the UNICAL, the peach class 1 *KNOPE1* gene was demonstrated to be triggered in affected leaves and hypothesised to activate the process of uncoordinated cell division and stimulate the de-novo cytokinin synthesis (Testone *et al*., unpublished; see also Frugis *et al.*, 2001; Ori *et al.*, 2000). *KNOPE1* is a meristem specific *KNOX* gene inactive in healthy leaves and prompted in palisade layer at very early stages of infection. Class 1 *KNOX* genes are proposed to maintain the cell identity undefined (Hake *et al.*, 2004) and this would be consistent with the histological pattern of curly sectors, generated by the distinct division speed between the upper and the lower cell layers. In chapter IV, all the *in situ* experiments showed that *KNOPE3* message was absent in meristem cells, including the SAM and stem cambium. We proposed that *KNOPE3* acted subsequently to class 1 *KNOX* genes and that the transcript domain of these latter excluded *KNOPE3*. In simple words, *KNOPE3* accompanies the late events of cell differentiation and/or of organ development (e.g: maturation), though a phloem specific role must be considered. Taking into account these back up data, we speculate that the re-activation of meristem identity of the mesophyll cell in curly leaves inhibits the *KNOPE3* expression.

The results of these experiments stimulate to investigate on the factors that repress *KNOPE3* in the mesophyll, the role of *KNOPE3* in plant response and the relationships between the gene and Z during the disease course. Several "signals" may establish the *KNOPE3* down-regulation in mesophyll cells: *T.d*. produces cytokinins and auxin (Johnston and Trione, 1974; Yamada *et al.*, 1990) likely to play roles in hyperplasia and hypertrophy. Consequently, the mesophyll cells may respond to this stimuli with *KNOPE3* activity turn down, which was as also observed in leaf hormone responsive assays. Our experiments showed that zeatin signal mainly localised to the vascular bundles of healthy leaves, whereas labelled in a scattered manner the mesophyll cells of abnormal areas, where Z abundance was estimated to increase per surface unit (though not measured in this work). As said before, *T.d.* was reported to produce cytokinin and auxin like compounds in very low amounts per litre of culture medium (Johnston and Trione, 1974). The anti zeatine antibodies used in our experiments were not tested on *T.d.* culture extract to ascertain the specificity for plant Z so as to exclude cross reactions. However, the patterns of localisation evidenced that the Z signal occurred inside the plant cells rather than in the intercellular spaces (apoplast) where the fungus lay. *T.d* is an ectoparasite, which has not austoria, contrary to most of the ascomycetes. Several attempts to provide plant tissues with *T.d.*

cytokinin and auxin-like compounds failed to reproduce those cellular disorders observed in affected leaves (Johnston and Trione, 1974). In this context, we propose that misallocated and increased cytokinins observed in infected peach leaves are produced by the host.

It is hard to hypothesise a role for *KNOPE3* in mechanisms of defence or susceptibility against the pathogen, though transcription factors are involved several and overlapping genetic programmes (see pages 23 and on in the chapter I). The fungus is speculated to benefit from cytokinin excess for two reasons: a) kinetin mobilizes (Muller and Leopold, 2004) metabolites as nutrition supply for the fungus and b) dividing cells with non thick walls and dense cytoplasm favour the colonisation of an apoplastic parasite. In this context, if class 1 *KNOX* up-regulation causes cell division by cytokinin mediation, then the pathogen would benefit from the gene activation. Hence, the meristem *KNOX* may represent genes for susceptibility and pathogen targets. After class 1 *KNOX* triggering and reactivation of cell division, class 2 *KNOX* members, including *KNOPE3*, would be consequently repressed.

Summarizing results from this work, literature and unpublished data, we can outline the following points: a) *KNOPE1* acts in SAM domains (Testone et al., unpublished) which exclude *KNOPE3* (chapter IV of this thesis) and cytokinins accumulate in peach SAM (Chiappetta et al., unpublished, UNICAL); b) *KNOPE1* ectopic expression was associated to cytokinin overproduction in transgenic Arabidopsis and *KNAT3* (*KNOPE3* ortholog) was down regulated (Testone et al., unpublished); c) *KNOPE1* triggering was followed by zeatine synthesis in leaf affected by the curl disease (Testone et al., unpublished), whereas *KNOPE3* message drop was associated to cells accumulating Z (chapter V of this thesis) and d) exogenous cytokinin supply repressed *KNOPE3* expression in leaf (chapter V of this thesis). As a consequence, we would like to propose that *KNOPE3* transcription, and that of class 2 *KNOX*, may be regulated (in a negative fashion) by class 1 *KNOX* through a mediation of cytokinins. This hypothesis requires a sound set of supporting experiments which represent one of our future goals.

MATERIALS AND METHODS

Leaf Transcriptional Response to Cytokinin and Auxin Exogenous Treatments.

Fully expanded leaves (mid vein average length 8 cm) borne on the primary axis of 3 month old seedlings (average height 15 cm), grown in the green house, were excised at the petiole-stem join point (August, 4.00 p.m.). Leaves were gently immerged into sterile tubes filled with 50 ml buffer containing a) Benzyl Amino Purine 10μM (BAP)/0,1 μM NaOH and b) Indol Acetic Acid 50μM (IAA)/0,1 μM and c) 0,1 μM NaOH (control buffer). The 0,1 μM NaOH was necessary to dissolve the hormones. Samples were kept at 22°C under light intensity of 100 μmol $m^{-2}s^{-1}$ PAR and removed from the buffers after 30 min, 2 and 4 h and frosted into liquid nitrogen. Total RNA was

extracted from cold pulverized tissue (0,5 gr) of two leaves, DNAse treated and reverse transcribed as previously described. The cDNA of untreated leaves were used as control. The assay was repeated using the leaves borne on two other distinct plants and the same procedure as described above was carried out. PCR parameters were those reported in the previous chapters: starting from 3 µl of the cDNA batch, 40 PCR cycles were performed to detect *KNOPE3* and *CycD3* transcripts, whereas 20 cycles for *26S* rRNA; 15 µl was electrophoresed in a 1% agarose gel. Reverse transcribed *26S* rRNA appeared to be equal in all the tissues tested

Cytological-histological analyses and zeatin immuno-cytolabelling in leaves.

Leaves affected by the curl disease exhibiting clear symptoms and healthy leaves (n=3) were sampled from 3 distinct adult plants in the open field. As for cyto-histological exams, procedures were described in Giannino *et al.*, 2000. Concerning zeatin localisation, the leaf portions were pre-embedded, cut using the vibratome (Leica VT1000E, Bensheim, Germany) and incubated with primary antibody against zeatin according to the procedure described by Dewitte *et al.* (1999). Colloidal gold (<1 nm)-labelled secondary antibodies (1:40, Aurion, Wageningen, The Netherlands) were used. The fixation procedure hampered the detection of conjugate zeatin and hence the antibodies specifically recognised free cytokinin in the tissues.

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CHAPTER VI

Unraveling *KNOPE 3* function: approaches and perspectives

(With: Giulio Testone, Elisabetta di Giacomo, Domenico Mariotti , Maria Beatrice Bitonti and Donato Giannino)

Summary

To better investigate on the *KNOPE3* biological function we followed two approaches: the *KNOPE3* overexpression in *A. thaliana,* and the virus induced down-regulation in peach. *Arabidopsis thaliana* ecotype Landsberg was genetically transformed with *35S:KNOPE3* construct and the T_1 progeny examined. Six plants were confirmed to express the peach gene and appeared to be stunt with different degrees of severity as compared to controls. Major altered traits were: reduced number and lamina surfaces of leaves, thickened and enlarged petioles, highly prolonged vegetative phase, abnormal flowers which did not produce siliques. These mutants are under a more accurate characterization.

In order to test the potential efficiency of virus induced gene silencing (VIGS) technology, peach plants were inoculated with constructs derived form tobacco tobravirus and engineered to trigger silencing of the phytoene desaturase gene (*PDS*). Infected plants exhibited leaf chlorosis three weeks after treatment as compared to controls; the peach *PDS* down regulation correlated to the bleaching severity and the presence of virus was associated to the phenotype. These results suggest that VIGS is feasible in peach by using tobraviruses, hence constructs to silence *KNOPE3* were prepared and are ready to be used.

INTRODUCTION

Class 2 KNOX function: a challenge in the model species.

A role for Arabidopsis *STM*, *KNAT1* and *KNAT2* has been established and class 1 *KNOX* genes are widely explored in several crops, whereas little information has been produced about class 2 *KNOX* functions. In late 90's transgenic lines in which *KNAT3* was over-expressed or repressed did not produce strong phenotypes. One explanation for why decreased *KNAT3* levels did not result in a clear phenotype was that the class 2 *KNOX* genes were at least partially redundant in function, whereas a possible explanation for the lack of an overexpression phenotype was that *KNAT3* interacts with specific partners which are present in limiting amounts (Serikawa *et al.*, 1997). In the 15th International Conference on Arabidopsis Research (11/7/2004, Berlin) a new data set for *KNAT3* and *KNAT4* mutants were presented (Chandler and Werr, 2004; abstract-poster: T02-050, http://www.arabidopsis.org/news/15ArabAbstract.pdf). The authors characterised Arabidopsis lines in which *KNAT3* and *KNAT4* function were repressed by a) the chimeric repressor interference system (CHRIS; Chandler and Werr, 2003) and b) knock-out T-DNA insertion which produced null mutants for each gene. CHRIS-*KNAT3* and knocked out lines showed longer hypocotyls, petioles, leaf blades (due to more elongated cell) and increased plastochrone as compared to controls. The decreased transcript levels of GA3ox1 in the *knat3* mutant had was proposed to represent increased gibberellin levels and cause the cell elongation phenotype Some floral and inflorescence phenotypes were also observed. The *KNAT3* expression pattern correlated with the tissues where the phenotypes were observed and authors pinpointed that KNAT3 transcript was also revealed in floral meristem and floral organs. As for CHRIS *KNAT4* and *knat 4* mutant, they appeared wild type except for an increased plastochrone, and redundancy in the function of both genes was proposed, for their proteins share 79% identity up to 98% within the homeodomains. Finally, the plant *Kalanchoe daigremontiana* was transformed with a class 2 *KNOX* gene driven by 35S promoter and transgenic lines exhibited shorter internodes, enhanced lateral branching, overall reduced height; smaller leaves with spoon shaped and with irregular margin as compared to controls (Regis *et al*., presented at the 50th SIGA congress, 2006, Naples, http://www.siga.unina.it/Convegni06.html).

Gene function in fruit trees: a second challenge for the future.

Knocking out genes is the most frequently used strategy of reverse genetics to address gene function. Peach is a recalcitrant species to genetic transformation mediated by *Agrobacteria* due to the low regeneration efficiency of infected tissues (a synopsis is in chapter 1), as also experienced in our laboratory (data not presented). Consequently, to understand the function of

peach genes we used two approaches : a) the over–expression of peach *KNOX* in *Arabidopsis thaliana* and b) virus induced gene silencing (VIGS) mediated by Agrobacteria infiltration performed on peach plants. Both approaches, if successful, offer the advantages to lead to phenotypes in shorter periods as compared to transformation of other non recalcitrant fruit trees (e.g.: cherry). VIGS technology offers the advantages to a) overcome transformation-regeneration procedures, b) repress meristem specific genes *in vivo* and c) score out phenotypes in few weeks. These aspects lead us to test the system by using tobraviruses. In this chapter we present preliminary data on a) Arabidopsis phenotypes overexpressing the peach *KNOPE3* and b) peach phenotypes in which the phytoene desaturase gene (PDS) was repressed by VIGS.

RESULTS AND DISCUSSION

KNOPE3 overexpression produces stunt phenotypes in *Arabidospis thaliana*.

The construct *35S:KNOPE3* was transferred into *A. thaliana* Ler by *Agrobacterium tumefaciens*, using the method of flower vacuum infiltration. Seeds were plated on selective media and phosphinthricin resistant plants were transferred in soil. Six plants from T_1 population were found to over express *KNOPE3* and exhibited abnormal phenotypes as compared to the wild type (Fig. 1). The latter also derived from seedlings grown *in vitro* without selective antibiotics so as to exclude that phenotypical differences of the transformants could be due to *in vitro* stress effects. The six plants had very slow growth speed, were stunted and persisted in the vegetative phase (Fig. 1B-C) Only two of them produced abnormal flowers which did not set fruits (not shown). The number and the surface of leaves were reduced and petioles thickened (Fig. 1E), the main axis and its internodes were shortened (Fig. 1D). Leaf shape appeared unaffected with respect to controls.

Serikawa et al. (1997) reported that no evident phenotype could be scored out in Arabidopsis over-expressing *KNAT3* and *KNAT4*. *KNAT3* knock out phenotypes were described (Chandler and Werr, 2004; poster abstract: T02-050, http://www.arabidopsis.org/news/15ArabAbstract.pdf): the elongated petiole and leaf lamina were hypothesized to derive from a gibberellin accumulation putatively caused by the down regulation of the GA3ox1. The *KNOPE3* Arabidopsis mutants represent a novelty and strongly suggest that *KNOPE3* has a role in development since their plant organs are affected. However, *KNOPE3* may not share functional homology with *KNAT3* and cause phenotypes other than those induced by *KNAT3* overexpression. The two genes share an identity of only 36% in the N terminus and this divergence may be responsible for species-specific roles and regulatory tasks.

In the end, *35S:KNOPE3* Arabidopsis plants deserve further and more accurate characterization, including transcript profiling of several key genes. To dissect *KNOPE3* role in organ development, genetic constructs with hormone inducible promoters are envisaged and likely to be our next tool.

Tobravirus-based vectors may be effective tools to induce gene silencing in peach.

Tobravirus (TRV)-based vectors were injected into peach to determine whether they are suitable tools to trigger post transcriptional gene silencing. The *TRV:tPDS* construct carried a fragment of tomato *phytoene desaturase* (*tPDS*) gene, which was identical to peach *PDS* (*PpPDS*), was inoculated by Agrobacteria mediated infiltration in leaflets of: a) 2-3 week old seedlings and b) one year old plants at the vegetative resumption. Approximately 21 days post-infiltration a photo-bleached phenotype was observed in the newly emerging leaves of *TRV*:*tPDS*-treated plants. Mock-treated samples were inoculated with *TRV:RNA1* and the empty vector *TRV*:*RNA2* and plants grew normally as compared to non inoculated plants and no viral symptoms featured. To confirm that the photo-bleached phenotypes were caused by silencing events, we monitored the peach *PDS* transcript abundance by RT-PCR, using a primer couple which fell outside the region of the *tPDS* fragment transcribed by the *TRV:tPDS* (Fig. 2A). Photo-bleached leaves exhibited a down-regulation of *PpPDS* mRNA levels proportionally to the severity of the mutant phenotype (Fig. 2E). In addition, both the viral *RdRP* (expressed by *TRV:RNA1*) and the *TRV*:*tPDS* transcripts were revealed in mutants, but not in controls inoculated with the empty *TRV:RNA2* (Fig. 2E). To address *KNOPE3* gene function in peach by *tobravirus*-based vectors, *TRV*:*KNOPE3* constructs were prepared, transferred into *Agrobacteria* and ready to be inoculated onto seedlings next season (Fig. 2B).

The *PpPDS* silencing is an important outcome for the study of gene function in peach, known to be recalcitrant to genetic transformation. Most of the VIGS systems exploit the engineering of viruses naturally hosted by the target plant. In our case, we managed to silence peach *PDS* by using a virus of *Solanaceae* and a highly conserved fragment of tomato *PDS*. The plum pox virus of *Prunus*. spp. is known to act with PTGS mechanisms (Scorza et al., 2001) in peach and is also responsible for the tremendous SHARKA disease (Agrios, 1997) and strong limitations for its use are imposed to the scientific community.

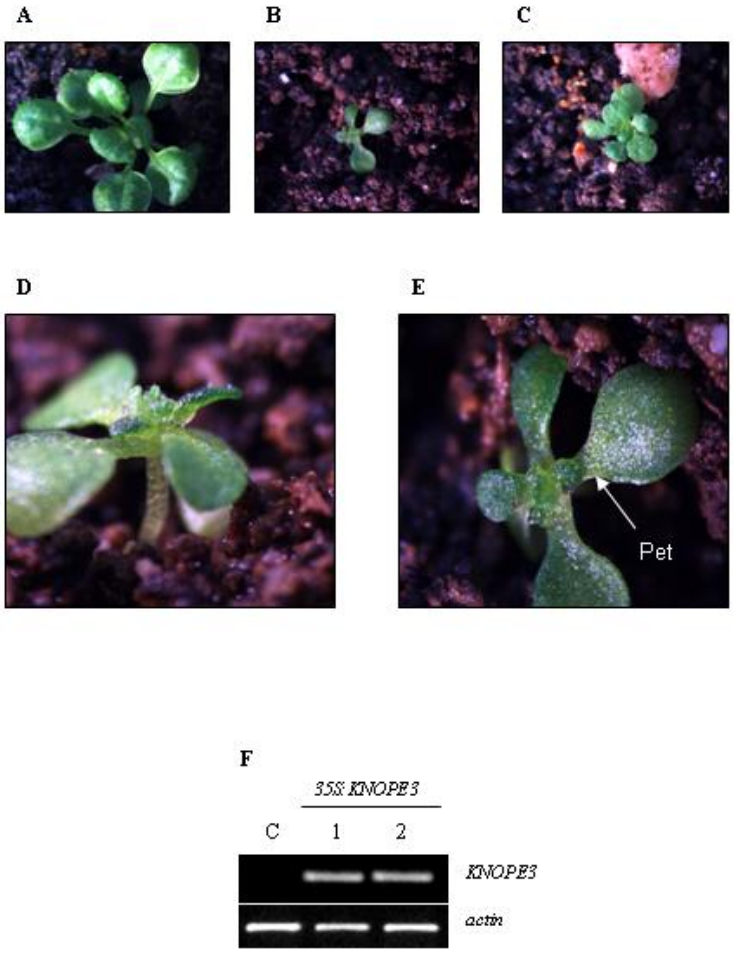

Figure 1. Phenotypes of *35S:KNOPE1* over-expressing Arabidopsis plants. Rosette from wild type *Ler* (**A**) and severe (**B**) and mild (**C**) *35S:KNOPE3* transformants. **(D-E)** Top view of rosette *35S:KNOPE3* severe phenotypes. The main axis and its internodes were shortened (D). The number and the surface of leaves were reduced and petioles (Pet) thickened (E). **(F)** RT-PCR analysis was performed on *35S:KNOPE3* transgenic lines. *Actin* of Arabidopsis was used as control for cDNA synthesis and loading. **C**, control; **1**, mild phenotype; **2**, severe phenotype.

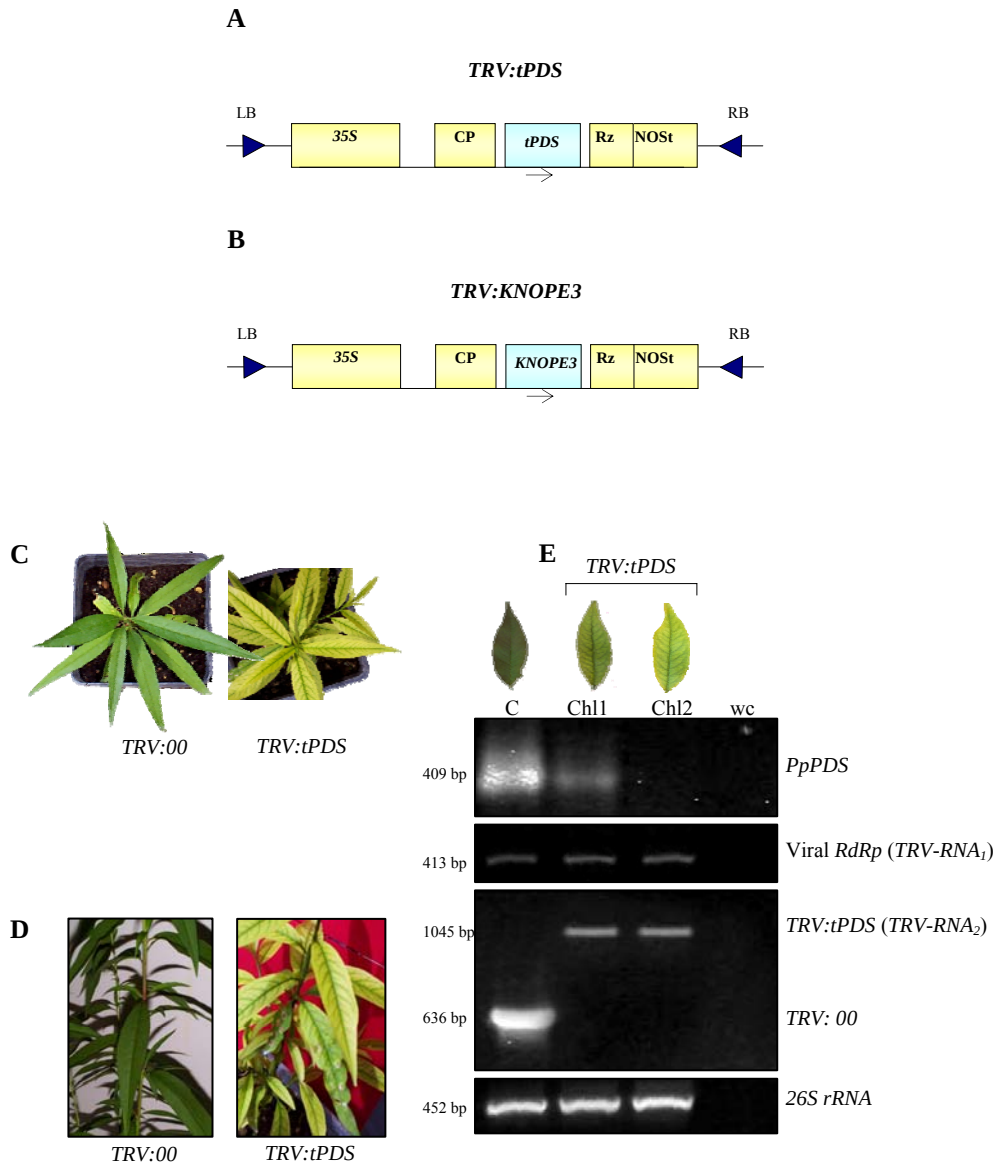

Figure 2 – Peach phenotypes using tomato *PDS (tPDS).* (A) The *phytoene desaturase* (*PDS*) gene is involved in carotenoid biosynthesis and, when silenced, results in photo-bleached phenotype. A *TRV:tPDS* construct that carries a 409bp fragment of tomato *PDS* (*tPDS*) gene was used in VIGS experiments. *tPDS* gene is sense-oriented (arrow) and it is identical to *PDS* of peach (*PpPDS*, see material and methods). **LB-RB**, left and right T-DNA borders; ***35S***, CaMV *35S* promoter**; CP**, capside protein; **Rz**, self-cuting ribozime; **NOSt**, nopaline synthase terminator.
(B) *TRV:KNOPE3*.
(C-D) Leaflets of peach plants were transformed by Agrobacteria-mediated infiltration with the *TRV*:*tPDS* construct. About 3 weeks after the infiltration photo-bleached phenotypes were observed. **(C)** Phenotypes resulting from the infection of leaves on 2-3 weeks old seedlings with empty *TRV* vector (***TRV:00***, left) or *TRV* carrying the *tPDS* (right). **(D)** Phenotypes resulting from the infection of leaves on one-year-old plants at the vegetative resumption with empty *TRV* vector (left) or *TRV*:*tPDS* (right).
(E) RT-PCR analysis on photo-bleached leaves with increasing chlorotic severity degree. *PpPDS* transcript abundance analysis was performed using a primer couple which fell outside the region of the *tPDS* fragment transcribed by the *TRV:tPDS*. **C**, leaf from plant inoculated with empty *TRV* vector; **Chl1** and **Chl2**, chlorotic leaves; **wc**, water control of the PCR. 26S transcripts were used to check for an effective reverse transcription and that an equal cDNA synthesis from distinct tissues occurred. The sizes of amplified fragments are reported.

MATERIALS AND METHODS

Plant materials and growth conditions.

Arabidopsis thaliana ecotypes Landsberg-*erecta* (Ler) were grown in a growth chamber under long day conditions (16h light and 8h dark) with steady temperature (21°C). The plants were sprinkled tree times a week, one of this with fertilizing solution. After 4-6-weeks the inflorescences were cut (Clough and Bent, 1998) and the plants were treated with the infiltration solution. Seeds were yelded (T_1 generation), sterilized (50% v/v NaClO and 0,01% v/v SDS) for 8 min., washed 10 times with sterile water and plated on selective media to select out transgenic from non transformed lines. Germination medium was 1,5% (v/v) agar, 1% (v/v) saccharose, 0,5x Murashige & Skoog media, Gamborg vitamins (SIGMA), pH 5,7, supplemented with 10mg/L BASTA. To synchronize seed germination, the plate remained at 4°C for 3 days before growth chamber. The plants grew on plate for 10-14 days (fluorescent white light 150 $\mu mol \cdot m^{-2} \cdot s^{-1}$) at 21°C, then they were moved into pots with 40% sand, 35% peat (EINHEITSERDE) and 25% soil.

***Arabidopsis thaliana* transformation.**

The *KNOPE3* cDNA was amplified using the high fidelity Platinum Taq (INVITROGEN) primers (KN3.15FW: 5'-GTCTAGAGTATTCATGGCGTACCACAACC-3'; KN3.18BW: 5'-CAATGACCTGAGCATGGTTCCCTGCTCGAG.-3') equipped with the XbaI and Xho sites (underlined) and cloned in oriented verse into the pBA002® binary vector (Kost *et al.*, 1998) under the control of the *35S* promoter of *Cauliflower mosaic virus* (CaMV). Recombinant plasmid was introduced into the *Agrobacterium tumefaciens* strain GV3101(pMP90) via electroporation. Arabidopsis ecotype Landsberg-*erecta* was transformed by vacuum-infiltration (Bechtold *et al.*, 1993).

VIGS procedure in peach.

pTRV:RNA1, *pTRV:RNA2* (Acc. numbers: AF406990.1 and AF406991) and *pTRV:tPDS* VIGS vectors derive from the tobravirus Tobacco Rattle Virus (described in Liu *et al.*, 2002) and were gently provided by Dr. Dinesh-Kumar (Yale University, New Haven, Connecticut, USA). Recombinant and empty vectors were introduced into *A. tumefaciens* strain GV3101 (tetracycline resistant, 5mg/L) by the freeze and thaw method (Holstein *et al.*, 1978). Recombinant *A. tumefaciens* strains were selected at 28°C either on Luria-bertani (LB) medium with appropriate antibiotics (kanamycin 50 mg/L, rifampicine 50mg/L, gentamicine 50 mg/L). The bacterial cells were harvested by centrifugation and resuspended into Agrobacterium inoculation buffer (10 mM $MgCl_2$, 10 mM 2-Morpholinoethanesulfonic acid pH 5.6, 150μM acetosyringone) to a final OD_{600} of 1.0 (for both *TRV1* and *TRV2*). A 1:1 ratio mixture of Agrobacteria strains containing *TRV1* and *TRV2* was shaken for 4–6 h at room temperature before infiltration. Infiltration was performed using a needle-less syringe (1 ml of the mixture) on leaves borne: a) on 2-3 week old seedlings and b) on 1 year old shoots after the vegetative resumption. Accumulation of virus in the freshly grown part of the plant was RT-PCR detected 3 weeks after inoculation. Eight to ten replications were performed for each experiment and the experiment was repeated at least two times.

RT-PCR analyses.

The procedures to isolate RNA and synthesise cDNA are described in the materials and methods of chapter 2 and 4. The presence of viral *RNA1* was detected by primers for RNA dependent RNA polymerase, RdRPFw (5'-CCGAGGAGGTCTCTATCATCGC-3') and RdRPBw (5'-CCCAGCACATCAGTCAATGACG-3'), which yielded

a PCR product of 413 nt. The empty or *tPDS* containing viral *RNA2* was evidenced by pTV00Fw (5'-CTGCTAGTTCATCTGCAC-3') and pTV00Bw (5'-CACGGATCTACTTAAAGAAC-3'), which produced 636 nt and 1045 nt bands, respectively. The tomato *PDS* (acc n. AY484445) fragment (858-1266 nt from the ATG) cloned into the TRV vector shared the 77% identity with that of *Prunus persica* cloned in our labs, of which no acc. number is available yet. However you may compare *tPDS* fragment with the same region of apricot *PDS*, acc n. AY822065.1. Apricot and peach *PDS* genes are 95% identical at the nucleotide level. A partial cDNA sequence of the peach *PDS* (*PpPDS*) is available in EST databank (PP_LEa0015G15f, http://www.itb.cnr.it/estree), and primers were designed in the 3'downstream region which included 91 nt of the ORF and 318 of the 3'UTR so as to discriminate the endogenous *PpPDS* transcript from the *tPDS* mRNA produced by the silencing viral vector. *PpPDS* primers were: *PpPDSFw* (5'-CTGTCAGGGAAACTTTGTGC-3') and *PpPDSBw* (5'-CAAAAATACATATTCATTTGG -3') and yielded a 409 nt PCR signal. The peach *26S* rRNA primers are reported in chapter 4. The final PCR conditions were: 200 ng of cDNA, 1 mM of each primer, 0.5 mM dNTPs, Taq DNA polymerase (Quiagen) 2.5 U, 1/10 of 10X Taq Buffer (Quiagen), 2.5 mM $MgCl_2$, in a final volume of 50 µl. Cycling conditions included an initial cycle at 95°C for 5 min followed by either 35 cycles or 15 cycles (only for 26S rRNA detection) at 95°C for 30s, 55°C for 1 min and 72°C for 45s, final extension at 72°C for 5 min. 15µl of each sample was electrophoresed in a 0,8% agarose gel.

Tobravirus-based construct for KNOPE3 gene silencing (TRV:KNOPE3).

To address *KNOPE3* gene function by *TRV*-based vectors a construct containing *KNOPE3* fragments was prepared. A 391 nt *KNOPE3* fragment was amplified by primers: KN3.0 FW/KN3.9 BW. PCR product was ligated into *TRV:RNA2* and checked by sequencing.

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